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**PRODUCTIVITY AND QUALITY OF DEHESA
PASTURES IN RELATION TO GLOBAL CHANGE:
INCREASING TROPOSPHERIC OZONE AND
NITROGEN DEPOSITION**

PhD Thesis

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**PRODUCTIVITY AND QUALITY OF DEHESA PASTURES IN
RELATION TO GLOBAL CHANGE: INCREASING
TROPOSPHERIC OZONE AND NITROGEN DEPOSITION**

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“Instead of controlling the environment for the benefits of population, perhaps it is time to control the population to allow the survival of the environment”

David Attenborough

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RESUMEN

La contaminación atmosférica representa una amenaza para la salud de los ecosistemas y la biodiversidad vegetal en todo el mundo; en particular para el área mediterránea donde con frecuencia se registran niveles elevados de ozono (O_3) troposférico y de depósito de nitrógeno (N) atmosférico. Los pastos anuales mediterráneos se encuentran entre los ecosistemas de mayor importancia del sur de Europa debido a su gran biodiversidad y extensa distribución. Estos pastos constituyen de forma mayoritaria el sotobosque de los bosques perennes mediterráneos y de agrosistemas forestales tradicionales como la dehesa, que se encuentran protegidos bajo la Directiva Hábitat 92/43/EEC e incluidos en la red Natura 2000.

El objetivo general de la presente tesis es el estudio de la respuesta de las comunidades herbáceas anuales mediterráneas al incremento del O_3 troposférico y del depósito de N atmosférico, principales contaminantes atmosféricos presentes de forma crónica en su área natural de distribución. Para ello se han recogido y analizado datos de campo, durante dos ciclos de crecimiento, de un pastizal experimental, constituido por especies representativas (*Trifolium striatum*, *T. cherleri*, *Briza maxima*, *Ornithopus compressus*, *Cynosorus echinatus*, *Silene gallica*), bajo distintos niveles de O_3 y N. Esta fase experimental se ha realizado en condiciones de campo en una instalación experimental de Cámaras de Techo Descubierta (*Open Top Chamber*, *OTC*). Se han considerado además de parámetros de respuesta relacionados con la producción y el intercambio gaseoso, los relacionados con la calidad forrajera menos estudiados. En base a la cuantificación de los efectos y a un ejercicio de modelización para calcular del O_3 absorbido por las plantas se ha desarrollado un nuevo enfoque del modelo de intercambio estomático de O_3 DO_3SE con nuevas relaciones de exposición y dosis –respuesta de O_3 proponiendo nuevos umbrales límite (niveles críticos) de este contaminante para la protección de comunidades anuales a escala de dosel. Esto supone un avance novedoso en este tipo de modelizaciones hasta ahora solo basadas en aproximaciones mono-específicas. Al mismo tiempo, se ha realizado un seguimiento en campo durante 3 años consecutivos de la producción, estructura de especies y fenología de una dehesa natural. Finalmente, en base a esta información se ha realizado un ejercicio de análisis de riesgo por O_3 del pasto de dehesa.

A escala de dosel, los diferentes parámetros de rendimiento analizados presentaron un patrón de respuesta al O₃ semejante durante las dos estaciones de crecimiento analizadas: este contaminante provocó una reducción de la producción del pasto parcialmente mitigada por la fertilización nitrogenada; simultáneamente, el O₃ redujo la eficiencia del fertilizante. Estos efectos se relacionan con una reducción de la producción primaria bruta del pasto debido a la reducción del intercambio de CO₂ del ecosistema y al incremento de su respiración ecosistémica.

Los resultados a escala de especie mostraron un comportamiento heterogéneo ante los dos factores analizados, O₃ y N. Las leguminosas no reaccionaron al N pero fueron las más sensibles al O₃: El género *Trifolium* respondió negativamente al O₃, mientras que el *Ornithopus* lo hizo positivamente adquiriendo ventaja competitiva frente a los tréboles sensibles. Por ello, la competición leguminosa-leguminosa jugó un papel clave dentro del sistema. Las no leguminosas fueron más tolerantes al O₃ pero, especialmente las gramíneas, mostraron una respuesta clara al N. Las interacciones significativas entre factores siguieron el patrón descrito para el dosel, una pérdida de eficiencia del N en una atmósfera contaminada por O₃ y una capacidad del fertilizante para contrarrestar el daño inducido por el O₃, pero ambos efectos fueron dependientes de los niveles de O₃ y N. De hecho, el sentido de la interacción pudo revertirse a niveles elevados del fertilizante, como se observó en la raíz de *T. striatum* creciendo en el tratamiento de O₃ más elevado; en este caso, una entrada elevada de N incrementó la reducción de la biomasa radicular provocada por el O₃. Los resultados indican que tanto el O₃ como el N pueden afectar las relaciones de competencia entre las especies debido a su respuesta heterogénea a estos factores; por ello deben considerarse como factores de estrés que afectan a la estructura y composición de los pastos mediterráneos.

El incremento de O₃ y la entrada de N afectaron a la distribución de carbono (C) y N en los distintos órganos de *Briza maxima* y *Trifolium striatum*, las especies seleccionadas como representativas de las principales familias botánicas de los pastos analizados. El O₃ provocó un incremento de contenido de N en los órganos aéreos de ambas especies y en los subterráneos de la gramínea, reduciendo con ello las tasas C/N correspondientes. La entrada de N también afectó a este

contenido de N en la misma dirección que el O₃, aunque en el caso de la gramínea el efecto se moduló por la disponibilidad de N. Aunque coincidiendo en la dirección de su respuesta al O₃ y el N, la diferente intensidad observada entre la respuesta de la gramínea y la leguminosa, provocó un desequilibrio en la distribución de la proteína cruda (PC) dentro del ecosistema; por ejemplo, la entrada de N favoreció la acumulación de PC en la fracción correspondiente a las gramíneas. La alteración de la tasa de PC Gramínea/Trébol puede estar relacionada con los cambios observados en la estructura de especies del pasto de acuerdo con las alteraciones observadas en la proporción de sus biomásas inducidas por los distintos tratamientos.

El O₃ afectó a la calidad nutricional del trébol *T. striatum* debido a un incremento en el contenido de lignina en los órganos aéreos, lo que esta negativamente relacionado con la digestibilidad del forraje; pero al mismo tiempo, el aumento de N provocó una reducción del contenido de fibras foliares del trébol, lo que favoreció el incremento de su valor alimentario en base al índice RFV (*Relative Feed Value*). En los parámetros de *B. maxima* solo se observó una respuesta muy limitada a los tratamientos. El efecto de estos resultados en la calidad nutritiva de un pasto dependerá de la intensidad de cada factor y de la proporción entre especies tolerantes y sensibles. Sin embargo, debe continuarse investigando estos efectos en relación con los cambio de calidad de forraje determinados por el estado fenológico de las plantas, las condiciones ambientales de crecimiento y la variabilidad en la composición de especies.

Los niveles críticos (CLE, *Critical Levels*) son valores límite de los contaminantes atmosféricos por debajo de los cuales se asegura la salud de la vegetación de acuerdo con el conocimiento actual; se trata de valores de gran utilidad que están siendo empleados para analizar la eficacia de las políticas medioambientales de la EU. Sin embargo, los CLE de O₃ para vegetación herbácea, y concretamente, para pastos anuales mediterráneos, están aún poco desarrollados. Los CLE actuales están basados en la respuesta de especies consideradas individualmente, y no reflejan la diversidad multi-específica natural del dosel herbáceo. Además, tampoco consideran los efectos potenciales de otros factores moduladores como la entrada de N en el sistema, por ello, su empleo en los análisis

de riesgo de ecosistemas herbáceos naturales es incierto. El modelo de Depósito de O_3 e Intercambio Estomático (DO_3SE) ha sido modificado para darle un enfoque multi-específico considerando las seis especies sembradas durante dos estaciones de crecimiento, en el pasto experimental con diferentes tasas de intercambio gaseoso y tolerancia al O_3 . Los datos de biomasa se emplearon para ponderar los flujos de absorción de O_3 en función de las especies dentro del flujo total del dosel. Las funciones basadas en concentración de O_3 ($AOT40$) solo fueron estadísticamente significativas considerando el tratamiento de N más elevado. En cambio, las funciones basadas en flujos de absorción de O_3 (POD_{y-MS}) fueron todas significativas excepto para los niveles de N de fondo. A pesar de que hasta ahora no se ha tenido en cuenta en las metodologías para el establecimiento de los CLe, la disponibilidad de N, afecta a las funciones dosis respuesta, lo que implica que afecta también a los CLe derivados. Considerando los valores medios entre los niveles de N, la dosis fitotóxica de O_3 umbral, considerando la aproximación multi-específica (POD_{MS-1}), se ha estimado en 7.9 mmol m^{-2} acumulada a lo largo de mes y medio, con un intervalo de confianza del 95% de (5.9-9.8). Se requiere analizar como aunar los CL_{ef-MS} surgidos de esta aproximación multi-específica, de los actuales CL_{ef} basados en la respuesta individual de las especies.

El análisis de riesgo realizado en un pastizal natural durante 3 años consecutivos, ha permitido comparar años con diferentes condiciones meteorológicas (húmedo, normal y seco); y en consonancia con distintos niveles de O_3 (los valores más elevados se registraron en el año seco, mientras que los más bajos lo fueron en el húmedo). Las diferencias en las condiciones ambientales, provocaron diferencias en el crecimiento del pasto, en su producción, en la composición de especies y en las dosis absorbidas de O_3 , que fueron modelizadas para las distintas especies ($PODs$) y para el dosel (POD_{-MS}). La humedad del suelo fue identificada como un factor clave para explicar la variabilidad interanual de los valores estimados de POD , debido a su influencia en el intercambio gaseoso y en la duración del periodo de crecimiento. Los resultados mostraron que bajo condiciones hídricas limitantes el riesgo de sufrir daños por O_3 es bajo a pesar de haber niveles ambientales de O_3 elevados. Sin embargo, el riesgo es mayor cuando las condiciones son más favorables para el crecimiento, aunque estén asociadas a niveles de O_3 más moderados. Los efectos del O_3 están más ligados al O_3 absorbido

que a los niveles ambientales del contaminante. Por ello, se recomienda que los análisis de riesgo de O_3 para pastos anuales se basen siempre en niveles críticos basados en flujos (POD). El uso de modelos de crecimiento del pasto, ligados a la aproximación multi-específica del DO_3SE para el cálculo de la absorción de O_3 a escala de dosel, son mas precisos para refinar los análisis de riesgo que los basados en índices de concentración como el AOT40.

ABSTRACT

Air pollution represents a threat to ecosystems health and biodiversity throughout the world and particularly at the Mediterranean area, where high tropospheric ozone (O₃) concentrations and atmospheric nitrogen (N) deposition are frequently recorded. Mediterranean annual pastures are among the most important ecosystems in southern Europe due to their high biodiversity and widespread distribution. They mainly constitute the understory of broadleaf evergreen forests and Dehesa traditional agroforestry systems, protected by the 92/43/EEC Habitat Directive and included in the Nature 2000 network.

The aim of this Thesis is to study the responses of the Mediterranean annual pasture communities to increased levels of surface O₃ and N-deposition, the main atmospheric pollutants affecting their natural distribution area. Data from two years of field work with experimental annual communities, a mixture of representative annual species (*Trifolium striatum*, *T. cherleri*, *Briza maxima*, *Ornithopus compressus*, *Cynosorus echinatus*, *Silene gallica*), over two growing seasons, under different levels of O₃ and N were analyzed. The experiments under field conditions were developed in an Open Top Chamber (OTC) facility. Yield, biomass species composition and gas exchange response parameters were measured, as well as forage quality parameters that are usually less studied. Based on the quantification of the effects, and the modelling exercises for calculating O₃ uptake inside the plants through a new multi-species approach of the Deposition of Ozone and Stomatal Exchange (DO₃SE) model, new O₃ exposure- and dose-response relationships were derived and threshold limits (critical levels) for this pollutant were proposed for the protection of annual communities. The canopy approach is a novel consideration which brings forward the current individual species approach. Moreover, three years of field data on growth, phenology and species composition from a natural Dehesa pasture were collected and analyzed. Finally, based on all the information available, a 3-years O₃-risk assessment study was performed for a natural Dehesa pasture.

The pasture yield at canopy level showed similar response patterns to the treatments for the two growing seasons analysed: O₃ caused a yield loss that was partially mitigated by N-fertilization; simultaneously, O₃ reduced the fertilization effect of N. Accordingly, O₃ decreased the community Gross Primary Production

due to both a global reduction of ecosystem CO₂ exchange and an increase of ecosystem respiration.

The results at the species level showed heterogeneous responses to both factors, O₃ and N. Legumes did not react to N but were very sensitive to O₃: *Trifolium* species responded negatively, while *Ornithopus* responded positively, taking advantage of the greater sensitivity of clovers to O₃. Thus, the legume-legume competition plays an important role in the system under polluted environments. The grasses and the herb were more O₃-tolerant than the *Trifolium* but more responsive to N, especially the grasses. The significant interactions between factors followed the general pattern described at canopy scale, a loss of effectiveness of N in O₃-polluted atmospheres and an ability of the fertilizer to counterbalance the damage induced by O₃; but both effects were dependent on the species, and on the O₃ and N levels. The direction of the interaction can reverse if N is too high at the highest O₃ levels, as it was found for *T. striatum* roots. In this situation, the fertilizer increased the O₃-induced root reduction. Both factors might affect the competitive relationships among species through their heterogeneous responses to O₃ and N, affecting the structure and composition of Mediterranean annual pastures.

Ozone and N-inputs also affected the carbon (C) and N allocation within plant organs of *Briza maxima* and *Trifolium striatum*, the species selected as representatives of the main botanical families of the pasture. Ozone induced an increase in the N-content of the aerial organs for both species, and also in the belowground organs for the grass, reducing the corresponding C/N ratios. N-inputs also affected the N-content in the same direction than O₃; but the O₃ effect on the grass was modulated by N availability. Although coincidental in the direction, the different intensity in the response between species caused an unbalance in the crude protein (CP) distribution within the ecosystem; i.e. increasing N-inputs favored the accumulation of CP in the grass fraction. The alterations of the Grass/Clover CP ratio must be interpreted in the context of observed changes in the pasture species structure and biomass effects induced by the experimental treatments.

Ozone exposure affected the nutritive quality of the clover *T. striatum* through an increase in the lignin content of the aerial organs, which is negatively related with forage digestibility. However N improved the quality of the clover through reductions in leaf fiber content and increases in Relative Feed Value (RFV). *B. maxima* quality parameters only showed subtle responses to the treatment effects. The final effect on the nutritive quality of the pasture would depend on the levels of each factor and the proportion between O₃-tolerant and O₃-sensitive species. However, further research is needed taking also into account the forage quality changes induced by plant phenology, environmental growing conditions and species composition.

The critical levels (CL_e) are limit values of atmospheric pollutants to ensure the vegetation health according to current knowledge; and constitute useful policy target values for the protection of vegetation from air pollution effects in the UE. However, CL_e for O₃ are still poorly developed for herbaceous vegetation. They are currently based on single species responses, which do not reflect the multi-species nature of semi-natural vegetation communities neither the potential effects of other factors like N-input, making their use uncertain under natural conditions. The DO₃SE model was modified for a new multi-species approach and applied to the six species of the experimental pasture, with different gas exchange rates and O₃-tolerance, through the two growing seasons. The biomass data was used for weighting the fluxes of each species in the total canopy flux. The O₃ exposure-response functions using the AOT40 index were not statistically significant except when the highest N input was considered alone. In contrast, the O₃ dose-response functions, derived using POD_{y-MS} indices, were all significant but for the lowest N input level. The results showed that O₃ effects were more closely related to the O₃ uptake than to the external O₃ exposure. Thus, it is recommended that O₃ risk assessments are based on O₃ stomatal fluxes. The influence of the N input on the exposure- and dose-response functions implies that N can modify the O₃ CL_e. However, this is an aspect that has not been considered so far in the methodologies for establishing O₃ CL_e. Averaging across N input levels, a multi-species O₃ CL_e (CL_{eF-MS}) is proposed POD_{1-MS}=7.9 mmol m⁻², accumulated over 1.5 month with a 95% confidence interval of (5.9, 9.8). Further efforts will be needed for comparing the CL_{eF-MS} with current O₃ CL_{eF} based on single species responses.

The O₃-risk assessment for annual pastures was based on 3 consecutive years of intensive monitoring of a natural pasture community. The three years presented contrasting meteorological conditions and O₃ concentrations. The first year was classified as humid compared with the long-term average rainfall for the region, showing the lowest O₃ concentrations of the three years studied. The second presented an average rainfall and intermediate O₃ concentrations while the third was dry with high O₃. The differences in environmental conditions induced changes in the pasture growth and in the species composition. This resulted also in inter-annual differences in the modelled absorbed doses of the pollutant for the different species (POD) and for the canopy (POD_{-MS}). Soil moisture was identified as a key variable for explaining the inter-annual variability in estimated POD values due to its key influence on gas exchange rates and on growing season duration. The results show that under limiting soil moisture conditions the risk of O₃ damage is low even under high O₃ exposure. However, the risk is higher when favorable conditions for plant growth are associated with moderate to high O₃ concentrations. Therefore, O₃ effects are more linked to O₃ uptake than environmental concentration levels. So, it is recommended that O₃ risk assessments for annual grasslands are always based on flux critical levels (POD). The use of pasture growth models coupled with the multi-species approach of the DO₃SE model helped to describe these changes and to refine the risk assessment results as compared with concentration-based indices like the AOT40.

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CHAPTER 1

General Introduction

1.1. Tropospheric ozone and nitrogen deposition, two main factors of Global Change

The chemical composition of the atmosphere, and particularly tropospheric ozone (O_3) and its precursors, have been rising since the Industrial Revolution up to now in many parts of the Northern Hemisphere (Stevenson et al., 2006). During the last century, O_3 background concentrations have been doubled from levels of the pre-industrial period, and have been continuously rising at an annual rate of 0.5-2% (Vinzargan, 2004). Although extremely high-concentration events have been ameliorated in Europe over the last two decades due to the implementation of environmental policies (Klingberg et al., 2014), air quality is still far from achieving acceptable levels for human and environmental health (EEA, 2014).

Tropospheric O_3 is mainly formed from photochemical reactions between their precursors such as nitrogen oxides (NO_x), hydrocarbons and volatile organic compounds (VOCs) that are generated mostly by anthropogenic activities related to energy consumption, transport and agricultural intensification (Crutzen et al., 1999). Under favorable weather conditions of high temperature and solar radiation, and stable atmospheric conditions, these compounds react to increase natural background O_3 levels in the lower layer of the atmosphere. This increase is directly related to climate change and global warming due to the radiation-absorption capacity of this gas (Mickley et al., 2001); but most important, considering the present Thesis, are the toxic effects that it potentially can cause, affecting human health, materials and ecosystems (EEA, 2014). Currently, O_3 is considered the most phytotoxic air pollutant, inducing negative effects on growth, productivity and yield quality of crops, forests and grasslands (Ainsworth et al., 2012; Fuhrer, 2009).

Within the European scenario, highest O_3 levels are usually recorded in the Mediterranean countries. This is the particular situation of the Iberian Peninsula where O_3 precursor emissions have increased steadily since the 1950s due to the large industrial development and the increase of road transport; also, meteorological conditions and orography in the region benefit O_3 formation and transport. Consequently, ground-surface O_3 levels frequently exceed current thresholds established for plant protection (critical levels) according to the EU Air

Quality Directive 2008/50/EC (Fernández-Fernández et al., 2011). Recorded O₃ levels in the area are sufficiently high to affect the physiological activity and metabolism of plants, notably crops and herbaceous or tree species, reducing their growth and yield (Gimeno et al., 1999; Alonso et al., 2001, Gimeno et al., 2004; Sanz et al., 2007, 2011, Calvo et al., 2007, 2009; Calatayud et al., 2011).

In parallel with rising tropospheric O₃ concentrations, anthropogenic N emissions have increased dramatically over the second half of the last century. The increasing use of fossil fuels for transport and industrial processes are the main cause of rising N oxides compounds (NO_x) emissions; whereas the increasingly intensive farming systems, both agricultural and livestock, have been responsible for the generation of reduced nitrogen compounds (NH₃) (Sutton et al., 2011). As a result, an alteration of the global N cycle has been detected, inducing a cascade of environmental effects such as the formation of tropospheric O₃, direct toxicity, ecosystem acidification and eutrophication, and loss of biodiversity (Galloway et al., 2008; Bobbink et al., 2010).

Natural and semi-natural ecosystems impacted by N-deposition are typically uncultivable and have high biodiversity value through the presence of specialist species. Such species often possess resilient traits that confer tolerance to grazing and low nutrient availability, but are frequently unable to respond to additional nutrient inputs and are at risk of losing competitiveness with more nutrient-demanding species (Maskell et al., 2010). At regional scale, plants adapted to the local conditions are frequently replaced by fast-growing nitrophilous plants (Emmett, 2007). These shifts in component species and loss of species richness following N inputs have been observed in different terrestrial ecosystems in Central Europe like heathland, acid, calcareous and mesotrophic grasslands (Maskell et al., 2010; Stevens et al., 2010; Bobbink et al., 2010).

The Mediterranean basin is a hotspot region at global scale threatened by N deposition (Sala et al., 2000). The total atmospheric N deposition measured in Spain reaches values around 23-30 kg N ha⁻¹ year⁻¹ (Avila and Roda, 2012; Garcia-Gomez et al., 2014). Although the threshold for Mediterranean pasture protection (critical load) regarding N-deposition is still uncertain, according to the provisional

value established and based on expert criteria, the measured values in Spain exceed this limit (Bobbink et al., 2010).

Recent studies have shown that increasing N loads to natural Mediterranean ecosystems alters soil cation availability that might have implications in terms of plant nutrition and soil microbial communities (Ochoa-Hueso et al., 2014). Nevertheless, Mediterranean ecosystems are characterized by a high spatial and temporal heterogeneity in terms of plant cover and soil resources, which favor different responses to N fertilization even across small spatial scales within the same zone (Ochoa-Hueso et al., 2011); thus, more research is needed for finding consistent responses.

The co-occurrence in the same area of chronically high O₃ levels and N deposition is considered one of the most harmful environmental stress combinations for plant communities (Fowler et al., 1999). This interaction is responsible for some of the most crucial changes observed in the C and N cycles in the forests of California (Takemoto et al., 2001; Fenn et al., 2003). The coincidence of both pollutants is currently occurring in extensive natural regions of the Iberian Peninsula which are under the influence of urban or industrial areas. This is the case of the southern slopes and base of the Guadarrama Mountains in the Spanish Central Range located 50 km northwest of Madrid city. This area is chronically affected by high O₃ levels and moderate levels of N deposition due to its topographic and climatic conditions and, especially, the emissions from the city (Plaza et al., 1997; García-Gómez et al., 2016). One of the most characteristic ecosystems occupying the area is pasture communities dominated by annual species that grown as an understory of broadleaf evergreen forests or, mainly, dehesa agroforestry systems.

1.2. Ozone effects on therophytic pastures

The dehesa system is a managed open oak forest that currently covers an area of 3.5-4 million hectares in the Iberian Peninsula (Olea & San Miguel-Ayanz, 2006) (Figure 1.1). The dehesa includes different herbaceous communities mainly composed of annual species that provide fodder for wildlife and livestock. Their

great annual and seasonal variability, in terms of production and nutritional quality, is related to the inter-annual variability of the climatic conditions characteristic of the Mediterranean area (Vazquez Aldana et al., 2008). Also, these pastures represent a high floristic diversity including up to 230 species, with the most predominant families being Gramineae, Leguminoseae and Compositae (García del Barrio et al., 2014). The structure and species composition of the pasture is also related to the climatic conditions of the year, making these pastures among the most complex in terms of their seasonal dynamic and competition relationships (Peco et al., 1998). Due to the high biodiversity and expanse of this ecosystem, it is protected under the 92/43/EEC Habitat Directive and included in the Nature 2000 network.

Figure 1.1. Distribution Map of the dehesa in the Iberian Peninsula (source Corine Land Cover 2006).

Since the last decade, significant effort is ongoing for assessing the O₃ responses of annual species belonging to these Mediterranean pastures. Ozone has been recognized as an important negative factor causing foliar damage and affecting their growth, yield, consumable food value and reproductive capacity (Bermejo et al., 2003; Gimeno et al., 2004a; Sanz et al., 2005, 2007, 2011, 2013, 2014). To investigate the pollutant effects on pasture, these studies employed Open Top Chambers (OTC) as experimental units (adapted from the original OTC design used in the National Crop Loss Assessment Program of the Environmental Protection Agency, USA; Heck et al., 1982) for which more detailed description is presented in Chapters 3, 4 and 5 (Figure 1.2). Table 1.1 summarizes the annual species studied up to now in previous experimental works and the variables affected by the pollutant. However, all these studies are based on monocultures (individual species), or simple 2-species mixtures, and on experimental designs in which plants were grown in pots and not under water restriction, leaving considerable uncertainty when the results have to be extrapolated to natural conditions.

Figure 1.2. Open Top Chamber (OTC) field facility located in the Spanish central plateau at Santa Olalla (Toledo, 450 m.a.s.l.; 40°3'N, 4°26'W), in the research experimental farm “La Higuera” (MNCN-CSIC). Photos from the experiments developed in the present work during the 2011 and 2012 growing seasons.

Table 1.1. Summary of the experimental studies about O₃ effects on Mediterranean annual species. All experiments were developed under non water limitation and grown in pots. The variables affected and the O₃ treatments considered are indicated; CFA= charcoal filtered air; NFA= non-filtered air; NFA+= non-filtered air supplemented with O₃.

Experimental Year	Species	O ₃ treatment	Other factors	Variables	References
2000	<i>Anthyllis cornicina</i> , <i>Anthyllis lotoides</i> , <i>Biserrula pelecinus</i> , <i>Medicago minima</i> , <i>Ornithopus compressus</i> , <i>Trifolium angustifolium</i> , <i>Trifolium cherleri</i> , <i>Trifolium glomeratum</i> , <i>Trifolium striatum</i> , <i>Trifolium subterraneum</i> , <i>Aegilops geniculata</i> , <i>Aegilops triuncialis</i> , <i>Avena sterilis</i> , <i>Briza maxima</i> , <i>Bromus hordeaceus</i> , <i>Bromus sterilis</i> , <i>Cynosurus echinatus</i> , <i>Lolium rigidum</i> , <i>Vulpia myuros</i>	CFA, NFA, NFA+	Monocultures	Aerial and subterranean biomass, Visible injury, Senescence biomass	Bermejo et al., 2003, Gimeno et al., 2004a
2001	<i>Trifolium cherleri</i> , <i>Trifolium striatum</i> , <i>Trifolium subterraneum</i>	CFA, NFA, NFA+	2-species mixture	Flower production, Seed production	Gimeno et al., 2004b
2002	<i>Trifolium cherleri</i> , <i>Trifolium striatum</i> , <i>Trifolium subterraneum</i> , <i>Briza maxima</i> , <i>Bromus hordeaceus</i>	CFA, NFA, NFA+	Nitrogen	Visible injury, Phenology, Aerial and subterranean biomass, Forage quality, Seed production	Sanz et al., 2005, Sanz et al., 2007, Sanz et al., 2011, Sanz et al., 2013, Sanz et al., 2014

1.2.1. Foliar visible injury

Herbaceous species can develop foliar visible injury under O₃ exposure (Heagle et al., 1994; Novak et al., 2003; Bergmann et al 1999). These specific symptoms caused by the pollutant appear when damage at cellular and physiological scales has already occurred. The O₃-induced foliar injuries have also

been described for some O₃-sensitive therophytic species. An assessment based on visible injury considering 22 native annual species from dehesa pastures indicated that 70% of the analyzed species were foliar-damaged by the pollutant (Bermejo et al., 2003). The appearance of leaf damage on O₃-sensitive annuals takes place under relative low O₃ exposures compared with perennial species from central and northern Europe. In fact, the annual species are considered the most O₃-sensitive among all herbaceous communities analyzed thus far under experimental conditions (Bassin et al., 2007a).

The most abundant families constituting annual pastures are legumes and grasses. Although there are O₃-sensitive species in both families, regarding the development of specific foliar symptoms under O₃ exposure, the frequency and intensity of the symptomatology are greater in legumes, particularly in the genus *Trifolium* (Bermejo et al., 2003). Figure 1.3 illustrates the evolution of O₃-induced foliar injury on the leaves of *Trifolium cherleri* under progressive increments of O₃ exposure during the experiment conducted in 2011 in the present Thesis.

However, in grasses, instead of leaf symptoms, injury is more characteristic of an O₃-induced increment of senescence, a more nonspecific damage. Therefore, for this family the parameters related to senescence processes are frequently considered for determining their O₃-sensitivity than are specific visible injuries (Bermejo et al., 2003, Sanz et al., 2011).

Figure 1.3. Leaves of *Trifolium cherleri* exposed to different O₃ treatments, from left to right; FA = charcoal filtered air, NFA = non filtered air, NFA+ = non filtered air supplemented with 20 nl l⁻¹ of O₃, NFA++ = non filtered air supplemented with 40 nl l⁻¹ of O₃. Photos from the experiments developed in the present Thesis during the 2011 growing season.

1.2.2. Effects on growth and biomass production

Ozone has been identified as an important factor that negatively affects growth rates and biomass production of therophytic pasture species. Accordingly with the visible injury effects, annual legumes in general show higher O₃-sensitivity than annual grasses when biomass effects are evaluated, even when the underground biomass is considered; although there are also tolerant legumes like the *Ornithopus* or *Anthyllus* genus (Gimeno et al., 2004a).

Based on monoculture studies, the reduction of the total biomass of sensitive clovers can be as great as 20% due to the O₃ ambient levels compared with filtered air; but the losses increase to around 30% when plants are exposed to the highest O₃ levels, which are maximum levels currently recorded in some rural areas of the Iberian Peninsula (Sanz et al., 2007). Gimeno et al. (2004a) indicated that, under high O₃ levels, 70% of the assayed legume species experienced a reduction in their relative growth; meanwhile, 75% of the studied grasses showed no effect in terms of growth. These heterogeneous responses among species can potentially alter the structure and composition of the pastures in areas under chronic O₃ pollution, although no studies up to the present work have been done that consider the O₃ effects at community scale. Studies that considering other grassland communities have shown this kind of effects where tolerant species successfully compete against the sensitive (Davison and Barnes 1998; Hayes et al. 2009; Wedlich et al. 2012).

Another interesting effect caused by O₃ exposure is the different response between aerial and subterranean biomass. This effect has been detected in five different annual legumes species (Gimeno et al., 2004, Sanz et al., 2007). In these species, the O₃ negative effect is frequently more pronounced in the roots than in the aerial biomass, increasing the aboveground/belowground ratio; i.e., under O₃ exposure, the clovers *Trifolium subterraneum*, *T. striatum* and *T. glomeratum* increased this ratio within a range 33 to 60%. The O₃ effects on belowground biomass have major relevance in annual species due to the important function of their roots as a reservoir of storage photoassimilates during their maximum physiological activity in early spring for use later in the season when the more adverse conditions of summer drought lead to a lack of nutrient availability (Chaves et al., 2002). Therefore, any alteration in the root system could compromise the plant's resistance and survival under water-stress conditions.

1.2.3. Effects on seed production

One of the most relevant results regarding annual species is the reduction of plant reproductive capacity caused by the pollutant (Figure 1.4). The studies of Gimeno et al. (2004b) and Sanz et al. (2007) indicated an O₃-induced decrease in flower and seed production of *Trifolium striatum*, *T. subterraneum* and *T. glomeratum*; the loss of seed production can be as high as 30% in *T. striatum*.

Loosing reproductive capacity has very important implications for annual species by compromising their competitive capacity and even their survival. The composition and structure of an annual community is defined by its soil seed bank composition, which is maintained by the annual contribution of seeds from all the species (Peco et al., 1998). Considering this, O₃ might have a direct effect on ecosystem biodiversity. However, while most of the threshold limits for plant protection (critical levels) currently developed for risk assessment are based on biomass loss, seed and flower production in annual communities are also key parameters to consider.

Figure 1.4. Seed production of *Trifolium striatum* plants (means $\pm$ se) for the different O₃ treatments (g plant⁻¹). CFA= charcoal-filtered air; NFA= Non-filtered air; NFA+= Non-filtered air supplemented with O₃ (reproduced from Gimeno et al., 2004b).

1.2.4. Effect on nutritive quality

The adverse effects that O₃ can cause to pastures strongly affect one of the main ecosystem services of the grasslands: the loss of yield and quality of the forage, the main food source in livestock farming. Ozone induces changes in nutritional quality of pasture that could affect productivity of ruminant herbivores that feed on the pasture, either directly via grazing, or through silage and hay production (Krupa et al., 2004).

Forage quality is determined by a combination of several factors, including the content of highly digestible nutrients such as proteins and less digestible nutrients such as structural carbohydrates that form the cell walls (among other constituents), and must be broken down in the digestive process in order for nutrients to be released. Some studies with grasslands showed that the loss of nutritional quality is an important response after O₃ exposure (Hayes et al., 2016). The pollutant generally causes an increase in the foliar fiber content, mainly in the

lignin fraction, resulting in decreased pasture quality in terms of the digestible energy content of the vegetation (Muntifernig et al., 2006).

Annual Mediterranean species are especially sensitive to O₃ effects on forage quality. Several studies indicate a negative effect on nutritive quality, not only in O₃-sensitive legumes (Sanz et al., 2005, 2014) in addition to effects on their biomass and growth, but the pollutant also reduced the quality of the more tolerant grasses, even without producing effects on biomass. Sanz et al. (2010) showed that under O₃ exposure, *Briza maxima*, a relative O₃-tolerant grass, has increased lignin and fiber contents in the leaves without altering their biomass.

The O₃ effects on forage quality in annuals are usually associated with an acceleration of plant senescence (Sanz et al., 2007, 2010). These effects, together with the loss of biomass in legumes which are the major contributors to the protein content of the forage stand in Mediterranean pastures (Vazquez-de-Aldana et al. 2008), can seriously compromise the palatability and the nutritive value of the pasture for wild herbivores and grazing cattle.

1.2.5. Effect on plant phenology

Another effect caused by O₃ exposure is the reduction of the life span of plants such as González-Fernández et al., (2013) have shown for Mediterranean wheat. Regarding therophytic species, the pollutant increases the senescence of both legumes and grasses, accelerating the phenological pattern and reducing their life span (Sanz et al., 2007, 2010).

In addition to nutritive quality parameters, plant phenology appears to be a very O₃-sensitive parameter for annual species. The pollutant can alter the phenology pattern of the more tolerant annuals without affecting other parameters like foliar symptoms, growth or biomass production. Sanz et al. (2010) indicated a shortening in the life span of *Briza maxima* (from emergence to seed maturity) of 11 days due to O₃ exposure, without showing effects on their growth rate or biomass production.

This kind of effect is of great interest due the potential impact at ecosystem scale. Any alteration on plant phenology may disrupt the temporal complementarity between species, which optimizes the use of resources and reduces competition among species allowing their coexistence (Cleland et al. 2006; Gross et al. 2007). Thus, effects on plant phenology can be of major importance in the Mediterranean pasture communities dominated by annuals, with a high floristic richness maintained by complex competitive relationships among species (Peco et al., 1998).

1.2.6. Effects on ecosystem scale

Studies with Central European perennial pastures found that interspecific competition modifies the O₃ effects (Volk et al., 2006; Stampfli and Fuhrer, 2010). Volk et al. (2011) did not find any O₃ response at community level after 5 years of O₃ exposure of a highly diverse alpine perennial grassland. Similarly, only small O₃ effects on community composition were observed in calcareous grassland turfs after three growing seasons (Thwaites et al., 2006).

Regarding Mediterranean pastures, no previous work has been done on responses to O₃ in annual communities. There is only one previous experiment that utilized simple mesocosms with 2-species mixtures in Gimeno et al. (2004a). This study evaluated the O₃ effects on the reproductive capacity of three sensitive clovers planted in competition with the relatively resistant grass *Briza maxima*. The study showed no significant interactions between O₃ and competition factors; clover plants in mesocosms produced fewer flowers than in monoculture, but the response to O₃ was similar in both cases. These results indicate a similar response of the species to the pollutant in both experimental designs, monocultures and species mixtures, allowing hypothesis-testing of the same high O₃ sensitivity in a more complex community. On the contrary, as already commented, studies developed with perennials determined a major resilience of the community compared with the species grown without inter-specific competence (Bassin et al., 2007a).

There is a lack of experimental work with O₃ effects on annual communities. Nevertheless, with reference to the previous experimental studies based on monocultures or simple mixture species, some mechanisms can be identified related to their potential responses at community scale. Probably the main mechanism is based on the heterogeneous response of the annuals species to the pollutant. The different O₃ sensitivity of the species and families inhabiting the same community, as has been found between clovers and grasses, might drive the pastures under chronic pollutant exposure to structural and compositional changes. The high O₃-sensitivity of the clovers, which can develop visible symptoms, yield less and especially reduce seed production when grown under high O₃ levels (Bermejo et al., 2003; Gimeno et al., 2004a; Sanz et al., 2005, 2007), may compromise the competitiveness of this family and diminish its presence in the annual pastures under the pressure of the more tolerant species.

1.3. Nitrogen counterbalances ozone effects on therophytic pastures

The observed O₃-responses of the annual Mediterranean species are modulated by environmental factors such as atmospheric N-deposition that increases soil N-availability. In general, plant response to N-inputs follows the opposite direction of the O₃ response; whereas N stimulates plant production, O₃ decreases it (Bassin et al., 2009). However, for annuals, depending on the levels of the factors and on the parameter assayed, the direction of the interaction can be different.

Under moderate O₃ levels, N can counterbalance the negative O₃-induced effect on seed and flower production of the annual clover *Trifolium striatum* (Sanz et al., 2007). However, Sanz et al. (2005) concluded that N fertilization intensified O₃ effects on forage quality parameters, like the O₃-induced increase of the lignocellulose fraction in the *Trifolium subterraneum* cells wall; whereas at the same time, N fertilizer partially ameliorated the detrimental effects caused by O₃ on the green/senescence biomass ratio. Some interactive effects have been also found for annual grasses. In the case of *Bromus hordeaceus*, O₃ induced an increase in foliar senescence, with the consequent imbalance in the senescent/green

biomass ratio; but this response was modulated by N availability, which mitigated the effect of the pollutant for intermediate O₃ levels (Sanz et al., 2013).

In summary, the experimental studies completed to date with annuals do not report a common response pattern of the O₃ and N interaction, indicating their complexity. Moreover, there is no information about the direction of this interaction within more complex communities in which the competitive relationships can play an important role in the ecosystem functioning. Thus, more experimental research is needed in this regard.

1.4. Risk assessment strategies against environmental pollution at European scale

The Convention on Long-range Transboundary Air Pollution (CLRTAP; UNECE, 1979) was signed by 34 governments (including Spain) and the European Community, and it represented the first international legally binding instrument within the framework of UNECE to deal with problems of air pollution on a broad regional basis. This Convention implies exchanges of information, consultation, research and monitoring among the Parties to protect human beings and the environment against air pollution and endeavors to limit and, as far as possible, gradually reduce and prevent air pollution including long-range transboundary air pollution by means of policies and strategies (UNECE, 1979). Currently, the CLRTAP has 51 Parties, and eight different Protocols have been developed in its framework.

The UNECE is leading the process of setting O₃ critical levels (CLE) as policy target values for the protection of vegetation. Since the 1980s, O₃ risk assessments have been performed by means of exposure indices based exclusively on the atmospheric O₃ concentration (CLE_c) (Kärenlampi and Skärby, 1996). More recently, the estimation of O₃ effects in relation to stomatal uptake, or flux-based CLe (CLE_f), has been recognised as a more suitable index to evaluate real impacts of O₃ in plants (Emberson et al., 2000a; Mills et al., 2011). The flux approach for CLE_f derivation relies on the ability to adequately estimate stomatal conductance (g_{sto}) and the availability of species-specific dose-response relationships. The stomatal

uptake of O_3 at leaf level over the sensitive phase of plant development, or Phytotoxic O_3 Dose (POD_y), is modelled within the CLRTAP framework using the Deposition of Ozone and Stomatal Exchange model (DO_3SE) (CLRTAP, 2010). DO_3SE is currently included within the European Monitoring and Evaluation Programme (EMEP) photo-oxidant chemical transport model, which is used by the CLRTAP to inform the European air pollution abatement strategies (Simpson et al., 2007). The DO_3SE model is based on a big-leaf approach parameterized for a single species (Emberson et al., 2000a). However, this approach should be optimized for plant communities like grasslands that comprise a high number of different species, presenting a great range of specific g_{sto} (González-Fernández et al., 2010b).

CHAPTER 2

Objectives and General Methodology

2.1. Objectives

General objective

To characterize and quantify the responses of annual Mediterranean communities to increased levels of tropospheric ozone and atmospheric nitrogen deposition in order to develop response functions for risk assessment analysis.

For achieving this general objective, data from two years of experimental work with annual communities in an OTC facility, and three years of field data from a natural dehesa pasture, have been collected and analyzed; based on these results, modelling exercises and a risk assessment study has been developed. Laboratory analysis corresponding with the forage quality parameters have been conducted in the Department of Animal Sciences at Auburn University in Auburn, Alabama USA through a 3-month stay.

This work has been organized into 5 chapters corresponding to 5 manuscripts for their publication in SCI journals: 2 already published, 1 under revision and 2 ready for sending to publish. Each Chapter presents its own specific objective and sub-objectives in order to reach the general objective of the thesis.

Objective 1 (developed in chapter 3 and 6)

To characterize at canopy level the responses of an experimental annual community under field conditions exposed to increased ozone levels and atmospheric nitrogen deposition.

