Effects of climate change on native plant communities in semiarid gypsum ecosystems.

Programa de Doctorado: Biología Fundamental y de Sistemas

Universidad de Granada

María Guadalupe León Sánchez

El doctorando / The *doctoral candidate* [**María Guadalupe León Sánchez**] y los directores de la tesis / and the thesis supervisor/s: [**José Ignacio Querejeta Mercader**]

Garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por el doctorando bajo la dirección de los directores de la tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

/

Guarantee, by signing this doctoral thesis, that the work has been done by the doctoral candidate under the direction of the thesis supervisor/s and, as far as our knowledge reaches, in the performance of the work, the rights of other authors to be cited (when their results or publications have been used) have been respected.

Lugar y fecha / Place and date:

Murcia, 27/07/2016

Director/es de la Tesis / *Thesis supervisor/s;*   Doctorando / *Doctoral candidate*:

J. I. Querejeta

Firma / Signed   Firma / Signed

AGRADECIMIENTOS

A mi padre, mi referente de vida, mi modelo a seguir. Ojalá hubieras estado aquí estos años, ¡lo hubiera hecho todo mil veces mejor! Te echo de menos cada día de mi vida. Sé que hubieras estado orgulloso de mí, así que estoy feliz.

A mi madre, por animarme siempre con la tesis. Eres muy exigente, pero precisamente eso me ha salvado en infinidad de momentos en los que lo fácil era tirar la toalla. Gracias por contribuir a que esto sea una realidad, mamá.

A mis hermanos Toni, Javi, David y Beluca, por vuestro apoyo incondicional, por hacerme sentir que no estaba sola y por ayudarme siempre que lo he necesitado. Belu, eres mi hermana del alma y siempre estaremos juntas, a pesar de los kilómetros. Gracias por venir al campo a ayudarme y por los viajes curativos, pero sobre todo, gracias por preocuparte tanto por mí.

A mis sobrinos Javi, Mercedes, Toni, Marta, Álvaro, Candela, Sofía y Alicia, por llenar mi vida de una forma tan sumamente preciosa. Porque cada momento a vuestro lado viéndoos crecer es un regalo de la vida que me da fuerzas para comerme el mundo.

A David, por abrirme la mente, por comprenderme y empatizar tanto conmigo, por molar tantísimo, vamos. Me has ayudado mucho en estos años y conocerte y tener tu amistad ha sido un regalazo de la vida.

A Isabel María, Mavi y Ana, por ser mis amigas. Si os parece poco motivo es que no sabéis lo difícil que es mantener y cuidar las amistades con el tiempo. Sois geniales y especiales a más no poder. Gracias por estar ahí.

A Elena, por ser una grata sorpresa de la vida y el sol de Manchester a distancia. Hemos vivido mil cosas juntas y hemos aprendido mucho la una de la otra. Poco queda ya de aquellas pipiolas que llegaron al CEBAS desde Graná en 2011, ¡por suerte! Muchas gracias por tu apoyo, sobre todo en la última etapa, ha significado mucho para mí.

A Victoria, mi amiga y confidente. No te imaginas cuánto me has ayudado en estos años. Gracias por tus consejos, por preocuparte por mí y por hacerme sentir tan especial.

A Iván, compañero de aventuras científicas en la última etapa. Espero que podamos trabajar juntos en el futuro.

A Nacho Querejeta, por su dedicación a esta tesis. Gracias a ti he podido trabajar durante casi 5 años en algo que me apasiona profundamente.

A Emilio, compañero de aventuras ecofisiológicas. He aprendido mucho sobre ecofisiología gracias a ti en estos años y no he podido trabajar más a gusto contigo.

Y quiero agradecer especialmente a Salva, por ser mi euforia y mi incondicional. Has estado ahí siempre, apoyándome y animándome. Cada vez que iba a tirar la toalla, tú has estado ahí para darme fuerzas y motivación. Sin tu ayuda y tu apoyo incondicional esto hubiera sido mil veces más difícil. Gracias por creer en mí, bueno, gracias es una palabra que se queda muy corta…It has been overwhelming!

"Manifiesto delirista" leerás en tu pared,

Son mis versos homenaje, tú ya sabes para quién.

Qué suerte que aún hay gente que lo hace fácil

Aquéllos que consiguen que fluya bien,

Los que convierten todo en estimulante,

Y entonces ves que hay más allá y vuelve a amanecer.

Delirio amanecer…cuando ya piensas que no hay más…

He vuelto a amanecer.

(Manifiesto Delirista, Love of Lesbian)

Table of contents

ABSTRACT / RESUMEN

"If you only read the books that everyone else is reading, you can only think what everyone else is thinking."

Haruki Murakami, *Norwegian Wood*

Abstract / Resumen

ABSTRACT

Anthropogenic greenhouse gas emissions are expected to increase global mean temperature by 2-6ºC by the end of the XXI century. The Mediterranean region will be one of the most responsive to global climate change (Giorgi, 2006), and current climatic models predict drastic changes including temperature increases and reduced amount and frequency of rainfall relative to current climate conditions. Semiarid Mediterranean ecosystems might be particularly vulnerable to climate change, since both increased temperature and decreased precipitation will tend to reduce soil moisture availability, which is already the major limiting factor for primary productivity under current climate conditions. These ecosystems have a high conservation value as one of the most important biodiversity hotspots of the Earth and provide multiple ecosystem goods and services to society, but they could be at increased risk of vegetation cover loss, land degradation and desertification under the forecasted climate change scenario. Moreover, semiarid shrubland communities growing on gypsum soils are very rich in rare and endemic species (several of our target shrub species are considered vulnerable in the IUCN Red List of Spanish Vascular Flora). In this thesis, we have simulated the climate conditions projected for the second half of XXI century, by using open top chambers (1-2ºC temperature increase in the wintertime and 4-6ºC in the summertime; W treatment), rainout shelters (-30% rainfall exclusion; RR treatment) and their combination (W+RR treatment) in order to assess the effects of forecasted climate change conditions on the performance of three semiarid Mediterranean shrubland communities located in central (Aranjuez) and southeastern Spain (Sorbas and Sax) throughout 4 hydrological years (2011-2015). Our target species are *Helianthemum squamatum* (present at the three study sites)*, Helianthemum syriacum, Gypsohila struthium, Santolina viscosa, Teucrium turredanum* and *Coris hispanica*, which are native shrub species with different sizes/biovolumes, life history traits, phenology, stoichiometry, water use strategies and mycorrhizal association types (ectomycorrhizal or arbuscular mycorrhizal). For this purpose, we measured leaf gas exchange parameters (photosynthesis rate, stomatal conductance, transpiration, maximum efficiency of photosystem II under light conditions, the quantum efficiency of photosystem II and water use efficiency), carbon isotope ratio (δ^{13}C), foliar nutrient status, leaf mass per unit area, shoot dry biomass production, shoot elongation, shoot growth phenology and survival rate at the end of the 4-year study period, as well as mycorrhizal fungal community composition and relative

Abstract / Resumen

abundances. We hypothesized that warming, rainfall reduction and their combination would reduce soil water availability to an extent that would significantly impair plant nutrient uptake and status, while at the same time increasing stomatal limitation of photosynthesis, thus negatively affecting photosynthesis, productivity and survival across coexisting plant species in these three semiarid ecosystems. Moreover, we predicted that the climate manipulation treatments would impair the performance of both mycorrhizal fungi and their host plants due to the adverse effects of increased heat and drought stress on fungal and plant physiology.

Warming sharply reduced the net photosynthesis rate and water use efficiency of the target plants across the three experimental sites throughout the 4-year study period, without any evidence of photosynthetic acclimation to warming. The target plants also showed a significant downregulation of photosystem II, likely to match a reduced carboxylation capacity linked to decreased leaf nutrient (N, P) status. Stomatal conductance was unaffected by warming in the experimental site of Sax, likely due to the presence of a *Pinus halepensis* overstory that provides shade to understory *H. squamatum* shrubs and thus moderates temperature and vapor pressure deficit extremes. In the sites of Aranjuez and Sorbas, stomatal conductance was generally increased by warming, probably as an adaptive mechanism to prevent leaf overheating and photosynthetic machinery damage through enhanced evaporative leaf cooling. The leaf $\delta^{13}C$ values of the target shrub species were consistently lower in plants exposed to warming across the three study sites, indicating decreased time-integrated water use efficiency under warming and demonstrating the validity of stable isotope techniques as a useful tool in ecophysiological studies evaluating the impacts of climate manipulation on vegetation. Warming also consistently reduced leaf N and P concentrations relative to control plants across study sites over the 4-years study period, which could be the result of a hindered soil nutrient mineralization, solubilization, diffusion and/or uptake by roots under warming, owing to a more rapid and severe drying of the upper soil layers where the majority of nutrients available for plants are located. In the site of Sorbas, we found that the foliar concentrations of other important nutrients for photosynthesis such as K, Fe, Cu and Zn were also strongly decreased by warming and moderately decreased by rainfall reduction. Warming strongly reduced shoot dry biomass production relative to control plants in all the target species across study sites. Warmed plants showed an advanced shoot growth phenology, concentrating vegetative growth in the earlier part of spring. Simulated climate change sharply reduced the

Abstract / Resumen

diversity and relative abundance of EMF communities in the rhizosphere of *H. squamatum* in the site of Aranjuez, likely due to detrimental effects of soil warming and drying on mycorrhizal fungi and to reductions in belowground carbon allocation by host plants. The deeply interdependent responses of plants and their EMF partners to experimental climate change had mutually amplifying effects that strongly reduced plant nutrient status, photosynthesis, water use efficiency, shoot biomass production and drought survival. The post-summer survival rate of *H. squamatum* was not affected by warming at the Sax site, likely due to the buffering effects of the pine overstory on microclimatic conditions for understory plants. In the coolest and wettest site, Aranjuez, the post-summer survival rate of *H. squamatum* shrubs remained high and similar across treatments in years with above- or near-average rainfall, likely due to the wide range of adaptive phenotypic plasticity mechanisms that enable plants to adjust their physiology to warmer and/or drier conditions. However, in a dry year, warming exceeded plant phenotypic plasticity capacity, leading to sharp declines in post-summer survival rate, probably due to plant hydraulic impairment caused by xylem embolism, carbon starvation, or the combination of both factors. In the warmest and driest site (Sorbas), plant survival rate at the end of the 4 year study period was sharply reduced by experimental warming across target species (especially when in combination with rainfall reduction).

Across species and experimental sites, the detrimental effects of warming on plant performance were generally stronger than those of rainfall reduction, likely because native plant species are well preadapted to nearly chronic drought stress. The detrimental effects of the combination of warming and rainfall reduction on plant performance tended to be additive (or not even that) rather than synergistic across species and sites.

Overall, the results of this thesis highlight the potential vulnerability of Mediterranean-type native semiarid shrublands (and their ectomycorrhizal fungal partners in the case of *H. squamatum*) to forecasted climate change, which will likely cause multiple detrimental feedback loops that could lead to an alternative state of decreased vegetation productivity and push these ecosystems to a degradation and desertification pathway. Therefore, the findings reported in thesis will hopefully contribute to a better understanding and anticipation of the detrimental impacts of ongoing climate warming and aridification on the structure and functioning of plant and mycorrhizal fungal

Abstract / Resumen

communities in semiarid gypsum ecosystems, which will ultimately aid the long-term management and conservation of biodiversity in these vulnerable habitats.

Abstract / Resumen

RESUMEN

Las emisiones de gases de efecto invernadero van a aumentar la temperatura media global entre 2 y 6º C a finales del siglo XXI. La Cuenca Mediterránea será una de las regiones más sensibles al cambio climático global, ya que los actuales modelos climáticos predicen cambios drásticos que incluyen un aumento de la temperatura y una reducción de la cantidad y frecuencia de las precipitaciones respecto a las condiciones climáticas actuales. Los ecosistemas Mediterráneos semiáridos podrían ser particularmente vulnerables al cambio climático, ya que este aumento de la temperatura y reducción de la precipitación reducirán la disponibilidad de agua del suelo, lo cual es ya el principal factor limitante para la productividad primaria en estos ecosistemas bajo las actuales condiciones climáticas. Estos ecosistemas tienen un alto valor de conservación como uno de los "puntos calientes" de biodiversidad más importantes de la Tierra, y proporciona multitud de bienes ecosistémicos y servicios a la sociedad. Sin embargo, podrían estar en creciente riesgo de pérdida de la cubierta vegetal, degradación del suelo y desertificación bajo el escenario de cambio climático proyectado. Además, las comunidades de matorrales semiáridos que viven en suelos de yesos son muy ricas en especies raras y endémicas (algunas de nuestras especies de matorral estudiadas son consideradas como vulnerables en la Lista Roja de la Flora Vascular Española de la UICN). En esta tesis, hemos simulado las condiciones climáticas proyectadas para la segunda mitad del siglo XXI, usando para ello "open top chambers" (OTCs: 1-2ºC de aumento promedio de temperatura en invierno y hasta 4-6ºC de aumento en verano; tratamiento W), casetas de exclusión parcial de lluvia (30% exclusión de lluvia; tratamiento RR) y la combinación de ambos tratamientos (tratamiento W+RR), con el fin de evaluar los efectos de las condiciones de cambio climático proyectadas sobre el funcionamiento ecofisiológico de 3 comunidades de matorral semiárido Mediterráneo localizadas en el centro (Aranjuez) y sureste de España (Sorbas y Sax) a lo largo de 4 años hidrológicos (2011-2015). Nuestras especies estudiadas son *Helianthemum squamatum* (presente en los tres sitios de estudio)*, Helianthemum syriacum, Gypsohila struthium, Santolina viscosa, Teucrium turredanum* y *Coris hispanica*, las cuales son especies nativas de matorral con diferentes tamaños, biovolúmenes, rasgos foliares, fenología, estequiometría, estrategias del uso del agua y tipos de asociación micorrícica (ectomicorrizas o micorrizas arbusculares). Con este fin, llevamos a cabo medidas de intercambio de gases (tasa fotosintética, conductancia

Abstract / Resumen

estomática, eficiencia máxima del fotosistema II bajo condiciones de luz, eficiencia cuántica del fotosistema II y eficiencia en el uso del agua), composición isotópica en carbono de las hojas ($\delta^{13}C$), concentraciones foliares de nutrientes, peso de la hoja por unidad de área, producción de biomasa seca de tallos, elongación de los tallos, fenología del crecimiento de los tallos y tasa de supervivencia al final del estudio de 4 años de duración, así como la composición de la comunidad de hongos micorrícicos y sus abundancias relativas (esto último en Aranjuez solamente). Nuestra hipótesis es que el calentamiento, la exclusión de lluvia y su combinación reducirán la disponibilidad de agua del suelo hasta un punto en el que se podría reducir significativamente la absorción de nutrientes por las plantas y su status nutricional, al mismo tiempo que aumentará la limitación estomática de la fotosíntesis, afectando así negativamente a las tasas de asimilación de carbono, productividad y supervivencia en todas las especies de plantas coexistentes en estos 3 ecosistemas semiáridos. Además, predecimos que los tratamientos de manipulación del clima podrían reducir la diversidad y abundancia relativa de los hongos micorrícicos, debido a los efectos adversos del creciente estrés por calentamiento y sequía sobre la fisiología de los hongos micorrícicos y de sus plantas huésped.

El calentamiento redujo drásticamente la tasa fotosintética neta y la eficiencia en el uso del agua de las plantas en los 3 sitios experimentales y a lo largo de los 4 años del estudio, sin evidencia alguna de aclimatación de la fotosíntesis al calentamiento. Las plantas también mostraron una regulación a la baja del fotosistema II, probablemente para adaptarse a la reducida capacidad de carboxilación ligada a la reducción del status nutricional de las hojas (N, P). La conductancia estomática no se vio afectada por el calentamiento en el sitio experimental de Sax, probablemente debido a la presencia de un dosel arbóreo de *Pinus halepensis* que proporciona sombra a los *H. squamatum* del estrato inferior, moderando así los extremos de temperatura y déficit de presión de vapor. En los sitios de Aranjuez y Sorbas, la conductancia estomática generalmente se vio aumentada por el calentamiento, probablemente como mecanismo de adaptación para prevenir el sobrecalentamiento foliar y los consiguientes daños en la maquinaria fotosintética, a través de un mayor enfriamiento evaporativo de la hoja. Los valores de $\delta^{13}C$ foliar de las especies estudiadas fueron consistentemente más bajos en las plantas expuestas a calentamiento en los 3 sitios experimentales, indicando una reducción de la eficiencia en el uso del agua integrada en el tiempo como consecuencia del

Abstract / Resumen

calentamiento y demostrando la validez de las técnicas de isótopos estables como una herramienta útil en estudios ecofisiológicos que persigan evaluar los impactos de la manipulación del clima sobre la vegetación. El calentamiento también redujo de forma consistente las concentraciones foliares de N y P con respecto a las plantas control en todos los sitios experimentales y a lo largo de los 4 años de estudio, lo cual podría ser el resultado de una reducida mineralización de los nutrientes del suelo, solubilización, difusión y/o consumo por parte de las raíces bajo calentamiento, debido a un más rápido y severo secado de las capas más superficiales del suelo donde se encuentra la mayoría de nutrientes disponibles para las plantas. En el sitio de Sorbas, encontramos que las concentraciones foliares de otros importantes nutrientes para la fotosíntesis como son el K, Fe, Cu y Zn también se redujeron fuertemente bajo calentamiento y más moderadamente bajo reducción de lluvia. El calentamiento redujo fuertemente la producción de biomasa de los tallos con respecto a las plantas control en todas las especies estudiadas y sitios experimentales. Las plantas calentadas mostraron un adelanto fenológico del crecimiento de los tallos, concentrando su crecimiento vegetativo en la primera parte de la primavera. El calentamiento y la reducción de lluvia redujeron drásticamente la diversidad y abundancia relativa de las comunidades de hongos ectomicorrícicos en la rizosfera de *H. squamatum* en el sitio de Aranjuez, debido probablemente a los efectos negativos del calentamiento y secado del suelo sobre los hongos micorrícicos y a la reducción de la asignación de carbono a micorrizas por parte de las plantas huésped. Las respuestas interdependientes de las plantas y sus hongos ectomicorrícicos frente al cambio climático experimental tuvieron efectos negativos multiplicativos que redujeron en gran medida el status nutricional de las plantas, la fotosíntesis, la eficiencia en el uso del agua, la producción de biomasa aérea y la supervivencia frente a la sequía. La tasa de supervivencia tras el verano de *H. squamatum* no se vio afectada por el calentamiento en Sax, debido probablemente a que los pinos del dosel arbóreo ejercieron un afecto amortiguador del microclima de los matorrales del estrato inferior. En el sitio más frío y húmedo, Aranjuez, la tasa de supervivencia de *H. squamatum* tras el verano se mantuvo alta y sin diferencias entre tratamientos en años con precipitación anual cercana o superior al promedio histórico, seguramente por la amplia variedad de mecanismos de plasticidad fenotípica que permitieron a las plantas adaptarse ajustando su fisiología a las nuevas condiciones más cálidas y secas de cambio climático. Sin embargo, en un año seco, el calentamiento excedió la capacidad de plasticidad fenotípica de las plantas, dando lugar a una drástica

Abstract / Resumen

reducción de la tasa de supervivencia despúes del verano. Esto fue debido probablemente a una insuficiencia hidráulica causada por embolismo del xilema, agotamiento de las reservas de carbono de la planta, o a la combinación de ambos factores. En el sitio más cálido y seco, Sorbas, la tasa de supervivencia al final de los 4 años de estudio se vio fuertemente reducida por el calentamiento experimental en todas las especies de la comunidad, especialmente en el tratamiento de combinación de calentamiento y exclusión de lluvia.

En todas las especies y sitios experimentales, los efectos perjudiciales del calentamiento sobre la ecofisiología, crecimiento y supervivencia de las plantas fueron generalmente más fuertes que los de la exclusión parcial de lluvia, posiblemente porque las especies de matorral nativas de ecosistemas semiáridos están bien pre-adaptadas al estrés hídrico crónico. Los efectos perjudiciales de la combinación de calentamiento y exclusión de lluvia sobre las plantas fueron más bien aditivos (o ni siquiera) que sinérgicos en todas las especies y sitios experimentales.

En conjunto, los resultados de esta tesis destacan la alta vulnerabilidad potencial de las comunidades de matorrales nativos del Mediterráneo semiárido (y de sus hongos ectomicorrícicos en el caso de *H. squamatum*) frente al cambio climático previsto, el cual probablemente causará múltiples bucles de realimentación que podrían dar lugar a un estado alternativo de productividad primaria reducida que llevaría a estos ecosistemas hacia una senda de degradación y desertificación progresivas. Se espera que los resultados de esta tesis contribuyan a una mejor comprensión y anticipación de los impactos perjudiciales del calentamiento y aridificación del clima sobre la estructura y funcionamiento de las comunidades de plantas y hongos micorrícicos en los ecosistemas semiáridos de yesos, lo cual ayudará en último término a la gestión y conservación a largo plazo de la biodiversidad en estos hábitats vulnerables.

Abstract / Resumen

References consulted / Bibliografía consultada

Bussotti F, Ferrini F, Pollastrini M, Fini A (2014) The challenge of Mediterranean sclerophyllous vegetation under climate change: From acclimation to adaptation. *Environmental and Experimental Botany*, **103**, 80-98.

Cernusak LA, Ubierna N, Winter K, Holtum JA, Marshall JD, Farquhar GD (2013) Environmental and physiological determinants of carbon isotope discrimination in terrestrial plants. *New Phytologist*, **200**, 950-965.

Collins M, Knutti R, Arblaster J *et al.* (2013) Climate change 2013: the physical science basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Long-term Climate Change: Projections, Commitments and Irreversibility, Cambridge University Press, Cambridge, UK, and New York.

Dawson TE, Mambelli S, Plamboeck AH, Templer PH, Tu KP (2002) Stable isotopes in plant ecology. *Annual review of ecology and systematics*, 507-559.

Giorgi F (2006) Climate change hot-spots. *Geophysical research letters*, **33**.

Giorgi F, Lionello P (2008) Climate change projections for the Mediterranean region. *Global and Planetary Change*, **63**, 90-104.

Gunderson CA, O'hara KH, Campion CM, Walker AV, Edwards NT (2010) Thermal plasticity of photosynthesis: the role of acclimation in forest responses to a warming climate. *Global Change Biology*, **16**, 2272-2286.

Leake JR, Johnson D, Donnelly DP, Muckle GE, Boddy L, Read DJ (2004) Networks of power and influence: the role of mycorrhizal mycelium in controlling plant communities and agroecosystem functioning. *Canadian Journal of Botany*, **82**, 1016–1045.

McDowell NG, Beerling DJ, Breshears DD, Fisher RA, Raffa KF, Stitt M (2011) The interdependence of mechanisms underlying climate-driven vegetation mortality. *Trends in Ecology & Evolution*, **26**, 523-532.

Moreno JC (2010) Lista Roja 2008 de la flora vascular española.

Abstract / Resumen

Myers N, Mittermeier RA, Mittermeier CG, Da Fonseca GA, Kent J (2000) Biodiversity hotspots for conservation priorities. *Nature*, **403**, 853-858.

Nardini A, Lo Gullo MA, Trifilò P, Salleo S (2014) The challenge of the Mediterranean type climate to plant hydraulics: responses and adaptations. *Environmental and Experimental Botany,* **103**, 68–79.

Nicotra AB, Atkin OK, Bonser SP, Davidson AM, Finnegan EJ, Mathesius U *et al.* (2010) Plant phenotypic plasticity in a changing climate. *Trends in plant science*, **15**, 684-692.

Reich PB, Oleksyn J, Wright IJ (2009) Leaf phosphorus influences the photosynthesis–nitrogen relation: a cross-biome analysis of 314 species. *Oecologia*, **160**, 207–212.

Rodgers VL, Hoeppner SS, Daley MJ, Dukes JS (2012) Leaf-level gas exchange and foliar chemistry of common old-field species responding to warming and precipitation treatments. *International Journal of Plant Sciences*, **173**, 957-970.

Sage RF, Kubien DS (2007) The temperature response of C3 and C4 photosynthesis. *Plant, Cell & Environment*, **30,** 1086–1106.

Sardans J, Peñuelas J (2013) Plant-soil interactions in Mediterranean forest and shrublands: impacts of climatic change. *Plant and Soil*, **365**, 1-33.

Smith SE, Read DJ (2008) Mycorrhizal symbiosis. Edn. 3rd., Academic Press.

Wu Z, Dijkstra P, Koch GW, Peñuelas J, Hungate BA (2011) Responses of terrestrial ecosystems to temperature and precipitation change: a meta-analysis of experimental manipulation. *Global Change Biology*, **17**, 927-942.
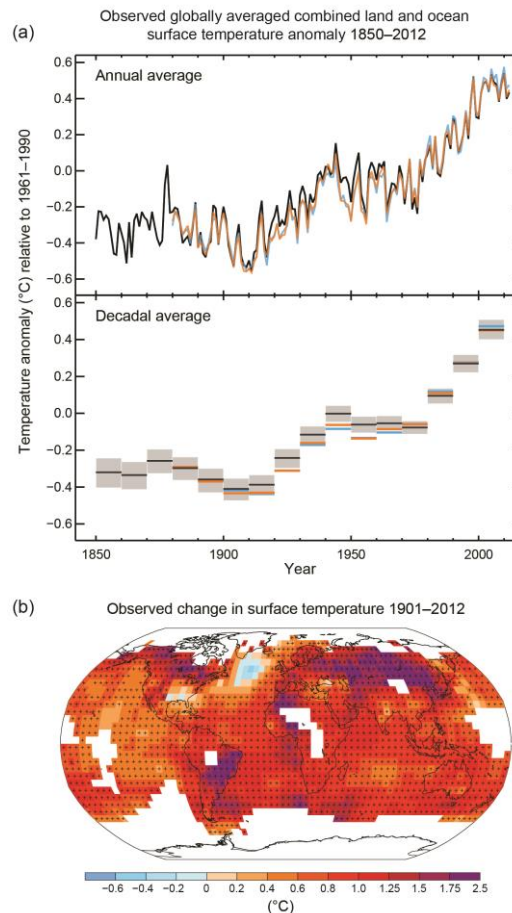
GENERAL INTRODUCTION

"A certain type of perfection can only be realized through a limitless accumulation of the imperfect."

Haruki Murakami, *Kafka on the Shore*
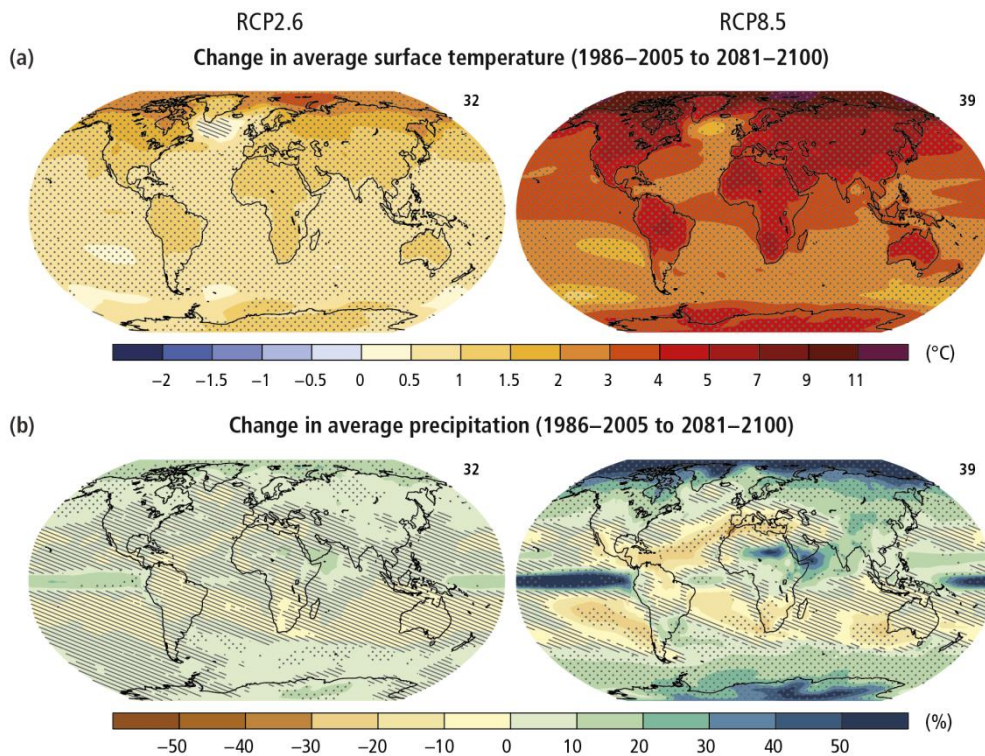
GENERAL INTRODUCTION

Atmospheric carbon dioxide concentrations have increased by 40% since pre-industrial times due to anthropogenic fossil fuel combustion and land use change emissions, with strong consequences for the climate system. As a consequence of increasing concentrations of greenhouse gases of anthropogenic origin, the atmosphere and oceans temperatures have increased, snow and ice have been reduced and sea level has risen (Alexander et al., 2013). According to the fifth assessment report of the IPCC, climate warming is unequivocal and changes observed since the 1950s are unprecedented (Figure 1, IPCC 2013). Since 1900, temperature of the Earth surface has been increasing steadily, with nearly every decade being warmer that any preceding decade. Indeed, 2015 was the warmest year globally by the widest margin on record (1880-2015) (NOAA, 2015). These rapid changes may exceed the capacities of plant individuals, populations and communities to adjust and adapt to them (Peñuelas et al., 2013).

General Introduction

Figure 1. (a) Observed global mean combined land and ocean surface temperature anomalies, from 1850 to 2012 from three data sets. Top panel: annual mean values. Bottom panel: decadal mean values including the estimate of uncertainty for one dataset (black). Anomalies are relative to the mean of 1961−1990. (b) Map of the observed surface temperature change from 1901 to 2012 derived from temperature trends determined by linear regression from one dataset (orange line in panel a) (from IPCC 2013).

The Coupled Model Intercomparison Project Phase 5 (CMIP5) used in the fifth assessment report of ICPP predicts that global mean temperatures will continue to rise over the 21st century if greenhouse gas emissions persist (0.3-4.8℃ for the period 2016-2100, depending on the RCP scenario), and this rise will not be regionally uniform. Changes in precipitation patterns will not be uniform either. Average precipitation will be more contrasted between wet and dry regions and between wet and dry seasons. In many mid-latitude and subtropical dry regions, mean precipitation will likely decrease, while in high latitudes and the equatorial Pacific many mid-latitude wet regions, mean precipitation will likely increase. Extreme precipitation events over most of the mid-latitude land masses and over wet tropical regions will very likely become more intense and more frequent (Figure 2) Furthermore, climate change will lead to increases in intensity and/or duration of drought and heat waves events (Alexander et al., 2013).

General Introduction

Figure 2. Change in average surface temperature (a) and change in average precipitation (b) based on multi-model mean projections for 2081–2100 relative to 1986–2005 under the RCP2.6 (left) and RCP8.5 (right) scenarios. The number of models used to calculate the multi-model mean is indicated in the upper right corner of each panel. Stippling (i.e., dots) shows regions where the projected change is large compared to natural internal variability and where at least 90% of models agree on the sign of change. Hatching (i.e., diagonal lines) shows regions where the projected change is less than one standard deviation of the natural internal variability (from IPCC 2013).

Several meta-analyses have assessed plant responses to warming (Rustad et al., 2001; Lin et al., 2010; Wu et al., 2011), altered precipitation (Gerten et al., 2008; Wu et al., 2011) and their combination (Wu et al., 2011) at global scale. General trends point to a stimulated plant biomass and productivity in response to increased temperature, likely due to an increase in net photosynthesis rate and extended growing season and/or an enhanced nutrient availability resulting from increased rates of decomposition and N mineralization (Rustad et al., 2001). Another general pattern found is that decreased precipitation reduced plant biomass production and ecosystem primary productivity, owing to a decreased photosynthesis rate caused by increased stomatal limitation. However, few of the studies included in these meta-analyses were carried out in Mediterranean-type ecosystems, and even fewer in semiarid climates. Lin et al., (2010) found a quadratic response of plant biomass production to climate warming along a mean annual temperature gradient analysing 127 studies worldwide. They found that, in areas with mean annual temperature above 18ºC, plant biomass production generally decreases with further increases in temperature. This indicates that areas that are already above (or near) this mean annual temperature threshold will not necessarily follow the general global trend of increasing primary productivity with warming, and that vegetation in these areas will thus be threatened by the largely negative impacts of climate change. Therefore, it is necessary to gain additional experimental knowledge on the responses of these vulnerable ecosystems to climate change, in order to establish policies to prevent and reverse the expansion of desertification processes at global scale.

The Mediterranean area will be one of the most responsive regions to global climate change (Giorgi, 2006) and current GCMs projections warn about the predictably strong negative impacts of the forecasted climate change scenarios for this region. A marked warming, more frequent heat wave events and a pronounced decrease in the frequency and amount of precipitation are projected, especially in the summer season (Pausas,

2004; Giorgi, 2006). Predicted climate warming will exceed 4-5ºC and annual precipitation will decrease by 25-30% across the Mediterranean Region (Giorgi & Lionello, 2008; see Figures 3 and 4). Therefore, the Mediterranean region will be particularly vulnerable to climate change, since these predicted changes will increase aridity conditions and reduce soil moisture availability (Dai 2013, Huang et al. 2015), thus reducing plant primary productivity and vegetation cover (Delgado-Baquerizo et al. 2013; Williams et al., 2013; Ahlström et al., 2015), which will increase the risk of soil erosion, land degradation and desertification (Le Houérou 1996; Safriel & Adeel 2005).
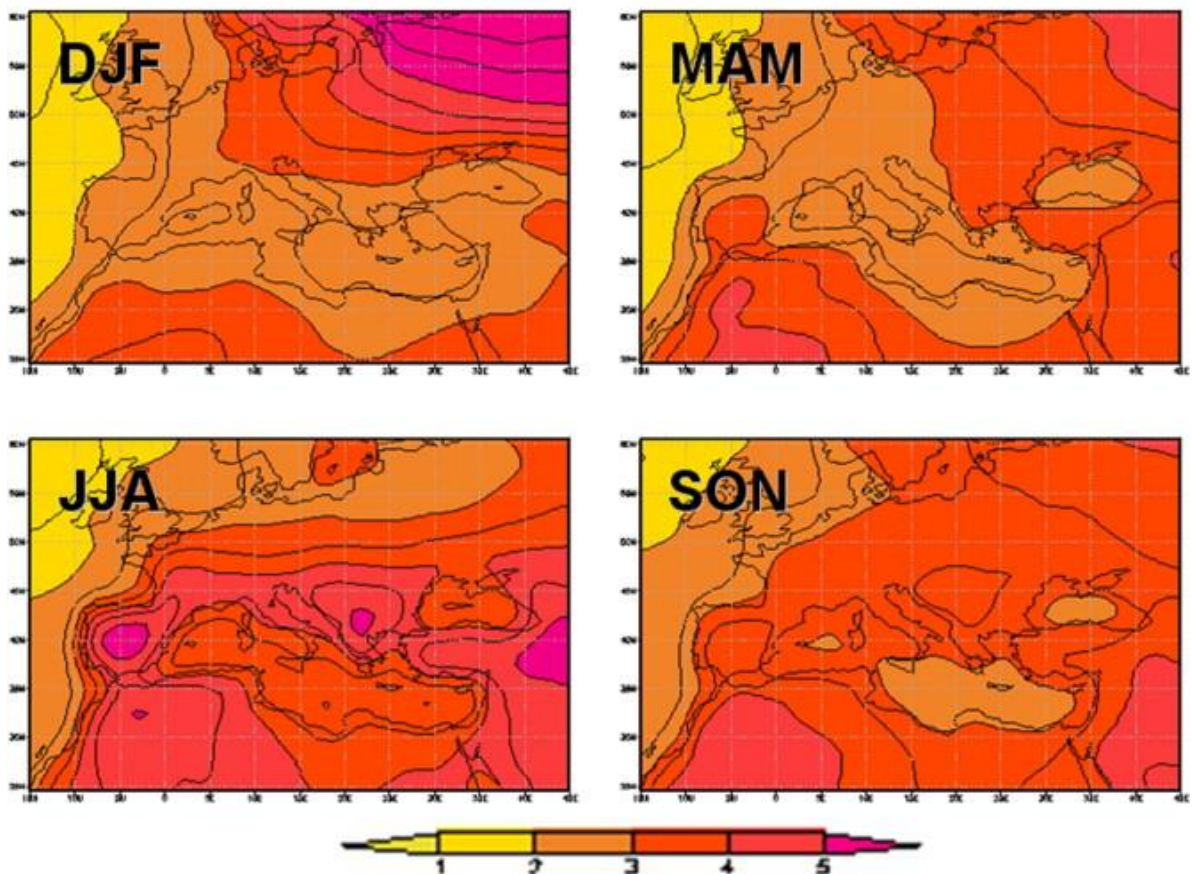

Figure 3. MGME ensemble average change in surface air temperature for the four seasons, 2071–2100 minus 1961–1990, A1B scenario. Units are °C. DJF is December–January–February, MAM is March–April–May, JJA is June–July–August, SON is September–October–November (from Giorgi & Lionello 2008).

General Introduction

Precipitation change (%, 2071-2100 minus 1961-1990), MGME ensemble average, A1B scenario
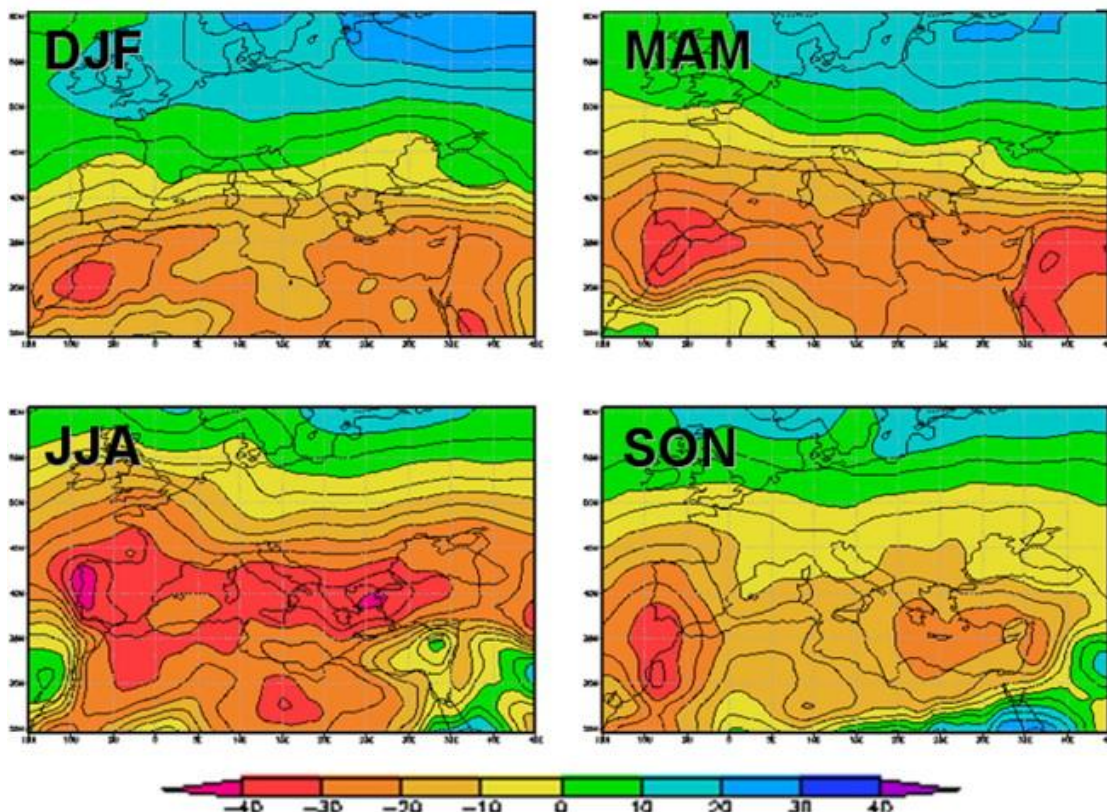

Figure 4. MGME ensemble average change in precipitation for the four seasons, 2071–2100 minus 1961–1990, A1B scenario. Units are % of 1961–1990 value. DJF is December–January–February, MAM is March–April–May, JJA is June–July–August, SON is September–October–November (from Giorgi & Lionello 2008).

The Mediterranean area is one of the most important biodiversity hotspots in the world with 4.3% of endemic plants, thus representing a conservation priority, since it occupies less than 5% of the Earth surface but contains almost 20% of the vascular plant species (Cowling et al., 1996; Myers et al., 2000). Mediterranean-type ecosystems, and drylands in general, often behave as net carbon sinks under current climate conditions, but may turn into net carbon sources as a consequence of land degradation resulting from the combined effects of anthropogenic land use change and climate aridification (Ciais et al., 2005; Luo et al., 2007; Pereira et al., 2007; Aires et al., 2008; Padilla et al., 2010), which is a major threat since drylands occupy 41% of the total terrestrial surface.

In order to better understand and anticipate how natural and semi-natural Mediterranean plant communities will respond to climate change, manipulative field studies need to be carried out that accurately simulate projected climatic conditions. Recent research based

on manipulative field experiments has documented the response of Mediterranean-type plant communities to increased temperature or to decreased precipitation. These pioneering studies have reported significant effects of altered temperature or precipitation on a wide array of ecosystem properties or processes, including: plant nutrient status and physiological activity (Llorens et al., 2003; Sardans et al., 2008); plant growth and biomass accumulation (Peñuelas et al., 2007; Prieto et al., 2009a); or plant community composition, structure, diversity and successional dynamics (Prieto et al., 2009b; Lloret et al., 2009). However, these studies have been largely carried out in ecosystems located in dry or sub-humid Mediterranean ombroclimates, where water is not as severely limiting for primary productivity as in Mediterranean semiarid ecosystems, where temperatures during the summer drought period are less extreme. Therefore, there is a lack of knowledge about how semiarid Mediterranean ecosystems might respond to climate warming and aridification. It is well known that the magnitude of the effects of experimental climate change on plant performance can differ greatly among different levels of soil-water availability (Peñuelas et al., 2013). The general trends emerging from these studies carried out in non-semiarid Mediterranean ecosystems point to moderate and contrasting growth responses to warming among coexisting species, and a more consistent reduction in plant growth and improved water use efficiency in response to drought across species. These studies suggest that plant sensitivity to extreme climate conditions is highly species-specific (Llorens et al., 2004; Prieto et al., 2009; Ogaya et al., 2011), albeit general trends points towards an enhanced resource use efficiency by plants under climatic stress (Peñuelas et al., 2013) and stronger detrimental effects of drought relative to those of warming.

Moreover, the majority of manipulative field studies conducted to date in Mediterranean-type ecosystems have attempted to evaluate the effects of increased temperature or reduced precipitation separately, without considering potential interactions between these factors. Only a few studies have assessed the combined effects of warming and rainfall reduction on plant performance, with smaller responses than expected from the single-factor responses (Luo et al., 2008; Wu et al., 2011; Rodgers et al., 2012), thus suggesting non-synergistic but rather additive effects of the combination of warming and rainfall reduction on plant performance. On the other hand, research conducted in other climatic regions has shown that the combined effect of simultaneous changes in two or more of the major drivers of global change

(temperature, precipitation, rise in the atmospheric concentration of CO2) on terrestrial ecosystems can be markedly different from the effects of changes in a single driver (e.g. Hanson et al. 2005; Dermody et al., 2007), likely due to the often opposite sign of the effects of these climate change drivers on plant response variables. Plants from semiarid ecosystems have evolved a wide range of adaptive mechanisms to cope with the prevailing harsh climatic conditions, which could buffer the predictably negative impacts of climate change on plant performance (Nicotra et al., 2010; Bussotti et al., 2014; Nardini et al., 2014). It is therefore necessary to design and carry out new field studies in which two or more climate change drivers are manipulated simultaneously, in order to gain insight into how these drivers interact with each other to affect the structure and functioning of Mediterranean ecosystems.

Ongoing climate change strongly affects the C and nutrient cycles, resulting in feedbacks to the global climate system (Heimann & Reichsten, 2008). Nitrogen and phosphorus are essential macronutrients for primary producers in terrestrial ecosystems (Dijkstra et al., 2012) and their deficiency is well known to inhibit photosynthesis, plant growth and thus, plant biomass production (Chandler & Dale 1995; Huang et al., 2004; Wright et al 2004, Thomas, Montagu & Conroy 2006, Reich, Oleksyn & Wright 2009). There is a reported relationship between climate variables and plant elemental composition (N, P, K), as leaf nutrient concentrations generally decrease with increasing temperatures and increase with increasing rainfall at broad geographical scales (Reich & Oleksyn., 2004; Sardans et al., 2016). Hence, in semiarid Mediterranean ecosystems we expect a strong decline in the concentrations of these essential elements in plant foliar tissues under a warmer and drier climate, with severe consequences for plant nutrient status and photosynthetic capacity.

Gypsum soils are particularly stressful for plants due to their high salt concentration, low fertility and unfavourable physical properties (Porta et al., 2003; Escudero et al., 2015). Semiarid plant communities growing on gypsum soils are rich in rare and endemic species of high conservation value (Rubio & Escudero 2000; Escudero et al., 2015). For example, gypsum ecosystems of the southern part of the Madrid province show higher biodiversity and greater number of endemic plant species (many with small and shrinking distribution ranges) than any other ecosystems in this region, and are therefore a top priority for conservation efforts at the national and European levels. The

General Introduction

European Union included this habitat in Annex II of the Habitats Directive (European Union 1992) as one of the most threatened habitats in Spain and Europe. Recent studies have shown that the soil microbial communities present in gypsum soils are also very rich in rare and endemic species (e.g. mycorrhizal fungal communities, Alguacil et al., 2009).