The sub-objectives of this chapter are: (1) To determine the O₃ and N effects on the gas exchange parameters at canopy scale: primary production and ecosystem respiration; (2) To investigate the O₃ and N effects on the total yield; (3) To assess how O₃ and N interact on the yield response of the pasture.

Objective 2 (developed in chapter 4)

To identify at species level the responses of an experimental annual community under field conditions exposed to increased ozone levels and atmospheric nitrogen deposition.

The sub-objectives of this chapter are: (1) To determine the relative sensitivity of the annual species to O₃ and N based on their biomass responses; (2) to analyze at species level how N inputs modulate the O₃ response; (3) To assess whether O₃ can affect the N-use efficiency of the fertilizer; (4) To resolve whether species growing in the presence of competition and under field conditions are more resilient to O₃ and N than expected from species tested individually; (5) to determine whether the effects of O₃ and N at species scale can drive the competitive capacity of the species affecting the structure and biodiversity of the pasture.

Objective 3 (developed in chapter 5)

*To determine nitrogen and ozone impacts on carbon and nitrogen allocation and on the nutritive quality of *Briza maxima* and *Trifolium striatum*, as representative species of the annual community.*

The sub-objectives of this chapter are: (1) to analyze if O₃ and N can unbalance the C and N-allocation within plant organs; (2) to determine if the individual responses of the species can unbalance the C and N in the ecosystem; (3) to analyze the O₃ and N effects on the forage quality of the pasture; (4) to determine if the heterogeneous responses of the species regarding C and N allocation can be related with the observed changes in species composition and forage quality.

Objective 4 (developed in chapter 6)

To develop a new multispecies approach of the DO_3SE model for calculating the O_3 uptake at canopy scale and the influence of N on the pasture response to O_3 , in order to derive dose-response functions and critical levels of yield for the protection of the Mediterranean annual pastures.

The main sub-objectives of this chapter are: (1) to present a new experiment on the response of an annual community to O_3 and N; (2) to set a parametrization of the multiplicative algorithm of DO_3SE for g_{sto} modelling combining the six assayed species; (3) to estimate the POD_y for a multi-species canopy (POD_{y-MS}); (4) to develop exposure- and dose- response functions for yield considering the multi-species approach and the influence of N availability, and suggesting CLe_c and CLe_f for annual pastures based on this method.

Objective 5 (developed in chapter 7)

To develop an O_3 risk assessment for a Mediterranean annual pasture, through three consecutive years with contrasted meteorological conditions, based on the multi-species approach of the DO_3SE model ensemble with two different growth pasture models, DEHESA and MEDPAS.

The main sub-objectives of this chapter are: (1) To model the yield of an annual pasture and their seasonality throughout three consecutive hydrological years using the growth models MEDPAS and DEHESA; (2) to validate the output of the models based on the phenological stage and aboveground biomass samples collected over the three consecutive years in a natural dehesa in Central Spain; (3) to ensemble MEDPAS and DEHESA models with the multispecies approach of the DO_3SE model for calculating the stomatal O_3 flux within the canopy during the three years; (4) to carry out an O_3 -risk assessment for annual pastures in Central Spain based on concentration and flux based critical levels derived from the multi-species approach.

2.2. General methodology

Experimental assays

During the growing season of 2011 and 2012, a simplified annual community was exposed to increased levels of O₃ and N inputs. The multi-species design of the experiment allowed competition relationships among species and close to field conditions regarding soil and meteorological parameters.

The experiments were developed in an Open Top Chamber facility located in Santa Olalla (Toledo) in La Higuera Research Farm (MNCN/CSIC). The facility has been described in the chapters 3, 4 and 5 (Figure 1.2).

The analysis of the effects on gas exchange and biomass parameters are presented in chapter 3 and 6 (effects at canopy scale) and chapter 4 (effects at species scale). The analyses of the effects on the forage quality (protein and fiber content) are presented in chapter 5.

Modelling

The gas exchange and biomass parameters acquired during the experimental work have provided the basis for developing a multi-species O₃ flux model based in the DO₃SE model of the CLRTAP (CLRTAP, 2010) to calculate the absorbed O₃ dose of the canopy for the different O₃ treatments. Dose response functions were built, and O₃ critical levels (threshold values) were calculated for the protection of the annual Mediterranean pastures. These results are presented in chapter 6.

Field studies and risk analysis

The yield and phenology together with the meteorological conditions of an open holm-oak forest located in Tres Cantos (705 m.a.s.l; 3°43' 59"O; 40°35' 17" N) were followed during the growing seasons of 2010, 2011 and 2012. This data were used to validate the output of the MEDPAS and DEHESA pasture growth

models for the three years. Both models were ensemble with the multi-species approach of the DO₃SE model (CLRTAP, 2010) for calculating the O₃ absorption within the canopy. Based on the threshold values for Mediterranean annual pastures developed in chapter 6, the O₃-risk assessment for the annual pasture of the Tres Cantos dehesa was developed for three consecutive years in chapter 7.

CHAPTER 3

Current ozone levels threaten gross primary production and yield of Mediterranean annual pastures and nitrogen modulates the response

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Abstract

Pastures are among the most important ecosystems in Europe considering their biodiversity and distribution area. However, their response to increasing tropospheric ozone (O_3) and nitrogen (N) deposition, two of the main drivers of global change, is still uncertain. A new Open-Top Chamber (OTC) experiment was performed in central Spain, aiming to study annual pasture response to O_3 and N in close to natural growing conditions. A mixture of six species of three representative families was sowed in the field. Plants were exposed for 40 days to four O_3 treatments: filtered air, non-filtered air (NFA) reproducing ambient levels and NFA supplemented with 20 and 40 $nl\ l^{-1}\ O_3$. Three N treatments were considered to reach the N integrated doses of “background”, +20 or +40 $kg\ N\ ha^{-1}$. Ozone significantly reduced green and total aboveground biomass (maximum reduction 25%) and increased the senescent biomass (maximum increase 40%). Accordingly, O_3 decreased community Gross Primary Production due to both a global reduction of ecosystem CO_2 exchange and an increase of ecosystem respiration. Nitrogen could partially counterbalance O_3 effects on aboveground biomass when the levels of O_3 were moderate, but at the same time O_3 exposure reduced the fertilization effect of higher N availability. Therefore, O_3 must be considered as a stress factor for annual pastures in the Mediterranean areas.

3.1. Introduction

Tropospheric ozone (O₃) and atmospheric nitrogen (N) deposition are two of the main air pollutants affecting natural and seminatural areas and causing harmful ecological effects (EEA, 2011; Sutton et al., 2011). In the Mediterranean area, high solar radiation, temperature and prevailing stable atmospheric conditions favor photochemical O₃ formation (Millán et al., 2000; Cristofanelli and Bonasoni, 2009), resulting in some of the highest surface O₃ concentrations in Europe (EEA, 2011). Ozone concentrations in Spain frequently exceed current thresholds established for plant protection according to the EU Air Quality Directive 2008/50/EC or the Convention on Long-Range Transboundary Air Pollution of the UN/ECE (CLRTAP) (Fernández-Fernández et al., 2011). Moreover, O₃-induced effects have been reported in crops and natural vegetation (Ferretti et al., 2007; Bermejo et al., 2011). Total atmospheric N deposition in Spain reaches values up to 23-30 kg N ha⁻¹ year⁻¹ (Àvila and Rodà, 2012; García-Gómez et al., 2014). These values are a relatively lower than deposition levels recorded in central Europe. However, since changes in species composition occurs early in the sequence of N saturation (Emmett, 2007), N deposition effects could be occurring in Spanish natural ecosystems. Unfortunately, despite the presence of extraordinary biological richness in the Mediterranean Basin, very little information is available on the threat that air pollution and in particular reactive N, can pose to biodiversity in this area (Ochoa-Hueso et al., 2011).

Most of the research on ecosystems responses to air pollution has been focused on the effects of a single pollutant while air pollutants seldom occur individually. Interactive effects of O₃ and N deposition are still poorly understood. Higher N availability can increase plant productivity providing higher resource availability for investing in defense, repair and compensation processes counterbalancing O₃ effects, or may exacerbate O₃ damage due to stimulated growth and higher specific leaf area (Bytnerowicz, 2002; Ashmore, 2005; Bassin et al., 2007). On the other hand, O₃ can inhibit some N-related enzyme activities and reduce the allocation of assimilates to roots and root growth (Bytnerowicz, 2002; Ashmore, 2005), thus diminishing the fertilization effects of higher N levels.

Grasslands communities represent an important contribution to global biodiversity. Experiments with single species have shown a wide range of O₃ sensitivity (Bassin et al., 2007; Hayes et al., 2007). However, the response of plant communities cannot readily be predicted from the response of their component species, and little information is available on the effects of O₃ on pasture communities (Bassin et al., 2007; Cape, 2008). Communities of temperate perennial grasslands have shown higher resilience to O₃ compared to individual species (Thwaites et al., 2006; Volk et al., 2011). On the other hand, chronic N deposition is reducing plant species richness of European temperate acid grasslands (Stevens et al., 2004; Dupre et al., 2010). The combination of O₃ and N deposition on perennial grasslands shows contrasting results with no interactive effects or an additive response in C allocation in some species (Volk et al., 2011; Wyness et al., 2011).

Fewer works have been focused on the response of annual pastures to air pollution. Annual grasslands constitute the understorey of broadleaf evergreen forests, and dehesa traditional agroforestry systems. These ecosystems are protected by the 92/43/EEC Habitat Directive and included in the Nature 2000 network. Previous experiments with individual species grown in pots showed that O₃ induces visible injury, accelerates senescence and reduces biomass production and consumable food value of some representative species (Bermejo et al., 2003; Gimeno et al., 2004a,b; Sanz et al., 2005, 2007, 2011). Interestingly, nitrogen fixing legumes, with high nutritive quality for herbivorous feeding, were more O₃ sensitive than grasses (Bermejo et al., 2003; Gimeno et al., 2004a). Moreover, O₃ strongly reduced flower and seed production of some sensitive legumes (Gimeno et al., 2004b; Sanz et al., 2007) which could interfere with species perpetuation and competitive fitness. For some species, N fertilization counterbalanced O₃ effects on senescence and plant and flower biomass, when plants were exposed to moderate O₃ concentrations (Sanz et al., 2005, 2007, 2011). Experiments with annual plant communities are needed to test if results obtained with individual species grown in pots are applicable for O₃ risk assessment to annual grassland communities.

An experiment has been carried out to study the interactive effects of O₃ and N fertilization on a simplified annual community. The main hypotheses were that O₃ affects community gas exchange rates reducing biomass production, and that N can modulate the negative response to this pollutant. To our knowledge this is the first experiment providing results of effects of O₃ and N fertilization on an annual grassland community.

3.2. Materials and methods

3.2.1. Experimental site

The experiment was performed in a new Open Top Chamber (OTC) facility located in the Spanish central plateau at Santa Olalla (450 m.a.s.l.; 40°3'N, 4°26'W), in the scientific experimental farm "La Higuera" (CSIC). The area presents a Thermo-Mediterranean temperate climate, with moderate winters and dry summers. The field site is far from local sources of air pollution and at 80 km downwind of Madrid City. The soil is a calcic luvisol, characterized by differentiation pedogenetic of clay (11.4% clay, 24% silt, 64.6% sand). Further information of edaphic and physic-chemical characteristics of Ap horizon is provided in Appendix (Table A.I.1.)

3.2.2. Plant material

A simplified pasture community was sown on the ground using 6 representative annual species: 3 legumes (*Trifolium striatum*, *Trifolium cherleri*, *Ornithopus compressus*), 2 grasses (*Briza maxima*, *Cynosurus echinatus*) and 1 forb (*Silene gallica*) (Table 3.1). The family composition followed a proportion of 45% leguminous, 45% grasses and 10% caryophyllaceae. Legumes and grasses are the most abundant species in natural annual pastures (Montoya et al., 1988). Species selection was based on natural abundance, relatively homogeneous seed size to allow homogeneous sowing, seed availability, high seed germinative capacity and O₃-sensitivity based on previous studies (Bermejo et al., 2003; Gimeno et al., 2004; Sanz et al., 2007). Before the experiment, in order to reduce plants growing from

the natural soil seed bank inside the experimental plots, seeds present in the soil were successively allowed to germinate and seedlings were eliminated afterwards turning over 20 cm of top soil. Experimental seeds were sown on February 11th 2011, aiming for a density of 1000 plant m⁻² using the proportion of species seeds indicated in Table 3.1; the sowing mixture was adjusted according to seed weight and germination capacity tested previously. The homogeneity of species germination and distribution inside the chambers was tested and confirmed 5 days after emergence (DaE). Every week, alien species were manually removed

Table 3.1 Species composition of the experimental annual pasture community.

Species	Family	Seed Source	Percentage in the mix ^c	Seed germinative rate (%)	100 Seed weight (g)	Ozone sensitivity	Reference
<i>Trifolium striatum</i>	<i>Leguminosae</i>	Seed Bank ^a	15	88	0.234	Sensitive	Sanz et al., 2007
<i>Trifolium cherleri</i>	<i>Leguminosae</i>	Seed Bank ^a	15	96	0.285	Sensitive	Sanz et al., 2007
<i>Ornithopus compressus</i>	<i>Leguminosae</i>	Seed Bank ^a	15	57	0.156	Resistant	Bermejo et al. 2003
<i>Cynosurus echinatus</i>	<i>Gramineae</i>	D. Moncalvillo ^b	22.5	88	0.133	Resistant	Gimeno et al., 2004a,b
<i>Briza maxima</i>	<i>Gramineae</i>	D. Moncalvillo ^b	22.5	93	0.273	Rel. resistant	Sanz et al., 2011
<i>Silene gallica</i>	<i>Carifoliaceae</i>	D. Moncalvillo ^b	10	76	0.041	Unknown	

^a Bank of Extremadura Community Agriculture Department (seeds from central-western areas of the Iberian Peninsula; *T. striatum*: 38°55'N, 05°60'W; *T. cherleri*: 38°22'N, 05°64'W; *O. compressus*: 38°55'N, 05°06'W).

^b Dehesa de Moncalvillo, Guadalix de la Sierra, Madrid (40°40'N, 03°46'W).

^c Weight based.

3.2.3. Experimental design

The experiment followed a split-plot design conducted in 15 plots with four O₃ treatments three times replicated (i.e. a total of 12 OTCs with 3 OTCs per O₃ treatment) and three ambient plots without chamber. Four O₃ treatments were considered: charcoal filtered air (FA), non-filtered air (NFA) reproducing ambient levels, non-filtered air supplemented with 20 nl l⁻¹ O₃ (NFA+) and nonfiltered air supplemented with 40 nl l⁻¹ O₃ (NFA++). Ambient air chamberless plots (AA) were considered to evaluate the chamber effect. The experimental unit was the NCLAN-type OTC (adapted from the original OTC design used in the National Crop Loss Assessment Program of the Environmental Protection Agency, USA; Heck et al., 1982). Ozone supply for NFA+ and NFA++ treatments was applied during 8 h day⁻¹ (7:00 to 15:00 GTM) 7 day week⁻¹ by mean of an O₃ generator (Model 16, A2Z Ozone Systems Inc., USA) system fed with pure oxygen. Ozone (ML® 9810B, Teledyne, USA), sulfur dioxide (SO₂; ML®9850B UV, Teledyne, USA), and nitrogen oxides (NO₂ and NO; ML®9841, Teledyne, USA) concentration inside each chambers and AA plots were monitored continuously above the canopy (50 cm above the soil) using an automated time-sharing system which sampled each plot for 10 min. The accumulated O₃ exposure was characterized by the AOT40 index representing the accumulated exposure over a threshold of 40 nl l⁻¹ during daylight hours. Micro-meteorological stations were installed inside the AA plots, FA and NFA++ chambers for continuously monitoring air relative humidity (RH) and temperature (HOBO® Pro v2, Onset), photosynthetic active radiation (PAR; OSO-SUN HOBO®, Onset, USA), soil relative humidity (ECHO 10, Decagon Devices, USA) and soil temperature (TMC6-HD HOBO®, Onset, USA) at 10-15 cm depth.

Immediately after seed sowing (February 11, 2011), all experimental plots were divided in three sectors (1.4 m² each) delimiting the three N input treatments: N0 (soil N background), N20 (20 kg N ha⁻¹) and N40 (40 kg N ha⁻¹). To reach these integrated doses, N supplementation was applied every 2-weeks using an ammonium nitrate (NH₄NO₃) solution (Table 3.2). Trying to keep the natural limited water conditions of annual pastures, watering was only lightly applied when strictly necessary, i.e. after sowing, with N applications (3 l water m⁻² for each N application) or when an excessive water stress was detected that could

compromise the experiment. Nitrogen content of the water supplied was periodically controlled being always close to zero. A total volume of 48 l water m⁻² during the whole experimental period was applied; thus, incrementing about 30% the natural precipitation experienced during the growing season.

Table 3.2 Timetable of the experimental events.

Event	Date	DaE ^a	DaS ^b
Sowing	11-Feb		
Emergence	25-Feb	1	
First harvest (vegetative stage)	11-Apr	46	0
Start of ozone exposure	12-Apr	47	1
Gas exchange sampling (vegetative stage)	12/14 Apr	47 (46-48)	0-2
First N fertilization	13-Apr	48	2
		61	15
Second N fertilization	26-Apr	62 (62-63)	16-
Gas exchange sampling (reproductive stage)	27/28 April		17
Third N fertilization	10-May	75	34
	20-May	85	39
Second harvest (reproductive stage)/leaf damage quantification		89	43
Fourth N fertilization	24-May	91 (89-93)	44-
Gas exchange sampling (senescence stage)	25-27 May		48
End of ozone exposure	30-May	95	49
Third harvest (senescence and seed maturity)	20-Jun	116	70

Numbers in brackets indicate the real range of the sampling period for a particular parameter.

^a DaE = Days after emergence.

^b DaS = Days after start of ozone exposure.

Plant exposure to the different O₃ treatments started in April, 47 days after emergence (DaE; Table 3.2). Almost at the same time (48 DaE), the first N fertilization was applied. Pasture was exposed to O₃ for 49 days until the community reached its maximum development and productivity, then the O₃ exposure system was switched off allowing plants to dry up and complete seed maturation which happened at the end of May.

Total soil mineral N (N_{min}) content (NO₃⁻ NH₄⁺) was analyzed throughout the experiment within the top 20 cm of the upper soil horizon. After the extraction

of 8 g of mixed fresh soil with 50 ml of water and 50 ml of 1 M KCl solution, NO_3^- and NH_4^+ concentrations were measured with an Orion 720A electrode (Thermo Fisher Scientific, Beverly, MA, USA) and with a Technicon AAI Auto-analyser (Technicon Hispania, Madrid, Spain) respectively.

3.2.4. Visible injury

Visible leaf damage was evaluated on the second harvest (Table 3.2) based on the percentage of damaged leaves per plant. All the plants inside one sampling harvest ring (5 dm²) per chamber and N treatment were evaluated. Ozone-induced foliar damage was quantified as the percentage of damaged leaves per plant relative to the total considering four classes: no injury, 0-25%, 25-50%, 50-75% and 75-100% of leaves affected. A leaf damage index for the community (LDI_c) was calculated based on the foliar mean damage for each individual species weighted by its own abundance. Abundance per species was estimated from their green biomass proportion inside the sampling ring.

3.2.5. Biomass harvest

Aboveground biomass production was harvested three times throughout the life cycle of the pasture (Table 3.2). The early harvest was done just before the start of O_3 treatments and N applications (46 DaE), signaling the initial stage of the pasture (Vegetative Stage). The second harvest was collected after 39 days of O_3 exposure (85 DaE), when the pasture reached its maximum biomass and flowering development (Reproductive Stage). After this harvest, the O_3 fumigation system was switched off coinciding with the start of pasture natural decline. The last harvest was done when plant biomass was completely dried and seeds reached maturity (116 DaE, Senescence and seed maturity Stage). For each harvest, all plants within a 5 dm² sampling ring were cautiously collected per chamber and N treatment. Plant material was classified into species (data not shown) and immediately weighed to obtain fresh weight biomass. Afterwards, samples were dried to constant weight at 60°C. Additionally, in the second harvest, green and

senescent leaves were separated and root biomass was extracted by hand from the first 10 cm of soil collected inside the sampling ring.

3.2.6. Gas exchange at canopy level

Gas exchange at canopy level was measured using a steady-state custom-made cuvette coupled to the Li-Cor 6400 portable gas exchange system (Li-Cor, Nebraska, USA) following the company technical recommendations (García et al., 1990). A cylindrical cuvette was made of transparent methacrylate (24.5 cm radius, 29.4 cm height), covered with a polypropylene film to moderate temperature increments. The inside walls of the cuvette were coated with Teflon® tape to avoid condensation and water absorption and desorption on the walls. The open bottom allows the cuvette to be placed over the pasture. Two fans were set inside to facilitate air mixing. PAR (LI-190, LI-COR Inc, USA), air temperature and RH (Vaisala Inc. USA) were continuously monitored inside the cuvette. Air flow to the cuvette was propelled using an external pump (KNF NMP 830 KVDE 12V, KNF) and the inlet air flow was continuously monitored with an air velocity transducer (TSI 8455, TSI Inc., USA).

Gas exchange measurements were performed between 9:00e11:00 h GMT on clear sunny days at the three different pasture development stages (Table 3.2). During the Reproductive Stage, NFA was not measured due to bad weather conditions. Gas exchange measures were acquired only when the steady-stationary state was achieved which took around 2 min. Afterwards, instantaneous values were averaged for 2 min. Bare soil measures without plants inside the sampling ring were done at Senescence Stage in plots previously harvested and left without plants for one month. Ecosystem CO₂ Exchange (NEE) was calculated on ground surface area basis ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) and on dry biomass basis ($\mu\text{mol CO}_2 \text{ g dw}^{-1} \text{ s}^{-1}$) using the biomass harvest data collected at the different phenological stages. After each full-light steady-state NEE measurement, cuvette was completely darkened with a black cloth to record the steady-state ecosystem Dark Respiration (R_{eco}). Gross Primary Production (GPP) was estimated according to: $\text{GPP} = \text{NEE} -$

R_{eco} . Two measures per OTC and N treatment were done. After the measurements, plants inside the cuvette were harvested for biomass analyses.

3.2.7. Statistical analysis

The effects of N fertilization and O_3 exposure on the different response parameters and harvests were evaluated with Analysis of Variance (ANOVA) using a split-plot design, considering O_3 as the main factor, N as sub-factor and their interaction. A block effect was also included as a random factor. A phenological stage factor was considered to analyze gas exchange rates. Annual pastures did not grow again after harvest, thus different sampling rings were selected for each harvest assuring its independence for statistical purposes. When significant differences among treatments were detected ($p < 0.05$), mean differences were assessed with the Tukey Honestly Significant Difference test (HSD). Normal probability plots and scatter plots of residuals were used to test normality. The Levenne test was applied to check variance homoscedasticity. When data did not fulfill model requirements, data were log-transformed. Outliers were rejected based on studentized residuals procedure (if greater than 3). All statistical analyses were carried out using the Statistica v.11 (StatSoft Inc., USA).

Table 3.3 Summary of the meteorological conditions and O₃ exposure indexes for the different O₃ treatments during the growing season (from February 25 to June 20) and during the maximum growth period (April-June). RH = relative air humidity; PAR = Photosynthetic Active Radiation; VPD = Atmospheric Vapor Pressure Deficit; FA = charcoal filtered air; NFA = non filtered air; NFA+ = non filtered air + 20 nl l⁻¹ of O₃; NFA++ = non filtered air + 40 nl l⁻¹ of O₃.

Meteorological conditions						
	February-June			April-June		
	24 h-mean	Max	Min	24 h-mean	Max	Min
Temperature (°C)	16.1	36.4	-1.5	18.8	36.4	6.1
RH (%)	65.6	99.6	17.5	61.1	99.2	17.5
PAR (μml m ⁻² s ⁻¹)	476.9	2248.2	0	550.7	2248.2	0
VPD (Kpa)	1.08	6.33	0	1.35	6.33	0.03
Soil Temp. (°C)	17.2	31.6	4.2	20.1	31.6	13.2
Soil Humidity (%)	21.7	40.1	18	20.1	27.1	18.0
Precipitation (mm)	148.1 ^a			97.7		
Ozone indexes						
	24 h-mean (nl l ⁻¹)	Hourly maximum (nl l ⁻¹)	8 h-mean (nl l ⁻¹)	Febr.-June AOT40 (nl l ⁻¹ h)	April-June AOT40 (nl l ⁻¹ h)	
AA	29	68	36	3780	3737	
FA	20	48	23	174	158	
NFA	28	65	34	2805	2798	
NFA+	33	138	45	9205	9173	
NFA++	34	189	50	14257	14245	

^a Total precipitation

3.3. Results

3.3.1. Ozone exposure, meteorological and soil conditions

Ozone was the most important air pollutant in this area with SO₂ and NO_x concentrations always within the range of monitor detection limits (1 nl l⁻¹; data not shown). Ambient O₃ concentrations increased from March to June (Figure 3.1) reaching the hourly maximum of 68 nl l⁻¹ in April (Table 3.3, Figure 3.1). Ozone exposure indices and mean daily profiles for the different O₃ treatments are presented in Table 3.3 and Figure 3.1 respectively. No significant differences among chambers or O₃ treatments were detected on prevailing meteorological conditions (Table 3.3).

Figure 3.1 Mean daily profiles of ozone concentration in the different treatments during the experimental period: AA = Ambient plots without chamber, FA = charcoal filtered air, NFA = non filtered air, NFA+ = non filtered air supplemented with 20 nl l^{-1} of O_3 , NFA++ = non filtered air supplemented with 40 nl l^{-1} of O_3 .

Total soil mineral N background represented in N0 treatment was stable throughout the experiment averaging 10 $\text{mg N}_{\text{min}} \text{kg}^{-1}$ (Figure 3.2). Soil N content increased with each N dose, and values remained slightly higher than before the N addition. At the end of the experiment, N20 and N40 presented 18 $\text{mg N}_{\text{min}} \text{kg}^{-1}$ and 27 $\text{mg N}_{\text{min}} \text{kg}^{-1}$ respectively (means across O_3) compared to the background 10 $\text{mg N}_{\text{min}} \text{kg}^{-1}$.

Figure 3.2 Total soil N mineral content ($N_{\min}$) for the different N treatments (means across O_3 treatments $\pm$ se). Arrows indicate the addition of the different N dose supplementation. N0 = soil background; N20 = 20 kg N ha⁻¹; N40 = 40 kg N ha⁻¹.

3.3.2. Visible foliar injury

Different O_3 -injury typology was observed depending on plant family: *Leguminosae* species showed brown-reddish necrotic spots on the upper surface of mature leaves; *Gramineae* species exhibited necrotic leaf tips and an obvious increase in foliar senescence; *Silene* showed similar symptoms to clover although very scarce. No O_3 -induced visible injury was registered on plants exposed to ambient O_3 levels (NFA treatment), nevertheless O_3 significantly increased LDI_c by 31% and 48% in NFA+ and NFA++ treatments respectively compared with FA (mean across N treatments; Table 3.4). No significant differences were observed among N treatments, neither N modulated the foliar injury response to ozone (Table 3.4).

Table 3.4 Growth related parameters (mean $\pm$ se) corresponding to the second harvest for the different O₃ and N treatments (after 39 days of O₃ exposure). FA = charcoal filtered air, NFA = non filtered air, NFA+ = non filtered air + 20 nl l⁻¹ of O₃, NFA++ = non filtered air + 40 nl l⁻¹ of O₃. Different letters indicate significant differences among treatments.

	Canopy leaf damage index (%)	Senescent biomass (gdw m ⁻²)	Total aboveground biomass (gdw m ⁻²)	Root biomass (gdw m ⁻²)	Fresh/dry aerial biomass ratio	Senescent/green biomass ratio	Root/aerial biomass ratio
<i>Experimental factors</i>							
Ozone	< 0.005	< 0.05	< 0.05	ns	ns	< 0.05	ns
Nitrogen	ns	ns	ns	ns	ns	ns	ns
Nitrogen x Ozone	ns	ns	< 0.05	ns	ns	ns	ns
Block	ns	ns	ns	ns	ns	ns	ns
<i>Mean values</i>							
FA N0	0	20.1 $\pm$ 3.1	417.9 $\pm$ 17.0 ^{abc}	37.7 $\pm$ 8.5	19.2 $\pm$ 2.0	0.05 $\pm$ 0.01	0.09 $\pm$ 0.02
NFA N0	0	20.8 $\pm$ 6.9	382.6 $\pm$ 14.6 ^{bc}	36.3 $\pm$ 5.6	20.9 $\pm$ 1.9	0.06 $\pm$ 0.02	0.10 $\pm$ 0.02
NFA+ N0	30.3 $\pm$ 3.9	48.5 $\pm$ 7.5	414.1 $\pm$ 11.1 ^{bc}	38.3 $\pm$ 7.4	17.7 $\pm$ 2.0	0.13 $\pm$ 0.02	0.10 $\pm$ 0.02
NFA++ N0	45.1 $\pm$ 2.7	42.5 $\pm$ 2.3	380.4 $\pm$ 10.6 ^{bc}	44.4 $\pm$ 14.9	21.1 $\pm$ 1.0	0.13 $\pm$ 0.01	0.13 $\pm$ 0.05
FA N20	0	32.9 $\pm$ 7.5	495.2 $\pm$ 20.3 ^a	39.1 $\pm$ 2.4	19.7 $\pm$ 0.4	0.07 $\pm$ 0.02	0.09 $\pm$ 0.01
NFA N20	0	16.1 $\pm$ 6.0	404.5 $\pm$ 23.5 ^{bc}	36.5 $\pm$ 3.1	20.2 $\pm$ 1.8	0.04 $\pm$ 0.01	0.09 $\pm$ 0.00
NFA+ N20	36.7 $\pm$ 3.4	35.7 $\pm$ 17.5	393.4 $\pm$ 46.6 ^{bc}	42.9 $\pm$ 8.2	20.4 $\pm$ 0.4	0.1 $\pm$ 0.04	0.12 $\pm$ 0.03
NFA++ N20	47.3 $\pm$ 4.1	36.6 $\pm$ 7.6	349.4 $\pm$ 39.5 ^c	23.6 $\pm$ 0.9	22.1 $\pm$ 1.1	0.12 $\pm$ 0.03	0.08 $\pm$ 0.01
FA N40	0	28.3 $\pm$ 10.5	442.0 $\pm$ 19.2 ^{ab}	47.0 $\pm$ 14.6	20.3 $\pm$ 3.0	0.07 $\pm$ 0.02	0.11 $\pm$ 0.04
NFA N40	0	17.5 $\pm$ 1.8	450.6 $\pm$ 18.9 ^{ab}	36.6 $\pm$ 0.6	21.3 $\pm$ 0.7	0.04 $\pm$ 0.01	0.08 $\pm$ 0.01
NFA+ N40	26.1 $\pm$ 2.6	30.4 $\pm$ 5.3	404.0 $\pm$ 26.0 ^{bc}	33.5 $\pm$ 4.7	20.0 $\pm$ 1.1	0.08 $\pm$ 0.02	0.09 $\pm$ 0.01
NFA++ N40	51.1 $\pm$ 3.9	35.4 $\pm$ 6.1	344.4 $\pm$ 25.1 ^c	28.9 $\pm$ 5.4	22.1 $\pm$ 1.5	0.11 $\pm$ 0.01	0.10 $\pm$ 0.02

3.3.3. Biomass

The pasture life cycle experienced a slow growing period during the first 45 days (February-March, growth rate of $1.7 \text{ g dw day}^{-1}$; Figure 3.3), then a very fast and short growing period during April until mid-May (46-85 DaE, mean growth rate across O_3 and N treatments of $8.5 \text{ g dw day}^{-1}$), and finally pasture growth rate declines until mid-June (86-116 DaE, average growth rate of $4.4 \text{ g dw day}^{-1}$), when plants were completely dry.

Figure 3.3 Total aboveground biomass of the pasture for the different O_3 treatments since emergence till the end of the life span (means across N treatments $\pm$ se). FA = charcoal filtered air, NFA = non filtered air, NFA+ = non filtered air supplemented with 20 nl l^{-1} of O_3 , NFA++ = non filtered air supplemented with 40 nl l^{-1} of O_3 . Arrows indicate the time of N addition.

The first aboveground biomass harvest was sampled before starting the O_3 exposure, in order to test pasture growth homogeneity in all the experimental plots. At this time, seedlings showed a very low yield with a mean value across O_3 and N treatments of 76 g dw m^{-2} , representing 14% of the total pasture yield at the end of the growing cycle. No significant differences were found among chambers,

although differences between OTCs and AA plots were significant at this early time (see Annex I).

At the second aboveground biomass harvest (Table 3.4), the mean yield of the pasture was 406 g dw m⁻² (overall mean across O₃ and N treatments); thus, the accumulated biomass from the emergence represented 75% of the total yield at the end of the life of the pasture. Ozone caused stronger effects than N in all the biomass parameters evaluated since the O₃ factor accounted for 43-55% (depending of the parameter) of the total variability of the data compared with the <3% assigned to the N factor, 16-17% to the interaction and 14% to the block effect caused by the soil heterogeneity of the field. Green, senescent and total aboveground biomass were negatively affected by O₃ exposure (Table 3.4). Significant reductions on green biomass of 7%, 14% and 25% were detected in NFA, NFA+ and NFA++ treatments respectively compared with FA (means across all N treatments), although the differences between FA and NFA treatments were not statistically significant. Ozone increased 40% the senescent biomass in both NFA+ and NFA++ compared to FA, resulting in a strong increment up to 90% in the NFA++ treatment of the senescent/green biomass ratio (Figure 3.4). When total yield was considered, O₃ exposure induced 9%, 11% and 21% reductions in NFA, NFA+ and NFA++ respectively (mean across N treatments). No significant O₃ effects were detected on the fresh/dry weight ratio of green biomass (Table 3.4).

Figure 3.4 Total aboveground biomass (g dw m⁻²) and senescent/green biomass ratio for the different O₃ treatments at the second harvest (mean across N treatments ± se). FA = charcoal filtered air, NFA = non filtered air, NFA+ = non filtered air supplemented with 20 nl l⁻¹ of O₃, NFA++ = non filtered air supplemented with 40 nl l⁻¹ of O₃. Different letters indicate significant differences among means.

Nitrogen fertilization treatments did not cause any significant effect on biomass parameters in the second harvest. However, a significant interaction between O₃ and N was detected on green and total aboveground biomass (Table 3.4). While no differences among O₃ treatments were detected in the N0 treatment, NFA+ and NFA++ green biomass were significantly lower than FA in N20, and NFA++ biomass was significantly lower than FA and NFA in the N40 treatment (Figure 3.5). Increasing N availability enhanced green biomass production mainly in FA and NFA, becoming higher than in the treatments supplemented with O₃. Thus, O₃ exposure reduced the fertilization effect of N additions. On the other hand, higher N availability in N40 decreased the O₃ induced effects on green biomass when O₃ concentrations were moderate, but not with high O₃ levels since green biomass in NFA++ was still significantly lower than in FA. Total biomass followed exactly the same statistical pattern as green biomass. The observed differences between OTCs and AA found in the earlier harvest disappeared in the second harvest (See Annex I).

Figure 3.5 Green biomass for the different O₃ and N treatments at the second harvest (mean across N treatments $\pm$ se). FA = charcoal filtered air, NFA = non filtered air, NFA+ = non filtered air supplemented with 20 nl l⁻¹ of O₃, NFA++ = non filtered air supplemented with 40 nl l⁻¹ of O₃. N0 = soil N background, N20 = 20 kg N ha⁻¹; N40 = 40 kg N ha⁻¹. Different letters indicate significant differences among O₃ treatments.

A third aboveground biomass harvest was collected at the end of the life span of the pasture when all the species were completely dry. At this stage, a 19% yield reduction was observed in NFA++ compared with FA (Figure 3.3), although this difference represented only a statistical trend ($p < 0.1$). No significant effect of the N treatments was observed on pasture yield at this harvest, neither a significant O₃ x N interaction.

Mean pasture root biomass per square meter collected at the second harvest was 37 g dw m⁻² giving a mean root/aerial biomass rate of 0.1 (means across O₃ and N treatments). No significant effects caused by O₃, N or their interaction could be detected on root biomass or root/aboveground biomass ratio (Table 3.4).

3.3.4. Canopy gas exchange

NEE was affected by both O_3 and phenological stage factors without interactive effects between them (Table 3.5). No significant effects of the different N treatments were detected. NEE showed maximum values at the Reproductive Stage, when the pasture reached its maximum growth development. At this stage, mean values across N treatment averaged $26 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ (Figure 3.6). During the Senescence Stage, NEE was reduced to $9 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$, thus 64% lower compared to the maximum (means across O_3 and N treatments). Ozone exposure induced a reduction of 28% of NEE throughout the growing cycle compared with FA (mean across N treatments and phenological stages), and O_3 ambient levels (NFA) were enough to produce this effect (Table 3.5). When NEE was expressed in terms of dry biomass ($\mu\text{mol CO}_2 \text{ g dw}^{-1} \text{ s}^{-1}$), the O_3 induced differences were significant only at the Vegetative Stage (Figure 3.6b). Interestingly, maximum NEE values based on dry biomass were reached in the earlier stages of the pasture, diminishing progressively until pasture senescence (Figure 3.6).

Table 3.5 Mean values of the canopy gas exchange parameters expressed in terms of ground surface area ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) corresponding to the second harvest period for the different O_3 treatments and phenological stages (mean $\pm$ se). Values are averages across N treatments. FA = charcoal filtered air, NFA = non filtered air, NFA+ = non filtered air supplemented with 20 nl l^{-1} of O_3 , NFA++ = non filtered air supplemented with 40 nl l^{-1} of O_3 . NEE = Ecosystem CO_2 Exchange; R_{eco} = Ecosystem Dark Respiration; GPP = Gross Primary Production (GPP = NEE- R_{eco}). DaE = Days after Emergence. Different letters indicate significant differences among O_3 treatments (across N treatments and phenological stages) and differences among phenological stages (across N and O_3 treatments).

	NEE ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)	R_{eco} ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)	GPP ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)
<i>Experimental factors</i>			
Ozone	< 0.05	ns	<0.01
Nitrogen	ns	ns	ns
Phenological stage	<0.001	ns	<0.001
$\text{O}_3 \times$ Phenological stage	ns	<0.1	ns
<i>Mean values per ozone treatment (across N treatments and phenological stages)</i>			
FA	17.31 $\pm$ 1.60 ^a	-6.26 $\pm$ 0.56	8.77 $\pm$ 1.63 ^a
NFA	11.97 $\pm$ 1.66 ^b	-7.82 $\pm$ 0.65	3.78 $\pm$ 2.08 ^{ab}
NFA+	13.50 $\pm$ 1.10 ^b	-7.04 $\pm$ 0.52	6.22 $\pm$ 1.66 ^{ab}
NFA++	12.01 $\pm$ 2.10 ^{ab}	-10.19 $\pm$ 1.91	1.76 $\pm$ 3.14 ^b
<i>Mean values per phenological stage (across N and O_3 treatments)</i>			
Vegetative stage (47 DaE)	16.4 $\pm$ 1.14 ^b	-7.33 $\pm$ 0.84	9.08 $\pm$ 1.53 ^a
Reproductive stage (62 DaE)	25.81 $\pm$ 1.56 ^c	-8.05 $\pm$ 0.86	16.3 $\pm$ 2.67 ^a
Senescence stage (91 DaE)	9.20 $\pm$ 0.70 ^a	-7.82 $\pm$ 0.48	1.28 $\pm$ 1.12 ^b

Dark respiration (R_{eco}) was relatively stable across O_3 and phenological stages (Table 3.5; Figure 3.6) and no differences were detected caused by N treatments. However, when it is expressed in terms of dry weight biomass, both O_3 and phenological stage factors affected R_{eco} (Figure 3.6): at the Vegetative Stage, R_{eco} in NFA++ treatment doubled the value of the FA control (mean across N treatments). This effect was also observed during the Senescence Stage. Average respiration of bare soil (CO_2 flux without plants inside the sampling ring) was $-2.2 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$, representing about 28% of the average R_{eco} of the canopy pasture ($-7.82 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$, average value across O_3 and N treatments). GPP presented the same phenological pattern as NEE, with maximum values recorded during the peak growing season (Reproductive Stage). Ozone reduced pasture GPP, being the NFA++ values 80% significantly lower than FA (Table 3.5). At the end of the

growing cycle (Senescence Stage), due to the O_3 -induced increment of R_{eco} , only FA sustained a positive gas exchange balance (Figure 3.6)

Figure 3.6 Net Ecosystem gas exchange (NEE; gray bar) and Ecosystem Respiration (R_{eco} ; dark bar) for the different O_3 treatments at three phenological stages. Values are expressed in terms of ground surface area ($\text{mmol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$, left graphs) and dry weight biomass ($\mu\text{mol CO}_2 \text{ g dw}^{-1} \text{ s}^{-1}$, right graphs). FA = charcoal filtered air, NFA = non filtered air, NFA+ = non filtered air supplemented with 20 nl l^{-1} of O_3 , NFA++ = non filtered air supplemented with 40 nl l^{-1} of O_3 . Values are means across N treatments $\pm$ se. Different letters indicate significant differences among means for NEE and R_{eco} ; letters within brackets indicate a trend ($p < 0.1$).