Recent studies suggest that climate aridification could lead to important changes in the composition and structure of mycorrhizal fungal communities in Mediterranean-type ecosystems, including displacement of ectomycorrhizal fungi (EMF) by arbuscular mycorrhizal fungi (AMF) in systems that are co-dominated by both groups of symbiotic fungi under current climatic conditions (Querejeta et al., 2009). This shift in the composition of soil mycorrhizal fungal communities has been reported for dryland ecosystems in North America (Querejeta et al., 2009), and might represent an important adaptive mechanism of the vegetation to more xeric environmental conditions, because the AMF symbiosis appears to be better adapted to heat and drought stress, and is thus more efficient and more cost-effective for plants (in terms of carbon investment in exchange for nutrients received) in ecosystems where primary productivity is severely limited by low water availability (Vargas et al., 2010). The dwarf scrub communities growing on gypsum soils in semiarid Spain are rich in plant species of the genus *Helianthemum* (e.g. *H. squamatum, H. syriacum*) that are capable of forming tripartite symbiotic associations with both EM and AM fungi simultaneously (Gutiérrez et al., 2003), so they are a suitable system to assess whether the abovementioned pattern of response of mycorrhizal fungal communities to climate change might also occur in Mediterranean-type semiarid ecosystems.

General Introduction

References

Ahlström A, Raupach MR, Schurgers G *et al.* (2015) The dominant role of semi-arid ecosystems in the trend and variability of the land CO2 sink. *Science*, **348**, 895–899.

Aires LMI, Pio CA, Pereira JS (2008) Carbon dioxide exchange above a Mediterranean C3/C4 grassland during two climatologically contrasting years. *Global Change Biology*, **14**, 539-555.

Alexander L *et al.* (2013) Summary for policymakers, in Climate Change 2013: The Physical Science Basis. Working Group I Contribution to the IPCC 5th Assessment Report, Stockholm, Sweden.

Alguacil MM, Roldán A, Torres MP (2009) Assessing the diversity of AM fungi in arid gypsophilous plant communities. *Environmental Microbiology*, **11**, 2649-2659.

Bussotti F, Ferrini F, Pollastrini M, Fini A (2014) The challenge of Mediterranean sclerophyllous vegetation under climate change: From acclimation to adaptation. *Environmental and Experimental Botany*, **103**, 80-98.

Chandler JW, Dale JE (1995) Nitrogen deficiency and fertilization effects on needle growth and photosynthesis in Sitka spruce (*Picea sitchensis*). *Tree Physiology*, **15**, 813-817.

Ciais P, Reichstein M, Viovy N, Granier A, Ogée J, Allard V *et al.* (2005) Europe-wide reduction in primary productivity caused by the heat and drought in 2003. *Nature*, **437**, 529-533.

Cowling RM, Rundel PW, Lamont BB, Arroyo MK, Arianoutsou M (1996) Plant diversity in Mediterranean-climate regions. *Trends in Ecology & Evolution*, **11**, 362-366.

Dai A (2013) Increasing drought under global warming in observations and models. *Nature Climate Change*, **3**, 52-58.

General Introduction

Delgado-Baquerizo M, Maestre FT, Gallardo A, Bowker MA, Wallenstein MD, Quero J.L *et al.* (2013) Decoupling of soil nutrient cycles as a function of aridity in global drylands. *Nature,* **502**, 672-676.

Dermody O, Weltzin JF, Engel EC, Allen P, Norby RJ (2007) How do elevated [CO2], warming, and reduced precipitation interact to affect soil moisture and LAI in an old field ecosystem? *Plant and Soil*, **301**, 255-266.

Dijkstra FA, Pendall E, Morgan JA, Blumenthal DM, Carrillo Y, LeCain D. R, Follett RF, Williams DG (2012) Climate change alters stoichiometry of phosphorus and nitrogen in a semiarid grassland. *New Phytologist*, **196**, 807-815.

Escudero A, Palacio S, Maestre FT, Luzuriaga AL (2015) Plant life on gypsum: a review of its multiple facets. *Biological Reviews*, **90**, 1-18.

Gerten D, Luo Y, Le Maire G, Parton WJ, Keough C, Weng E *et al.* (2008). Modelled effects of precipitation on ecosystem carbon and water dynamics in different climatic zones. *Global Change Biology*, **14**, 2365-2379.

Giorgi F (2006) Climate change hot-spots. *Geophysical research letters*, **33**.

Giorgi F, Lionello P (2008) Climate change projections for the Mediterranean region. *Global and Planetary Change*, **63**, 90-104.

Gutiérrez A, Morte A, Honrubia M (2003) Molecular identification of the mycorrhiza formed by *Helianthemum almeriense* Pau with *Terfezia claveryi Chatin* and *Picoa lefebvrei* (Pat.) Maire. *Mycorrhiza*, **13**, 299-307.

Habitats EU Directive (1992) Office for Official Publication of the European Union. CONSLEG: 1992LOO43-01.05.

Hanson PJ, Wullschleger SD, Norby RJ, Tschaplinski TJ, Gunderson CA (2005) Importance of changing CO_2, temperature, precipitation, and ozone on carbon and water cycles of an upland-oak forest: incorporating experimental results into model simulations. *Global Change Biology*, **11**, 1402-1423.

General Introduction

Heimann M, Reichstein M (2008) Terrestrial ecosystem carbon dynamics and climate feedbacks. *Nature*, **451**, 289-292.

Huang J, Yu H, Guan X, Wang G, Guo R (2016) Accelerated dryland expansion under climate change. *Nature Climate Change*, **6**, 166–71.

Huang ZA, Jiang DA, Yang Y, Sun JW, Jin SH (2004) Effects of nitrogen deficiency on gas exchange, chlorophyll fluorescence, and antioxidant enzymes in leaves of rice plants. *Photosynthetica*, **42**, 357-364.

Le Houérou HN (1996) Climate change, drought and desertification. *Journal of Arid Environments*, **34**, 133-185.

Lin, D., Xia, J., & Wan, S. (2010). Climate warming and biomass accumulation of terrestrial plants: a meta-analysis. New Phytologist, 188(1), 187-198.

Llorens L, Penuelas J, Estiarte M (2003). Ecophysiological responses of two Mediterranean shrubs, *Erica multiflora* and *Globularia alypum*, to experimentally drier and warmer conditions. *Physiologia plantarum*, **119**, 231-243.

Llorens L, Penuelas J, Estiarte M, Bruna P (2004) Contrasting growth changes in two dominant species of a Mediterranean shrubland submitted to experimental drought and warming. *Annals of Botany*, **94**, 843-853.

Lloret F, Peñuelas J, Prieto P, Llorens L, Estiarte M (2009) Plant community changes induced by experimental climate change: seedling and adult species composition. Perspectives in Plant Ecology, Evolution and Systematics 11: 53-63.

Luo H, Oechel WC, Hastings SJ, Zulueta R, Qian Y, Kwon H (2007) Mature semiarid chaparral ecosystems can be a significant sink for atmospheric carbon dioxide. *Global Change Biology*, **13**, 386-396.

Luo Y, Gerten D, Le Maire G, Parton WJ, Weng E, Zhou X *et al.* (2008) Modeled interactive effects of precipitation, temperature, and [CO2] on ecosystem carbon and water dynamics in different climatic zones. *Global Change Biology*, **14**, 1986-1999.

General Introduction

Myers N, Mittermeier RA, Mittermeier CG, Da Fonseca GA, Kent J (2000) Biodiversity hotspots for conservation priorities. *Nature*, **403**, 853-858.

Nardini A, Lo Gullo MA, Trifilò P, Salleo S (2014) The challenge of the Mediterranean type climate to plant hydraulics: responses and adaptations. *Environmental and Experimental Botany*, **103**, 68–79.

Nicotra AB, Atkin OK, Bonser SP, Davidson AM, Finnegan EJ, Mathesius U. *et al.* (2010) Plant phenotypic plasticity in a changing climate. *Trends in Plant Science*, **15**, 684–692.

NOAA National Centers for Environmental Information, State of the Climate: Global Analysis for Annual 2015, published online January 2016, retrieved on June 13, 2016 from http://www.ncdc.noaa.gov/sotc/global/201513.

Ogaya R, Penuelas J, Asensio D, Llusià J (2011) Chlorophyll fluorescence responses to temperature and water availability in two co-dominant Mediterranean shrub and tree species in a long-term field experiment simulating climate change. *Environmental and Experimental Botany,* **73**, 89-93.

Padilla FM, Vidal B, Sánchez J, Pugnaire FI (2010) Land-use changes and carbon sequestration through the twentieth century in a Mediterranean mountain ecosystem: implications for land management. *Journal of environmental management*, **91**, 2688-2695.

Pausas JG (2004) Changes in fire and climate in the eastern Iberian Peninsula (Mediterranean basin). *Climatic change*, **63**, 337-350.

Peñuelas J, Prieto P, Beier C, Cesaraccio C, De Angelis P, de Dato, G *et al.* (2007). Response of plant species richness and primary productivity in shrublands along a north–south gradient in Europe to seven years of experimental warming and drought: reductions in primary productivity in the heat and drought year of 2003. *Global Change Biology,* **13**, 2563-2581.

Peñuelas J, Sardans J, Estiarte M *et al.* (2013) Evidence of current impact of climate change on life: a walk from genes to the biosphere. *Global Change Biology*, **19**, 2303–2338.

Pereira JS, Mateus JA, Aires LM, Pita G, Pio C, David JS, Andrade V, Banza J, David TS, Paço TA, Rodrigues A (2007) Net ecosystem carbon exchange in three contrasting Mediterranean ecosystems? The effect of drought. *Biogeosciences,* **4**, 791-802.

Porta J, López-Acevedo M, Roquero C (2003) Edafología para la agricultura y el medio ambiente, 3ª Edición. Ed. Mundiprensa, Madrid.

Prieto P, Peñuelas J, Llusià J, Asensio D, Estiarte M (2009a) Effects of experimental warming and drought on biomass accumulation in a Mediterranean shrubland. *Plant Ecology*, **205**, 179-191.

Prieto P, Peñuelas J, Lloret F, Llorens L, Estiarte M (2009b) Experimental drought and warming decrease diversity and slow down post-fire succession in a Mediterranean shrubland. *Ecography*, **32**, 623-636.

Querejeta JI, Egerton-Warburton LM, Allen MF (2009) Topographic position modulates the mycorrhizal response of oak trees to interannual rainfall variability. *Ecology*, **90**, 649-662.

Reich PB, Oleksyn J (2004) Global patterns of plant leaf N and P in relation to temperature and latitude. *Proceedings of the National Academy of Sciences of the United States of America,* **101**, 11001-11006.

Reich PB, Oleksyn J, Wright IJ (2009) Leaf phosphorus influences the photosynthesis–nitrogen relation: a cross-biome analysis of 314 species. *Oecologia*, **160**, 207–212.

Rodgers VL, Hoeppner SS, Daley MJ, Dukes JS (2012) Leaf-level gas exchange and foliar chemistry of common old-field species responding to warming and precipitation treatments. *International Journal of Plant Sciences*, **173**, 957-970.

Rubio A, Escudero A (2000) Small scale spatial soil-plant relationships in semiarid gypsum environments. *Plant and Soil*, **220**, 139-150.

General Introduction

Rustad LEJL, Campbell J, Marion G, Norby R, Mitchell M, Hartley A, Gurevitch J (2001) A meta-analysis of the response of soil respiration, net nitrogen mineralization, and aboveground plant growth to experimental ecosystem warming. *Oecologia*, **126**, 543-562.

Safriel UN, Adeel Z (2005) Dryland Systems. In Ecosystems and Human Well-being: Current State and Trends. Vol. 1, edited by R. Hassan, R. Scholes, & N. Ash, 623–662.

Sardans J, Peñuelas J, Prieto P, Estiarte M (2008) Drought and warming induced changes in P and K concentration and accumulation in plant biomass and soil in a Mediterranean shrubland. *Plant and Soil*, **306**, 261-271.

Sardans J, Alonso R, Carnicer J, Fernández-Martínez M, Vivanco MG, Peñuelas J (2016) Factors influencing the foliar elemental composition and stoichiometry in forest trees in Spain. *Perspectives in Plant Ecology, Evolution and Systematics*, **18**, 52-69.

Thomas DS, Montagu KD, Conroy JP (2006) Leaf inorganic phosphorus as a potential indicator of phosphorus status, photosynthesis and growth of Eucalyptus grandis seedlings. *Forest Ecology and Management*, **223**, 267-274.

Vargas R, Baldocchi D, Querejeta JI, Curtis PS, Hasselquist NJ, Janssens IA, Allen MF, Montagnani L (2010) Ecosystem CO2 fluxes of arbuscular and ectomycorrhizal dominated vegetation types are differentially influenced by precipitation and temperature. *New Phytologist*, **185**, 226-236.

Williams AP, Allen CD, Macalady AK, Griffin D, Woodhouse CA, Meko DM, Swetnam TW, Rauscher SA, Seager R, Grissino-Mayer HD, Dean JS, Cook ER, Gangodagamage C, Cai M, McDowell NG (2013) Temperature as a potent driver of regional forest drought stress and tree mortality. *Nature Climate Change,* **3,** 292–297.

Wright IJ, Reich PB, Westoby M, Ackerly DD, Baruch Z, Bongers F, Cavender-Bares J, Chapin T, Cornelissen JHC, Diemer M, Flexas J, Garnier E, Groom PK, Gulias J, Hikosaka K, Lamont BB, Lee T, Lee W, Lusk C, Midgley JJ, Navas M-L, Niinemets U, Oleksyn J, Osada N, Poorter H, Poot P, Prior L, Pyankov VI, Roumet C, Thomas SC, Tjoelker MG, Veneklaas EJ, Villar R (2004) The worldwide leaf economics spectrum. *Nature*, **428**, 821–827.

General Introduction

Wu Z, Dijkstra P, Koch GW, Peñuelas J, Hungate BA (2011) Responses of terrestrial ecosystems to temperature and precipitation change: a meta-analysis of experimental manipulation. *Global Change Biology*, **17**, 927-942.

General Introduction

OBJECTIVES

"Life is not like water. Things in life don't necessarily flow over the shortest possible route."

Haruki Murakami, *1Q84*

OBJETIVES

The overarching goal of this thesis is to assess the effects of a realistic (based on current IPCC projections) combination of temperature increase and rainfall reduction on the mid-term performance of native plant communities growing on gypsum soils in semiarid ecosystems in the central and south-eastern Iberian Peninsula. In order to achieve this overarching objective, the following specific objectives were proposed:

- To evaluate the ecophysiological response of coexisting plant species with divergent water use strategies to increased temperature and/or decreased precipitation. In particular, identify potential physiological and phenological adaptive mechanisms that might buffer or moderate the negative effects of climate change on the water and nutrient status of dwarf scrub species living on semiarid gypsum soils.
- To characterize potential species-specific changes in plant carbon isotopic composition, leaf stoichiometry and nutrient status, biomass production and mortality rates that may occur in response to increased temperature and/or decreased precipitation in dwarf scrub communities of semiarid gypsum ecosystems.
- To evaluate and characterize potential changes in the composition and structure of soil mycorrhizal fungal communities (both ectomycorrhizal and arbuscular) that may occur in response to increased temperature and/or decreased precipitation, using cutting-edge molecular techniques (pyrosequencing).
- To determine whether changes in soil mycorrhizal fungal communities might be associated with changes in host plant ecophysiological performance.

MATERIALS AND METHODS (OVERVIEW)

"In this world, there are things you can only do alone, and things you can only do with somebody else. It's important to combine the two in just the right amount."

Haruki Murakami, *After Dark*

MATERIALS AND METHODS

Study Sites

This study has been carried out in three experimental sites located in southeast (Sax and Sorbas) and central Spain (Aranjuez). The soils are derived from gypsum and are classified as Gypsiric Leptosols. The vegetation at the site of Sax is dominated by *Pinus halepensis* and the other two experimental sites are dominated by a native grassland/shrubland community. The tussock grass *Stipa tenacissima* is present at the three sites. The target plant has been the shrub *Helianthemum squamatum* species across all the study sites. In the site of Sorbas, the target vegetation is formed by the main species of the native shrubland community, including *Helianthemum squamatum, Helianthemum syriacum, Gypsophila struthium, Teucrium turredanum, Santolina viscosa* and *Coris hispanica* (see Figure 1). The climate is semi-arid Mediterranean in the three study sites, however, there are some important differences among locations. Mean annual temperature varies from 15ºC in Sax and Aranjuez, to 17ºC in Sorbas. Mean annual rainfall is similar in Sax and Aranjuez (approximately 350 mm), while in Sorbas it is 275 mm, thus being the warmest and driest experimental site of this study. Moreover, Sax and Sorbas are located near the sea (< 40 km) while Aranjuez is located further away from the sea and therefore has a continental Mediterranean climate, with more contrasting seasons.

To assess the effects of forecasted climate change conditions on the performance of native plants of *Helianthemum squamatum* in Sax and Aranjuez, and *Helianthemum squamatum, Helianthemum syriacum, Gypsophila struthium, Teucrium turredanum, Santolina viscosa* and *Coris hispanica* in Sorbas, we conducted a randomized field experiment in the three study sites simulating the predicted climate conditions according to the average predictions of six atmosphere general circulation models (GCMs) for the second half of the twenty-first century in the Mediterranean region. The experiments were established during 2011 (between February and October). In Sax, only ventilated open top chambers (OTCs) were installed, in order to achieve 1-2ºC increase in mean annual air temperature during wintertime and 4-7ºC increase in mean annual temperature during summertime.

Materials and Methods (Overview)

Figure 1. Target species *Helianthemum squamatum* (a), *Helianthemum syriacum* (b), *Gypsophila struthium* (c), *Teucrium turredanum* (d), *Santolina viscosa* (e) and *Coris hispanica* (f).

In Sorbas and Aranjuez, three different climate manipulation treatments were established. In addition to the warming treatment achieved with the OTCs (W

treatment), passive rainout shelters were installed to intercept and exclude approximately 30% of the total precipitation, in order to simulate the forecasted reductions in precipitation (RR treatment). For more detailed information about the design of open top chambers and rainout shelters, see the "Materials and Methods" sections of Chapters I, II and III. The combined warming plus rainfall reduction (W+RR) treatment is achieved by installing both OTCs and rainout shelters over the same experimental plot (see Figure 2). In order to measure the effects of treatments on microclimatic variables, air and soil temperature, relative humidity and soil moisture were continuously monitored using replicated automated sensors. (HOBO ® U23 Pro v2 Temp/RH and TMC20-HD sensors, Onset Corp., Pocasset, MA, USA, and EC-5 soil moisture sensors, Decagon Devices Inc., Pullman, WA, USA, respectively).
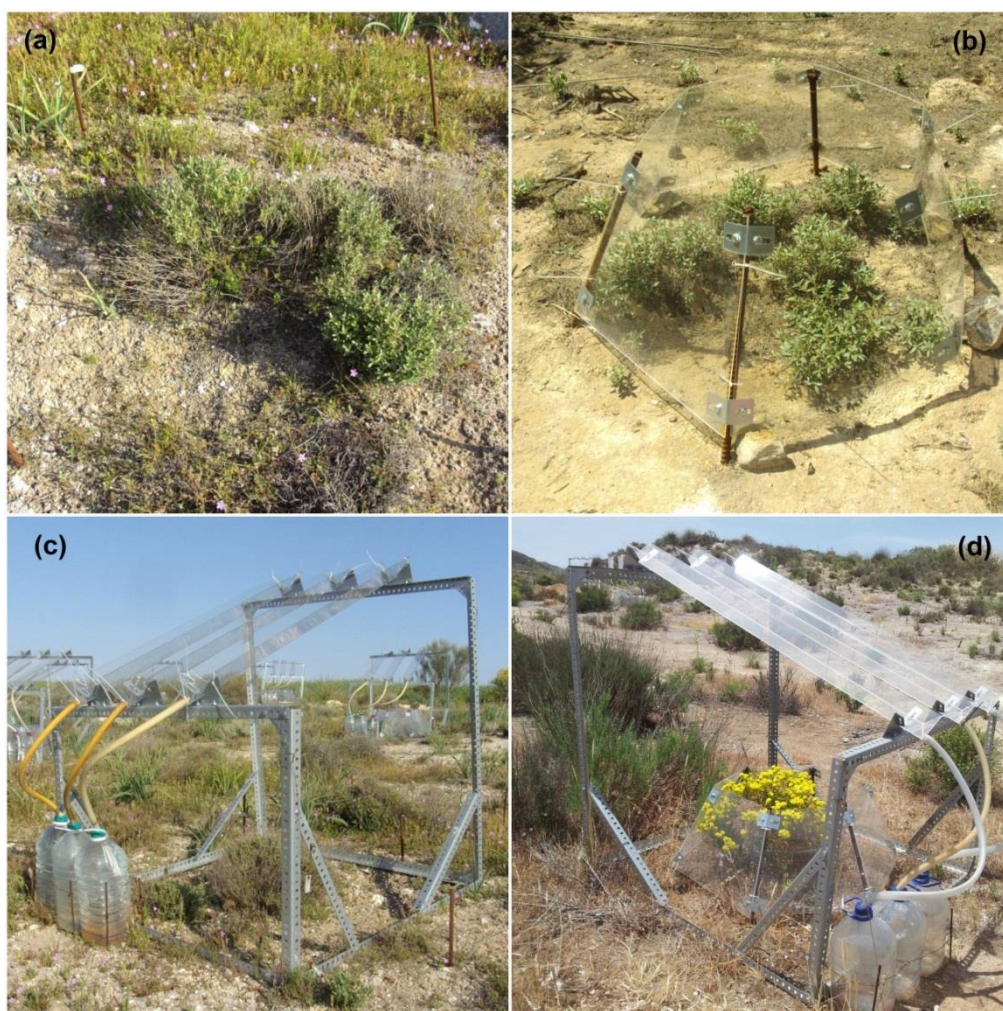

Figure 2. Experimental plots: a) *Control* treatment, with plants exposed to current climate conditions; b) *Warming* treatment, achieved by installing an open top chamber (OTC); c) *Rainfall Reduction* treatment,

Materials and Methods (Overview)

achieved by installing a rainout shelter; d) *Warming + Rainfall Reduction* treatment, achieved by installing an open top chamber (OTC) and a rainout shelter on the same plot.

Plant measurements

Leaf gas exchange parameters (A, gs, E, Fv': Fm' and ØPSII) of *H. squamatum* (in all the 3 sites), *H. syriacum* and *G. struthium* (in the site of Sorbas only) were measured multiple times in 2012, 2013, 2014 and 2015 along the growing season with as LI-6400-40 Leaf Chamber Fluorometer and a LICOR 6400-01 CO_2 injector. Intrinsic water use efficiency (WUE_i) was calculated as the ratio between net photosynthetic rate and stomatal conductance (A/g_s). For more detailed information about leaf-gas exchange measurements, see the "Materials and Methods" sections of Chapters I, II and III.

Across all study sites, fully sun-exposed adult leaves of *H. squamatum* in Sax and Aranjuez, and *Helianthemum squamatum, Helianthemum syriacum, Gypsophila struthium, Teucrium turredanum, Santolina viscosa* and *Coris hispanica* in Sorbas were collected each year in Spring in order to determine their carbon isotope ratios ($\delta^{13}C$). Leaf $\delta^{13}C$, $\delta^{18}O$ (only in *H. squamatum* shrubs at Sax) and C and N concentrations were measured using elemental analyzer/continuous flow isotope ratio mass spectrometry (ANCA/SL elemental analyzer coupled with a Finnigan MAT Delta PlusXL IRMS) and these analyses were conducted at the Centre for Stable Isotope Biogeochemistry, University of California, Berkeley (USA). For more detailed information about leaf $\delta^{13}C$ measurements, see the "Materials and Methods" sections of Chapters I, II and III. Foliar P (in all study sites), K, Fe, Cu and Zn concentrations (in Sorbas) were measured by inductively coupled plasma optical emission spectrometry (ICP-OES, Thermo Elemental Iris Intrepid II XDL, Franklin, MA, USA) after a microwave-assisted digestion with $HNO_3:H_2O_2$ (4:1, v:v) in the ionomics facility at CEBAS-CSIC.

In Spring 2013, 2014 and 2015, four leaves per target *H. squamatum* shrub were collected to measure their area (cm^2) and dry mass (mg) to calculate their dry mass per unit leaf area (LMA, cm^2 mg^{-1}) in the three experimental sites. LMA was calculated as the ratio between leaf dry weight and leaf area. In late winter 2012 and 2013, 3-4 terminal shoots of each target shrub were labeled with red tape to measure their elongations during the growing season. We measured shoot elongation in mid-Spring (April) and again at the end of the growing season (June). The late/early Spring growth

ratio was then calculated as the quotient between the shoot elongations recorded during the latter and earlier parts of the Spring growing season (May-June and March-April, respectively) in order to indicate changes in shoot growth phenology with the different climate manipulation treatments, relative to the controls. At the end of the 4 year study period (October 2015), one representative terminal shoot of approx. 10 cm length per target *H. squamatum* shrub in all study sites, and *Helianthemum squamatum, Helianthemum syriacum, Gypsophila struthium, Teucrium turredanum* and *Santolina viscosa* shrubs in Sorbas were destructively sampled to evaluate the effects of the climate manipulation treatments on shoot dry biomass production. Total dry mass, number of leaves and total leaf area of these shoots were measured, as described above, and values were standardized per 10 cm shoot length.

Each year, post-summer plant survival rates were measured after the first Autumn rainfalls (October 2012, 2013, 2014, 2015) in the three study sites. In Sax and Aranjuez, plant survival rate was estimated as the percentage of individuals of *H. squamatum* present in Spring in each experimental plot that were still alive at the end of the Summer drought period. In Sorbas, the 4-year survival of *Helianthemum squamatum, Helianthemum syriacum, Gypsophila struthium, Teucrium turredanum, Santolina viscosa* and *Coris hispanica* in each treatment was assessed in October 2015, at the end of the study period. Survival was calculated as the proportion of plants that were still alive in each experimental plot relative to the total number of plants that were present in each plot in March 2012 (one year after setting up the climate manipulation treatments).

Characterization of the soil mycorrhizal fungal community

In Aranjuez, surface (0-5 cm) soil samples were collected on 30 June 2014 from the rhizosphere of *H. squamatum* individuals, by inserting a hand auger corer (5 cm diameter) into the soil under each target shrub (8 replicate plots per treatment). Thereafter, pyrosequencing analyses were conducted. For a detailed description of molecular methods, see the "Materials and Methods" section of Chapter II. The total number of sequences corresponding to each fungal OTU and experimental plot were obtained, and the relative abundance of each OTU in each plot was calculated based on the total number of sequences in the same plot. We selected those OTUs most closely related to known ectomycorrhizal and arbuscular mycorrhizal fungi (EMF and AMF, respectively), based on a literature review. Fungal OTUs in the Ascomycota and

Materials and Methods (Overview)

Basidiomycota were classified as putatively ectomycorrhizal (or non-mycorrhizal) according to the scientific literature. All fungal OTUs identified as members of the Glomeromycota were considered AMF. However, we acknowledge that the primers and classification methods employed may not capture all potential mycorrhizal fungal diversity in the system.

Statistical analyses

In Sax and Aranjuez, repeated measures ANOVAs (RM-ANOVAs) were used in order to assess the effects of experimental warming and/or rainfall reduction on leaf gas exchange parameters (A, gs, E, Fv'/Fm', ØPSII), foliar nutrient concentrations, δ^{13}C, δ^{18}O (in Sax), leaf dry mass and area, LMA, shoot growth phenology and survival rate. At the Sorbas site, multiple-way ANOVAs were used to analyze the abovementioned plant response variables across target species, due to high and progressively increasing plant mortality within and across years. Plant survival in Sorbas was analyzed using a simple binary logistic regression where survival was the dependent variable and simulated climate warming (W), rainfall exclusion (RR) and their combination (W+RR) were the predictor factors.

The relationship between A and gs in Sax and Aranjuez was examined by using a linear regression analysis whereby the intercepts and slopes of the fitted regression lines were compared among all the climate manipulation treatments with ANCOVA analyses. ANOVA analyses were used to test the effects of the climate manipulation treatments on shoot dry biomass production (and mean leaf number and area, leaf biomass and stem biomass per unit shoot length) for *H. squamatum* in Sax and Aranjuez, and for *Helianthemum squamatum, Helianthemum syriacum, Gypsophila struthium, Teucrium turredanum* and *Santolina viscosa* in Sorbas. Mycorrhizal OTU richness and relative abundance of EM and AM fungal communities under *H. squamatum* shrubs were also analyzed by ANOVA in Aranjuez.

We used SPSS 22.0 software (SPSS Inc., Chicago, IL, USA) to perform ANOVA analyses in all sites, StatgraphicsPlus 5.1 (Statgraphics Plus 5.1. for Windows, 2000) to carry out ANCOVA analyses in Sax and Aranjuez, and the R software (v. 2.15.3, R Development Core Team 2013) for survival analyses in Sorbas.

CHAPTER I

PHOTOSYNTHESIS AND GROWTH REDUCTION WITH WARMING ARE DRIVEN BY NONSTOMATAL LIMITATIONS IN A MEDITERRANEAN SEMI-ARID SHRUB

"You have to overcome the fear and anger inside you. Let a bright light shine in and melt the coldness in your heart. That's what being tough is all about."

Haruki Murakami, *Kafka on the Shore*

Chapter I

Photosynthesis and growth reduction with warming are driven by non-stomatal limitations in a Mediterranean semiarid shrub

Lupe León-Sánchez[1], Emilio Nicolás[1], Pedro A. Nortes[1], Fernando T. Maestre[2], José I. Querejeta[1]

[1]Centro de Edafología y Biología Aplicada del Segura (CEBAS-CSIC), Campus Universitario de Espinardo, PO Box 164, 30100, Murcia, Spain; [2]Biodiversity and Conservation Unit, Department of Biology and Geology, Universidad Rey Juan Carlos, Móstoles, 28933, Madrid, Spain.

Author for correspondence: José I. Querejeta

Address: Campus Universitario de Espinardo. Espinardo. Murcia. E-30100 (SPAIN)

Tel: +34 968396341

Email: querejeta@cebas.csic.es

Chapter I

ABSTRACT

Whereas warming enhances plant nutrient status and photosynthesis in most terrestrial ecosystems, dryland vegetation is vulnerable to the likely increases in evapotranspiration and reductions in soil moisture caused by elevated temperatures. Any warming-induced declines in plant primary production and cover in drylands would increase erosion, land degradation and desertification. We conducted a four-year manipulative experiment in a semiarid Mediterranean ecosystem to evaluate the impacts of a ~2ºC warming on the photosynthesis, transpiration, leaf nutrient status, chlorophyll content, isotopic composition, biomass growth and post-summer survival of the native shrub *Helianthemum squamatum*. We predicted that warmed plants would show reduced photosynthetic activity and growth, primarily due to the greater stomatal limitation imposed by faster and more severe soil drying under warming. On average, warming reduced net photosynthetic rates by 36% across the study period. Despite this strong response, warming did not affect stomatal conductance and transpiration. The reduction of peak photosynthetic rates with warming was more pronounced in a drought year than in years with near-average rainfall (75% and 25-40% reductions relative to controls, respectively), with no indications of photosynthetic acclimation to warming through time. Warmed plants had lower leaf N and P contents, $\delta^{13}C$ and sparser and smaller leaves than control plants. Warming reduced shoot dry mass production by 31%. However, warmed plants were able to cope with large reductions in net photosynthesis, leaf area and shoot biomass production without changes in post-summer survival rates. Our findings highlight the key role of non-stomatal factors (biochemical and/or nutritional) in reducing net carbon assimilation rates and growth under warming, which has important implications for projections of plant carbon balance under the warmer and drier climatic scenario predicted for drylands worldwide. Projected climate warming over the coming decades could reduce net primary production by about one-third in semiarid gypsum shrublands dominated by *H. squamatum*.

Key words: climate change, dryland ecosystems, *Helianthemum squamatum,* plant nutrient status, leaf trait plasticity, plant-climate interactions, plant survival and growth, stable isotopes.

Chapter I

INTRODUCTION

Anthropogenic greenhouse gas emissions are expected to raise global mean temperature by 2–6 °C by the end of the XXI century (Collins et al. 2013). Manipulative field experiments have shown that warming generally enhances plant photosynthetic activity, primary production and reproductive output in most ecosystem types (Rustad et al. 2001; Lin, Xia & Wan 2010; Wu et al. 2011; Liang et al. 2013; Peñuelas et al. 2013). The positive effects of warming on primary production have been largely attributed to the kinetic sensitivity of photosynthesis to temperature, and to extended growing seasons and enhanced nutrient availability (Luo 2007). Moreover, plants tend to increase their photosynthetic thermal optimum under warming, so photosynthesis acclimation to warming can further contribute to increase primary production when other resources, such as nutrients and water, are not limiting (Yamori, Noguchi & Terashima 2005; Gunderson et al. 2010; Peñuelas et al. 2013).

Several lines of evidence indicate that plants may benefit more from warming in humid climates than in drier habitats (Rustad et al. 2001; Peñuelas et al. 2013; Xia et al. 2014; Tan et al. 2015). However, the generality of this rule has been questioned by both manipulative field studies and meta-analyses (Perfors, Harte & Alter 2003; Dijkstra et al. 2010, 2012; Lin et al. 2010; Wu et al. 2011). Compared with the large number of field warming studies conducted in tundra, alpine, boreal and mesic temperate ecosystems, the impacts of warming on the nutrient status and photosynthesis of dryland vegetation have received less attention (but see Niu et al. 2008a, b; Dijkstra et al. 2010, 2012), even though dryland vegetation is particularly vulnerable to climate change (Peñuelas et al. 2013; Sardans & Peñuelas 2013). Arid, semiarid and dry-subhumid ecosystems (drylands hereafter) occupy 41% of the global land area (Safriel & Adeel 2005) and include about 20% of the major centers of global plant biodiversity (White & Nackoney 2003). Additional studies are thus urgently needed to improve our current understanding of the net impacts of warming on dryland plants, as any significant warming-induced declines in primary production and vegetation cover in drylands would greatly increase erosion, land degradation and subsequent desertification (Safriel & Adeel 2005; Wang et al. 2012).

Chapter I

The Mediterranean Basin will be particularly affected by climate change, as current general circulation models predict a 2-5ºC warming during the coming decades, as well as longer, hotter and drier summers with sharp reductions in late spring rainfall (up to 40%) and higher frequency and intensity of extreme droughts and heat waves (Giorgi & Lionello 2008; Collins et al. 2013). It is thus widely assumed that the net impacts of climate warming on the primary production of Mediterranean vegetation will be primarily dominated by increases in stomatal limitations on photosynthesis (Sardans & Peñuelas 2013; Nardini et al. 2014; Bussotti et al. 2014). In contrast to more northern latitudes, temperatures during the growing season in large parts of the Mediterranean Region may already be at or near the thermal optimum for plant photosynthesis (20-30º C for most species; Larcher 2000). Climate warming could enhance photorespiratory reactions (von Caemmerer & Quick 2000), impair the functioning of the photosynthetic biochemistry (Galmés et al., 2013) or even damage the leaf photosynthetic apparatus of many native plant species, particularly during summer drought periods when evaporative leaf cooling cannot prevent excessive leaf overheating (in which case leaf temperatures can reach up to 8-10º C higher than ambient temperatures; Larcher 2000).

Previous studies conducted in subhumid Mediterranean ecosystems have reported largely neutral or even positive effects of moderate night-time warming (1ºC) on the photosynthesis, nutrient status and growth of native shrubs (Llorens, Peñuelas & Estiarte 2003; Peñuelas et al. 2004, 2007; Sardans et al. 2008a, b; Prieto et al. 2009a, b). However, the conclusions obtained from night-time warming studies conducted in subhumid ecosystems cannot be extrapolated to semiarid environments, where photosynthesis is more severely water-limited, and where vegetation could thus be more vulnerable to the desiccating effects of climate warming (Niu et al. 2008b). Moreover, plants can respond very differently to day-time vs. night-time warming (i.e. to increases in maximum vs. minimum daily temperatures), as increases in maximum daily temperatures can impose a greater constraint on both stomatal conductance and photosynthesis, and can thus be considerably more stressful for plants (Peng et al. 2013; Tan et al. 2015).

We conducted a four-year manipulative field study in a semiarid Mediterranean ecosystem in Southeast Spain to evaluate the effects of a 2º C warming on the leaf gas

exchange, foliar N and P concentrations and isotopic composition (δ^{13}C, δ^{18}O), chorophyll content, leaf dry mass and area, shoot biomass production and post-summer survival of the native shrub *Helianthemum squamatum* (L.) Dum. Cours. Since water is by far the most limiting factor for primary productivity in semiarid Mediterranean ecosystems, we predicted that warmed plants would show reduced photosynthetic activity and growth, primarily due to the greater stomatal limitation imposed by faster and more severe soil drying and higher evaporative demand under warming (Maestre et al. 2013). Furthermore, we predicted that plant nutrient status would also be negatively affected by warming in this semiarid ecosystem, as the soil-drying effects of warming can hamper organic N and P mineralization (Allison & Treseder 2008; Sardans & Peñuelas 2013) and decrease the geochemical desorption and dissolution of inorganic P (Dijkstra et al. 2012). The soil-drying effects of warming would also be expected to reduce transpiration, and thus the diffusion of nutrients to roots and the transport of nutrients from roots to leaves via the transpiration stream (He & Dijskstra 2014), a response with potential negative feedbacks on plant photosynthetic capacity (Wright et al. 2004).

MATERIALS AND METHODS

Study site and experimental design

This study was carried out near the town of Sax, in Southeast Spain (38° 32' 42" N - 0° 50' 42" W; 474 m.a.s.l.). The soil is derived from gypsum, has pH values ~7, and is classified as Gypsiric Leptosols (IUSS Working Group WRB, 2006). The vegetation is dominated by *Pinus halepensis*, which was planted in the 1950´s, and also contains grasses and shrubs, such as *Stipa tenacissima*, *Anthyllis cytisoides* and *H. squamatum.* The climate is semiarid Mediterranean, with a mean annual temperature of 15°C and mean annual rainfall of 359 mm (28 yr average; Villena weather station; 38° 37' 46" N - 0° 51' 40" W; 486 m.a.s.l.).

We established a randomized field experiment to evaluate the effects of warming on the performance of *H. squamatum* shrubs. A passive warming treatment was achieved by installing open top chambers (OTCs) on vegetated patches dominated by *H.*

Chapter I

squamatum. The OTCs have a hexagonal design with sloping sides of 40 cm × 50 cm × 32 cm, and were made of transparent methacrylate. The methacrylate sheets used in our experiment transmit about 92% of visible light and over 85% of incoming energy, and have a reflection of incoming solar radiation of 4% (according to the manufacturer; Decorplax S. L., Humanes, Spain). The methacrylate sheets used filtered up to 15% of UV radiation (Maestre et al. 2013). These OTCs have been used in previous field warming experiments (Maestre et al. 2013, 2015; Ladrón de Guevara et al. 2014). Upon installation in the field, the OTCs were suspended ~3 cm above the ground level by a metal frame to allow free air circulation and exchange with the surrounding environment (Fig.S1), which minimizes undesirable experimental effects, such as reduced wind and unnatural gas and humidity concentrations (Hollister & Webber 2000). The 2ºC increase in mean annual air temperature achieved within the OTCs at our experimental site simulates warming levels within the range of predictions given by Atmosphere-Ocean General Circulation Models for the second half of the 21st century in south-eastern Spain (De Castro, Martin-Vide & Alonso 2005; Giorgi & Lionello 2008; Collins et al. 2013). Moreover, the OTCs used promote more intense warming effects during the summer than during winter (Maestre et al. 2013), which is also in good agreement with climate change model predictions for the region.

In October 2011, we installed nine OTCs in areas dominated by *H. squamatum* individuals. The same number of control plots (i.e. ambient temperature) was randomly established in adjacent areas containing *H. squamatum* individuals of similar size. To increase the number of replicates of each treatment, we also monitored *H. squamatum* individuals existing within OTCs that were established in 2008 in the framework of another study (Maestre et al. 2015), and which had exactly the same design and dimensions as those we installed. This increased the number of replicated control and warmed plots used in our study to 15, resulting in a total of 30 experimental plots. The leaf gas exchange rates of warmed *H. squamatum* shrubs did not differ significantly between OTCs established in 2008 vs. OTCs established in 2011 (see Table S1 in Supporting Information).

To measure the effects of the OTCs on microclimatic parameters, we continuously monitored air temperatures, relative humidity, soil temperature and soil moisture content (0-5 cm) in both warmed and control plots using replicated automated sensors

Chapter I

(HOBO$^®$ U23 Pro v2 Temp/RH sensors, Onset Corp., Pocasset, MA, USA, and EC-5 soil moisture sensors, Decagon Devices Inc., Pullman, WA, USA, respectively).

Plant measurements

Net photosynthesis rate (A), stomatal conductance (g_s), transpiration rate (E), maximum efficiency of photosystem II under light (Fv'/Fm') and the actual photochemical efficiency of photosystem II ($\varnothing$PSII) were measured multiple times during 2012 (February, March, April, June and November), 2013 (February, April, June and November), 2014 (April) and 2015 (April) with a LI-6400XT photosynthesis system (Li-Cor, Inc., Lincoln, NE, USA) equipped with a LI-6400-40 Leaf Chamber Fluorometer and a LICOR 6400-01 CO_2 injector. Leaf gas exchange was measured on fully sun-exposed leaves that were placed in a 2 cm^2 leaf cuvette. During these measurements, air CO_2 concentration was controlled using the injection system and compressed CO_2-cylinders with a CO_2 concentration of 390 µmol mol^{-1} CO_2. Measurements were done at a saturating light of 1.500 µmol m^{-2} s^{-1}, and at ambient air temperature and relative humidity. The air flow was set to 250 µmol s^{-1}. All leaf-gas exchange measurements were conducted between 8:00 and 11:00 am (GMT), when the peak of maximum photosynthetic rates was found at each survey. For warmed plants, all leaf gas exchange measurements were conducted under the prevailing microclimatic conditions within the OTCs (i.e., elevated temperature relative to ambient). All the leaves used for gas exchange measurements were collected thereafter to measure their area using an image scanner program (Image Pro Plus, Media Cybernetics, Inc. Rockville, MD, USA). On each date, leaf gas exchange measurements (A, g_s, E, Fv'/Fm', $\varnothing$PSII) were conducted on 10-15 *H. squamatum* individuals per each temperature treatment. Intrinsic water use efficiency (WUE$_i$) and instantaneous water use efficiency (WUE$_{Inst}$) were determined as A/g_s and A/E, respectively. Additionally, leaf respiration rates in the dark were measured in 9 control and 12 warmed *H. squamatum* individuals at the peak of the 2014 growing season (April) to elucidate whether the large reduction in net photosynthetic rates found in warmed plants might be caused by increased leaf respiration rates. For calculations of mitochondrial respiration (non-photorespiratory CO_2 release), leaves were placed in the dark by covering them with aluminum foil for 30 min before CO_2 exchange measurements, which were

conducted at the same CO_2 concentration as above, and with the air flow set to 150 μmol s^{-1}.

The leaves used for gas exchange measurements at Spring (April) in each study year were collected thereafter to measure their carbon and oxygen isotope composition ratios (δ^{13}C and δ^{18}O, respectively). Samples were dried at 60ºC and finely ground with a ball mill before being weighed and placed into tin capsules for these analyses. The δ^{13}C values and C and N concentrations of leaf material were measured by elemental analyzer/continuous flow isotope ratio mass spectrometry (ANCA/SL elemental analyzer coupled with a Finnigan MAT Delta PlusXL IRMS). The δ^{18}O of leaf material was determined with a Finnigan MAT Delta Plus XL IRMS (Finnigan MAT, Bremen, Germany) following the method described in Farquhar, Henry & Styles (1997). Isotope analyses were conducted at the Centre for Stable Isotope Biogeochemistry, University of California, Berkeley (USA). Isotope ratios are expressed in delta notation (‰) relative to an internationally accepted reference standard: V-PDB for δ^{13}C and V-SMOW for δ^{18}O. Long-term (3+ years) external precisions for δ^{13}C and δ^{18}O measurements of leaf material are 0.14 and 0.23‰, respectively. Leaf P concentrations were measured by inductively coupled plasma optical emission spectrometry (ICP-OES, Thermo Elemental Iris Intrepid II XDL, Franklin, MA, USA) after a microwave-assisted digestion with HNO_3:H_2O_2 (4:1, v:v) in the Ionomics laboratory at CEBAS-CSIC (Spain).

In April 2013, 2014 and 2015 we collected four leaves per plant (one individual per plot) to measure their dry mass, area and leaf dry mass per unit area (LMA). Foliar areas were calculated using the Image Pro Plus software, and the leaves were thereafter oven dried at 60ºC to determine their dry weight. LMA was calculated as the ratio between leaf dry weight and leaf area. In April 2015, we collected leaf material for chlorophyll content determinations. Briefly, 30 mg of leaves were sampled avoiding major veins, and chlorophyll was extracted from the leaves by submerging them in 3 mL of N, N-dimethylformamide in the dark for at least 72 h. Absorbance was read at 647 nm and 664.5 nm with a Thermo Spectronic device (Helios alpha, UVA No. 092009, England) and used to calculate fresh mass-based chlorophyll content (mg · g fresh mass^{-1}) according to Inskeep & Bloom (1985).

Chapter I

Four years into the study (September 2015), we collected one representative terminal shoot per target plant (10 cm long, 15 plants per treatment) to evaluate the long-term impacts of warming on the leaf and stem biomass production of *H. squamatum*. We measured total dry biomass (leaves plus stems) per unit shoot length, as well as the number of leaves and total leaf area per unit shoot length. More extensive destructive harvest of above- or below ground plant biomass (e.g. whole-plant harvest) was ruled out to ensure the integrity of the target plants in this long-term field experiment. Finally, we evaluated post-summer plant mortality after the first Autumn rainfalls in each study year (October 2012-2015). Plant survival was measured as the percentage of surviving *H. squamatum* individuals in each experimental plot after the summer drought.