3.4. Discussion

To our knowledge, the present experiment is the first one studying the response of an experimental annual pasture community to increased O_3 and N levels growing under natural soil conditions and allowing plant competition. Although growing inside OTCs, the pasture followed a similar development to pastures growing under natural conditions in the Central Iberian Peninsula. The average yield of the experimental pasture at maximum development was 406 g dw m^{-2} , equivalent to 4060 kg dry matter ha^{-1} . This value is within the range of 500-5400 kg dry matter ha^{-1} year $^{-1}$ reported under natural conditions (Olea and San-Miguel-Ayanz, 2006; Vázquez-De-Aldana et al., 2008; Hussain et al., 2009). Likewise, NEE and dark respiration rates were similar to values measured in natural annual pastures in Portugal (Hussain et al., 2009). Therefore, our experimental annual community can be considered representative of natural annual pastures.

Ozone clearly affected the annual pasture community causing visible foliar injury, accelerating plant senescence and reducing green and total aboveground biomass. Ozone induced foliar visible damage in 30%-48% of the canopy and decreased up to 25% of green biomass and up to 21% of total aboveground biomass. Interestingly, even ambient O_3 levels (NFA treatment) showed a clear trend to reduce total aboveground biomass of the annual community (9%). These results agree with previous studies performed with individual annual species or two-species mesocosms showing O_3 -induced reductions on growth parameters within the range 20-30% in some sensitive species (Gimeno et al., 2004a,b; Sanz et al., 2005, 2007). Thus, the response to O_3 observed in an annual community was in the range of the effects reported for individual species. By contrast, well established perennial grassland communities have shown higher resilience to O_3 effects when comparing to individual component species (Volk et al., 2006; Stampfli and Fuhrer, 2010). On the other hand, our experimental annual community seemed to be more sensitive to O_3 than perennial grasslands since significant effects were detected after few weeks of O_3 exposure. Volk et al. (2011) did not find any O_3 -response at community level after 5 years of O_3 exposure of a high diverse alpine perennial grassland. Similarly, only small O_3 effects on

community composition were observed in calcareous grassland turfs after three growing seasons (Thwaites et al., 2006). The fact that annual communities regenerate each growing season from the seed bank with a fast growing rate when meteorological conditions are optimal, provides annual grasslands its characteristic state of non-maturity. In this sense, our results support the idea of Grime et al. (2000) that mature ecosystems exhibit greater inertia to stress and disturbance than newly established communities. Other sown model perennial communities have also shown quick and strong responses to O_3 suggesting that high growing rates is among the main plant trait related to O_3 -sensitivity (see review in Bassin et al., 2007).

In addition to reductions in green and total aboveground biomass, O_3 significantly increased the senescent biomass of the annual pasture. The O_3 -induction of premature senescence has been frequently observed in different vegetation types (Ashmore, 2005) and was also reported in previous experiments performed with annual single species (Bermejo et al., 2003; Sanz et al., 2007). Premature senescence together with reduction of aboveground biomass would imply lower plant resources for flower and seed development determining annual community viability. In fact, the O_3 -induced reduction of the reproductive capacity of some annual legumes has been demonstrated in experiments with individual species (Gimeno et al., 2004b). Results of the O_3 effects on biomass, flower and seed production of the individual species forming this annual community are presented elsewhere (Calvete-Sogo et al., 2013).

The O_3 effects on pasture biomass were related to the observed response of gas exchange rates since reductions of NEE up to 28% were recorded. Similar results were observed when NEE was expressed based on dry biomass or on ground surface area. Thus O_3 effects on NEE at canopy level were partially explained by the O_3 -induced reduction in biomass, but photosynthetic and/or respiration rates of the pasture were also affected. Indeed, O_3 increased dark respiration when calculated on biomass weight basis, but effects on photosynthetic rates cannot be disregarded (O_3 effects on photosynthesis at leaf level are presented elsewhere: Calvete-Sogo et al., 2013). Dark CO_2 fluxes include aboveground plant respiration, plant root respiration and soil microbial

respiration, representing a combination of plant and soil processes. Since aboveground biomass was responsible of 70% of the observed dark respiration at the late phenological stage, effects on plant metabolism could mask the effects on soil processes. A test with a limited amount of replicated measurements showed that O_3 strongly increased bare soil dark respiration ($-3.9 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ in NFA+ compared to $-1.78 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ in FA; data not shown) suggesting that O_3 could be affecting both plant and soil processes. Further research is needed to clarify possible O_3 effects on soil metabolism. The combined effects of reducing NEE and increasing dark respiration resulted in a significant O_3 -induced reduction of GPP. Recent works indicate that O_3 can reduce C sequestration in tree living biomass (Harmens and Mills, 2012). However, quantifying the C budget of grassland systems is still challenging and further investigation is needed to quantify O_3 effects on C sequestration in annual grasslands.

Increasing N availability slightly affected annual pasture development. The low N doses used in this experiment, selected to reproduce the potential range of atmospheric N deposition in this area, increased soil mineral N content but these doses were not enough to significantly affect the yield or gas exchange rates of the annual pasture. The background soil N content of the soil ($10 \text{ mg N}_{\text{min}} \text{ kg}^{-1}$ i.e. $33 \text{ kg N}_{\text{min}} \text{ ha}^{-1}$) seemed to be enough to cover pasture N nutritional demand, explaining the lack of response to low N additions. Natural annual pastures usually grow in low fertility soils (Vázquez-De-Aldana et al., 2008) and a soil N content representing one tenth of the background soil N content of our experiment has been reported in natural annual pastures in Portugal (Hussain et al., 2009). Other nutrients, such as phosphorus, could be limiting plant development in this ecosystem type.

Despite the low responsiveness to N, a significant interactive response between N and O_3 was detected in green and total aboveground biomass production. On the one hand, O_3 reduced the fertilization effect of higher N availability since yield responses to N addition were positive only under low O_3 levels. This effect was not biomass ratio, suggesting that O_3 could be affecting plant metabolism related to nitrogen. On the other hand, N seemed to counterbalance O_3 yield reductions when pollutant levels were moderate, but this compensation was

not observed in the high O₃ exposure treatment. A similar positive influence of N on O₃ effects on yield and quality parameters has been already detected when annual plants have been tested individually (Sanz et al., 2005, 2007, 2011). Other authors have highlighted the importance of nutrient availability in modifying O₃ responses of semi-natural vegetation (Bassin et al., 2007) but these interactions need further characterization in different vegetation types.

Ozone exposure-response functions were built based on the results obtained in this experiment using the relative yield loss of green and total aboveground biomass in the second harvest as the response parameter, and the accumulated O₃ exposure from the start of the O₃ exposure until the harvest (from 47 till 85 DaE, i.e. 39 days). Considering a 10% yield loss, the exposure-based critical level for annual communities would be 3545 nl l⁻¹ h and 4249 nl l⁻¹ h for green and total aboveground biomass respectively ($y = -0.0021x + 97.44$, $R^2 = 0.94$, $p < 0.05$ for green biomass and $y = -0.0016x + 96.80$, $R^2 = 0.84$, $p < 0.05$ for total aboveground biomass). These values are slightly above the critical level of 3000 nl l⁻¹ h currently proposed for the protection of grasslands dominated by annuals species (CLRTAP, 2010). In any case, O₃ concentrations in the Iberian Peninsula where annual pastures grow frequently exceed these values (Fernández-Fernández et al., 2011). Also, critical levels are above the long-term objective of 3000 nl l⁻¹ h established by the EU Air Quality Directive for plant protection, but below the current target of 9000 nl l⁻¹ h defined by the Air Quality Directive to be met since 2010.

3.5. Conclusions

Ozone exposure induced visible injury and reduced the yield and gross primary production of a model annual community. Even ambient O₃ levels were enough to decrease aboveground biomass and NEE. Small increments of soil N availability slightly altered pasture growth but a significant interactive effect with O₃ exposure was detected. While O₃ limited the fertilization effect of higher soil N availability, higher N could compensate O₃ effects on yield only when concentrations were moderate, but not under high O₃ levels. The response of the

annual community was related to the cumulative O_3 exposure during the experiment suggesting an O_3 critical level of $4000 \text{ nl l}^{-1} \text{ h}$ to protect this vegetation type against a 10% yield loss. Since O_3 concentrations in the Mediterranean area frequently exceed that level, O_3 must be considered as a stress factor for annual pastures that can affect the yield, structure and composition of these ecosystems.

CHAPTER 4

Heterogeneous responses to ozone and nitrogen alter the species composition of Mediterranean annual pastures

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Abstract

Air pollution represents a threat to biodiversity throughout the world and particularly in the Mediterranean area, where high tropospheric ozone (O_3) concentrations and atmospheric nitrogen (N) deposition are frequently recorded. Mediterranean annual pastures are among the most important ecosystems in southern Europe due to their high biodiversity and extension. Aiming to study the responses of these communities to the main atmospheric pollutants in the Mediterranean region, an experimental study was performed in an open-top chamber (OTC) facility. A mixture of six species representative of annual pastures was grown under field conditions inside the OTC. Plants were exposed for 39 days to four O_3 treatments and three doses of N. The species responded heterogeneously to both factors. Legumes did not react to N but were very sensitive to O_3 : *Trifolium* species responded negatively, while *Ornithopus* responded positively, taking advantage of the greater sensitivity of clovers to O_3 . The grasses and the herb were more tolerant of O_3 and grasses were the most responsive to N. Significant interactions between factors indicated a loss of effectiveness of N in O_3 -polluted atmospheres and an ability of N to counterbalance the damage induced by O_3 , but both effects were dependent on O_3 and N levels. The inclusion of plant competition in the experimental design was necessary to reveal results that would otherwise be missed, such as the positive growth responses under elevated O_3 levels. Surprisingly, competition within the legume family played the most important role in the overall response of the annual community to O_3 . Both tropospheric O_3 and N deposition should be considered important drivers of the structure and biodiversity of Mediterranean annual pastures.

4.1. Introduction

Changes in community structure or species abundance caused by global change drivers are difficult to detect and reverse, despite their direct repercussions on ecosystem properties, conservation status, and ecosystem services (Zimov et al. 1995; Hooper et al. 2012). The study of regional biodiversity loss has mainly focused on land-use changes, which are currently considered the most important threat to biodiversity in Southern European countries (Cuttelod et al. 2008; Pereira et al. 2009). Nevertheless, air pollutants such as tropospheric ozone (O_3) and atmospheric nitrogen (N) deposition represent an additional risk to biodiversity throughout the world due to their effects on plant growth and ecosystem functioning. This threat is particularly important for the Mediterranean Basin, which is recognized as one of the global biodiversity hotspots for which conservation must be prioritized (Myers et al. 2000), and is where high tropospheric O_3 and moderate N deposition levels frequently occur (Dentener et al. 2006).

Tropospheric O_3 is causing damage to the growth and reproduction of vegetation, crop yields, and grassland biodiversity in many parts of the world (Royal Society 2008; Fuhrer 2009; Mills et al. 2009; Leisner and Ainsworth 2012). The effects of ozone on herbaceous species have been described, although such descriptions are based mainly on results from individual species or simple species mixtures (Hayes et al. 2007; Bassin et al. 2007a). Field experiments with complex mixtures or intact plant communities are scarce and generally show that complex plant communities are less sensitive to O_3 than expected from studies in which the component species were tested individually or in simple mixtures (Thwaites et al. 2006; Bassin et al. 2007b). Several reasons have been proposed to explain this inconsistency at the plant community level: functional redundancy, genetic variability, and species interactions (Bassin et al. 2007a). Other factors can also modify the O_3 sensitivity of an individual species when growing within a community, such as the canopy structure or the micrometeorological growing conditions, as plants of a particular species in a community may be less exposed to O_3 compared with isolated individuals of the same species (Bassin et al. 2007a). For instance, Gonzalez-Fernandez et al. (2008) described how O_3 -induced

decreases in *Trifolium repens* biomass were reduced when this species was growing in competition with the O₃-tolerant grass *Lolium perenne*, as compared with the growth reductions seen in *T. repens* monocultures.

Nitrogen is usually a limiting nutrient for primary production in a great number of ecosystems. However, excessive N input can produce a cascade of effects, including the alteration of biogeochemical processes in soil and vegetation (Galloway et al. 2008; Sutton et al. 2011; Bobbink et al. 2010). These changes have been associated with reductions in biodiversity in some herbaceous communities (Stevens et al. 2004; Dupre et al. 2010). The effects of N deposition on Mediterranean vegetation are still barely understood, and results are contradictory: some studies have indicated that N deposition increases the diversity of vascular plants (Dias et al. 2011), whereas others did not find any change (Bonanomi et al. 2009). The scarcity of empirical data has prevented the definition of experimental N deposition thresholds (critical load, CL) for the conservation of Mediterranean grasslands so far, although a provisional CL of 15–25 kg N ha⁻¹ has been proposed based on expert knowledge (Bobbink et al. 2010).

Mediterranean annual pastures are very valuable ecosystems that present high biodiversity (Peco et al. 1998) and cover large areas of Southern Europe. In the Iberian Peninsula, they mainly constitute the understory of the dehesa ecosystem, representing 7–10% of the territory (Vazquez-de-Aldana et al. 2008). Due to their importance, dehesas are currently protected under the 92/43/ EEC Habitat Directive. Efforts have been made to understand the main drivers affecting the productivity, quality, and sustainability of Mediterranean annual pastures (Lloret et al. 2004; Diaz et al. 2007; Carmona et al. 2015). Ozone has been identified as an important negative factor affecting the growth and reproductive capacity of these pastures, especially for some legume species (Sanz et al. 2007, 2014). Furthermore, the interspecific variability of O₃ sensitivity (Gimeno et al. 2004a, b) suggests that O₃ may be affecting the structure and biodiversity of these pastures. On the other hand, N enrichment can modulate the O₃ response in both directions: increasing the effects of O₃ (Sanz et al. 2014) or, more commonly, mitigating them (Sanz et al. 2007, 2015), depending on the species and parameters analyzed. However, so far, the study of the response of annual pastures to N and O₃

has been based on data from individual species or two-species mixtures. In 2011, a new open-top chamber (OTC) experiment was set up in central Spain to expose a representative mixture of annual Mediterranean species growing under close to natural field conditions to different O₃ concentrations under a range of N-supplementation regimes. A previous paper presented the effects observed at canopy scale, showing that O₃ reduced the community gross primary production (GPP) due to a global reduction in ecosystem CO₂ exchange and an increase in ecosystem respiration (Chapter 3). These effects resulted in an increase in community senescence and a decline in pasture yield of up to 25%, in contrast to previous statements for perennial species, which present a higher resilience of the community compared with the species assayed individually (Bassin et al. 2007a). In the present study, the effects of O₃ and N are presented at species level. The following hypotheses are tested: (1) ozone affects the species composition of annual Mediterranean pastures through its stronger negative effects on legume species; (2) N enrichment similar to atmospheric N deposition levels can mitigate O₃-induced effects in O₃-sensitive species; (3) species grown in the presence of competition and under field conditions are more resilient to O₃ and N than expected from species tested individually.

4.2. Materials and methods

4.2.1. Experimental site

The OTC facility was located at the La Higuera/CSIC agricultural research farm in the Santa Olalla municipality (Toledo, Spain; 450 m a.s.l., 40°3'N, 4°26'W), under continental Mediterranean climatic conditions.

In February 2011, six representative annual species were hand sown in natural soil inside the OTC at a density of 1000 seeds m⁻²: 3 legumes (*Trifolium striatum*, *Trifolium cherleri*, *Ornithopus compressus*), 2 grasses (*Briza maxima*, *Cynosurus echinatus*), and 1 herb (*Silene gallica*). The targeted proportion was 45% legumes, 45% grasses, and 10% herbs. The species were selected based on seed availability, natural abundance, germination capacity, and homogeneity, as well as their sensitivities to O₃, based on previous work (Bermejo et al. 2003 ; Gimeno et

al. 2004a). The proportion of each species in the mixture was based on seed weight but weighted according to its germination capacity (Chapter 3). A summary of the edaphic and physicochemical characteristics of the soil is provided in Chapter 3. 47 day-old plants were exposed for 39 days to four O₃ treatments, replicated in 3 OTCs: charcoal-filtered air (FA), nonfiltered air reproducing ambient levels (NFA), nonfiltered air supplemented with 20 nl l⁻¹ O₃ (NFA+), and nonfiltered air supplemented with 40 nl l⁻¹ O₃ (NFA++). Ozone exposure was expressed in terms of the accumulated AOT40 index, currently used for O₃ risk assessment. AOT40 was calculated as the sum of the hourly average of O₃ concentrations in excess of 40 nl l⁻¹ during daylight hours over the O₃-fumigation period (CLRTAP 2014). OTCs were divided into three sectors of 1.4 m² each in order to apply the three N treatments reproducing atmospheric N deposition: soil N background ("N-low"), addition of 20 kg N ha⁻¹ ("N-medium"), and 40 kg N ha⁻¹ ("N-high"). N treatment position was randomized among the blocks. Nitrogen supplementation was applied biweekly using a solution of ammonium nitrate (NH₄NO₃) in four equal applications during the experiment, starting one day after the onset of O₃ treatment. At the time of the biomass harvest, only three of them had been applied. Ozone, NO₂, and SO₂ were continuously and sequentially monitored in every OTC by means of a time-sharing system. Meteorological conditions inside the OTC were also continuously recorded. Plant-available water (PAW) was calculated as the difference in water content between field capacity and permanent wilting point. A more detailed description of the experimental field site, instrumentation, experimental treatment events, and some of the parameters such as the soil N mineral content can be consulted in Chapter 3.

4.2.2. Aboveground and subterranean biomass

Plant harvest was performed at the time of maximum green biomass, 85 days after emergence (DaE). Plants from one 5-dm² sampling ring per O₃ and N treatment were collected, sorted into the different species, and divided into aerial and subterranean biomass. Green and senescent leaves were separated to calculate the green/senescent biomass ratio. Samples were dried to a constant weight at 60 °C and weighed. Biomass parameters were expressed in terms of dry weight per

square meter (gdw m^{-2}). All plants inside the sampling ring were assessed for foliar damage (FD). A description of O_3 symptoms and their quantification, based on the intensity of the damage per leaf, is provided in Chapter 3.

4.2.3. Statistical analysis

The effects of N fertilization, O_3 exposure, and their interaction on different species and biomass parameters were analyzed via GLM analyses in which O_3 was considered the main factor in a completely randomized block design, and N the split-plot factor. The block effect was also included as a random factor. Planned (a priori) comparisons were performed to test the statistical significance of the observed trends through the levels of the factors (increase or decrease) and their interaction for the different species, considering both linear and quadratic responses. Due to the high variability of the data caused by the environmental conditions of the experiment, a p value of <0.1 was considered significant. All data were first checked for normality and homogeneity of variance; when these assumptions were not fulfilled, the data were log-transformed. Statistical analyses were performed using Statistica v.12 (StatSoft Inc., Tulsa, OK, USA). Aiming to understand the patterns of the O_3 response of the whole community, a multivariate approach was carried out, considering biomass parameters from all of the species by means of constrained ordinations (redundancy analysis, RDA, in CANOCO, version 4.5, Centre for Biometry, Wageningen, The Netherlands). The significance of the constrained axis was tested by performing Monte Carlo permutation tests. Centroid trajectories of species composition were calculated in an RDA with O_3 and N as explanatory variables to visualize changes in the studied plots, and using block as a covariable.

4.3. Results

4.3.1. Meteorological conditions and ozone exposure

During the growing cycle, watering was avoided as much as possible to allow natural plant phenology development, and was only applied to prevent seedling desiccation at early stages. The total water input was 48 l m^{-2} for the growing cycle, and precipitation amounted to 148 l m^{-2} (Figure 4.1). During the period at the maximum growth rate (from early April to late May), the mean daily temperature (8:00–17:00 h GMT) inside the chambers was 25.5°C . Ozone exposure followed the natural daily and seasonal patterns (Figure 4.2). Ozone levels expressed as AOT40 when accumulated from the start of O_3 exposure to the harvest were 3, 760, 5771, and 10316 ppb.h for the FA, NFA, NFA+, and NFA++ treatments, respectively. The 3-month AOT40 index (April to June) for the ambient level at the farm (NFA treatment) was 2798 ppb.h, close to the threshold of 3000 ppb.h indicated by the European Directive of Air Quality for the protection of vegetation (long-term objective, 2008/50/EC).

Figure 4.1 Daily mean values of air temperature ($^\circ\text{C}$) and plant-available water (%) inside the OTCs. Daily precipitation and watering (l m^{-2}) from the emergence of the pasture (25 February) until maturity (20 June). The gray arrow indicates the harvest (20 May), while small arrows indicate N-supplemented inputs (13 April, 26 April, 10 May, and 24 May). The ozone exposure period is shown in the graph from the start (11 April) until the end (30 May)

Figure 4.2 Mean daily profiles for the different ozone treatments during the experiment (April–May). FA charcoal-filtered air, NFA nonfiltered air, NFA+ nonfiltered air + 20 nl l⁻¹ of O₃, NFA++ nonfiltered air + 40 nl l⁻¹ of O₃.

4.3.2. Foliar damage

Specific O₃ symptoms were developed in the upper blades of the leaves in all of the species growing in the NFA+ and NFA++ treatments (Table 4.1). Nitrogen or the N by O₃ interaction did not have any effect on the FD. The strongest O₃-induced FD was found in *T. striatum*, which presented 56 and 86% injured leaves in the NFA+ and NFA++ treatments, respectively (mean across N treatments). *T. cherleri* presented FD percentages of 31 and 53% for these same treatments. Among the grasses, *C. echinatus* was the most sensitive, showing no significant difference between NFA+ and NFA++: in these treatments, 33–44% of the leaves were injured by O₃. *B. maxima* was more tolerant of O₃: symptoms only developed in the NFA++ treatment (in 28% of the leaves). The most tolerant species regarding FD were the legume *O. compressus* and *S. gallica* (of the Caryophyllaceae), both of which presented damage percentages of <11%.

Table 4.1 Foliar damage for the different species at 85 DaE (39 days after starting the treatments), expressed as the percentage of leaves that were injured (means across N treatments $\pm$ SE).

	<i>T. striatum</i>	<i>T. cherleri</i>	<i>O. compressus</i>	<i>B. maxima</i>	<i>C. echinatus</i>	<i>S. gallica</i>
Means (%)						
FA	0	0	0	0	0	0
NFA	0	0	0	0	0	0
NFA+	55.6 $\pm$ 5.6	30.6 $\pm$ 2.8	5.6 $\pm$ 5.0	5.6 $\pm$ 3.7	33.3 $\pm$ 8.2	8.3 $\pm$ 5.2
NFA++	86.1 $\pm$ 5.6	52.8 $\pm$ 2.8	5.6 $\pm$ 5.0	27.8 $\pm$ 3.7	44.4 $\pm$ 8.2	11.1 $\pm$ 5.2
p-values						
O ₃	0.005	0.000	1.000	0.003	0.367	0.715
N	0.412	0.412	0.644	0.531	0.574	1.000
N x O ₃	0.412	0.412	0.644	0.531	1.000	1.000

Statistical differences between NFA+ and NFA++ are presented in the lower part of the table; significant differences for $p < 0.1$ are marked in bold FA charcoal-filtered air, NFA nonfiltered air, NFA+ nonfiltered air + 20 nl l⁻¹ of O₃, NFA++ nonfiltered air + 40 nl l⁻¹ of O₃.

4.3.3. Biomass parameters

In accord with the FD results, not all of the legume species showed the same O₃ sensitivity (Table 4.2). *Trifolium striatum* was also the most sensitive to O₃ regarding biomass parameters. Increasing the pollutant levels linearly decreased the green biomass by 38, 26, and 45% for the NFA, NFA+, and NFA++ treatments, respectively, compared with the FA treatment (mean across N treatments). The effect was similar when considering the total aerial biomass (losses of 37, 22, and 42%) (Figure 4.3a). Foliar senescence was strongly enhanced by the pollutant it exhibited a significant linear pattern. Accordingly, the senescent/green ratio was trebled in the NFA+ and NFA++ treatments compared with the control (Figure 4.3c). Significant negative effects of O₃ were also found for the root biomass of this clover, with almost the same response observed for it as for the aerial biomass: reductions of 38, 26, and 43% in the NFA, NFA+, and NFA++ treatments, respectively (Figure 4.3b). Moreover, a significant O₃ by N interaction was found for root biomass, indicating a loss of N efficiency with increasing O₃, and even an enhancement in O₃-induced root reduction in the NFA++ treatment (Figure 4.4a). *Trifolium cherleri* was the second most O₃-sensitive species. We measured

reductions in green biomass of 12 and 29% for the NFA+ and NFA++ treatments, respectively, but the high variability of the data did not allow us to find a statistically significant response. However, when the green biomass was expressed as the dry weight per plant (gdw plant⁻¹), the pollutant was found to linearly decrease the aerial biomass of this species ($p = 0.06$, data not shown). Ozone also enhanced leaf senescence and the senescent/green ratio (Figure 4.3c) of this clover, with the same intensity and pattern attained as seen for *T. striatum*. The senescent biomass of *T. cherleri* was the only parameter of the legume species that responded to N, albeit with a quadratic pattern: the maximum increase was observed for the N-medium dose, not for the N-high dose. This is in agreement with the direction of the O₃ by N interaction found for the root biomass of *T. striatum*. However, the variability in the data led to a p value of 0.101 for the O₃ and N interaction. The senescent/green biomass ratio was not unbalanced by the fertilizer.

Figure 4.3 Effects of ozone on biomass parameters of *T. striatum*, *T. cherleri*, *O. compressus*, *B. maxima*, *C. echinatus*, and *S. gallica* for the different O₃ treatments (mean $\pm$ SE): **a** total aerial biomass, **b** root biomass, **c** senescent/green biomass ratio. FA charcoal-filtered air, NFA nonfiltered air, NFA+ nonfiltered air + 20 nl l⁻¹ of O₃, NFA++ nonfiltered air + 40 nl l⁻¹ of O₃

Table 4.2 Statistical results for growth-related parameters: *p* values for the planned comparisons to test the statistical significance of the observed trend through the levels of the factors (increase or reduction) and their interaction for the different species, considering both linear and quadratic responses.

Species	Factor	Green (g dw m ⁻²)		Senescent (g dw m ⁻²)		Total aerial (g dw m ⁻²)		Root (g dw m ⁻²)		Senescent/Green ratio	
		Linear	Quadratic	Linear	Quadratic	Linear	Quadratic	Linear	Quadratic	Linear	Quadratic
<i>T. cherleri</i>	N	0.532	0.396	0.476	0.059	0.508	0.321	0.317	0.914	0.706	0.173
	O ₃	0.129	0.678	0.001	0.797	0.272	0.674	0.848	0.639	0.000	0.960
	N x O ₃	0.101		0.725		0.830		0.985		0.789	
<i>T. striatum</i>	N	0.483	0.928	0.484	0.612	0.576	0.988	0.886	0.636	0.138	0.852
	O ₃	0.001	0.158	0.080	0.253	0.003	0.240	0.081	0.471	0.002	0.356
	N x O ₃	0.336		0.622		0.337		0.027		0.881	
<i>O. compressus</i>	N	0.608	0.394	0.210	0.464	0.751	0.376	0.740	0.238	0.285	0.471
	O ₃	0.149	0.063	0.064	0.817	0.116	0.076	0.102	0.042	0.114	0.722
	N x O ₃	0.112		0.904		0.140		0.199		0.266	
<i>C. echinatus</i>	N	0.102	0.809	0.626	0.804	0.158	0.795	0.861	0.563	0.015	0.587
	O ₃	0.118	0.617	0.936	0.737	0.215	0.775	0.282	0.572	0.317	0.349
	N x O ₃	0.126		0.329		0.143		0.319		0.587	
<i>B. maxima</i>	N	0.945	0.078	0.640	0.109	0.900	0.070	0.478	0.046	0.428	0.736
	O ₃	0.253	0.310	0.600	0.043	0.266	0.230	0.398	0.279	0.169	0.097
	N x O ₃	0.763		0.030		0.972		0.706		0.035	
<i>S. gallica</i>	N	0.144	0.316	0.462	0.600	0.185	0.314	0.851	0.338	0.550	0.480
	O ₃	0.172	0.259	0.097	0.013	0.255	0.419	0.516	0.305	0.020	0.030
	N x O ₃	0.942		0.186		0.835		0.531		0.190	

Significant differences for *p* < 0.1 are marked in bold

Figure 4.4 Significant effects of the $O_3 \times N$ interaction (mean $\pm$ SE): **a** root biomass of *T. striatum*, **b** senescent biomass of *B. maxima*, **c** senescent/green rate of *B. maxima*. N-low soil N background, N-medium +20 kg N ha⁻¹, N-high +40 kg N ha⁻¹.

No negative effects of O₃ were detected on any of the biomass parameters for the legume *O. compressus*, but a general positive stimulus was induced by the pollutant. Increased levels of O₃ caused >50% enhancement of the green and total aboveground biomass compared to the control treatment (Table 4.2; Figure 4.3a). When the increase was related to clover species through the *O. compressus*/clover biomass ratio, this rate increased by more than 100% in all O₃ treatments ($p = 0.05$). The *O. compressus*/nonlegume species ratio also increased with O₃ exposure level on the subterranean biomass of *O. compressus* was also evident (Figure 4.3b). The root biomass increased by 100% in the NFA++ compared with the FA treatment. The pollutant also enhanced the foliar senescence of this legume, but without significantly affecting the senescence/green rate. In accord with the other clovers, N fertilization did not have any effect, and no significant interaction between N and O₃ was found for this species.

The effect of O₃ on *S. gallica* was related to the senescence parameters. The senescent biomass and senescent/green ratio of this member of the Caryophyllaceae showed significant responses to the pollutant, although these responses presented a notable quadratic component (Table 4.2; Figure 4.3c): medium O₃ levels resulted in a reduction in leaf senescence. The biomass of *S. gallica* tended to increase with increasing N level, but this effect was not statistically significant. The interaction between N and O₃ did not alter any biomass parameter.

Grasses were in general less sensitive to O₃ than legumes, but they were more responsive to N. Although the response pattern of *C. echinatus* indicated that aerial biomass reduced with increasing O₃, none of the biomass parameters showed significant responses to this pollutant (Table 4.2; Figure 4.3). However, this species was the most responsive to N. Fertilizer increased the green biomass by 25 and 40% in the N-medium and N-high treatments, respectively, compared with N-low (the mean values for the O₃ treatments were compared), without affecting the senescent biomass. Thus, the senescence/green biomass ratio was reduced by 25 and 37% in the N-medium and N-high treatments, respectively. *Briza maxima* was slightly more responsive to O₃ regarding senescence-related parameters. The response followed a similar pattern to that of *S. gallica*, with the

pattern showing a significant quadratic component: medium O₃ levels (the NFA and NFA+ treatments) presented a reduction in senescent biomass compared with the FA treatment, but the higher levels of O₃ in the NFA++ treatment increased senescent biomass by around 30%. This is related to the significant O₃ by N interaction found for this parameter (Figure 4.4b, c), similar to that found for clover root. N-medium treatment reduced the O₃-induced increase in the senescent/green ratio obtained with the NFA+ treatment, and N-high treatment compensated for the increase in this ratio in the NFA++ treatment, although the presence of the pollutant reduced the effectiveness of N supplementation.

4.3.4. Multivariate analysis

In order to capture the general patterns of response to O₃ and N, all species were examined collectively in RDA multivariate analyses (Table 4.3). The statistical model was significant ($p = 0.062$) for green biomass, with a significant effect of the factor O₃ ($p = 0.007$), which explained 11% of the variability found for the first axis. The adjusted variance for each species was: *T. striatum* -69%, *T. cherleri* -32%, *S. gallica* -13%, *B. maxima* -10%, *C. echinatus* -8%, and *O. compressus* +21%. Thus, the species for which green biomass was most affected by O₃ were the two clover species (*T. striatum* and *T. cherleri*), which were negatively associated with O₃ (Figure 4.5), whereas the association of green biomass with O₃ became less strong for the grasses and the herb species in the following order (due to their lower O₃ sensitivities): *S. gallica* > *B. maxima* > *C. echinatus*. *Ornithopus compressus* was the only species that was positively associated with O₃ and thus showed a positive value of adjusted variance, indicating that the presence of the pollutant was relatively advantageous to this species. Although the factor N did not have a significant effect, the RDA pattern was in accord with the results found for the species when they were analyzed individually. The grass *C. echinatus* was the species that was most responsive to increased N. In terms of senescent biomass, *C. echinatus* was the most responsive to increased N, followed by *B. maxima* and *S. gallica*, while the senescent biomass of the legumes was associated with the factor O₃. The N by O₃ interaction was not significant in the model.

Figure 4.5 Trajectories of the centroids of species composition considering the factors O_3 and N , corresponding to the redundancy analysis (RDA) for **a** green biomass, **b** senescent biomass, and **c** total aerial biomass. Biplot with explanatory variables (O_3 , N , and $O_3 \times N$) for the six species studied is shown.

Table 4.3 Effects of O₃, N, and the O₃ × N interaction in constrained ordinations (redundancy analysis; RDA) for green, senescent, and total aboveground biomass: percentage of variability explained by each variable (variability), *f* ratio, and *p* value obtained using a Monte Carlo test with 999 random permutations, as well as the total variation explained by the model (TVE).

Forward stepwise RDA	Explanatory variable	Covariable	Monte Carlo test		
			Variability (%)	<i>f</i>	<i>p</i>
Green biomass					
Step 1	O ₃	Block	11.1	4.46	0.007
Step 2	N	Block	2.3	0.43	0.786
Step 3	O ₃ x N	Block	0.4	0.69	0.573
Reduced RDA model	$\lambda_{\text{axis 1}}$	$\lambda_{\text{axis 2}}$	TVE	<i>f</i>	<i>p</i>
	11.1	2.3	13.8	1.815	0.062
Senescence biomass					
Step 1	O ₃	Block	10.6	3.57	0.012
Step 2	N	Block	1.2	0.51	0.734
Step 3	O ₃ x N	Block	0.3	0.42	0.803
Reduced RDA model	$\lambda_{\text{axis 1}}$	$\lambda_{\text{axis 2}}$	TVE	<i>f</i>	<i>p</i>
	10.6	1.2	12.1	1.45	0.152
Total aboveground biomass					
Step 1	O ₃	Block	8.4	3.28	0.027
Step 2	N	Block	1.9	0.35	0.868
Step 3	O ₃ x N	Block	0.4	0.60	0.646
Reduced RDA model	$\lambda_{\text{axis 1}}$	$\lambda_{\text{axis 2}}$	TVE	<i>f</i>	<i>p</i>
	8.4	1.9	10.8	1.32	0.180

Significant differences for *p* < 0.1 are marked in bold

4.4. Discussion

The potential impacts of increased levels of tropospheric O₃ and atmospheric N deposition on Mediterranean annual pasture species have been described based on previous studies of plants growing individually in well-irrigated pots (Sanz et al. 2005, 2007, 2013). However, these experimental designs make it difficult to assess the effects of O₃ at community level, especially due to the absence of plant–plant interactions. The present study is the first attempt to assay the response to O₃ and N of an annual Mediterranean community growing under close to natural conditions.

Ozone exposure and N fertilization levels of the experiment were within the range found in the field. Three-month AOT40 values in the range 10000–20000 ppb.h are common in the central areas of the Iberian Peninsula, where annual dehesa pastures grow (Notario et al. 2012; Fernandez-Fernandez et al. 2011; Monteiro et al. 2012), and are in the same range as the 39-day AOT40 registered for the NFA+ and NFA++ treatments during the exposure period. Reported atmospheric N deposition levels in Spain of up to 25 kg N ha⁻¹ year⁻¹ (Garcia-Gomez et al. 2014) are also in the range of the N added in this experiment. Besides, maximum growth and phenological events coincided with the development of natural pastures in the area (Chapter 3). Thus, the experiment represents the growing conditions and present pollution climate of the annual dehesa pastures reasonably well.

4.4.1. Response patterns of the species

Modeling the responses of complex plant communities such as grasslands to future scenarios of global climate change requires the detection of general response patterns. This is essential if we are to deal properly with the complexity of these communities. However, finding these general patterns requires the analysis of multiple independent community experiments, which are scarce, preventing any advances in this direction. The annual pasture experiment reported here represents a contribution to this important issue.

In the present study, we clearly established—by analyzing individual species and by performing multivariate RDA—that annual species present heterogeneous responses to O₃ and N, which alter the competitive relationships among component species and thus the structural composition of the community. Although both clovers showed similar response patterns, the response patterns observed within the legume family were surprisingly varied.

The clover species were the most sensitive to O₃, showing a negative linear response to it, but they did not present any significant response to N. Their well-developed specific FD and O₃-induced effects on yield parameters were the highest amongst the species tested. Up to 35% reductions in biomass were observed under

above-ambient O₃ concentrations. When both clovers were analyzed together (data not shown), the individual response pattern was strengthened, leading to a robust linear pattern where yield losses increased with O₃ exposure. Both clovers also presented an O₃-induced increase in senescent biomass, which is a common response of O₃-sensitive species (Franzaring et al. 2000; Booker et al. 2009), including annuals (Bermejo et al. 2003; Sanz et al. 2007, 2014). Therefore, regarding FD and yield, a high sensitivity of annual clover species to O₃ was apparent when they were grown in a community and under field conditions.

The non-clover legume *O. compressus* presented a unique response pattern. This species showed no response to N, in accord with the other legumes, but also minor FD and significant increases in aerial and root biomass with increasing O₃. This pattern contrasts with those of the clovers, even though all of these species belong to the same group. The quadratic component of the responses to O₃ indicates that medium O₃ levels caused similar effects to the highest levels, pointing to a high positive O₃ sensitivity. Preceding studies in which annual species were grown individually also noted that some legume genera are tolerant to O₃, including *Ornithopus* and *Anthyllis* (Gimeno et al. 2004a), but a positive response to this pollutant has not been reported before. In the present experiment, *O. compressus* may have benefited from not only its O₃ tolerance but also from decreased competition for light, water, space, and nutrients, mainly at the expense of O₃-sensitive clover species. Ozone-induced changes in plant–plant relationships have been found in other grassland community studies, and have usually been assigned to the greater sensitivity of legumes compared to grasses (Davison and Barnes 1998; Hayes et al. 2009; Wedlich et al. 2012). In the present work, the interesting legume–legume competition described above played an important role in the response of the annual pasture to O₃, highlighting the importance of considering plant–plant interactions when attempting to gain a true understanding of community-scale responses to atmospheric pollutants.

The grasses *B. maxima* and *C. echinatus* and the herb *S. gallica* can be considered more tolerant of O₃ than clovers. Ozone-specific symptoms appeared in the aboveambient O₃ treatments but at a lower intensity than seen for the clovers. Although the pollutant reduced the green and total aerial biomass in all of these

species to some extent, this effect was not statistically significant (due also to the high variability of the field data). However, all these species except for *C. echinatus* presented a noticeable response to O_3 regarding foliar senescence. Other studies focusing on individual species or simple mixtures have also indicated that biomass-related parameters of the annual grasses are relatively tolerant of O_3 (Gimeno et al. 2004a; Sanz et al. 2011). These three species, in contrast to legumes, responded positively to the fertilizer, although the effect was not significant for *S. gallica*, and the response of *B. maxima* was masked by the effect of the interaction between O_3 and N, causing a quadratic pattern in the response of this species to N. When all three nonlegume species were analyzed together (data not shown), the response pattern was strengthened: the O_3 -induced reduction in aerial biomass became significant (although it was less intense than seen for the clover group), as did the N-induced increase. Moreover, the senescent biomass response of the group was driven by the interaction of O_3 with N: O_3 reduced the fertilization effect of N, while increasing levels of N mitigated the negative effect of the highest O_3 treatment.

4.4.2. General ozone response

The heterogeneous responses of the different species found in the present study show that O_3 has the ability to alter the competitive relationships among species, thus affecting the structure of Mediterranean annual pastures. Moreover, the effects of O_3 on biomass and species composition have consequences for livestock exploitation of the pasture, an important ecosystem service provided by grasslands. The reduction of biomass production together with the increase in senescent biomass and the alteration of the clover fraction, which is the major source of protein in Mediterranean pastures (Vazquez-de-Aldana et al. 2008), directly lead to reductions in pasture quality and palatability for herbivore animals. Considering yield quality when analyzing the effects of O_3 substantially increases the impacts of this pollutant (Krupa et al. 2004).

RDA analysis showed that O_3 was the most significant factor, explaining around 10% of the variance in the data of this experiment. This is an important

value considering the high number of environmental variables involved in experiments performed under field conditions. By contrast, field studies with other grassland communities found that O_3 explained only around 5% of the variability (Thwaites et al. 2006). This high variability in the response of individuals of the same species prevented the statistical tests from finding a greater number of significant effects, and is consistent with the high phenotypic plasticity that annual species require to endure the variable Mediterranean climatic conditions (Peco et al. 1998). A high O_3 sensitivity of annual Mediterranean pasture species was suggested based on a meta-analysis of 83 European grassland species (Hayes et al. 2007), which concluded that Mediterranean therophytes were the most O_3 -sensitive life forms based on the effects on the aboveground biomass of individual species growing in pots. The high O_3 sensitivity of annual species might be related to their high growth rates during their short life spans. Indeed, a high growth rate has been considered an important trait that is linked with O_3 sensitivity (Bassin et al. 2007b). Besides, annual pastures experience rapid growth in spring, coinciding with high levels of O_3 in the Iberian Peninsula (Notario et al. 2012) under nonlimiting soil water availability, allowing the absorption of high O_3 fluxes during a short period of time (Alonso et al. 2007; Gonzalez-Fernandez et al. 2010b).

The present study in which species were grown in a field-based community confirmed previous assessments based on pot experiments. Biomass losses of the O_3 -sensitive species in the present experiment were within a similar range to those reported for the species when they were assayed without interspecific competition (Gimeno et al. 2004a; Sanz et al. 2007, 2014). These findings do not support the third hypothesis considered, as species growing in a community did not present increased resilience to O_3 compared with the species tested individually. Neither did the whole community respond in this way, considering the total yield (Chapter 3). These results contrast with the responses observed in other well-established perennial grasslands (Thwaites et al. 2006; Bassin et al. 2007b). The differences between the behaviors of the annual and perennial communities might be related to the lack of a stable maturity stage in the annuals, which start to senesce immediately after reaching maximum growth development.

4.4.3. General nitrogen response

The species assayed also responded heterogeneously to the supplemented N inputs, although the effects of N were less intense than those induced by O₃. Grass species were the most responsive to the fertilizer. In agreement with this result, previous studies with herbaceous species showed that N supplementation can give a competitive advantage to non-N-fixing species (Ledgard and Steele 1992; Vallano et al. 2012). Tilman (1993) suggested that, under N-limited conditions, N deposition would give a competitive advantage to fast-growing species, thus explaining the differences in the response. However, the species tested in this experiment showed similar growth rates (Gimeno et al. 2004a) but responded in different ways to N addition. The low N response of the legume species observed in this experiment contrasts with previous results where plants growing in pots showed greater effects under similar levels of added N (Sanz et al. 2005, 2007, 2014). The background soil N content (33 kg N_{min} ha⁻¹; Chapter 3) and the ability of legumes to fix N could provide enough N to cover their nutritional demands, this leading to a lack of response of these plants to N supplementation. Legumes are usually reduced in fertilized plant communities, especially in the presence of grasses (Dupre et al. 2010), so interspecific competition might also have played an important role in this lack of response to N. This behavior contrasts with results from a highly diverse subalpine perennial grassland community that showed an intense response to N but a lack of response to O₃ after 5 years of treatment (Volk et al. 2011). Nevertheless, large differences in response are expected between these communities due to the different growth rates and life forms of subalpine perennial grasslands as compared to Mediterranean annual pastures.

4.4.4. Effects of the interaction of ozone and nitrogen

Information on the interactive effects of atmospheric pollutants on vegetation is scarce. Some studies have shown that N can exacerbate O₃-induced effects, such as the enhanced reduction of belowground carbon allocation detected in *Dactylis glomerata* (Wyness et al. 2011). In annual species grown in pots without

interspecific competition, species- and parameter-specific responses to the interaction between N and O₃ were noted. Intensification of the effects of O₃ on the nutritive quality of *T. subterraneum* (Sanz et al. 2005) and the leaf senescence of *T. cherleri* (Sanz et al. 2014) were observed upon N addition. This phenomenon contrasts with the ability of N to mitigate the effects of O₃ on the biomass and seed production of *T. striatum* (Sanz et al. 2007) or the leaf senescence of *T. subterraneum* (Sanz et al. 2005). In the present experiment, N addition partially mitigated the O₃-induced reduction of biomass seen at the community level in the NFA+ but not the NFA++ treatment (Chapter 3). However, heterogeneous responses were found when data were examined at the species level. The pattern found for the senescence-related parameters of *B. maxima* pointed to a loss of N efficiency under above-ambient O₃ concentrations and to an ability of N to counterbalance the effects of O₃ at medium levels of nitrogen. This pattern agrees with previous works using pot-based experimental designs (Bermejo et al. 2003; Sanz et al. 2011). On the other hand, the effect of the interaction of O₃ and N noted for the clover *T. striatum* showed a slightly different nonlinear response pattern: a medium N input mitigated the negative effects of O₃ on biomass, while a high N input intensified them. These results indicate that the effects of the interaction of O₃ and N depend not only on O₃ exposure but also on the N input level. Therefore, the second hypothesis tested—that atmospheric N deposition can mitigate O₃-induced effects—cannot be considered a general response, with such mitigation depending on the levels of O₃ and N input.

Annual Mediterranean pastures are characterized by their high species diversity and complex population dynamics (Peco et al. 2014; Pineda and Montalvo 1995). This high biodiversity is linked to the complementarity in resource use efficiency among the species coexisting in the community, which reduces competition among neighbors and facilitates species coexistence (Cleland et al. 2006; Gross et al. 2007). According to the present study, O₃ and N are factors that affect this complementarity, changing the competitive relationships among species through their heterogeneous responses to O₃ and N. The chronic exposure to current O₃ concentrations and N deposition levels occurring in the Iberian Peninsula may already be driving changes to the structure and composition of

Mediterranean annual pastures. Further experimental effort is needed to find general response patterns of Mediterranean annual pastures to air pollution.

4.5. Conclusions

Annual species responded heterogeneously to increasing levels of O₃ and N input. Grasses and the herb were more tolerant of O₃, but grasses were the most responsive to N. Legumes did not respond to N but were very sensitive to O₃, although contrasting responses were seen within the family: *Trifolium* species responded negatively, leading to reduced biomass, while *O. compressus* increased its biomass by taking advantage of the sensitivity of clovers to O₃. Therefore, competition within the legume family played the most important role in O₃-induced shifts in the annual community composition. Significant effects of O₃ by N interactions were found for some species and parameters, but the combined effect depended on the levels of O₃ and N. The general pattern indicated that O₃ limited the effects of N while N partially counterbalanced the effects of O₃. However, the direction of the interaction can reverse if N is too high, as found for *T. striatum* roots at the highest O₃ levels. The resilience of the species growing under plant competition to N increased when compared with monoculture-based experiments, but this was not the case for O₃.

Some of the observed effects occurred under chronic O₃ and N levels, as found within the natural distribution of the annual pastures in the Iberian Peninsula. Therefore, both O₃ and N enrichment should be considered important drivers of alterations to the structure and species composition of Mediterranean annual pastures via changes in the competitive relationships among species.

CHAPTER 5

**Nitrogen and ozone impacts on carbon and
Nitrogen allocation and on nutritive quality of
Briza maxima and *Trifolium striatum***

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Abstract

Two annual species, *Briza maxima* and *Trifolium striatum*, representative of the legume and grass families of the Mediterranean pastures, were selected to assay the effects of increased ozone (O_3) levels and nitrogen (N) atmospheric deposition on the carbon (C) and nitrogen (N) allocation within plant parts, and whether these effects can be related to the observed changes in species composition and forage quality. The experiment was conducted in an OTC facility located in the Mediterranean continental climate characteristic of the natural distribution area of the pasture community. Four O_3 treatments (filtered air (FA), non-filtered air (NFA) reproducing ambient levels and NFA supplemented with 20 and 40 $nl\ l^{-1}\ O_3$) and three N-treatments (background, 20 and 40 $kg\ N\ ha^{-1}$) were considered. The assay under field conditions considered a mixture of 6 annual species directly sown in the soil. Carbon and N concentrations within plant organs and nutritive quality analysis of aerial organs were analyzed for the selected species.

Ozone induced an increase in the N-content of the aerial organs for both species, and also in the belowground organs for the grass, reducing the corresponding C/N ratios. N-inputs also affected the N-content in the same direction as O_3 . However, the O_3 effect on the grass was modulated by N availability, because O_3 -induced increment in the N-content was observed only for N-inputs over the background levels. This interaction also implied a loss of N-use efficiency under increasing O_3 exposure. Although coincidental in the direction, the different intensity in the crude protein (CP) responses to O_3 and N between species caused an imbalance in the CP distribution within the ecosystem. Increasing N-inputs favored the accumulation of CP in the grass fraction. Increasing O_3 levels changed the CP balance, following the more complex pattern of the O_3 by N interaction of the grass. The alterations of the Grass/Clover CP ratio can be related to the observed changes in the pasture species structure based on biomass effects.

Ozone exposure increased the lignin content in aerial organs of the clover, which is negatively related to forage digestibility; but N improved the quality of the clover through reductions in leaf fiber content and increase in relative feed value (RFV). The grass quality parameters only showed subtle responses to the treatments. The implications of the observed effects in the nutritional quality of

the pasture need further research, taking also into account forage quality changes through plant phenology, environmental growing conditions and species composition.

5.1. Introduction

Due to the capacity of terrestrial vegetation to both assimilate and release carbon and water, understanding the influence of global change on vegetation has been a key issue in the last decade. As a result, a great number of studies analyzing the effects on carbon allocation to increased atmospheric CO₂ (Franks et al., 2013), drought (Allen et al., 2010) and temperature (Rustad et al., 2001) have been conducted in order to determine the impacts and feedbacks between vegetation and global change drivers. As global change drivers do not occur independently, and responses to individual factors frequently have opposite directions and interactive effects (Way et al., 2013; Sevanto et al., 2015), the combined effects of two or more factors are required for modelling adequately future vegetation changes.

Atmospheric pollutants are main factors of global change that can cause alterations in plant physiology and growth, affecting processes at local, regional and/or global scale (Grimm et al., 2008). Plants, due to their physiology and continuous gas exchange with the atmosphere, are one of the most sensitive organisms to air pollutants; particularly to tropospheric ozone (O₃) and nitrogen (N) deposition. These two are considered the most harmful atmospheric pollutants on vegetation (Ashmore, 2005). The Mediterranean basin is chronically affected by the highest O₃ concentrations in Europe (EEA, 2014) due to the intense atmospheric photochemistry, caused by the high precursor emission rates and the favorable meteorological conditions of the area (Millán et al., 1997; Cristofanelli and Bonasoni, 2009). At the same time, the Mediterranean area is considered one of the world regions threatened by N-deposition. Although it cannot be considered as a high N-deposition region, the high sensitivity of their valuable ecosystems allows this classification (Sala et al., 2000; Ochoa-Hueso et al., 2011).

The main reported O₃ effects on herbaceous species include parameters related with plant production like biomass reduction (Gimeno et al., 2004a; Sanz et al., 2005, Hayes et al., 2007, Chapter 3), senescence acceleration (Dawnay and Mills, 2009; Sanz et al., 2015) or alteration of carbon allocation (Vollsnes et al., 2010), showing a great range of species sensitivity. These heterogeneous responses to the pollutant among species competing for space and resources can lead to changes in the structure and composition of the grassland communities (Hayes et al., 2009; Chapter 4).

Mediterranean communities dominated by annuals are considered among the most sensitive to O₃ (Hayes et al., 2007), but a wide range of susceptibilities have also been found among their component species. Assays with monocultures showed that annual clovers are among the most O₃-sensitive species, whereas the annual grass species can be considered tolerant to O₃ (Bermejo et al., 2003; Gimeno et al., 2004b). The pollutant can reduce the biomass production of the annual clovers *Trifolium subterraneum*, *T. cherleri* and *T. striatum*, and even more important for the annuals, O₃ can reduce their reproductive capacity (Sanz et al., 2005, 2016). *Briza maxima*, a representative annual grass of these pastures, has been considered a relatively tolerant species compared with the clovers. No biomass loss has been detected under above-ambient O₃ exposures (Gimeno et al., 2004a). However, the pollutant can increase the leaf senescence and shorten the life span of this species (Sanz et al., 2011). The O₃-sensitivity of the species is consistent when results are compared between monoculture experiments and community scale experiments (Chapter 4).

These O₃-induced effects might also affect other main ecosystem services related to forage yield and nutritive quality for livestock farming, either directly via grazing or through conserved-forage (e.g., hay or silage) production. In spite of their importance, these quality effects are much less studied and have been reported for a few forage plants and plant communities (Muntifering et al., 2000; Powell et al., 2003; Hayes et al., 2016).

The nutritive quality of the forage is determined by a combination of several factors including the content of desirable nutrients such as proteins, which constitute the cell solubles and are hydrolyzed during the digestive process. The

content of fibers is the main constituent of the cell walls that can limit the digestive process. The proportion of fiber and protein in the forage has been frequently used for determining the forage quality. One of the measures that integrate the nutrient content and digestibility aspects of forage quality is the Relative Feed Value (RFV) (Rohweder et al., 1978).

The exposure to increased O₃ levels can directly influence the nutritive quality of herbaceous vegetation as a result of altered concentrations of minerals, protein, readily available carbohydrates and cell-wall constituents (cellulose, hemicellulose and lignin) (Krupa et al., 2004). The enhancement of the lignin biosynthesis due to the effects of O₃ on the cell secondary metabolism can derive in alterations in cell walls, loss of turgor and elasticity as shown with chronic sulfur dioxide exposures (Mayo et al., 1992). Such changes in the physical properties of the leaves can affect the tissue digestibility. The increase of the lignin and fiber content seems also to be a consistent O₃-response of annual species when assayed in monoculture experiments for both clovers and grasses (Sanz et al., 2011, 2014). Interestingly, these effects can occur without significant reductions on the species yield, like in *B. maxima* and other perennials grasses like *Lolium perenne* (González-Fernández et al., 2008; Sanz et al., 2011). These results highlight the high O₃-sensitivity of forage quality parameters.

Other response to O₃ regarding quality parameters is the reduction in the foliage protein content, which has frequently been reported in crops (Lechowicz, 1987, Mulchi et al., 1992). However, the experiments with individual annual species do not always follow this pattern, and different responses between clovers and grasses have been found: although O₃ increased the fiber and lignin content in clover species, the pollutant also increased the foliage protein content rather than decreasing it (Sanz et al., 2005, 2014). In the opposite direction, the response pattern of the grass *B. maxima* was decreased leaf Crude Protein (CP) concentration under O₃ exposure (Sanz et al., 2011).

Blaser et al. (1986) displayed a conceptual model where the quality of the pasture, based on the relative lignin and protein content of legumes and grasses, changed through the different development stages of the pasture. This model has been modified adding the expected response of annual legumes and grasses to O₃

according to the literature (Figure 5.1): In the figure model, the pollutant enhanced the natural evolution of fiber compounds for clovers, intensifying the age-induced increase in lignin content, but also ameliorating the protein loss caused by the aging; meanwhile for grasses, it would increase foliage lignin while intensifying the natural decrease in protein.

Figure 5.1. Figure model of the forage quality through the different stages of the pasture development (adapted from Blaser et al., 1986) modified by ozone for annual species.

Anthropogenic N emissions have increased dramatically in parallel with rising tropospheric O₃ concentrations over the second half of the last century (Sutton et al., 2011). As a result, the global N-cycle has been altered, inducing a cascade of environmental effects including direct toxicity, ecosystem acidification and eutrophication, and loss of biodiversity (Galloway et al., 2008; Bobbink et al., 2010).

Nitrogen deposition had resulted in widespread loss of species richness in heathland, acid, calcareous and mesotrophic grasslands in Central Europe (Maskell et al., 2010; Stevens et al., 2010; Bobbink et al., 2010). Specialist species of grassland communities that are usually tolerant to grazing and low nutrient availability are frequently unable to respond to additional nutrient inputs. Thus, in

regions impacted by N-deposition, specialist species cannot compete with nutrient-demanding species (Makel et al., 2010). Current studies have not reported consistent responses of Mediterranean pastures to N-deposition, which is probably due to its characteristic high spatial and temporal heterogeneity in terms of plant cover and soil resources (Ochoa-Hueso et al., 2011).

Despite N-induced effects on ecosystem biodiversity loss, increased N-inputs in grasslands usually result in greater dry matter production (Gough et al., 2000). Furthermore, N has been shown to increase the foliar protein content in some annual clover species (*T. subterraneum* and *T. cherleri*) (Sanz et al., 2005, 2014). These effects, increasing biomass yield and foliar protein content, might seem favorable from the point of view of cattle feeding. However, the growth stimulation is mostly observed in the grass fraction of the pasture, due to the lesser response of legumes to N addition. Because legumes constitute the main contributor to the sward protein content (Collins, 1988). N-deposition can decrease the legume ratio in the botanical composition of the sward, reducing the forage protein and therefore the pasture quality for cattle and herbivorous feeding (Aydin and Uzun, 2005).

In recent years, research interests have been focused on the combined effects of O₃ and N-deposition on vegetation, especially for grassland ecosystems, but the knowledge about the interactive effects of the pollutants is still scarce regarding biomass parameters, the most frequent interactive pattern indicates that N can ameliorate the negative impacts of O₃ on yield (Mills et al., 2009). This same pattern has been reported for the biomass response of *T. striatum*, at least when O₃ levels were moderated (Sanz et al., 2007); and for *B. maxima* regarding the O₃-induced increase in senescence (Sanz et al., 2011).

At community scale, the pasture yield also followed this pattern of response to the combination of O₃ and N (Chapter 3). However, this response was the result of the different behavior of the component species to both factors: clovers species were more responsive to O₃, whereas grasses showed a greater effect from N (Chapter 4). Understanding the inter- and intra-specific imbalances of N and C concentration and partitioning is key for comprehending the mechanisms

underlying changes in species competitiveness and pasture community structure caused by atmospheric pollutants and its repercussion to forage quality.

An experiment considering a simplified annual community was developed for studying the effects of O₃ and N on annual Mediterranean pastures (Chapter 3 and 4). Two species in the experiment, the legume *Trifolium striatum* and the grass *Briza maxima*, were selected as representative of the legume and grass families, the dominant families in the annual pastures (Peco et al., 1998). Carbon and N concentrations in the tissues of these species were measured in order to determine if O₃ and N can perturb the balance of C and N concentrations within plant organs and between species, as the underlying mechanisms related with the observed changes observed in species structure and on forage quality.

The objectives of the present study were: 1- Determine if O₃ and N can perturb the balance of C and N-allocation within the plant organs of both species; 2-Determine if the individual responses of the species can perturb the balance of inter-specific C and N in the system; 3- Determine the O₃ and N effects on the forage quality of the pasture; 4- Determine if the heterogeneous responses of the species regarding C and N allocation can be related to the observed changes in species composition and forage quality.

5.2. Materials and methods

5.2.1. Plant culture, nitrogen and ozone treatments

The experiment was conducted in an OTC field site located at the agricultural research farm “La Higuera/CSIC” in Santa Olalla municipality (Toledo, Spain; 450 m a.s.l., 40°3’N, 4°26’W); a site under continental Mediterranean climatic conditions. Twelve Open Top Chambers (OTC), were placed on a calcic luvisol soil, the natural soil of the farm.

In winter of 2011, a simplified pasture community formed by 6 representative annual species (3 legumes (*Trifolium striatum*, *Trifolium cherleri*, *Ornithopus compressus*), 2 grasses (*Briza maxima*, *Cynosurus echinatus*) and 1 forb (*Silene gallica*)) was sown on natural soil. The botanical composition followed a

proportion of 45% leguminous, 45% grasses and 10% Caryophyllaceae. The sowing mixture was adjusted to a density of 1000 plants m⁻² according to seed weight and germination capacity tested previously. Forty-seven days after emergence, plants were exposed for 39 days to four O₃ treatments, replicated in 3 OTC following a randomized complete block design: Charcoal Filtered Air (FA), Non-Filtered Air reproducing ambient levels (NFA), Non-Filtered Air supplemented with 20 nl l⁻¹ O₃ (NFA+) and Non-Filtered Air supplemented with 40 nl l⁻¹ O₃ (NFA++). Plots were divided into 3 sectors of 1.4 m² each for applying the three N treatments reproducing different atmospheric N-deposition inputs: soil N background (N-low), addition of 20 Kg N ha⁻¹ (N-medium) and 40 Kg N ha⁻¹ (N-high). The position of the N-treatments inside the OTCs was randomized among blocks. Nitrogen supplementation was applied biweekly using a solution of ammonium nitrate (NH₄NO₃) in four equal applications during the experiment starting one day after initiation of the O₃ treatments. Meteorological conditions were monitored inside each OTC. Ozone, NO₂ and SO₂ were sequentially monitored by means of a time-sharing system that checked the pollutant levels inside all the OTCs sequentially. A detailed description of the experimental field site, instrumentation, and experimental events can be consulted elsewhere (Chapter 3 and 4).

5.2.2. Elemental analysis

Forage biomass was harvested on 20th May when plants presented the maximum greenness and development, before the start of the senescence period. All the biomass growing 1 cm above-ground within a sampling ring (25 cm diameter) was collected per OTC and N treatment. The harvested plant material was separated by species, then by the different plant organs (flower, leaf, stem and root), dried to constant weight at 60° C and ground to 1-mm size. Carbon and N content for the different parts were analyzed for the legume *T. striatum* and the grass *B. maxima*. Elemental composition analysis was performed at the CIEMAT Chemistry Division using a Leco TruSpec CHN-S (LECO Corporation, St. Joseph, Michigan) by total combustion at 1000°C in pure oxygen, using 150-mg samples.

The lab considered two replicates per each sample. Values are expressed as percentage of total dry weight mass for N, C and the C to N ratio (C/N).

5.2.3. Forage quality parameters

Crude Protein (CP) content was defined as $CP = \%N \times 6.25$. Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) fractions were determined sequentially, following the procedures described by Van Soest et al., (1991). The NDF fraction contains insoluble constituents of the cell wall (hemicellulose, cellulose, lignin and other partially recalcitrant materials); the ADF fraction consists of lignocellulosic material and other recalcitrant materials; and the ADL fraction consists of lignin and other totally recalcitrant materials.

Parameters relating forage quality to animal performance, such as percentage digestible dry matter (%DDM) (Linn and Martin, 1989) and dry matter intake (DMI), calculated from NDF (Mertens, 1987), were also determined. The Relative Feed Value (RFV) was calculated by reference to the digestible DMI of standard forage containing 53% NDF and 41% ADF which as a RFV of 100 (Linn and Martin, 1989).

5.2.4. Statistical analysis

The effects of O₃ and N inputs and their interaction on the quality parameters assayed were analyzed via GLM analyses. Ozone was considered as the main factor in a completely randomized block design, and N as the split-plot factor; the block effect was also included as a random factor. Planned (a priori) comparisons were performed to test the statistical significance of the observed trends through the levels of the factors (increment or decrement) and their interactions considering both linear and quadratic responses. Due to the high variability of the data caused by the field conditions of the experiment, a p-value under 0.1 was considered significant.

Statistical differences between treatments were assessed with the Tukey Honestly Significant Difference test (HSD). All data were first checked for normal distribution and homogeneity of variance, and when assumptions were not fulfilled data were log-transformed. Statistical analyses were performed using Statistica v13 (StatSoft Inc., Tulsa, USA).

5.3. Results and discussion

5.3.1. Carbon and nitrogen allocation

Allocation of carbon to different plant organs is a key issue to understand impacts and feedbacks between vegetation and atmospheric pollutants. The term allocation has been used to describe processes acting at different scales, from fine-scale carbon dynamics within a plant to coarse-scale carbon dynamics within an ecosystem (Litton et al., 2007, Epron et al., 2012). Three constituents of C-allocation have been considered: biomass (the amount of material presented), flux (carbon flow to a component per unit of time) and partitioning (the fraction of gross primary productivity used by a component) (Litton et al, 2007). At the individual plant scale, it is also important to distinguish between the mass of all organic components (biomass) and the carbon quantity in a component (concentration) which is a subset of the biomass (Sevanto et al., 2015).

In the present work, at the individual plant scale, measured changes in biomass and concentration have been used as indicators of the C and N movement in *B. maxima* and *T. striatum*, which is a common assumption due to complexity of capturing fluxes (Sevanto et al., 2015). Ozone and N effects on biomass partitioning will be discussed here in the context of carbon partitioning and redistribution of C and N within plant organs. The effects on biomass parameters of these species can be found in Chapter 4.

Carbon and N allocation within plant organs differed between the legume and the grass (Figure 5.2). The carbon content of the legume was relatively homogenous among plant parts because aboveground and belowground organs presented similar values (considering mean values across O₃ and N treatments);

on the contrary, the grass presented the maximum C-content in the leaves, followed by the stems and the roots, whose C-content was around 8% less than in the leaves.

For both species, N was mainly located in the aboveground organs. The N percentage in flowers and leaves of *T. striatum* almost doubled the values found in stems and roots; this different distribution, compared with that of C, caused lower C/N rates in the legume for flowers and leaves, but higher values for stems and especially roots. The N-distribution pattern of *B. maxima* reproduced the C-pattern; thus, the C/N rate increased from leaves to roots.

Comparing the C and N distribution patterns in plant organs of *T. striatum* and *B. maxima*, the greatest differences between the two species were found in the roots and the stems. The root C content was 10 % lower in the grass. However, the higher N-content compared with the legume counterbalanced the lower C, allowing similar root C/N rates for both species. The stem N-content was greater in the grass, which also caused lower C/N rate for this organ. The C and N distribution in the leaves was remarkably similar comparing the two species. The C/N ratio was slightly higher in *B. maxima* than in *T. striatum* as a result of higher C content and lower N.

Figure 5.2. Mean values (across O_3 and N treatments) of carbon (C) and nitrogen (N) content, and C/N rate within the different plant organs for the annual legume *Trifolium striatum* and the annual grass *Briza maxima*.

5.3.1.1 Effects of O_3 and N on *Briza maxima*

The allocation priorities for C and N within plant organs changed significantly due to the effect of O_3 and N. Ozone induced a linear reduction of the leaf C-content, thus affecting the plant organ with the greatest C-proportion (Table 5.1, Figure 5.3). The C and N distribution within the plant were also affected by N-

input in all the plant organs. The aboveground parts, stems and leaves, increased significantly their N-content and reduced their C-content with increasing N supplementation, causing a linear reduction in the C/N ratio (Table 5.1, Figure 5.3). The foliage presented 15% and 25% lower C/N rate in the N-medium and N-high treatments, respectively, compared with the control (mean across O_3 treatments). This reduction was stronger in stems (19% for N-medium and 35% for N-high compared with the N-background). The response of belowground C was in the opposite direction than in aerial organs. Belowground C increased with the fertilizer, but due to the more pronounced N-induced increment in the N-content (around 35% in N-high compared with N-low), a reduction in the C/N rate was also observed.

Figure 5.3. Leaf response patterns to O_3 exposure and N-inputs for the annual grass *Briza maxima*. FA= charcoal filtered air; NFA= non filtered air; NFA+=non filtered air + 20 nl l⁻¹ of O_3 ; NFA++=non filtered air +40 nl l⁻¹ of O_3 (mean $\pm$ se); N-low=N background levels; N-medium= supplemented with 20 kg N ha⁻¹; N-high= supplemented with 40 kg N ha⁻¹ (mean $\pm$ se).

Table 5.1. Ozone (O₃) and nitrogen (N) effects on C and N allocation and C/N rate of *Briza maxima* within plant organs after 39 days after the start of the treatments. Letters indicate differences among levels of the each factor based on the Tukey test.

Species		Factor	C (%)		N (%)		C/N	
			Linear	Quadratic	Linear	Quadratic	Linear	Quadratic
B. maxima	Leaves	N	0.050	0.167	<0.001	0.776	<0.001	0.444
		O ₃	0.039	0.149	0.158	0.576	0.226	0.674
		N x O ₃	0.812		0.081		0.186	
		FA	42.46±0.17 ^{ab}		3.20±0.13		13.61±0.55	
		NFA	42.75±0.17 ^a		3.37±0.13		12.86±0.55	
		NFA+	42.27±0.17 ^{ab}		3.22±0.13		13.56±0.55	
		NFA++	42.03±0.17 ^b		3.53±0.13		12.34±0.55	
		N-low	42.70±0.15 ^a		2.89±0.11 ^a		15.10±0.48 ^a	
		N-medium	42.20±0.15 ^b		3.36±0.11 ^b		12.78±0.48 ^b	
		N-high	42.24±0.15 ^{ab}		3.75±0.11 ^c		11.39±0.48 ^b	
	Stems	N	0.071	0.058	<0.001	0.463	<0.001	0.791
		O ₃	0.779	0.495	0.714	0.189	0.785	0.908
		N x O ₃	0.437		0.034		0.068	
		FA	39.15±0.35		3.03±0.15		13.89±0.86	
		NFA	38.36±0.39		2.92±0.15		13.24±0.97	
		NFA+	39.20±0.35		2.85±0.15		14.55±0.86	
		NFA++	38.63±0.35		3.14±0.15		13.67±0.86	
		N-low	39.45±0.30 ^a		2.48±0.13 ^a		16.81±0.75 ^a	
		N-medium	38.26±0.30 ^b		2.91±0.13 ^b		13.56±0.75 ^b	
		N-high	38.84±0.33 ^{ab}		3.56±0.13 ^c		10.95±0.81 ^c	
	Roots	N	0.033	0.535	0.002	0.360	0.001	0.079
		O ₃	0.416	0.931	0.905	0.081	0.272	0.354
		N x O ₃	0.627		0.043		0.013	
		FA	36.48±0.76		1.48±0.09		26.71±1.71	
		NFA	35.15±0.76		1.36±0.09		26.18±1.71	
		NFA+	37.95±0.76		1.29±0.09		31.60±1.71	
		NFA++	36.49±0.76		1.49±0.09		27.80±1.71	
		N-low	35.60±0.66 ^a		1.17±0.08 ^a		33.36±1.48 ^a	
		N-medium	36.17±0.66 ^a ^{ab}		1.47±0.08 ^b		25.81±1.48 ^b	
		N-high	37.77±0.66 ^b		1.58±0.08 ^b		27.05±1.48 ^b	

Significant and consistent interactions between O₃ and N were also found for the N-content of leaves, stems and roots, and for the C/N rate of stems and roots (Table 5.1). These interactions modulate the effects observed in the N-content and C/N ratios, showing that O₃ effects were counterbalanced by the fertilizer inputs.

Ozone increased the N-content of the aboveground organs, but only when the N availability exceeded background levels (N-low). Under N-low, the N-content

of the leaves and stems did not respond to O_3 . However, when the N-inputs were increased, their N-content increased with O_3 exposure (Figure 5.4). For belowground organs, O_3 exposure caused a reduction in the N-content under N-low, but this effect was counterbalanced by the N-input. Since O_3 did not affect the root C-content, the same interactive pattern was also observed for the C/N rate (Figure 5.4).

From another point of view, the interaction between O_3 and N shows that, under high O_3 levels, maintaining high N-content in the aerial biomass, and thus protein content required for the pasture quality, required greater N-inputs than under low O_3 exposure. Thus, the pollutant reduced the N-use efficiency.

Figure 5.4 *Briza maxima* interactive responses to N and O_3 for: leaves (a) and root (b) N-content, and leaves (c) and root (d) C/N rate. FA= charcoal filtered air; NFA= non filtered air; NFA+=non filtered air + 20 nl l⁻¹ of O_3 ; NFA++=non filtered air + 40 nl l⁻¹ of O_3 ; N-low=N background levels; N-medium= supplemented with 20 kg N ha⁻¹; N-high= supplemented with 40 kg N ha⁻¹. (mean $\pm$ se). p-values<0.1 indicate significant interaction between factors.

It is interesting to note that the effects of O₃ on C and N -contents of the different organs occurred without showing any significant effect on biomass parameters (Chapter 4). This species was previously classified as relatively O₃-tolerant regarding growth parameters compared with annual clovers (Gimeno et al., 2004a). However, Sanz et al. (2011) observed an O₃-induced increase in plant senescence and a shortened life span in their experiments. The results reported here in relation with C and N allocation confirm the O₃ sensitivity of this species and, more interestingly, that O₃ effects were modulated by N availability.

The effects of O₃ on plant growth can consist not only of net C-input reductions due to the alteration of gas exchange processes and carbon fixation, but also to the disruption of source-sink C-partitioning (Andersen, 2003). Carbon and other nutrients may be retained in damaged leaves in order to repair O₃ injury, sometimes reducing the C allocation to the root system (Long and Naidu, 2002). The root C-content was not affected by O₃ in this experiment. Thus, the reduction of the C-content in *B. maxima* leaves might be related to enhanced respiration for repair mechanisms at cellular level that expend C resources. This capacity could be related to the greater O₃-tolerance of this species compared with annual clovers. Blocking the oxidative burst caused by the pollutant on plant tissues have been related with O₃ tolerance mechanisms (Schraudner et al., 1998).

Although not found in the previous potted experiment with *B. maxima* (Sanz et al., 2011), the increment of the N-content in the aerial organs due to the greater N-availability is in agreement with the positive effect of N on the biomass parameters (Sanz et al., 2011, Chapter 4). Changes in N-content of plant organs, especially leaves, may alter metabolic functions such as enzyme and photosynthetic pigment production, which require nitrogenous compounds, favoring photosynthesis and carbon fixation (Marek and Frank, 1984). This effect has also been found in annual clovers even with active nodulation (Sanz et al., 2014).

The modulation of the N-induced effect on foliar N-content by O₃ is a new finding for this species. Some O₃-sensitive annual clovers have shown that the atmospheric oxidant can cause an increment in the foliar N-content. This was associated with a concentration effect caused by the concurrent biomass loss

induced by O_3 rather than a direct effect of O_3 (Sanz et al, 2005; 2014). However, this was not the case in *B. maxima*.

The positive effect of N-inputs and of O_3 exposure, provided that enough N is available, on leaf N-content may induce a higher physiological activity of *B. maxima* plants. These changes could give the grass a competitive advantage over the clover, contributing to O_3 -induced changes in the species structure as observed at the end of the growing season (Chapter 4).

The changes observed in the grass root system can have consequences at the ecosystem level, especially considering the major belowground biomass of the annual grass compared with the clover (Sánchez-Martín et al., 2016). The O_3 induced increases in root N-content have been related to an enhancement of soil N_2O emissions measured during the same experiment (Sánchez-Martín et al., 2016). Thus, what happen to be small changes at species level might have important repercussions at the ecosystem level.

5.3.1.2 Effects of O_3 and N on *Trifolium striatum*

The O_3 exposure and N-inputs altered significantly the C and N allocation within clover organs, but followed a different response pattern compared with the grass. Ozone did not affect the C allocation in any organ, but increased the N-content of the aerial structures, leaves, stems and flowers (table 5.2, figure 5.5). These organs almost doubled the N-content in the roots. The increment pattern was linear for leaves and stems, whereas it was quadratic for flowers due to a high N content increase in the NFA++ treatment.

The imbalanced O_3 effect on the N-content of flowers, leaves and stems significantly reduced the C/N ratio for these organs. Leaves showed 2%, 6% and 9% reduction in the C/N ratio for NFA, NFA+ and NFA++ treatments, respectively, compared with FA (mean across N treatments); the percentages of reduction for stems were in the range of 7% to 14% for the different O_3 treatments. Contrary to results observed for *B. maxima*, no modulation of the O_3 -induced effects by N was observed in *T. striatum*.

Trifolium striatum has been described in previous monoculture experiments as an O₃-sensitive species, showing O₃-induced reductions in the aboveground and root biomass, and in flower and seed production (Gimeno et al., 2004a,b; Sanz et al., 2005). The present experiment confirmed that *T. striatum* is very O₃-sensitive also when it grows in a multi-species canopy. The pollutant induced alterations in the gas exchange (Llusia et al., 2014) and biomass parameters (Chapter 4). Some of them were found at O₃ levels frequently registered in their natural distribution area in the Iberian Peninsula.

Surprisingly, despite the observed reduction in aerial and root biomass (losses in the range 26-43 % depending on the O₃ levels) during the same experiment (Chapter 4), no effects were observed on C allocation within the organs. This could be related to the incapacity of clover for activating the detoxification mechanisms at the cellular level and blocking the free radicals generated by the pollutant at the plant tissue level. Such response would have caused a respiration-induced C loss. In order to confirm this hypothesis, it would be necessary to analyze the differences in the response of the antioxidant cellular systems in the legume and the grass.

Figure 5.5. Leaf response patterns to O_3 exposure and N-inputs for the annual clover *Trifolium striatum*. FA= charcoal filtered air; NFA= non filtered air; NFA+=non filtered air + 20 nl l⁻¹ of O_3 ; NFA++=non filtered air + 40 nl l⁻¹ of O_3 (mean $\pm$ se); N-low=N background levels; N-medium= supplemented with 20 kg N ha⁻¹; N-high= supplemented with 40 kg N ha⁻¹ (mean $\pm$ se).

Table 5.2. Ozone (O₃) and nitrogen (N) effects on C and N allocation among the different plant organs of *Trifolium striatum* 39 days after starts of the O₃ exposure.

Species		Factor	C (%)		N (%)		C/N		
			Linear	Quadratic	Linear	Quadratic	Linear	Quadratic	
T. striatum	Flowers	N	0.018	0.253	0.576	0.086	0.148	0.048	
		O ₃	0.560	0.272	0.106	0.029	0.158	0.051	
		N x O ₃	0.936		0.247		0.226		
		FA	41.50±0.25		3.13±0.07 ^{ab}		13.31±0.28 ^a		
		NFA	41.26±0.25		3.06±0.07 ^a		13.58±0.28 ^a		
		NFA+	41.36±0.25		3.05±0.07 ^a		13.59±0.28 ^a		
		NFA++	41.70±0.25		3.30±0.07 ^b		12.69±0.28 ^a		
		N-low	41.15±0.22 ^a		3.11±0.06 ^a		13.24±0.24 ^{ab}		
		N-medium	41.24±0.22 ^a		3.22±0.06 ^a		12.87±0.24 ^a		
		N-high	41.97±0.22 ^b		3.07±0.06 ^a		13.76±0.24 ^b		
		Leaves	N	0.112	0.706	0.020	0.707	0.018	0.495
			O ₃	0.114	0.723	0.010	0.667	0.013	0.791
			N x O ₃	0.256		0.424		0.980	
			FA	40.94±0.52		3.15±0.11 ^a		13.09±0.32 ^a	
			NFA	40.32±0.52		3.16±0.11 ^{ab}		12.86±0.32 ^{ab}	
	NFA+		41.93±0.52		3.41±0.11 ^{ab}		12.33±0.32 ^{ab}		
	NFA++		41.69±0.52		3.52±0.11 ^b		11.93±0.32 ^b		
	N-low		40.76±0.45		3.13±0.09 ^a		13.14±0.28 ^a		
	N-medium		41.08±0.45		3.34±0.09 ^{ab}		12.39±0.28 ^{ab}		
	N-high		41.83±0.45		3.46±0.09 ^b		12.11±0.28 ^b		
	Stems		N	0.133	0.614	0.659	0.228	0.592	0.368
			O ₃	0.477	0.725	0.014	0.982	0.041	0.468
			N x O ₃	0.860		0.108		0.207	
			FA	40.59±0.40		1.55±0.07 ^a		26.47±1.01 ^a	
			NFA	40.25±0.45		1.78±0.07 ^{ab}		23.32±1.13 ^{ab}	
		NFA+	40.99±0.40		1.67±0.07 ^{ab}		24.71±1.01 ^{ab}		
		NFA++	40.70±0.40		1.89±0.07 ^b		22.37±1.01 ^b		
		N-low	40.24±0.35		1.67±0.06		24.73±0.88		
		N-medium	40.42±0.35		1.79±0.06		23.36±0.88		
		N-high	40.33±0.38		1.71±0.06		24.69±0.95		
Roots		N	0.420	0.780	0.162	0.005	0.177	0.007	
		O ₃	0.202	0.762	0.402	0.731	0.356	0.738	
		N x O ₃	0.099		0.929		0.773		
		FA	40.92±0.24		1.30±0.05		31.69±1.04		
		NFA	40.89±0.24		1.36±0.05		30.31±1.04		
	NFA+	40.69±0.24		1.33±0.05		30.75±1.04			
	NFA++	40.50±0.24		1.37±0.05		30.07±1.04			
	N-low	40.90±0.21		1.33±0.04 ^{ab}		30.94±0.90 ^{ab}			
	N-medium	40.70±0.21		1.45±0.04 ^a		28.43±0.90 ^a			
	N-high	40.65±0.21		1.25±0.04 ^b		32.74±0.90 ^b			

In spite of the N fixation capacity of the clover, the fertilizer linearly increased the foliar N-content but not in the stems. This effect resulted in a significant linear reduction of the leaf C/N ratio. Nitrogen addition also induced a quadratic decrease in the root N-content that caused the C/N ratio to increase in the roots. The N effects on the root N-content could be related to a loss of the N-fixation capacity. The activity of the *Rhizobium* system can be inactivated under high N availability (Carlsson and Huss-Danell 2003). Although O₃ can also induce this type of effect (Hewitt et al., 2014). No significant effects of O₃ have been detected for the root elemental composition, nor an interaction between factors that could suggest this type of effect. However, the O₃-induced reduction in the belowground biomass of the clover was intensified by the fertilizer (Sanchez-Martín et al., 2016).

The treatment effects found in *T. striatum* contrast with the ones observed in *B. maxima*. The effects of N-input on the N pools of *T. striatum* revealed that legume species can also be affected directly by N. Previously reported effects on biomass parameters (Chapter 4) showed that the grass was more N-sensitive than the clover. However, including the effects on the C and N partitioning, both species can be considered as N-sensitive.

5.3.2. Nutritive quality

The nutritive quality of the two species was evaluated on the basis of their CP and fiber contents in the aboveground biomass. Legume species are usually considered to improve the quality of a pasture due to their high CP and low fiber content (Van Soest, 1994). However, the grass and the clover assayed in the present study presented similar CP (Figure 5.6). The fiber content measured in the present study was also lower in the grass than in the clover, resulting in higher RFV values. These values do not support the agronomic generalization of greater forage quality of the clover compared with the grass. However, this observation is in accordance with the CP and fiber content values reported for different annual clovers and grasses in previous studies. These studies considered the species in the present study, but also *T. cherleri*, *T. subterraneum* and *Bromus hordeaceus* (Sanz et

al., 2005, 2007, 2011, 2014) reported CP and fiber values within the same range, regardless of their belonging to the grass or legume family.

Figure 5.6. Mean values for the forage quality parameters of *B. maxima* and *T. striatum* across nitrogen and ozone treatments (means $\pm$ se). a) CP=Crude Protein, NDF=Neutral Detergent Fiber, ADF=Acid Detergent Fiber; b) ADL=Acid Detergent Lignin, RFV=Relative Feed Value

5.3.2.1. Protein content

As CP is calculated on the base of the N-content, the results have been already presented in section 5.3.1. However, the main results are summarized here only for the aerial organs of both species in order to discuss how the alterations caused by the experimental treatments in the protein pools can affect the forage quality of the pasture.