Statistical analyses

We used repeated measures analysis of variance (RM-ANOVA) to evaluate the effects of experimental warming on leaf gas exchange (A, g_s, E, Fv':Fm', $\varnothing$PSII, WUE_i and WUE_{Inst}; 10-11 sampling dates) and the variables that were measured at the peak of the growing season in each study year (leaf N and P concentrations, $\delta^{13}C$, $\delta^{18}O$, leaf dry mass and area, LMA and post-summer plant survival). Temperature treatment (warming vs. control) and time were used as between-subject and within-subject factors in these analyses, respectively. Student's t tests were used to evaluate the effects of warming on shoot biomass production and leaf number and area. The relationships of leaf nutrient status with net photosynthetic rates and leaf biomass production were examined using linear regression analyses. To compare the intercepts and slopes of the regressions between A and g_s in the warming and control treatments, we used analysis of covariance (ANCOVA). RM-ANOVA and ANCOVA analyses were performed using the software SPSS 19.0 (SPSS Inc., Chicago, IL, USA) and StatgraphicsPlus 5.1 (Statgraphics Plus 5.1. for Windows, 2000), respectively.

Chapter I

RESULTS

Treatment effects on microclimatic variables and surface soil moisture content

Throughout the study period, the warming treatment (OTC) increased mean annual air temperature by ~2°C relative to ambient conditions (Fig. S2). Whereas minimum daily air temperatures were not significantly affected by the warming treatment, maximum daily air temperatures were on average 2.9°, 3.7°, 5.7° and 3.7° C higher within the OTCs during winter, spring, summer and autumn, respectively. Mean annual vapor pressure deficit was moderately but significantly higher in warmed plots (854 Pa) than in control plots (775 Pa), and this was attributable to differences in temperatures rather than to differences in mean annual relative humidity (which was similar in both treatments: 68.2% in control plots vs. 68.7% in warmed plots). Mean annual surface soil temperature (0-5 cm) was also higher in warmed plots (19.2° C) than in control plots (18.1° C). Mean daily surface soil temperatures were higher in warmed than in control plots by 0.7° in winter, by 1.3° in spring, by 2.1° in summer and by 1.1 in autumn. The warming treatment increased maximum daily soil temperatures by 0.9° in winter, by 2.4° in spring, by 5.0° in summer and by 1.2° C in autumn. Volumetric soil water content in the upper soil layer (0-5 cm depth) was lower in the warming treatment (8.0%) than in the control treatment (9.7%) across the study period (Fig. S3). Lower surface soil moisture content in the warming treatment was largely due to faster soil drying during rainless periods, rather than to any alteration of rainfall water inputs by the OTCs (as soil water content did not differ between warmed and control plots immediately after rain events).

Rainfall variability during the study period and its relationship to the performance of *Helianthemum squamatum*

Rainfall varied dramatically during the study period (Fig. S3). Mean annual rainfall from October 2011 to September 2012 (hydrological year) was 307.2 mm (15% below average), whereas annual rainfall in the same period in 2012-2013 was 405 mm (13% above average). The 2013-2014 hydrological year was extremely dry, as total annual rainfall (141 mm) was 60% below average. The 2014-2015 hydrological year was near the average (378.6 mm). The study period included an unusually wet autumn (threefold

higher rainfall than average in November 2012), a wet growing season (nearly twofold higher rainfall than average between February and April 2013), an exceptionally dry growing season (~27% of average rainfall during February-April 2014), as well as the second hottest summer in the last 60 years (24.1º C mean temperature in Summer 2015). Across temperature treatments, stomatal conductance and net photosynthetic rates were strongly limited by soil water availability (Fig. 1). Moreover, all the variables measured in *H. squamatum* at the peak of the growing season were strongly affected by the high inter-annual climatic rainfall variability during the study period (Table S2). Across temperature treatments, net photosynthetic rates were highest during wet periods in early spring when ambient air temperature at mid-day was between 17-27º C, and photosynthesis decreased sharply above 30º C.

Warming impacts on leaf gas exchange

The warming treatment consistently reduced the net photosynthetic rates (A), intrinsic water use efficiency (WUEi) and instantaneous water use efficiency (WUE$_{Inst}$) of *H. squamatum* throughout the study period (P<0.001 in all cases, Table S2; Fig. 2a, c and Fig. 4c, respectively). Averaged across growing seasons and measurement dates, mean A and WUEi values were ~36% and 41% lower, respectively, in warmed than in control plants. The strong detrimental effects of warming on A and WUE were exacerbated during drought periods, as indicated by their respective significant *Warming x Year* interactions (Table S2). The reduction of peak photosynthetic rates during Spring in warmed plants was more pronounced in a severe drought year (75% reduction relative to controls in April 2014) than in years with near-average rainfall (25%, 35% and 40% reductions in April 2012, 2013 and 2015, respectively). Interestingly, the only time when A and WUE values did not differ between warmed and control plants was during a cool and unusually rainy period in autumn (November 2012, with 15.7ºC mean monthly temperature, 16 rainy days and three-fold higher rainfall than average; Fig. 2a, c). In contrast to the large and consistent differences in A and WUEi between warmed and control plants, stomatal conductance (g$_s$) and transpiration rate (E) did not vary significantly with warming (P=0.400 and P=0.690, respectively, Table S2; Fig. 2b and Fig. 4b, respectively). When pooling leaf gas exchange data across measurement dates,

Chapter I

the linear regression between net photosynthetic rate and stomatal conductance had lower intercept and slope values for warmed plants than for control plants (Fig. 3).

The maximum efficiency of photosystem II (Fv':Fm') was not affected by the warming treatment (P=0.870, Table S2; Fig S4). However, the warming treatment marginally reduced the quantum efficiency of photosystem II ($\varnothing$PSII) in *H. squamatum*, although this effect was much smaller and less consistent through time than the effects on A and WUEi (P=0.058, Table S2; Fig 4a). Across study years, $\varnothing$PSII values at the peak of the growing season (April) were on average 21% lower in warmed plants than in control plants. Leaf respiration rates in the dark (mitochondrial respiration) did not differ between warmed and control plants at the peak of the growing season in 2014 (means $\pm$ standard errors = -1.43 $\pm$ 0.33 vs. -1.33 $\pm$ 0.38 μmol CO_2 m^2 s^{-1}, respectively; F= 0.033; P= 0.858; n= 21), although this result must be interpreted with caution due to the limited scope of the measurements.

Warming impacts on leaf nutrients, $\delta^{13}C$, $\delta^{18}O$, chlorophyll contents, leaf dry mass and area.

The warming treatment consistently reduced leaf N and P concentrations at the peak of the growing season (P=0.024 and P=0.005, respectively, Table S3; Table 1). Across study years, mean leaf N and P were on average 5.2% and 12.7% lower in warmed plants than in control plants, respectively. As a result, mean leaf C/N and C/P ratios were up to 11% and 16% higher, respectively, in warmed than in control plants. However, during a cool and unusually rainy period in autumn 2012, foliar N and P concentrations did not differ between control and warmed plants (N = 2.1 $\pm$ 0.1 % in both treatments; P = 1.3 $\pm$ 0.1 and 1.4 $\pm$ 0.1 mg g^{-1} in control and warmed plants, respectively). Across study years, peak photosynthetic rates during Spring correlated positively with leaf N and P concentrations in warmed plants (r= 0.536, P< 0.001 and r= 0.294, P= 0.024, respectively; N= 60), suggesting nutritional limitation of photosynthesis under warming. In contrast, inter-annual variation in peak photosynthetic rates was unrelated to leaf N or P status for control plants (r= -0.017, p= 0.899 and r= 0.173, p= 0.187, respectively; N= 60).

Chapter I

Across study years, warmed plants showed consistently lower leaf $\delta^{13}C$ values than control plants (P=0.016, Table S3; Fig. 5). However, the leaf $\delta^{18}O$ values of *H. squamatum* shrubs were not consistently affected by the warming treatment across years (P=0.735, Table S3; Table 1). Warmed plants showed a higher leaf chlorophyll b content than control plants (0.300 ± 0.007 and 0.275 ± 0.010 mg/g, respectively; F= 4.194; P= 0.055) in April 2015. Chlorophyll a content was not significantly affected by warming (means ± standard errors= 0.979 ± 0.034 mg/g in control plants and 0.976 ± 0.026 mg/g in warmed plants; F= 0.006; P= 0.941). The chlorophyll a:b ratio was thus significantly lower in warmed plants than in plants exposed to ambient temperature conditions (3.275 ± 0.034 and 3.524 ± 0.047, respectively; F= 13.366; P= 0.001).

Leaf dry mass at the peak of the growing season (April) was negatively correlated with leaf C:N (r= -0.445; P< 0.001; N= 89) and C:P (r= -0.263; P= 0.013; N= 89) ratios across years and temperature treatments, suggesting nutrient-limited leaf growth. Leaf area at the peak of the growing season also correlated negatively with leaf C:N (r= -0.641; P<0.001; N=89; Fig 6) and C:P (r= -0.246; P= 0.021; N=89) ratios across years and treatments. Averaged across years, mean leaf dry mass and area were 13% and 11% lower, respectively, in warmed plants than in control plants (P=0.044 and P=0.093, respectively, Table S3; Table 1). The relative decreases in mean leaf dry mass and area under warming were greatest during the drought year (2014, 17.5% and 21% decreases, respectively). The simultaneous decreases in leaf dry biomass and leaf N and P concentrations under warming translated into large relative decreases in mean leaf N and P contents across years (18% and 25% decreases, respectively).

Warming impacts on shoot biomass growth and plant post-summer survival

 Four years after the start of the experiment, the total dry biomass of terminal shoots (foliage plus stems) per unit length was 31% lower in warmed plants than in control plants (Table 2). Warmed plants had on average 35% lower leaf dry mass, 19% lower stem dry mass, 23% lower number of leaves and 29% smaller leaf area per unit shoot length than control plants (Table 2). However, post-summer plant survival rates did not differ between warmed and control plots within or across years (P= 0.832, Table S3; Table 1).

Chapter I

DISCUSSION

Warming effects on leaf gas exchange, nutrient and chlorophyll concentrations

Simulated climate warming caused large reductions of net photosynthetic rates in *H. squamatum*, particularly during dry periods, whereas stomatal conductance and transpiration rates remained largely unchanged. Therefore, decreased net photosynthetic rates in warmed plants were not caused by increased stomatal limitations on A, but rather by non-stomatal constraints (nutritional, biochemical, metabolic and/or diffusional; Flexas & Medrano 2002; Galmés, Medrano & Flexas 2007; Flexas et al. 2014). Warming reduced the carbon assimilation capacity of *H. squamatum* leaves at any given stomatal aperture (Fig. 3), except during a cool and unusually rainy period in November 2012. Furthermore, there was no evidence of photosynthesis acclimation to warming over the four years studied (Gunderson et al. 2010); on the contrary, the negative effects of warming on A and WUEi were most pronounced in the last two years of the study (Fig. 2a, c). In agreement with leaf gas exchange data, warmed plants showed consistently lower leaf $\delta^{13}C$ values than control plants across years (Fig. 5), which indicates that warming decreased time-integrated water use efficiency through non-stomatal reductions in net photosynthesis (Scheidegger et al. 2000; Seibt et al. 2008; Cernusak et al. 2013). Moreover, leaf $\delta^{18}O$ was not consistently affected by warming across years, which further suggests that neither time-integrated stomatal conductance (Fig 2b), nor the time-integrated $\delta^{18}O$ of the source water (which is primarily determined by rooting depth and soil water uptake depth) may have differed greatly between warmed and control plants (Barbour 2007).

Identifying and disentangling the underlying physiological mechanisms responsible for the large reduction of net photosynthetic rates under moderate warming in *H. squamatum* shrubs is beyond the scope of this study. Decreased photosynthetic rates with moderate temperature increases above the photosynthetic thermal optimum (see Galmés et al. 2013 for a physiologically-based definition of "moderate" vs. "severe" plant heat-stress) have been previously reported by many glasshouse studies (Sage & Kubien 2007; Galmés et al. 2013). Decreased photosynthesis under moderate warming has been related to biochemical limitations, including, among others, a) enhanced

photorespiration due to decreased Rubisco specificity for CO_2 relative to its alternative substrate oxygen and increased solubility of oxygen relative to CO_2 with rising temperature (von Caemmerer & Quick 2000); b) heat-induced inhibition of ribulose-1.5-bisphosphate regeneration (Kubien & Sage 2008); c) down-regulation of Rubisco activation state caused by increased catalytic inactivation of Rubisco at elevated temperatures, and by the high thermal lability of the enzyme Rubisco activase (Crafts-Brandner & Salvucci 2000, Sage & Kubien 2007); d) decreases in leaf protein content and Rubisco concentration under warming (Galmés et al., 2013); and, e) reduced electron transport capacity of photosystem II at supraoptimal temperatures for photosynthesis (Sage & Kubien 2007).

Warmed plants exhibited large reductions in A and WUEi even at the peak of the spring growing season, which is a period of mild temperatures in our study area (Fig S2) and considered optimal for photosynthesis (Galmés et al. 2013). The moderate increase in mean air temperature achieved by the warming treatment during spring (~1.5-2ºC) is thus unlikely to have caused any severe or irreversible heat-induced damage of the leaf photosynthetic machinery in warmed plants. Leaf gas exchange measurements were always conducted on young leaves formed during the current spring, so carry-over heat stress damage from the previous summer (maximum daily temperatures during summer within the OTCs increased up to 6ºC) is also an implausible explanation for the large reductions of A in warmed plants during the subsequent growing season. Interestingly, the maximum efficiency of photosystem II under light conditions (Fv':Fm'; Baker & Rosenqvist 2004) was not negatively affected by the warming treatment (Fig S4), which does not support a damaged PSII phytochemistry in warmed plants. Therefore, marginally decreased ∅PSII in warmed plants probably reflects PSII downregulation in response to a reduced carboxylation capacity (Loik et al. 2000; Sage & Kubien 2007; Baker & Rosenqvist 2004).

The tight negative correlations found between leaf dry mass/ area production and C:N and C:P ratios indicate nutrient limitation of foliar growth in *H. squamatum*. The simultaneous decreases of leaf N and P concentrations in warmed plants thus likely caused interactive and mutually reinforcing detrimental effects on their photosynthetic performance, given the key importance of N and P for Rubisco carboxylation capacity

Chapter I

and for ribulose-1.5-bisphosphate regeneration, respectively (Warren & Adams 2002; Campbell & Sage 2006; Reich, Oleksyn & Wright 2009*)*. Leaf photosynthetic capacity is strongly correlated with leaf N concentration because of the crucial role that N-rich enzymes, particularly Rubisco, play in the biochemical fixation of CO_2 (Field & Mooney 1986; Wright et al. 2004). Low leaf P concentration further limits photosynthesis through reductions in ribulose 1,5 bisphosphate regeneration, photochemical efficiency of photosystem II, mesophyll conductance to CO_2 and stomatal conductance (Jacob & Lawlor 1991, 1992; Loustau et al. 1999*;* Thomas, Montagu & Conroy 2006). Interestingly, the single measurement date when A and WUE values did not differ between warmed and control plants (during a cool and unusually wet period in November 2012) coincided with the only time when leaf N and P concentrations did not differ between treatments either. This result supports a role of decreased foliar N and P status in reducing photosynthesis in warmed plants at all other measurement dates.

Faster and more severe drying of the fertile upper soil layers (0-5 cm) where plant-available nutrients are most abundant may have hampered nutrient mineralization, solubilisation, diffusion and/or uptake by roots in the warmed plots (Sardans & Peñuelas 2013), which could explain the lower leaf N and P status of warmed plants relative to control plants. Decreased leaf N and P concentrations in warmed plants (along with lower leaf A, WUEi, $\delta^{13}C$ and biomass growth) would also be consistent with a decreased mycorrhizal contribution to nutrient uptake, since mycorrhizal fungi usually enhance the nutrient status, photosynthesis and water use efficiency (through C sink-stimulation), $\delta^{13}C$ and growth of their host plants in semiarid ecosystems (Querejeta et al. 2006; Mohan et al. 2014). Further research is warranted to resolve the precise mechanisms responsible for the moderate but consistent decreases in leaf N and P found under warming.

Contrary to expectations, warming did not alter the stomatal conductance and transpiration rates of *H. squamatum* (Table S2; Fig. 2b and Fig. 4b), despite faster and more severe surface soil drying (0-5 cm, Fig. S3) and higher evaporative demand in warmed plots, thus suggesting anisohydric behaviour in this species (Tardieu & Simonneau 1998). *H. squamatum* shrubs likely have the ability to extract water from

deeper (> 5 cm) soil layers that are less intensely affected by the drying effects of warming than surface layers. Mediterranean woody plants have evolved a wide array of adaptive homeostatic mechanisms to cope with fast and intense soil drying (e.g., adjustments in root:shoot biomass ratios, rooting depth or volume, and/or hydraulic architecture; Nardini et al. 2014), which may have enabled *H. squamatum* plants to sustain unchanged g_s and E under warming. In addition, the ability of *H. squamatum* shrubs to use the crystallization water of gypsum rocks and soils as a water source (Palacio et al., 2014) may have further helped them to maintain unchanged g_s and E under warming, given that the release of gypsum crystallization water is favoured by warming. Moreover, the 25% reduction in leaf area per unit shoot length in warmed plants resulted in sparser foliage, which may have allowed them to sustain unchanged g_s and E rates on a leaf area basis, while at the same time reducing total canopy transpiration (Limousin et al., 2009).

Leaf photosynthetic pigment ratios, such as the chlorophyll a:b ratio, provide useful indicators for plant stress detection, including heat, drought and nutrient stresses and their combination (Zhang et al. 2008; Thompson et al 1992). The chlorophyll a:b ratio decreased significantly in the warmed plants as compared with the controls, which is an indication of severe stress and is in accordance with the large reductions in net photosynthetic rates and shoot biomass production found under warming.

Warming effects on plant growth and survival

Interestingly, warmed plants were able to achieve similar post-summer survival rates as control plants throughout the study, despite their lower leaf area and biomass, foliar nutrient contents and photosynthetic rates. Given that the study period encompassed both the driest hydrological year on record (2013-2014) and the second hottest summer on record (2015) across Southeastern Spain, the unchanged survival rates under warming highlight the remarkable resistance and resilience of *H. squamatum* shrubs against forecasted climate change conditions. However, plant growth was strongly reduced by moderate warming, as warmed plants had about one-third lower shoot dry biomass production (leaves plus stems) and leaf area at the end of the study period. The lower carbon cost of building thinner stems and sparser and smaller leaves (Table 2)

may be a key adaptive mechanism that helped maintain the carbon balance and survival of *H. squamatum* shrubs under warming, despite the large reductions in their net carbon assimilation rates (Nicotra et al. 2010; Bussotti et al. 2014). Reducing leaf numbers and leaf area per unit shoot length may also be an effective adaptive mechanism to decrease total canopy transpiration under warming, in order to minimize the risk of catastrophic hydraulic failure under enhanced vapor pressure deficit and soil drying (Limousin et al., 2009).

Comparison with other warming experiments

The strong detrimental effects of simulated climate warming on photosynthesis and growth in this semiarid ecosystem contrast to the overwhelmingly positive or neutral plant responses to warming found in most terrestrial ecosystems (including drylands; Rustad et al. 2001; Xia, Niu & Wan 2009; Raich et al. 2006; Lin et al. 2010; Wu et al. 2011; Peñuelas et al. 2013). Few (if any) field studies have so far reported such large reductions in peak photosynthesis rates (25-75%) and plant biomass growth (31%) linked to simultaneous decreases in leaf N and P concentrations because of warming. The vast majority of field studies conducted to date report increased leaf N and/or P concentrations (attributed to increased soil nutrient mineralization) and photosynthesis under warming (Rustad et al. 2001; Melillo et al. 2002; Butler et al. 2012; Bai et al. 2013; Dijkstra et al. 2012). Although a few studies found decreases in leaf N concentrations under warming, this was generally attributed to dilution effects caused by enhanced photosynthesis and growth (Dijkstra et al. 2010; An et al. 2005), or to changes in proportional nutrient allocation to leaves vs. woody tissue in warmed plants (Sardans et al. 2008a).

In a series of field studies investigating the responses of native shrubland species to passive night-time warming in a subhumid Mediterranean environment, Llorens et al. (2003) found few consistent changes in A, WUEi, foliar N or leaf $\delta^{13}C$ values in warmed relative to control plants. However, the nutrient status and photosynthetic performance of some shrub species were moderately enhanced by warming, as indicated by increased foliar P, higher Rubisco activity and carboxylation efficiency, enhanced photochemical efficiency of photosystem II and increased biomass production (Llorens

et al. 2003; Sardans et al. 2008b; Prieto et al. 2009a, b). The discrepancies between our study and previous work thus demonstrate that climate warming can be much more detrimental to plant nutrient status, photosynthesis and growth in semiarid Mediterranean ecosystems than previously reported for subhumid Mediterranean sites (Llorens et al. 2003; Peñuelas et al. 2004, 2007; Prieto et al. 2009a, b). Differences in the timing (day-time vs night-time) and intensity of warming ($2º$ C vs. $1º$ C) could in part explain the contrasting results with these previous studies. However, the large differences in annual rainfall between study sites likely play a more pivotal role in determining plant vulnerability to warming (Swarbreck et al. 2011): whereas mean annual temperature was very similar between our semiarid site and the abovementioned subhumid site (around 15ºC; Llorens et al. 2003), mean annual rainfall during the respective study periods was much lower at our site (308 vs. 580 mm; Llorens et al. 2003). In support of this interpretation, we found that the detrimental effects of warming on the A, WUE and leaf N and P concentrations of *H. squamatum* shrubs disappeared transiently during an unusually rainy period in November 2012.

Concluding remarks

A moderate (~$2º$ C) warming exerted strong negative effects on the net photosynthetic rate and shoot biomass growth of *H. squamatum*, especially (but not only) during dry periods. Warming reduced peak photosynthetic rates during Spring by 25-40% in near-average rainfall years, and by 75% in a severe drought year, which has important implications for projections of plant carbon balance under the warmer climatic scenario predicted for the Mediterranean (Giorgi & Lionello 2008) and other dryland regions. However, warmed plants were able to cope with large reductions in net photosynthetic rates, leaf area and shoot biomass production without significant changes in post-summer plant survival. Contrary to expectations, stomatal conductance and transpiration rates remained unchanged under warming, thus highlighting the key role of non-stomatal limitations on photosynthesis (e.g. biochemical and nutritional; Flexas et al., 2014) in this Mediterranean shrub. Our findings indicate that warming could significantly reduce net primary production (by about one-third) and potentially alter other key ecological processes such as plant-herbivore relationships, leaf litter

decomposition and nutrient cycling (through changes in leaf N and P) in semiarid gypsum shrublands dominated by *H. squamatum*.

References

Allison, S.D. & Treseder, K.K. (2008) Warming and drying suppress microbial activity and carbon cycling in boreal forest soils. *Global Change Biology*, **14**, 2898–2909.

An, Y., Wan, S., Zhou, X., Subedar, A.A., Wallace, L.L. & Luo, Y. (2005) Plant nitrogen concentration, use efficiency, and contents in a tallgrass prairie ecosystem under experimental warming. *Global Change Biology*, **11**, 1733-1744.

Bai E., Li, S., Xu, W., Li W., Dai W. & Jiang, P. (2013) A meta-analysis of experimental warming effects on terrestrial nitrogen pools and dynamics. *New Phytologist*, **199**, 441–451.

Baker N.R. & Rosenqvist E. (2004) Applications of chlorophyll fluorescence can improve crop production strategies: an examination of future possibilities. *Journal of Experimental Botany* 55, 1607–1621.

Chapter I

Barbour, M.M. (2007) Stable oxygen isotope composition of plant tissue: a review. *Functional Plant Biology*, **34**, 83–94.

Bussotti, F., Ferrini, F., Pollastrini, M., & Fini, A. (2014) The challenge of Mediterranean sclerophyllous vegetation under climate change: From acclimation to adaptation. *Environmental and Experimental Botany*, **103**, 80-98.

Butler, S.M., Melillo, J.M., Johnson, J.E., Mohan, J., Steudler, P.A., Lux, H. *et al.* (2012) Soil warming alters nitrogen cycling in a New England forest: implications for ecosystem function and structure. *Oecologia*, **168**, 819-828.

von Caemmerer S. & Quick W.P. (2000) Rubisco: physiology in vivo. In Photosynthesis: Physiology and Metabolism (eds R.C. Leegood, T.D. Sharkey & S. von Caemmerer), pp. 85–113. Kluwer Academic, Dordrecht, the Netherlands.

Campbell, C.D. & Sage, R.F. (2006) Interactions between the effects of atmospheric CO_2 content and P nutrition on photosynthesis in white lupin (*Lupinus albus L.*). *Plant, Cell and Environment,* **29**, 844-853.

Cernusak, L.A., Ubierna, N., Winter, K., Holtum, J.A., Marshall, J. D., & Farquhar, G. D. (2013) Environmental and physiological determinants of carbon isotope discrimination in terrestrial plants. *New Phytologist*, **200**, 950-965.

Crafts-Brandner, S.J., & Salvucci, M.E. (2000) Rubisco activase constrains the photosynthetic potential of leaves at high temperature and CO_2. *Proceedings of the National Academy of Sciences*, **97**, 13430-13435.

Castro, M. D., Martín-Vide, J., & Alonso, S. (2005) El clima de España: pasado, presente y escenarios de clima para el siglo XXI. In: ed. Moreno JM. *Evaluación Preliminar de los Impactos en España por Efecto del Cambio Climático*. Madrid: Ministerio de Medio Ambiente, 1–64.

Collins, M., Knutti, R., Arblaster, J., Dufresne, J., Fichefet, T., Friedlingstein, P. *et al.* (2013) Climate change 2013: the physical science basis. Contribution of Working

Chapter I

Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. *Long-term Climate Change: Projections, Commitments and Irreversibility*, Cambridge University Press, Cambridge, UK, and New York.

Dijkstra, F.A., Blumenthal, D., Morgan, J.A., Pendall, E., Carrillo, Y., & Follett, R.F. (2010) Contrasting effects of elevated CO2 and warming on nitrogen cycling in a semiarid grassland. *New Phytologist*, **187**, 426-437.

Dijkstra, F.A., Pendall, E., Morgan, J.A., Blumenthal, D.M., Carrillo, Y., LeCain, D.R. *et al.* (2012) Climate change alters stoichiometry of phosphorus and nitrogen in a semiarid grassland. *New Phytologist*, **196**, 807-815.

Farquhar, G.D., Henry, B.K., & Styles, J.M. (1997) A rapid on-line technique for determination of oxygen isotope composition of nitrogen-containing organic matter and water. *Rapid Communications in Mass Spectrometry*, **11**, 1554-1560.

Field C., Mooney, H.A. (1986) The photosynthesis-nitrogen relationship in plants. *On the economy of plant form and function* (eds. GivnishTJ), pp. 25–55. Cambridge University Press, Cambridge.

Flexas, J., Diaz-Espejo, A., Gago, J., Gallé, A., Galmés, J., Gulías, J., & Medrano, H. (2014) Photosynthetic limitations in Mediterranean plants: a review. *Environmental and Experimental Botany*, **103**, 12-23.

Flexas, J. & Medrano, H. (2002) Drought-inhibition of photosynthesis in C3 plants: stomatal and non-stomatal limitations revisited. *Annals of Botany*, **89**, 183-189.

Galmés, J., Medrano, H. & Flexas, J. (2007) Photosynthetic limitations in response to water stress and recovery in Mediterranean plants with different growth forms. *New Phytologist*, **175**, 81-93.

Galmés, J., Aranjuelo, I., Medrano, H. & Flexas, J. (2013) Variation in Rubisco content and activity under variable climatic factors. *Photosynthesis research*, **117**, 73-90.

Chapter I

de Guevara, M.L., Lázaro, R., Quero, J.L., Ochoa, V., Gozalo, V., Berdugo, M. *et al.* (2014) Simulated climate change reduced the capacity of lichen-dominated biocrusts to act as carbon sinks in two semi-arid Mediterranean ecosystems. *Biodiversity and Conservation*, **23**, 1787-1807.

Giorgi, F. & Lionello, P. (2008) Climate change projections for the Mediterranean region. *Global and Planetary Change*, **63**, 90-104.

Gunderson, C.A., O'HARA, K.H., Campion, C.M., Walker, A.V. & Edwards, N.T. (2010) Thermal plasticity of photosynthesis: the role of acclimation in forest responses to a warming climate. *Global Change Biology*, **16**, 2272-2286.

He, M. & Dijkstra, F.A. (2014) Drought effect on plant nitrogen and phosphorus: a meta-analysis. *New Phytologist*, **204**, 924–931.

Hollister, R.D. & Webber, P.J. (2000) Biotic validation of small open-top chambers in a tundra ecosystem. *Global Change Biology*, **6**, 835–842.

Inskeep, W.P. & Bloom, P.R. (1985) Extinction coefficients of chlorophyll a and b in N, N-dimethylformamide and 80% acetone. *Plant Physiology*, **60**, 606-608.

IUSS Working Group WRB. (2006) World reference base for soil resources 2006: World Soil Resources Reports No. 103. FAO, Rome.

Jacob, J. & Lawlor, D.W. (1991) Stomatal and mesophyll limitations of photosynthesis in phosphate deficient sunflower, maize and wheat plants. *Journal of Experimental Botany*, **42**, 1003–1011.

Jacob, J. & Lawlor, D.W. (1992) Dependence of photosynthesis of sunflower and maize leaves on phosphate supply, ribulose-1,5-bisphosphate carboxylase/oxygenase activity, and ribulose-1,5-bisphosphate pool size. *Plant Physiology,* **98**, 801–807.

Kubien, D.S. & Sage, R.F. (2008) The temperature response of photosynthesis in tobacco with reduced amounts of Rubisco. *Plant, Cell and Environment*, **31**, 407–418.

Chapter I

Larcher, W. (2000) Temperature stress and survival ability of Mediterranean sclerophyllous plants. *Plant Biosystems*, **134**, 279-295.

Liang, J., Xia, J., Liu, L. & Wan, S. (2013) Global patterns of the responses of leaf-level photosynthesis and respiration in terrestrial plants to experimental warming. *Journal of Plant Ecology*, **6**, 437-447.

Limousin JM, Rambal S, Ourcival JM, Rocheteau A, Joffre R, Rodríguez-Cortina R. (2009). Long-term transpiration change with rainfall decline in a Mediterranean *Quercus ilex* forest. *Global Change Biology,* **15**, 2163-2175.

Lin, D., Xia, J. & Wan, S. (2010) Climate warming and biomass accumulation of terrestrial plants: a meta-analysis. *New Phytologist*, **188**, 187–198.

Llorens, L., Peñuelas, J. & Estiarte, M. (2003) Ecophysiological responses of two Mediterranean shrubs, *Erica multiflora* and *Globularia alypum*, to experimentally drier and warmer conditions. *Physiologia Plantarum*, **119**, 231–243.

Loik, M.E., Redar, S.P. & Harte J. (2000). Photosynthetic responses to a climate-warming manipulation for contrasting meadow species in the Rocky Mountains, Colorado, USA. *Functional Ecology* **14,** 166–175.

Loustau, D., Ben Brahim, M., Gaudillére, J.P. & Dreyer, E. (1999) Photosynthetic responses to phosphorus nutrition in two-year-old maritime pine seedlings. *Tree Physiology*, **19**, 707–715.

Luo, Y. (2007) Terrestrial carbon cycle feedback to climate warming. *Annual Review of Ecology Evolution and Systematics*, **38**, 683-712.

Maestre, F.T., Escolar, C., de Guevara, M.L., Quero, J.L., Lázaro, R., Delgado-Baquerizo, M. *et al.* (2013) Changes in biocrust cover drive carbon cycle responses to climate change in drylands. *Global Change Biology*, **19**, 3835–3847.

Maestre, F. T., Escolar, C., R. Bardgett, J. A. D. Dungait, B. Gozalo & V. Ochoa. 2015. Warming reduces the cover and diversity of biocrust-forming mosses and lichens, and

increases the physiological stress of soil microbial communities in a semi-arid *Pinus halepensis* plantation. *Frontiers in Microbiology* 6: 865.

Melillo, J.M., Steudler, P.A., Aber, J.D., Newkirk, K., Lux, H., Bowles, F.P. *et al.* (2002) Soil warming and carbon-cycle feedbacks to the climate system. *Science*, **298**, 2173–2176.

Mohan, J.E., Cowden, C.C., Baas, P., Dawadi, A., Frankson, P.T., Helmick, K. *et al.* (2014) Mycorrhizal fungi mediation of terrestrial ecosystem responses to global change: mini-review. *Fungal Ecology*, **10**, 3-19.

Nardini, A., Lo Gullo, M.A., Trifilò, P. & Salleo, S. (2014) The challenge of the Mediterranean type climate to plant hydraulics: responses and adaptations. *Environmental and Experimental Botany*, **103**, 68–79.

Nicotra, A.B., Atkin, O.K., Bonser, S.P., Davidson, A.M., Finnegan, E.J., Mathesius, U. *et al.* (2010) Plant phenotypic plasticity in a changing climate. *Trends in Plant Science*, **15**, 684–692.

Niu, S., Li, Z., Xia, J., Han, Y., Wu, M., & Wan, S. *et al.* (2008a) Climatic warming changes plant photosynthesis and its temperature dependence in a temperate steppe of northern China. *Environmental and Experimental Botany*, **63**, 91–101.

Niu, S., Wu, M., Han, Y., Xia, J., Li, L. & Wan, S. (2008b) Water-mediated responses of ecosystem carbon fluxes to climatic change in a temperate steppe. *New Phytologist*, **177**, 209–219.

Palacio S, Azorín J, Montserrat-Martí G, Ferrio JP. 2014. The crystallization water of gypsum rocks is a relevant water source for plants. *Nature Communications* **5**: 4660.

Peng, S., Piao, S., Ciais, P., Myneni, R. B., Chen, A., Chevallier, F. *et al.* (2013) Asymmetric effects of daytime and night-time warming on Northern Hemisphere vegetation. *Nature*, **501**, 88–92.

Chapter I

Peñuelas, J., Gordon, C., Llorens, L., Nielsen, T., Tietema, A., Beier, C. *et al.* (2004) Non-intrusive field experiments show different plant responses to warming and drought among sites, seasons, and species in a North-South European gradient. *Ecosystems*, **7**, 598–612.

Peñuelas, J., Prieto, P., Beier, C., Cesaraccio, C., De Angelis, P., De Dato, G. *et al.* (2007) Response of plant species richness and primary productivity in shrublands along a north–south gradient in Europe to seven years of experimental warming and drought: reductions in primary productivity in the heat and drought year of 2003. *Global Change Biology*, **13**, 2563–2581.

Peñuelas, J., Sardans, J., Estiarte, M., Ogaya, R., Carnicer, J., Coll, M. *et al.* (2013) Evidence of current impact of climate change on life: a walk from genes to the biosphere. *Global Change Biology*, **19**, 2303–2338.

Perfors, T., Harte, J. & Alter, S.E. (2003) Enhanced growth of sagebrush (*Artemisia tridentata*) in response to manipulated ecosystem warming. *Global Change Biology*, **9**, 736–742.

Prieto, P., Peñuelas, P., Llusia, J., Asensio, D. & Estiarte, M. (2009a) Effects of long-term experimental night-time warming and drought on photosynthesis, Fv/Fm and stomatal conductance in the dominant species of a Mediterranean shrubland. *Acta Physiologiae Plantarum*, **31**, 729–739.

Prieto, P., Peñuelas, J., Llusia, J., Asensio, D. & Estiarte, M. (2009b) Effects of experimental warming and drought on biomass accumulation in a Mediterranean shrubland. *Plant Ecology*, **205**, 179–191.

Querejeta, J.I., Allen, M.F., Caravaca, F. & Roldán, A. (2006) Differential modulation of host plant $\delta^{13}C$ and $\delta^{18}O$ by native and nonnative arbuscular mycorrhizal fungi in a semiarid environment. *New Phytologist*, **169**, 379–387.

Raich JW, Russell AE, Kitayama K, Parton WJ, & Vitousek PM. (2006). Temperature influences carbon accumulation in moist tropical forests. *Ecology* **87,** 76–87.

Chapter I

Reich, P.B., Oleksyn, J. & Wright, I.J. (2009) Leaf phosphorus influences the photosynthesis–nitrogen relation: a cross-biome analysis of 314 species. *Oecologia*, **160**, 207–212.

Rustad, L.E., Campbell, J.L, Marion, G.M, Norby, R.J, Mitchell, M.J, Hartley, A.E. *et al.* (2001) A meta-analysis of the response of soil respiration, net nitrogen mineralization, and aboveground plant growth to experimental ecosystem warming. *Oecologia*, **126**, 543–562.

Safriel, U., Adeel, Z. (2005) Dryland systems. In: Hassan R, Scholes R, Ash N (eds) *Ecosystems and human well-being, current state and trends, vol 1*. Washington, USA: Island Press, 625–658.

Sage, R.F. & Kubien, D.S. (2007). The temperature response of C3 and C4 photosynthesis. *Plant, Cell & Environment* **30,** 1086–1106.

Sardans, J., Peñuelas, J., Estiarte, M. & Prieto, P. (2008a) Warming and drought alter C and N concentration, allocation and accumulation in a Mediterranean shrubland. *Global Change Biology*, **14**, 2304–2316.

Sardans, J., Peñuelas, J., Prieto, P. & Estiarte, M. (2008b) Drought and warming induced changes in P and K concentration and accumulation in plant biomass and soil in a Mediterranean shrubland. *Plant and Soil*, **306**, 261-271.

Sardans, J. & Peñuelas J. (2013) Plant-soil interactions in Mediterranean forest and shrublands: impacts of climatic change. *Plant and Soil*, **365**, 1-33.

Scheidegger, Y., Saurer, M., Bahn, M. & Siegwolf, R. (2000) Linking stable oxygen and carbon isotopes with stomatal conductance and photosynthetic capacity: a conceptual model. *Oecologia*, **125**, 350–357.

Seibt, U., Rajabi, A., Griffiths, H. & Berry, J.A. (2008) Carbon isotopes and water use efficiency: sense and sensitivity. *Oecologia*, **155**, 441–454.

Chapter I

Swarbreck, S., Sudderth, E.A., StClair, S.B., Salve, R., Casthanha, C., Torn, M.S. *et al.* (2011) Linking leaf transcript levels to whole plant analyses provides mechanistic insights to the impact of warming and altered water availability in an annual grass. *Global Change Biology*, **17**, 1577–1594.

Tan, J., Piao, S., Chen, A., Zeng Z., Ciais, P., Janssens, I.A. *et al.* (2015) Seasonally different response of photosynthetic activity to daytime and night-time warming in the Northern Hemisphere. *Global Change Biology*, **21**, 377–387

Tardieu F, Simonneau T. 1998. Variability of species among stomatal control under fluctuating soil water status and evaporative demand: modeling isohydric and anisohydric behaviours. *Journal of Experimental Botany* **49**:419–432.

Thomas, D.S., Montagu, K.D. & Conroy, J.P. (2006) Leaf inorganic phosphorus as a potential indicator of phosphorus status, photosynthesis and growth of *Eucalyptus grandis* seedlings. *Forest Ecology and Management*, **223**, 267–274

Thompson, W. A., Huang, L. K., & Kriedemann, P. E. (1992). Photosynthetic response to light and nutrients in sun-tolerant and shade-tolerant rainforest trees. II. Leaf gas exchange and component processes of photosynthesis. *Functional Plant Biology*, **19**, 19-42.

Warren, C.R., Adams, M.A. (2002) Phosphorus affects growth and partitioning of nitrogen to Rubisco in *Pinus pinaster*. *Tree Physiology*, **22**, 11–19.

Wang, L., D'Odorico, P., Evans, J.P., Eldridge, D.J., McCabe, M.F., Caylor, K.K. *et al.* (2012) Dryland ecohydrology and climate change: critical issues and technical advances. *Hydroloy and Earth System Sciences*, **16**, 2585-2603.

White, R.P., Nackoney, J. (2003) *Drylands, people, and ecosystem goods and services: a web-based geospatial analysis.* World Resources Institute. [WWW document] URL http://pubs.wri.org [accessed 30 January 2012].

Chapter I

Wright IJ, Reich PB, Westoby M, Ackerly DD, Baruch Z, Bongers F, Cavender-Bares J, Chapin T, Cornelissen JHC *et al.* (2004) The worldwide leaf economics spectrum. *Nature*, **428**, 821–827.

Wu, Z., Dijkstra, P., Koch, G.W., Peñuelas, J. & Hungate, B.A. (2011) Responses of terrestrial ecosystems to temperature and precipitation change: a meta-analysis of experimental manipulation. *Global Change Biology*, **17**, 927-942.

Xia, J., Niu, S. & Wan, S. (2009) Response of ecosystem carbon exchange to warming and nitrogen addition during two hydrologically contrasting growing seasons in a temperate steppe. *Global Change Biology*, **15**, 1544–1556.

Xia, J.Y., Chen, J.Q., Piao, S.L., Ciais, P., Luo, Y.Q. & Wan, S.Q. (2014) Terrestrial carbon cycle affected by non-uniform climate warming. *Nature Geoscience*, **7**, 173–180.

Zhang, X., Wollenweber, B., Jiang, D., Liu, F. & Zhao, J. (2008) Water deficits and heat shock effects on photosynthesis of a transgenic *Arabidopsis thaliana* constitutively expressing ABP9, a bZIP transcription factor. *Journal of Experimental Botany*, **59**, 839-848.

Chapter I

Table 1. Leaf N and P concentrations, leaf dry mass, area and leaf mass per unit area (LMA) values, leaf $\delta^{13}C$ and $\delta^{18}O$ and post-summer plant survival values in the four years of the study. Data represent means ± SE (n = 10-15).

|		Leaf N (%)	Leaf P (mg g^{-1})	Leaf mass (mg)	Leaf area (cm^2)	LMA (mg/cm^2)	Leaf $\delta^{18}O$	Survival
2012	**CONTROL**	2.60 ± 0.07	1.27± 0.06	-	-	-	26.42 ± 0.35	63.6
	WARMING	2.22 ± 0.07	1.06 ± 0.06	-	-	-	26,16 ± 0.35	55.3
2013	**CONTROL**	2.20 ± 0.07	1.01 ± 0.05	12.12 ± 0.59	0.74 ± 0.04	16.5 ± 0.5	25.35 ± 0.24	94.7
	WARMING	2.15 ± 0.08	0.87 ± 0.06	10.28 ± 0.70	0.69 ± 0.05	14.6 ± 0.6	25.25 ± 0.27	100.0
2014	**CONTROL**	1.73 ± 0.06	0.91 ± 0.06	10.69 ± 0.714	0.60 ± 0.05	17.9 ±0.5	25.60 ± 0.39	33.3
	WARMING	1.57 ± 0.06	0.79 ± 0.05	8.83 ± 0.66	0.55 ± 0.05	18.2 ± 0.5	26.98 ± 0.35	28.9
2015	**CONTROL**	2.44 ± 0.05	0.89 ± 0.04	13.73 ± 1.06	1.11 ± 0.08	12.5 ± 0.6	24.10 ± 0.22	94.4
	WARMING	2.34 ± 0.06	0.79 ± 0.05	12.58 ± 1.10	1.05 ± 0.08	11.9 ± 0.5	23.41 ± 0.21	89.2

Chapter I

Table 2. Mean number of leaves, total leaf area, total dry mass (leaves plus stem), leaf dry mass and stem dry mass of terminal shoots (10 cm long) in control and warmed *Helianthemum squamatum* plants. Data represent means ± SE (n = 15 plants per treatment). P-values <0.05 indicate significant differences between temperature treatments (t test).