Ozone and N increased linearly the CP content of the aerial organs (leaves and stems) of *B. maxima* (table 5.4). However, the effect of O₃ on CP was only observed in N-medium and N-high fertilization levels, which resulted in a significant interaction between O₃ and N input. On the other hand, the N-efficiency, evaluated on the base of the N-inputs effect on foliage CP, was reduced by the pollutant (Figure 5.7 a,b). Higher N inputs were needed to increase leaf CP values under elevated O₃ levels than at low O₃ exposure.

O₃ and N also affected the CP content of *T. striatum* leaves, whereas the CP in the stems was only affected by O₃. The linear increase of CP in leaves and stems in response to the experimental treatments was similar to that for the grass. However, no interactions between O₃ and N were found for the clover (Figure 5.7 c,d).

Figure 5.7 Ozone and N interactive effect (mean $\pm$ se) on Crude Protein (%) for: a) leaves of *B. maxima*; b) stems of *B. maxima*; c) leaves of *T. striatum*; d) stems of *T. striatum*. FA= charcoal filtered air; NFA= non filtered air; NFA+=non filtered air + 20 nl l⁻¹ of O₃; NFA++=non filtered air + 40 nl l⁻¹ of O₃; N-low = soil N background; N-medium= +20 Kg N ha⁻¹; N-high= + 40 Kg N ha⁻¹.

Although both species responded to the factors in the same direction, there were differences in the magnitude of the effects. *Briza maxima* showed more pronounced increases in CP content than *T. striatum*. This difference may be indicative of an imbalance in the CP distribution within the ecosystem which can be evaluated through the Grass/Clover CP ratio. Under N-low, a greater fraction of the CP was located in the clover (Figure 5.8a). As the N-availability increased, a greater proportion of the CP was found in the grass. Thus, increasing N-inputs favored the accumulation of CP in the grass fraction. This pattern agrees with the greater N-responsiveness of *B. maxima* biomass parameters compared with *T. striatum* (Chapter 4).

Increasing O₃ levels also changed the CP balance between the legume and the grass (Figure 5.8b), but in a more complex pattern than the N-inputs due to the existing interaction between O₃ and N on CP levels in *B. maxima*. At N-low level, the Grass/Clover CP ratio decreased with O₃ exposure (Figure 5.9). This response resulted from increasing CP levels with O₃ in the clover fraction, whereas no changes were observed in the grass fraction. In N-medium and N-high input levels, O₃ induced increases in the CP content of both species, but this effect was more pronounced in the clover fraction, yielding the mean NFA+ and NFA++ Grass/Clover ratios lower than in FA or NFA O₃ levels. Interestingly, under the lowest and the highest levels of the pollutant (FA and NFA++), both species presented a similar proportion of CP (Figure 5.8).

Figure 5.8. Unbalances observed in the Grass (*B. maxima*)/Clover (*T. striatum*) Protein rate due to the N inputs (a) and the increasing O₃ levels (b). N-low = soil N background; N-medium= +20 Kg N ha⁻¹; N-high= + 40 Kg N ha⁻¹; FA= charcoal filtered air; NFA= non filtered air; NFA+=non filtered air + 20 nl l⁻¹ of O₃; NFA++=non filtered air + 40 nl l⁻¹ of O₃

Figure 5.9. Unbalances observed in the Grass (*B. maxima*)/Clover (*T.striatum*) Protein rate due to the combination of N inputs and increasing O_3 levels. N-low = soil N background; Mean N-medium+N-high= mean values for the treatments N-Medium (+20 Kg N ha⁻¹) and N-high (+ 40 Kg N ha⁻¹); FA= charcoal filtered air; NFA= non filtered air; NFA+=non filtered air + 20 nl l⁻¹ of O_3 ; NFA++=non filtered air + 40 nl l⁻¹ of O_3 .

These results indicate that both factors, O_3 and N-inputs, can unbalance the apportionment of protein within the grass-legume system. The ultimate effect on the protein content of the pasture will depend on the species biomass proportion. The inter-annual variability in the species composition is a characteristic of annual Mediterranean pastures. This variability is related to the high variability of the meteorological conditions in the Mediterranean climate, especially the soil water availability (Peco et al., 1998). Years with greater abundances of legumes or grasses are commonly reported in the literature (Vázquez de Aldana et al., 2008). Thus, any effect of O_3 and N input on CP pools of annual pasture species must be interpreted in the wider context of inter-annual changes on the species composition. Multi-species approaches could provide more insightful conclusions on the effects of O_3 and N on the CP of annual pastures.

Table 5.3. Ozone (O₃) and nitrogen (N) effects on nutritive quality-related parameters of *Trifolium striatum* 39 days after start of the treatments (Mean values ±se). CP =Crude Protein; NFD = Neutral Detergent Fiber; ADF = Acid Detergent Fiber; ADL = Acid Detergent Lignin; RFV = Relative Feed Value; FA = charcoal Filtered Air; NFA = Non-Filtered Air; NFA+ = Non-Filtered Air+20 nl l⁻¹ O₃; NFA++ = Non-Filtered Air+40 nl l⁻¹ O₃; N-low = soil N background; N-medium= +20 Kg N ha⁻¹; N-high= + 40 Kg N ha⁻¹. Different letters indicate significant effects (HSD) for each factor, O₃ and N at p<0.1.

Species		Factor	CP (%)		NDF (%)		ADF (%)		ADL (%)		RFV	
			Linear	Quadratic	Linear	Quadratic	Linear	Quadratic	Linear	Quadratic	Linear	Quadratic
T. striatum	Leaves	N	0.020	0.707	0.014	0.828	0.007	0.048	0.251	0.187	0.005	0.467
		O ₃	0.010	0.667	0.424	0.006	0.301	0.452	<0.001	0.459	0.273	0.264
		N x O ₃	0.424		0.181		0.276		0.360		0.302	
		FA	19.66±0.66 ^a		37.54±0.55 ^a		23.16±0.49		3.91±0.24 ^a		177.11±4.03	
		NFA	19.77±0.66 ^{ab}		38.93±0.61 ^{ab}		23.18±0.49		3.73±0.24 ^a		175.01±4.03	
		NFA+	21.33±0.66 ^{ab}		39.71±0.55 ^b		24.31±0.49		4.99±0.24 ^b		166.03±4.03	
		NFA++	22.01±0.66 ^b		38.21±0.55 ^{ab}		23.57±0.49		5.17±0.24 ^b		173.27±4.03	
		N-low	19.55±0.57 ^a		39.81±0.47 ^a		24.11±0.43 ^a		4.51±0.21		165.83±3.49 ^a	
		N-medium	20.87±0.57 ^{ab}		38.70±0.47 ^a		24.30±0.43 ^a		4.69±0.21		170.73±3.49 ^a	
		N-high	21.65±0.57 ^b		37.14±0.52 ^b		22.25±0.43 ^b		4.16±0.21		182.01±3.49 ^b	
	Stems	N	0.659	0.228	0.011	0.193	0.796	0.681	0.186	0.787	0.025	0.353
		O ₃	0.014	0.982	0.141	0.010	0.612	0.195	0.005	0.004	0.212	0.010
		N x O ₃	0.108		0.312		0.927		0.446		0.675	
		FA	9.69±0.46 ^a		40.68±0.38 ^{ab}		28.45±0.42		4.52±0.11 ^a		152.87±2.29 ^{ab}	
		NFA	11.11±0.46 ^{ab}		39.92±0.43 ^a		28.13±0.59		4.05±0.13 ^b		158.28±2.56 ^a	
		NFA+	10.41±0.46 ^{ab}		39.99±0.38 ^a		27.73±0.42		4.66±0.11 ^a		156.88±2.29 ^{ab}	
		NFA++	11.81±0.46 ^b		41.54±0.38 ^b		29.03±0.42		4.85±0.11 ^a		149.11±2.56 ^b	
		N-low	10.43±0.39		40.05±0.33 ^a		28.26±0.40		4.38±0.10		157.13±1.98 ^a	
		N-medium	11.16±0.39		40.17±0.36 ^a		28.20±0.40		4.65±0.11		155.31±2.16 ^{ab}	
		N-high	10.68±0.39		41.40±0.33 ^b		28.56±0.36		4.58±0.10		150.16±1.98 ^b	

Table 5.4. Table 5.3. Ozone (O₃) and nitrogen (N) effects on nutritive quality-related parameters of *Briza maxima* 39 days after start of the treatments (Mean values $\pm$ se). CP =Crude Protein; NFD = Neutral Detergent Fiber; ADF = Acid Detergent Fiber; ADL = Acid Detergent Lignin; RFV = Relative Feed Value; FA = charcoal Filtered Air; NFA = Non-Filtered Air; NFA+ = Non-Filtered Air+20 nl l⁻¹ O₃; NFA++ = Non-Filtered Air+40 nl l⁻¹ O₃; N-low = soil N background; N-medium= +20 Kg N ha⁻¹; N-high= + 40 Kg N ha⁻¹. Different letters indicate significant effects (HSD) for each factor, O₃ and N at p<0.1.

Species		Factor	CP (%)		NDF (%)		ADF (%)		ADL (%)		RFV	
			Linear	Quadratic	Linear	Quadratic	Linear	Quadratic	Linear	Quadratic	Linear	Quadratic
B. maxima	Leaves	N	<0.001	0.776	0.356	0.067	0.722	0.426	0.191	0.309	0.557	0.159
		O ₃	0.158	0.576	0.969	0.018	0.901	0.081	0.834	0.122	0.989	0.021
		N x O ₃	0.081		0.645		0.425		0.107		0.781	
		FA		20.02±0.79		35.52±0.62 ^a		18.49±0.51 ^a		1.22±0.12		196.25±4.56 ^a
		NFA		21.09±0.79		33.40±0.56 ^b		16.76±0.46 ^b		1.12±0.11		212.13±4.08 ^b
		NFA+		20.11±0.79		33.46±0.62 ^{ab}		17.59±0.51 ^{ab}		0.94±0.12		210.10±4.56 ^{ab}
		NFA++		22.09±0.79		34.95±0.62 ^{ab}		18.27±0.51 ^{ab}		1.24±0.12		199.86±4.56 ^{ab}
		N-low		18.05±0.68 ^a		34.40±0.52 ^a		17.84±0.43		1.06±0.10		204.49±3.85
		N-medium		20.99±0.68 ^b		35.03±0.52 ^a		17.50±0.43		1.03±0.10		200.72±3.85
		N-high		23.45±0.68 ^c		33.49±0.52 ^a		17.91±0.43		1.30±0.10		209.23±3.85
	Stems	N	<0.001	0.463	0.296	0.034	0.603	0.016	0.864	0.361	0.330	0.035
		O ₃	0.714	0.189	0.390	0.910	0.302	0.483	0.837	0.597	0.477	0.685
		N x O ₃	0.034		0.788		0.850		0.839		0.767	
		FA		18.94±0.91		38.69±5.78		18.15±2.67		0.71±0.29		198.44±42.03
		NFA		18.27±0.91		39.05±4.57		15.23±2.11		1.06±0.23		213.71±33.22
		NFA+		17.79±0.91		37.31±4.57		15.96±2.11		0.88±0.24		180.16±35.16
		NFA++		19.60±0.91		37.93±5.78		17.35±2.67		0.74±0.29		198.30±42.03
		N-low		15.52±0.79 ^a		44.58±4.06 ^a		19.18±1.87 ^a		0.78±0.22		157.52±29.48 ^a
		N-medium		18.17±0.79 ^b		30.55±5.24 ^b		12.24±2.42 ^b		1.07±0.26		254.37±39.76 ^a
		N-high		22.26±0.79 ^c		37.38±4.34 ^{ab}		17.10±2.00 ^{ab}		0.78±0.22		203.61±31.65 ^a

5.3.2.2. Fiber content

O₃ and N affected the different fiber contents of *T. striatum* and *B. maxima*. The clover was the most sensitive species to both O₃ and N. In this species, the two factors induced opposite effects on fiber contents, without statistically significant interactions between them. Ozone generally caused a reduction in the forage quality of the clover. The strongest response to O₃ was an increase in the foliar lignin content. The foliar lignin was enhanced by 28% and 32% in NFA+ and NFA++, respectively, compared with FA (means across N treatments). O₃ effects were also observed in the leaf NDF fraction following a quadratic pattern, with the highest NDF contents recorded in NFA and NFA+. These variations in the composition of leaf fibers did not induce a change in the forage digestible energy content evaluated as RFV (Table 5.3). Similarly, O₃ induced a linear increase in stem lignin content and a quadratic effect on the NDF content and the RFV. N-inputs tended to improve the overall quality of the leaves of clover. Nitrogen addition significantly decreased the foliar fiber concentrations of ADF and NDF by 7% and 8%, respectively, in N-high compared with N-low. These positive changes increased the leaf RFV by 17% in N-high compared with N-low. However N-inputs also reduced the forage quality of the clover stems through a 3.4% increase in NDF and a 4.4% reduction in RFV in N-high compared with N-low (Table 5.3).

The forage quality of *B. maxima* was less sensitive to O₃ and N, and no interactions between these two factors were identified. Foliar NDF and ADF responded to the increased levels of O₃ following a quadratic pattern, that in turn resulted in a quadratic effect on the RFV (Table 5.4). However, the pollutant did not affect the leaf lignin content. Although *B. maxima* was N-sensitive in terms of biomass and CP responses, the leaf fiber content of the grass did not respond to N-inputs. Only the NDF showed a N-induced change following a quadratic pattern such that the highest fiber content was found in N-medium. The fiber content of the stems did not respond to O₃, but effects were found for N. Nitrogen inputs induced a quadratic response in NDF and ADF such that the lowest fiber contents were measured in N-medium. These changes caused a quadratic response in RFV such that the highest RFV was observed in N-medium.

The NDF and ADF fiber contents measured in this study were in the same range of values as reported previously for annual species such as *T. subterraneum*, *T. cherleri*, *B. maxima* and *Bromus hordeaceus* (Sanz et al., 2005, 2011, 2014, 2015). However, it must be noted that fiber contents measured in *B. maxima* were considerably lower than in previous experiments with the same species or with other annual or perennial grass species (González-Fernández et al., 2008, Sanz et al. 2011, 2015). Thus, the RFV for *B. maxima* was higher than values generally reported for grass species, and even greater than the RFV measured in *T. striatum*. This was unexpected considering that legumes are traditionally considered to have superior forage quality compared with grasses. Higher RFV have been measured in clover species than in grasses growing in the same experiment (e.g. González-Fernández et al., 2008, Sanz et al., 2005, 2011, 2014, 2015).

In terms of lignin content, the values reported in this study were larger than data from other pasture species (Muntifering et al., 2006; Sanz et al., 2005, 2011, 2014, 2015), but they were within the same range of values reported for perennial grassland species such as *T. repens* and *Lolium perenne* (González-Fernández et al., 2008). Higher lignin contents have also been reported for perennial communities growing in the UK (Hayes et al., 2016).

Forage quality parameters are affected by many environmental variables that may explain the differences observed between studies. The nutritive quality of pastures declines largely with plant maturity (Blaser, 1986, Linn and Martin, 1989, Van Soest, 1994). The biomass harvested in the different experiments, and from the different species in one experiment, may correspond to different growth stages, which makes comparisons between studies difficult. Unfortunately, this information is frequently not reported in forage quality studies. The different meteorological conditions can also cause differences in the forage quality parameters (Van Soest 1994, Lin et al., 2007, Vázquez de Aldana et al., 2008). Vazquez de Aldana et al. (2008) studied the quality and yield of a Dehesa annual pasture over a 20 years period, showing that the protein content was negatively correlated with the spring precipitation, whereas ADF and lignin contents were positively correlated with spring precipitation. The different growing conditions

between the experiments can help to explain the differences found in forage quality parameters.

Ozone and N effects on fiber content in this experiment were comparatively lower than effects reported in other studies. The RFV, which integrates treatment effects on the dry matter voluntary intake (affected by NDF content) and digestibility (affected by the ADF fraction), was significantly reduced by O₃ exposure in *B. maxima* and *T. cherleri* (Sanz et al., 2011, 2014). RFV was probably reduced also in *T. subterraneum* due to O₃-induced increases in NDF and ADF levels, though the RFV was not determined (Sanz et al., 2007). No effects of O₃ were found in RFV of *T. repens*, *Bromus hordeaceus* or *Lolium perenne* (González-Fernández et al., 2008; Sanz et al., 2015). However, RFV was reduced by twice-ambient O₃ exposure in *T. repens* when this species was growing in competition with typical Piedmont region (southeastern USA) grass species in OTC (Gilliland et al., 2016). In the latter experiment, the grass species *Lolium arundinacea*, *Paspalum dilatatum* and *Cynodon dactylon* showed a 5% increase in RFV under twice-ambient O₃ exposures. The NDF levels measured on *T. pratense* and *T. repens* in a free-air O₃-fumigation facility were again not affected by O₃ (Muntifering et al., 2006). On the contrary, the only N effects reported on the RFV have shown an increase in *B. maxima* forage quality with increasing N-availability (Sanz et al., 2011).

Lignin content is generally considered one of the main factors limiting forage digestibility, influencing cell wall degradability (Van Soest, 1994). Ozone exposure has been shown to increase the lignification of many annual herbaceous species such as *B. maxima*, *T. subterraneum*, *T. cherleri*, *Bromus hordeaceus* (Sanz et al., 2005, 2011, 2014, 2015). Among perennial species, the results varied between experiments and species. The lignin content of *T. repens* and *Lolium perenne* was not affected by O₃ in an OTC O₃ fumigation study in northern England (González-Fernández et al., 2008). However, lignin was increased by O₃ exposure in *T. repens* and *T. pratense* in a free-air O₃ fumigation facility in Wisconsin (Muntifering et al., 2006), and in *T. repens* and *Paspalum notatum* in two OTC experiments in Alabama USA (Muntifering et al., 2000, Gilliland et al., 2016). A combined analysis of several pasture communities from the UK showed a moderate increase in lignin content

with increasing O₃ exposure (Hayes et al., 2016). These results are in agreement with the ones presented in this study. Ozone exposure induced an increase in the lignin content of *T. striatum*, but not in *B. maxima*. This grass is normally more tolerant to O₃ than the clover in different biomass parameters, which is consistent with the differences observed in lignin content. However, the sensitivity of *B. maxima* to O₃ effects on lignin content cannot be ruled out because O₃-induced increases in lignin content were observed in a previous experiment with the same species (Sanz et al., 2011). A similar variation of O₃ effects in lignin content between studies has been also observed with *T. repens*, as described above.

The differences observed in the response of forage quality parameters to O₃ between studies might be related to the growing conditions experienced by plants. The environmental conditions can modulate the effects of O₃ on forage quality parameters. For example, Sanz et al. (2016) found a stronger positive correlation between the CFV, an index that integrates effects on biomass yield and quality, of O₃-sensitive annual pasture species and the O₃ flux absorbed by the leaves than with the concentration-based index AOT40. The O₃ flux is modulated by environmental variables such as temperature, VPD or soil moisture, as well as by O₃ concentration (Emberson et al., 2000a). Thus, under similar exposure levels, O₃ effects would be generally greater under more favorable conditions for plant growth and O₃ uptake. The same interaction between O₃ and environmental growing conditions has been found in many different species and response parameters (e.g., tomato and wheat yield and yield quality, tree growth, etc., González-Fernández et al., 2014, Mills et al., 2011), and in aboveground biomass in the present experiment (Chapter 4). The majority of the results regarding O₃ effects on forage quality reported in the literature, particularly those for annual Mediterranean species, have been based on individual species growing in pots with a plentiful supply of water and nutrients. These conditions can favor the effect of O₃ in comparison with the more stressful conditions that plants experienced in the present experiment. This hypothesis is consistent with the results reported for *Medicago sativa* over five growing seasons in three sites from west-central Alberta (Canada) (Lin et al., 2007). Air quality parameters (mainly O₃) explained a higher proportion (86%) of the variability in RFV in high-yielding harvests than in low-

yielding harvests (25%), which shows that O₃ effects on forage quality of *M. sativa* were more important when the growing conditions were more favorable.

Ozone and N effects on forage quality parameters are caused not only by direct effects on the biochemical composition of plant tissues but also through indirect processes that are not considered in this study. The forage nutritive value can be affected by the green to senescent biomass and the leaf to stem biomass ratios, the phenological stage or the species composition of the pasture (Van Soest 1994, Muntifering et al., 2006). These factors have been reported to be affected by O₃ and N inputs in pasture species growing individually, in simple mixtures or in intact communities (Sanz et al., 2005, 2007, 2011, 2014, 2015, Stevens et al., 2010, Wedlich et al., 2012, Chapter 3 and 4). These effects could potentially result in indirect alterations of the forage quality at the community level. The analysis of indirect effects will require more detailed information that integrates the multi-species nature of pasture canopies. These assessments will need to consider also the important interactions existing between plant community dynamics and the environmental growing conditions, especially for annual Mediterranean pastures.

5.4. Conclusions

Ozone exposure and N-inputs induced changes in the C and N-distribution within plant organs. The general response was an O₃-induced increase in the N-content of the aerial organs for both species, and also in the belowground organs for the grass. The N-inputs affected the N-content in the same direction as O₃. The O₃ effect on the grass was modulated by N availability. O₃-induced increases in N-content were observed only in N-medium and N-high, but not in N-low. This interaction also implied a loss of N-use efficiency under increasing O₃ exposure. The observed effects on N-content caused an imbalance in the corresponding C/N ratios for the different organs.

Although both species responded to O₃ and N, the magnitude of the effect was different between species and factors, causing an imbalance in the CP distribution within the ecosystem. Increasing N-inputs favored the accumulation of CP in the grass fraction. Increasing O₃ levels also changed the CP balance between

the legume and the grass, but in a more complex pattern as a result of the O₃ by N interaction found in the grass CP content. These alterations of the Grass/Clover CP ratio can be related to changes in the species competitiveness and with the observed changes in the pasture species structure based on biomass effects.

Ozone and N showed potential to change the forage quality of the pasture through direct changes in the biochemical composition of the aboveground biomass of *Trifolium striatum*. Ozone exposure increased the lignin content in leaves and stems of the clover, which is negatively related with biomass digestibility. On the contrary, N tended to improve the quality of the clover through reductions in leaf fiber content and increases in leaf RFV, although this effect can be counterbalanced by the increase in fiber content in the stems. However, no interactions were found between the two factors. *B.maxima* quality parameters only showed subtle responses to the treatment effects.

The indirect effects of O₃ and N on forage quality through changes in community composition, plant phenology or plant senescence need to be addressed, as much as the interaction between O₃ and N effects and the environmental growing conditions, to understand the overall effect on pasture nutritive before a more general conclusion can be reached.

CHAPTER 6

Developing ozone critical levels for multi-species canopies of Mediterranean annual pastures

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Abstract

Ozone (O_3) critical levels (CLE) are still poorly developed for herbaceous vegetation. They are currently based on single species responses which do not reflect the multi-species nature of semi-natural vegetation communities. Also, the potential effects of other factors like the nitrogen (N) input are not considered in their derivation, making their use uncertain under natural conditions.

Exposure- and dose-response relationships were derived from two open-top chamber experiments exposing a mixture of 6 representative annual Mediterranean pasture species growing in natural soil to 4 O_3 fumigation levels and 3 N inputs. The Deposition of O_3 and Stomatal Exchange model was modified to account for the multi-species nature of the canopy following a big-leaf approach. This new approach was used for estimating a multi-species phytotoxic O_3 dose (POD_{y-MS}). Response relationships were derived based on O_3 exposure (AOT40) and flux (POD_{y-MS}) indices.

The treatment effects were similar in the two seasons: O_3 reduced the aboveground biomass growth and N modulated this response. Gas exchange rates presented a high inter-specific variability and important inter-annual fluctuations as a result of varying growing conditions during the two years. The AOT40-based relationships were not statistically significant except when the highest N input was considered alone. In contrast, POD_{y-MS} relationships were all significant but for the lowest N input level. The influence of the N input on the exposure- and dose-response relationships implies that N can modify the O_3 CLE. However, this is an aspect that has not been considered so far in the methodologies for establishing O_3 CLE. Averaging across N input levels, a multi-species O_3 CLE (CLE_{f-MS}) is proposed $POD_{1-MS}=7.9 \text{ mmol m}^{-2}$, accumulated over 1.5 month with a 95% confidence interval of (5.9, 9.8). Further efforts will be needed for comparing the CLE_{f-MS} with current O_3 CLE_f based on single species responses.

6.1. Introduction

Tropospheric Ozone (O_3) is an important greenhouse gas that is also considered the most phytotoxic air pollutant, inducing negative effects on growth, productivity and yield quality of forests, crops and pastures (Ainsworth et al., 2012; Fuhrer, 2009).

Background O_3 concentrations have been raising since the Industrial Revolution in many parts of the Northern Hemisphere (Stevenson et al., 2006). Although the implementation of environmental policies in the last two decades has decreased peak O_3 concentrations (Klingberg et al., 2014), the air quality in Europe is still not acceptable for protecting the human health and the environment (EEA, 2014).

The Convention of Long-Range Transboundary Air Pollution (CLRTAP) of the United Nations Economic Commission for Europe (UNECE) is leading the process of setting O_3 critical levels (CLE) as policy target values for the protection of vegetation. Initially, O_3 CLE made use of exposure indices based on the atmospheric O_3 concentration (CLe_c) (Kärenlampi and Skärby, 1996). More recently, O_3 stomatal uptake or flux-based CLE (CLe_f) have been developed in recognition that effects are more related with the amount of O_3 being absorbed by the leaves (Emberson et al., 2000a; Mills et al., 2011). CLe_f are used within the CLRTAP for performing O_3 risk assessments where the O_3 stomatal fluxes, also known as Phytotoxic O_3 Doses (POD_y), are computed by the Deposition of Ozone and Stomatal Exchange model (DO_3SE).

The current DO_3SE model configuration computes POD_y values for a big-leaf of a single species (Emberson et al., 2000a; CLRTAP, 2010). However, this represents a limitation when the model is used to assess the risk of O_3 effects on plant communities composed by different species like pastures. The community-level response to O_3 is the aggregated response of the component species and the existing interactions between them (Bassin et al., 2007; González-Fernández et al., 2008; Chapter 4). Besides, pasture species often present a great range of stomatal conductance (g_{sto}) values (e.g. González-Fernández et al., 2010). Thus, the O_3 risk

assessment methodology for pastures could be improved by using POD_y estimates that integrate the species composition of the pasture.

Mediterranean annual pastures are one the most valuable ecosystems in Southern Europe. In the Iberian Peninsula, annual pastures constitute the understory of Dehesas, a traditional agroforestry system (Montado in Portugal) covering nearly 20.000 km² (Díaz et al., 2007). Dehesas present a high vascular plant richness estimated at 230 species (Garcia del Barrio et al., 2014). This diversity is comparatively higher than other forest and agroforestry ecosystems from temperate climates (Díaz et al., 1999; Ojeda et al., 2000; Wagner et al., 2000). Dehesas are located in areas frequently exceeding current European air quality limit values (EU Air Quality Directive 2008/50/EC) for the protection of ecosystems. The particular climatic conditions of the Mediterranean area with high temperature and solar radiation and stable atmospheric conditions favour photochemical reactions that produce O₃ (Millán et al., 2000; Cristofanelli and Bonasoni, 2009). This results in some of the highest surface O₃ concentrations in Europe (EEA, 2014).

Ozone has been recognized as an important negative factor affecting the yield, reproductive capacity and species composition of annual pasture species (Bermejo et al., 2003; Gimeno et al., 2004a, b; Chapter 3 and 4). These studies also showed how atmospheric nitrogen (N) deposition interacts with O₃, where N can mitigate the O₃-induced effects and O₃ reduces the efficiency of the N fertilization. Nonetheless, there is still the need to expand the experimental basis for evaluating the effect of O₃ and its interaction with N under close to field conditions and to increase the information available about the ecophysiology and gas exchange behavior of annual species for developing more robust CLe_f.

In this study, a new O₃ and N experiment with annual Mediterranean pastures following the same methodology than in Chapter 3 is presented. The combined experimental results from the two seasons are analyzed considering an improved O₃ flux model considering a multi-species approach and the influence of N on the response of the pasture to O₃. The inclusion of the specific O₃-sensitivity and gas exchange rates of the component species in this new approach will allow us to propose more realistic CLe for Mediterranean annual pastures communities.

The hypotheses were that higher CLe will be obtained with multiple species compared with individual sensitive species due the introduction of O₃-resistant species in the model and that nitrogen availability will modify the response to O₃. The main objectives of this work are: (1) to present a new experiment on the response of an annual community to O₃ and N; (2) to set a parametrization of the multiplicative algorithm of DO₃SE for g_{sto} modelling combining the six assayed species; (3) to estimate the POD_y for a multi-species canopy (POD_{y-MS}); (4) to develop exposure- and dose- response functions for yield considering the multi-species approach and the influence of N availability, and suggesting CLe_c and CLe_f for annual pastures based on this method.

6.2. Material and methods

6.2.1. Experimental design

In February 2011 and 2012 seeds were sown on the ground of 12 open-top chambers (OTC) located in central Spain (450 m.a.s.l.; 40°3'N, 4°26'W). Plants were exposed to four O₃ treatments from 7:00 to 15:00 GMT, seven days a week (3 OTC per O₃ treatment): charcoal filtered air (FA), non-filtered air (NFA) reproducing ambient levels, non-filtered air supplemented with 20 nl l⁻¹ O₃ (NFA+) and non-filtered air supplemented with 40 nl l⁻¹ O₃ (NFA++). Three ambient plots (AA) were also considered. Ozone was supplemented by a generator (Model 16, A2Z Ozone Systems Inc., USA) system fed with pure oxygen. A time sharing system registered the pollutants levels inside the OTCs and AA plots (O₃, SO₂ and NO_x) above the canopy (50 cm above the soil). All plots were divided in three sectors (1.4 m² each) following a split-plot design with three N treatments: N-low (soil N background), N-medium (N addition equivalent to 20 kg N ha⁻¹ y⁻¹) and N-high (40 kg N ha⁻¹ y⁻¹). To reach these integrated doses, N supplementation was applied every 2-weeks using a NH₄NO₃ solution.

Micro-meteorological conditions were continuously monitored inside the different chambers: air RH and temperature, photosynthetic active radiation (PAR), soil relative humidity and temperature at 10-15 cm depth. More details of the experimental design can be found in Chapter 3.

6.2.2. Plant material and harvests

The simplified pasture community was composed of six representative species from Dehesa acidic annual grasslands. Three legumes (*Trifolium striatum*, *Trifolium cherleri*, *Ornithopus compressus*), two grasses (*Briza maxima*, *Cynosurus echinatus*) and one forb (in 2011 *Silene gallica*; in 2012 *Petrorhagia nanteuili*) were sown by hand using a mixture of seeds on 11th and 27th February in 2011 and 2012 respectively. More details about the plant material preparation and sowing can be found elsewhere (Chapter 3). Plant emergence was in 25th February 2011 and 9th March 2012 and considered as day 0 after emergence (DaE). Exposure to the different O₃ treatments started in April, 47 DaE (2011) and 57 DaE (2012). On the same date the first N fertilization dose was applied. The pasture was exposed to O₃ for 49 and 56 days in 2011 and 2012 respectively until the community reached its maximum development and productivity. Then the O₃ exposure system was switched off allowing plants to dry up and complete seed maturation, which happened in late May and early June in 2011 and 2012 respectively.

Aboveground biomass was harvested three times throughout the life span of the pasture in both years. The first harvest was done just before the start of O₃ treatments and N applications. The homogeneity of the species distribution between the sub-plots was tested at this harvest. The second harvest was collected after 39 (2011) and 38 (2012) days of O₃ exposure, at 84 DaE in both years, when the pasture reached its maximum biomass and blooming development; and the last harvest was done when plant biomass was completely dry and seeds had reached maturity, 116 DaE (2011) and 115 (2012). For each harvest, all plants within a 5 dm² sampling ring were cautiously collected in the central part of each treatment to avoid any border effect in the sub-plot. The biomass was sorted into species and immediately weighed to obtain the fresh weight. Afterwards, samples were dried to constant weight at 60 °C. The canopy yield was the sum of the dry weight of the different species expressed as biomass per square meter.

6.2.3. Gas exchange measurements

Stomatal conductance to water vapor was measured in both seasons using a LICOR-6400 infrared gas analysis system (LiCor Inc., Lincoln, NE, USA). The devices were calibrated by the company every year before the start of the measurements. Gas exchange measures were performed throughout the growing seasons under different environmental conditions. Only fully expanded leaves were considered. At each gas exchange sampling campaign, 2 leaves per plant and 3 plants per species were measured per each O₃ and N plot. Plants were measured under temperatures in the range 15-35 °C, 0.6-5.5 kPa of vapor pressure deficit (VPD) and photosynthetic active radiation (PAR) at saturating light (1000-1500 µmol m⁻² s⁻¹). Light curves were also measured following the PAR sequence: 2000, 1500, 1000, 500, 300, 100, 30, 0 µmol m⁻² s⁻¹. A final database of 1670 individual leaf measurements (35% *T. cherleri*, 24% *T. striatum*, 18% *B. maxima*, 9% *O. compressus*, 9% *P. nanteuili* and 4% *S. gallica*) was used to characterize the stomatal conductance of the species tested in this study. Leaf area inside the chamber was determined using WinFOLIA Pro 04 software (Régent Instruments Inc., Canada).

6.2.4. Stomatal conductance modeling

The g_{sto} was modelled for individual species using a multiplicative algorithm based on the methodology described by Jarvis (1976) and modified by Emberson et al. (2000b) and CLRTAP (2010) (Equation 1):

$$g_{Spi} = g_{max} \cdot f_{light} \cdot f_{phen} \cdot \max\{f_{min}, (f_{temp} \cdot f_{VPD} \cdot f_{PAW})\} \quad \text{Equation 1.}$$

Where g_{Spi} is the stomatal conductance of the species i (mol H₂O m⁻² s⁻¹); g_{max} is the species-specific maximum g_{sto} (mol H₂O m⁻²s⁻¹) expressed on a projected leaf surface area basis. g_{max} is calculated as the mean maximum g_{sto} values above the 90th percentile. The remaining parameters are all expressed in relative terms

ranging from 0 to 1 representing the modifying factors of $g_{\max}$: PAR (f_{light}), plant phenology (f_{phen}), relative minimum g_{sto} that occurs during daylight hours (f_{min}), air temperature (f_{temp}), VPD (f_{VPD}), and plant available soil water content (f_{PAW}). The species-specific limiting functions were characterized by boundary line analysis. The boundary lines were adjusted using non-linear regression following the method in González-Fernández et al. (2008). The model was fully re-parameterized according to the g_{sto} measurements of the six species for calculating the O_3 fluxes inside plants.

6.2.5. Ozone exposure and stomatal O_3 fluxes

The O_3 exposure was expressed using the cumulative hourly O_3 concentration at the top of the canopy above 40 nl l^{-1} for all daylight hours (AOT40) between the start of the fumigation and the second harvest (maximum green yield), that is 39 and 38 days in 2011 and 2012 respectively.

Accumulated stomatal O_3 fluxes for multiple species ($\text{POD}_{\text{y-MS}}$) were estimated using the DO_3SE deposition model following the methodology described in the chapter 3 of the CLRTAP Mapping Manual (CLRTAP, 2010). The DO_3SE model was modified to account for multiple species following the big-leaf approach as in the Mapping Manual (CLRTAP, 2010). The multi-specific big-leaf stomatal conductance (g_{sto}) was calculated using the g_{spi} estimates weighted by the relative biomass abundance of each species (AB_{spi}) (Equations 2 and 3).

$$\text{POD}_{\text{y-MS}} = \sum (c_{\text{O}_3} \cdot g_{\text{sto}} \cdot \frac{r_c}{r_b + r_c}) \quad \text{Equation 2.}$$

$$g_{\text{sto}} = \sum (g_{\text{spi}} \cdot \text{AB}_{\text{spi}} \cdot D_{\text{H}_2\text{O}/\text{O}_3}) \quad \text{Equation 3.}$$

Where c_{O_3} is the hourly O_3 concentrations (nmol m^{-3}) measured at the top of the canopy in the different O_3 treatments; $D_{\text{H}_2\text{O}/\text{O}_3}$ represents the difference in

diffusivity between O₃ and H₂O vapor in air, 0.663 (Massman, 1998). The r_b (s m⁻¹) represents the quasi-laminar boundary layer resistance (CLRTAP, 2010), which was also modified to account for the different leaf shapes of the species present in the canopy (equation 4).

$$r_b = 1.3 \cdot 150 \cdot \sqrt{\frac{\sum(L_{spi} \cdot AB_{spi})}{u(z)}} \quad \text{Equation 4.}$$

The values of L_{spi} (m) are the cross-wind leaf dimension of each species weighted by their biomass abundance; and u is the constant wind speed inside the OTC $u(z) = 0.55$ m s⁻¹ (Heagle et al., 1988). r_c (s m⁻¹) is the leaf surface resistance accounting for both non-stomatal and stomatal deposition of O₃ (equation 5).

$$r_c = \frac{1}{g_{sto} + g_{ext}} \quad \text{Equation 5.}$$

The value for g_{ext} has been simply scaled as $g_{ext} = 1/2500$ (m s⁻¹) as recommended in the Mapping Manual (CLRTAP, 2010). POD_{y-MS} values were accumulated from the start of the fumigation period until the second harvest just before the start of the senescence period.

6.2.6. Response functions and ozone critical level calculation

Dose and exposure-response relationships were established based on linear regressions between modelled POD_{y-MS} or AOT40 respectively and the total aboveground biomass (green+senescent) averaged across N treatments and also for individual N input levels. Yield losses were expressed in relative terms to the control treatment FA (relative yield 100%). Dose-response functions for the average response were calculated considering a range of flux thresholds (y), from 0 to 6 nmol m⁻²s⁻¹, in order to test the best correlation. These response functions

and their confidence intervals were used to calculate O_3 critical levels following the methodology of the CLRTAP for semi-natural vegetation (CLRTAP, 2010), considering a range of effect thresholds from 5 to 20% of yield loss. 95% Confidence intervals of the CLe were obtained following the methodology described in González-Fernández et al. (2014).

6.2.7. Statistical analysis

The treatment effects on the aerial biomass were analyzed considering O_3 as the main factor in a randomized block design and N as the split-plot. A-priori comparisons were used to test the statistical significance of the trends between treatment levels considering both linear and quadratic responses. A p-value under 0.1 was considered significant due the high variability of the data. Normal distribution and homogeneity of data variance were checked before the statistical analyses and data were log-transformed when assumptions were not fulfilled.

The goodness of fit of the individual species parameterizations to estimate gs_{spi} was evaluated using metrics commonly employed in model evaluation (Yu et al., 2006; García-Gómez et al., 2014). Based on the information available in the literature, the tipping points used to evaluate the parameterization performance were: index of agreement (IOA) ≥ 0.70 (EMEP, 2012); mean fractional bias (MFB), $-30\% \leq MFB \leq 30\%$ (Boyland et al., 2006); and Pearson correlation coefficient $r > 0.7$ (DEFRA, 2013). Finally, the root mean square error (RMSE) is a not normalized error metric expressed in the units of the measurement. Statistical analyses were performed using Statistica v12 (StatSoft Inc., Tulsa, USA).

6.3. Results

6.3.1. Inter-annual variability of meteorological conditions and O_3 concentrations

The two growing seasons presented contrasting environmental conditions for plant development (figure A.II.1a,b; table A.II.1): 2011 was wetter (148.1 l m^{-2}) compared with 2012 (78.8 l m^{-2}) but both were below the long-term average for

the site during the same season (1982-2010; 155 l m⁻²) (AEMET, 2012). The first year was also colder (16.1°C) than the second year (16.4°C), 1.3 and 1.6°C respectively above the long-term average (14.8°C) for this site. As a result, contrasting soil moisture regimes were observed during the two growing seasons: in 2011, PAW values inside the OTCs stayed above 45% over the whole season; on the contrary, mean PAW values in 2012 were below 50%, a threshold where it is considered that drought induced growth restrictions start. Thus, 2012 can be described as a dry year.

Ozone levels expressed as 24-hour mean from nascence to maturity (February-June) were higher during 2012 for all the O₃ treatments (table 6.1); the ambient levels for this period were 24% higher compared with the first year. The AOT40 value in 2012 from the start of the O₃ treatments until the second harvest more than doubled the value of the index in 2011 in ambient air (1251 and 3218 nl l⁻¹ h for 2011 and 2012 respectively). Charcoal filtration reduced the mean O₃ ambient levels in FA by 28.6% and 33.3% for 2011 and 2012 respectively. It should be considered that in 2012, the OTCs remained open until the start of the fumigation, contributing to the greater differences found between years when the period between nascence and the start of the fumigation is considered.

Table 6.1. Ozone concentrations and AOT40 exposure indexes for the different O₃ treatments throughout both growing seasons and two periods: 1- from nascence to senescence of the pasture and seed maturity (February 25 – June 20 in 2011; and March 9 – July 2 in 2012); 2- from start of the fumigation to the second harvest (April 12 – May 20 in 2011; April 24 and June 1 in 2012). AA = Ambient plots; FA = charcoal filtered air; NFA = non filtered air; NFA+ = non filtered air + 20 nl l⁻¹ of O₃; NFA++ = non filtered air + 40 nl l⁻¹ of O₃.

Ozone indexes	24 h-mean (nl l ⁻¹)		Hourly maximum (nl l ⁻¹)		Nascence-Maturity AOT40 (nl l ⁻¹ h)		Start fumigation- 2 nd Harvest AOT40 (nl l ⁻¹ h)	
	2011	2012	2011	2012	2011	2012	2011	2012
AA	29	36	68	73	3780	9907	1251	3218
FA	20	24	48	70	174	4911	3	14
NFA	28	36	65	78	2805	8585	760	2080
NFA+	33	39	138	120	9205	16925	5771	8945
NFA++	34	43	189	169	14257	26310	10316	13873

6.3.2. Aboveground biomass

The pasture growth before the start of the treatments (harvest 1) was homogenous for all the plots in both growing seasons (table 6.2). The differences between years started from the first harvest: at this time the yield in the first year (86 ± 5 gDW m⁻², mean value across O₃ and N treatments) was lower than the second (100 ± 6 gDW m⁻²). The pattern changed in spring and the second harvest yielded a higher biomass in 2011 (406 ± 9 gDWm⁻²) compared with 2012 (377 ± 7 gDWm⁻²) (figure A.II.1). At this second harvest, when the pasture showed its maximum green biomass, the exposure to O₃ induced a significant loss in both years following a linear pattern, though the effects were less severe in 2012 (table 6.2). The first year, the pollutant caused a 21% loss in NFA++ compared with FA while in the second year this reduction was just a 12%. The N fertilization did not cause any significant effect on the yield. However, a significant O₃ and N interaction was found following the same pattern on both years (figure 6.1 and table 6.2), although differences among levels of the factors were lower for 2012. As detailed below, the fertilizer modified effect of O₃ on the aboveground biomass and, simultaneously, the pollutant reduced the N efficiency. Treatment effects on yield during the first growing season are described in detail elsewhere (Chapter 3).

Figure 6.1. Total aboveground biomass for the different O₃ and N treatments at the second harvest for both growing seasons (2011 and 2012). FA= charcoal filtered air, NFA=non filtered air, NFA+=non filtered air supplemented with 20 nl l⁻¹ of O₃, NFA++ = non filtered air supplemented with 40 nl l⁻¹ of O₃. N-low = soil N background, N-medium = +20 kg N ha⁻¹; N-high = +40 kg N ha⁻¹.

Table 6.2. Treatment effects (p-values) on total aboveground biomass across the three harvests: 1-harvest at the start of the O_3 treatments, 2-harvest at maximum green biomass development, 3-harvest at seed maturity, plants dry. P-values were calculated using planned comparisons to test the statistical significance of the observed trend through the levels of the factors (increment or reduction) and the interaction. Significant differences for $p < 0.1$ are highlighted in bold.

Harvest	Factors	2011		2012	
		Linear	Quadratic	Linear	Quadratic
1	<i>N</i>				
	O_3	0.808	0.951	0.835	0.432
	$N \times O_3$				
2	<i>N</i>	0.260	0.800	0.176	0.877
	O_3	<0.001	0.950	0.012	0.450
	$N \times O_3$	0.016		0.091	
3	<i>N</i>	0.373	0.397	0.234	0.322
	O_3	0.001	0.018	0.978	0.171
	$N \times O_3$	0.120		0.964	

A third aboveground biomass harvest was collected at the end of the life span when all the biomass was completely dry. The mean values across O_3 and N treatments were 442 ± 16 (se) g dw m^{-2} in 2011 and 334 ± 11 (se) g dw m^{-2} in 2012. At this stage, in 2011 a 19% yield reduction was observed in NFA++ compared with FA. No significant effects were observed in 2012, neither N effects nor interaction between both factors.

6.3.3. DO_3SE model parameterization

The parametrizations of the g_{spi} model for the six annual species is shown in table 6.3. Due to the scarcity of gas exchange measurements on the grass *C. echinatus*, the multiplicative model was not parameterized for this species. Different g_{max} values were obtained. The mean g_{max} was $1.33 \text{ mol H}_2\text{O m}^{-2}\text{s}^{-1}$ with a range (1.13, 1.49) for all the species except for the *Gramineae B. maxima* with a $g_{max} = 0.29 \text{ mol H}_2\text{O m}^{-2}\text{s}^{-1}$.

The performance metrics (table A.II.2) showed that the parameterized models fitted generally well with the field measurements and can be considered acceptable for modelling $g_{s_{spi}}$, although some differences were found among species. The IOA values were above 0.70 (good agreement) except for *T. cherleri* and *S. gallica*, though for the second species this figure was 0.67. However, the MFB for this two species were 22 and -27%, both within the good performance threshold. For all the species modeled, the MFB values ranged -35% to 22%, which is considered as an acceptable to good performance. RMSE values averaged $0.30 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ (23% of the averaged g_{max}), excluding *B. maxima*, which obtained a RMSE of $0.05 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ (16% of its g_{max}). The Pearson correlation coefficient was considered good except in *T. cherleri*, *T. striatum* and *P. nanteuili*, though all $r \geq 0.47$.

Table 6.3. Parameterization of the multiplicative model to estimate g_{sto} for the annual species tested growing under field conditions.

Function	Parameter	Units	<i>T. cherleri</i>	<i>T. striatum</i>	<i>O. compressus</i>	<i>B. maxima</i>	<i>S. gallica</i>	<i>P. nanteuili</i>
g_{max}		mol H ₂ O m ⁻² s ⁻¹	1.35	1.13	1.44	0.29	1.26	1.49
f_{min}		-	0.040	0.055	0.030	0.060	0.027	0.040
f_{light}	α	-	0.007	0.002	0.007	0.002	0.001	0.008
f_{phen}	A_{start}	DoY	56	56	56	56	56	68
	A_{end}	DoY	216	216	216	216	216	228
	$f_{phen\ a}$	DaE	20	20	40	40	20	20
	$f_{phen\ b}$	DaE	60	60	55	60	45	60
	$f_{phen\ c}$	DaE	70	80	65	65	70	90
	$f_{phen\ d}$	DaE	120	120	120	120	120	120
	L	m	0.02	0.02	0.02	0.006	0.0065	0.0065
f_{temp}	T_{min}	°C	13	15	11	14	18	11
	T_{opt}	°C	22	21	18	19	25	25
	T_{max}	°C	36	38	35	42	30	35
f_{VPD}	VPD_{max}	kPa	1.1	1.9	1	0.5	1	2.1
	VPD_{min}	kPa	4.8	4.4	2.9	4.2	2.8	4.3
f_{PAW}	PAW_{max}	%	90	103	90	91	76	85
	PAW_{min}	%	-30	41	-2	-22	-33	-15

g_{max} is the maximum g_{sto} expressed on projected leaf surface area basis. f_{min} is the fraction of g_{max} at minimum g_{sto} ; α is the rate of saturation of g_{sto} in response to photosynthetic active radiation; A_{start} and A_{end} are the start and the end of the growing season corresponding to the emergence and senescence of the plants in days of the year (DoY). $f_{phen\ a}$ is the date after emergence with minimum f_{phen} ; $f_{phen\ b}$ is the date after emergence when starts maximum f_{phen} ; $f_{phen\ c}$ the date after emergence when ends maximum f_{phen} ; $f_{phen\ d}$ is the date after emergence with minimum f_{phen} again; L is the leaf surface area; T_{min} and T_{max} denote the temperatures below and above g_{sto} is limited to f_{min} ; T_{opt} is non-limiting temperature for g_{sto} ; VPD_{max} and VPD_{min} define the level when vapour pressure deficit starts to limiting g_{sto} and f_{min} are reached respectively; PAW_{max} and PAW_{min} are the minimum non-limiting soil water content and the value below which g_{sto} is limited to f_{min} respectively.

6.3.4. Accumulated stomatal O_3 fluxes

During the first growing season POD_{0-MS} fluxes increased steadily from the start of the O_3 fumigation until the 2nd harvest (figure 6.2a). The maximum increment was between DoY 112 and 128, following an intense precipitation event and coinciding with the maximum growth rate of the plants. The 2012 season showed a distinct pattern (figure 6.2c); increasing fluxes also coincided with maximum plant growth until DoY 132 but a sudden interval of high temperatures (24 h-mean 25°C, temp max 39°C) caused the POD_{0-MS} to stabilize between DoY 132 and 140. Towards the end of this hot period the pasture was watered, decreasing the water stress and resuming the increment of the O_3 flux accumulation until the harvest. Modeled POD_{0-MS} values from fumigation start until the 2nd harvest were higher during 2011 than in 2012. POD_{0-MS} reached fluxes of 5.3 and 2.8 in FA; 8.6 and 6.6 in NFA; 12.7 and 7.6 in NFA+, and 14.8 and 11.6 mmol m⁻² in NFA++ in 2011 and 2012 respectively. The differences between years cannot be explained by the differences in the O_3 levels; for example, the AOT40 index for NFA was 2.7 times higher in 2012 than 2011 over the same period (table 6.1), while POD_{0-MS} was 1.3 times smaller.

The relative contributions of each species to the total POD_{0-MS} in the NFA treatment were shown in figure 6.2 b,d. *T. cherleri* represented a 50% and a 33% of the total flux, *T. striatum* a 16% and 6%, *O. compressus* 25% and 8%, *B. maxima* 3% and 6% and *S. gallica* or *P. nanteuli* a 5% or 46% in 2011 and 2012 respectively. These percentages varied depending on the O_3 treatment the POD_{y-MS} flux threshold or year considered. Nevertheless, the species with the highest g_{max} and biomass abundance such as *T. cherleri*, *O. compressus* or *P. nanteuli* had the greatest relevance to the total flux. The relative contribution of the species to the POD_{0-MS} in the rest of the O_3 treatments is shown in figure 2.II.A of the Appendix.

Figure 6.2. Phytotoxic O_3 dose following the multi-specific big-leaf approach without threshold (POD_{MS-0}) in 2011 (a) and 2012 (c). Contribution of individual species to the POD_{0-MS} modelled in NFA from the start of the fumigation until the second harvest in 2011 (b) and 2012 (d). FA = charcoal filtered air, NFA = non filtered air, NFA+ = non filtered air supplemented with 20 $nl\ l^{-1}$ of O_3 , NFA++ = non filtered air supplemented with 40 $nl\ l^{-1}$ of O_3 .

6.3.5. Ozone exposure- ($AOT40$) and dose- (POD_{y-MS}) yield response functions

Averaging across N treatments, the response functions based on the $AOT40$ index showed a poorer performance compared with flux based POD_{y-MS} functions (figure 6.3, table A.II.3). The $AOT40$ index showed a better agreement with the effects on biomass in 2011 ($R^2=0.84$, $p=0.08$) compared with 2012 ($R^2=0.36$, $p=0.40$). Considering both years, the R^2 value was 0.41 but the function was not statistically significant at the 95% confidence level ($p=0.09$) (table A.II.3). All the POD_{y-MS} indices (considering flux thresholds ranging 0-6 $nmol\ m^{-2}\ s^{-1}$) performed better than the $AOT40$. The POD_{3-MS} showed the best R^2 and p-value for the two years (table A.II.3). However, the differences among POD_{y-MS} indices were slight, R^2 ranging from 0.76 till 0.80, and with all the p-values significant ($p<0.005$).

Figure 6.3. Exposure (AOT40, nl l⁻¹ h) and dose (POD_{MS-1} mmol m⁻²) response functions for the aboveground biomass of the canopy. Dashed lines indicate the 95% confidence interval.

The exposure- and dose-response relationships changed depending on the N treatment level considered. When AOT40-based relationships were considered, the N-low and N-medium sub-plots did not yield a statistically significant relationship ($p=0.405$ and $p=0.255$). However, in N-high, a linear relationship was

found between the relative aboveground total biomass and AOT40: $y = -0.011 \cdot \text{AOT40} + 0.980$ (slope confidence interval $(-0.022, 0.000)$, $R^2 = 0.52$, $p = 0.045$). $\text{POD}_{1\text{-MS}}$ relationships were again not significant in N-low ($p = 0.502$) but yielded significant linear regressions in N-medium, $y = -0.026 \cdot \text{POD}_{1\text{-MS}} + 1.07$ (slope confidence interval $(-0.038, -0.014)$, $R^2 = 0.83$, $p = 0.002$), and in N-high, $y = 0.015 \cdot \text{POD}_{1\text{-MS}} + 1.03$ (slope confidence interval $(-0.030, -0.001)$, $R^2 = 0.52$, $p = 0.043$).

Different CLe were obtained, averaging the effect of N, depending on the % effect relative to the control treatment considered (table A.II.3). Ozone CLe_f using the multi-species approach (CLe_{f-MS}) increased between 26% and 3-fold with each 5% increment in the effect. The smallest CLe_{f-MS} change for a 5% increase in the effect (26% increase) was obtained using the $\text{POD}_{0\text{-MS}}$ between the 15 and 20% effect. The highest change (3-fold increase) was found using the $\text{POD}_{6\text{-MS}}$ when moving from the 5 to 10% effect. The ranges of the 95% confidence intervals of the CLe_{f-MS} were the smallest for a 10% effect and decreased with increasing flux threshold from $\text{POD}_{0\text{-MS}}$ to $\text{POD}_{6\text{-MS}}$.

Accordingly with the current CLRTAP methodology for calculating CLe_f for the protection of the semi-natural vegetation (CLRTAP, 2010), and in agreement with the results, a $\text{POD}_{1\text{-MS}}$ and an effect threshold of 10% were chosen to derive the CLe_{f-MS}. The CLe_{f-MS}, following the multi-species modelling approach proposed in the present study, was set at $\text{POD}_{1\text{-MS}} = 7.9 \text{ mmol m}^{-2}$ with a 95% confidence interval of $\pm 25\%$ (5.9-9.8) (figure 6.3). No AOT40-based CLe_{c-MS} can be proposed due to the lack of statistical robustness.

6.4. Discussion

The O_3 CLe set by the CLRTAP for the protection of herbaceous communities (semi-natural vegetation), including Mediterranean pastures, are considered as the most uncertain (CLRTAP, 2010). Several causes have been recognized to explain this uncertainty: the high diversity of herbaceous communities and the low number of studies about their O_3 -sensitivity, the inter-specific variability in the gas exchange rates (and thus in O_3 flux), the genotypic variability in O_3 -sensitivity between and within species, the importance of soil

moisture (which is frequently poorly characterized in the flux models), and the interactions between O₃ effects and plant competition, management or other pollutants (Bassin et al., 2007; Davison and Barnes 1998; Gonzalez-Fernandez et al 2010). The present study contributes to filling some of these gaps for Dehesa annual pastures developing O₃ CLe based on a multi-species approach and considering the influence of N availability.

6.4.1. Ozone and nitrogen effects on yield

Previous studies have shown differences in O₃ sensitivity among Mediterranean annual species (Bermejo et al., 2003; Gimeno et al., 2004a). These studies based on species tested individually or in simple mixtures generally showed that species from the *Gramineae* family are more tolerant to O₃ than *Leguminosae* species, though some legumes are also tolerant to the pollutant (Gimeno et al., 2004a). However, the interspecific competition can modulate the response to O₃ at the community level (Bassin et al., 2007) and it needs to be considered for a more realistic assessment of O₃ effects under field conditions.

The present study was based on a mixture of representative species of Mediterranean annual Dehesa pastures with different sensitivities to O₃. Negative O₃ effects were found in both years, although they were slighter in the second one. These differences, as discussed below, cannot be explained by inter-annual variations in O₃ levels because higher O₃ concentrations were recorded in 2012. Other factors like the different growing conditions affecting the stomatal O₃ flux can help to explain the variability in the response to O₃.

Interestingly, the N input also modulated the response of the pasture to the O₃ exposure. Ozone and N interactions have been reported for several annual species growing under Mediterranean conditions, *Trifolium striatum*, *T. cherleri*, *Briza maxima* and *Bromus hordeaceus*, but not in *T. subterraneum* (Sanz et al., 2005, 2007, 2011, 2014, 2015). In this experiment with multiple species the fertilizer also modified the effect of O₃ on the aboveground biomass: differences between O₃ levels were not found in N-low but were present in N-medium and N-high in 2011 (Chapter 3). Although weaker, a similar trend was observed in 2012,

as indicated by the significant O₃ and N interaction (Table 6.2). At the same time, the exposure to O₃ reduced the N-efficiency. Nitrogen induced increases in aboveground biomass observed under FA and NFA O₃ treatments disappeared under NFA++ O₃ exposures. These are interesting findings because current studies for assessing the impact of O₃ do not consider the availability of N as a potential modifier of the response of semi-natural vegetation communities, probably due to the contrasting results found in the literature (Bassin et al., 2007).

During the first year, O₃ effects on the yield of the pasture can be explained through an alteration of the competitiveness among species, stressing the importance of the multi-species studies for assessing the effect of O₃ under field conditions. The O₃-induced biomass loss in the sensitive clover *T. striatum* was partially counterbalanced by the increment of the O₃-tolerant legume *O. compressus* (Chapter 4). This legume to legume interaction is in contrast with previously published studies that report O₃ induced changes in grass-legume competition due to the usually higher O₃-tolerance of the grasses (Davison and Barnes 1998; Hayes et al., 2009; Weldlich et al., 2012). Several reasons have been proposed to explain the inter-specific variability in O₃ sensitivity, such as differences in maximum g_{sto} (Bassin et al., 2007). In the present experiment, the variability in the O₃ sensitivity of the different species does not seem to be related with their g_{sto} values and pollutant uptake rates, at least at the maximum growth rate season, when the measurements were recorded. For instance, similar g_{sto} values were recorded for *T. striatum* and *O. compressus*, and even the later presented the highest g_{max}. Nevertheless, the annual species measured in this study presented in general high g_{max} values compared with other perennial herbaceous species (CLRTAP, 210). This is in agreement with their fast growing rate in spring and can be the cause of the relatively high O₃ sensitivity of the annuals compared with other grassland communities (Bassin et al., 2007). Other variables like the detoxification capacity, the specific leaf area or the resource allocation and partitioning are also important in determining plant responses to O₃ (Heath and Taylor, 1997; Ashmore, 2005; Bassin et al., 2007) and should be addressed in the future to identify the traits related with O₃ tolerance.

6.4.2. Stomatal conductance and multi-species O_3 flux modelling

This study presents 6 new species-specific parameterizations of the DO₃SE model based on over 1600 individual leaf measurements. This constitutes an important contribution because the estimation of g_{sto} is one of the key issues for developing CLe_f and for performing O_3 flux-based risk assessments. Based on the performance metrics, the model estimations fitted the measurements reasonably well and, therefore, the parametrizations can be considered statistically adequate. The poorest estimation was achieved for *T. cherleri* with a low correlation coefficient ($r=0.47$) between modelled and measured values. However, the index of agreement for this species was similar to the other species (IOA=0.64) and the mean fractional bias was at acceptable levels (MFB=22%). The parametrizations which yielded the best agreement with observations based on all the metrics were those for *O. compressus* and *B. maxima* though presenting mean biases over 30%.

Previous efforts have been done for developing species-specific parameterizations of the DO₃SE model for modelling O_3 stomatal fluxes for annual species (Alonso et al., 2007; González-Fernández et al., 2010). However, these single species approaches have limitations when complex plant communities like annuals are considered (González-Fernández et al., 2010). The flux modelling scale should be related to the nature of the impacts to be assessed and new methods for up-scaling the models for complex ecosystems are needed (Ashmore et al., 2007). The approach followed in the present study is based on the big-leaf method, following the current DO₃SE model configuration, but it has been modified for taking into account the inter-specific variability in the gas exchange rates of the different species present in the canopy. Each species is represented by a single parameterization showing its own g_{spi} range and response to the environmental variables but weighted using the biomass abundance of the species in the calculation of a composite big-leaf g_{sto} . The different contribution of each species to the final flux was not strictly related to their biomass abundance. The g_{max} and the response to the growing conditions played also an important role. *T. cherleri* was the most abundant species in both growing seasons, but it was the highest contributor to the final flux only in 2011, while *P. nanteuili* took this position in 2012. The higher g_{max} of the latter and its greater tolerance to the high VPD

conditions of the 2012 drier season can explain this result. The clover *T. striatum* decreased its abundance by around 20% in 2012 compared with 2011, but the corresponding change in O_3 -fluxes reached only a 12% of reduction. On the contrary, the grass *B. maxima* increased its abundance by 15% in 2012 compared with 2011, favored by the more stressful conditions of the second season, while its contribution to the final flux in 2012 only increased by 2.5%. Due to its low g_{max} the contribution of this grass was only 3.1 and 5.6% to the final flux for 2011 and 2012 respectively.

The different meteorological conditions and O_3 concentrations observed in the two years reflected the high inter-annual variability of the plant growing conditions typical of the Mediterranean climate. These differences had an influence on the biomass growth which was 7% and 24% lower for the 2nd and 3rd harvest respectively in 2012 compared with 2011. These differences also caused great inter-annual variations in the gas exchange rates of the annual species. This has been already observed under field conditions, where inter-annual differences in gas exchange rate in one species can be of the same range than the differences observed between species growing in the same pasture (González-Fernández et al., 2010).

The inter-annual differences were also observed in O_3 concentrations. AOT40 values recorded in the drier season of 2012 in NFA were 3.1 times higher compared with the year 2011 whereas the resulting POD_{0-MS} was 23.5% lower, mostly caused by the soil water shortage in 2012.

6.4.3. Response functions and critical levels

It is widely acknowledged that O_3 effects are more closely linked to the stomatal uptake of O_3 , than to the O_3 concentration in the air (Musselman and Massman, 1999; Massman et al., 2000; Wieser, 1997; Matyssek et al., 2004). In the present study the lower POD_{y-MS} modelled for the year 2012 was related with the lower O_3 effects for that year. As a result, the POD_{y-MS} values showed a better correlation with the pasture relative yield than the AOT40 index over the two years. In fact, the exposure-response function based on the AOT40 index was not

statistically significant, because of the weak relationship between AOT40 and biomass in 2012. This result stresses the advantage of using flux-based O₃ risk assessments, especially under water limiting conditions.

The N input also influenced the response functions in different ways depending on the O₃ index considered. Although the averaged biomass response across N treatments was not related with the AOT40, the N-high data resulted in a significant exposure-response relationship. In contrast, the POD_{1-MS} functions showed a stronger and more O₃ sensitive relationship for N-medium data than for N-high, though 95% confidence intervals of both slopes overlapped substantially. The influence of N inputs on O₃ exposure- and dose-response relationships implies that N can modify the O₃ CLe. However, this is an aspect that has not been considered so far in the methodologies for establishing O₃ CLe. The CLe_{f-MS} and the confidence intervals were derived in this study from the dose-response relationships averaging across N input levels and, thus, they are considered to include the effect of N.

Once the dose-response relationship has been estimated, there are two more key parameters for deriving a CLe_f: the effect threshold and the flux threshold (y-value). Their selection affects greatly the value of the CLe_f and, thus, the conclusion of the risk assessments. The CLe_{f-MS} derived in this study used a 10% effect in agreement with the recommendations of the CLRTAP for semi-natural vegetation receptors (CLRTAP, 2010). However, the high variability of the CLe_{f-MS} for every change in the effect threshold highlights the need of an open debate about the ecological relevance of the effect thresholds considered for setting O₃ CLe_{f-MS} for grassland communities. The second factor affecting the derivation of CLe_f is the flux threshold. Here, a range of flux thresholds were tested. Although the POD_{3-MS} was the index which presented the best correlation with the relative yield, the results presented here show that the CLe_{f-MS} are more stable using lower flux thresholds for every change in the effect relative to the control treatment, suggesting that risk assessments performed using the lowest flux threshold would yield the most robust results. Thus, in agreement with the current CLe_f for perennial grassland species of the CLRTAP (CLRTAP, 2010), it is proposed

to keep the flux threshold at $1 \text{ nmol m}^{-2} \text{ s}^{-1}$ for $\text{CLe}_{\text{f-MS}}$ derivation for Mediterranean annual pastures.

The new $\text{CLe}_{\text{f-MS}}$ for Mediterranean annual pastures was set at $\text{POD}_{1\text{-MS}}=7.9 \text{ mmol m}^{-2}$ (5.9, 9.8, CI 95%) for a 10% of biomass reduction. This $\text{CLe}_{\text{f-MS}}$ is higher than the CLe_{f} currently set by UNECE for the protection of perennial herbaceous vegetation, $\text{POD}_1=2$ and $\text{POD}_1=6 \text{ mmol m}^{-2}$ based on results for *Trifolium repens* and *Viola* spp respectively. The differences in the physiological behaviour between perennial and annual species can explain partially the differences in the CLe_{f} : annuals present a great spring growth rate supported by their high gas exchange rates. g_{max} values estimated for annual clover species in this study ranged $1.13\text{--}1.35 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$, greater than the g_{max} of $0.64 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ estimated for white clover growing under Atlantic and Continental climatic conditions (CLRTAP, 2010). These gas exchange rates of Mediterranean species combined with high O_3 concentrations accumulate large $\text{POD}_{\text{y-MS}}$ values in short periods of time.

The $\text{CLe}_{\text{f-MS}}$ is also lower than the recently proposed CLe_{f} for annual Mediterranean species growing individually or in two species mixtures, $\text{POD}_1=12.2 \text{ mmol m}^{-2}$ (8.9, 15.5 CI 95%) (Sanz et al., 2016), though the confidence intervals of the CLe_{f} and $\text{CLe}_{\text{f-MS}}$ for annual species show some overlapping. It should be taken into account that the proposed $\text{CLe}_{\text{f-MS}}$ is not directly comparable with the CLe_{f} for individual species because O_3 -tolerant species have been included in the calculation of the $\text{POD}_{\text{y-MS}}$ and the response functions, while only O_3 -sensitive species are considered in the individual species approach. Further efforts will be needed in the future for coordinating both approaches. For the moment, the advantage of this multi-species approach is that it is able to incorporate the high diversity present in semi-natural vegetation communities regarding biomass production, gas exchange and O_3 sensitivity of the different component species and thus providing a more realistic O_3 risk assessment.

6.5. Conclusions

Ozone exposure caused biomass losses in the annual pasture during the two experimental growing seasons and this effect was modulated by the N input, confirming the first hypothesis of the study. The O_3 effect was greater in N-medium and N-high treatment levels compared to N-low. At the same time, O_3 reduced the N fertilization effect in the pasture. The interaction existing between O_3 and N affected the derivation of the exposure- and dose-response relationships. This result implies that N can modify the O_3 CLe. However, this is an aspect that has not been considered so far in the methodologies for establishing O_3 CLe.

Based on the average response of the pasture across N input levels, the POD_{1-MS} index outperformed the AOT40 in fitting the effects of O_3 on biomass over the two years. A new CLe_{f-MS} for Mediterranean annual pastures based on the POD_{1-MS} accumulated over 1.5 months was set at 7.9 mmol m^{-2} (5.9, 9.8 CI 95%) for a 10% of biomass reduction. This value is lower than the CLe_f proposed for sensitive annual species. However, the two values are not directly comparable because of differences in the calculation of the O_3 fluxes and the influence of inter-specific competition interactions. This multi-species approach can help to incorporate the high diversity of semi-natural vegetation into the CLe derivation and can produce more realistic risk assessment analysis. Further efforts will be needed for comparing the CLe_{f-MS} with the current O_3 CLe based on single species responses.

CHAPTER 7

Ozone risk assessment on Mediterranean annual pastures under contrasting meteorological conditions using MEDPAS and DEHESA pasture growth models

Abstract

Annual Dehesa pastures comprise a valuable ecosystem in southern Europe due to its high biodiversity and extension. Current tropospheric ozone (O_3) concentrations are affecting negatively the yield and quality of component species of Mediterranean annual pastures. The annual pastures' growing period is highly variable between years due to the characteristic high inter-annual variability in meteorological conditions of the Mediterranean area. This variability makes difficult to run models characterizing the pasture life span, gas exchange behavior and to perform air pollution risk assessments.

This study presents a three year experiment set in an intensively monitored Dehesa in the Central Iberian Peninsula. Aboveground biomass samples were collected throughout three growing seasons. Two growth models, MEDPAS and DEHESA, were used to simulate the plant growth and were coupled with another model (DO_3SE) estimating the O_3 stomatal dose. Phytotoxic O_3 dose (POD_y) estimates and measured O_3 concentrations accumulated over 40 ppb during daylight hours (AOT_{40}) were used to perform a risk assessment based on the critical levels (CL_e) developed in O_3 fumigation experiments on open-top chambers.

High inter-annual variability in the meteorological conditions was observed over the three years, which were characterized as humid, normal and dry. Ozone concentrations varied also between years; the highest concentrations were recorded during the dry year and the lowest during the humid year. The differences in environmental conditions induced changes in the pasture growth and composition. Taking into account the growing period the normal year was the only one to exceed the 5% CL_e based on AOT_{40} of $1563 \text{ nl l}^{-1} \text{ h}$. During the dry year, only the lower bound of the CL_e confidence interval was exceeded; and while in the humid year none of the thresholds were reached. Otherwise, the CL_e based on POD_1 for a 5% effect, 4.7 mmol m^{-2} was also exceeded in the normal year. The lower bound of the 5% CL_e was also exceeded during the humid year but not the CL_e itself. The dry year did not exceed any of the O_3 CL_e , which represents a situation of low risk of O_3 induced yield losses based on the POD_1 .

Soil moisture was identified as a key variable for explaining the inter-annual variability in estimated POD_1 values due to the influence on gas exchange rates and growing season duration. The results of the present study shows that under limiting soil moisture conditions the risk of O_3 damage is low even under high O_3 exposure.

The two growth models used to simulate the biomass growth were unable to perform well in all the years but MEDPAS adequately described the start and end of the three growing seasons. Although improvable, both models in combination with the DO_3SE model were useful to estimate the risk by O_3 damage considering the modulation of environmental variables on the growth period and the O_3 stomatal uptake. The POD_y index has greater adaptation than the AOT40 to the high inter-annual variability present in the Mediterranean climate and is the recommended index to perform O_3 risk assessments for annual Mediterranean pastures like dehesas.

7.1. Introduction

Current tropospheric ozone (O_3) concentrations can cause negative effects on the yield, the nutritive quality and the species composition of Mediterranean annual pastures (Chapter 3, 4 and 5). Mediterranean annual pastures are among the most important ecosystems in southern Europe due to its high biodiversity and extension, besides they are currently protected under the 92/43/EEC Habitat Directive. However, habitat conservation plans do not usually consider the impacts of air pollutants.

The deleterious impact of O_3 on the yield of annual pasture species growing individually or in simple mixtures has been previously demonstrated in different studies (e.g. Bermejo et al., 2003; Gimeno et al., 2004a, 2004b; Sanz et al., 2005, 2007). These studies have shown a generally higher sensitivity in the *Leguminosae* species compared with the *Gramineae*, although tolerant species are also found among legumes. In a recent study with a mixture of six representative species of annual pastures exposed to O_3 and nitrogen (N) doses in open-top chambers (OTC), O_3 was able to induce a reduction of the whole canopy biomass even in the presence of O_3 tolerant species (Chapter 3). The differences in the O_3 sensitivity of the species induced a change in the composition of the pasture when it was exposed to high O_3 concentrations and sensitive legume species were outperformed by more tolerant species (Chapter 4).

The critical levels (Cl_e) concept, the concentration or deposition above which deleterious effects of a pollutant can be found on a particular receptor (CLRTAP, 2010), has been used as policy target value for the protection of vegetation in Europe within the Convention on Long-Range Transboundary Air Pollution (CLRTAP) of the United Nations Economics Commission for Europe (UNECE). These Cl_e have been used to perform O_3 risk assessments to evaluate the benefits associated with the air pollution emission reduction Protocols established by the CLRTAP. Since the 1980s the O_3 risk assessments have been performed by means of exposure indices based exclusively on the atmospheric O_3 concentration (Kärenlampi and Skärby, 1996). Different concentration-based indices have been developed but the most commonly used among European researchers is the AOT40 index. AOT40 is the sum of the differences between the hourly mean O_3

concentration at the top of the canopy and 40 nl l^{-1} for all daylight hours during the growing season. More recently, O_3 indices based on the estimation of the stomatal uptake, or flux-based Cl_e has been recognized as a more suitable index to evaluate the real impact of O_3 in plants because it is more biologically sound, it is better related with O_3 effects and considers the modulating effect of different environmental conditions on the O_3 sensitivity of the plant (Emberson et al., 2000a; Mills et al., 2011). The flux approach for Cl_e derivation relies on the ability to adequately estimate stomatal conductance (g_s) and the availability of species-specific dose-response relationships. The stomatal uptake of O_3 at leaf level over the sensitive phase of plant development, or Phytotoxic O_3 Dose (POD_y), is modelled within the CLRTAP framework using the Deposition of Ozone and Stomatal Exchange model (DO_3SE) (CLRTAP, 2010).

The DO_3SE model is currently included within the European Monitoring and Evaluation Programme (EMEP) photo-oxidant chemical transport model, which is used by the CLRTAP to inform the European air pollution abatement strategies (Simpson et al., 2007). The DO_3SE model is based on a big-leaf approach parameterized for a single species (Emberson et al., 2000a). This mono-specific parametrization is a simplification that may be unlikely in complex ecosystems like Mediterranean annual grasslands due to the multi-species composition comprising more than 200 species (Garcia del Barrio et al., 2014); and their great range of specific g_s where the different species may influence each other in the O_3 absorption (Alonso et al., 2007). A modification in the DO_3SE model has been proposed in Chapter 6 to include a multi-species framework that takes into account the species composition of the canopy and the inter-specific variability in gas exchange rates of the component species. Each species must be represented by a single parameterization showing its own g_s range and response to environmental variables. The species-specific g_s are weighted using its biomass abundance in order to obtain a composite big-leaf g_s which is used to calculate the O_3 stomatal flux of the whole canopy.

The calculation of O_3 indices should be performed over the growing season of the plant receptor. In the case of the Mediterranean annual pastures, the growing season start and duration is controlled by the beginning of the rains in

autumn and the onset of the summer drought. Nevertheless, this period is highly variable between years due to the characteristic high inter-annual variability in meteorological conditions of the Mediterranean area (Peñuelas et al., 2004; Montaldo et al., 2008). In consequence, no fixed dates for the calculation of the O_3 indices can be proposed. Hence, the use of models to detect the period of growth and stomatal O_3 deposition on annual pastures can be useful for this task.

The MEDPAS model is one of the two mechanistic models used in the present study to simulate the pasture growth (González-Fernandez et al., 2010b). The purpose of this model is simulate the growth of annual pasture communities and, coupled with the DO_3SE model to estimate the O_3 flux uptake by plants comprising the dehesa ecosystem (González-Fernandez et al., 2010b). The MEDPAS model is composed by two modules, the soil water content component and the pasture growth module, coupled with the DO_3SE model, estimating the stomatal conductance and the O_3 stomatal flux. This model is capable to reflect the inter-annual variability observed in annual pasture dynamics induced by the amount and different distribution of precipitation in the studied area where it was developed, a dehesa located in the Central of the Iberian Peninsula. However, it needs to be validated across different sites and authors identified some limitations related to the up-scaling from leaf to canopy gas exchange and from one species to community level O_3 deposition (González-Fernandez et al., 2010b).

The second mechanistic model used in this study to simulate the growth of an annual pasture community is the DEHESA model (Hernández Díaz-Ambrona et al., 2007). The DEHESA was built using the structural methodology of the dynamic systems using the software VENSIM® which is based in programming causal diagrams with a very visual presentation. The operating way of this program is aimed at the creation of the corresponding diagram representing the real system to be simulated; in this case the system model is "oak trees-grassland pasture-livestock" as biotic compounds, and "soil-climatic factors" and abiotic components of the system. These components are represented in the model as five modules: grassland, oaks, cattle, climate and soil. These main components have a structure of flow equations in order to simplify its complexity. In this experiment only the grassland, climate and soil modules will be considered.

The aims of this study are: (1) to simulate the biomass yield and to define the growing season of an annual Mediterranean pasture from the Central Iberian Peninsula affected chronically by O₃ pollution throughout three consecutive hydrological years using two pasture growth models specially developed for this kind of ecosystem, MEDPAS and DEHESA; (2) to compare the output of such models with samplings of aboveground biomass over the three consecutive years; (3) to integrate the MEDPAS and DEHESA model outputs with the modification of the DO₃SE model proposed in Chapter 6 to calculate the stomatal ozone flux of a multi-species canopy following a big-leaf approach over the three consecutive years with varying hydro-meteorological conditions; (4) to carry out a risk assessment of O₃ effects on aboveground biomass based on the AOT40 index and on modeled O₃ fluxes using the two model ensembles and the O₃ critical levels proposed by Sanz et al., (2016) and Chapter 6 for effects on aboveground biomass.

7.2. Materials and methods

7.2.1. Study site and environmental conditions

An intensive monitoring facility of air pollution concentration and deposition was set up in 2011 at an abandoned holm-oak (*Quercus ilex* L.) dehesa in the Central Iberian Peninsula (705 m.a.s.l; 3°43' 59"O; 40°35' 17" N). The study site is an open forest surrounded by "El Monte de El Pardo" historically managed as a traditional dehesa until 1941 and left without grazing for around 100 years. The site is located in a natural protected area 9 km North of Madrid and 1.5 km away from the town of Tres Cantos. In the middle-upper land, the understory and open spaces between the trees are dominated by *Cistus ladanifer* shrubs and therophytes (annual pasture species), while in the lower areas this pasture predominates, with the most prevalent families being *Gramineae*, *Leguminoseae* and *Compositae*.

The site presents a Mediterranean semi-arid climate, characterized by relatively cold winters followed by warm and dry summers. The historical mean annual rainfall (1860-2013) is 465 mm (Comunidad de Madrid, 2014) with a very irregular distribution throughout the year. The characteristic high inter-annual

weather variability makes unpredictable the start and length of the summer drought that controls the duration of the growing season of the annual pastures. Meteorological parameters were continuously monitored at 2 meters above ground level including air relative humidity (RH) and temperature (T) (HOBO®, Onset), photosynthetic active radiation (PAR) (S-LIA-M003, Onset), wind speed and direction (S-WCA-M003, Onset), and precipitations (Tipping bucket rain gauge 52203, Young Co.).

Hourly O₃ concentration was also monitored (SIR O₃ S-5014 NS/090) at 3 meters above ground level on-site during the whole study period. When there was a lack of O₃ data, two windward and leeward monitoring stations from the Air Quality Network of the Madrid Community (Colmenar Viejo and Alcobendas) were used to complete the data taking into account the wind direction.

The soil was characterized in the Edaphology Department, Farmacy School (Universidad Complutense de Madrid) at 10-20 cm intervals up to 2 meters. The texture of the upper layer is an acidic sandy loam with 82, 8 and 10% of sand, loam and clay respectively. The soil relative humidity at 20 cm depth (Capacitance Probe Sentek™ Enviroscan SOLO; Sentek Pty. Ltd.) was recorded from April 24th 2010 until the end of the experiment. The sensor logged also humidity at 50, 100 and 200 cm but only the most superficial soil layer was considered for the risk assessment. The field capacity and wilting point were calculated based on Walczak et al., (2006).

Three growing seasons were followed throughout the hydrological years from 2009 to 2012, starting every 1st October until 30th September of the following year: the 2009/10 growing season with 514 mm of rainfall, hereafter referred as humid year; the 2010/11 growing season with 472 mm of rainfall, hereafter referred as normal year, was within the average of rainfall in the study site; and the 2011/12 growing season with 327 mm of rainfall, hereafter referred as dry year, was dryer, warmer and sunnier than the other two.

7.2.2. Aboveground biomass sampling

The sampling area was checked once a week after the autumn rains during the three growing seasons to visually identify the pasture emergence, which happened on October 8th, 7th and 28th in the humid, normal and dry years respectively. Aboveground biomass samples were collected on 13, 8 and 8 different dates in each year respectively with a frequency of two or three weeks throughout the life cycle of the pasture until the last harvest of each year, which was done when the plant biomass was completely dry: July 9th, 11th and June 4th in the humid, normal and dry years respectively. On each harvest date all plants within a 5 dm² sampling ring, 3 random replicates per date, were cautiously collected at 1 cm aboveground level. In the maximum growth point (May 18th, 13th and June 7th) each sample was manually sorted into its main botanical components: *Gramineae*, *Leguminosae*, *Cruciferae*, *Compositae* and other non-legume *forbs* (the last three were joined and presented as *forbs*). Then, samples were dried at 60 °C to constant weight for at least 48 hours and the dry weight (DW) biomass per sampling ring was determined. A total of 87 biomass samples were collected throughout these experimental campaigns.

Figure 7.1 Diagram of the MEDPAS- and DEHESA- DO₃SE ensemble models. Soil water content (SWC) and pasture growth modules were used at a daily time scale, and the stomatal conductance (g_s) module at an hourly time scale. Variables coupling the modules are depicted by arrows. Growth and g_s modules compute values for day 'i' using the SWC at the end of day 'i-1'. Input variables of each model is named in each own box. ET_0 , reference evapotranspiration; ET_c , actual evapotranspiration; θ soil water content at 0-30 and 0-20 cm; LAI, leaf area index; h, canopy height; f_{phen} , plant phenology; PAR, photosynthetic active radiation; T, temperature; RH, relative humidity; VPD, vapour pressure deficit; [O₃], ozone concentration.