	Leaf Number	Leaf Area (cm^2)	Shoot Dry Mass (mg)	Leaf Dry Mass (mg)	Stem Dry Mass (mg)
CONTROL	81.7 ± 4.9	17.0 ± 1.4	360 ± 26	268 ± 22	91 ± 5
WARMING	62.6 ± 4.8	12.0 ± 1.3	248 ± 25	173 ± 21	74 ± 4
p-value	0.010	0.014	0.004	0.004	0.013

Figure 1. Linear regressions between net photosynthesis rate (a)/stomatal conductance (b) and soil water content in control and warmed plants across measurement dates. Each point represents the mean value of 10-15 replicated plants from separate plots. Vertical and horizontal error bars represent standard errors. Linear regression equations for control and warmed plants are also shown.
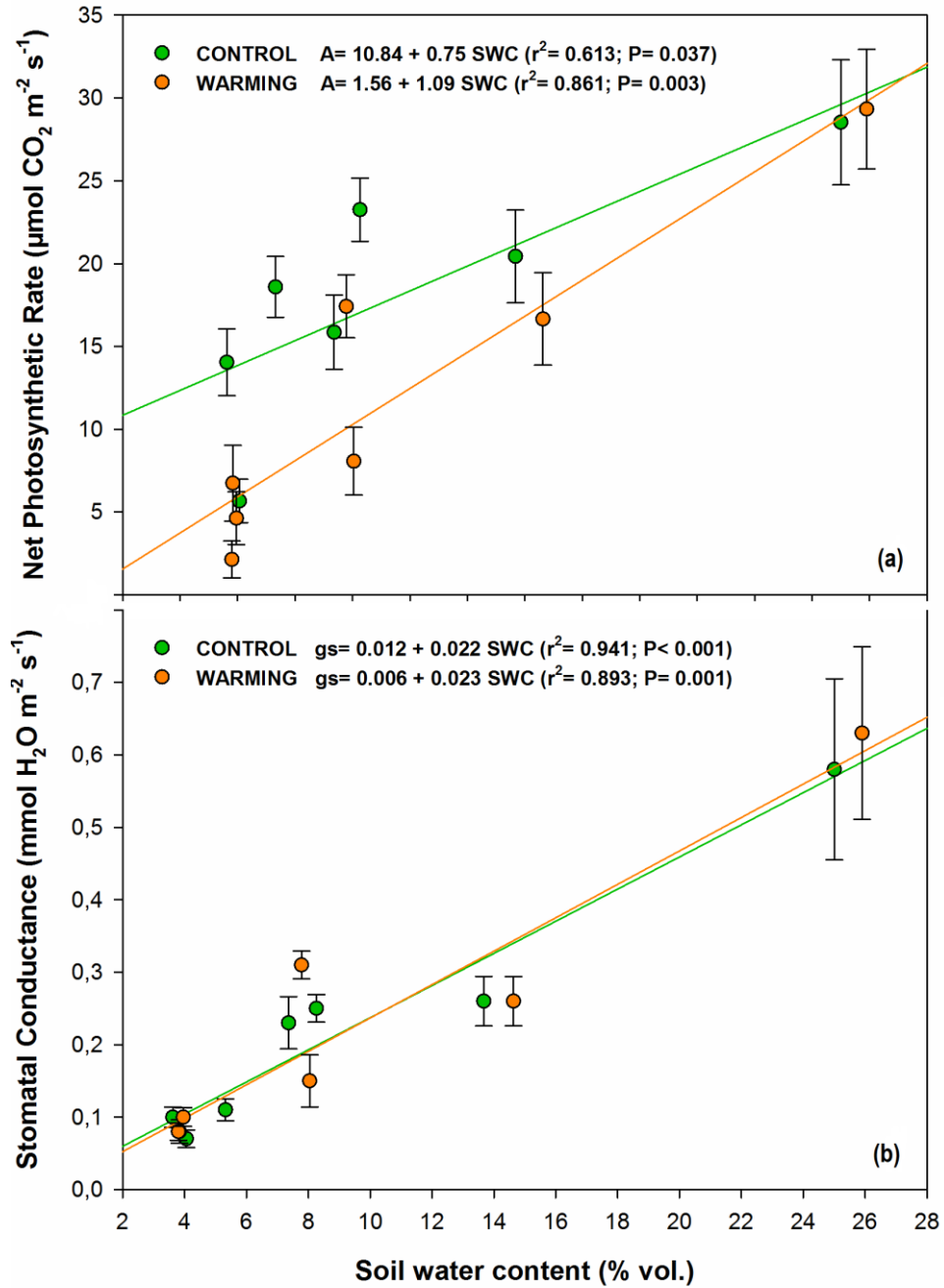
Figure 2. Mean net photosynthetic rates (a), stomatal conductance (b) and intrinsic water use efficiency values (c) in warmed and control plants at 11 different measurement dates spanning four growing seasons. Data represent means ± SE (n = 10-15).
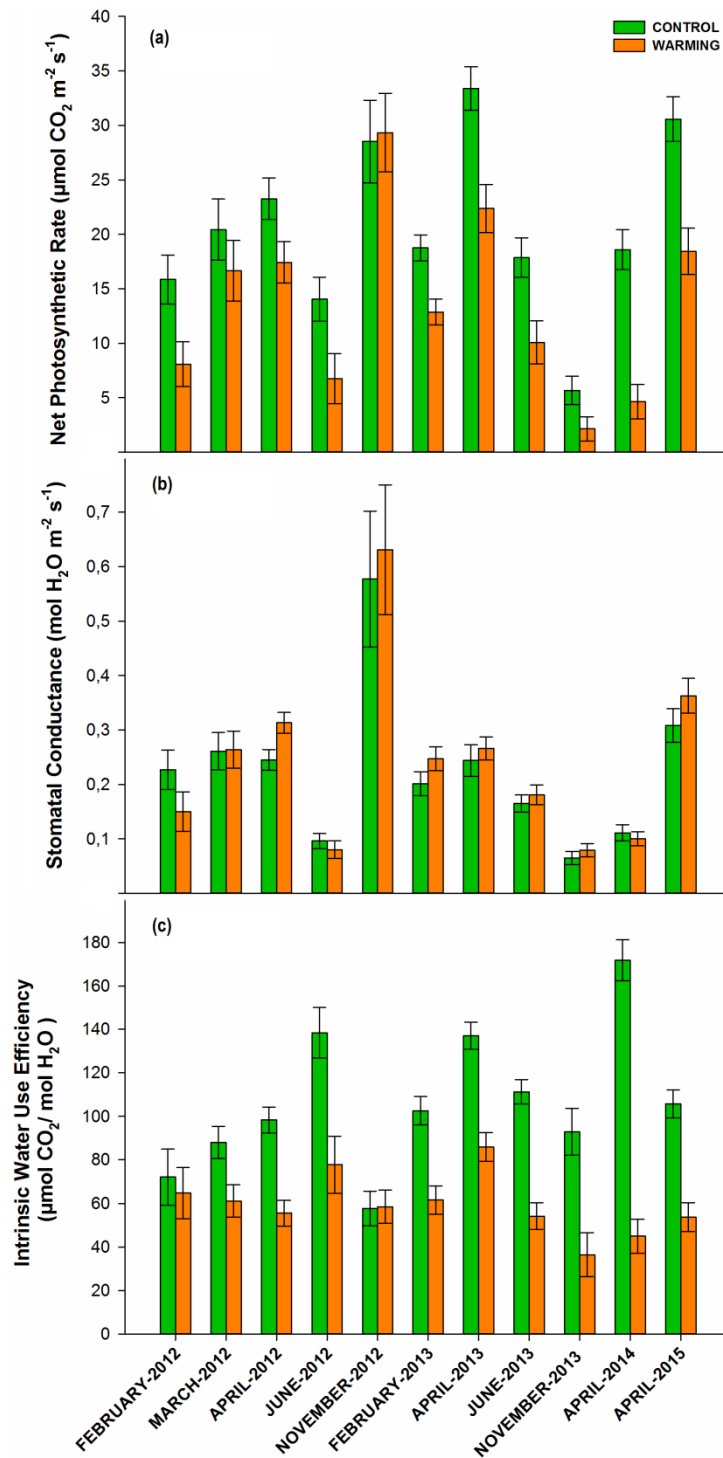
Figure 3. Linear regressions between mean net photosynthesis rates (A) and mean stomatal conductance (g_s) in control and warmed plants across measurement dates. Each point represents the mean value of 10-15 replicated plants from separate plots. Vertical and horizontal error bars represent standard errors. Fittted linear regressions for control and warmed plants are also shown. Analysis of covariance indicated that the regression lines of control and warmed plants were significantly different (P= 0.001). Data recorded in November 2012 were excluded from the regression analysis, as there were no significant differences in A or g_s between temperature treatments at this time.
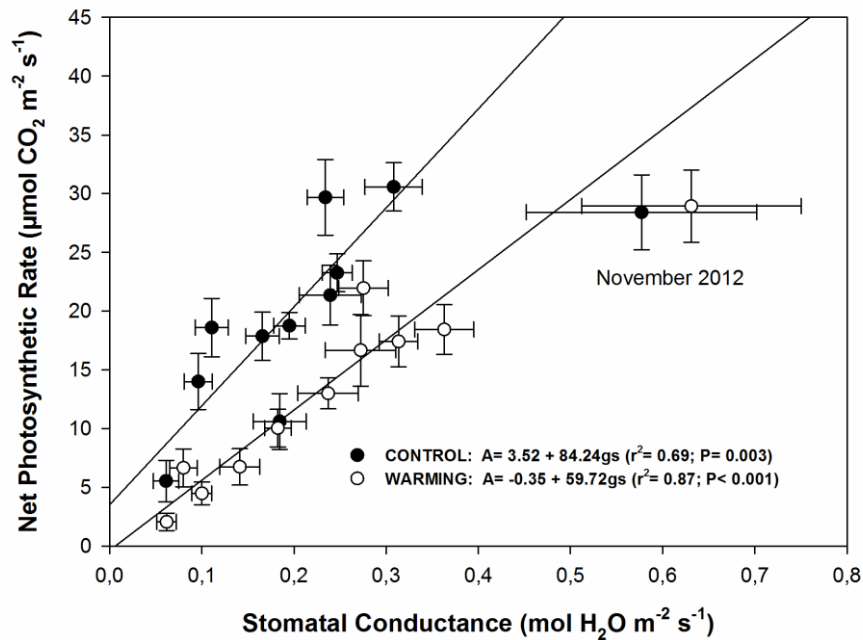
Figure 4. Quantum efficiency of photosysytem II (a), transpiration rates (b) and instantaneous water use efficiency values (c) in warmed and control plants at 10-11 different measurement dates spanning four consecutive growing seasons. Data represent means ± SE (n = 10-15).
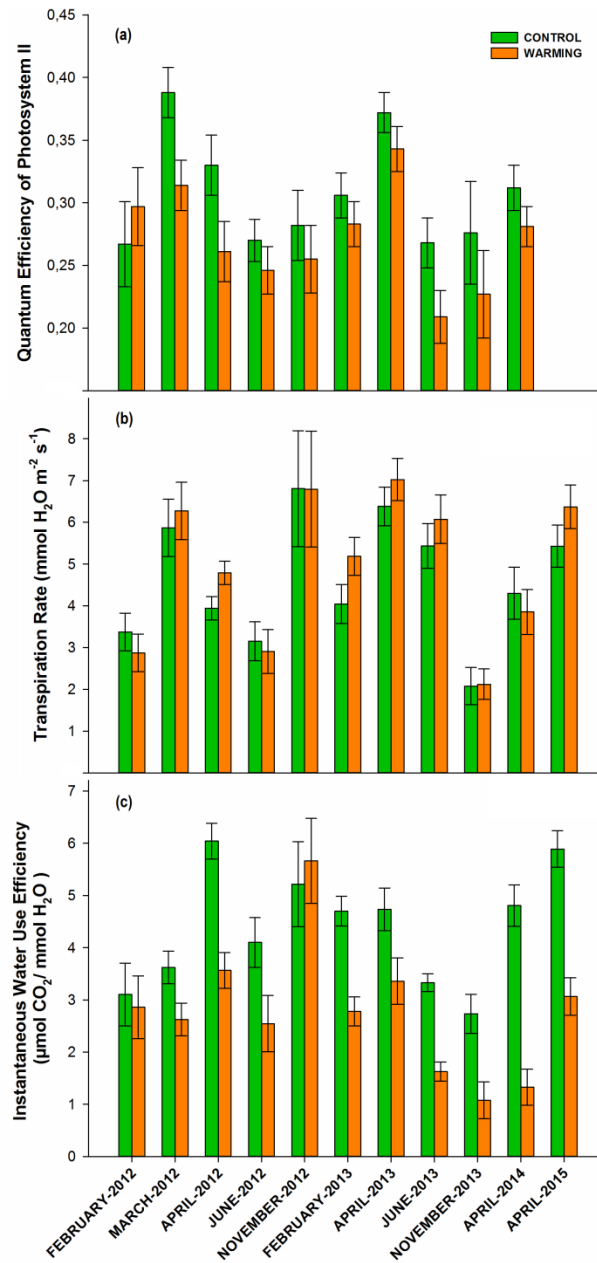
Figure 5. Mean leaf $\delta^{13}C$ values in control and warmed plants at the peak of the growing season (April) in the 4 years of the study. Data represent means $\pm$ SE (n = 10-15).
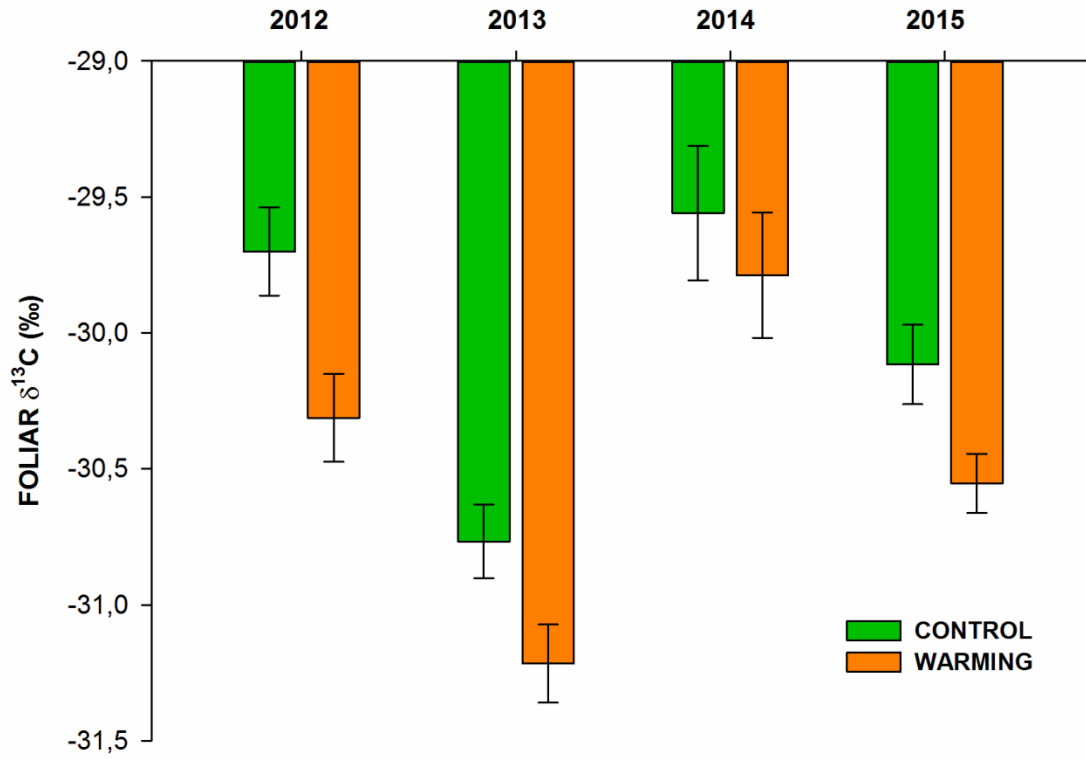
Figure 6. Negative relationship between leaf areas and C:N ratios at the peak of the growing season (April 2013, 2014, 2015). Green circles represent control plants and orange circles represent warmed plants. N= 10-15 individual plants per treatment x year combination.
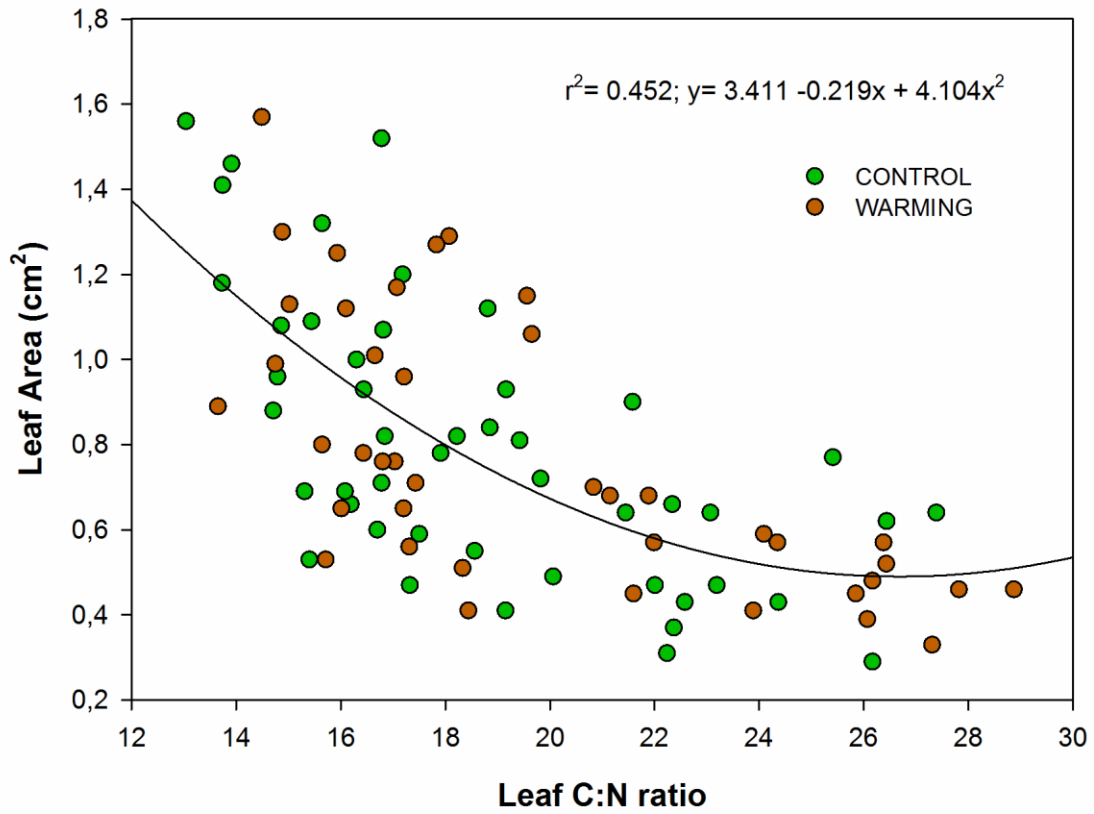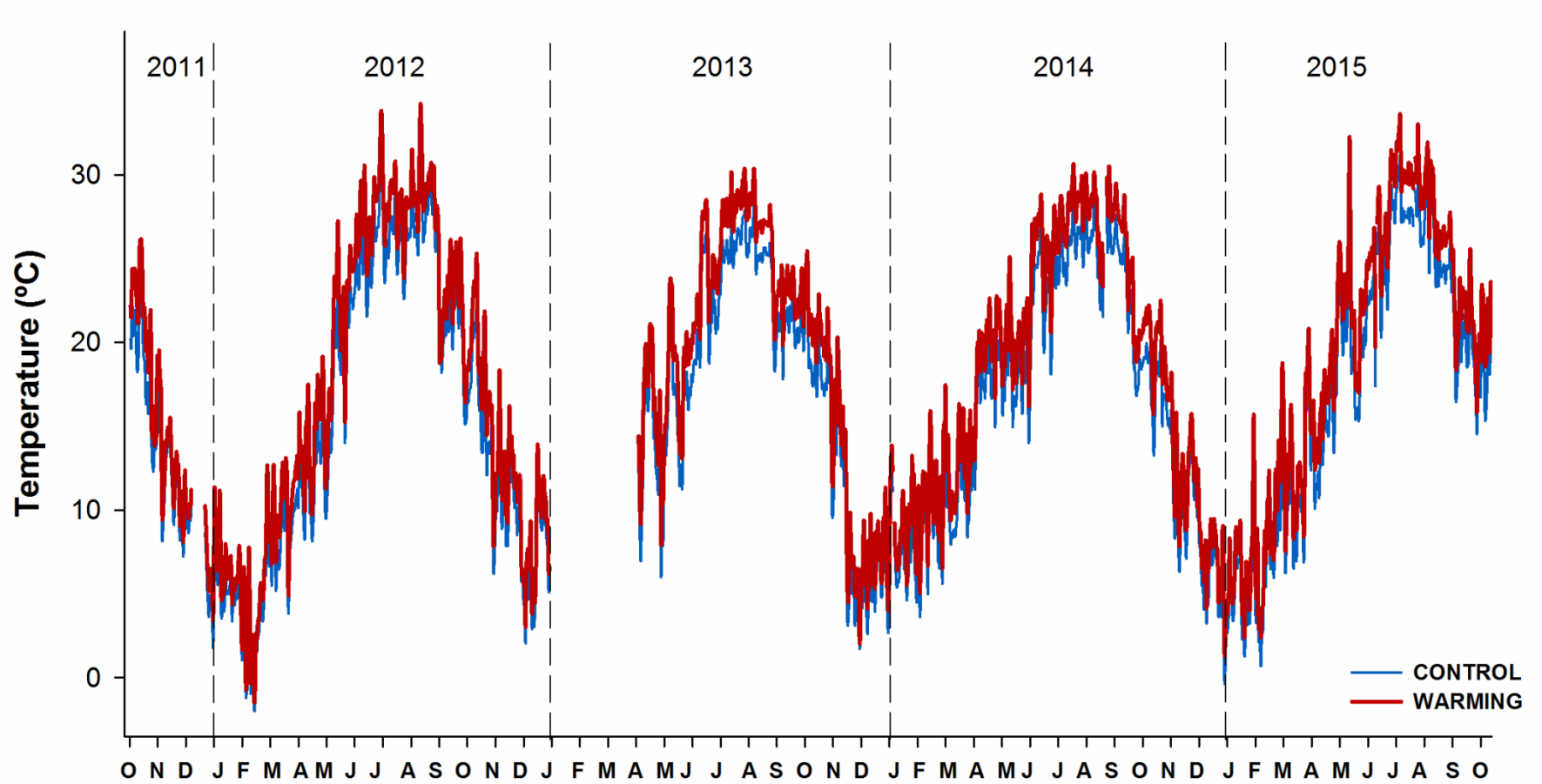
Chapter I

SUPPORTING INFORMATION

Figure S1. Detailed view of an open top chamber (OTC), including several *H. squamatum* shrubs. The bottom of the OTC is elevated ~3 cm above the ground level to allow ventilation and avoid overheating.
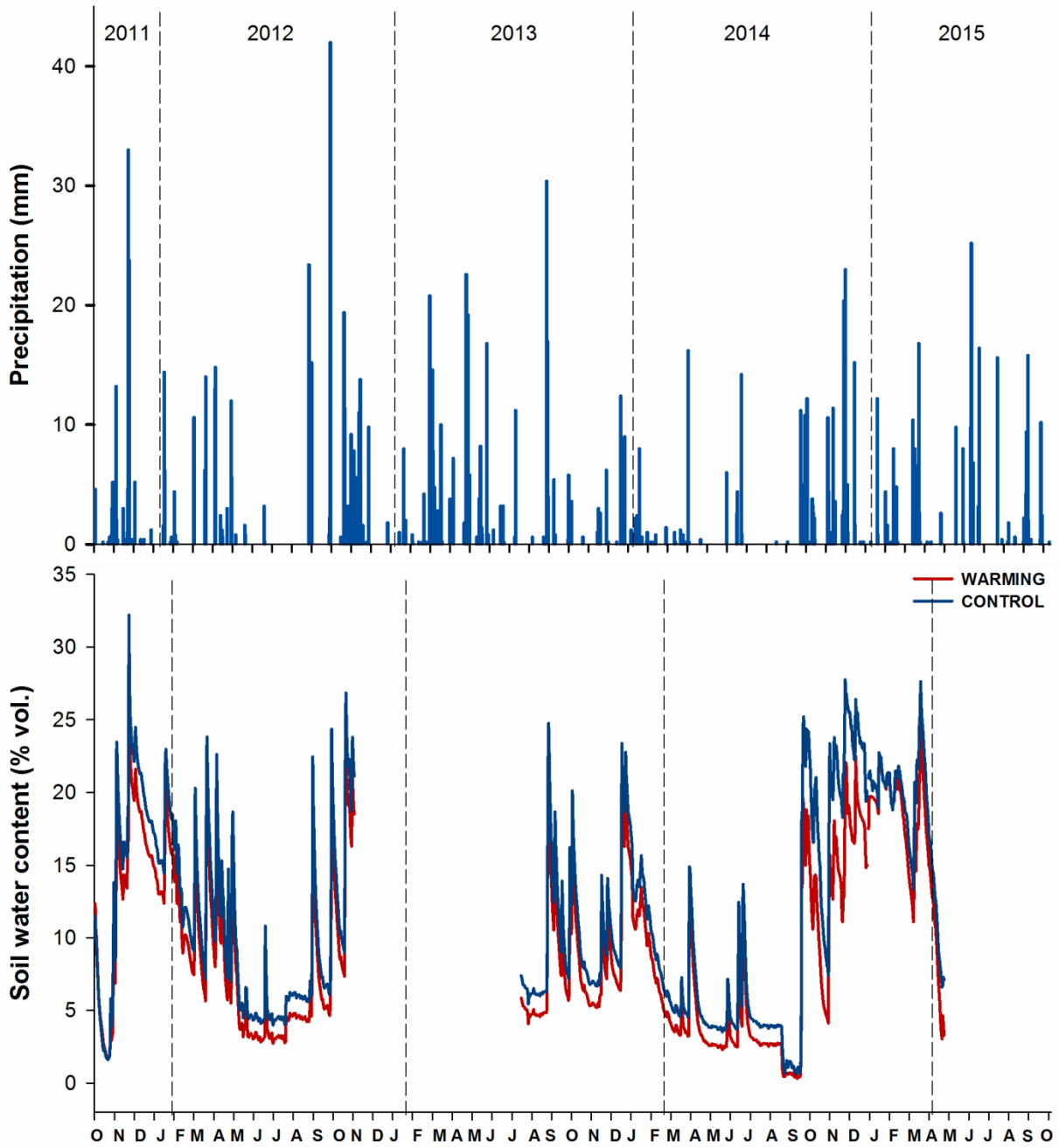
Figure S2. Air temperature in the control and warming treatment throughout the experiment, from October 2011 until October 2015.

Figure S3. Rainfall distribution (blue bars) and soil water content (0-5 cm depth) measured by automated sensors on warmed and control plots throughout the 4-yr study period.

Figure S4. Maximum efficiency of photosystem II under light conditions (Fv':Fm') in control and warmed plants at 10 different measurement dates spanning three consecutive growing seasons (data not available for 2015). Data represent means ± SE (n = 10-15).
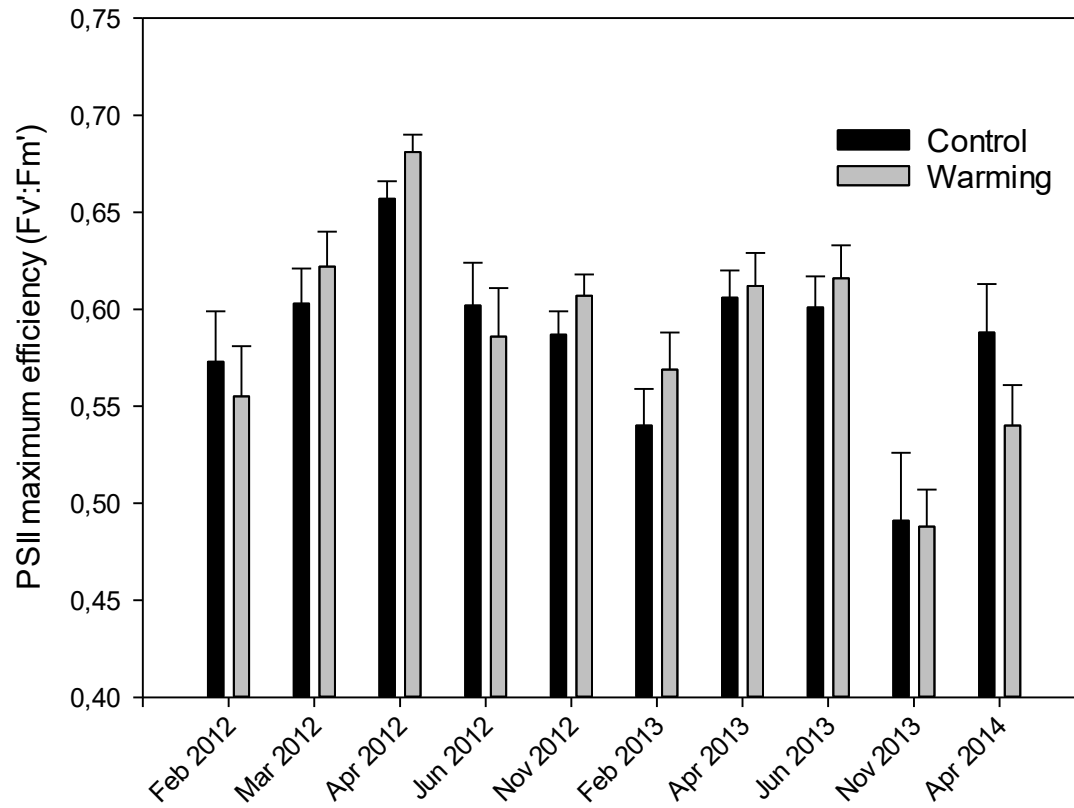
Chapter I

Table S1. Mean net photosynthetic rate (A), stomatal conductance (g_s) and intrinsic water use efficiency (WUE_i) at 11 different measurement dates spanning 4 growing seasons in warmed plants of OTCs installed in 2008 vs. warmed plants of OTCs installed in 2011. Mean values across measurements dates ± standard errors are shown (in each measurement date, N=7 for OTCs installed in 2011; N=5 for OTCs installed in 2008). The P values of the repeated measures ANOVA (using "year when OTCs were installed" as between-subject factor, and "study year" as within-subject factor) are also shown, indicating that neither leaf gas exchange parameter was significantly different between OTCs installed in 2008 and OTCs installed in 2011.

	A	g_s	WUE_i
	($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)	($mol\ H_2O\ m^{-2}\ s^{-1}$)	($\mu mol\ CO_2/\ mol\ H_2O$)
OTC (2008)	13.168 ± 0.796	0.285 ± 0.035	55.898 ± 3.700
OTC (2011)	13.933 ± 0.941	0.250 ± 0.041	59.576 ± 4.378
P	0.549	0.537	0.535

Chapter I

Table S2. Results of the repeated measures analysis of variance (RM-ANOVA) of net photosynthetic rate (A), stomatal conductance (g_s), transpiration rate (E), intrinsic water use efficiency (WUE_i), instantaneous water use efficiency (WUE_{Inst}), maximum efficiency of photosystem II under light (Fv':Fm'), and the actual photochemical efficiency of photosystem II ($\varnothing$PSII). In these analyses, Warming and Time were the between-subject and within-subject factors, respectively. P values below 0.05 are highlighted in bold. All leaf gas exchange parameters were measured on 11 dates spanning 4 consecutive growing seasons (except for Fv':Fm' and ØPSII, which were not measured in April 2015).

		A	g_s	E	WUE_i	WUE_{inst}	Fv':Fm'	ØPSII
Warming	F	75.207	0.740	0.164	91.820	59.529	0.028	4.072
	df	1,19	1,19	1,19	1,19	1,19	1.19	1,19
	P	**< 0.001**	0.400	0.690	**< 0.001**	**< 0.001**	0.870	0.058
Time	F	31.157	14.851	13.365	10.367	15.538	6.253	7.212
	df	10,190	10,190	10,190	10,190	10,190	9.171	9,171
	P	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
Warming*Time	F	2.026	0.619	0.611	6.569	3.891	1.361	1.048
	df	10,190	10,190	10,190	10,190	10,190	9.171	9,171
	P	**0.033**	0.797	0.524	**< 0.001**	**< 0.001**	0.210	0.404

Table S3. Results of the repeated measures analysis of variance (RM-ANOVA) of leaf N and P concentrations, leaf mass, area and mass per unit area (LMA, data not available for April 2012), leaf isotopic composition ($\delta^{13}C$, $\delta^{18}O$, measured in April 2012, 2013, 2014, 2015), and post-summer plant survival (measured in October 2012, 2013, 2014 and 2015). Rest of legend as in Table S1.

		Leaf N	Leaf P	Leaf mass	Leaf area	LMA	$\delta^{13}C$	$\delta^{18}O$	Plant survival
Warming	F	5.908	9.876	4.394	3.009	2.200	7.061	0.118	0.047
	df	1.21	1.23	1.31	1.31	1.32	1.18	1.18	1.17
	P	**0.024**	**0.005**	**0.044**	0.093	0.148	**0.016**	0.735	0.832
Time	F	66.511	19.211	14.834	63.970	100.311	37.368	23.813	10.719
	df	3.63	3.69	2.62	2.62	2.64	3.54	3.54	3.51
	P	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
Warming*Time	F	2.475	1.186	0.099	0.629	2.320	1.317	2.890	0.107
	df	3.63	3.69	2.62	2.62	2.64	3.54	3.54	3.51
	P	0.070	0.322	0.906	0.537	0.107	0.278	**0.044**	0.956

CHAPTER II

MYCORRHIZAL MEDIATION OF PLANT RESPONSES TO WARMING AND RAINFALL REDUCTION IN A SEMIARID SHRUBLAND

"And once the storm is over, you won't remember how you made it through, how you managed to survive. You won't even be sure, whether the storm is really over. But one thing is certain. When you come out of the storm, you won't be the same person who walked in. That's what this storm's all about."

Haruki Murakami, *Kafka on the Shore*

Chapter II

Responses of *Helianthemum squamatum* and its associated mycorrhizal fungal community to warming and rainfall reduction in a semiarid shrubland

Authors: *Lupe León-Sánchez[1], Emilio Nicolás[1], Marta Goberna[2], Iván Prieto[1], Fernando T. Maestre[3], José Ignacio Querejeta[1].*

[1] Centro de Edafología y Biología Aplicada del Segura (CEBAS-CSIC), Murcia, Spain.

[2] Centro de Investigaciones sobre Desertificación (CIDE-CSIC, UVEG, GV), Moncada, Valencia, Spain

[3] Departamento de Biología y Geología, Área de Biodiversidad y Conservación, Escuela Superior de Ciencias Experimentales y Tecnología, Universidad Rey Juan Carlos, Móstoles, Spain

Chapter II

ABSTRACT

Warmer and drier conditions associated with climate change could reduce vegetation productivity and cover in Mediterranean-type drylands, thus increasing the risk of land degradation and desertification. We conducted a four-year manipulative study in a semiarid shrubland to assess the effects of experimental warming and rainfall reduction on the performance of native shrubs and their symbiotic mycorrhizal fungi. *Helianthemum squamatum* shrubs were exposed to a ~2.5°C temperature increase using open top chambers (Warming, W), a ~30% rainfall reduction using rainout shelters (Rainfall Reduction, RR), and their combination (W+RR). Warming reduced the net photosynthetic rates of shrubs by ~30%, despite concurrent increases in stomatal conductance and transpiration. The combination of decreased photosynthesis with increased stomatal conductance led to a drastic reduction (~50%) of water use efficiency under warming. W and W+RR plants had lower leaf N and P concentrations and leaf mass area than control plants, and experienced a 32-38% reduction, respectively, in shoot biomass production. RR plants showed moderate reductions (10-20%) in net photosynthesis, stomatal conductance and shoot biomass production relative to control plants. Warming reduced post-summer plant survival during a dry year, especially in the W+RR treatment (40% vs. 70% in the controls). Ectomycorrhizal fungal (EMF) OTU richness and relative abundance under shrubs were drastically reduced (40-60%) by the W, RR and W+RR treatments. We suggest that the interdependent responses of native plants and EMF to climate change may have led to large decreases in plant nutrient status, growth and drought survival through a detrimental feedback loop mediated by EMF, thus stressing the need to evaluate the coupled responses of plants and their microbial symbionts to global change. These results highlight the vulnerability of ectomycorrhizal plant and fungal species to forecasted climate warming and drying, which could send dryland EM ecosystems into a degradation pathway leading to large decreases in primary productivity.

Chapter II

INTRODUCTION

Current climatic models predict drastic changes in the climate of the Mediterranean region over the next decades as a consequence of anthropogenic greenhouse gas emissions (Collins *et al.,* 2013). Forecasted changes include temperature increases of 2-5ºC, reduced amount and frequency of rainfall, increased vapor pressure deficit and potential evapotranspiration, and more frequent occurrence of extreme climatic events towards the second half of the 21st century (Meehl & Tebaldi 2004; Giorgi & Lionello, 2008; NOAA 2015). These projected changes will increase aridity conditions and reduce soil moisture availability in Mediterranean drylands (Dai 2013, Huang *et al.,* 2016), thus reducing plant primary productivity and vegetation cover (Delgado-Baquerizo *et al.,* 2013; Williams *et al.,* 2013; Ahlström *et al.,* 2015) and increasing the risk of soil erosion, land degradation and desertification (Le Houérou 1996; Safriel & Adeel 2005).

Most studies assessing climate change effects on Mediterranean-type vegetation have evaluated the effects of warming (Peñuelas 2004; Prieto *et al.,* 2009; León-Sánchez *et al.,* 2016) and rainfall reduction (Peñuelas *et al.,* 2001; Miranda *et al.,* 2011; Peñuelas *et al.,* 2013; Tielbörger *et al.,* 2014) in isolation from each other. Furthermore, few of them have investigated the response of Mediterranean semiarid ecosystems to predicted climate change, as most research conducted so far in this region has focused on systems with dry or sub-humid climate (e.g., Peñuelas *et al.,* 2004; Sardans *et al.,* 2008; Prieto *et al.,* 2009; but see Tielbörger *et al.,* 2014). Field studies have shown that warming modifies plant phenology by advancing the onset of the growing season during the winter-spring transition (Gordo & Sanz 2009). Warming enhances plant nutrient status, photosynthesis and growth in Mediterranean plant communities during winter and early spring, but has neutral or negative effects during late spring and summer (Llorens *et al.,* 2003; Prieto *et al.,* 2009). Rainfall reduction has predominantly negative effects on plant nutrition, photosynthesis, growing season length, and biomass accumulation in Mediterranean-type ecosystems (Sardans *et al.,* 2008; Prieto *et al.,* 2009; Wu *et al.,* 2011), although coexisting plant species may differ widely in their sensitivity to this factor (Llorens *et al.,* 2003).

Field studies have shown that the effects of warming and rainfall reduction on key ecosystem processes are often of opposing sign: for example, whereas warming

stimulates soil nutrient cycling and often enhances photosynthesis and plant growth, rainfall reduction usually induces opposite effects (e.g. Rustad *et al.,* 2001; Lin *et al.,* 2010; Wu *et al.,* 2011). The net effects of simultaneous warming and rainfall reduction will therefore depend on complex temporal interactions between them, as well as on which factor exerts the dominant control on each ecosystem process at different times of the year (Zavaleta *et al.,* 2003; Sardans & Peñuelas 2013). Moreover, native plant species from semiarid ecosystems have evolved a wide array of adaptive phenotypic plasticity mechanisms to cope with heat and drought stress, which could buffer any negative impacts of climate warming and drying on plant performance (Nicotra *et al.,* 2010; Bussotti *et al.,* 2014; Nardini *et al.,* 2014).

Rhizosphere microbial interactions and feedbacks are an often overlooked crucial aspect of ecosystem functioning that could aggravate the vulnerability of dryland plant communities to forecasted climate warming and drying, thereby increasing the risk of accelerated land degradation and desertification. Symbiotic mycorrhizal fungi are key drivers of terrestrial ecosystem processes that mediate plant nutrient uptake, carbon dynamics and vegetation responses to environmental change (Allen *et al.,* 2003; Leake *et al.,* 2004; van der Heijden *et al.,* 2008; Vargas *et al.,* 2010). However, relatively few studies have examined in detail the coupled responses of dryland plants and their associated mycorrhizal fungi to climate manipulation (Compant *et al.,* 2010; Mohan *et al.,* 2014), so further experimental research in this field is needed. There is potential for multiple mycorrhizal-mediated feedbacks on the primary productivity of dryland ecosystems if both plants and their mycorrhizal symbionts are negatively affected by warming and rainfall reduction (Allen *et al.,* 2003; Barea *et al.,* 2011), which could amplify the detrimental impacts of climate change on the functioning of drylands and the provision of key services by these ecosystems.

Woodland, shrubland and scrubland communities composed of ectomycorrhizal (EM) sclerophyllous woody plants in the genera *Cistus, Halimium, Helianthemum, (*Cistaceae*), Arbutus (*Ericaceae*), Quercus* (Fagaceae) and *Pinus* (Pinaceae), among others, are widespread throughout the Mediterranean Region and other dryland areas (Thanos *et al.,* 1992; Brundrett 2009). Previous studies suggest that climate warming and drying could lead to drastic changes in the composition of mycorrhizal fungal communities in EM dryland ecosystems (Querejeta *et al.,* 2009; Büntgen *et al.,* 2012; de

la Varga *et al.,* 2013). Most (but not all) ectomycorrhizal fungal (EMF) taxa show rather low tolerance to soil drying and/or warming, which can lead to large decreases in EMF root colonization, extraradical mycelial production and physiological activity with even moderate heat or drought stress (Cline *et al.,* 1987; Coleman *et al.,* 1989; Runion *et al.,* 1997; Jany *et al.,* 2003; Kennedy & Peay 2007). Several studies have shown that the abundance and diversity of EMF communities are strongly negatively affected by heat and drought stress in Mediterranean-type ecosystems (Querejeta *et al.,* 2009; de la Varga *et al.,* 2013; Salerni *et al.,* 2014; Ágreda *et al.,* 2015), and elsewhere (Swaty *et al.,* 1998; Gehring *et al.,* 2006; Valdés *et al.,* 2006). In contrast, arbuscular mycorrhizal fungi (AMF) are the dominant mycorrhizal type in drylands worldwide (Allen *et al.,* 1995) and are thus better adapted and more resilient to heat and drought stress than EMF (Compant *et al.,* 2010; Mohan *et al.,* 2014).

Semiarid plant communities growing on gypsum soils are rich in rare and endemic plant and mycorrhizal fungal species of high conservation value (Alguacil *et al.,* 2009; Escudero *et al.,* 2015, and references therein). Thus, gypsum ecosystems are a top priority for conservation efforts in Europe and elsewhere. Gypsum soils are particularly stressful for plants and soil microbiota due to their high salt concentration, low fertility and poor physical properties (Escudero *et al.,* 2015). However, very little is known about the potential impacts of forecasted climate change on the diversity, composition and functioning of native plant and soil microbial communities from gypsum habitats. To advance in this direction, we carried out a four year manipulative field experiment in a semiarid shrubland in Central Spain dominated by the gypsophilous shrub *Helianthemum squamatum* (L.) Dum. *Helianthemum squamatum* is a member of the Cistaceae family that is capable of forming symbiotic mycorrhizal associations with both EMF and AMF simultaneously (Gutiérrez *et al.,* 2003; Alguacil *et al.,* 2009; Brundrett 2009), making this species a suitable model system to evaluate the impacts of climate warming and drying on a rich and complex mycorrhizal fungal community. We simulated the climate conditions projected for the second half of the 21st century (Collins *et al.,* 2013) by using open top chambers (~2.5°C temperature increase), rainout shelters (~30% rainfall reduction), and their combination, and assessed the effects of climate manipulation on plant gas exchange, nutrient status, growth and survival, as well as on the composition and relative abundance of mycorrhizal fungal communities.

Chapter II

The overarching hypothesis of this study is that the deeply interdependent physiological responses of plants and their symbiotic rhizosphere microorganisms to increased climatic stress will have multiplicative effects on nutrient cycling and primary productivity through strong plant-soil feedback mechanisms (Ehrenfeld *et al.,* 2005). We test two competing hypotheses: 1) The diversity and relative abundance of mycorrhizal fungi remain unchanged (or increase) under simulated climate change conditions, which would be expected to buffer any negative impacts of warming and/or rainfall reduction on plant nutrient status and photosynthesis; or 2) Alternatively, climate change conditions reduce the diversity and relative abundance of mycorrhizal fungi, which would be expected to amplify any negative impacts of warming and/or rainfall reduction on plant nutrient status, photosynthesis and overall performance. Hypothesis 1 implies that mycorrhizal fungal communities are tolerant to increased heat and drought stress, and also that host plants are capable of maintaining or increasing carbon allocation to mycorrhizae under stressful climatic conditions (if reciprocal rewards stabilize or enhance cooperation between the symbionts under climatic stress; Kiers *et al.,* 2011). In contrast, hypothesis 2 implies that mycorrhizal fungal communities are vulnerable to increased heat and drought stress in soil, and/or that carbon allocation to mycorrhizae decreases significantly in climatically-stressed host plants, with negative consequences for the nutrient uptake capacity of mycorrhizal fungi and for plant nutrient status. We predict that warming, rainfall reduction and their combination will impair the performance of both plants and mycorrhizal fungi due to the adverse effects of increased heat and drought stress on plant and fungal physiology. Moreover, we predict that warming, rainfall reduction and their combination will thereby lead to multiple detrimental feedbacks on the nutrient status, productivity and drought survival of dryland vegetation, through decreases in carbon and nutrient fluxes and exchange between climatically stressed plants and mycorrhizal fungi.

MATERIALS AND METHODS

Study site and experimental design

The study was carried out near Aranjuez, in central Spain (40°02′N–3°32′W, 495 m altitude). Soils are derived from gypsum, have pH values ca. 7 (Table S1), and are

classified as Gypsiric Leptosols (IUSS Working Group WRB). Vegetation is a native grassland/shrubland community dominated by the tussock grass *Stipa tenacissima* L. and gypsophilous shrubs such as *H. squamatum* and *Gypsophila sp.* Perennial vegetation cover is less than 40%. The study area has a continental Mediterranean climate, with a mean annual temperature of 15°C and average rainfall of 349 mm.

To evaluate the effects of projected climate change conditions on the performance of native plants and mycorrhizal fungi, we conducted a randomized factorial experiment with two factors: warming (W) and rainfall reduction (RR). We established the replicated field experiment in February 2011 to examine the effects of three different climate manipulation treatments (W, RR, W+RR) on pre-existing *H. squamatum* shrubs and their associated mycorrhizal fungi. The experiment includes 10 plots per each climate manipulation treatment plus 30 control plots, making a total of 60 experimental plots. The size of each experimental plot is ~1 m^2. The target shrub *H. squamatum* was the overwhelmingly dominant (often the only) plant species present in the experimental plots across treatments. Each experimental plot contained 1-4 adult individuals in all the treatments.

The warming treatment simulates the predictions derived from six atmosphere general circulation models for the second half of the twenty-first century in the Western Mediterranean region (Collins et al., 2013), and was achieved by installing ventilated open-top chambers (OTCs) which increase mean air and soil temperature through a greenhouse effect (Fig. S1). OTCs are hexagonal chambers with sloping slides of 40 cm x 50 cm x 32 cm. They are made of transparent methacrylate that transmits about 92% of visible light, has a reflection of incoming radiation of 4%, and passes on ca. 85% of incoming energy (information provided by the manufacturer; Decorplax S. L., Humanes, Spain). These OTCs have been used in previous field warming experiments (Maestre *et al.,* 2013, 2015). Upon installation in the field, the OTCs were suspended ~3 cm above the ground level by a metal frame to allow free air circulation and exchange with the surrounding environment, which minimizes undesirable experimental effects, such as reduced wind and unnatural gas and humidity concentrations (Hollister & Webber 2000). The mean air and soil temperature increases achieved within the OTCs range between 1-2° C during wintertime to 4-7° C during summertime, which is in good agreement with climate change projections for Spain which predict greater temperature increases during summertime than during wintertime across the Mediterranean region (Collins *et al.,* 2013).

Chapter II

To simulate projected reductions in precipitation (Collins *et al.,* 2013), we used passive rainout shelters that intercept and exclude ~30% of the incoming rainfall from the plots. The permanent (non-moveable) rain exclusion shelters are made of transparent methacrylate troughs (made of the same material as the OTCs) that cover ~30% of the area of the experimental plots. Rainfall reduction is achieved by suspending the methacrylate troughs over an aluminum frame above the experimental plots (height 130 cm, width 100 x 100 cm, see Fig. S1). Intercepted rainwater is diverted through pipes and collected in storage tanks placed next to the experimental plots, and is thereafter removed after each rainfall event. Finally, the combined W+RR treatment is achieved by installing both OTCs and rainfall exclusion shelters over the same experimental plot (Fig. S1).

The experimental plots were instrumented with continuous temperature, relative humidity and soil moisture recording systems. Air temperature and relative humidity and soil moisture and temperature were continuously monitored using replicated automated sensors (HOBO® U23 Pro v2 Temp/RH and TMC20-HD sensors, Onset Corp., Pocasset, MA, USA, and EC-5 soil moisture sensors, Decagon Devices Inc., Pullman, WA, USA, respectively).

Plant measurements

Net photosynthetic rate (A), stomatal conductance (g_s), transpiration rate (E), maximum efficiency of photosystem II under light conditions (Fv'/Fm') and the quantum efficiency of photosystem II ($\varnothing$PSII, which provides a measure of the proportion of light absorbed by chlorophyll associated with photosystem II that is used for photosynthesis (Baker & Rosenqvist 2004) were measured in 2012 (February, May, June and July), 2013 (February, April, June and October), 2014 (May) and 2015 (May) with a LI-6400-40 Leaf Chamber Fluorometer and a LICOR 6400-01 CO_2 injector. Leaf gas exchange was measured on fully expanded leaves that were placed in a 2 cm^2 leaf cuvette. During these measurements, air CO_2 concentration was controlled using the injection system and compressed CO_2-cylinders with a CO_2 concentration of 390 µmol mol^{-1} CO_2. Measurements were conducted at a saturating light of 1.500 µmol m^{-2} s^{-1}, and at ambient air temperature and relative humidity. The air flow was set to 250 µmol s^{-1}. All leaf-gas exchange measurements were conducted between 8:00 and 11:00 am (GMT), when maximum photosynthetic rates were found at each survey. For warmed

plants, all leaf gas exchange measurements were conducted under the prevailing microclimatic conditions within the OTCs (i.e., elevated temperature and VPD relative to ambient). All the leaves used for gas exchange measurements were collected thereafter to measure their area using an image scanner program (Image Pro Plus, Media Cybernetics, Inc. Rockville, MD, USA). On each date, leaf gas exchange measurements (A, g_s, E, Fv'/Fm', $\varnothing$PSII) were conducted on 8-10 *H. squamatum* individuals per climate manipulation treatment. Intrinsic water use efficiency (WUE$_i$) was calculated as the ratio between net photosynthetic rate and stomatal conductance (A/g_s).

In each study year, fully sun-exposed adult leaves were collected in Spring (April-May) to determine their carbon isotope ratios ($\delta^{13}C$). Samples were oven dried at 60ºC and finely ground with a ball mill before being weighted and encapsulated into tin capsules for analyses $\delta^{13}C$ analysis. The $\delta^{13}C$ and C and N concentrations of leaf material were measured using elemental analyzer/continuous flow isotope ratio mass spectrometry (ANCA/SL elemental analyzer coupled with a Finnigan MAT Delta PlusXL IRMS). Isotope analyses were conducted at the Centre for Stable Isotope Biogeochemistry, University of California, Berkeley (USA). Leaf $\delta^{13}C$ values are expressed in delta notation (‰) relative to the reference standard V-PDB. Long-term external precision for $\delta^{13}C$ analyses is 0.14‰. Foliar P concentration was measured by inductively coupled plasma optical emission spectrometry (ICP-OES, Thermo Elemental Iris Intrepid II XDL, Franklin, MA, USA) after a microwave-assisted digestion with HNO_3:H_2O_2 (4:1, v:v) in the ionomics facility at CEBAS-CSIC.

In April 2013, 2014 and 2015, four leaves per target shrub were collected to measure their area (cm^2) and dry mass (g) to calculate their dry mass per unit leaf area (LMA, cm^2 g^{-1})**.** A picture of the fresh leaves was taken using a Canon 3000D (Canon Inc, Tokyo, Japan) and the resulting image was processed using Image Pro Plus software to calculate the total leaf area. Leaves were thereafter oven dried at 60ªC for at least 24h to determine their dry weight. LMA was calculated as the ratio between leaf dry weight and leaf area (Poorter *et al.,* 2009). In late winter 2012 and 2013, 3-4 terminal shoots of each target shrub were labeled with red tape to measure their elongations during the growing season. We measured shoot elongation in mid-Spring (April) and again at the end of the growing season (June). The late/early Spring growth ratio was then calculated as the quotient between the shoot elongations recorded during the latter and earlier parts of the Spring growing season (May-June and March-April, respectively). This ratio

provides an indication of changes in shoot growth phenology with the different climate manipulation treatments, relative to the controls.

At the end of the 4 year study period (October 2015), one representative terminal shoot of approx. 10 cm length per target *H. squamatum* shrub was destructively sampled to evaluate the effects of the climate manipulation treatments on shoot dry biomass production. We measured the total dry mass (48 hours at 60ºC), number of leaves and total leaf area of these shoots, as described above, and standardized the values per 10 cm shoot length. Finally, we measured post-summer plant survival rates after the first Autumn rainfalls in each study year (October 2012, 2013, 2014, 2015). Plant survival rate was estimated as the percentage of *H. squamatum* individuals present in Spring in each experimental plot that were still alive at the end of the Summer drought period.

Characterization of the soil mycorrhizal fungal community

Our target shrub *H. squamatum* is the only known ectomycorrhizal host present in the experimental plots and in the local plant community at large, as all the other coexisting plant species in the community are thought to be strictly arbuscular mycorrhizal, according to current knowledge (Brundrett 2009). Sampling of roots to assess mycorrhizal root colonization and to characterize the composition of the mycorrhizal fungal community colonizing the roots was impractical and unfeasible in this long-term field experiment, as it would have implied destructive excavation and severe disruption of our experimental system (given that *H. squamatum* is a deep-rooted species), so we chose to focus instead on the mycorrhizal fungal community present in rhizosphere soil. Surface (0-5 cm) soil samples were collected on 30 June 2014 from the rhizosphere of *H. squamatum* individuals, by inserting a hand auger corer (5 cm diameter) into the soil under each target shrub (8 replicate plots per treatment). Soil samples were thereafter transported to the laboratory on ice, immediately sieved through a 2 mm mesh and stored at 4 ºC. Soil DNA was extracted from 1 g sieved soil subsamples using the UltraClean^{TM} DNA isolation kit (MO BIO Laboratories, CA, USA). Extracted DNA was checked in 1 % agarose gels run in 0.5 X TAE buffer (Tris-acetate-EDTA; 100 V, 15 min). To analyze the soil fungal communities we used a tag-encoded FLX-titanium amplicon pyrosequencing (TEFAP) approach. The internal transcribed spacer (ITS) region was PCR amplified by using the fungal universal primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4R (5'-

TCCTCCGCTTATTGATATGC-3') and a HotStarTaq Plus Master Mix Kit (Qiagen, CA, USA) under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. Amplicons from all samples were mixed in equal concentrations, purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA) and sequenced with Roche 454 FLX titanium instruments and reagents.

Barcodes and primers were trimmed, and a total 176,702 raw fungal sequences were deposited in EMBL with the study accession number PRJEB12618 (http://www.ebi.ac.uk/ena/data/view/PRJEB12618). Low-quality sequences (shorter than 200 bp, including ambiguous base calls, or with homopolymer runs exceeding 9 bp) were removed. Sequences were then de-noised and chimeras removed. Operational taxonomic units (OTUs) were defined after removal of singleton sequences by clustering at 3% sequence divergence. OTUs were taxonomically classified using BLASTn. Sequencing and basic bioinformatics was performed by MR DNA (Shallowater, TX, USA).

We obtained the total number of sequences corresponding to each fungal OTU and experimental plot, and calculated the relative abundance of each OTU in each plot based on the total number of sequences in the same plot. We selected those OTUs most closely related to known ectomycorrhizal and arbuscular mycorrhizal fungi (EMF and AMF, respectively), based on a literature review. Fungal OTUs in the Ascomycota and Basidiomycota were classified as putatively ectomycorrhizal (or non-mycorrhizal) according to the comprehensive review by Tedersoo *et al.* (2010) on the ectomycorrhizal lifestyle in global fungi. All fungal OTUs identified as members of the Glomeromycota were considered AMF (Schussler *et al.,* 2001). However, we acknowledge that the primers and classification methods employed may not capture all potential mycorrhizal fungal diversity in the system (e.g. Abarenkov *et al.,* 2010).

Statistical analyses

Repeated measures ANOVAs (RM-ANOVAs) were used to evaluate the effects of warming, rainfall reduction, time, and their interactions, on leaf gas exchange

parameters (A, gs, WUE_i, ØPSII, Fv'/Fm', E), nutrient concentrations (N, P), dry mass and area, LMA and carbon isotope ratios ($\delta^{13}C$), shoot elongation during late spring, late/early spring growth ratio, and survival rate. Temperature (warming vs. ambient) and rainfall (reduced vs. ambient) treatments were used as between-subject factors, whereas time was the within-subject factor. Linear regression analysis was used to examine the relationship between A and g_s in each treatment, and we then compared the intercepts and slopes of the fitted regression lines between all the climate manipulation treatments with an analysis of covariance (ANCOVA). Analyses of variance (ANOVA) were used to test the effects of the climate manipulation treatments on shoot biomass production per unit length and its components (leaf number and area, leaf biomass and stem biomass), followed by post-hoc tests. Treatment effects on the richness and relative abundance of EMF and AMF communities under *H. squamatum* shrubs were also tested using ANOVAs. We used SPSS 22.0 software (SPSS Inc., Chicago, IL, USA) to perform RM-ANOVA and ANOVA analyses, and StatgraphicsPlus 5.1 (Statgraphics Plus 5.1. for Windows, 2000) to carry out ANCOVA analyses.

RESULTS

Treatment effects on microclimatic variables

Throughout the study period, the warming treatment increased air temperature by ~2.5ºC (Fig. S2). Warming effects were maximized during Summer (June–September), when temperatures were increased by warming up to 7 °C on some days. The warming treatment also increased surface soil temperature by ~2.5ºC on average. Vapor pressure deficit (VPD) was also higher in plots exposed to warming (W and W+RR) than in those exposed to current ambient temperature (Control and RR) throughout the study (1311 vs 1042 Pa, respectively), and this difference was greatest during Spring (1488 vs 1138 Pa, respectively) and Summer (2924 vs 2330 Pa, respectively). Rainout shelters did not substantially alter air/soil temperature, as average differences between RR and control treatments were consistently below 0.4 °C throughout the study period (Fig. S2). Topsoil water content (0-5 cm depth) closely followed the rainfall events registered, and was reduced by both the OTCs and the rainout shelters (by 2-3% on average, Fig. S3), with largest reductions observed during the Spring growing season (3-5%). Therefore,

warming-induced soil drying and partial rainfall reduction led to similar reductions in mean soil moisture content throughout the experimental period.

Changes in plant gas exchange with climate change

Across the study period, warming reduced the mean net photosynthetic rate (A) of *H. squamatum* shrubs by 29% and 32.5% in the W and W+RR treatments, respectively (Fig. 1a; P<0.001, Table S1), with no evidence of acclimation to warming through time. Net photosynthetic rates were consistently lower in shrubs exposed to warming than in those exposed to current ambient temperature throughout the study, except during a cold period in winter when the opposite occurred (i.e. higher A under warming in February 2012; Fig. 1a; significant W x Time interaction in Table S1). Across dates, warming (W and W+RR) also reduced ØPSII by 8.6% on average, relative to the controls (Fig. 2b; P<0.001, Table S1). However, Fv':Fm' was not significantly affected by warming across dates (Fig. 2a; P=0.836, Table S1).

Stomatal conductance and transpiration rate were higher under warming than in the controls plots across dates (Figs. 1b and 2c; P<0.001, Table S1), with mean increases of 32-34% in the W and W+RR plants relative to the controls. The combination of decreased A with increased g_s under warming led to a drastic reduction in WUEi throughout the study (Fig. 1c; P<0.001, Table S1), which was on average 55.2% and 46.6% lower in the W and W+RR plants, respectively, than in the controls. The linear regression between mean A and g_s values across dates had significantly lower intercept and slope in W and W+RR plants than in Control or RR plants (Fig. S4; ANCOVA, P<0.001 and P=0.008, respectively). Differences in leaf gas exchange between warmed (W and W+RR) and control plants were greatest during the peak of the growing season (April-May), when W plants had on average 30.5% lower A, 13.5% lower ØPSII, 41.2% higher g_s, 34.8% higher E and 52% lower WUEi values than control plants across years. W+RR plants had on average 42% lower A, 17.2% lower ØPSII, 24% higher g_s, 28.3% higher E and 51% lower WUEi than control plants during the peak of the growing season.

Across dates and treatments, RR moderately decreased net photosynthetic rates (Fig 1a; P=0.042) and stomatal conductance (Fig. 1b; P=0.049), and enhanced intrinsic water use efficiency (Fig. 1c; P= 0.004), but had no effects on the ØPSII or Fv':Fm' of *H.*

squamatum (Fig. 2a, b; Table S1). On average, RR plants had 9.6% lower A, 12.1% lower g_s, 9.2% lower E and 4% higher WUE_i than control plants across dates. The detrimental effects of the RR treatment tended to increase during the peak of the growing season (April-May), when RR plants had on average 17.2% lower A, 19.3% lower g_s and 16% lower E than the controls across years.

Changes in leaf nutrient status, $\delta^{13}C$, LMA, shoot growth and post-summer survival under climate change

Plants subjected to W and W+RR treatments had lower leaf N and P concentrations than plants under current ambient temperatures across years (Tables 1, S2; P=0.004 and P=0.008, respectively). Rainfall reduction had no significant effects on leaf N and P status across treatments and years (Tables 1, S2). However, in the last growing season of the study (May 2015), leaf N and P concentrations were 11-25% lower in all the climate manipulation treatments (W, RR, W+RR) than in controls plants (Table 1). These decreases in leaf N and P concentrations translated into even larger decreases in total foliar N and P pools due to reduced foliage biomass production under warming and/or rainfall reduction (see data below).

Net photosynthetic rate correlated positively with both leaf N (r= 0.506, P= 0.001) and P (r= 0.380, P= 0.014) concentrations across treatments in the last growing season of the study. Intrinsic water use efficiency also correlated positively with both leaf N (r= 0.508, P= 0.001) and P (r= 0.407, P= 0.008) concentrations. Across years, peak A and g_s values during Spring correlated positively with leaf P concentrations in warmed plants (W and W+RR treatments; r^2= 0.320, P= 0.016 for A; r^2= 0.534, P< 0.001 for g_s), but these correlations were absent from plants under ambient temperatures.

Throughout the study, leaf $\delta^{13}C$ values of *H. squamatum* were consistently lower in the W and W+RR treatments than in the control and RR treatments (Fig. 3; Table S2, P<0.001). Warming (W and W+RR) reduced LMA across years (Tables 1, S2, P<0.001). This response was due to a 21% reduction in their mean leaf dry mass (P=0.007) with no significant changes in mean leaf area (Tables 1, S2). Mean shoot elongation during the latter half of Spring (May-June) was significantly reduced by warming (24% mean decrease; P=0.019; Tables 1, S2). As a result, both W and W+RR plants had lower ratios of late spring/early spring shoot elongation than plants exposed

to ambient temperatures (Table S2, P= 0.001), which indicates an advanced shoot growth phenology and an earlier cessation of the growing season under warming. Shoot growth phenology was not significantly affected by rainfall reduction (Table S2).

Fifty-four months after the setup of the experiment, dry biomass production per unit shoot length (foliage plus stems) was 33% and 38% lower in W and W+RR plants, respectively, than in the controls (Fig. 4; P= 0.002). Decreased shoot dry biomass production under warming was primarily due to large reductions in leaf biomass (P=0.001) without significant changes in stem biomass (Table 2). Plants in the W and W+RR treatments had on average 28% lower number of leaves (P=0.011) and 36% smaller leaf area per unit shoot length (P=0.002) than control plants (Table 2). Plants in the RR treatment had lower number of leaves per unit shoot length than control plants (16% decrease; P= 0.036; Table 2). The RR treatment caused a marginally significant reduction in total shoot dry biomass production relative to the controls (19.8% decrease; P=0.092 after post hoc LSD test; Fig. 4).

Across treatments, post-summer plant survival rate was lower in 2012 (60%) than in other years (89% in 2013; 90% in 2014; 85% in 2015), probably because 2012 was the driest year of the study (262 mm annual rainfall, vs. 326-458 mm in the other years). Across study years, mean post-summer survival was marginally lower in W and W+RR plants than in plants under current ambient temperature (Control and RR; P=0.069; Table S2). However, post-summer survival was drastically reduced by warming during the driest year of the study (2012), particularly for plants in the W+RR treatment (Fig 5; significant *W x Time* interaction in Table S2). In contrast, post-summer plant survival was largely unchanged by warming in wetter years (2013, 2014 and 2015). Post-summer plant survival rate was consistently similar in RR and control plants within and across years.

Changes in mycorrhizal OTU richness and relative abundance with climate change

Pyrosequencing analyses detected a total of 59 putatively ectomycorrhizal fungal OTUs under *H. squamatum* across treatments. The most frequent EMF OTUs were members of the Pezizales (Ascomycota), including *Picoa* sp. (21 OTUs), *Helvella* sp. (16 OTUs), *Geopora* sp. (5 OTUs) and *Tuber* sp. (3 OTUs). Other less frequent EMF were

identified as members of the Cantharellales (9 OTUs including *Ceratobasidium sp.* and *Tulasnella sp.*), Sebacinales (3 OTUs including *Sebacina* sp.), and Helotiales (2 OTUs).

Forty months after the setup of the experiment, mean EMF OTU richness per plot was much lower in all the climate manipulation treatments than in the control plots (P<0.05; ANOVA, LSD post-hoc test). Mean EMF OTU richness per soil core was 23.1±8.4 (mean± standard deviation) in the control plots, but decreased to 14.4±6.6 in the W treatment, 11.9±6.5 in the RR treatment, and 13.6±6.4 in the W+RR treatment. The relative abundance of EMF OTUs was reduced by more than 60% in all the climate manipulation treatments compared to the control plots (Fig 6). This dramatic reduction was largely driven by sharp declines (> 65%) in the relative abundance of Pezizales, including the dominant OTU *Picoa lefebvrei*, as well as *Geopora* sp., *Helvella* sp. and *Tuber* sp. The relative abundance of other (rarer) EMF taxa (e.g. *Sebacina, Ceratobasidium*) was not significantly affected by the treatments.

Pyrosequencing analyses detected a total of 22 arbuscular mycorrhizal fungal OTUs under *H. squamatum*. The most frequent AMF were *Glomus* sp. (15 OTUs), *Entrophospora* sp. (2 OTUs) and *Rhizophagus* sp. (2 OTUs). The richness of AMF OTUs per soil sample was rather low across treatments (ranging from 0 to 6; mean ± standard deviation =1.63±1.66) and was not significantly affected by the treatments (P> 0.05). The mean relative abundance of AMF OTUs within the soil fungal community was below 1% in all the treatments (0.12±0.09% in the control treatment; 0.32±0.42% in the RR plots; 0.52±0.67% in the W plots; 0.34±0.39% in the W+RR plots), and was marginally higher in the W treatment than in the control treatment (P=0.089; ANOVA, LSD post-hoc test).

DISCUSSION

Large reductions in plant photosynthesis and water use efficiency with climate change.

Warming reduced the net photosynthetic rate of *H. squamatum* shrubs by nearly one-third on average throughout the study, despite concurrent increases in stomatal conductance and transpiration. The largest proportional reductions in A with warming were observed during the Spring growing season (37% on average across years), which

is a period of mild temperatures that are considered optimal for photosynthetic activity (Galmés *et al.,* 2013). Moreover, there was no indication of progressive photosynthetic acclimation to warming throughout the 4-year study period (Gunderson *et al.,* 2010). Warming reduced the carbon assimilation rate of *H. squamatum* shrubs at any given stomatal aperture, thus revealing the existence of strong non-stomatal limitations on photosynthesis under warming (nutritional, biochemical, metabolic and/or diffusional; Flexas & Medrano 2002).

Plant Fv'/Fm' was unaffected by warming (with the only exception of a very hot summer period in 2012), suggesting that the photosynthetic machinery of photosystem II was not severely damaged by the warming treatment (Baker & Rosenqvist 2007). However, ØPSII was consistently reduced by warming throughout the study. Similar to reductions in A, the warming-induced reductions in ØPSII were largest at the peak of the Spring growing season, which, together with unchanged Fv'/Fm', suggests photosystem II down-regulation to match reductions in the rates of downstream photosynthetic reactions (Baker & Rosenqvist 2004; Sage & Kubien 2007).

In the last growing season of the study (May 2015), net photosynthetic rates correlated strongly with leaf N and P concentrations across treatments, indicating a key role of nutrient limitation in reducing photosynthesis under climate change. The simultaneous decreases of leaf N and P in W and W+RR plants likely caused interactive and mutually reinforcing detrimental effects on their photosynthetic biochemistry, given the key importance of N and P for Rubisco carboxylation capacity and for ribulose-1.5-bisphosphate regeneration, respectively (Reich *et al.,* 2009*)*. Leaf photosynthetic capacity correlates tightly with leaf N concentration because of the crucial role that N-rich enzymes, particularly Rubisco, play in the biochemical fixation of CO_2 (Wright *et al.,* 2004). Phosphorus limitation further decreases net photosynthetic rates through reductions in ribulose 1,5 bisphosphate regeneration, photochemical efficiency of photosystem II and mesophyll conductance to CO_2 (Jacob & Lawlor 1991, 1992). In addition to decreased leaf N and P status, other non-stomatal limitations may have further contributed to reduce the net photosynthetic rates of *H. squamatum* under warming. Decreasing net photosynthesis with moderate warming has been related to several biochemical and/or metabolic mechanisms such as, among others, a) enhanced photorespiration (von Caemmerer & Quick 2000); b) inhibition of ribulose-1.5-

bisphosphate regeneration due to a reduced whole-chain electron transport capacity (Sage & Kubien 2007); c) down-regulation of Rubisco activation state caused by the high thermal lability of the enzyme Rubisco activase (Feller *et al.,* 1998; Crafts-Brandner & Salvucci 2000); d) decreases in leaf soluble proteins and Rubisco concentration (Galmés *et al.,* 2013); and/or e) enhanced mitochondrial respiration rates (Atkin & Tjoelker 2003; Lin *et al.,* 2012). However, disentangling the contributions of these various non-stomatal mechanisms to the observed photosynthesis decrease under moderate warming (Sharkey 2005) is beyond the scope of this study.

Stomatal conductance and transpiration rates were enhanced by warming, especially during rainy periods when soil moisture was plentiful (e.g. April and October 2013, May 2015), as previously reported in other studies (White *et al.,* 1999; Misson *et al.,* 2004). Increased g_s and E with warming may prevent or minimize leaf overheating and heat-induced damage of the photosynthetic machinery through increased evaporative leaf cooling (Crawford *et al.,* 2012). Increased g_s and E may also represent an adaptive mechanism for enhancing transpiration driven mass flow of dissolved mineral nutrients to roots in the nutrient-deprived W and W+RR plants (Cramer *et al.,* 2009; Matimati *et al.,* 2014). Warming favors the release of crystallization water contained in gypsum rocks and soil (which can account for up to 21% of gypsum's weight; Palacio *et al.,* 2014), thus rendering it available for plant water uptake, which may have further contributed to increased g_s and E in W and W+RR plants during Spring and Summer. Higher g_s and E rates in warmed plants likely accelerated soil moisture depletion, thereby shortening the growing season through an earlier onset of drought stress, as indicated by reduced shoot elongation during the latter half of Spring in W and W+RR plants.

Intrinsic water use efficiency was halved by warming as a result of the large decreases in A and increases in g_s found in W and W+RR plants. In agreement with leaf gas exchange data, W and W+RR plants had consistently lower leaf $\delta^{13}C$ values than plants under ambient temperature (Control and RR), thus confirming that warming decreased time-integrated WUEi throughout the study (Cernusak *et al.,* 2013). A plummeting WUEi under warming may represent a detrimental feedback whereby plants need to transpire more water to assimilate less carbon, which appears a maladaptive response in

the long term to cope with ongoing climate warming and aridification across the Mediterranean region (Collins *et al.,* 2013).

Large reductions in mean leaf dry mass and biomass production per unit shoot length in the W and W+RR treatments agree with recent findings from another ectomycorrhizal semiarid ecosystem in the US Southwest (Adams *et al.,* 2015). Despite the large decreases in A, WUEi, ØPSII, nutrient status and growth observed in W and W+RR plants, they achieved similarly high post-summer survival rates as control plants in years with above- or near-average rainfall. Several phenotypic plasticity mechanisms many have helped ensure plant survival under warming, such as advancing shoot growth phenology to escape late season drought and heat stress (Badeck *et al.,* 2004; Menzel *et al.,* 2006; Parmesan 2007), decreasing the carbon cost of growth by building fewer leaves with lower dry mass and LMA (Poorter *et al.,* 2009), or increasing g_s and E to reduce leaf overheating (Crawford *et al.,* 2012). However, the buffering capacity of these presumably adaptive responses was clearly overwhelmed during a dry year (2012), as indicated by large increases in summer mortality in W and, especially, W+RR shrubs. Hydraulic impairment due to drought-induced xylem embolism (likely favored by reduced soil moisture under warming), carbon starvation (due to chronic photosynthesis reduction with warming) and their mutually exacerbating effects could explain the drastic increase in summer mortality observed in W and W+RR shrubs during a dry year (Sala *et al.,* 2010). Given that the refilling and repair of embolized xylem conduits consumes carbohydrates (Zwieniecki & Holbrook 2009), the combination of chronic photosynthesis reduction under warming and periodic episodes of severe drought stress may lead to a feedback mechanism whereby the proportion of embolized vascular elements increases and plant hydraulic conductivity decreases, eventually leading to sharp increases in summer mortality during dry years (McDowell *et al.,* 2011; Williams *et al.,* 2013).

In contrast to the strong detrimental effects of warming, rainfall reduction by itself had moderate effects on the performance of *H. squamatum*. Shubs in the RR treatment experienced modest but significant reductions in stomatal conductance and net photosynthetic rates, as well as reductions in leaf number, dry mass and shoot biomass production (albeit some of these effects were only marginally significant). Interestingly, RR plants consistently achieved similar post-summer survival rates as the controls

throughout the study, which adds to the mounting evidence that Mediterranean semiarid shrubs are rather tolerant and resistant to rainfall reduction because they are well adapted to high precipitation variability (intra- and interannual) and chronic drought stress (Miranda *et al.,* 2011; Tielborger *et al.,* 2014). In addition to an enhanced WUE_i (which allows smaller decreases in A than in g_s during progressive drought), other adaptive mechanisms may have contributed to the remarkable resilience of *H. squamatum* against rainfall reduction, such as decreasing leaf number per unit shoot length to reduce whole canopy transpiration (Limousin *et al.,* 2009), increasing rooting depth or volume, and/or increasing root/shoot biomass ratios (Nardini *et al.,* 2014). No significant interactions between warming and rainfall reduction were found, suggesting that the combined detrimental effects of these climate change drivers are more additive than multiplicative.

Drastic reduction in ectomycorrhizal fungal richness and relative abundance under climate change

Warming, rainfall reduction and their combination led to sharp reductions in the diversity and relative abundance of EMF communities in the rhizosphere of *H. squamatum*. The reduced photosynthetic rate of shrubs subjected to warming and/or rainfall reduction likely led to decreasing carbon allocation to their mycorrhizal fungal partners, with potential feedback consequences for plant nutrient status due to the key role of EMF in plant N and P uptake (Leake *et al.,* 2004; Smith & Read 2008). Decreased EMF OTU richness per plot under warming and/or rainfall reduction may have reduced the multiple benefits to the host plants derived from functional complementarity among coexisting EMF (Jones *et al.,* 2010). Lower diversity of EMF hyphal exploration types (Agerer 2001) and less diverse EMF enzymatic capabilities (Courty *et al.,* 2005) under climate change conditions may have led to less efficient EMF scavenging for soil nutrients across space and time and may have reduced the array of organic and inorganic nutrient sources available to plants. Moreover, reduced relative abundance of EMF mycelia within the soil fungal community under climate change conditions suggests a shift in the competitive balance and partitioning of nutrients between the saprotrophic and ectomycorrhizal functional guilds (and their host plants), to the detriment of the latter as indicated by decreased leaf N and P concentrations and pool sizes in EMF host plants (Fernandez & Kennedy 2016).

Chapter II

Our findings are in agreement with the results of other studies reporting declines in EMF abundance and diversity under heat and drought stress in dryland ecosystems (Büntgen *et al.,* 2012; de la Varga *et al.,* 2013; Ágreda *et al.,* 2015). Navarro-Ródenas *et al.* (2011) found that even drought-adapted EMF such as the desert truffle *Picoa lefebvrei* (by far the most abundant and frequent EMF in our semiarid ecosystem) exhibit large reductions in mycelial growth under moderate drought stress. In contrast to EMF, the OTU richness and relative abundance of AMF under *H. squamatum* shrubs was unaffected by warming and/or rainfall reduction, probably due to their better adaptation and tolerance to drought and heat stress (Allen *et al.,* 1995; Soudzilovskaia *et al.,* 2015a) and their lower carbon requirements and cost to the host plant, compared to EMF (Jones *et al.,* 1998; Leake *et al.,* 2004; Gehring *et al.,* 2006; Soudzilovskaia *et al.,* 2015b). However, an important caveat to this finding is that the primers that we used for pyrosequencing apparently did a relatively poor job at amplifying AMF sequences, given that less than 1% of the total fungal sequences detected in the rhizosphere soil of *H. squamatum* shrubs belonged to fungi in the Glomeromycota. Nevertheless, this study adds to the mounting evidence that ectomycorrhizal plants and fungi might be more vulnerable and less resilient than arbuscular mycorrhizal plants and fungi to the detrimental impacts of climate warming and aridification in drylands, and elsewhere (Mueller *et al.,* 2005; Querejeta *et al.,* 2009; Compant *et al.,* 2010; Mohan *et al.,* 2014).

Potential for mycorrhizal modulation of plant responses to climate change in dryland ecosystems: a conceptual model.

We found strong support for our second hypothesis, as climate change conditions drastically reduced the diversity and relative abundance of ectomycorrhizal fungi, which is expected to amplify the negative impacts of warming and/or rainfall reduction on plant nutrient status, photosynthesis, growth and survival. Based on the results of this study, we propose a tentative conceptual model in which a warmer and drier Mediterranean climate triggers an EMF-mediated detrimental feedback loop on the primary productivity of EM and mixed EM/AM semiarid shrublands and woodlands (Fig. 7). Supraoptimal temperatures for native plants during the growing season reduce net photosynthetic rates through multiple non-stomatal mechanisms, whereas evapotranspiration and soil moisture depletion are enhanced through warming-induced increases in VPD and rainfall reduction. At the same time, EMF richness and relative

abundance decrease sharply due to the direct detrimental effects of soil warming and drying, and also indirectly through reductions in belowground carbon allocation by their drought- and heat-stressed host plants, thus resulting in large decreases in EMF carbon-sink strength (Wright *et al.,* 2000) and nutrient-uptake capacity (Leake *et al.,* 2004). Eventually, decreased host plant N and P status combined with decreased carbon-sink stimulation of photosynthesis by EMF further reduce carbon assimilation rates, WUE and biomass production (Guehl & Garbaye 1990), thereby closing the detrimental feedback loop on plant productivity in EM dryland ecosystems.

Rising atmospheric carbon dioxide concentration over the coming decades would be expected to counteract the negative impacts of climate warming and drying on plant WUE and EMF performance, through elevated-CO_2 stimulation of A and inhibition of g_s and E (Eamus 1991; Drake *et al.,* 1997, 2011; Curtis & Wang 1998; Wullschleger *et al.,* 2002; Donohue *et al.,* 2013). However, whether CO_2-induced increases in WUE can compensate for the detrimental effects of warming and rainfall reduction on plant performance is largely unknown. A recent study found that increases in aridity registered in a US grassland over the last four decades reduced plant productivity, irrespective of the CO_2-induced increases in WUE observed during this period (Brookshire & Weaver 2015). Long-term research is thus warranted to evaluate the extent of the buffering capacity of the CO_2 fertilization effect to counterbalance the detrimental impacts of climate warming and drying on plants and EMF in dryland ecosystems.

In conclusion, our results highlight the vulnerability of native plants and their EMF partners to forecasted climate change in Mediterranean-type semiarid shrublands. The deeply interdependent responses of plants and EMF to warming and rainfall reduction appear to have mutually amplifying effects that strongly reduce plant nutrient status, photosynthesis, WUE, biomass production, and drought survival. The mutually exacerbating responses of plants and EMF to warming and rainfall reduction might thus lead to large declines in vegetation nutrient capture and pool size, with potentially cascading detrimental effects on ecosystem nutrient retention and cycling (van der Heijden *et al.,* 2010). Reduced leaf N and P contents may decrease plant nutritional quality for herbivores, and slow down litter decomposition and nutrient recycling under forecasted climate change. Moreover, reduced EMF diversity, relative abundance and

Chapter II

nutrient capture (and transfer to their host plants) might stimulate soil organic matter decomposition through an increased nutrient availability for competing soil fungal and bacterial decomposers (Fernandez & Kennedy 2016), which would accelerate land degradation and desertification in drylands. This study illustrates how the negative impacts of forecasted climate change on plant physiology and rhizosphere-microbial interactions could send drylands into a degradation trajectory in which carbon and nutrient flows through fungal-plant mycorrhizal associations spiral downward to a new alternative state of decreased vegetation productivity. Overall, our findings indicate that a deeper understanding of plant-soil feedbacks is critical to accurately predicting dryland vegetation responses to forecasted climate change.

References

Abarenkov K, Henrik Nilsson R, Larsson KH, Alexander IJ, Eberhardt U, Erland S. *et al.* (2010) The UNITE database for molecular identification of fungi–recent updates and future perspectives. *New Phytologist*, **186**,281–285.

Adams HD, Collins AD, Briggs SP, Vennetier M, Dickman LT, Sevanto SA, McDowell NG (2015) Experimental drought and heat can delay phenological development and reduce foliar and shoot growth in semiarid trees. *Global Change Biology*, **21**, 4210-4220.

Chapter II

Agerer R (2001) Exploration types of ectomycorrhizae. A proposal to classify ectomycorrhizal mycelial systems according to their patterns of differentiation and putative ecological importance. *Mycorrhiza*, **11**, 107–114.

Ágreda T, Águeda B, Olano JM., Vicente-Serrano SM, Fernández-Toirán M (2015) Increased evapotranspiration demand in a Mediterranean climate might cause a decline in fungal yields under global warming. *Global Change Biology*, **21**, 3499–3510.

Ahlström A, Raupach MR, Schurgers G *et al.* (2015) The dominant role of semi-arid ecosystems in the trend and variability of the land CO2 sink. *Science*, **348**, 895–899.

Alguacil MM, Roldán A, Torres MP (2009) Assessing the diversity of AM fungi in arid gypsophilous plant communities. *Environmental Microbiology*, **11**, 2649-2659.

Allen EB, Allen MF, Helm DJ, Trappe JM, Molina R, Rincon E (1995) Patterns and regulation of mycorrhizal plant and fungal diversity. *Plant and Soil,* **170,** 47 – 62.

Allen MF, Swenson W, Querejeta JI, Egerton-Warburton LM, Treseder KK (2003) Ecology of mycorrhizae: a conceptual framework for complex interactions among plants and fungi. *Annual Review of Phytopathology*, **41**, 271-303.

Atkin OK, Tjoelker MG (2003) Thermal acclimation and the dynamic response of plant respiration to temperature. *Trends in Plant Science*, **8**, 343-351.

Badeck FW, Bondeau A, Böttcher K, Doktor D, Lucht W, Schaber J, Sitch S (2004) Responses of spring phenology to climate change. *New Phytologist*, **162**, 295-309.

Baker NR, Rosenqvist E (2004) Applications of chlorophyll fluorescence can improve crop production strategies: an examination of future possibilities. *Journal of Experimental Botany*, **55**, 1607–1621.

Barea JM, Palenzuela J, Cornejo P *et al.* (2011) Ecological and functional roles of mycorrhizas in semi-arid ecosystems of Southeast Spain. *Journal of Arid Environments,* **75**, 1292–1301.

Chapter II

Brookshire ENJ, Weaver T (2015) Long-term decline in grassland productivity driven by increasing dryness. *Nature Communications* 6:7148

Brundrett MC (2009) Mycorrhizal associations and other means of nutrition of vascular plants: understanding global diversity of host plants by resolving conflicting information and developing reliable means of diagnosis. *Plant Soil*, **320**, 37–77.

Büntgen U, Egli S, Camarero JJ *et al.* (2012) Drought-induced decline in Mediterranean truffle harvest. *Nature Climate Change*, **2**, 827–829.

Bussotti F, Ferrini F, Pollastrini M, Fini A (2014) The challenge of Mediterranean sclerophyllous vegetation under climate change: From acclimation to adaptation. *Environmental and Experimental Botany*, **103**, 80-98.

von Caemmerer S, Quick WP (2000) Rubisco: physiology in vivo. In Photosynthesis: Physiology and Metabolism (eds Leegood RC, Sharkey TD & von Caemmerer S), pp. 85–113, Kluwer Academic, Dordrecht, The Netherlands.

Cernusak LA, Ubierna N, Winter K, Holtum JAM, Marshall JD, Farquhar GD (2013) Environmental and physiological determinants of carbon isotope discrimination in terrestrial plants. *New Phytologist*, **200**, 950–965.

Cline ML, France RC, Reid CPP (1987) Intraspecific and interspecific growth variation of ectomycorrhizal fungi at different temperatures. *Canadian Journal of Botany*, **65**, 869–875.

Coleman MD, Bledsoe CS, Lopushinsky W (1989) Pure culture response of ectomycorrhizal fungi to imposed water stress. *Canadian Journal of Botany*, **67**, 29–39.

Collins M, Knutti R, Arblaster J *et al.* (2013) Climate change 2013: the physical science basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. *Long-term Climate Change: Projections, Commitments and Irreversibility*, Cambridge University Press, Cambridge, UK, and New York.

Chapter II

Compant S, van der Heijden MGA, Sessitsch A (2010) Climate change effects on beneficial plant–microorganism interactions. *FEMS Microbiology Ecology*, **73**, 197–214.

Courty PE, Pritsch K, Schloter M, Hartmann A, Garbaye J (2005) Activity profiling of ectomycorrhiza communities in two forest soils using multiple enzymatic tests. *New Phytologist,* **167,** 309–319.

Crafts-Brandner SJ, Salvucci ME (2000) Rubisco activase constrains the photosynthetic potential of leaves at high temperature and CO_2. *Proceedings of the National Academy of Sciences of the USA*, **97**, 13430–13435.

Cramer MD, Hawkins HJ, Verboom GA (2009) The importance of nutritional regulation of plant water flux. *Oecologia*, **161**, 15–24.

Crawford AJ, McLachlan DH, Hetherington AM, Franklin KA (2012) High temperature exposure increases plant cooling capacity. *Current Biology*, **22**, 396–397.

Curtis PS, Wang XZ (1998) A meta-analysis of elevated CO_2 effects on woody plant mass, form, and physiology. *Oecologia*, **113**, 299–313.

Dai A (2013) Increasing drought under global warming in observations and models. *Nature Climate Change,* **3,** 52-58.

de la Varga H, Águeda B, Ágreda T, Martínez-Peña F, Parladé J, Pera J (2013) Seasonal dynamics of *Boletus edulis* and *Lactarius deliciosus* extraradical mycelium in pine forests of central Spain. *Mycorrhiza*, **23**, 391–402.

Delgado-Baquerizo M, Maestre FT, Gallardo A, Bowker MA, Wallenstein MD, Quero J.L *et al.* (2013) Decoupling of soil nutrient cycles as a function of aridity in global drylands. *Nature,* **502**, 672-676.

Donohue RJ, Roderick ML, McVicar TR, Farquhar GD (2013) Impact of CO_2 fertilization on maximum foliage cover across the globe's warm, arid environments. *Geophysical Research Letters*, **40**, 3031–3035.

Chapter II

Drake BG, Gonzàlez-Meler MA, Long SP (1997) More efficient plants: a consequence of rising atmospheric CO_2? *Annual Review of Plant Physiology and Plant Molecular Biology*, **48**, 609–639.

Drake JE, Gallet-Budynek A, Hofmockel KS *et al.* (2011) Increases in the flux of carbon belowground stimulate nitrogen uptake and sustain the long-term enhancement of forest productivity under elevated CO_2. *Ecology Letters*, **14**, 349–357.

Eamus D (1991) The interaction of rising CO_2 and temperatures with water use efficiency. *Plant, Cell & Environment*, **14**, 843–852.

Ehrenfeld JG, Ravit B, Elgersma H (2005) Feedback in the plant-soil system. *Annual Review of Environmental Resources*, **30**, 75–115.

Escudero A, Palacio S, Maestre FT, Luzuriaga AL (2015) Plant life on gypsum: a review of its multiple facets. *Biological Reviews*, **90**, 1-18.

Feller U, Crafts-Brandner J, Salvucci ME (1998) Moderately high temperatures inhibit ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) activase-mediated activation of Rubisco. *Plant Physiology*, **116**, 539–546.

Fernandez CW, Kennedy PG (2016) Revisiting the 'Gadgil effect': do interguild fungal interactions control carbon cycling in forest soils? *New Phytologist,* **209,** 1382–1394.

Flexas J, Medrano H (2002) Drought-inhibition of photosynthesis in C3 plants: stomatal and non-stomatal limitations revisited. *Annals of Botany*, **89**, 183-189.

Galmés J, Aranjuelo I, Medrano H, Flexas J (2013) Variation in Rubisco content and activity under variable climatic factors. *Photosynthesis Research*, **117**, 73-90.

Gehring CA, Mueller RC, Whitham TG (2006) Environmental and genetic effects on the formation of ectomycorrhizal and arbuscular mycorrhizal associations in cottonwoods. *Oecologia*, **149**, 158–164.

Giorgi F, Lionello P (2008) Climate change projections for the Mediterranean region. *Global and Planetary Change*, **63**, 90-104.

Chapter II

Gordo O, Sanz JJ (2009) Long-term temporal changes of plant phenology in the Western Mediterranean. *Global Change Biology*, **15**, 1930–1948.

Gunderson CA, O'Hara KH, Campion CM, Walker AV, Edwards NT (2010) Thermal plasticity of photosynthesis: the role of acclimation in forest responses to a warming climate. *Global Change Biology*, **16**, 2272-2286.

Guehl JM, Garbaye J (1990) The effects of ectomycorrhizal status on carbon dioxide assimilation capacity, water-use efficiency and response of transplanting in seedlings of *Pseudotsuga menziesii* (Mirb) Franco. *Annales des Sciences Forestières* **21**: 551–553.

Gutiérrez A, Morte A, Honrubia M (2003) Molecular identification of the mycorrhiza formed by *Helianthemum almeriense* Pau with *Terfezia claveryi Chatin* and *Picoa lefebvrei* (Pat.) Maire. *Mycorrhiza*, **13**, 299-307.

Habitats EU Directive (1992) Office for Official Publication of the European Union. CONSLEG: 1992LOO43-01.05.

van der Heijden MGA (2010) Mycorrhizal fungi reduce nutrient loss from model grassland ecosystems. *Ecology,* **91**, 1163–1171.

van Der Heijden, MGA, Bardgett RD, Van Straalen NM (2008) The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecology Letters*, **11**, 296–310.

Hollister RD, Webber PJ (2000) Biotic validation of small open-top chambers in a tundra ecosystem. *Global Change Biology*, **6**, 835–842.

Huang J, Yu H, Guan X, Wang G, Guo R (2016) Accelerated dryland expansion under climate change. *Nature Climate Change,* **6,** 166–71.

IUSS Working Group WRB (2006) World Reference Base for Soil Resources 2006. World Soil Resources Reports No. 103. FAO, Rome.

Chapter II

Jacob J, Lawlor DW (1991) Stomatal and mesophyll limitations of photosynthesis in phosphate deficient sunflower, maize and wheat plants. *Journal of Experimental Botany*, **42**, 1003–1011.

Jacob J, Lawlor DW (1992) Dependence of photosynthesis of sunflower and maize leaves on phosphate supply, ribulose-1,5-bisphosphate carboxylase/oxygenase activity, and ribulose-1,5-bisphosphate pool size. *Plant Physiology,* **98**, 801–807.