7.2.3. MEDPAS model

The MEDPAS model was developed to describe the growth and phenology of Mediterranean annual pastures and, in combination with the DO₃SE model, their gas exchange behavior and the O₃ stomatal deposition during the growing season (González-Fernández et al., 2010b). A diagram with the main variables and modules of the model is presented in figure 7.1. The MEDPAS model was parameterized for the first time in an annual dehesa pasture based on two field campaigns of biomass growth and gas exchange measurements performed in a

dehesa in Miraflores de la Sierra (40° 48.40' N, 3° 48.40' W; Madrid, 1059 m.a.s.l.). In this study, the same model was applied at the site in Tres Cantos. The input variables of the model hourly and daily are: solar radiation, photosynthetic active radiation, temperature, daily maximum and minimum temperature, relative humidity, daily maximum and minimum relative humidity, vapour pressure deficit, precipitation, wind speed, O₃ concentration, soil texture, rooting depth, field capacity and wilting point. The model is composed of three modules: (1) The soil water content module which calculates daily SWC at two soil layers 0-15 and 15-30 cm deep following Nouvellon et al., (2000). Infiltrated rainfall is distributed down the soil profile filling the two layers successively from the top to the bottom. A soil water balance is computed for each soil layer independently following the scheme described in Allen et al. (1998). (2) The pasture growth module is a modified version of the vegetation dynamic model described by Montaldo et al. (2005, 2008). The growth module computes daily changes of biomass based on rates of carbon gain (photosynthesis) and carbon loss (respiration and senescence) that depend on the prevailing hydro-meteorological growing conditions. The dependence of carbon gain and loss rates on meteorological conditions were parameterized using gas exchange measurements performed under field conditions in a dehesa located in Miraflores de la Sierra, 60 Km North of Madrid (González-Fernández et al., 2010b). (3) The stomatal conductance and O₃ deposition module, as described in section 7.2.5 below.

7.2.4. DEHESA model

The DEHESA model (Figure 7.1) was built aiming for integrating the livestock, pasture yield, acorn production and cover of the evergreen oaks trees which traditionally forms part of the agroforestry dehesa systems (Hernández Díaz-Ambrona et al., 2007). The model is composed by five interacting modules: (1) the climate module contributes with daily data of temperature, solar radiation and precipitation, to calculate reference evapotranspiration (ET_o) using a simplification of the Priestley-Taylor equation which used the solar radiation and the mean daily temperature. (2) The soil module calculates a soil water balance

based in the cascade model of Campbell and Stockle (1993). This module is a simplification of the available water in the soil representing the water content in three horizons. There are some soil parameters to consider: number of horizons, depth and bulk density of each horizon, field capacity and wilting point. (3) The pasture module computes daily forage production. The biomass growth is modelled using the solar radiation interception and a maximum growth rate, limited by a radiation and a water use efficiency ratio. The computing daily sum of green biomass is transformed to senescent biomass when the pasture is limited by water stress and then to dry biomass, or can be consumed by animals if the livestock module is activated. Senescence is influenced by variables such as the leaf area index (LAI), water stress and temperature. Furthermore, the model can distinguish between the pasture under the canopy of trees and under the sunlight (in this case the simulation is without trees). (4) The evergreen oak module allows knowing oak and acorn biomass. (5) The livestock module can run with different stocking rates and kind of livestock such as cattle, sheep or swine. Modules 4 and 5 remained switched off in the model for comparison with the actual aboveground biomass data and the MEDPAS model output.

7.2.5. DO₃SE model

The accumulated stomatal O₃ fluxes were estimated for the growing period as determined by the two pasture growth models (MEDPAS and DEHESA) during the three years modeled. The stomatal conductance module is included in the Deposition of Ozone and Stomatal Exchange model (DO₃SE) (CLRTAP, 2010). DO₃SE is currently incorporated within the European Monitoring and Evaluation Programme (EMEP) photo-oxidant chemical transport model in order to calculate the stomatal deposition of O₃ over the vegetation. DO₃SE is designed to estimate the risk of effects of O₃ on plant communities, determining the flux of pollution from the atmosphere into the plant. This model was originally developed for mono-specific vegetation canopies. However, this situation contrast with the biodiverse nature of annual Mediterranean pastures. For this reason, a modification of the DO₃SE model that estimates the stomatal deposition of O₃ for

multiple species, following the methodology described in Chapter 6, was used here. Each species is represented by a single parameterization showing its own g_s range and response to environmental variables. The species-specific g_s is scaled up using the species biomass abundance at the maximum growth point of the pasture, in order to obtain a composite big-leaf g_s which is used to calculate the O_3 stomatal flux. More detailed information on the use of the DO_3SE model on annual Mediterranean pastures can be consulted in González-Fernández et al. (2010b) and Chapter 6.

7.2.6. Ozone risk assessment

A risk assessment has been done using exposure- and dose-based O_3 Cl_e considering, AOT40 and POD_1 indexes following the current methodology of the CLRTAP for semi-natural vegetation (CLRTAP, 2010). The AOT40-based Cl_e (Cl_{ec}) and its confidence interval was obtained from Sanz et al., (2016) based on the response of O_3 sensitive annual species from dehesa pastures. Two effect thresholds, 5% and 10% of yield loss, were considered, corresponding to a O_3 Cl_{ec} of 1562 (1315, 1923) $nl\ l^{-1}\ h$ and 3116 (2631, 3846) $nl\ l^{-1}\ h$ respectively, the 95% confidence intervals are provided between brackets.

The AOT40 was calculated over the three consecutive growing seasons using the O_3 concentration measurements performed on-site, which were accumulated over a running period of 45 days to match the duration of the OTC fumigation experiments that were used to obtain the Cl_{ec} (Sanz et al., 2016) The risk assessment was performed considering the running 45-days AOT40 values starting on the 1st October until the observed end of the growing season for each year but, taking into account for the definitive window period, the physiological activity of the pasture.

The O_3 flux-based Cl_e (Cl_{ef}) and their confidence intervals were obtained from Chapter 6, on the base of two experiments under close to field conditions, where a mixture of annual species were exposed to 4 different O_3 treatments. These authors considered a multi-species approach to establish new dose-response functions from which canopy flux critical levels (Cl_{MS-ef}) were derived:

$POD_{MS-1} = 4.7$ (2.5, 6.9) mmol m^{-2} and $POD_{MS-1} = 7.9$ (5.9, 9.8) mmol m^{-2} for 5 and a 10% effect thresholds respectively (95% confidence interval between brackets).

Risk analysis based of flux thresholds were done from the POD_1 values accumulated over 45-day periods for comparison with the Cl_{MS-ef} calculated for the three growing season using MEDPAS- DO_3SE and DEHESA- DO_3SE .

7.3. Results

7.3.1. Ozone concentrations and meteorological conditions

The meteorological parameters recorded in the study area during the three growing periods are presented in Table 7.1 and figure A.III.1. The hydrologic years in the region start on 1st October, thus all data are presented starting on this date. During the autumn (Oct.-Dec.), the meteorological conditions in the humid and normal years were reasonably similar. Nevertheless, during autumn of the dry year higher mean and maximum, and lower minimum temperatures were logged; besides precipitation amount was 33.6% lower than the mean of the season for the other two years. Throughout winter (Jan.-Mar.) and spring (Apr.-Jun.), where meteorology has greater influence in the plant development due the high relative growth rates of the pasture, the differences between years become greater. Moreover, the humid year was also much colder and cloudier than the other two, especially during winter recording considerably low temperatures and solar radiation (PAR) compared with the normal and dry years. Remarkably, the mean SWC during winter of the dry year was 64.4% lower than the mean of the other two years.

Table 7.1 Summary of the meteorological conditions and O₃ concentrations during the three growing seasons divided in seasons. RH = relative air humidity; PAR = Photosynthetic Active Radiation; SWC =Soil Water Content.

Hydrological years	Autumn			Winter			Spring			Summer		
Humid year	24 h-mean	Max	Min	24 h-mean	Max	Min	24 h-mean	Max	Min	24 h-mean	Max	Min
Temperature (°C)	7.2	26.7	-15.3	2.5	27.5	-14.3	14.2	33.5	-3.6	24.2	38.2	5.7
RH (%)	73.2	90.7	4.7	75.3	100.0	13.5	62.1	100.0	16.5	43.4	100.0	9.5
PAR (μmol m ⁻² s ⁻¹)	158.4	1493.4	0	161.8	1827.6	0	499.4	2108.6	0	546.4	2034.1	0
SWC (%)	13.9	22.0	7.3	16.4	22.0	6.3	10.0	22.0	2.7	10.5	22.0	7.7
O ₃ (nl l ⁻¹)	18.4	52.3	0.3	22.1	43.5	0.5	32.7	73.5	0.1	33.6	74.1	4.9
Precipitation (mm)	144.5			215.0			119.5			35.3		
Normal year	24 h-mean	Max	Min	24 h-mean	Max	Min	24 h-mean	Max	Min	24 h-mean	Max	Min
Temperature (°C)	8.1	26.7	-7.9	6.6	22.1	-5.5	17.9	37.3	3.7	23.2	38.6	6.4
RH (%)	76.3	100.0	11.1	78.8	100.0	14.0	60.5	100.0	14.2	39.9	99.8	7.0
PAR (μmol m ⁻² s ⁻¹)	211.3	1563.0	0	235.9	1754.6	0	551.0	2234.6	0	565.9	2069.2	0
SWC (%)	13.4	22.0	4.8	18.7	22.0	11.5	14.6	22.0	6.9	15.0	26.8	12.6
O ₃ (nl l ⁻¹)	21.9	55.2	0.7	21.8	46.2	0	39.7	76.6	0	38.1	76.8	4.8
Precipitation (mm)	179.2			132.3			151.9			8.2		
Dry year	24 h-mean	Max	Min	24 h-mean	Max	Min	24 h-mean	Max	Min	24 h-mean	Max	Min
Temperature (°C)	10.2	32.3	-5.2	6.3	25.0	-10.8	16.9	38.7	-1.5	23.8	41.8	6.1
RH (%)	73.5	100.0	9.9	58.2	100.0	5.0	53.6	100.0	7.8	34.3	100.0	3.0
PAR (μmol m ⁻² s ⁻¹)	215.3	1504.6	0	299.7	1695.5	0	529.8	2112.1	0	570.1	2015.9	0
SWC (%)	12.8	22.0	2.7	5.9	16.7	2.7	10.4	22.0	5.3	13.3	22.0	8.3
O ₃ (nl l ⁻¹)	21.6	63.4	0.7	27.5	64.4	1.6	43.9	105.7	2.0	46.1	97.5	2.7
Precipitation (mm)	107.5			29.5			105.7			84.5		

The mean and maximum O_3 concentrations ($nl\ l^{-1}$) are presented in table 7.1 and figure 7.2. Ozone levels followed the common seasonality of the pollutant, where the highest average and peak concentrations are found in spring and summer and the lowest in autumn and winter. However, important inter-annual variations were found comparing the three hydrologic years studied here. During the dry growing season higher values were recorded, particularly in spring, with an increment of between 34.4% and 10.6% on the 24 h-means, and between 43.9% and 38.8% on the maximum O_3 concentrations compared to the humid and normal years respectively.

Figure 7.2 Mean daily profiles of ozone concentration 20 cm above-ground in the study site throughout the three hydrological years (1st October to 30th September).

7.3.2. Biomass

The aboveground biomass measured throughout the growing period of the pasture during each hydrological year is presented in Figure 7.3. The growing pattern was different in each year due the different meteorological conditions. Maximum growth yields of 2712, 1995, and 778 kg DW ha⁻¹ were measured in the

humid, normal and dry years respectively. The growing period started on 23th Oct., 11th Oct., and 9th Nov., and the pasture was considered totally senescent on 25th Jul., 12th Jul., and 26th Jun. on the humid, normal and dry years respectively. Therefore, the humid and normal years accumulated 275 and 274 active growing days. Nevertheless, in the dry one the pasture was active 1.5 month less. Main botanical components at the maximum growth point showed a different distribution through the years (figure 7.4). In the humid year the most abundant group was the legumes, constituting up to 54% of the total biomass while grasses and non-legume forbs represented a 33.3% and a 12.7% of the total biomass respectively. The proportion of legumes decreased to 2.3 and 3.8% in the normal and dry years respectively while the proportion of grasses increased to 79.8 and 94.0% on the same years. Non-legume forbs represented 17.9 and 2.2% of the total biomass on the normal and dry years respectively.

Figure 7.3 Aboveground biomass samples (mean $\pm$ se). The pasture production biomass from the MEDPAS model output (green line) and the DEHESA model output (blue line) from: a) the humid year; b) the normal year; and c) the dry year.

Figure 7.4 Total aboveground biomass (Kg DW ha⁻¹) of the main botanical compounds: Leguminosae, Gramineae and Forb (*Cruciferae*, *Compositae* and other non-legume *forbs* joined and presented as forbs) (mean + se) in the maximum biomass growth point of each biomass campaigns. On May 18th of the humid year, May 13th of the normal year and on June 7th of the dry year.

7.3.3. MEDPAS Model output

The MEDPAS model simulation of the biomass growth dynamics throughout the humid, normal and dry growing seasons is presented in figure 7.3. Growth started after the first important autumn rains which eliminated the limitation imposed by low SWC on photosynthesis during the summer. The growth module established the leaf onset on October 24th in the humid and normal year and on October 21st in the dry year, well in agreement with the field observations. During autumn and winter months, growth was mainly limited by low temperature and solar radiation, especially in the humid year where 24 h-mean and minimum temperatures were substantially lower than the other two years. In the normal and dry years the simulated biomass followed the prevailing hydro-meteorological conditions and fitted generally well with the field measurements. However, during the humid year, the model output steadily underestimated the biomass yield

(Figure 7.3a). Early drought conditions during the winter months in the dry year resulted in an early senescence due to soil water shortage. The modelled end date of the growing season was set on May 14th in the dry year (Figure 7.3c). Nevertheless, in the humid and normal years, mid spring rains allowed the modelled pasture to grow further until July 9th and July 11th (Figure 7.3a,b). Inter-annual variability in the amount and distribution of precipitation in spring induced differences up to 56 days in the modelled length of the growing period between a dry and a humid year, variation that matched field observations. The model simulated the length of the pasture growth period considerably well compared with the field observations over the three growing seasons.

7.3.4. DEHESA Model output

Biomass growth simulations performed with the DEHESA model throughout the humid, normal and dry growing seasons are presented in Figure 7.3. Throughout the humid and normal seasons the simulated biomass fitted reasonably well with the field measurements in terms of absolute biomass growth, following the prevailing hydro-meteorological conditions. Nevertheless, the growth module established the leaf senescence onset on 28th and 12th July and consequently the length of the growing period was enlarged by 19 and 1 days after the end of the growing season compared with the observations. During the dry season the model output steadily overestimated the biomass yield of the pasture and under-predicted as well the length of the growing season. The end of the growing season estimated by the DEHESA model happened on 28th Jun, 24 days later than the observations.

7.3.5. AOT40 and DO₃SE model outputs

The AOT40 index showed a great inter-annual variability throughout the three years studied in response to the different hydro-meteorological conditions and O₃ levels prevailing during the humid, normal and dry hydrologic years (Figure 7.5). Due to the different accumulation periods resulting from MEDPAS and

DEHESA model runs during the three growing seasons, differences were found in the estimation of AOT40 over the growing season depending of the model selected. The humid year consistently showed the lowest AOT40 levels compared with the other two years, in agreement with the lower O₃ concentrations recorded that year. The accumulation of AOT40 values calculated using the estimate of the growing season length of the DEHESA model lasted for 26 more days, resulting in a 31% higher final AOT40 value in the humid year than the AOT40 estimated using the MEDPAS model. Very small differences in AOT40 values were found comparing the estimates for the normal year between the two models, with DEHESA predicting a 1% higher AOT40 than MEDPAS. The greatest differences between the two models were observed for the dry, high-O₃ year. The AOT40 from the DEHESA model was 38% higher than from the MEDPAS model. Accumulated AOT40 in the dry year using MEDPAS started earlier and showed consistently higher values over the spring but, as a result of an early stop of the growing season, the final value was just 10 % lower than in the normal year. The DEHESA model showed a similar pattern but the accumulation period of AOT40 lasted until 28 of June and the final AOT40 value was the greatest of the three years and 38% higher than the value estimated with MEDPAS.

Figure 7.5 Accumulated O₃ concentration over the threshold value of 40 nl l⁻¹ during daylight hours throughout the three annual pasture growing seasons as defined by the pasture growth models: a) MEDPAS model and b) DEHESA model.

The g_s modelled using the MEDPAS-DO₃SE and DEHESA-DO₃SE model ensembles showed a marked annual evolution in response to the seasonal changes of the environmental growing conditions (Figure 7.6). High g_s values, close to the maximum g_s , were estimated in autumn at the beginning of the growing season, when warm and sunny weather conditions coincided with soil water availability. Plants overwintered with low g_s induced by low temperatures and solar radiation and resumed their high g_s in spring, when the weather was warm and sunny again. The g_s declined abruptly at the start of the summer drought, characterized by high temperatures and prolonged rain-free periods. The g_s showed also a great inter-annual variability that was reflected by both model ensembles. Twelve hours (6 a.m to 6 p.m) mean daily g_s ($\text{mol m}^{-2} \text{s}^{-1}$) $\pm$ SE from the start of the hydrological year to the end of the growing season estimated for the humid year were $60.6 \cdot 10^{-3} \pm 2.0 \cdot 10^{-3}$ and $67.1 \cdot 10^{-3} \pm 2.2 \cdot 10^{-3} \text{ mol m}^{-2} \text{s}^{-1}$ using the MEDPAS and DEHESA models respectively. During the normal year, relatively similar values were recorded, $48.1 \cdot 10^{-3} \pm 1.2 \cdot 10^{-3}$ and $49.2 \cdot 10^{-3} \pm 1.2 \cdot 10^{-3} \text{ mol m}^{-2} \text{s}^{-1}$. Nevertheless, the twelve hours mean daily g_s throughout the dry year were considerably lower, $19.7 \cdot 10^{-3} \pm 0.7 \cdot 10^{-3}$ and $12.8 \cdot 10^{-3} \pm 0.5 \cdot 10^{-3} \text{ mol m}^{-2} \text{s}^{-1}$ using the MEDPAS and DEHESA models respectively.

Figure 7.6 Phytotoxic O_3 dose (POD) following a multi-species big-leaf approach without threshold (POD_0). Contribution of the main functional types to the total POD_0 throughout the three growing seasons as defined by both pasture growth models during the humid (a,b), normal (c,d) and dry (e,f) hydrological years (1st October to 30th September): a,c,e, growing seasons estimated using the MEDPAS model; b,d,f, growing seasons estimated using the DEHESA model. The black line represents the POD_0 of the whole canopy.

Figure 7.6 shows the accumulation of POD_0 values during the growing season of the humid, normal and dry years for the main functional type components of the canopy as estimated using the MEDPAS- and DEHESA- DO_3SE model ensembles. The high inter-annual variability showed in AOT40 values was found also in the estimated POD using both models. Total POD_0 estimates for the humid and normal years were similar using both models. The humid year showed an accumulated POD_0 at the end of the growing season of 14.1 and 16.6 $mmol\ m^{-2}$

using MEDPAS and DEHESA models respectively, 18% higher using DEHESA. The normal year reached a POD_0 for MEDPAS and DEHESA of 14.9 and 15.2 $mmol\ m^{-2}$, just 2% higher using DEHESA. However, the contribution of the functional types showed remarkable differences between the humid year, when the total flux to the canopy was dominated (81%) by the legume fraction, and the normal year, when the O_3 flux to *Gramineae* (65%) was the most important. Nonetheless, in the dry year, despite having the highest O_3 concentrations, the POD_0 values were the lowest of the three seasons studied, 5.0 $mmol\ m^{-2}$ for MEDPAS and 4.4 $mmol\ m^{-2}$ for DEHESA, dominated (82%) by the grasses fraction. Remarkably, the final POD_0 estimates using the DEHESA model were only 13% lower compared with the total POD_0 estimated using MEDPAS. These differences are due to estimation of the POD_0 accumulation period, which was different depending on the growth model used. The POD_0 estimated using the DEHESA-DO3SE model ensemble in the dry season started four months later compared with MEDPAS-DO3SE and also finished one month later than the other model.

7.3.6. Ozone risk assessment

The Figure 7.7.a presents the running values of the AOT40 index accumulated over 45-days periods through the three growing seasons, compared with the corresponding CL_{ec} (AOT40 based) for 5 and 10% effect thresholds and their corresponding 95% confidence intervals. The 45-days period was assumed according with the response functions and proposed CL_{ec} of Sanz et al., (2016). The start of the 45-days window period was set on 15th May to 30th June for the humid and normal years, and on 1st April to 15th May for the dry one. The start date for these window periods were established considering the increase in the pasture physiological activity after the wintering time.

Hence the maximum 45-days AOT40 for the humid, normal and dry years during the growing season were 1236, 2101 and 1412 $nl\ l^{-1}\ h$ respectively. Therefore, considering that these values are under the 10% CL_{ec} , it can be considered that there is no risk for the pasture at the study site of suffering a 10% yield loss. Nevertheless when the effect threshold is lowered, the risk of suffering a

5% of yield loss increase depending on the year (figure 7.7). The 5% Cl_{ec} was exceeded in the normal year, when AOT40 values were even higher than the upper boundary of the confidence interval, showing a high risk of 5% yield loss due to the pollutant. During the dry year, only the lower boundary of the Cl_{ec} confidence interval was exceeded, with a moderate risk of effect, while in the humid year none of the thresholds were reached, indicating no risk due to the ozone levels in the ambient.

Figure 7.7 MEDPAS Ozone risk assessment based on AOT40 (a) and POD1 (b) indexes accumulated over 45-days periods throughout the three hydrological years (1st October to 30th September) Concentration-based critical level (Cl_{ec}) (a) and canopy flux-based critical level (Cl_{MS-ef}) (b) for yield losses are represented by horizontal lines considering 5% and 10% of effect threshold; their 95% confidence intervals are represented by a grey shaded area around each critical level.

The estimated POD_1 values accumulated over running periods of 45 days using the MEDPAS- DO_3SE over the three years are presented in the figure 7.7b. The same figure shows, for comparison, the CL_{MS-ef} for 5 and 10% effect, together with their 95% confidence intervals (Chapter 4). The POD_1 -based risk assessment using the DEHESA- DO_3SE showed a close results with the MEDPAS- DO_3SE : both models matched the maximum POD_1 values, and their fluxes were strongly correlated for the three years ($r^2=0.79$, $r^2=0.99$, $r^2=0.71$ for the humid, normal and dry seasons respectively; for all $p<0.005$). The CL_{MS-ef} for a 10% effect was not exceeded in any year, only the normal year slightly exceeded the lowest bound of the confidence interval. Therefore, the studied pasture in the three modeled seasons showed no-risk of suffering a 10% of yield loss. However, when the effect threshold is lowered, the calculated POD_1 values during the normal year, clearly exceeded de 5% CL_{MS-ef} ; thus, causing a high risk of losing a 5% yield due to the pollutant exposure. The lower boundary of the 5% CL_{MS-ef} was also exceeded during the humid year but not the CL_{MS-ef} itself; the risk of O_3 damage in this case can be qualified as moderate. Finally, the dry year did not exceed any of the O_3 CL_{MS-ef} , which represents a situation of none or low O_3 -risk.

7.4. Discussion

7.4.1. Inter-annual variability in meteorology, ozone and pasture growth

The high inter-annual variability in the meteorological conditions, as well as on the O_3 surface concentrations, typical of Mediterranean climatic conditions is well represented by the three consecutive hydrologic years monitored in the present study. This variability, especially the amount of precipitation, and thus the soil water availability, affected the ecophysiology of the pasture, directly reflected by the maximum biomass production, the duration of the growing season and the species composition of the canopy. The highest yield was collected in the humid year harvesting 0.4 and 2.5 times more than in the normal and dry years respectively. This range of variation in biomass production was also found over a 20 year period in annual dehesa pastures of Salamanca, in Western Spain (Vazquez de Aldana et al., 2008). The yield of dehesa annual pastures and the start and end

of the growing season are strongly correlated with the amount and distribution of rainfall (Vázquez de Aldana et al., 2008; Peco et al., 2009). Furthermore, the distribution of the precipitations is also very important in determining the presence of different species in the pasture composition (Espigares and Peco, 1993; Peco et al., 1998, Vázquez de Aldana et al., 2008). In this experiment it was possible to observe how the legume species were the most abundant functional type in the humid year while its contribution to the total biomass was much reduced in the normal and dry years, which were dominated by grasses. These changes in the species composition complicates further the simulation of growth and gas exchange patterns during different growing seasons in this kind of plant communities. For instance, annual grass species growing in a dehesa 50 km North of Madrid presented maximum g_s values between 11% and 2.4 fold lower than legume or forb species growing in the same location, though considerable variability was found within functional types and plant families for this parameter (González-Fernández et al., 2010b). This has also consequences for flux-based risk assessments. A pasture dominated by grass species would present constitutively lower g_s than pastures dominated by legumes or forbs and therefore would have a lower risk of exceeding any of the Cl_{ef} .

7.4.2. MEDPAS and DEHESA model performance

The two models considered in this study were used to simulate the effect of the inter-annual variability in hydro-meteorological conditions on the onset and end of the growing season and on stomatal conductance of the main functional types present in annual pastures. The modelling approach followed represents a simplification of the real situation because is based on the stomatal conductance response of only 6 species to different environmental conditions and the proportion of them remain constant during each growing season.

As discussed in the previous paragraphs, the plant available soil water content is one of the most important parameters controlling the phenology, gas exchange and biomass growth of annual pastures. The two models tended to underestimate the SWC compared with field measurements (data not shown). SWC

estimations could be improved by a better characterization of soil properties, leading to a better estimation of evapotranspiration processes. Furthermore, plants could be extracting water from deeper soil layers, stressing the importance of rooting depth in soil water balance models. In this study rooting depth was considered to be 30 cm in the MEDPAS model and 20 cm in the DEHESA; although higher values up to 80 cm have been reported for annual grasslands of dehesa ecosystems (Moreno et al., 2005). Similar results have been described for other grassland communities where SWC from the top soil was not indicative of total plant available soil water, stressing the importance of rooting depth as a factor strongly influencing plant responses to soil water stress (Jäggi et al., 2005).

The differences in model performance observed between years might be related to changes in plant dynamics induced by water stress, including modified allocation of photoassimilates or acclimation responses that affect both plant and root growth. Chaves et al. (2002) showed how water stress strongly affects photosynthesis, growth and survival of plants species growing in semi-arid climates, such as the Mediterranean. In the summer, annual species rely mostly on rapid growth to escape drought stress as well as on fast responses of the photosynthetic and C metabolism machinery to early sign of stress, including storage of reserves in the stem or roots and producing seed as fast as possible. The DEHESA model failed to model the aboveground biomass growth and growing season duration during the dry year. This could be because DEHESA did not identify the complex and fast mechanisms that annuals turn on under water stress situations. MEDPAS specifically includes an algorithm describing this process, where biomass senescence rates are increased at the end of the growing season triggered by negative carbon balances over 15 consecutive days (González-Fernández et al., 2010b).

The air temperature has been identified as another factor that can explain differences in the floristic composition and biomass growth of the annual dehesa pastures (Espigares and Peco, 1993; Vázquez de Aldana et al., 2008). The low temperatures achieved in autumn and winter combined with the rainfall recorded during the humid hydrological year may have induced a change in the growth pattern, associated with the different floristic composition measured that year

compared with the normal and dry years that was not properly modelled by MEDPAS. This is an issue that has to be identified and solved.

The MEDPAS model computed successfully the start and the end of the growing seasons over the three years. The DEHESA model could be considered suitable in the sense of representing the maximum growth of the pasture during the humid and normal seasons, though not of the driest one. However, the start and end of the growing seasons as well as the timing for the maximum biomass growth of the annual pasture appears to be lagged in comparison with field observations in all three growing seasons and especially in the driest one. The estimation of the growing season duration is very important in O₃ risk assessments because plants appear to be sensitive to this pollutant while they are physiologically active. In annual species like wheat, the ozone exposures during the reproductive growing season, from flowering to seed maturity, are even the most relevant for explaining O₃ effects on yield (Pleijel et al., 1998). In the case of annual dehesa pastures, the highest O₃ levels that are usually recorded during the summer do not overlap with the actively growing season. This is depicted in Figure 7.7a, where the highest AOT40 values are found outside the growing season of the three years studied. The exposure to O₃ during the spring, when annual plant communities are growing fast, has been identified as the most relevant in terms of O₃ sensitivity (González-Fernández et al., 2010b). Considering the great variability observed in the growing season duration, growth models appear as useful tools for identifying the time of the year when O₃ risk assessments must be performed for annual dehesa pastures. By contrast, the majority of current approaches to evaluate the risk of O₃ effects are based on fixed time periods or temperature sums describing the typical duration of the growing season (e.g. CLRTAP, 2010).

7.4.3. Ozone risk assessment

Based on the modelled start and end of the growing season for the annual pasture, AOT40 values were accumulated for the three years throughout the complete growing seasons. Both models showed a great variability between years, reflecting the inter-annual variability in O₃ concentrations recorded over the three

year period ranging 4149-10208 $\text{nl l}^{-1} \text{h}$ using the MEDPAS growing season estimation and 5444-12664 $\text{nl l}^{-1} \text{h}$ using the DEHESA model estimation. Higher AOT40 values were estimated using the DEHESA model, as a result of the delayed end of the growing season compared with MEDPAS results and with field observations, which would result in an increased risk of O_3 effects if the DEHESA approach is selected. When AOT40 values are accumulated over 45-day periods during the whole hydrologic year (Figure 7.7) it is possible to observe that the highest peaks of this index occur during the summer months, when the pasture was not actively growing. This result stresses the need of a careful consideration of the growing season duration because O_3 levels tend to increase towards the summer and, thus, any delay in the modelled end of the growing season would result in higher estimates of the AOT40 and an overestimation of the risk of O_3 damage. The highest AOT40 accumulated over 45 days periods were reached during the summer months of all three years. Summer AOT40 values were especially high during the dry year, resulting in great exceedances of the 10% Cl_{ec} .

Sanz et al., (2016), aiming to cope with the inter-annual variability of the growing seasons for annual Mediterranean pastures, proposed an integration period to calculate the AOT40 for each year using a moving window of time depending on the meteorology and therefore the period with the highest plant growth rates. The proposed moving time window would be 45-days of O_3 accumulation from 1st February to 1st March for the start of the season and finishing from 31th May to 30th June. These time windows cover the inter-annual variability in the growing season duration observed in the present study. However, one of the limitations of this approach is the need of detailed phenological information at the local scale, which is not always available.

The AOT40 based O_3 risk assessment must be interpreted cautiously. Although Sanz et al. (2016) found a significant linear relationship between AOT40 values and aboveground biomass losses in sensitive species, the analysis of the exposure -response relationship using the AOT40 index to explain O_3 effects on aboveground biomass of an annual species canopy formed by a mixture of 6 species over two years did not show a statistically significant result (Chapter 6). In contrast, the dose-response relationships based on the POD_1 derived for the same

experiment found a significant relationship with effects. For this reason, the risk assessment based on the AOT40 is considered less reliable as compared with the flux-based risk assessment. It is generally acknowledged that the O_3 effects are more closely linked to O_3 uptake than to external O_3 exposure (Musselman and Massman, 1999; Massman et al., 2000; Wieser, 1997; Matyssek et al., 2004). POD_y indexes seem to have a greater adaptation to the high inter-annual variability present in the Mediterranean climate than the AOT40 because growing environmental conditions, an especially soil moisture, are considered in the calculation of O_3 stomatal fluxes (Chapter 6).

The two model ensembles used in this study, MEDPAS- DO_3SE and DEHESA- DO_3SE , helped to simulate the inter-annual differences in POD_1 as a result of the influence of hydro-meteorological conditions on stomatal conductance and growing season duration of the annual pasture as well as on O_3 concentrations. Thus, no detailed information on pasture phenology was needed to perform the risk assessment.

The inter-annual variability influenced the result of the flux-based risk assessment: the highest risk of and O_3 effect was found during the normal year, followed by the humid and the dry years. The POD_1 CL_{ef} for a 10% effect was always above the POD_1 estimates of the MEDPAS- DO_3SE model ensemble, showing that none of the three years risked of suffering a 10% yield loss. Nevertheless, the normal year showed a high risk of a 5% biomass loss because both the CL_{ef} and the upper bound of the confidence interval were exceeded during the growing season. During the humid year only the lower bound of the 5% CL_{ef} confidence interval was exceeded, showing a moderate risk of a 5% loss and the dry year did not exceed any of the O_3 CL_{ef} .

The risk assessment results performed using the POD_1 contrast with the AOT40 based risk assessment. The later was driven by inter-annual differences in O_3 concentrations during the growing season. Based on AOT40 the risk was the highest during the normal year, followed by the dry and humid years. Interestingly, using the POD_1 , the normal year showed also the highest risk of O_3 effects. Nevertheless, the humid year showed a moderate risk while the dry year presented a very low risk of effects. Differences between years were caused not

only by differences in O_3 concentrations but also by the plant physiology and phenology in each year, which are driven by the available soil water content. The lower gas exchange rates and shorter growing season during the dry year compared with the other two resulted in lower risks of O_3 effects despite higher O_3 concentrations were recorded that year.

It is interesting to note that the risk assessment reached the same result whether the MEDPAS-DO₃SE and DEHESA-DO₃SE model ensembles are used, despite differences were observed in the estimation of the growing season duration. These differences were evident when comparing the stomatal flux estimates over the whole growing season but for shorter accumulation periods like the 45-day period used for comparison with the CL_{ef} , the result was the same for both modelling approaches. This shows that inter-annual differences in POD_1 estimates for 45 day accumulation periods can be explained by differences in plant available soil water content, which drives gas exchange rates as well as the phenology of the annual pasture.

The results presented in this study shows that the high variability in the meteorological conditions, especially soil moisture, is one of the key issues to be considered when performing risk assessment exercises for dehesa and other annual Mediterranean pastures. Unfortunately, a number of uncertainties still persist to make spatially explicit and realistic O_3 risk assessment for Mediterranean annual pastures. Large-scale soil moisture modelling appears as one of the most limiting issues for assessing the risk of O_3 effects on annual Mediterranean pastures as well as other vegetation communities on a regional to continental scale (Büker et al., 2012; Simpson et al., 2012). Also, further investigations with different annual species, climatic conditions and management regimes are needed in order to develop more consistent CL_{ef} , growth and gas exchange models for Mediterranean annual pasture communities. However, the present approach shows some progress in assessing the risk of O_3 effects on semi-natural vegetation communities, which are considered as the most undertrained vegetation category for risk assessment within the CLRTAP (CLRTAP, 2010).

7.5. Conclusions

Mediterranean annual pastures are affected by current tropospheric O₃ concentrations as well as by other stresses under the Global Change present scenario. The three year experiment set in an intensive monitored dehesa in the Central Iberian Peninsula showed the high inter-annual variability in the meteorological conditions as well as on the O₃ concentrations, typical of Mediterranean climatic conditions during the pasture growth period. The POD₁ index has greater adaptation to this high inter-annual variability than AOT40 because growing environmental conditions controlling the physiological activity of annual pastures, in particular soil moisture, are considered in the calculation of O₃ stomatal fluxes. Soil moisture was identified as a key variable for explaining the inter-annual variability in estimated POD₁ values due to the influence on gas exchange rates and growing season duration. The results of the present study shows that under limiting soil moisture conditions the risk of O₃ damage is low even under high O₃ exposure. However, the risk is higher when favorable conditions for plant growth are associated with moderate to high O₃ concentrations. The use of pasture growth models coupled with the DO₃SE model helped to describe these changes and to refine the risk assessment results. Furthermore, O₃ effects are more closely linked to O₃ uptake than to external O₃ exposure. Thus, it is recommended that O₃ risk assessments are based on O₃ stomatal flux critical levels.

CHAPTER 8

General Discussion

8.1. Ozone and N effects at canopy level

Based on the results from two experimental growing seasons presented in Chapters 3 and 6, ozone (O₃) exposure affected the annual pasture at canopy level by causing foliar visible injury, accelerating plant senescence and reducing green and total aboveground biomass. These effects were also related to the reduction of the Ecosystem CO₂ Exchange (NEE) and the increase of the ecosystem respiration (R_{eco}).

Ozone induced foliar visible damage in 30%-48% of the canopy, and decreased up to 25% of green biomass and up to 21% of total aboveground biomass. Interestingly, even ambient O₃ levels (NFA treatment) showed a clear tendency to reduce total aboveground biomass of the annual community during the first experiment (9%). These results agree with previous studies performed with individual annual species (monocultures) or two-species mesocosms showing O₃-induced reductions in growth parameters on the order of 20-30% in some sensitive species (Gimeno et al., 2004a,b; Sanz et al., 2005, 2007). Thus, the response to O₃ observed in an annual community was within the range of the effects reported for individual species. By contrast, well-established perennial grassland communities have shown greater resilience to O₃ effects than individual component species (Volk et al., 2006; Stampfli and Fuhrer, 2010).

Indeed, our experimental annual community appeared to be more sensitive to O₃ than perennial grasslands, because significant effects were detected after a few weeks of O₃ exposure and in response to lower values of the O₃ index AOT40. Volk et al. (2011) did not find any O₃-response at canopy level in a highly diverse alpine perennial grassland after 5 years of O₃ exposure. Similarly, only small O₃ effects on the community composition were observed in calcareous grassland turfs after three growing seasons under exposure to the pollutant (Thwaites et al., 2006). The fact that annual communities regenerate each growing season from the seed bank with a fast-growing rate during early spring, when meteorological conditions are optimal and soil water is not a limiting factor, would allow high O₃ absorption rates favoring their high O₃-sensitivity. In agreement, other perennial communities at early stages of development, when they are not fully established, have also shown quick and strong responses to O₃, suggesting that high growth

rate is a key plant trait related to O₃-sensitivity (Bassin et al., 2007a). Our results also support the concept advanced by Grime et al. (2000) that mature ecosystems exhibit greater inertia to stress and disturbance than newly established communities, considering the characteristic state of non-maturity of the annual pastures.

Increasing nitrogen (N) availability slightly affected annual pasture development at canopy level. The low-N treatments used in the experiments presented in this thesis, selected to reproduce the potential range of atmospheric N deposition values in the area, increased soil mineral N-content (Chapter 3), but these doses were insufficient to significantly affect the yield or gas exchange rates of the annual pasture. The background soil N-content of the soil (10 mg N_{min}·kg⁻¹; i.e., 33 kg N_{min}·ha⁻¹) seemed sufficient to cover pasture N nutritional demand, explaining the lack of response to low levels of N addition. Natural annual pastures usually grow in low-fertility soils (Vázquez-De-Aldana et al., 2008), and a soil N-content representing one-tenth of the background soil N-content of our experiment has been reported in natural annual pastures in Portugal (Hussain et al., 2009). Nevertheless, other nutrients such as phosphorus could be limiting plant development in this ecosystem type.

Despite the low responsiveness to N, a significant interactive response between N and O₃ on the yield of the pasture at canopy level was detected in the two growing seasons. From one point of view, the fertilizer partially counterbalanced the O₃ effects on aboveground biomass when the O₃ levels were moderate. In this sense, N management might be considered as an adaptation strategy in areas under chronically high O₃ levels. Another interesting interpretation of the interaction is related to the reduction in N-use efficiency caused by the pollutant, as this effect would increase the negative effects of the pollutant added to the more typically reported losses in quality and yield. Other authors have also highlighted the importance of nutrient availability in modifying O₃ responses of herbaceous vegetation (Bassin et al., 2007a). Due to the important consequences of this interaction, even from the economic point of view, further characterization for different vegetation types is necessary in order to find how widespread is the pattern found for communities dominated by annuals.

8.2. Ozone and N effects at species level

Regarding the results reported in Chapter 4, annual Mediterranean pasture species evidenced heterogeneous responses to O₃ and N. Surprisingly, the responses were heterogeneous, even within species of the same family. *Trifolium* species showed similar response patterns, but they were very different from the response of *Ornithopus compressus*, the other legume analyzed.

The clover species were the most sensitive to O₃, showing a negative linear response to the pollutant, but they did not present any significant response to N. Their well-developed specific foliar damage and O₃-induced effects on yield parameters were the greatest among the tested species. Up to 35% reduction in biomass was observed under above-ambient O₃ concentrations. Both clovers also presented an O₃-induced increase in senescent biomass, which is a common response of O₃-sensitive species (Franzaring et al. 2000; Booker et al. 2009), including annuals (Bermejo et al. 2003; Sanz et al. 2007, 2014). Therefore, regarding foliar damage and yield, the high sensitivity of annual clover species to O₃ was apparent when they were grown in a community and under field conditions, in agreement with the previous studies considering single clover species of pot-grown monocultures (Sanz et al., 2005, 2007).

The non-clover legume *O. compressus* presented a unique response pattern. This species showed no response to N, in accord with the other legumes, but only minor foliar damage and significantly increased aerial and root biomass with increasing O₃ exposure. This pattern contrasts with those of the clovers, even though all of them belong to the same family. Preceding studies in which annual species were tested individually also noted that some legumes are tolerant to O₃, including *Ornithopus* and *Anthyllis* (Gimeno et al. 2004a), but a positive response to this pollutant has not been reported before. In our experiments, *O. compressus* may have benefited not only from its O₃ tolerance, but also from the decreased competition for light, water, space and nutrients, mainly at the expense of O₃-sensitive clover species. Ozone induced changes in plant–plant competitive relationships have been found in other grassland community studies, and have usually been attributed to the greater sensitivity of legumes compared with grasses (Davison and Barnes 1998; Hayes et al. 2009; Wedlich et al. 2012). The

interesting legume–legume competition described above played an important role in the response of the pasture to O₃, highlighting the importance of considering plant–plant interactions when attempting to gain a true understanding of natural community responses to atmospheric pollutants.

The grasses *Briza maxima* and *Cynosurus echinatus* and the herb *Silene gallica* can be considered more tolerant of O₃ than clovers. Ozone-specific symptoms appeared in the above-ambient O₃ treatments, but at lower intensity than clovers. Although there was a trend towards an aerial biomass reduction for all the three species, it was not statistically significant. However, these species except for *C. echinatus* presented a noticeable response to O₃ regarding foliar senescence. Other studies focusing on individual species or simple mixtures have also indicated that biomass related parameters of the annual grasses are relatively non-responsive to O₃, but in general the pollutant accelerated their senescence (Gimeno et al. 2004a; Sanz et al. 2011). These three species, in contrast to legumes, responded positively to N fertilizer, although the effect was not significant for *S. gallica*, and the response of *B. maxima* was masked by the effect of the interaction between O₃ and N that caused a quadratic pattern in their response. When the three non-legume species were analyzed together, the response pattern was strengthened, the O₃-induced reduction in aerial biomass became significant, although less intense than the clover group, and the N-induced increase was stronger.