Jany JL, Martin F, Garbaye J (2003) Respiration activity of ectomycorrhizas from *Cenococcum geophilum* and *Lactarius sp* in relation to soil water potential in five beech forests**.** *Plant and Soil*, **255**, 487–494.

Jones MD, Durall DM, Tinker PB (1998) Comparison of arbuscular and ectomycorrhizal *Eucalyptus coccifera*: growth response, phosphorus uptake efficiency and external hyphal production. *New Phytologist*, **140**, 125–134.

Jones MD, Twieg BD, Ward V, Barker J, Durall DM, Simard SW (2010) Functional complementarity of Douglas-fir ectomycorrhizas for extracellular enzyme activity after wildfire or clearcut logging. *Functional Ecology,* **24,** 1139–1151.

Kennedy PG, Peay KG (2007) Different soil moisture conditions change the outcome of the ectomycorrhizal symbiosis between *Rhizopogon* species and *Pinus muricata*. *Plant and Soil*, **291**: 155–165.

Kiers ET, Duhamel M, Beesetty Y. *et al.* (2011) Reciprocal rewards stabilize cooperation in the mycorrhizal symbiosis. *Science,* **333:** 880-882.

Le Houérou HN (1996) Climate change, drought and desertification. *Journal of Arid Environments*, **34**, 133-185.

Leake JR, Johnson D, Donnelly DP, Muckle GE, Boddy L, Read DJ (2004) Networks of power and influence: the role of mycorrhizal mycelium in controlling plant communities and agroecosystem functioning. *Canadian Journal of Botany*, **82**, 1016–1045.

Chapter II

León-Sánchez L, Nicolás E, Nortes P, Maestre FT, Querejeta JI (2016). Photosynthesis and growth reduction with warming are driven by non-stomatal limitations in a Mediterranean semiarid shrub. *Ecology & Evolution*, **6**, 2725-2738.

Limousin JM, Rambal S, Ourcival JM, Rocheteau A, Joffre R, Rodríguez-Cortina R. (2009) Long-term transpiration change with rainfall decline in a Mediterranean *Quercus ilex* forest. *Global Change Biology,* **15**, 2163-2175.

Lin D, Xia J,Wan S (2010) Climate warming and biomass accumulation of terrestrial plants: a meta-analysis. *New Phytologist*, **188**, 187-198.

Lin Y-S, Medlyn BE, Ellsworth DS (2012) Temperature responses of leaf net photosynthesis: the role of component processes. *Tree Physiology*, **32**, 219–231.

Llorens L, Penuelas J, Estiarte M (2003). Ecophysiological responses of two Mediterranean shrubs, *Erica multiflora* and *Globularia alypum*, to experimentally drier and warmer conditions. *Physiologia plantarum*, **119**, 231-243.

Maestre FT, Escolar C, Guevara ML, Quero JL, Lázaro R, Delgado-Baquerizo M, Ochoa V, Berdugo M, Gozalo B, Gallardo, A (2013) Changes in biocrust cover drive carbon cycle responses to climate change in drylands. *Global change biology*, **19**, 3835-3847.

Maestre FT, Escolar C, R. Bardgett RD, Dungait JAD, Gozalo B, Ochoa V (2015) Warming reduces the cover and diversity of biocrust-forming mosses and lichens, and increases the physiological stress of soil microbial communities in a semi-arid *Pinus halepensis* plantation. *Frontiers in Microbiology* **6**: 865.

Matimati I, Verboom GA, Cramer MD (2014) Nitrogen regulation of transpiration controls mass-flow acquisition of nutrients. *Journal of Experimental Botany*, **65**, 159–168.

McDowell NG, Beerling DJ, Breshears DD, Fisher RA, Raffa KF, Stitt M (2011) The interdependence of mechanisms underlying climate-driven vegetation mortality. *Trends in Ecology & Evolution,* **26,** 523–532.

Meehl GA, Tebaldi C (2004) More intense, more frequent, and longer lasting heat waves in the 21st century. *Science*, **305**, 994-997.

Menzel A, Sparks TH, Estrella N, Koch E, Aasa A, Ahas RN *et al.* (2006). European phenological response to climate change matches the warming pattern. *Global Change Biology*, **12**, 1969-1976.

Miranda JDD, Armas C, Padilla FM, Pugnaire FI (2011). Climatic change and rainfall patterns: effects on semi-arid plant communities of the Iberian Southeast. *Journal of Arid Environments*, **75**, 1302-1309.

Misson L, Panek JA, Goldstein AH (2004) A comparison of three approaches to modeling leaf gas exchange in annually drought-stressed ponderosa pine forest. *Tree Physiology*, **24**, 529–541.

Mohan JE, Cowden CC, Baas P, Dawadi A, Frankson PT, Helmick K. *et al.* (2014) Mycorrhizal fungi mediation of terrestrial ecosystem responses to global change: mini-review. *Fungal Ecology*, **10**, 3–19.

Mueller RC, Scudder CM, Porter ME, Talbot Trotter R, Gehrin CA, Whitham TG (2005) Differential tree mortality in response to severe drought: evidence for long-term vegetation shifts. *Journal of Ecology,* **93**, 1085–1093.

Nardini A, Lo Gullo MA, Trifilò P, Salleo S (2014) The challenge of the Mediterranean type climate to plant hydraulics: responses and adaptations. *Environmental and Experimental Botany*, **103**, 68–79.

Navarro-Ródenas A, Lozano-Carrillo MC, Pérez-Gilabert M, Morte A (2011) Effect of water stress on in vitro mycelium cultures of two mycorrhizal desert truffles. Mycorrhiza, **21**, 247-253.

Nicotra AB, Atkin OK, Bonser SP, Davidson AM, Finnegan EJ, Mathesius U. *et al.* (2010) Plant phenotypic plasticity in a changing climate. *Trends in Plant Science*, **15**, 684–692.

NOAA National Centers for Environmental Information, State of the Climate: Global Analysis for May, June and July 2015.

Chapter II

Palacio S, Azorín J, Montserrat-Martí G, Ferrio JP (2014) The crystallization water of gypsum rocks is a relevant water source for plants. *Nature Communications*, **5**, 4660.

Parmesan C (2007) Influences of species, latitudes and methodologies on estimates of phenological response to global warming. *Global Change Biology*, **13**, 1860-1872.

Peñuelas J, Lloret F, Montoya R (2001) Severe drought effects on Mediterranean woody flora in Spain. *Forest Science*, **47**, 214-218.

Peñuelas J, Gordon C, Llorens L, Nielsen T, Tietema A, Beier C, Gorissen A (2004) Nonintrusive field experiments show different plant responses to warming and drought among sites, seasons, and species in a north–south European gradient. *Ecosystems*, **7**, 598-612.

Peñuelas J, Sardans J, Estiarte M *et al.* (2013) Evidence of current impact of climate change on life: a walk from genes to the biosphere. *Global Change Biology*, **19**, 2303–2338.

Poorter H, Niinemets Ü, Poorter L, Wright IJ, Villar R (2009) Causes and consequences of variation in leaf mass per area (LMA): a meta-analysis. *New Phytologist*, **182**, 565–588.

Prieto P, Peñuelas J, Llusià J, Asensio D, Estiarte M (2009) Effects of experimental warming and drought on biomass accumulation in a Mediterranean shrubland. *Plant Ecology,* **205**, 179-191.

Querejeta JI, Egerton-Warburton LM, Allen MF (2009) Topographic position modulates the mycorrhizal response of oak trees to interannual rainfall variability. *Ecology*, **90**, 649–662.

Reich PB, Oleksyn J, Wright IJ (2009) Leaf phosphorus influences the photosynthesis–nitrogen relation: a cross-biome analysis of 314 species. *Oecologia*, **160**, 207–212.

Runion GB, Mitchell RJ, Rogers HH, Prior SA, Counts TK (1997) Effects of nitrogen and water limitation and elevated atmospheric CO_2 on ectomycorrhiza of longleaf pine. *New Phytologist*, **137**, 681–689.

Chapter II

Rustad LEJL, Campbell J, Marion G, Norby R, Mitchell M, Hartley A, Gurevitch J (2001) A meta-analysis of the response of soil respiration, net nitrogen mineralization, and aboveground plant growth to experimental ecosystem warming. *Oecologia*, **126**, 543-562.

Safriel U, Adeel Z (2005) Dryland systems. In: Hassan R, Scholes R, Ash N (eds) *Ecosystems and human well-being, current state and trends, vol 1*. Washington, USA: Island Press, 625–658.

Sage RF, Kubien DS (2007) The temperature response of C3 and C4 photosynthesis. *Plant, Cell & Environment*, **30,** 1086–1106.

Sala A, Piper F, Hoch G (2010) Physiological mechanisms of drought-induced tree mortality are far from being resolved. *New Phytologist*, **186**, 274–281.

Salerni E, Perini C, Gardin L (2014) Linking Climate Variables with *Tuber borchii* Sporocarps Production. *Natural Resources*, **5**, 408-418.

Sardans J, Peñuelas J, Estiarte M, Prieto P (2008) Warming and drought alter C and N concentration, allocation and accumulation in a Mediterranean shrubland. *Global Change Biology*, **14**, 2304-2316.

Sardans J, Peñuelas J (2013) Plant-soil interactions in Mediterranean forest and shrublands: impacts of climatic change. *Plant and Soil*, **365**, 1-33.

Schussler A, Schwarzott D, Walker C (2001) A new fungal phylum, the *Glomeromycota*: phylogeny and evolution. *Mycological Research*, **105**, 1413–1421

Sharkey TD (2005) Effects of moderate heat stress on photosynthesis: importance of thylakoid reactions, rubisco deactivation, reactive oxygen species, and thermotolerance provided by isoprene. *Plant, Cell & Environment*, **28**, 269–277.

Smith SE, Read DJ (2008) Mycorrhizal symbiosis. Edn. 3rd., Academic Press.

Soudzilovskaia NA, Douma JC, Akhmetzhanova AA *et al.* (2015a) Global patterns of plant root colonization intensity by mycorrhizal fungi explained by climate and soil chemistry. *Global Ecology and Biogeography*, **24**, 371–382.

Soudzilovskaia NA, van der Heijden MGA, Cornelissen JHC *et al.* (2015b) Quantitative assessment of the differential impacts of arbuscular and ectomycorrhiza on soil carbon cycling. *New Phytologist*, **208**, 280–293.

Swaty RL, Gehring CA, Van Ert M, Theimer TC, Keim P, Whitham TG (1998) Temporal variation in temperature and rainfall differentially affects ectomycorrhizal colonization at two contrasting sites. *New Phytologist*, **139**, 733–739.

Tedersoo L, May TW, Smith ME (2010) Ectomycorrhizal lifestyle in fungi: global diversity, distribution, and evolution of phylogenetic lineages. *Mycorrhiza*, **20**, 217–263.

Thanos CA, Georghiou K, Kadis C, Pantazi C (1992) *Cistaceae*: a plant family with hard seeds. *Israel Journal of Botany*, **41**, 251–263.

Tielbörger K, Bilton MC, Metz J, Kigel J, Holzapfel C, Lebrija-Trejos E, Sternberg M (2014) Middle-Eastern plant communities tolerate 9 years of drought in a multi-site climate manipulation experiment. *Nature Communications*, **5,** 5102.

Valdés M, Asbjornsen H, Gomez-Cardenas M, Juarez M, Vogt KA (2006) Drought effects on fine-root and ectomycorrhizal–root biomass in managed *Pinus oaxacana* Mirov stands in Oaxaca, Mexico. *Mycorrhiza*, **16**, 117–124.

Vargas R, Baldocchi DD, Querejeta JI, Curtis PS, Hasselquist NJ, Janssens IA, Allen MF, Montagnani L (2010) Ecosystem CO_2 fluxes of arbuscular and ectomycorrhizal dominated vegetation types are differentially influenced by precipitation and temperature. *New Phytologist,* **185,** 226–236.

White DA, Beadle CL, Sands PJ, Worledge D, Honeysett JL (1999) Quantifying the effect of cumulative water stress on stomatal conductance of *Eucalyptus globulus* and *Eucalyptus nitens*: a phenomenological approach. *Functional Plant Biology*, **26**, 17-27.

Chapter II

Williams AP, Allen CD, Macalady AK, Griffin D, Woodhouse CA, Meko DM, Swetnam TW, Rauscher SA, Seager R, Grissino-Mayer HD, Dean JS, Cook ER, Gangodagamage C, Cai M, McDowell NG. (2013) Temperature as a potent driver of regional forest drought stress and tree mortality. *Nature Climate Change,* **3,** 292–297.

Wright DP, Scholes JD, Read DJ, Rolfe SA (2000) Changes in carbon allocation and expression of carbon transporter genes in *Betula pendula* Roth. colonized by the ectomycorrhizal fungus *Paxillus involutus* (Batsch) Fr.. *Plant, Cell & Environment,* **23**, 39–49.

Wright IJ, Reich PB, Westoby M, Ackerly DD, Baruch Z, Bongers F, Cavender-Bares J, Chapin T, Cornelissen JHC *et al.* (2004) The worldwide leaf economics spectrum. *Nature*, **428**, 821–827.

Wu Z, Dijkstra P, Koch GW, Peñuelas J, Hungate BA (2011) Responses of terrestrial ecosystems to temperature and precipitation change: a meta-analysis of experimental manipulation. *Global Change Biology*, **17**, 927-942.

Wullschleger SD, Tschaplinski TJ, Norby RJ (2002) Plant water relations at elevated CO_2– implications for water-limited environments. *Plant, Cell & Environment*, **25**, 319–331.

Zavaleta ES, Thomas BD, Chiariello NR, Asner GP, Shaw MR, Field CB. (2003) Plants reverse warming effect on ecosystem water balance. *Proceedings of the National Academy of Sciences*, **100**, 9892–9893.

Zwieniecki MA, Holbrook NM (2009) Confronting Maxwell's demon: biophysics of xylem embolism repair. *Trends in Plant Science,* **14,** 530–534.

Table 1. Leaf nitrogen (N) and phosphorus (P) concentrations, leaf area, dry mass and leaf mas area, and shoot elongation during late Spring (May-June) of *Helianthemum squamatum* shrubs in the control (C), warming (W), rainfall reduction (RR) and warming + rainfall reduction (W+RR) treatments. Data represents means ± SE (n= 10).

YEAR	C	W	RR	W + RR
Leaf N (%)				
2012	1.74 ± 0.05	1.69 ± 0.08	1.92 ± 0.08	1.58 ± 0.08
2013	2.24 ± 0.05	2.16 ± 0.07	2.16 ± 0.06	2.08 ± 0.07
2014	2.07 ± 0.04	2.14 ± 0.07	2.13 ± 0.06	1.99 ± 0.07
2015	2.06 ± 0.05	1.65 ± 0.06	1.75 ± 0.06	1.75 ± 0.06
Leaf P (mg g^{-1})				
2012	0.85 ± 0.04	0.77 ± 0.07	0.98 ± 0.07	0.78 ± 0.07
2013	1.01 ± 0.04	0.95 ± 0.05	1.00 ± 0.05	0.91 ± 0.05
2014	0.70 ± 0.02	0.69 ± 0.03	0.74 ± 0.03	0.71 ± 0.03
2015	0.72 ± 0.03	0.54 ± 0.04	0.64 ± 0.04	0.63 ± 0.04
Leaf Area (cm^2)				
2013	0.69 ± 0.05	0.63 ± 0.06	0.52 ± 0.06	0.70 ± 0.06
2014	1.01 ± 0.06	0.82 ± 0.09	1.00 ± 0.09	0.96 ± 0.10
2015	0.72 ± 0.04	0.64 ± 0.05	0.64 ± 0.05	0.63 ± 0.05
Leaf Dry Mass (mg)				
2013	12.3 ± 0.8	9.4 ± 1.0	9.4 ± 1.0	10.7 ± 1.0
2014	18.4 ± 0.9	11.3 ± 1.4	15.4 ± 1.4	15.0 ± 1.5
2015	12.0 ± 0.7	10.7 ± 0.9	13.4 ± 1.0	10.6 ± 0.9
Leaf Mass Area (mg cm^{-2})				
2013	18.1 ± 0.5	15.0 ± 0.7	18.0 ± 0.7	15.4 ± 0.7
2014	18.3 ± 0.5	14.4 ± 0.8	15.6 ± 0.8	15.7 ± 0.9
2015	17.0 ± 0.7	17.0 ± 0.9	21.7 ± 1.0	17.1 ± 0.9
Shoot Elongation in late spring (cm)				
2012	4.8 ± 0.3	3.7 ± 0.6	4.5 ± 0.6	3.7 ± 0.6
2013	8.4 ± 0.6	4.7 ± 1.0	7.5 ± 0.9	7.2 ± 1.0

Table 2. Total leaf number, leaf area, leaf dry mass and stem dry mass per 10 cm shoot length of *Helianthemum squamatum* shrubs in the control, warming (W), rainfall reduction (RR) and warming + rainfall reduction (W+RR) treatments at the end of the four year study period. Data represents means ± SE (n= 10).

	Leaf Number	Leaf Area (cm^2)	Leaf Dry Mass (mg)	Stem Dry Mass (mg)
CONTROL	126.5 ± 7.7	18.5 ± 1.4	324.4 ± 20.3	128.5 ± 8.5
RR	100.0 ± 12.8	17.5 ± 2.4	259.6 ± 33.9	104.6 ± 14.2
W	94.8 ± 11.3	12.0 ± 2.1	196.9 ± 29.8	102.7 ± 12.5
W+RR	72.5 ± 12.9	11.7 ± 2.4	184.2 ± 33.9	98.9 ± 14.2

FIGURE CAPTIONS

Figure 1. Mean net photosynthesis rate, stomatal conductance and intrinsic water use efficiency of *Helianthemum squamatum* throughout the four years of the study. Data represents means ± SE (n= 10). Data on g_s and WUEi not available in February 2012.

Figure 2. Maximum efficiency of photosystem II (Fv':Fm'), quantum efficiency of photosystem II (ØPSII) and transpiration rate (E) of *Helianthemum squamatum* throughout the four years of the study. Data represents means ± SE (n= 10). Data not available for Fv':Fm' and ØPSII in May 2015.

Figure 3. Mean leaf carbon isotope ratios ($\delta^{13}C$) of *Helianthemum squamatum* throughout the four years of the study. Data represents means ± SE (n= 10).

Figure 4. Mean dry biomass production (leaves plus stems) per unit shoot length in *Helianthemum squamatum* at the end of the four year study period (October 2015). Data represents means ± SE (n= 10). W: warming; RR: rainfall reduction; W+RR: warming plus rainfall reduction.

Figure 5. Mean post-summer plant survival rate of *Helianthemum squamatum* during the driest year of the study (2012), in which survival was significantly reduced by warming (P=0.041). Data represents means ± SE (n= 10).

Figure 6. Mean relative abundance of ectomycorrhizal OTUs within the soil fungal community under *Helianthemum squamatum* shrubs. Data represents average values (n= 8). Standard errors of the mean for the control, warming (W), rainfall reduction (RR) and warming plus rainfall reduction (W+RR) treatments are 4.11, 1.66, 1.77 and 1.31%, respectively.

Figure 7. Conceptual model: mycorrhizal-mediated detrimental feedback loop on the primary productivity of ectomycorrhizal semiarid shrublands under a warmer and drier Mediterranean climate.
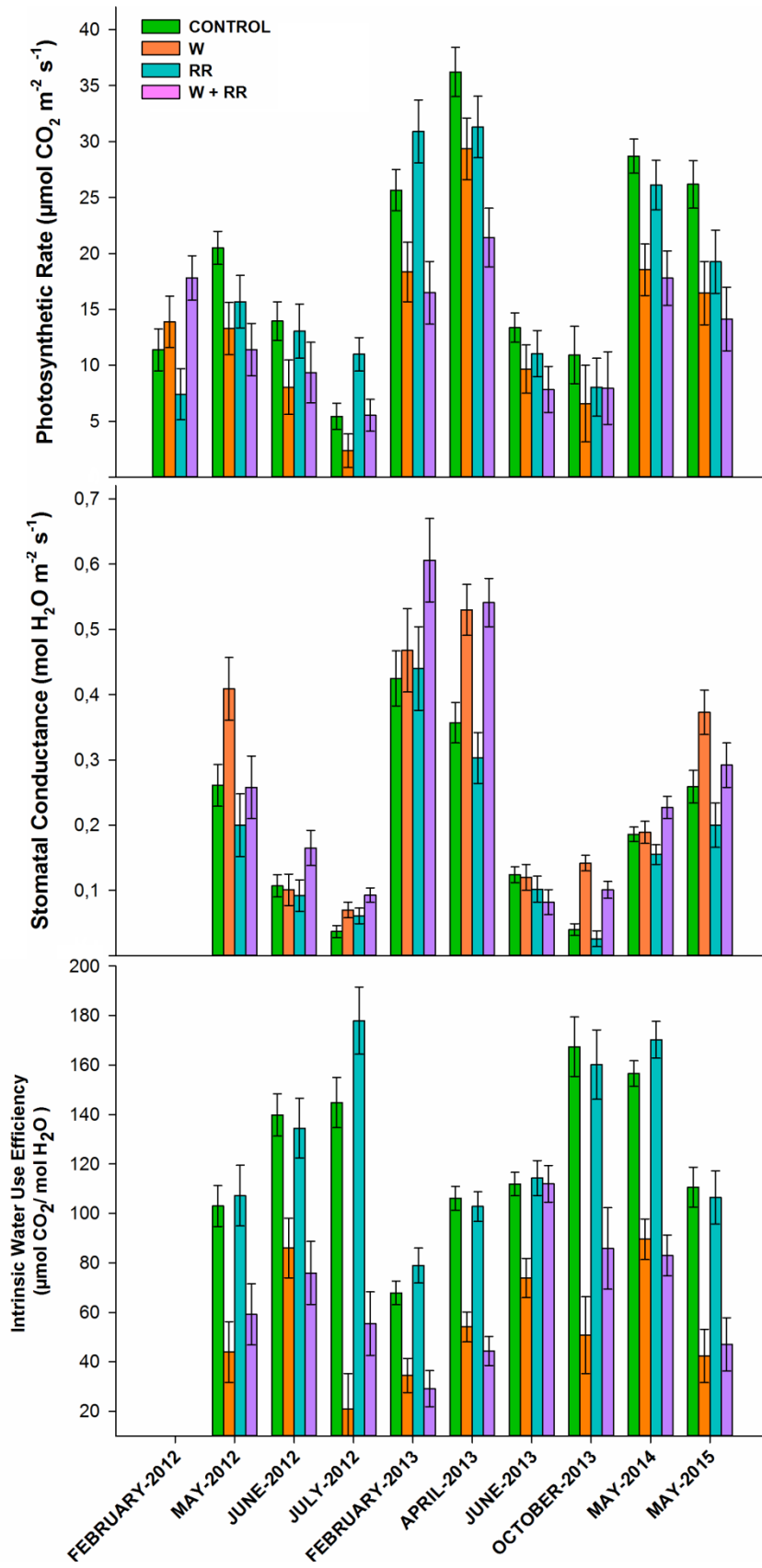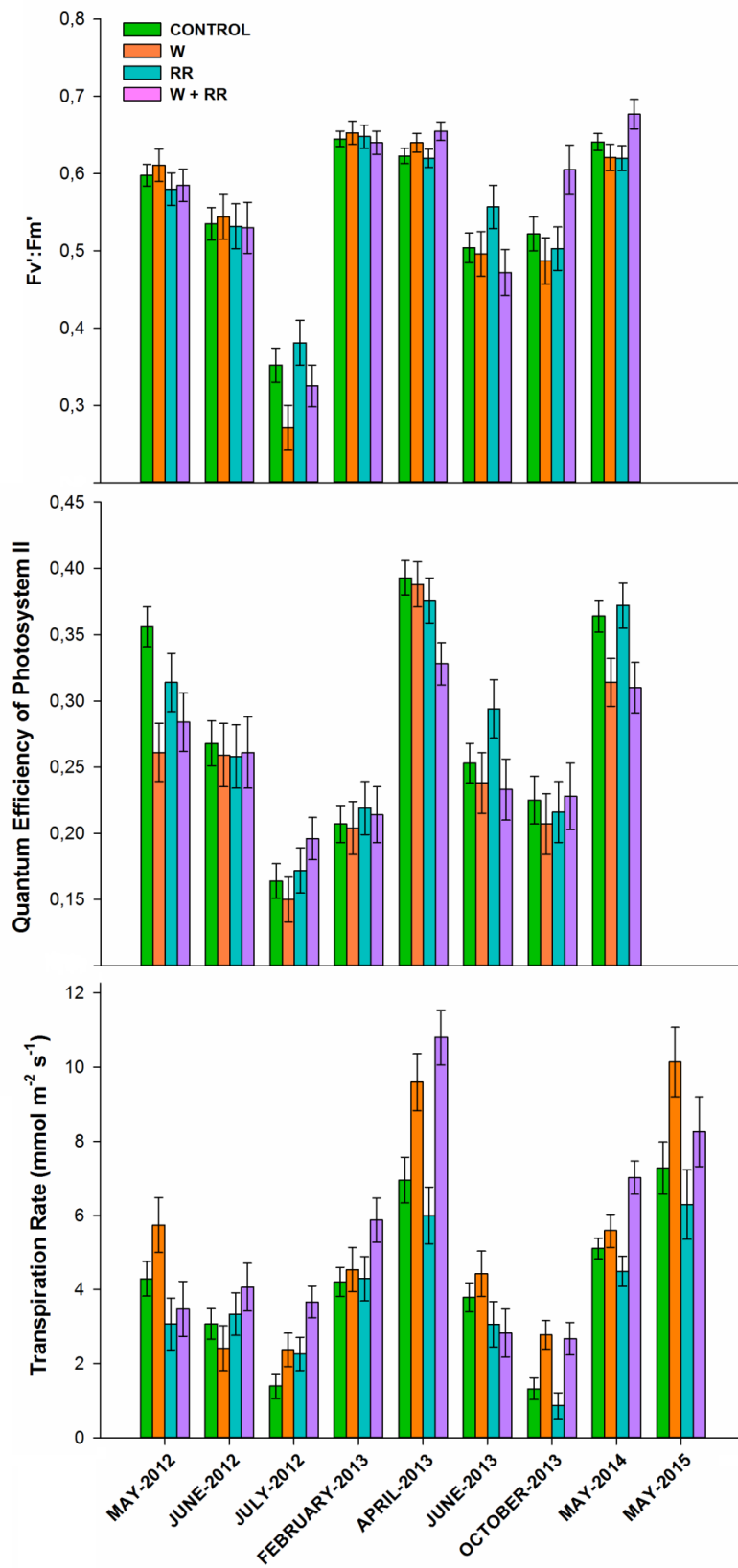
Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.
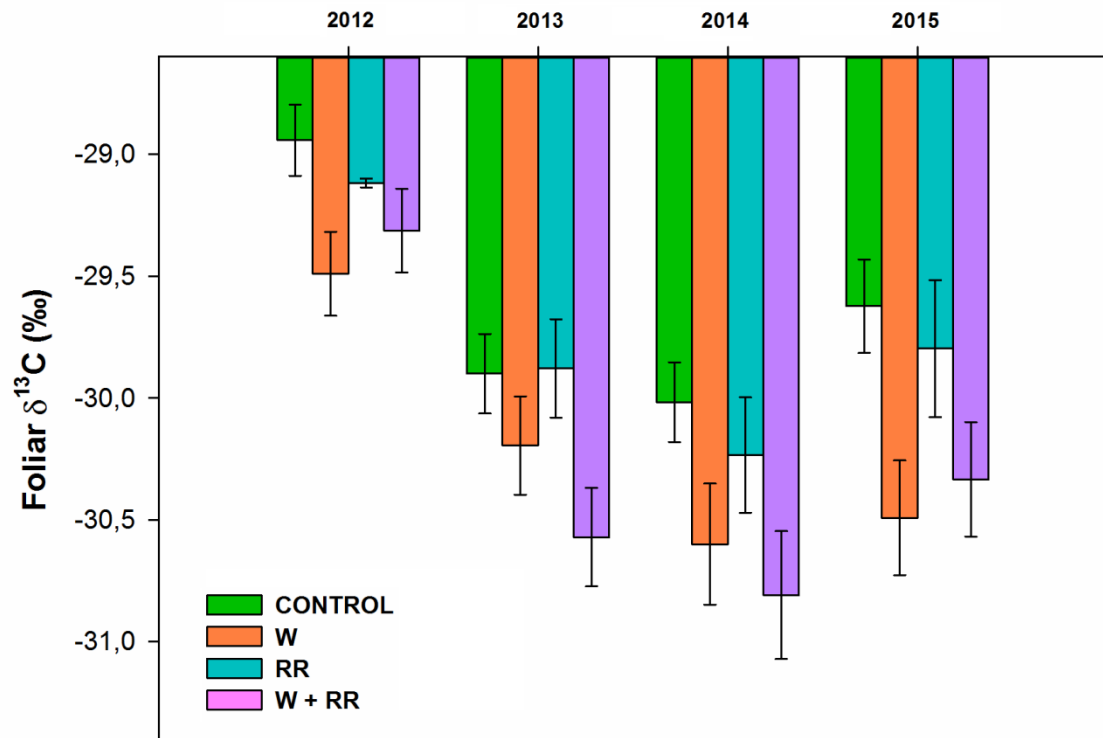
Figure 6.

MYCORRHIZAL-MEDIATED DETRIMENTAL FEEDBACK LOOP ON PLANT PHOTOSYNTHESIS

↓ Plant N and P status
↓ Carbon sink stimulation of photosynthesis by EMF

↓ Net photosynthetic rate
↑ Transpiration
↓↓ Water use efficiency

CLIMATE WARMING AND DRYING

↓ Belowground carbon allocation to EMF
↑ Faster soil water depletion

↓↓ EMF diversity and abundance
↓ N and P uptake capacity by EMF
↓ EMF carbon-sink strength
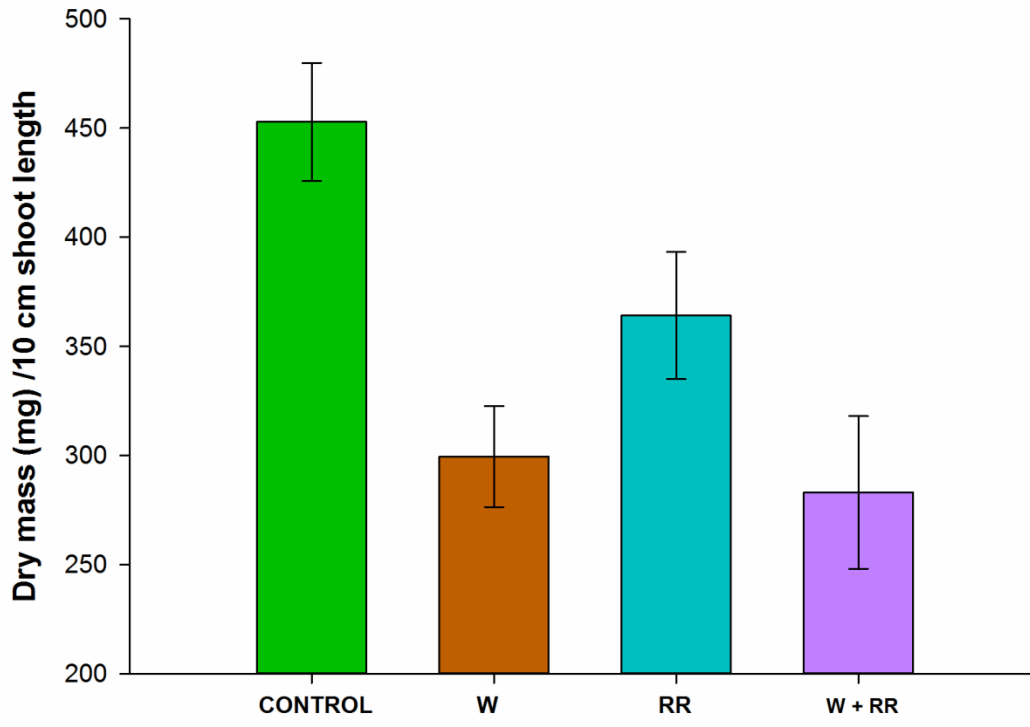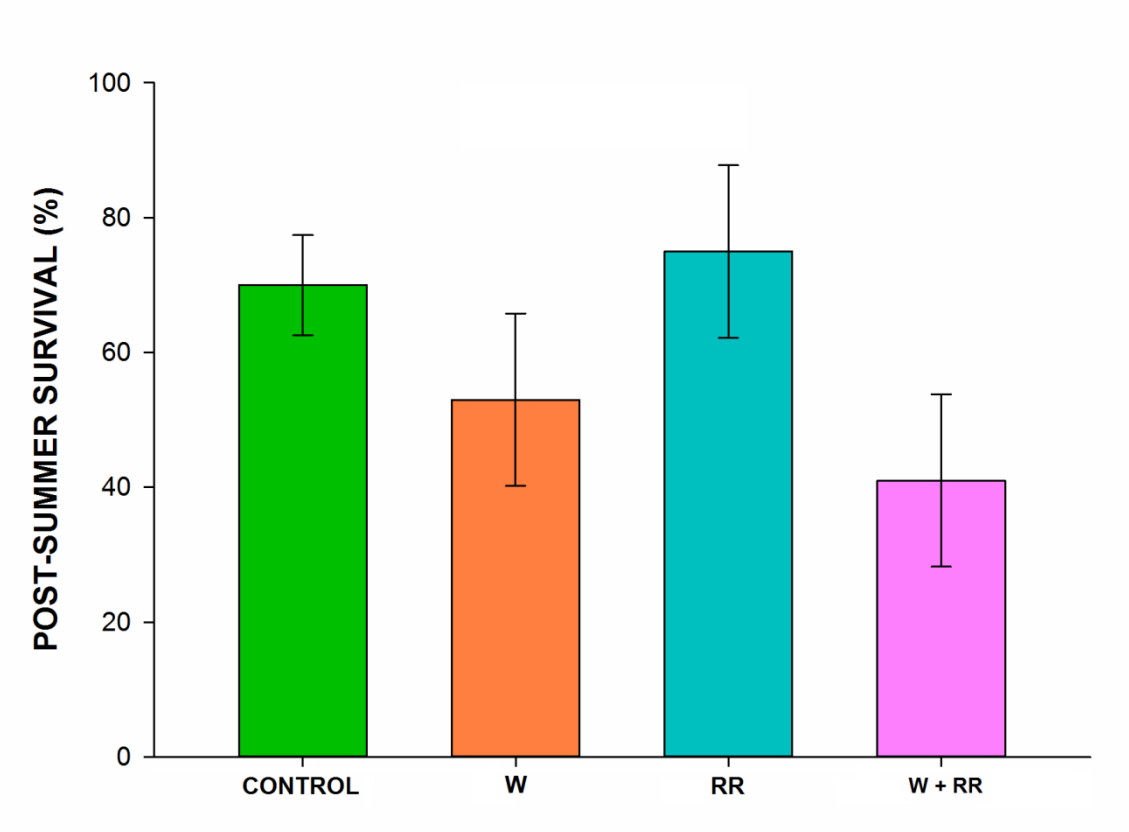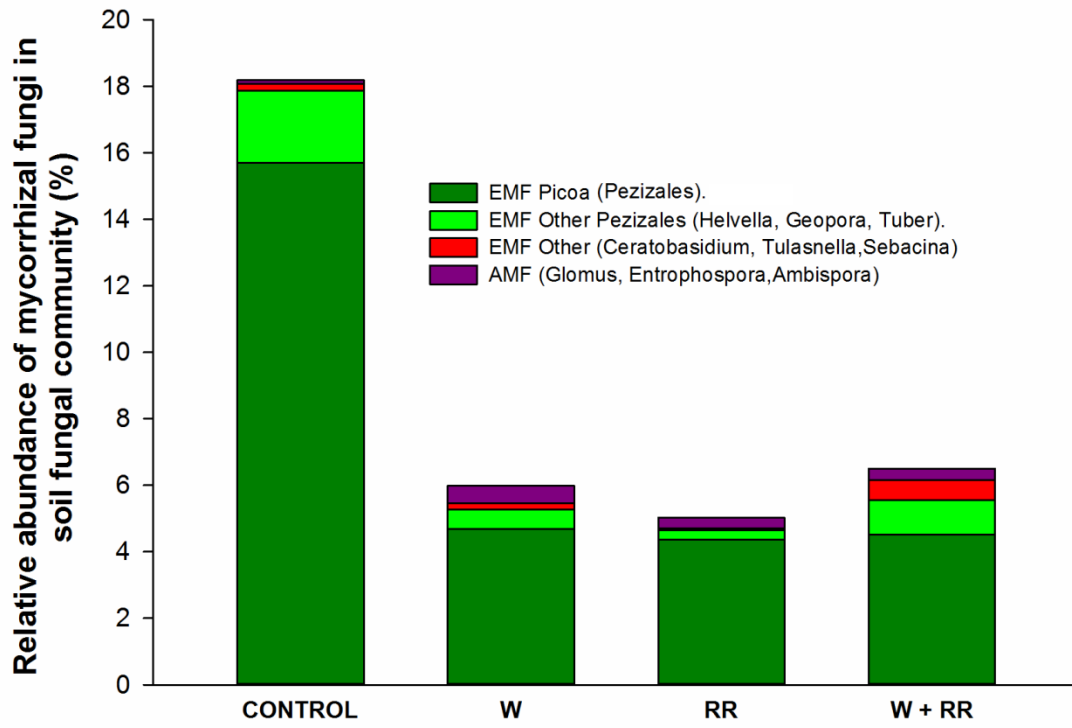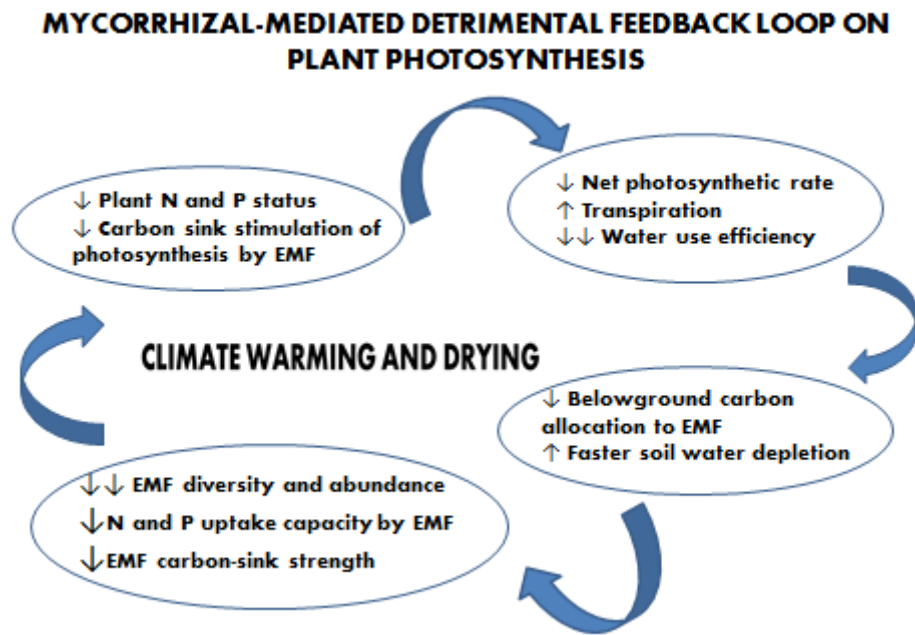
Figure 7.

SUPPORTING INFORMATION

Table S1. Results of the repeated measures analyses of variance (RM-ANOVA) of leaf gas exchange parameters measured on 9-10 measurement dates spanning four consecutive growing seasons (2012-2015). Leaf gas exchange parameters include net photosynthetic rate (A), stomatal conductance (g_s), transpiration rate (E) and maximum and quantum efficiency of photosystem II (Fv':Fm' and $\varnothing$PSII, respectively). Experimental factors are warming (W) and rainfall reduction (RR).

		A	g_s	E	WUE_i	ØPSII	Fv':Fm'
W	F	52.577	34.881	34.129	748.345	16.625	0.043
	P	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.836
RR	F	4.453	4.148	2.445	9.492	0.939	0.284
	P	0.042	0.049	0.126	0.004	0.339	0.598
W*RR	F	1.272	2.020	4.047	0.000	0.311	0.023
	P	0.267	0.164	0.052	0.994	0.580	0.881
Time	F	49.054	103.808	55.968	24.130	38.711	54.881
	P	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
W*Time	F	5.042	4.142	3.984	11.926	1.106	1.375
	P	< 0.001	< 0.001	< 0.001	< 0.001	0.360	0.218
RR*Time	F	2.409	4.155	4.752	1.228	0.946	0.884
	P	0.012	< 0.001	< 0.001	0.282	0.472	0.520

Table S2. Results of the repeated measures analyses of variance (RM-ANOVA) of leaf traits (leaf N and P concentrations, leaf dry mass, leaf mass per area, leaf carbon isotope ratio), shoot elongation in late Spring, shoot growth phenology and post-summer plant survival rate. Experimental factors are warming (W) and rainfall reduction (RR).

		Foliar N	Foliar P	Leaf Dry Mass	LMA	Leaf δ^{13}C	Shoot elongation in late spring	Shoot elongation phenology	Plant survival
W	F	9.127	8.008	8.161	30.647	17.451	5.950	11.830	3.431
	P	**0.004**	**0.008**	**0.007**	**< 0.001**	**< 0.001**	**0.019**	**0.001**	0.069
RR	F	2.417	0.802	0.311	3.795	0.972	0.004	0.268	0.045
	P	0.128	0.376	0.580	0.058	0.332	0.950	0.607	0.834
W*RR	F	0.744	0.065	3.796	0.176	0.613	2.129	0.317	0.345
	P	0.394	0.800	0.058	0.677	0.440	0.151	0.576	0.559
Time	F	25.925	39.374	21.165	9.765	18.673	30.654	1.928	11.846
	P	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	0.172	**< 0.001**
W*Time	F	1.567	1.389	1.003	0.519	0.162	1.639	0.727	2.711
	P	0.201	0.250	0.371	0.597	0.922	0.207	0.398	**0.047**
RR*Time	F	0.509	0.764	1.084	5.581	0.585	0.360	0.259	0.170
	P	0.677	0.516	0.343	**0.005**	0.626	0.551	0.613	0.917

Figure S1. Experimental plot of the *Warming + Rainfall Reduction* treatment, which is achieved by installing an open top chamber (OTC) and a rainout shelter on the same plot.
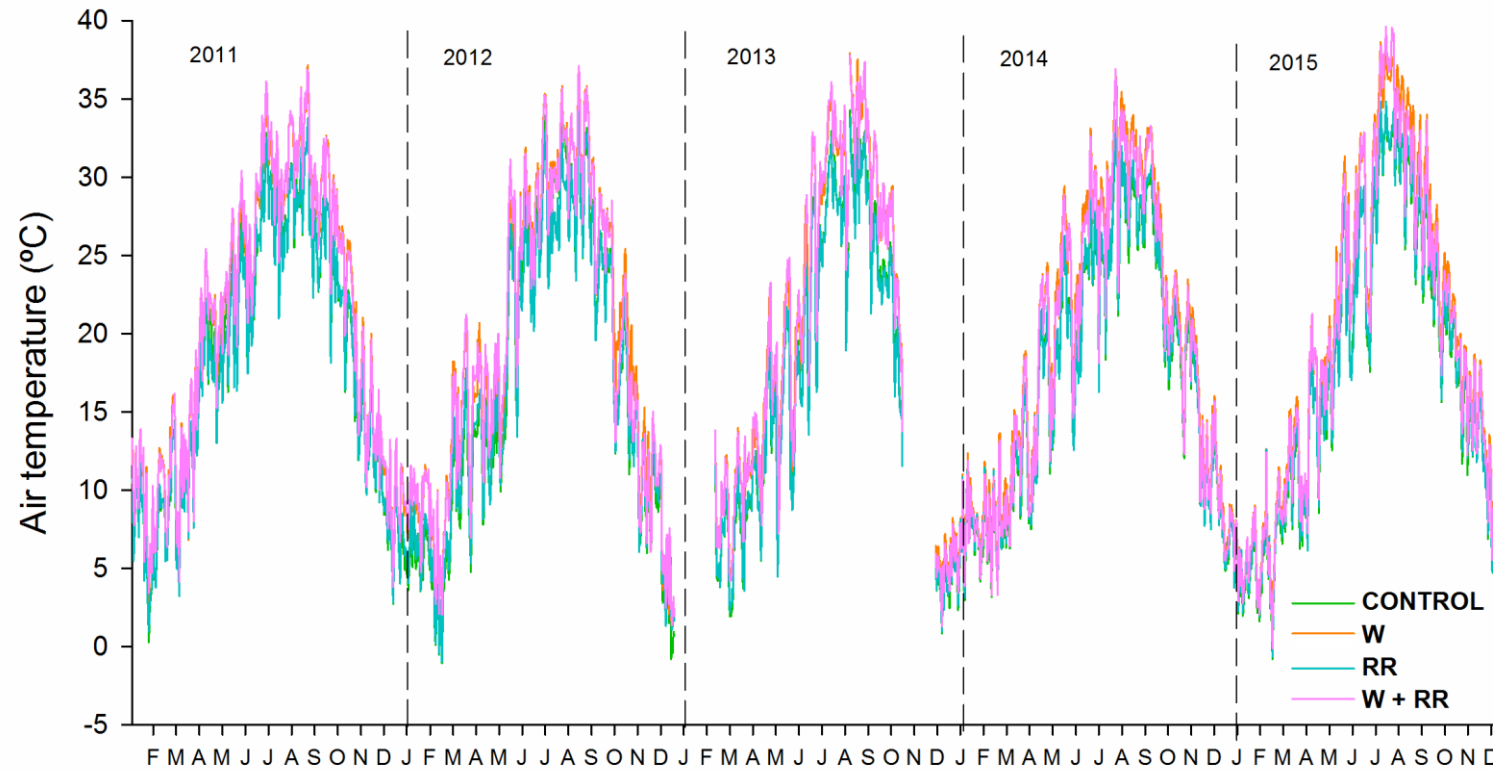
Figure S2. Air temperature in the different experimental treatments throughout the duration of the experiment, showing the effect of the treatments on this variable.
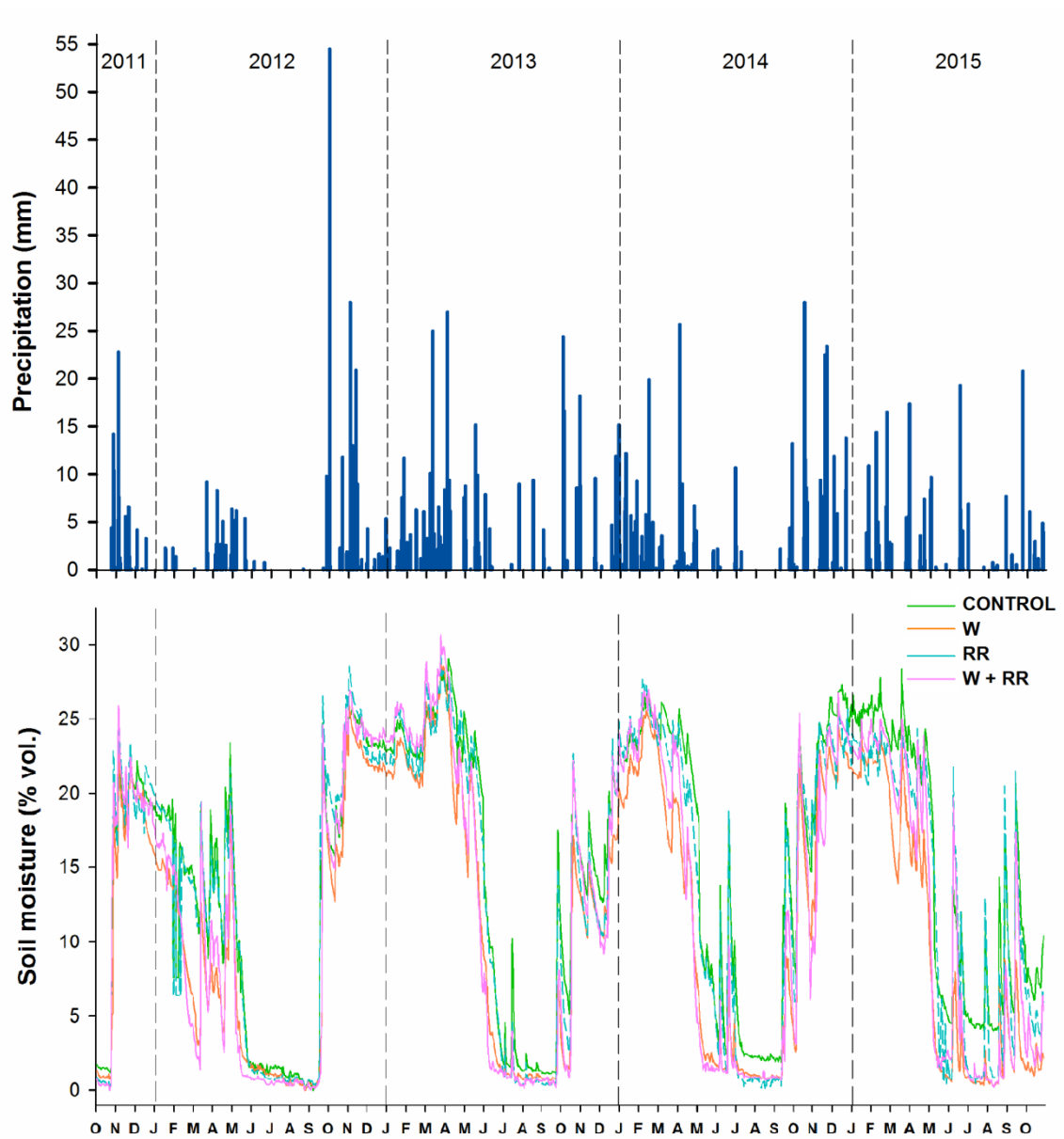
Figure S3. Rainfall amount and distribution throughout the study period, and temporal changes in soil moisture content (0-5 cm depth) in the different experimental treatments.
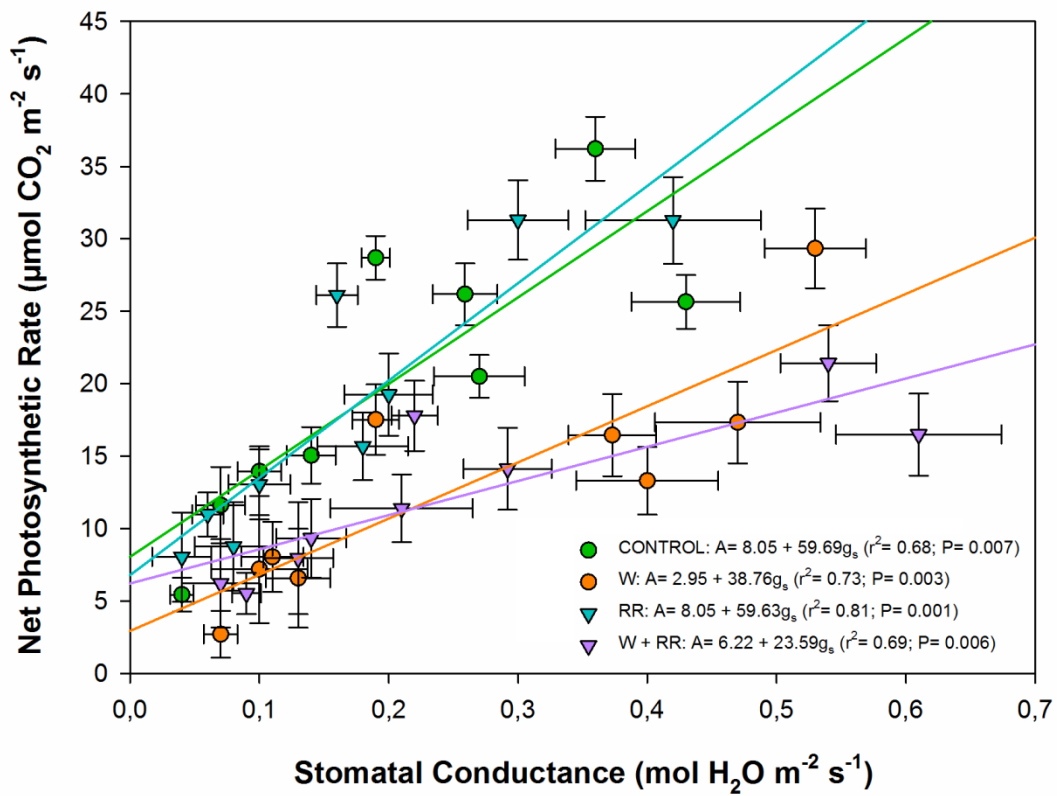
Figure S4. Linear regressions between mean net photosynthesis rate and mean stomatal conductance in all the climate manipulation treatments (control, warming, rainfall reduction, warming + rainfall reduction) across measurements dates. Error bars represent standard errors.

CHAPTER III

SHRUBLAND COMMUNITY RESPONSES TO EXPERIMENTAL WARMING AND RAINFALL REDUCTION IN A MEDITERRANEAN SEMIARID ECOSYSTEM

"In long-distance running the only opponent you have to beat is yourself, the way you used to be."

Haruki Murakami, *What I Talk About When I Talk About Running*

Chapter III

Shrubland community responses to experimental warming and rainfall reduction in a Mediterranean semiarid ecosystem

Authors: *Lupe León-Sánchez[1], Emilio Nicolás[1], Iván Prieto[1], Fernando T. Maestre[3], José Ignacio Querejeta[1].*

[1] Centro de Edafología y Biología Aplicada del Segura (CEBAS-CSIC), Murcia, Spain.

[3] Departamento de Biología y Geología, Área de Biodiversidad y Conservación, Escuela Superior de Ciencias Experimentales y Tecnología, Universidad Rey Juan Carlos, Móstoles, Spain

Chapter III

ABSTRACT

As a consequence of increasing anthropogenic greenhouse gas emissions, current climate models predict large increases in temperature and vapour pressure deficit and reductions in the amount and frequency of precipitation in the Mediterranean region, which will lead to large declines in soil water availability. Mediterranean semiarid ecosystems might be particularly vulnerable to the projected changes in climate, given that water is already the main limiting factor in these environments. We conducted a four-year manipulative field study to assess the effects of warming and/or rainfall reduction on the performance of six coexisting plant species in a native semiarid shrubland community. We used open top chambers (Warming, W) to expose plants to a ~2ºC temperature increase, rainout shelters to reduce annual rainfall by ~30% (Rainfall Reduction, RR), and their combination (W+RR). Across plant species and years, warming decreased foliar nutrient concentrations (N, P, K, Cu, Fe, Zn), net photosynthesis rate, water use efficiency, quantum efficiency of photosystem II and foliar $\delta^{13}C$ relative to control plants, whereas stomatal conductance was moderately enhanced. Warming advanced shoot growth phenology and strongly reduced shoot dry biomass production across target species, relative to control plants. Rainfall reduction decreased foliar K and Cu concentrations and shoot biomass production across species. In spite of the long-term evolutionary adaptation of Mediterranean semiarid plant species to heat and drought stress, survival rate at the end of the study was strongly reduced by warming (and more moderately by rainfall reduction) across all target species. The combination of warming and rainfall reduction had additive rather than synergistic detrimental impacts on plant performance. The consistently detrimental effects of warming (especially when in combination with rainfall reduction) on plant nutrient status, ecophysiological performance, growth and survival indicate that climate change will likely cause an exceedance of the threshold of plant phenotypic adaptation across coexisting native species, leading to a strong detrimental feedback mechanism whereby plants will need to transpire more water to gain less carbon under a climate change scenario. Ultimately, dry years will decrease plant survival and compromise the persistence of these vulnerable native plant communities, with severe implications for ecosystem nutrient cycling, primary productivity and service provision.

Chapter III

INTRODUCTION

General circulation models (GCMs) of the atmosphere predict increases in temperature, vapour pressure deficit (VPD) and potential evapotranspiration, and drastic reductions in the amount and frequency of precipitations in the Mediterranean region, as a consequence of the ongoing anthropogenic rise in atmospheric CO_2 concentration (Christensen *et al*., 2007; Giorgi & Lionello 2007; Collins *et al*., 2013). Mediterranean ecosystems are particularly vulnerable to climate change, as increased temperature and decreased precipitation will lead to a large decline in soil water availability (Miranda *et al*., 2011; Bussotti *et al*., 2014), which is already the main limiting factor for nutrient cycling, net primary productivity, plant growth and survival under the current climate conditions (Boyer 1982; Weltzin *et al*., 2003, Bussotti *et al*., 2014). Therefore, climate change may have serious negative consequences for the Mediterranean Region (Collins *et al*., 2013), since these ecosystems are already a dry and harsh environment for plants, especially during the hot and dry summer (Aschmann 1973). Several studies have focused on the response of plants to simulated climate change conditions in the Mediterranean Region, however, most of these studies have been carried out in areas with dry or sub-humid Mediterranean climates (Llorens *et al*., 2003; Llorens *et al*., 2004; Sardans *et al*., 2008a, Prieto *et al*., 2009) where water is not as severely limiting for plants as in semi-arid ecosystems. In order to better understand and anticipate how native semiarid vegetation will respond to ongoing climate change, it is necessary to carry out manipulative field experiments that simulate the forecasted climate conditions in these areas. Semi-arid Mediterranean ecosystems have a high conservation value as one of the most important biodiversity hotspots of the Earth (Myers *et al*., 2000) and provide multiple ecosystem goods and services to society, but they could be at increased risk of vegetation cover loss, land degradation and desertification under the forecasted climate change scenario (Safriel & Adeel 2005).

Native plant communities growing on semiarid gypsum soils are rich in rare and endemic plant species with a high conservation value (Rubio & Escudero 2000, Escudero *et al*., 2015) that are capable of coping with the stressful conditions of gypsum soils resulting from their high salt concentration and low fertility (Porta *et al*., 2003, Escudero *et al*., 2015). Semi-arid Mediterranean shrubland species are known to be well "pre-adapted" to drought and high temperatures, displaying a wide range of

physiological traits that enable them to escape, avoid or tolerate heat and drought stress (Nicotra *et al*., 2010; Bussotti *et al*., 2014, Nardini *et al*., 2014). However, rapid climate change towards harsher (warmer and drier) abiotic conditions may overwhelm their ability to acclimate through phenotypic plasticity and may exceed their adaptive capacity, thus potentially leading to large decreases in plant photosynthesis, biomass production and survival (Bussotti *et al*., 2014).

Ongoing climate warming affects the C and nutrient cycles, resulting in feedbacks to the global climate system (Heimann & Reichsten 2008). Nitrogen, phosphorus and potassium are key elements (macronutrients) in plant nutrition. Nitrogen is the key component of proteins and thus plays an essential role in all enzymatic and metabolic activity (including the major photosynthetic enzyme Rubisco), whereas phosphorus is involved in nucleic acid synthesis and in energy transfer in the cell (ATP and NADH) (Chapin 1999). Therefore, both are key nutrients for primary producers in terrestrial ecosystems (Dijkstra et al., 2012) and their deficiency is well-known to inhibit photosynthesis and thus, plant biomass production (Chandler & Dale 1995, Huang *et al*., 2004; Wright *et al*., 2004, Thomas, Montagu & Conroy 2006, Reich, Oleksyn & Wright 2009). Potassium has a key role in osmoregulation processes controlling stomatal regulation of transpiration, and activates more than 50 enzymatic systems (including photosynthetic enzymes).

Iron, copper and zinc are among the most important micronutrients for plants. Iron is essential for the biosynthesis of chlorophyll and plays a role in a large number of enzymatic processes (Marschner 2011). Copper is present in many proteins and enzymes involved in oxidation/reduction processes, and is also involved in photosynthesis as part of the enzyme plastocyanin. Finally, zinc has a stabilizing role on the molecule of chlorophyll, and plays a key role in a large number of enzymatic reactions in plants (Marschner 2011).

Large spatial-scale relationships between climate and plant elemental composition (N, P, K) indicate that leaf nutrient concentrations generally decrease with mean annual temperature and increase with mean annual rainfall (Reich & Oleksyn 2004; Sardans *et al*., 2016). Hence, in semi-arid Mediterranean ecosystems we expect that the forecasted climate change conditions (warmer and drier) will cause a decline in the foliar concentrations of these essential elements across coexisting plant species, with potential

detrimental consequences for plant nutrient status, photosynthetic capacity and productivity.

We carried out a 4-year manipulative field experiment in a semiarid shrubland in Southeastern Spain (Sorbas, Almería) with a plant community dominated by *Stipa tenacissima* and several gypsophilous shrub species, such as *Helianthemum squamatum, Helianthemum syriacum, Helianthemum alypoides, Helianthemum almeriense, Gypsophila struthium, Ononis tridentata, Anthyllis terniflora, Thymus sp., Teucrium turredanum, Santolina viscosa* and *Coris hispanica*. We simulated the warmer and drier climate conditions (~2-7ºC temperature increase and ~30% rainfall reduction) predicted by IPCC models (Collins *et al*., 2013) for the Mediterranean Region in the second half of the XXIst century, since these are the two key climate change drivers that will likely have the strongest impacts on vegetation performance across the Mediterranean Region (Bussotti *et al*., 2014). The main aim of the study was to evaluate the effects of warming, rainfall reduction and their combination on the performance of a native semiarid shrubland community typical of gypsum-rich soils, by evaluating potential changes in plant nutrient status, leaf gas exchange, carbon isotopic composition, shoot growth phenology, aboveground biomass production, and post-summer survival in contrasting plant functional types. Our pool of six target plant species included *Helianthemum squamatum* (Cistaceae)*, Helianthemum syriacum* (Cistaceae)*, Gypsophila struthium* (Caryophyllaceae), *Teucrium turredanum* (Lamiaceae)*, Santolina viscosa* (Compositae) and *Coris hispanica* (Primulaceae). This pool includes shrub species with different sizes/biovolumes, life history traits, phenology, stoichiometry, water use strategies and mycorrhizal association types (i.e., arbuscular mycorrhizal in *G. struthium, T. turredanum, S. viscosa* and *C. hispanica*; ectomycorrhizal or mixed arbuscular/ectomycorrhizal in *H. squamatum* and *H. syriacum;* Brundrett 2009). Moreover, *T. turredanum* and *C. hispanica* are considered vulnerable in the IUCN Red List of Spanish Vascular Flora (Moreno 2010). We hypothesized that warming, rainfall reduction and their combination would reduce soil water availability to an extent that would significantly impair plant nutrient uptake and status, while at the same time increasing stomatal limitation of photosynthesis, thus negatively affecting photosynthesis, productivity and survival across coexisting plant species in this semiarid shrubland community.

Chapter III

MATERIALS AND METHODS

Study site and experimental design

The experimental site is located in Sorbas (37°05'32.4"N 2°05'41.5"W, 409 m.a.s.l.), Southeastern Spain, in a shrubland community dominated by *Stipa tenacissima* and several gypsophilous shrub species such as *Helianthemum sp, Gypsophila struthium, Teucrium turredanum, Santolina viscosa* and *Coris hispanica*. The climate of the area is semiarid Mediterranean, with mean annual temperature of 17°C and mean annual precipitation of approximately 275 mm. Mean annual temperatures steadily increased from the first year (17.6ºC in 2011-2012) to the last year of the study (19.1ºC in 2014-2015). Mean annual precipitation varied widely during the study period. In the first two hydrological years (2011-2012 and 2012- 2013), annual rainfall was near the average (247 and 241 mm, respectively). The third hydrological year of the study (2013- 2014) was extremely dry (94 mm), and the last hydrological year (2014-2015) was also dry and below the average, with 180 mm registered.

We conducted a randomized factorial experiment with two experimental factors: warming (W) and rainfall reduction (RR). To simulate the predictions for the second half of the twenty-first century in the Mediterranean Region (IPCC 2013), open-top chambers (OTCs) were installed in order to achieve temperature elevation (1-2ºC during wintertime and 4-7ºC during summertime), whereas rainout shelters were set up with the purpose of reducing the total amount of precipitation reaching the plots by ~30%; plots combining both warming and rainfall reduction were also established (Figure 1). OTCs are hexagonal chambers, with sloping slides of 40 cm x 50 cm x 32 cm. They are made of methacrylate, which transmits about 92% of visible light, has a reflection of incoming radiation of 4%, and passes on ca. 85% of incoming energy (information provided by the manufacturer; Decorplax S. L., Humanes, Spain). OTCs were suspended ~3 cm above the ground level by a metal frame to allow free air circulation and exchange with the surrounding environment, thus minimizing undesirable experimental effects, such as reduced wind and unnatural gas and humidity concentrations (Hollister & Weber 2000). In order to simulated projected reductions in precipitation for the Mediterranean region (Collins *et al*., 2013), passive rainout shelters made of methacrylate troughs covering ~30% of the area of the experimental plots were installed. Rainfall reduction is achieved by suspending the methacrylate troughs over an

aluminum frame above the experimental plots (height 130 cm, width 100 x 100 cm, see Figure 1) Intercepted rainwater is diverted through pipes and collected in storage tanks placed next to the experimental plots, and is thereafter removed after each rainfall event. Finally, both OTCs and rainfall exclusion shelters were installed over the same experimental plot in order to achieve the combined W+RR treatment. Twelve plots with OTCs (W), 10 plots with rainout shelters (RR) and 10 plots combining OTCs with rainout shelters (W+RR), along with 32 adjacent paired control plots, were installed in May 2011, resulting in a total of 64 experimental plots.

Air and surface soil temperatures, relative humidity and soil moisture content were continuously monitored in all the climate manipulation treatments, using replicated automated sensors (HOBO® U23 Pro v2 Temp/RH and TMC20-HD sensors, Onset Corp., Pocasset, MA, USA, and EC-5 soil moisture sensors, Decagon Devices Inc., Pullman, WA, USA, respectively).

Plant measurements

We used a LI-6400XT photosynthesis system (Li-Cor, Inc., Lincoln, NE, USA) equipped with a LI-6400-40 Leaf Chamber Fluorometer and a LICOR 6400-01 CO_2 injector to measure net photosynthesis rate (A), stomatal conductance (gs) and quantum efficiency of photosystem II (ØPSII) throughout the study in *Helianthemum squamatum* (February, March, April, May and June 2012; February, April and June 2013; April 2014 and April 2015), *Helianthemum syriacum* (February, March, April, May and June 2012; March, May and June 2013; April 2014 and April 2015) and *Gypsophila struthium* (May, June, July, August and October 2012; August 2013; June 2014 and July 2015).

Leaf gas exchange was measured on fully sun-exposed leaves that were placed in a 2 cm^2 leaf cuvette. During these measurements, air CO_2 concentration was controlled using the injection system and compressed CO_2-cylinders with a CO_2 concentration of 390 μmol mol-1 CO_2. Measurements were done at a saturating light of 1.500 μmol m^{-2} s^{-1}, and at ambient air temperature and relative humidity. The air flow was set to 250 μmol s^{-1}. All leaf-gas exchange measurements were conducted between 8:00 and 11:00 am (GMT), when the peak of maximum photosynthetic rates was found at each survey. All leaf gas exchange measurements were conducted under the prevailing microclimatic

conditions in the each experimental treatment: control plots (ambient conditions), warmed plots (within the OTCs), rainfall reduction plots (under the rainout shelters) and the combined treatment plots (within the OTC and under the rainout shelters). Thereafter, leaves measured were collected to determine their area with an image scanner program in order to correct the LICOR outputs (Image Pro Plus, Media Cybernetics, Inc.Rockville, MD, USA). Intrinsic water use efficiency (WUEi) was determined as A/gs.

In October 2015 (i.e., four years after the beginning of the experiment), we collected leaves across target species to measure their carbon isotope composition ($\delta^{13}C$). Samples were dried at 60ºC and finely ground with a ball mill before being weighted and placed into capsules for these analyses. The $\delta^{13}C$ values of leaf material were measured by elemental analyzer/continuous flow isotope ratio mass spectrometry (ANCA/SL elemental analyzer coupled with a Finnigan MAT Delta PlusXL IRMS). Isotope analyses were conducted at the Centre for Stable Isotope Biogeochemistry, University of California, Berkeley (USA). Isotope ratios are expressed in delta notation (‰) relative to an internationally accepted reference standard: V-PDB. Long-term (3+ years) external precision for $\delta^{13}C$ measurements of leaf material is 0.14.

In spring 2012 and 2013, we collected leaf samples of all the six target species to evaluate the effects of climate treatments on plant nutrient status (N, P, K, Ca, Mg, S, Fe, Zn, Cu). However, *Coris hispanica* leaves were only collected in 2012 due to widespread mortality across treatments. We focused on N, P and K as macronutrients and Fe, Zn and Cu as micronutrients. Leaf samples were dried at 60ºC and thereafter finely ground with a ball mill before being weighed and placed into tin capsules for stoichiometry analyses. The C and N concentrations of leaf material were measured by elemental analyzer/continuous flow isotope ratio mass spectrometry (ANCA/SL elemental analyzer coupled with a Finnigan MAT Delta PlusXL IRMS). Leaf P, K and Fe concentrations were measured by inductively coupled plasma optical emission spectrometry (ICP-OES, Thermo Elemental Iris Intrepid II XDL, Franklin, MA, USA) after a microwave-assisted digestion with HNO3:H2O2 (4:1, v:v) at the Ionomics laboratory at CEBAS-CSIC (Spain).

In early March 2012 and 2013, three terminal shoots per individual of the target species present in the experimental plots were labeled using red tape in order to track shoot

elongations throughout the growing season. We measured shoot length in March, May and July/August and calculated early spring shoot growth as the difference between shoot length in May and shoot length in March, and late spring shoot growth as the difference between shoot length in July/August and shoot length in May. We calculated the quotient between late/early shoot elongations as an indicator of shoot growth phenology.

At the end of the study period, in October 2015, one 10 cm long terminal shoot per individual plant was collected in order to determine effects of climate manipulation on leaf and stem biomass production of five target species (*Helianthemum squamatum, Helianthemum syriacum, Gypsophila struthium, Santolina viscosa* and *Teucrium turredanum*). We measured total dry biomass (leaves plus stems) per unit shoot length, as well as the total number of leaves and total leaf area per unit shoot length.

Post-summer survival of each target species in each treatment was assessed in October 2015, at the end of the study period. Survival was calculated as the proportion of plants that were still alive in each experimental plot relative to the total number of plants that were present in each plot in March 2012 (one year after setting up the climate manipulation treatments). This reference date (March 2012) was chosen to account for the potential surge in plant mortality during the early stages of the experiment caused by a sudden change in the microclimatic conditions when establishing the climate manipulation treatments (i.e. treatment-shock mortality).

Statistical analyses

We used multiple-way ANOVAs (GLMs) to evaluate the effects of simulated climate warming, rainfall exclusion and their combination on plant performance. The plant variables analyzed were leaf nutrient concentrations (N, P, K, Ca, Mg, S, Fe, Cu and Zn), leaf gas exchange (A, gs, E, ØPSII and WUE$_i$), leaf δ^{13}C, shoot biomass production and shoot elongation.

Plant survival was analyzed using a simple binary logistic regression where survival was the dependent variable and simulated climate warming (W), rainfall exclusion (RR) and their combination (W+RR) were the predictor factors.

Chapter III

We used SPSS 22.0 software (SPSS Inc., Chicago, IL, USA) to perform ANOVA analyses and the R software (v. 2.15.3, R Development Core Team 2013) for survival analyses.

RESULTS

Effects of the experimental treatments on micro-climatic variables

The warming treatment (OTCs) increased mean annual air temperature by ~1.6ºC relative to ambient conditions (Figure 2). Mean annual temperature of surface soil (0-5 cm) was also raised inside OTCs by 2.2ºC on average (Figure 2). Mean annual vapor pressure deficit (VPD) was on average 14% higher within the OTCs than in the control plots (798 Pa in W and W+RR plots versus 703 Pa in non-warmed control and RR plots). Rainout shelters reduced the mean annual moisture content of surface soil (0-5 cm depth) by 1.24% in absolute terms (14% in relative terms) relative to the control treatment, but did not alter mean air and soil temperatures or VPD. During periods immediately following rainfall events, reductions in soil moisture content in the RR and W+RR plots reached up to 8% in absolute terms (36% in relative terms). The OTCs also reduced the mean annual soil moisture content in the upper soil layer (0-5 cm depth) by 1.6% in absolute terms (16% in relative terms) due to warming-induced enhancement of soil drying. However, there were no significant differences in soil moisture content among the different treatments during the summer, when soil water content was uniformly low across treatments (including the control treatment). For a more detailed description of the effects of the experimental treatments on microclimatic variables, see the supporting information provided in Maestre et al., 2013.

Plant stoichiometry

Across plant species and years (2102- 2013), foliar N, P, Fe, Zn and Cu concentrations were significantly decreased by warming (P= 0.003; P= 0.010; P< 0.001, P= 0.016, P=0.008 and P= 0.004, respectively; Table 1). Foliar N concentration was reduced by 7% on average in warmed plants (W and W+RR) relative to control plants across species (Figure 5), and therefore, leaf C/N ratios were on average 9% higher in warmed plants than in the controls across species. Foliar P concentration was on average 9% lower in warmed plants (W and W+RR) than in control plants across species (Figure 6).

Chapter III

Foliar Fe concentration showed the largest reduction of all nutrients, with on average 28% lower mean values in warmed plants (W and W+RR) than in control plants across species (Table 1; Figure 8). Foliar Zn concentration was on average 17% lower in plants exposed to climate warming (W and W+RR treatments) than in control plants across species (Table 1). Moreover, statistical analyses revealed synergistic detrimental effects of warming and rainfall reduction as regards plant Fe and Zn status, which led to largest decreases in leaf Fe and Zn concentrations in the W+RR treatment relative to the controls (39% and 22%, respectively; see significant *Warming x Rainfall Reduction* interactions in Table 1). Foliar Cu concentration was reduced by warming (W and W+RR treatments) by 23% on average across species, relative to control plants (Table 1). In addition, foliar Cu concentration was also significantly reduced by rainfall reduction across species (P= 0.008), hence all the climate manipulation treatments showed lower leaf Cu concentrations than control plants according to LSD post-hoc test (C vs W: P< 0.001; C vs RR: P< 0.001; C vs W+RR: P< 0.001). Finally, foliar K concentration was significantly decreased by rainfall reduction across species (P= 0.018), with a 16% average reduction relative to control plants (Figure 7). Warmed plants also showed on average 13% lower leaf K concentrations than control plants, albeit this effect was not statistically significant; however LSD post-hoc test revealed that all the climate manipulation treatments had significantly lower leaf K concentrations than the control treatment across species (C vs W: P= 0.004; C vs RR: P<0.001 and C vs W+RR P= 0.004).

In contrast to the abovementioned nutrients, the foliar concentrations of other important plant macronutrients (such as Ca, Mg and S) were not significantly altered by the climate manipulation treatments across species (see table 1 for results of statistical analyses; leaf Ca, Mg and S concentration data not shown).

There were relatively few statistically significant *Warming x Species* and *Rainfall Reduction x Species* interactions for foliar nutrient concentrations (Table 1), indicating that the detrimental effects of warming (and to a lesser extent, rainfall reduction) on plant nutrient status were rather uniform and consistent across coexisting shrub species.

Chapter III

Leaf-gas exchange

Throughout the study, warming (W and W+RR) consistently and strongly decreased net photosynthesis rate and water use efficiency (P< 0.001 and P< 0.001, respectively) across the three measured species (*H. squamatum*, *H. syriacum* and *G. struthium*), with average reductions of 32.3% in A and 41.5% in WUEi relative to the controls across species. Reductions in A and WUEi in warmed plants relative to the controls were slightly greater in the W+RR treatment (34.6% and 42.5%, respectively) than in the W treatment (30% and 40.5%, respectively) across the three target species. This pattern of response to warming was rather consistent across species, with no significant *Warming x Species* interactions for net photosynthetic rate. Across the study period, mean net photosynthesis rate was reduced on average by 36% in *H. squamatum*, 29% in *H. syriacum* and 36% in *G. struthium* in warmed plants (W and W+RR treatments) relative to control plants exposed to current climate conditions (Figure 9). Across the study period, intrinsic water use efficiency was also sharply reduced by warming in the three target species, with mean values that were on average 38% (*H. squamatum*), 33% (*H. syriacum*) and 54% (*G. struthium*) lower in warmed plants (W and W+RR treatments) than in control plants (Figure 11). However, there was a significant *Warming x Species* interaction for WUEi, indicating that the decrease in water use efficiency under warming was even greater in *Gypsophila struthium* than in the two *Helianthemum* species. Throughout the study period, warming also caused a significant decrease in the mean quantum efficiency of photosystem II across species (P< 0.001; data not shown), with average reductions of 11.4% in the W treatment and 17.3% in the W+RR treatment, relative to the control treatment (Table 2).

Stomatal conductance was significantly enhanced by warming across species (P= 0.020), with warmed (W and W+RR) plants showing on average 5% higher values than control plants throughout the study (Figure 10). Transpiration rate followed the same pattern as stomatal conductance throughout the study period, with an average increase of 7% in warmed plants (W and W+RR) relative to the controls across species (P= 0.028).

Chapter III

Leaf carbon isotopic composition

Foliar $\delta^{13}C$ values were measured at the end of the study period (October 2015) in five target species, with species mean values ranging from -26.3‰ in *Gypsophila struthium* to -31.1‰ in *Helianthemum squamatum* (Figure 12). Across the 5 measured species, foliar $\delta^{13}C$ values were significantly lower in plants exposed to warming (W and W+RR; P= 0.038; Table 3; Figure 12) than in plants exposed to current ambient temperatures (Control and RR), with no significant *Species x Warming* interaction.

Shoot biomass and elongation

Both warming (P< 0.001) and rainfall reduction (P= 0.001) decreased shoot dry mass production per unit length across species (Tables 3, 4; Figure 13). At the end of the study period, W+RR plants showed the largest mean decrease in shoot biomass production across species (39% relative to control plants), followed by W plants (28%) and RR plants (22%). Across species, total leaf dry mass per unit shoot length (foliage biomass production) was strongly reduced by both warming and rainfall reduction (P< 0.001 and P= 0.001, respectively). Stem dry mass production was only affected by warming (P= 0.004), with no significant reduction in plants exposed to rainfall reduction relative to control plants (Tables 3, 4).

RM-ANOVA analysis revealed a strong effect of warming on shoot growth phenology (P= 0.001) across years (2012-2013) and species. Shoot elongation during the early part of spring was significantly enhanced by warming across species (P= 0.003), with average values of 7.54 ± 0.33 cm in warmed plants (W, W+RR) versus 6.22 ± 0.24 cm in ambient plants (C, RR). In contrast, warming decreased shoot elongation during the latter part of the spring growing season across species (P= 0.001), with average values of 3.21 ± 0.44 cm in warmed plants (W and W+RR) vs. 5.20 ± 0.33 cm in plants exposed to ambient temperatures (C and RR). Across species, the late/early spring growth ratio was 1.29 ± 0.12 in control plants and 1.30 ± 0.17 in RR plants (1.30 ± 0.10 on average in ambient temperature plants), but this ratio decreased to 0.90 ± 0.18 in W plants and 0.51 ± 0.21 in W+RR plants.

Chapter III

Post-summer survival

Post-summer plant survival rate was significantly reduced by warming (W and W+RR) across species (P= 0.009; see Table 5). At the end of the study, (November 2015, four years since the start of the experiment), W+RR plants had the lowest survival rate of all the treatments (only 38.2% versus 71% in control plants), indicating 46% lower survival than control plants (Figure 14) across species. However, whereas *C. hispanica* showed 0% survival in the W +RR treatment at the end of the study, *G. sthrutium* showed 100% survival in the W+RR treatment (same as in the control treatment). The survival rate of W plants was on average 23% lower than that of control plants across species (54.5% versus 71% in control plants).

DISCUSSION

Climate change effects on plant stoichiometry and nutrient status

Throughout the study period, the target species showed a significant reduction in leaf N concentration due to simulated climate warming, while rainfall reduction did not affect leaf N concentration. Foliar P concentration followed the same pattern than N, being reduced by warming throughout the study and across all target species, however, it was not affected by rainfall reduction. Foliar K concentration was negatively affected by all the climate manipulation treatments, with W, RR and W+RR plants showing lower values than control plants exposed to current climate conditions. Nitrogen, phosphorus and potassium are key macronutrients for terrestrial plants playing essential roles in their physiology (Cakmak, 2005). Nitrogen plays a key role in protein synthesis and enzymatic activity, and is the major component of Rubisco (the enzyme responsible for photosynthetic CO_2 fixation), whereas P is involved in nucleic acid and phospholipid synthesis and in energy transfer in the cell (ATP and NAD/NADH phosphorylation/dephosphorylation; Chapin 1999). Therefore, both elements are essential macronutrients for primary producers in terrestrial ecosystems (Dijkstra et al., 2012) and it is widely reported that their deficiency strongly inhibits photosynthesis and plant growth and biomass production (Chandler & Dale 1995, Huang et al., 2004; Zhao et al 2005, Wright et al 2004, Thomas, Montagu & Conroy 2006, Reich, Oleksyn & Wright 2009). Our results are in agreement with these previous studies, since we have found a similar and consistent pattern of decreased foliar nutrient status, photosynthesis

and growth across all our target plant species. As a consequence of these reductions in foliar N and P concentrations, warmed plants (W and W+RR treatments) showed consistently higher leaf C/N and C/P ratios than control plants. It is becoming increasingly clear that warming and drought increase plant C:N and C:P ratios in warm-dry ecosystems, especially in Mediterranean-type ecosystems where the annual drought period occurs in summer coinciding with the highest annual temperatures (Reich & Oleksyn 2004; Sardans et al; 2008a; Sardans et al., 2016). This increase in the leaf C/N and C/P ratios of semiarid shrubland vegetation as a consequence of forecasted climate change conditions may thus lead to a decrease in the nutritional quality of plant foliage, which might have implications for plant-herbivore relationships (Sardans et al., 2008a).

Potassium activates more than 50 enzymatic systems and plays an outstanding role in plant osmoregulation and in stomatal opening and closure. Potassium is thus particularly important for controlling plant water losses and for achieving physiological acclimation to drought (Milla et al., 2005; Sardans et al., 2008b). Therefore, a reduction in leaf K status in plants exposed to rainfall reduction and warming may have a detrimental effect on stomatal regulation of transpiration water losses and on plant osmotic protection against drought stress (Rivas-Ubach et al., 2012).

Foliar Fe, Cu and Zn concentrations were also significantly reduced by warming, and Cu was reduced by all the treatments (W, RR and W+RR) relative to control plants. Iron, copper and zinc are among the most important micronutrients for plants. Iron is essential in the biosynthesis of chlorophyll and plays a key role in a large number of enzymatic processes (Marschner, 2011). Zinc has a stabilizing role on the chlorophyll molecule, and, is necessary as a catalyst for the activity of at least 80 enzymatic systems, many of them important in photosynthesis. Copper is present in various proteins and enzymes involved in oxidation/reduction processes, and is indispensable for photosynthetic electron flow as part of the proteins plastocyanin and cytochrome c oxidase. Therefore, a reduction of the foliar concentrations of all these microelements might negatively affect photosynthesis and water use efficiency, as well as biomass production and survival across coexisting species in this semiarid plant community. Moreover, together with decreased leaf N and P, decreased leaf K, Fe, Cu and Zn concentrations under warming may further contribute to reduce the nutritional quality of

plant foliage across plant species, thus potentially altering plant-herbivore interactions in this semiarid plant community under a climate change scenario.

Climate change effects on leaf-gas exchange

Simulated climate warming sharply reduced leaf-level net photosynthesis rate and caused a moderate but significant increase of stomatal conductance in the three measured species (Table 1). Therefore, warming imposed strong non-stomatal limitations on photosynthesis, since the carbon assimilation rates of the 3 species were strongly reduced regardless of increasing stomatal conductance (Flexas & Medrano 2002). Moreover, the quantum efficiency of photosystem II (ØPSII) was reduced by warming across species, although to a lesser extent than net photosynthetic rates, which suggests downregulation of photosystem II in response to a reduced carboxylation capacity caused by decreased leaf nutrient status (Sage and Kubien 2007). Leaf photosynthetic capacity correlates tightly with leaf N concentration because of the crucial role that N-rich enzymes, particularly Rubisco, play in the biochemical fixation of CO_2 (Wright *et al.,* 2004). Phosphorus limitation under warming can also further decrease net photosynthetic rates through reductions in ribulose 1,5 bisphosphate regeneration, photochemical efficiency of photosystem II and mesophyll conductance to CO_2 (Jacob & Lawlor 1991, 1992). Similarly, multiple nutrient co-limitation (including K, Fe, Cu, Zn, in addition to N and P) under warming likely has mutually reinforcing and multiplicative detrimental effects on plant photosynthetic capacity (Marschner 2011). In addition to decreased leaf nutrient status, other non-stomatal limitations (e.g. biochemical and metabolic) may have further contributed to reduce the net photosynthetic rates of *H. squamatum* under warming. Decreased net photosynthesis with moderate warming has been related to several biochemical and/or metabolic mechanisms such as, among others, a) enhanced photorespiration (von Caemmerer & Quick 2000); b) inhibition of ribulose-1.5-bisphosphate regeneration due to a reduced whole-chain electron transport capacity (Sage & Kubien 2007); c) down-regulation of Rubisco activation state caused by the high thermal lability of the enzyme Rubisco activase (Feller *et al.,* 1998; Crafts-Brandner & Salvucci 2000); d) decreases in leaf soluble proteins and Rubisco concentration (Galmés *et al.,* 2013); and/or e) enhanced mitochondrial respiration rates (Atkin & Tjoelker 2003; Lin *et al.,* 2012). However, disentangling the contributions of these various non-stomatal mechanisms to cross-

species photosynthesis decrease under moderate warming (Sharkey 2005) is beyond the scope of this study.

The combination of a large decrease in A and increased gs resulted in a dramatic reduction in the intrinsic water-use efficiency of warmed plants (Figure 11). Increased stomatal conductance and transpiration under warming may represent an adaptive physiological mechanism to prevent or decrease leaf overheating and damage of the photosynthetic machinery through evaporative leaf cooling. However, the large drop in WUEi found in plants exposed to simulated climate warming across species revealed a potential detrimental feedback on primary productivity, since this implies that dryland plants will need to transpire more water in order to assimilate less carbon under elevated temperatures and VPD, which appears to be a maladaptive response to progressive climate aridification in the mid- to long-terms. Moreover, there was no evidence of photosynthesis acclimation to warming with time, since reductions in A and WUE_i were rather consistent in magnitude and temporally persistent thorough the study across species (Figures 9, 11). Despite the large number of adaptive mechanism that Mediterranean plant species have developed to cope with extreme climate conditions (Sardans & Peñuelas., 2013; Bussotti et al., 2014, Escudero et al., 2015), our experimental warming treatment apparently exceeded their threshold of phenotypic plasticity, resulting in large reductions in A and WUE_i in warmed plants.

Climate change effects on leaf carbon isotope composition

Carbon isotope data indicate that our target shrubland community is comprised of plant species with sharply contrasting water-use strategies, ranging from *Gypsophila struthium*, which has the highest $\delta^{13}C$ values and therefore is the most water-conservative species, to *Helianthemum squamatum*, which showed the lowest $\delta^{13}C$ values and is thus the most water-profligate species (Moreno-Gutiérrez et al., 2012). The large differences in mean foliar $\delta^{13}C$ values among coexisting species (up to 4-5‰) are in agreement with leaf-gas exchange data, with *Gypsophila struthium* showing the lowest mean g_s and highest WUEi values, and *Heliathemum squamatum* showing the opposite strategy (high g_s and low WUEi). The profligate or opportunistic water use strategy typical of *H. squamatum* (high gs and low WUE) allows plants to take full advantage of the relatively short windows of opportunity when soil moisture is plentiful (generally after large rainfall pulses in late winter and early spring), by maximizing their

water uptake, net photosynthesis rate and growth when soil moisture. In contrast, the conservative water use strategy of *Gypsophila struthium* allows plants to maintain photosynthetic activity well into the dry season (thus extending the length of the growing season), and thus allows them to take advantage of sporadic rains during late spring and summer when profligate/opportunistic species are generally physiologically inactive or dormant (Moreno-Gutiérrez et al., 2012).

Across species, significantly lower foliar δ^{13}C values were found in warmed plants (W and W+RR) than in plants exposed to current temperatures (Control and RR) at the end of the 4-year study period, which indicates a reduction of time-integrated water-use efficiency in response to elevated temperature (Scheidegger et al., 2000; Cernusak et al., 2013). This interpretation of leaf δ^{13}C data is in good agreement with (and well supported by) leaf gas exchange data, thus confirming that leaf carbon isotope composition provides a useful tool to assess the time-integrated ecophysiological response of plants to environmental change across phylogenetically distant plant species with contrasting life history traits and water use strategies (Dawson et al., 2002; Ferrio et al., 2005).

Climate change effects on shoot biomass, elongations and post-summer survival

In agreement with a large body of literature (e.g. Peñuelas et al., 2009 and references therein), warming caused a phenological advance in shoot elongation rates across species. Thereby, plants exposed to climate warming (W and W+RR treatments) showed an advanced growth phenology relative to plants exposed to current ambient temperatures (Control and RR), in response to the optimal temperatures reached earlier in the growing season (late winter and early spring) within the OTCs . However, in the latter part of spring, plants in the W and W+RR treatments decreased their shoot elongation rates across species relative to plants in the Control and RR plots, due to an earlier onset of severe heat and drought stress during late spring compared to plants exposed to current ambient temperatures.

Across species, both total shoot dry biomass and leaf dry biomass production per unit shoot length were significantly reduced by all the climate manipulation treatments (W, RR and W+RR) relative to the controls. This finding is consistent with the cross-species decreases in leaf nutrient status and net photosynthetic rates reported above, and is in

agreement with the results of similar recent studies conducted in other semiarid ecosystems (Adams et al., 2015, Wertin et al., 2016). However, the large decreases in aboveground biomass production with warming (W and W+RR treatments) found across species in this semiarid shrubland community stand in stark contrast with the results of similar studies conducted in colder and wetter ecosystems (e.g. arctic, boreal, alpine and temperate areas), where climate warming usually enhances plant productivity and growth as a result of increases in soil microbial activity and nutrient mineralization (which increases nutrient cycling rates and availability for plants; Rustad et al., 2001), enhanced photosynthesis rates and/or extended growing seasons (Kudo & Suzuki 2003; Peñuelas et al., 2004; Hudson et al., 2009).

Plant survival rates were assessed in October 2015, after the warmest year (and hottest summer) of the study period (as well as the warmest year on record globally according to NOAA 2015). Plant survival rate was significantly reduced by experimental warming (W and, especially, W+RR treatments) across the 6 target species, with no significant *Warming x Species* interaction (despite some species-specific differences in this respect, see Fig. 6). Enhanced plant mortality in the W+RR and W treatments across species is presumably the final outcome of all the cumulative negative impacts of warming on plant performance that have been discussed above (including decreased leaf nutrient status, photosynthesis rates, water use efficiency and biomass growth) throughout the 4 year study period. In addition, warming-induced soil drying and faster and earlier depletion of soil moisture due to increased stomatal conductance and transpiration under warming may have further contributed to increased drought stress, hydraulic failure and drought mortality during the summer in the W and W+RR treatments (McDowell et al., 2011).