Information about the interactive effects of atmospheric pollutants on vegetation is scarce, especially considering O₃ and N-deposition. Some studies have shown that N can exacerbate O₃-induced effects, such as the enhanced reduction of the belowground carbon allocation detected in *Dactylis glomerata* (Wyness et al. 2011). From previous assays with annuals grown in monocultures, and thus without interspecific competition, species- and parameter-specific responses to the interaction between N and O₃ were noted, but the pattern was not consistent. Intensification of the effects of O₃ on the nutritive quality of *T. subterraneum* (Sanz et al. 2005) and on the leaf senescence of *T. cherleri* (Sanz et al. 2014), were observed upon N addition. These responses contrast with the ability of N to mitigate the effects of O₃ on the biomass and seed production of *T. striatum* (Sanz

et al. 2007) or on the leaf senescence of *T. subterraneum* and *Briza maxima* (Sanz et al., 2005, 2011). The latter responses are more in agreement with those reported in Chapter 3 for canopy biomass (N addition partially mitigated the O₃-induced reduction on canopy yield).

When data were examined at species level (Chapter 4), some interactive effects between the pollutant and the fertilizer were also found; this was the case for senescent biomass of *B. maxima* and for the green biomass of *T. cherleri*. The pattern found for the senescence-related parameters of the grass pointed to a loss of N efficiency under above-ambient O₃ concentrations and to an ability of N to counterbalance the effects of O₃ at intermediate levels of N. Thus, this pattern agrees with previous work based on potted clovers (Sanz et al., 2005, 2007), or potted grasses (Sanz et al., 2011).

On the other hand, the effect of the interaction noted for root biomass of *T. striatum* clover showed a slightly different non-linear response pattern: intermediate N-input mitigated the negative effects of O₃ on biomass, whereas high N-input intensified them. These results indicate the complexity of the O₃ and N interaction, depending not only on O₃ exposure but also on the N-input level.

Results showed clearly the heterogeneous yield responses of the annual species assayed, some more responsive to O₃ exposure (towards both directions positively or negatively) and others more responsive to N-inputs; and also showed the presence of responses caused by the interaction between both factors. All of these alter the competitive relationships among component species and thus the structural composition of the community.

Annual Mediterranean pastures are characterized by their high species diversity and complex population dynamics (Peco et al. 2014; Pineda and Montalvo 1995). This high biodiversity is linked to the complementarity in resource use efficiency among the species coexisting in the community, which reduces competition among neighbors and facilitates species coexistence (Cleland et al. 2006; Gross et al. 2007). According to the results reported in Chapter 4, O₃ and N are factors that affect this complementarity, changing the competitive

relationships among species through their heterogeneous sensitivity to the pollutants.

The chronic exposure to current O₃ concentrations and N-deposition levels occurring in the Iberian Peninsula might be already driving changes to the structure and composition of Mediterranean annual pastures. Further experimental effort is needed to extend the research including more species allowing the generalization of the response patterns to air pollution found in this work.

8.3. Different effects of N and O₃ on nutritive quality

The results presented in the Chapter 5 with two representative species of the annual legume and grass families, *Trifolium striatum* and *Briza maxima*, will help to discern the effects of O₃ and N fertilization, and their interaction, on C and N allocation and quality-related parameters of annual species when these are grown under field conditions and with inter-specific competition.

Carbon and N allocation within plant organs differed between the legume and the grass. The carbon content of the legume was relatively homogenous among plant parts. On the contrary, the grass presented the maximum C-content in the leaves, followed by the stems and the roots. For both species, N was mainly located in the aboveground organs. The allocation priorities for C and N within plant organs changed significantly due to the effect of O₃ and N. Significant and consistent interactions between O₃ and N were also found for the N-content of leaves, stems and roots, and for the C/N rate of stems and roots. These interactions modulate the effects observed in the N-content and C/N ratios, showing that O₃ effects were counterbalanced by the fertilizer inputs. From another point of view, the interaction between O₃ and N shows that, under high O₃ levels, maintaining high N-content in the aerial biomass, and thus protein content required for the pasture quality, required greater N-inputs than under low O₃ exposure. Thus, the pollutant reduced the N-use efficiency.

It is interesting to note that the effects of O₃ on C and N -contents of the different organs occurred without showing any significant effect on biomass parameters (Chapter 4). *Briza maxima* was previously classified as relatively O₃-tolerant regarding growth parameters compared with annual clovers (Gimeno et al., 2004a). However, Sanz et al. (2011) observed an O₃-induced increase in plant senescence and a shortened life span in their experiments. The results reported here in relation with C and N allocation confirm the O₃ sensitivity of this species and, more interestingly, that O₃ effects were modulated by N availability.

The O₃ exposure and N-inputs altered significantly the C and N allocation within clover organs, but followed a different response pattern compared with the grass. Ozone did not affect the C allocation in any organ, but increased the N-content of the aerial structures, leaves, stems and flowers. *Trifolium striatum* has been described in previous monoculture experiments as an O₃-sensitive species, showing O₃-induced reductions in the aboveground and root biomass, and in flower and seed production (Gimeno et al., 2004a,b; Sanz et al., 2005). The present experiment confirmed that *T. striatum* is very O₃-sensitive also when it grows in a multi-species canopy. The pollutant induced alterations in the gas exchange (Llusia et al., 2014) and biomass parameters (Chapter 4). Some of them were found at O₃ levels frequently registered in their natural distribution area in the Iberian Peninsula.

The nutritive quality of the two species was evaluated on the basis of their CP and fiber contents in the aboveground biomass. Legume species are usually considered to improve the quality of a pasture due to their high CP and low fiber content (Van Soest, 1994). However, the grass and the clover assayed in the present study presented similar CP (Figure 5.6). The fiber content measured in the present study was also lower in the grass than in the clover, resulting in higher RFV values. These values do not support the agronomic generalization of greater forage quality of the clover compared with the grass. However, this observation is in accordance with the CP and fiber content values reported for different annual clovers and grasses in previous studies. These studies considered the species in the present study, but also *T. cherleri*, *T. subterraneum* and *Bromus hordeaceus* (Sanz et

al., 2005, 2007, 2011, 2014) reported CP and fiber values within the same range, regardless of their belonging to the grass or legume family.

These results indicate that both factors, O₃ and N-inputs, can unbalance the apportionment of protein within the grass-legume system. The ultimate effect on the protein content of the pasture will depend on the species biomass proportion. The inter-annual variability in the species composition is a characteristic of annual Mediterranean pastures (Peco et al., 1998). Years with greater abundances of legumes or grasses are commonly reported in the literature (Vázquez de Aldana et al., 2008). Thus, any effect of O₃ and N input on CP pools of annual pasture species must be interpreted in the wider context of inter-annual changes on the species composition. Multi-species approaches could provide more insightful conclusions on the effects of O₃ and N on the CP of annual pastures.

Ozone and N affected the different fiber contents of *T. striatum* and *B. maxima*. The clover was the most sensitive species to both O₃ and N. In this species, the two factors induced opposite effects on fiber contents, without statistically significant interactions between them. Ozone generally caused a reduction in the forage quality of the clover. The forage quality of *B. maxima* was less sensitive to O₃ and N, and no interactions between these two factors were identified.

The NDF and ADF contents measured in this study were in the same range of values as reported previously for annual species such as *T. subterraneum*, *T. cherleri*, *B. maxima* and *Bromus hordeaceus* (Sanz et al., 2005, 2011, 2014, 2015). However, it must be noted that fiber contents measured in *B. maxima* were considerably lower than in previous experiments with the same species or with other annual or perennial grass species (González-Fernández et al., 2008, Sanz et al. 2011, 2015). Thus, the RFV for *B. maxima* was higher than values generally reported for grass species, and even greater than the RFV measured in *T. striatum*. This was unexpected considering that legumes are traditionally considered to have superior forage quality compared with grasses. Higher RFV have been measured in clover species than in grasses growing in the same experiment (e.g. González-Fernández et al., 2008, Sanz et al., 2005, 2011, 2014, 2015).

In terms of lignin content, the values reported in this study were larger than data from other pasture species (Muntifering et al., 2006; Sanz et al., 2005, 2011, 2014, 2015), but they were within the same range of values reported for perennial grassland species such as *T. repens* and *Lolium perenne* (González-Fernández et al., 2008). Higher lignin contents have also been reported for perennial communities growing in the UK (Hayes et al., 2016).

Forage quality parameters are affected by many environmental variables that may explain the differences observed between studies. The nutritive quality of pastures declines largely with plant maturity (Blaser, 1986, Linn and Martin, 1989, Van Soest, 1994). The biomass harvested in the different experiments, and from the different species in one experiment, may correspond to different growth stages, which makes comparisons between studies difficult. Unfortunately, this information is frequently not reported in forage quality studies.

Lignin content is generally considered one of the main factors limiting forage digestibility, influencing cell wall degradability (Van Soest, 1994). Ozone exposure has been shown to increase the lignification of many annual herbaceous species such as *B. maxima*, *T. subterraneum*, *T. cherleri*, *Bromus hordeaceus* (Sanz et al., 2005, 2011, 2014, 2015). Among perennial species, the results varied between experiments and species (González-Fernández et al., 2008; Muntifering et al., 2006; Gilliland et al., 2016; Hayes et al., 2016).

Ozone and N effects on forage quality parameters are caused not only by direct effects on the biochemical composition of plant tissues but also through indirect processes that are not considered in this study. The forage nutritive value can be affected by the green to senescent biomass and the leaf to stem biomass ratios, the phenological stage or the species composition of the pasture (Van Soest 1994, Muntifering et al., 2006). These factors have been reported to be affected by O₃ and N inputs in pasture species growing individually, in simple mixtures or in intact communities (Sanz et al., 2005, 2007, 2011, 2014, 2015, Stevens et al., 2010, Wedlich et al., 2012, Chapter 3 and 4). These effects could potentially result in indirect alterations of the forage quality at the community level. The analysis of indirect effects will require more detailed information that integrates the multi-species nature of pasture canopies. These assessments will need to consider also

the important interactions existing between plant community dynamics and the environmental growing conditions, especially for annual Mediterranean pastures.

8. 4. Modelling O₃ uptake dose by the canopy and defining O₃ critical levels for Mediterranean annual pastures

The O₃ CLe set by the CLRTAP for the protection of herbaceous communities (semi-natural vegetation), including Mediterranean pastures, are considered as the most uncertain (CLRTAP, 2010). Several causes have been recognized to explain this uncertainty: the high diversity of herbaceous communities and the low number of studies about their O₃-sensitivity, the inter-specific variability in the gas exchange rates (and thus in O₃ flux), the genotypic variability in O₃-sensitivity between and within species, the importance of soil moisture (which is frequently poorly characterized in the flux models), and the interactions between O₃ effects and plant competition, management or other pollutants (Bassin et al., 2007; Davison and Barnes 1998; Gonzalez-Fernandez et al 2010). The Chapter 6 contributes to filling some of these gaps for Dehesa annual pastures developing O₃ CLe based on a multi-species approach and considering the influence of N availability.

The variability in the O₃ sensitivity of the different species does not seem to be related with their g_{sto} values and pollutant uptake rates, at least at the maximum growth rate season, when the measurements were recorded. For instance, similar g_{sto} values were recorded for *T. striatum* and *O. compressus*, and even the later presented the highest g_{max} . Nevertheless, the annual species measured in Chapter 6 presented in general high g_{max} values compared with other perennial herbaceous species (CLRTAP, 210). This is in agreement with their fast growing rate in spring and can be the cause of the relatively high O₃ sensitivity of the annuals compared with other grassland communities (Bassin et al., 2007). Other variables like the detoxification capacity, the specific leaf area or the resource allocation and partitioning are also important in determining plant responses to O₃ (Heath and Taylor, 1997; Ashmore, 2005; Bassin et al., 2007) and should be addressed in the future to identify the traits related with O₃ tolerance.

Previous efforts have been done for developing species-specific parameterizations of the DO₃SE model for modelling O₃ stomatal fluxes for annual species (Alonso et al., 2007; González-Fernández et al., 2010). However, these single species approaches have limitations when complex plant communities like annuals are considered (González-Fernández et al., 2010). The flux modelling scale should be related to the nature of the impacts to be assessed and new methods for up-scaling the models for complex ecosystems are needed (Ashmore et al., 2007). The approach followed in the Chapter 6 is based on the big-leaf method, following the current DO₃SE model configuration, but it has been modified for taking into account the inter-specific variability in the gas exchange rates of the different species present in the canopy. Each species is represented by a single parameterization showing its own g_{spi} range and response to the environmental variables but weighted using the biomass abundance of the species in the calculation of a composite big-leaf g_{sto} .

The different meteorological conditions and O₃ concentrations observed in the two years reflected the high inter-annual variability of the plant growing conditions typical of the Mediterranean climate. These differences had an influence on biomass and gas exchange rates of the annual species. This has been already observed under field conditions, where inter-annual differences in gas exchange rate in one species can be of the same range than the differences observed between species growing in the same pasture (González-Fernández et al., 2010).

The better performance of POD_y than AOT40 is in accordance with the general acknowledgement that the O₃ effects are more closely linked to O₃ uptake than to external O₃ exposure (Musselman and Massman, 1999; Massman et al., 2000; Wieser, 1997; Matyssek et al., 2004). Thus, POD_y indexes seem to have a greater adaptation to the high inter-annual variability present in the Mediterranean climate than the AOT40 because growing environmental conditions, an especially soil moisture, are considered in the calculation of O₃ stomatal fluxes.

The new CLe_{f-MS} for Mediterranean annual pastures was set at POD_{1-MS}=7.9 mmol m⁻² (5.9, 9.8, CI 95%) for a 10% of biomass reduction. This CLe_{f-MS} is higher than the CLe_f currently set by UNECE for the protection of perennial herbaceous

vegetation, $POD_1=2$ and $POD_1=6$ mmol m⁻² based on results for *Trifolium repens* and *Viola* spp respectively. The differences in the physiological behaviour between perennial and annual species can explain partially the differences in the CLe_f : annuals present a great spring growth rate supported by their high gas exchange rates. g_{max} values estimated for annual clover species in this study ranged 1.13-1.35 mol H₂O m⁻² s⁻¹, greater than the g_{max} of 0.64 mol H₂O m⁻² s⁻¹ estimated for white clover growing under Atlantic and Continental climatic conditions (CLRTAP, 2010). These gas exchange rates of Mediterranean species combined with high O₃ concentrations accumulate large POD_{y-MS} values in short periods of time.

The CLe_{f-MS} is also lower than the recently proposed CLe_f for annual Mediterranean species growing individually or in two species mixtures, $POD_1=12.2$ mmol m⁻² (8.9, 15.5 CI 95%) (Sanz et al., 2016), though the confidence intervals of the CLe_f and CLe_{f-MS} for annual species show some overlapping. It should be taken into account that the proposed CLe_{f-MS} is not directly comparable with the CLe_f for individual species because O₃-tolerant species have been included in the calculation of the POD_{y-MS} and the response functions, while only O₃-sensitive species are considered in the individual species approach. Further efforts will be needed in the future for coordinating both approaches. For the moment, the advantage of this multi-species approach is that it is able to incorporate the high diversity present in semi-natural vegetation communities regarding biomass production, gas exchange and O₃ sensitivity of the different component species and thus providing a more realistic O₃ risk assessment.

8. 5. Ozone risk assessment for a Mediterranean annual pasture through three consecutive years with contrasting meteorological conditions

Based on the O₃-dose response function and CLe_{MS-f} developed in Chapter 6, and on the exposure response functions of Sanz et al., (2016), a risk assessment study through three growing seasons for a natural pasture of a holm-oak (*Quercus ilex*) dehesa in the Central Iberian Peninsula has been reported in Chapter 7.

The high inter-annual variability in meteorological conditions and seasonal O₃ levels typical of Mediterranean climatic conditions are well represented by the

three consecutive hydrologic years monitored at the dehesa. The three years were differently classified accordingly with their precipitation: dry, humid and normal. This variability, especially the amount of precipitation, affected the ecophysiology of the annual pasture as reflected by the variability in the maximum biomass production, the duration of the growing season and the species composition of the canopy during the 3-year study. Accordingly with previous studies, the yield of pastures and the start and end of the growing season were strongly correlated with the amount and distribution of rainfall (Vázquez de Aldana et al., 2008; Peco et al., 2009). Moreover, not only yield but species composition of the community depends on the meteorological conditions of the year. In Chapter 7 it was possible to observe how legume species were the most abundant functional type in the humid year, whereas its contribution to the total biomass was much reduced in the normal and dry years that were dominated by grasses. The variability in the species composition, life span and yield of the pasture complicates further the simulation of growth and gas exchange patterns of the canopy during the different growing seasons, which are directly correlated with the O_3 -absorbed fluxes.

Two models were considered for simulating the growth pattern of the pasture through the three growing seasons, which were the basis for modelling the stomatal O_3 -fluxes for the O_3 risk assessment, MEDPAS (González-Fernandez et al., 2010b) and DEHESA (Hernández Díaz-Ambrona et al., 2007). Before assembling these models with the DO_3SE , both were validated base on the yield samples collected in the field. The MEDPAS model computed successfully the start and the end of the growing seasons over the three years. The DEHESA model could be consider suitable in the sense of representing the maximum growth of the pasture during the humid and normal seasons, but not the driest one. However, the start and end of the growing seasons as well as the timing for the maximum biomass growth of the annual pasture appears to lag in comparison with field observations in all three growing seasons, especially in the driest one. The results highlight the need for careful consideration of growing season duration because O_3 levels tend to increase towards the summer and, thus, any delay in the modelled end of the growing season would result in higher estimates of the O_3 exposure dose and an overestimation of the risk of O_3 damage. However, both growth models are better tools for identifying the time of the year when O_3 risk assessments must be

performed, at maximum activity of the pasture, than the approaches considered for grasslands based on fixed time periods or temperature sums for describing the duration of the growing season (e.g. CLRTAP, 2010).

The risk assessments based on O_3 uptake flux (POD_1) contrasted with the assessment based on O_3 exposure (AOT40). The latter was driven by the inter-annual differences in O_3 concentrations during the growing season. Based on AOT40, the risk was the highest during the normal year, followed by the dry and humid years. However, using POD_1 the normal year also showed the highest risk of O_3 effects, the humid year showed a moderate risk, and the dry year presented a very low risk of effects. Based on O_3 uptake flux, differences among years were caused not only by differences in O_3 levels, but also by the plant physiology and phenology driven mainly by the available soil water content. The lower gas exchange rates and shorter growing season during the dry year compared with the other two resulted in lower risks of O_3 effects despite its higher O_3 concentrations.

The O_3 risk assessment based on AOT40 must be interpreted cautiously. This analysis is based on the statistically significant function and CLe_c derived from Sanz et al. (2016) for annual species, but analysis of the community experiments (Chapter 6) revealed that the AOT40 function was not significant. In contrast, the dose-response function based on POD_1 derived from the same experiments was highly significant. For this reason, the risk assessment based on the AOT40 is considered less reliable than the flux-based risk assessment. It is generally acknowledged that the O_3 effects are more closely linked to O_3 uptake than to external O_3 exposure (Musselman and Massman, 1999; Massman et al., 2000; Wieser, 1997; Matyssek et al., 2004), and POD_y index capture better the variation in the physiological activity of the annual pasture determining the O_3 uptake dose.

Results indicated that, among the yearly variable conditions, soil moisture especially is key to performing risk assessment exercises for annual Mediterranean pastures. Unfortunately, soil moisture modelling appears as one of the most limiting factors for assessing the risk of O_3 effects, especially when regional to continental scale analysis are implemented (Büker et al., 2012; Simpson et al., 2012).

Up to now, herbaceous communities are considered as the most unknown vegetation category for risk assessment within the CLRTAP (CLRTAP, 2010). Further research is needed, considering more species, climatic conditions and management regimes, in order to develop more robust response functions and CL_e for pastures dominated by annuals. However, Chapter 7 demonstrates that this thesis provides new tools for improving the O_3 -risk assessments of herbaceous Mediterranean communities.

8. 6. Future research needs

During the development of the present thesis where the O_3 and N effects on Mediterranean annual pastures have been analyzed, a great number of future research lines has been identified; some of them already under study, some others that will be addressed by future experimental field work and modelling exercises.

Through the present work, only few species of the Mediterranean annual communities have been assayed. Increasing the number species studied is necessary for finding general patterns for the different herbaceous Mediterranean communities. To accomplish this task, a significant effort is needed due the large number of herbaceous Mediterranean species and communities. Therefore, identifying general responses of different plant families or groups and selecting distinctive traits related to O_3 tolerance is mandatory.

Although biomass and nutritive quality have been identified as key response parameters for analyzing the O_3 and N effects, there are others parameters that should also be studied. Variables related with the reproductive capacity, like seed production and viability, are especially important for annual species. Although not presented in this thesis, this work is already ongoing, and germination and seed viability/seedling vitality are now under analysis based on the seeds produced in the OTCs experiments of the thesis in collaboration with the Ecology Department of the UAM.

Plant response to the pollutants should be considered also in the more complex atmospheric-plant-soil system. The effects of the pollutants in the soil

processes are scarcely assayed and need to be studied in relation with plant processes. Following this line of research, collaboration with the Agronomic School of the UPM allowed the analysis of the effects of O_3 and N on soil gas emissions of the pasture. These novel results will be the matter of an almost ready publication, and the start of a new research line that will deep on the effects of the atmospheric pollutants in the so close related soil-plant processes.

In the present study the O_3 and N effects on some of the main ecosystems services provided by annual pastures like yield, biodiversity or forage quality, have been addressed; but the Dehesa agro-forestry system offers further ecosystem services that can be affected by atmospheric pollution like carbon sequestration, climate regulation, genetic resources or food and shelter for domestic and wild animals, that need to be considered in future research.

It is also necessary to understand how other drivers of the Global Change, like water deficit or temperature increase, can modulate the plant responses to O_3 and N. In the near-term scenario of climate change and global warming, the Mediterranean basin appears to be one of the most affected areas in the globe. However, our ability to predict the effects of potential changes of O_3 exposure and N-inputs in the context of global change is still limited. Therefore, studding interactions between pollutants and other drivers are essential for realistic predictions of the Global Change effects in the ecosystems through modelling. In the last months, some steps studying the interaction between O_3 and water deficit on gas exchange and plant yield are on development in the OTC facility.

The limitations found for the two growth models (MEDPAS and DEHESA), laid on the definition of the start and end of the growing season, and in the timing of the maximum biomass growth; these are key factors that should be optimized in the models. The soil water content simulation has also been identified as a weakness step for both models, despite its greater important for identifying the pasture growing period in the Mediterranean climate. Further studies have to focus in this problem.

The uptake of O_3 by the stomata depends on the turbulence intensity above and within the canopy, the diffusive transfer in the quasi-laminar boundary layer

over the leaf surface, the biological properties of the plants, surface wetness condition and soil type. The progression in modeling the uptake throughout the canopy of different types of vegetation should be developed with multi-layer models which integrate the height of the vegetation and the O₃ absorption in 3-dimensions. The big-leaf approach presented in this thesis simplified the canopy in just 2-dimensions, which has to be improved.

It is necessary to improve O₃ risk assessment for herbaceous communities under current and future O₃ levels. However, the established critical levels for risk assessment are based on an individual species approach. This approach is very uncertain when applied to complex and diverse communities like the annual pastures. Ozone critical level derivation should be based, for these complex ecosystems, on composite multi-species canopy like the proposed approach in this thesis. Thus, the present work opens a new line for modeling pollutants absorption by vegetation that will have important repercussion in environmental policy aspects.

CHAPTER 9

Conclusions

Based on the research objectives described in Chapter 2, the following conclusions can be drawn:

1. Mediterranean annual pastures growing in competence are affected by N and O₃ through their direct and interactive effects. Since high O₃ concentrations and N deposition in the Mediterranean area are frequently recorded, both pollutants must be considered as stresses factors for annual pastures that can affect the yield, structure, quality and species composition of these ecosystems.

2. Ozone exposure induced visible injury and reduced the yield and gross primary production of the experimental annual community. Even ambient O₃ levels were sufficient to decrease aboveground biomass and NEE. Small increments of soil N availability slightly altered pasture growth, but a significant interactive effect with O₃ exposure was detected. Whereas O₃ limited the fertilization effect of higher soil N availability, higher N could compensate O₃ effects on yield only when concentrations were moderate, but not under high O₃ levels.

3. Annual species responded heterogeneously to increasing levels of O₃ and N inputs. The grasses and the herb were more O₃-tolerant, but the grasses were the most responsive to N. Legumes did not respond to N but were very O₃-sensitive, although contrasting responses were seen within the family: *Trifolium* species responded negatively, leading to reduced biomass, whereas *O. compressus* increased its biomass by taking advantage of the sensitivity of clovers to O₃. Therefore, competition within the legume family played the most important role in O₃-induced shifts in the annual community composition. The general pattern indicated that O₃ limited the effects of N while N partially counterbalanced the effects of O₃. However, the direction of the interaction can be reversed if N is too high, as evidenced by the effect on *T. striatum* roots at the highest O₃ levels.

4. Ozone exposure and N-inputs induced changes in the C and N-distribution within plant organs. The general response was an O₃-induced increase in the N-content of the aerial organs for both species. The N-inputs affected the N-content in the same direction as O₃. The O₃ effect on the grass was modulated by N availability. O₃-induced increases in N-content were not observed under low N availability. The observed effects on N-content caused an imbalance in the

corresponding C/N ratios for the different organs. The differences in the intensity of the effects between species caused an imbalance in the CP distribution within the ecosystem. Increasing N-inputs favored the accumulation of CP in the grass fraction. The alterations of the Grass/Clover CP ratio can be related to changes in the species competitiveness and with the observed changes in the pasture species structure based on biomass effects.

5. Ozone exposure increased the lignin content in leaves and stems of the clover, which is negatively related with biomass digestibility. On the contrary, N tended to improve the quality of the clover through reductions in leaf fiber content and increases in leaf RFV, although this effect can be counterbalanced by the increase in fiber content in the stems. The indirect effects of O_3 and N on forage quality through changes in community composition, plant phenology or plant senescence need to be addressed, as much as the interaction between O_3 and N effects and the environmental growing conditions, to understand the overall effect on pasture nutritive before a more general conclusion can be reached.

6. The multi-species approach of the DO_3SE for developing a canopy O_3 flux considering all the species, regardless their O_3 tolerance, and taking into account the interspecific variability in their gas exchange rates, improve the current individual-species approach of the model. The interaction existing between O_3 and N affected the derivation of the exposure- and dose-response relationships. This result implies that N can modify the O_3 CLe. Based on the average response of the pasture across N input levels, the POD_{1-MS} index outperformed the AOT40 in fitting the effects of O_3 on biomass over the two years. A new CLe_{f-MS} for Mediterranean annual pastures based on the POD_{1-MS} accumulated over 1.5 months was set at 7.9 mmol m^{-2} (5.9, 9.8 CI 95%) for a 10% of biomass reduction. This multi-species approach can help to incorporate the high diversity of semi-natural vegetation into the CLe derivation and can produce more realistic risk assessment analysis.

7. Monitoring 3-year a natural Dehesa pasture in the Central Iberian Peninsula showed the high inter-annual variability in meteorological conditions and O_3 concentrations during the pasture growth periods. The POD_1 index has greater adaptation to this high inter-annual variability than AOT40 because growing environmental conditions controlling the physiological activity of annual pastures,

in particular soil moisture, are considered in the calculation of O_3 stomatal fluxes. Under limiting soil moisture conditions, the risk of O_3 damage was low, even under high O_3 exposure. However, the risk was greater when favorable conditions for plant growth were associated with moderate to high O_3 concentrations. The use of pasture growth models coupled with the DO_3SE model helped to describe these changes and to refine the risk assessment results. Furthermore, O_3 effects were more closely linked to O_3 uptake than to external O_3 exposure. Thus, it is recommended that O_3 risk assessments be based on O_3 stomatal flux critical levels.

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ANNEX I.

Table A.I.1. Edaphic and physico-chemical characteristics of Ap horizon.

Edaphic characteristics	Organic matter by oxidation (%)	1.26
	% Clay	11.4
	% Silt	24.0
	% Sand	64.6
Physic-chemical characteristics	pH	6.9
	Olsen P (ppm)	39
	Changes N (ppm)	62
	Changes K (ppm)	244
	Changes Ca (ppm)	1780
	Changes K (ppm)	160
N mineral Content	NO ₃ ⁻ (mg N/kg soil)	17.26
	NH ₄ ⁺ (mg N/kg soil)	0.16

Assessing the chamber effect: AA Plots vs. NFA OTCs

AOT40 values in NFA chambers were slightly lower (average difference of 25%) than in ambient air (Table 3.3). In regards to meteorological conditions, an increment of 1.6 of temperature and a decrease of 148 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in PAR were recorded inside the OTC compared to AA plots since plant emergence till the second harvest. The other meteorological parameters did not present important differences to be highlighted (differences in soil temperature $<1^\circ \text{C}$, in SWC $<0.2 \text{ m}^{-3}$ and in air relative humidity $<4\%$). The growing pattern and canopy gas exchange values were compared between pasture growing in NFA chambers and in AA plots in order to control the chamber effect (Fig. A.I.1). Although no differences were recorded on species germination and distribution between AA and NFA plots, significant differences ($p < 0.05$) in total aboveground biomass were detected at the early stage of pasture development (Vegetative Stage, 46 DaE), when biomass inside the OTCs was almost 50% higher than in AA plots (mean value across N treatments; Fig. A.I.1). Similar results were found for green biomass. The same pattern was found in NEE values (Fig. A.I.1b), although these differences represented only a statistical trend ($p < 0.1$). This early delay in pasture development found in AA plots disappeared during the spring peak growth and no significant differences were recorded in the following biomass harvests or gas exchange assessments.

Figure A.I.1. Total aboveground biomass (a) and gas exchange (NEE and R_{eco}) (b) for ambient plots (AA) and non-filtered air chambers (NFA) to test the chamber effect. Different letters indicate significant differences between means (mean $\pm$ se).

ANNEX II.

Table A.II.1. Summary of the meteorological conditions for the ambient plots (AA) and chamber plots (mean across OTCs) during the whole growing season (February-June) and during the maximum growth period (April-June). RH = relative air humidity; PAR = Photosynthetic Active Radiation; PAW = Plant Available Water.

	February-June						April-June					
	24 h-mean		Max		Min		24 h-mean		Max		Min	
	AA	OTC	AA	OTC	AA	OTC	AA	OTC	AA	OTC	AA	OTC
2011												
Temperature (°C)	16.1	17.6	36.4	40.7	-1.5	-1	18.8	20.6	36.4	40.7	6.1	7
RH (%)	65.6	62.7	99.6	99.7	17.5	13.5	61.1	58	99.2	97.8	17.5	13.5
PAR ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	476.9		2248		0		550.7		2248		0	
Soil Temp (°C)	17.2	17.8	31.6	30.5	4.2	7.2	20.1	20.2	31.6	30.5	13.2	14.5
PAW (%)	87.1	75.7	110.6	104.5	71.5	55	82.1	67.3	110.6	100.4	71.5	55
Precipitation (mm)	148.1						97.7					
Watering (mm)	48.1						35					
2012												
Temperature (°C)	16.4	17.8	42	45.1	-4.5	-3	18.7	20.2	42	45.1	0.4	2
RH (%)	54.3	52.7	99.7	97.7	9.1	9.3	53.9	51.6	99.2	97.2	9.1	9.3
PAR ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	522.1		2076		0		568.5		2076		0	
Soil Temp (°C)	15.9	18	30.4	34.3	5.2	7.2	18.1	20.2	30.4	34.3	8.1	10
PAW (%)	73.1	55.6	109.5	113.6	42.4	20.4	73.7	53.4	109.5	113.6	42.4	20.4
Precipitation (mm)	78.8						62.1					
Watering (mm)	391.5						315.5					

Table A.II.2. Metrics used for evaluating the agreement of the stomatal conductance values modelled (M_i) by DO₃SE with the field measurements (observed; O_i). N corresponds to the pairs data compared. $\bar{O}$ and $\bar{M}$ correspond to the arithmetic means of the observed and modelled values, respectively.

Metrics	Statistics	Species					
	Equations	<i>T. cherleri</i>	<i>T. striatum</i>	<i>O. compressus</i>	<i>B. maxima</i>	<i>S. gallica</i>	<i>P. nanteuili</i>
Index of agreement	$IOA = 1 - \frac{\sum(M_i - O_i)}{\sum(M_i - \bar{O} + O_i - \bar{O})^2}$	0.64	0.75	0.88	0.82	0.67	0.76
Mean fractional bias	$MFB = \frac{1}{N} \sum \left(\frac{M_i - O_i}{\frac{M_i + O_i}{2}} \right)$	22%	-15%	-32%	-35%	-27%	-5%
Root mean square error	$RMSE = \left[\frac{1}{N} \sum (M_i - O_i)^2 \right]^{\frac{1}{2}}$	0.38	0.31	0.19	0.05	0.22	0.41
Pearson coefficient	$r = \frac{\sum(O_i - \bar{O})(M_i - \bar{M})}{\sqrt{\sum(O_i - \bar{O})^2 \sum(M_i - \bar{M})^2}}$	0.47	0.61	0.84	0.78	0.72	0.61

Table A.II.3. Pasture yield response functions considering the concentration-based index AOT40 ($\text{nl l}^{-1} \text{h}$) and the flux-based index $\text{POD}_{y\text{-MS}}$ (mmol m^{-2}) through a range of thresholds from 0 to 6 mmol m^{-2} averaged across nitrogen treatments. The O_3 critical levels presented were derived from the equations considering a range of yield losses from 5 to 20%. Values between brackets show the 95% confidence intervals of the $\text{CLe}_{\text{F-MS}}$.

Exposure- and dose-response functions				O_3 critical levels (multi-species)			
Ozone index	Equation	R^2	p-value	Effect relative to control			
				5%	10%	15%	20%
AOT40 ($\text{nl l}^{-1} \text{h}$)	$y = -0.008x + 0.957$	0.413	0.0858	0.9 (-0.7, 8.8)	6.9 (0.6, 13.2)	12.9 (2.0, 23.9)	19.0 (1.5, 36.4)
$\text{POD}_{0\text{-MS}}$ (mmol m^{-2})	$y = -0.015x + 1.048$	0.790	0.0032	6.4 (4.1, 8.7)	9.7 (7.7, 11.6)	12.9 (10.0, 15.8)	16.2 (11.9, 20.5)
$\text{POD}_{1\text{-MS}}$ (mmol m^{-2})	$y = -0.016x + 1.023$	0.789	0.0032	4.7 (2.5, 6.9)	7.9 (5.9, 9.8)	11.1 (8.2, 13.9)	14.2 (10.0, 18.5)
$\text{POD}_{2\text{-MS}}$ (mmol m^{-2})	$y = -0.017x + 1.009$	0.797	0.0034	3.5 (1.4, 5.5)	6.4 (4.7, 8.2)	9.4 (6.8, 11.9)	12.3 (8.5, 16.1)
$\text{POD}_{3\text{-MS}}$ (mmol m^{-2})	$y = -0.019x + 0.998$	0.801	0.0027	2.6 (0.8, 4.4)	5.3 (3.7, 6.9)	8.0 (5.6, 10.3)	10.6 (7.2, 14.1)
$\text{POD}_{4\text{-MS}}$ (mmol m^{-2})	$y = -0.021x + 0.989$	0.797	0.0029	1.9 (0.2, 3.6)	4.3 (2.9, 5.8)	6.8 (4.7, 8.9)	9.2 (6.1, 12.4)
$\text{POD}_{5\text{-MS}}$ (mmol m^{-2})	$y = -0.023x + 0.981$	0.782	0.0035	1.4 (-0.2, 2.9)	3.6 (2.2, 5.0)	5.8 (3.8, 7.8)	8.0 (5.0, 11.0)
$\text{POD}_{6\text{-MS}}$ (mmol m^{-2})	$y = -0.025x + 0.974$	0.762	0.0046	1.0 (-0.6, 2.5)	3.0 (1.7, 4.3)	5.0 (3.1, 6.9)	7.0 (4.2, 9.9)

Figure A.II.1. a) and b): seasonal profiles of meteorological parameters during the two growing cycles of the pasture (2011 and 2012) from the emergence till plant senescence. Daily mean values of: air temperature in the ambient plots (AA) and inside the OTCs (°C), plant available water (PAW, %) in AA and inside the OTCs; and water input through precipitation and watering (mm). Ozone exposure period is indicated in the graph from the starting till the end; c) and d): Total aboveground biomass of the pasture for the different O₃ treatments since emergence till the end of the life span (means across N treatments $\pm$ se). FA = charcoal filtered air, NFA = non filtered air, NFA+ = non filtered air supplemented with 20 nl l⁻¹ of O₃, NFA++ = non filtered air supplemented with 40 nl l⁻¹ of O₃.

Figure A.II.2. Individual contribution of the component species to the $\text{POD}_{\text{MS-0}}$ (from the start of the fumigation until the second harvest): a) FA 2011 (a), 2012 (d); NFA+ 2011 (b), 2012 (e), NFA++ 2011 (c), 2012 (f). FA = charcoal filtered air, NFA = non filtered air, NFA+ = non filtered air supplemented with 20 nl l^{-1} of O_3 , NFA++ = non filtered air supplemented with 40 nl l^{-1} of O_3 .

ANNEX III.

Figure A.III.1. Daily mean values of air temperature (°C) and Soil Water Content (SWC %). Daily precipitation and watering (mm) in the study area throughout the three hydrological years (1st October to 30th September).

PUBLICATIONS ARISING FROM THIS TESIS

Journals articles

Calvete-Sogo H, Elvira S, Sanz J, González-Fernández I, García-Gómez H, Sánchez-Martín L, Alonso R, Bermejo-Bermejo V (2014). Current ozone levels threaten gross primary production and yield of Mediterranean annual pastures and nitrogen modulates the response. *Atmospheric Environment* 95, 197-206

Calvete-Sogo H, González-Fernández I, Sanz J, Elvira S, Alonso R, García-Gómez H, Ibáñez-Ruiz MA, Bermejo-Bermejo V (2016). Heterogeneous responses of component species to ozone and nitrogen deposition shift the structure of Mediterranean annual pastures. *Oecologia* DOI 10.1007/s00442-016-3628-Z

Calvete-Sogo H, González-Fernández I, García-Gómez H, Alonso R, Elvira S, Sanz J, Bermejo-Bermejo V (2016). Developing ozone critical levels for multi-species canopies of Mediterranean annual pastures. *Environmental Pollution*. (Under Review)

Conference presentations / posters

Calvete-Sogo H, Sanz J, Sánchez-Martin L, Elvira S, Garcia-Torres L, de la Cruz A, González-Fernández I, Alonso R, Vallejo A, Bermejo-Bermejo V (2012). Response to ozone and nitrogen of Mediterranean annual pasture sown in natural soil: gas exchange yield and biodiversity. 25th Task Force Meeting and one-day ozone workshop. ICP Vegetation, 31th January – 2nd February. Brescia, Italia. (Oral Presentation)

Calvete-Sogo H, Sanz J, Sánchez-Martin L, Elvira S, Garcia-Torres L, González-Fernández I, Alonso R, Vallejo A, Bermejo-Bermejo V (2012). Gas exchange, Yield and Biodiversity Response to O₃ and Nitrogen of Annual Pasture Sown in Natural Soil. ÉCLAIRE 2nd Annual Meeting and GA, 15-18 October, Edinburg, UK (Oral Presentation)

Calvete-Sogo H, González Fernández I, Bermejo V, Sanz J, García Gómez H, Alonso R (2013). Modelización del desarrollo de un pasto anual de dehesa afectado por ozono.. XI Congreso Nacional de la Asociación de Ecología Terrestre, 6-10 May. Pamplona/Iruña. Spain (Oral Presentation)

Calvete-Sogo H, González-Fernández I, Sanz J, Muntifering R, Lin J, García-Gómez H, Sánchez-Martin L, Elvira S, Alonso R, Bermejo-Bermejo V (2013). Yield and quality responses of annual pastures to nitrogen and ozone. NECC-1013 (Northwest Coordinating Committee) Annual meeting. New Hampshire, USA (Oral Presentation)

Calvete-Sogo H, Bermejo-Bermejo V, Sánchez L, Muntifering R, Sanz J, Elvira S, González-Fernández I, Vallejo A, Alonso R (2013). Ozone and nitrogen effects on the annual understory vegetation of open holm oak forests. so. Vegetation response climate change and air pollution-unifying research and evidence across northern and southern hemisphere. International Union of Forest Research Organization (IUFRO) International Conference, 1 – 6 September, Ilhéus, Bahia, Brazil. (Oral Presentation)

Calvete-Sogo H (2014). Productividad y calidad de pastos de dehesa en relacion con el cambio global: efectos del ozono y del nitrógeno. Ciclo de Seminarios de Jóvenes Investigadores CIEMAT 10 October (ISBN 978-84-7834-734-6) (Oral Presentation)

Calvete-Sogo H, González-Fernández I, Sanz J, Elvira S, García-Gómez H, Valiño F, Bermejo V, Alonso R (2015). Interactive effects of ozone, nitrogen and climate on annual understory pastures of Holm oak forests. IUFRO 2015 International Congress, GreenIngUrbs and IUFRO RG7.01. 1-5 Junio Niza (Francia) (Oral Presentation)

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“The mountains are calling me and I must to go”

John Muir