Final remarks

In cool and humid ecosystems, community-level studies found that climate warming generally has positive effects on plant nutrient status, net photosynthesis and primary production (Chapin et al., 1995; Kudo & Suzuki 2003). However, in these mesic ecosystems, water is not the major limiting factor for plant productivity, furthermore, climate warming would push plants closer to their optimum temperature for photosynthesis, which would be beneficial for them. Likewise, in Mediterranean-type semiarid ecosystems where plants are already near their optimum temperature for

photosynthesis, a rise in temperature might still lead to an enhanced photosynthetic activity in winter (Galmés et al., 2012). However, climate warming would exacerbate the negative effects of heat and drought stress during the rest of the year and would push temperatures above the optimum for photosynthetic activity, thus resulting in strong negative impacts on the physiological performance and productivity of native vegetation. Accordingly, enhanced photosynthesis activity and growth during winter caused by warming might not offset the increased heat and drought stress suffered during spring and summer by warmed plants, since chronic drought is a much more important limitation of annual carbon gain than cold winter temperatures in Mediterranean ecosystems (Flexas et al., 2001).

Several studies carried out on Mediterranean plant communities have found that coexisting plant species usually respond differently to climate change (Llorens et al., 2003; Llorens et al., 2004; Peñuelas et al., 2004, Sardans et al., 2008a, b). However, in our study, we have found that 6 coexisting plant species belonging to different families and functional groups (with contrasting plant size, life history traits, stoichiometry, water use strategy and mycorrhizal association type) showed rather similar and consistent performance patterns in response to simulated climate change. Despite some relatively minor species-specific differences, all the target species showed roughly similar patterns of response to experimental warming, including decreased leaf nutrient concentrations, advanced growth phenology, decreased late spring shoot elongation, reduced time-integrated water use efficiency (as indicated by decreased leaf $\delta^{13}C$), decreased shoot biomass production and decreased post-summer survival rates. In addition, three of the target species also showed an altered leaf gas exchange pattern throughout the study, characterized by strong reductions in net photosynthesis rates, quantum efficiency of photosystem II and intrinsic water use efficiency, and by moderate increases in stomatal conductance.

The combination of warming and rainfall reduction (W+RR treatment) generally had the strongest detrimental effects on plant performance across species, although there were no statistically significant differences between these plants and plants exposed only to climate warming (W treatment) according to post-hoc tests. The most detrimental effects of the combination of warming and rainfall reduction were found on shoot biomass production and, especially, post-summer survival. Across species, W+RR

plants had on average 15% lower shoot dry biomass production and 35% lower post-summer survival rate than W plants exposed only to climate warming. Average foliar N, Fe, Cu and Zn concentrations were also generally lowest in W+RR plants across species. The lowest average values of net photosynthesis rate and intrinsic water-use efficiency were also found in W+RR plants across species (albeit leaf gas exchange data are available for only 3 species). However, we did not find much evidence that the combination of warming and rainfall reduction had synergistic (mutually reinforcing) detrimental effects on plant performance. Instead, the combined effects of warming and rainfall reduction on most plant response variables were additive (or not even that) rather than synergistic.

In conclusion, the long-term evolutionary adaptation of Mediterranean semiarid plant species to nearly chronic drought stress enables them to survive under severe drought conditions for long time periods under current temperature conditions (Bussotti et al., 2014). However, the predicted temperature rise with climate change might cause the exceedance of the threshold of plant phenotypic adaptation/plasticity across coexisting native species, leading to a strong detrimental feedback mechanism whereby plants will need to transpire more water in order to gain less carbon in the forecasted climatic scenario characterized by warmer and drier conditions. The large and consistent cross-species decreases in plant nutrient status, photosynthetic activity and water use efficiency encountered in this study in response to experimental climate warming will thus likely have strong negative impacts on the growth and survival of native semiarid plant species in the mid- to long-terms.

Chapter III

References

Adams HD, Collins AD, Briggs SP, Vennetier M, Dickman LT, Sevanto SA, McDowell NG (2015) Experimental drought and heat can delay phenological development and reduce foliar and shoot growth in semiarid trees. *Global Change Biology*, **21**, 4210-4220.

Aschmann H (1973) Distribution and peculiarity of Mediterranean ecosystems. In Mediterranean type ecosystems (pp. 11-19). Springer Berlin Heidelberg.

Boyer, J. S (1982) Plant productivity and environment. *Science*, **218**, 443-448.

Brundrett MC (2009) Mycorrhizal associations and other means of nutrition of vascular plants: understanding the global diversity of host plants by resolving conflicting information and developing reliable means of diagnosis. *Plant Soil*, **320**, 37–77.

Bussotti F, Ferrini F, Pollastrini M, Fini A (2014) The challenge of Mediterranean sclerophyllous vegetation under climate change: From acclimation to adaptation. *Environmental and Experimental Botany*, **103**, 80-98.

Cakmak I (2005) The role of potassium in alleviating detrimental effects of abiotic stresses in plants. *Journal of Plant Nutrition and Soil Science*, **168**, 521–530.

Cernusak LA, Ubierna N, Winter K, Holtum JA, Marshall JD, Farquhar GD (2013) Environmental and physiological determinants of carbon isotope discrimination in terrestrial plants. *New Phytologist*, **200**, 950-965.

Chandler JW, Dale JE (1995) Nitrogen deficiency and fertilization effects on needle growth and photosynthesis in Sitka spruce (Picea sitchensis). *Tree Physiology*, **15**, 813-817.

Chapin III FS, Shaver GR, Giblin AE, Nadelhoffer KJ, Laundre JA (1995) Responses of arctic tundra to experimental and observed changes in climate. *Ecology*, **76**, 694-711.

Collins M, Knutti R, Arblaster J *et al.* (2013) Climate change 2013: the physical science basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. *Long-term Climate Change: Projections,*

Chapter III

Commitments and Irreversibility, Cambridge University Press, Cambridge, UK, and New York.

Dawson TE, Mambelli S, Plamboeck AH, Templer PH, Tu KP (2002) Stable isotopes in plant ecology. *Annual review of ecology and systematics*, 507-559.

Dijkstra FA, Pendall E, Morgan JA, Blumenthal DM, Carrillo Y, LeCain D. R, Follett RF, Williams DG (2012) Climate change alters stoichiometry of phosphorus and nitrogen in a semiarid grassland. *New Phytologist*, **196**, 807-815.

Escudero A, Palacio S, Maestre FT, Luzuriaga AL (2015) Plant life on gypsum: a review of its multiple facets. *Biological Reviews*, **90**, 1-18.

Ferrio JP, Resco V, Williams DG, Serrano L, Voltas J (2005) Stable isotopes in arid and semi-arid forest systems. *Forest Systems*, **14**, 371-382.

Flexas J, Gulías J, Jonasson S, Medrano H, Mus M (2001) Seasonal patterns and control of gas exchange in local populations of the Mediterranean evergreen shrub *Pistacia lentiscus L. Acta Oecologica*, **22**, 33-43.

Flexas J, Medrano H (2002) Drought-inhibition of photosynthesis in C3 plants: stomatal and non-stomatal limitations revisited. *Annals of botany*, **89**, 183-189.

Galmés J, Flexas J, Medrano H, Niinemets Ü, Valladares F (2012) Ecophysiology of photosynthesis in semi-arid environments. Terrestrial Photosynthesis in a Changing Environment: A Molecular, Physiological, and Ecological Approach, 453-469.

Giorgi F, Lionello P (2008) Climate change projections for the Mediterranean region. *Global and Planetary Change*, **63**, 90-104.

Heimann M, Reichstein M (2008) Terrestrial ecosystem carbon dynamics and climate feedbacks. *Nature*, **451**, 289-292.

Huang ZA, Jiang DA, Yang Y, Sun JW, Jin SH (2004) Effects of nitrogen deficiency on gas exchange, chlorophyll fluorescence, and antioxidant enzymes in leaves of rice plants. *Photosynthetica*, **42**, 357-364.

Chapter III

Hudson JM, Henry GHR (2009) Increased plant biomass in a High Arctic heath community from 1981 to 2008. *Ecology*, **90**, 2657-2663.

Kudo G, Suzuki S (2003) Warming effects on growth, production, and vegetation structure of alpine shrubs: a five-year experiment in northern Japan. *Oecologia*, **135**, 280-287.

Llorens L, Penuelas J, Estiarte M (2003) Ecophysiological responses of two Mediterranean shrubs, Erica multiflora and Globularia alypum, to experimentally drier and warmer conditions. *Physiologia plantarum*, **119**, 231-243.

Llorens L, Penuelas J, Estiarte M, Bruna P (2004) Contrasting growth changes in two dominant species of a Mediterranean shrubland submitted to experimental drought and warming. *Annals of Botany*, **94**, 843-853.

Maestre FT, Escolar C, Guevara ML, Quero JL, Lázaro R, Delgado-Baquerizo M, Ochoa V, Berdugo M, Gozalo B, Gallardo A (2013) Changes in biocrust cover drive carbon cycle responses to climate change in drylands. *Global change biology*, **19**, 3835-3847.

Marschner H (2011). Marschner's mineral nutrition of higher plants. Academic press.

McDowell NG, Beerling DJ, Breshears DD, Fisher RA, Raffa KF, Stitt M (2011) The interdependence of mechanisms underlying climate-driven vegetation mortality. *Trends in Ecology & Evolution,* **26**, 523-532.

Milla R, Castro-Díez P, Maestro-Martínez M, Montserrat-Martí G (2005) Relationship between phenology and the remobilization of nitrogen,phosphorus and potassium in branches of eight Mediterranean evergreens. *New Phytologist*, **168**, 167–178.

Miranda JDD, Armas C, Padilla FM, Pugnaire FI (2011) Climatic change and rainfall patterns: effects on semi-arid plant communities of the Iberian Southeast. *Journal of Arid Environments*, **75**, 1302-1309.

Moreno JC (2010) Lista Roja 2008 de la flora vascular española.

Chapter III

Moreno-Gutiérrez C, Dawson TE, Nicolás E, Querejeta JI (2012) Isotopes reveal contrasting water use strategies among coexisting plant species in a Mediterranean ecosystem. *New Phytologist*, **196**, 489-496.

Myers N, Mittermeier RA, Mittermeier CG, Da Fonseca GA, Kent J (2000) Biodiversity hotspots for conservation priorities. *Nature*, **403**, 853-858.

Nardini A, Lo Gullo MA, Trifilò P, Salleo S (2014) The challenge of the Mediterranean type climate to plant hydraulics: responses and adaptations. *Environmental and Experimental Botany*, **103**, 68–79.

Nicotra AB, Atkin OK, Bonser SP, Davidson AM, Finnegan EJ, Mathesius U. *et al.* (2010) Plant phenotypic plasticity in a changing climate. *Trends in Plant Science*, **15**, 684–692.

NOAA National Centers for Environmental Information, State of the Climate: Global Analysis for Annual 2015, published online January 2016, retrieved on July 4, 2016 from http://www.ncdc.noaa.gov/sotc/global/201513.

Peñuelas J, Gordon C, Llorens L, Nielsen T, Tietema A, Beier C, Gorissen A (2004) Nonintrusive field experiments show different plant responses to warming and drought among sites, seasons, and species in a north–south European gradient. *Ecosystems*, 7, 598-612.

Peñuelas, J. & Filella, I (2009) Phenology feedbacks on climate change. *Science*, **324**, 887.

Porta J, López-Acevedo M, Roquero C (1994) Edafología para la agricultura y el medio ambiente. 3ª Edición. Ed. Mundi-Prensa, Madrid.

Prieto P, Peñuelas J, Llusià J, Asensio D, Estiarte M (2009) Effects of experimental warming and drought on biomass accumulation in a Mediterranean shrubland. *Plant Ecology*, **205**, 179-191.

R Development Core Team. 2013. R: A Language and Environment for Statistical Computing. Vienna, Austria.

Chapter III

Reich PB, Oleksyn J (2004) Global patterns of plant leaf N and P in relation to temperature and latitude. *Proceedings of the National Academy of Sciences of the United States of America*, **101**, 11001-11006.

Reich PB, Oleksyn J, Wright IJ (2009) Leaf phosphorus influences the photosynthesis–nitrogen relation: a cross-biome analysis of 314 species. *Oecologia*, **160**, 207–212.

Rivas-Ubach A, Sardans J, Pérez-Trujillo M, Estiarte M, Peñuelas J (2012) Strong relationship between elemental stoichiometry and metabolome in plants. *Proceedings of the National Academy of Sciences*, **109**, 4181-4186.

Rubio A, Escudero A (2000) Small-scale spatial soil-plant relationship in semi-arid gypsum environments. *Plant and Soil*, **220**, 139-150.

Safriel UN, Adeel Z (2005) Dryland Systems. In Ecosystems and Human Well-being: Current State and Trends. Vol. 1, edited by R. Hassan, R. Scholes, & N. Ash, 623–662.

Sage RF, Kubien DS (2007) The temperature response of C3 and C4 photosynthesis. *Plant, Cell & Environment*, **30,** 1086–1106.

Sardans J, Peñuelas J, Estiarte M, Prieto P (2008) Warming and drought alter C and N concentration, allocation and accumulation in a Mediterranean shrubland. *Global Change Biology*, **14**, 2304-2316.

Sardans J, Peñuelas J, Prieto P, Estiarte M (2008) Drought and warming induced changes in P and K concentration and accumulation in plant biomass and soil in a Mediterranean shrubland. *Plant and Soil*, **306**, 261-271.

Sardans J, Rivas-Ubach A, Peñuelas J (2012) The C: N: P stoichiometry of organisms and ecosystems in a changing world: a review and perspectives. *Perspectives in Plant Ecology, Evolution and Systematics*, **14**, 33-47.

Sardans J, Peñuelas J (2013) Plant-soil interactions in Mediterranean forest and shrublands: impacts of climatic change. *Plant and Soil*, **365**, 1-33.

Chapter III

Sardans J, Alonso R, Carnicer J, Fernández-Martínez M, Vivanco MG, Peñuelas J (2016) Factors influencing the foliar elemental composition and stoichiometry in forest trees in Spain. *Perspectives in Plant Ecology, Evolution and Systematics*, **18**, 52-69.

Scheidegger Y, Saurer M, Bahn M, Siegwolf R (2000) Linking stable oxygen and carbon isotopes with stomatal conductance and photosynthetic capacity: a conceptual model. *Oecologia*, **125**, 350-357.

Thomas DS, Montagu KD, Conroy JP (2006) Leaf inorganic phosphorus as a potential indicator of phosphorus status, photosynthesis and growth of Eucalyptus grandis seedlings. *Forest Ecology and Management*, **223**, 267-274.

Wertin TM, Belnap J, Reed SC (2016) Experimental warming in a dryland community reduced plant photosynthesis and soil CO2 efflux although the relationship between the fluxes remained unchanged. *Functional Ecology*. doi:10.1111/1365-2435.12708.

Weltzin JF, Loik ME, Schwinning S, Williams DG, Fay P, Haddad B, Harte J, Huxman TE, Knapp AK, Lin G, Pockman WT, Shaw MR, Small E, Smith MD, Smith SD, Tissue DT, Zak JC (2003) Assessing the response of terrestrial ecosystems to potential changes in precipitation. *BioScience*, **53**, 941–952.

Wright IJ, Reich PB, Westoby M, Ackerly DD, Baruch Z, Bongers F, Cavender-Bares J, Chapin T, Cornelissen JHC *et al.* (2004) The worldwide leaf economics spectrum. *Nature*, **428**, 821–827.

Wright IJ, Reich PB, Cornelissen JHC, Falster DS, Groom PK, Hikosaka K, Lee W, Lusk CH, Niinemets Ü, Oleksyn J, Osada N, Poorter H, Warton DI, Westoby M (2005) Modulation of leaf economic traits and trait relationships by climate. *Global Ecology & Biogeography*, **14**, 411–421.

Zhao D, Reddy KR, Kakani VG, Reddy VR (2005) Nitrogen deficiency effects on plant growth, leaf photosynthesis, and hyperspectral reflectance properties of sorghum. *European Journal of Agronomy*, **22**, 391-403.

Figure 1. Experimental plot of the Warming + Rainfall Reduction treatment, which is achieved by installing an open top chamber (OTC) and a rainout shelter on the same plot.
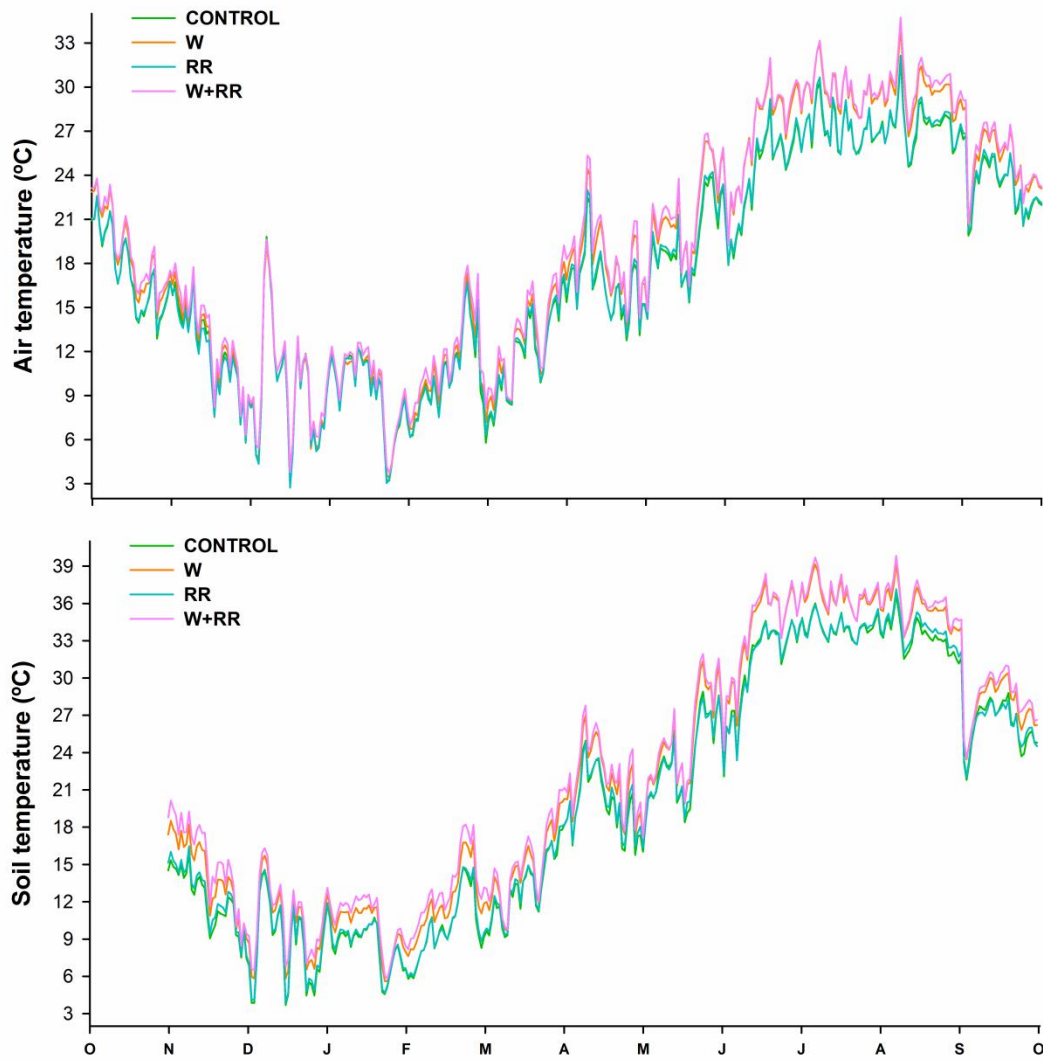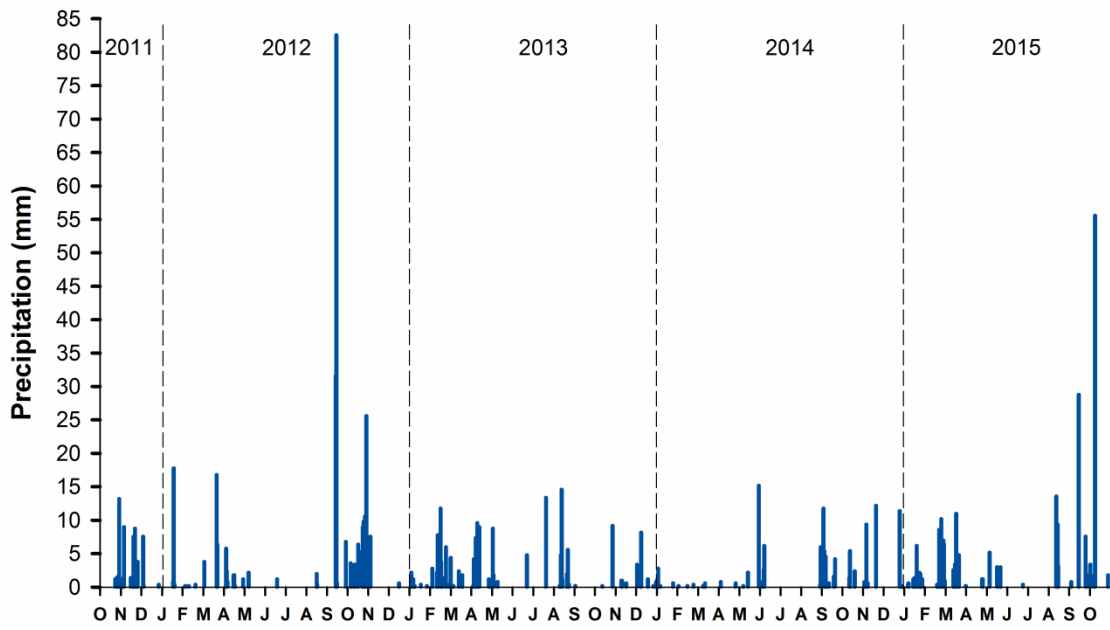
Figure 2. Air and soil (0-5 cm depth) temperature in the different experimental treatments in the hydrological year 2010-2011, showing the effect of the treatments on these variables. W: warming; RR: rainfall reduction; W+RR: warming plus rainfall reduction.
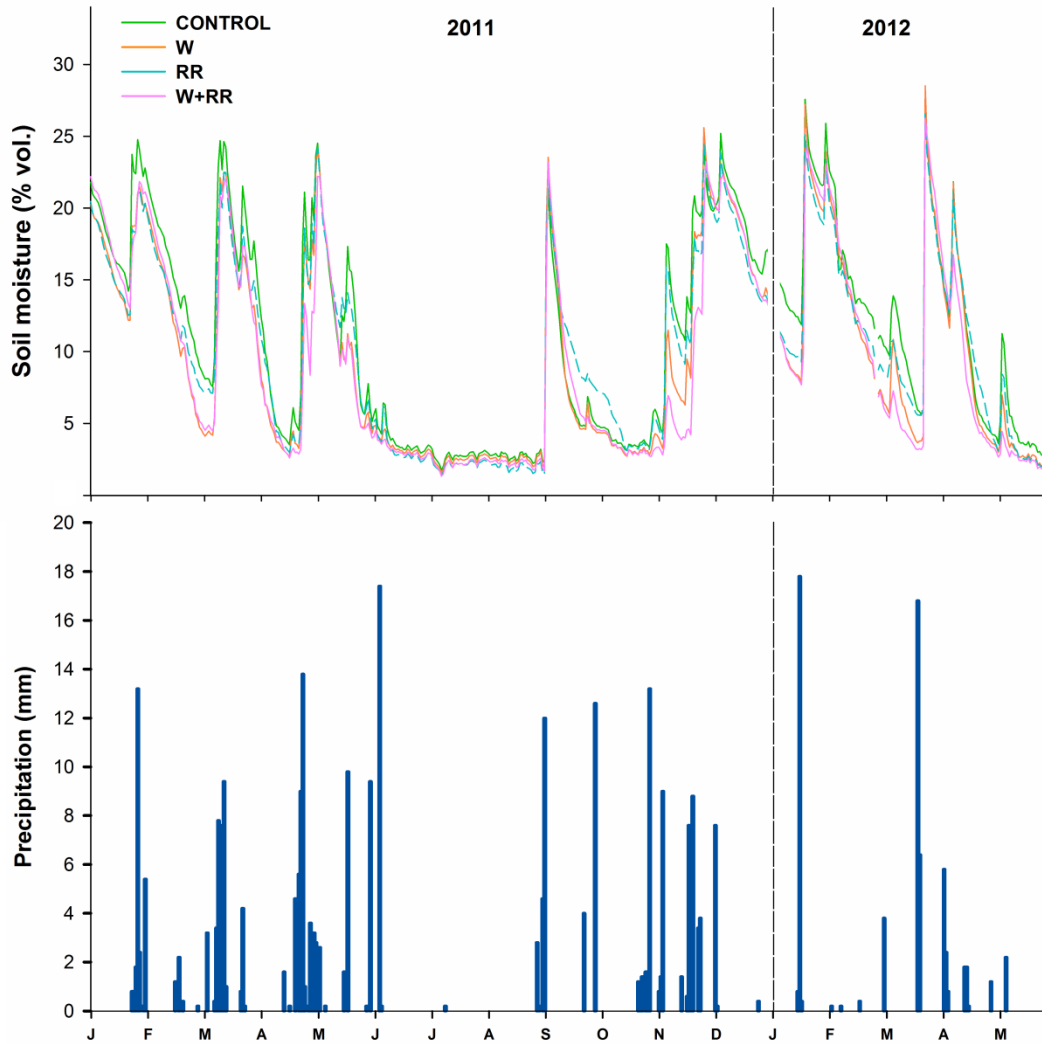
Figure 3. Rainfall amount and distribution throughout the study period.

Figure 4. Rainfall amount and distribution from January 2011 to March 2012, and temporal changes in soil moisture content (0-5 cm depth) in the different experimental treatments. W: warming; RR: rainfall reduction; W+RR: warming plus rainfall reduction.
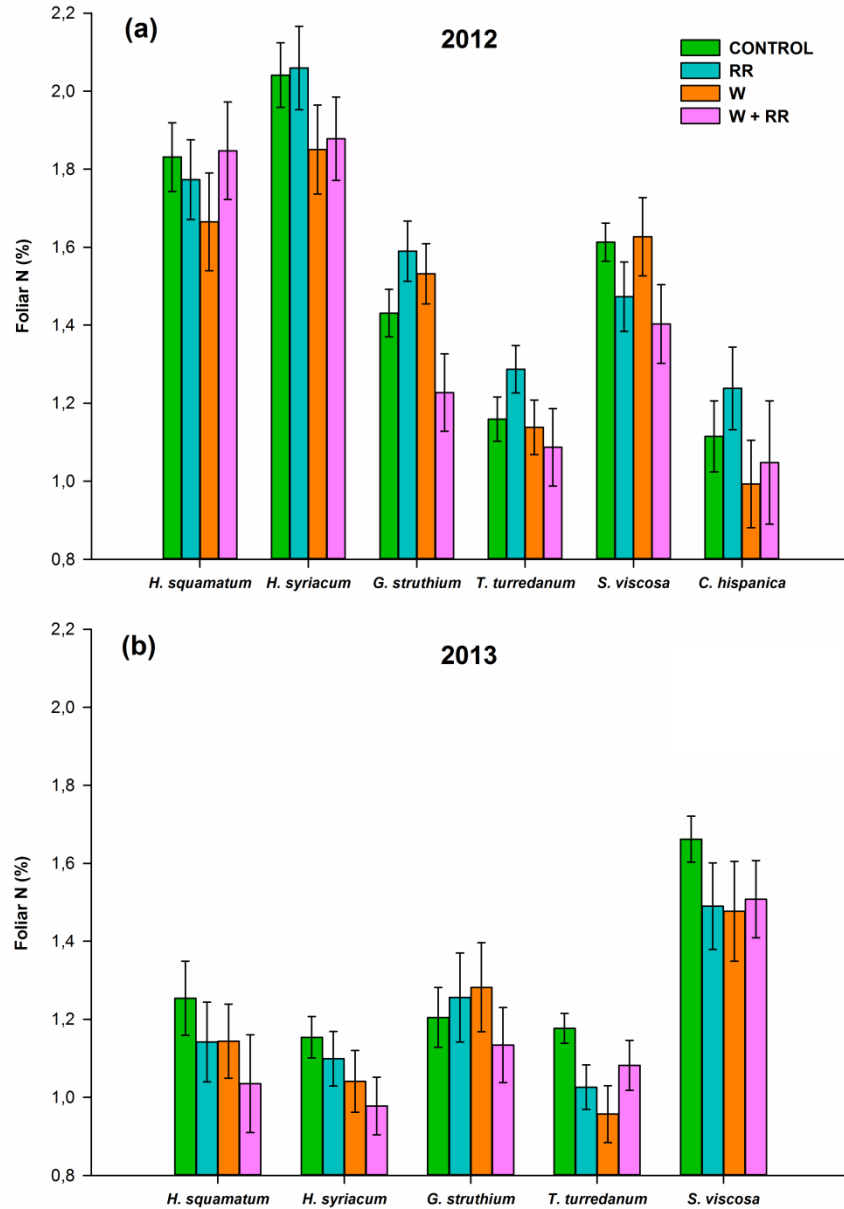
Figure 5 a, b. Leaf nitrogen (N) concentration measured at the peak of the growing season in 2012 and 2013 in *Helianthemum squamatum* (n= 33 in 2012, 26 in 2013), *Helianthemum syriacum* (n= 41, 40)*, Gypsophila struthium* (n= 22, 28), *Teucrium turredanum* (n= 27, 24), *Santolina viscosa* (n= 32, 26) and *Coris hispanica* (only measured in 2012; n= 33) shrubs in the control (C), warming (W), rainfall reduction (RR) and warming + rainfall reduction (W+RR) treatments.
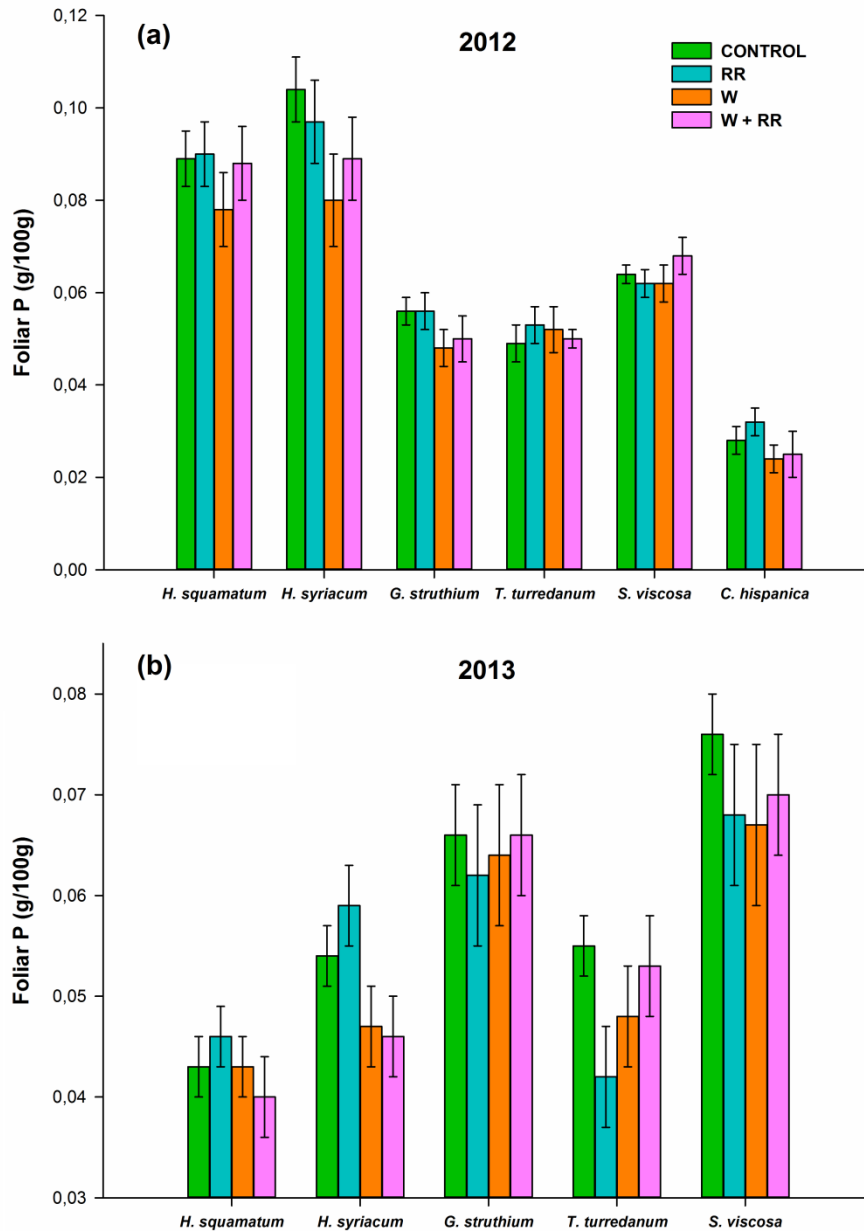
Figure 6 a, b. Leaf phosphorus (P) concentration measured at the peak of the growing season in 2012 and 2013 in *Helianthemum squamatum* (n= 33 in 2012, 26 in 2013), *Helianthemum syriacum* (n= 41,40)*, Gypsophila struthium* (n= 22, 28), *Teucrium turredanum* (n= 27, 24), *Santolina viscosa* (n= 32, 26) and *Coris hispanica* (only measured in 2012; n= 33) shrubs in the control (C), warming (W), rainfall reduction (RR) and warming + rainfall reduction (W+RR) treatments.
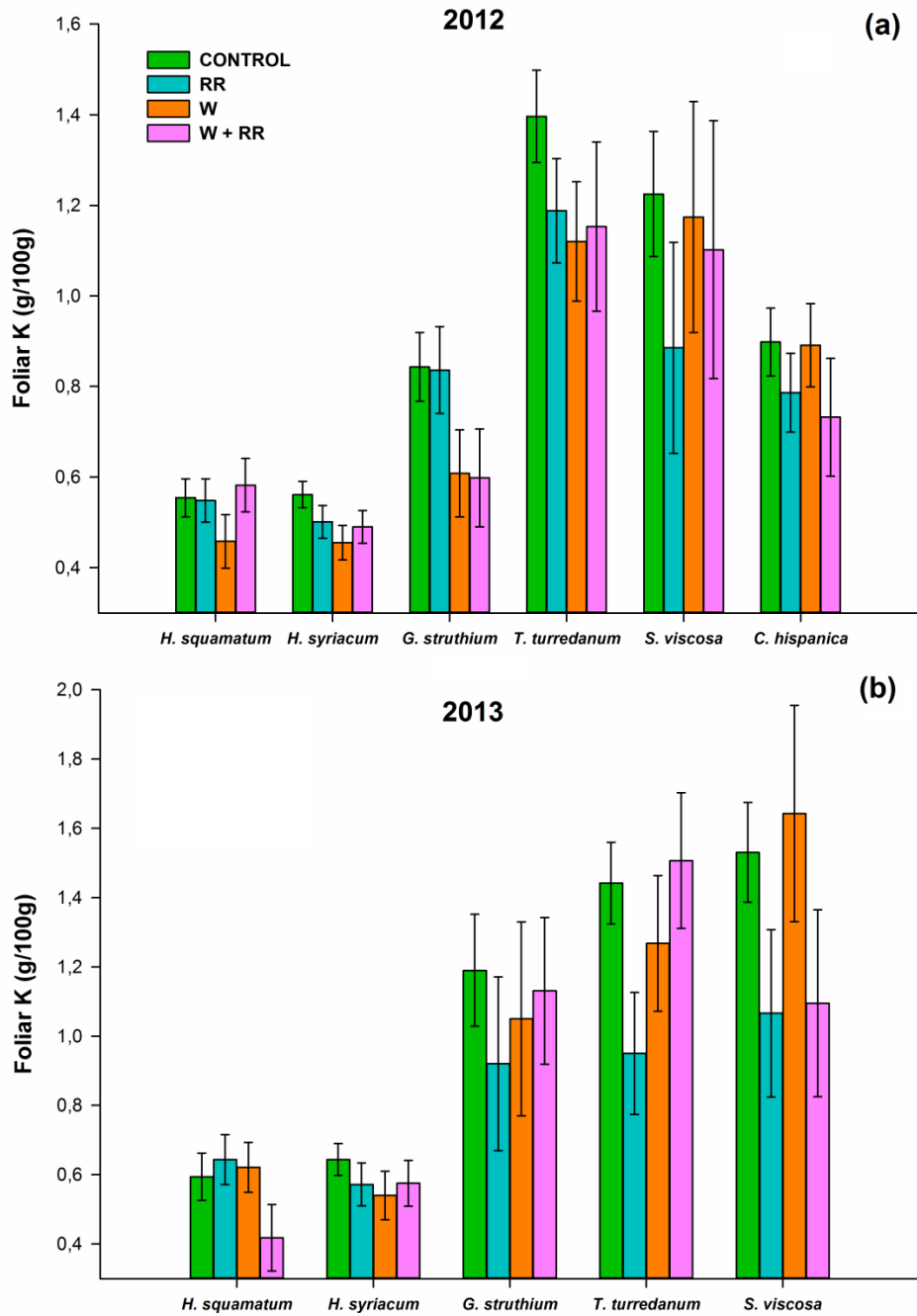
Figure 7 a, b. Leaf potassium (K) concentration measured at the peak of the growing season in 2012 and 2013 in *Helianthemum squamatum* (n= 33 in 2012, 26 in 2013), *Helianthemum syriacum* (n= 41,40)*, Gypsophila struthium* (n= 22, 28), *Teucrium turredanum* (n= 27, 24), *Santolina viscosa* (n= 32, 26) and *Coris hispanica* (only measured in 2012; n= 33) shrubs in the control (C), warming (W), rainfall reduction (RR) and warming + rainfall reduction (W+RR) treatments.
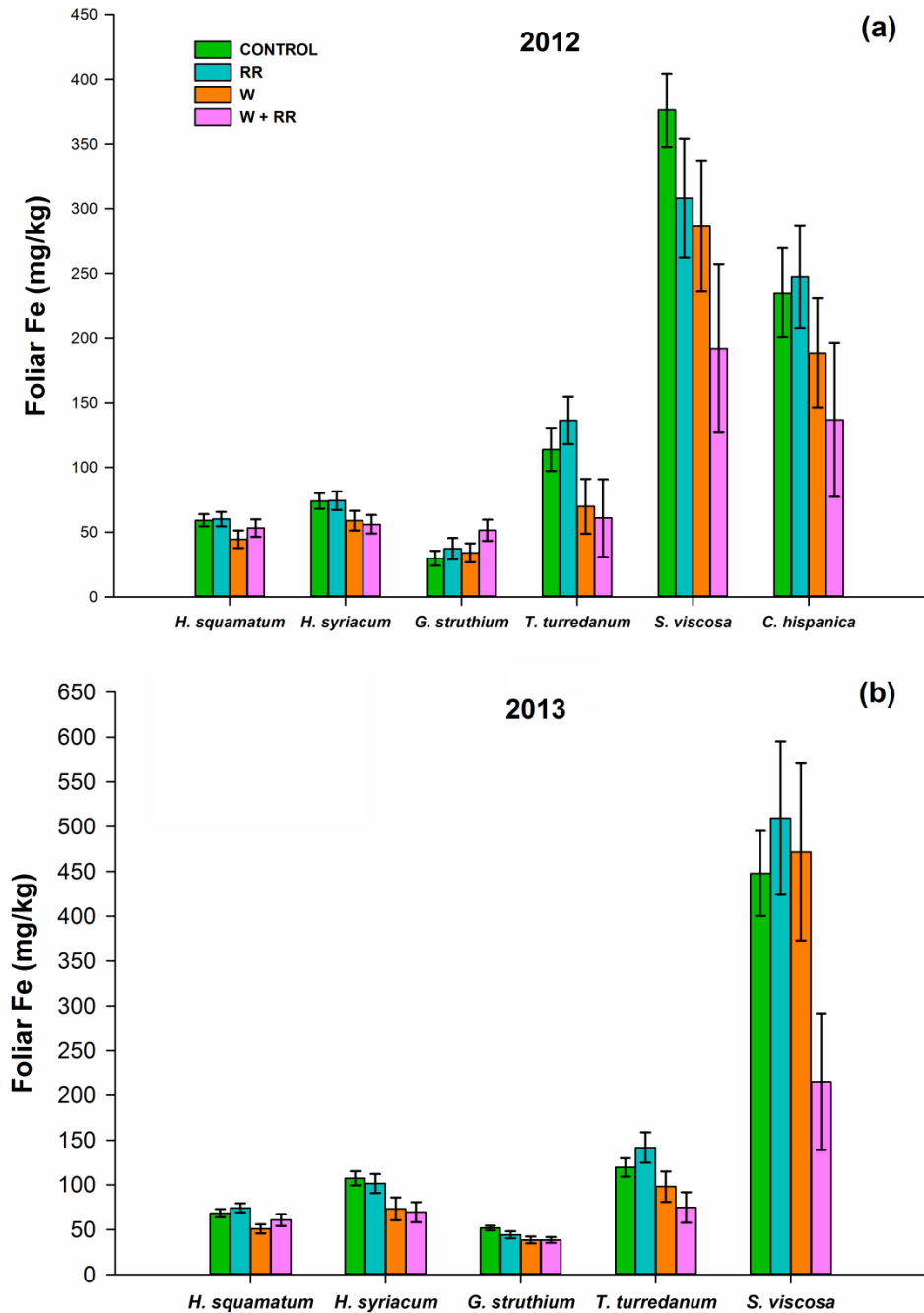
Figure 8 a, b. Leaf iron (Fe) concentration measured at the peak of the growing season in 2012 and 2013 in *Helianthemum squamatum* (n= 33 in 2012, 26 in 2013), *Helianthemum syriacum* (n= 41,40)*, Gypsophila struthium* (n= 22, 28), *Teucrium turredanum* (n= 27, 24), *Santolina viscosa* (n= 32, 26) and *Coris hispanica* (only measured in 2012; n= 33) shrubs in the control (C), warming (W), rainfall reduction (RR) and warming + rainfall reduction (W+RR) treatments.
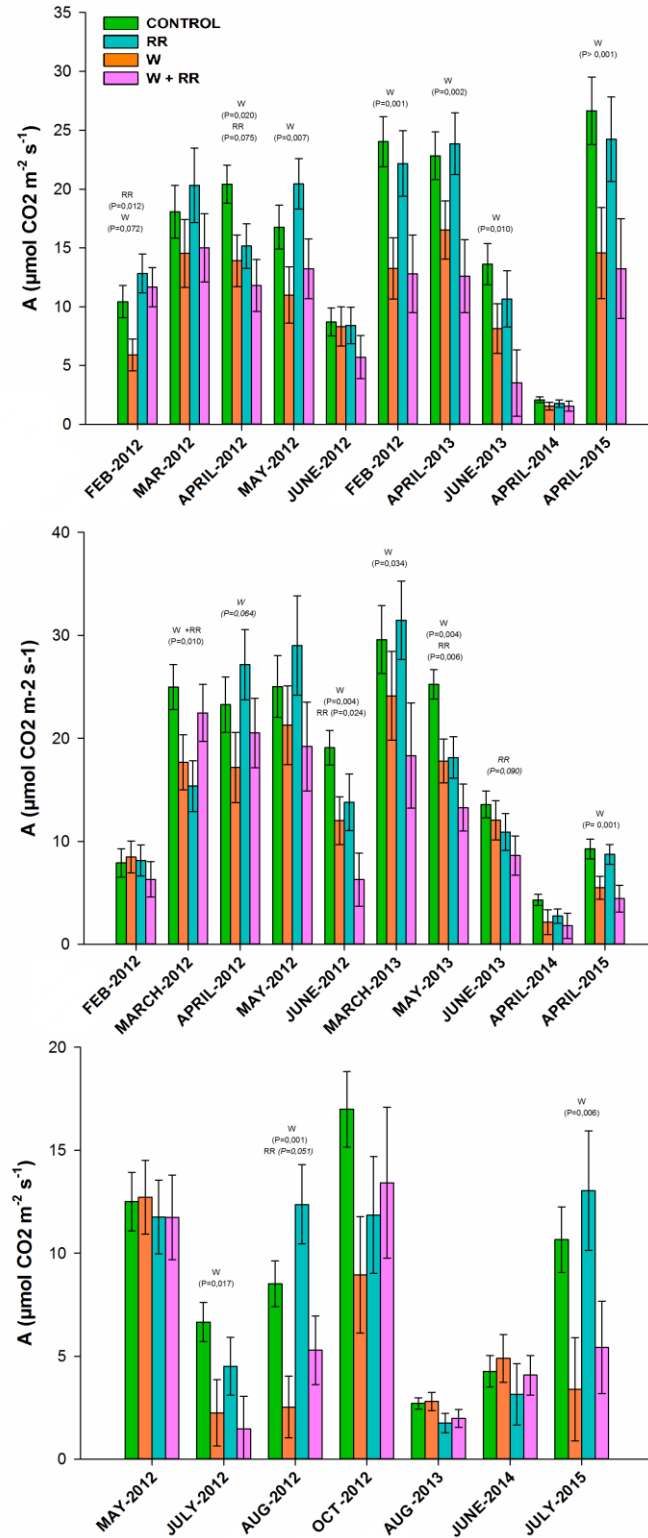
Figure 9 a, b, c. Mean net photosynthesis rate of *Helianthemum squamatum* (n= 30-35), *Helianthemum syriacum* (n= 30-40) and *Gypsophila struthium* (n= 20-25) throughout the four years of the study. W: warming; RR: rainfall reduction; W+RR: warming plus rainfall reduction. Significant factors are shown above columns.
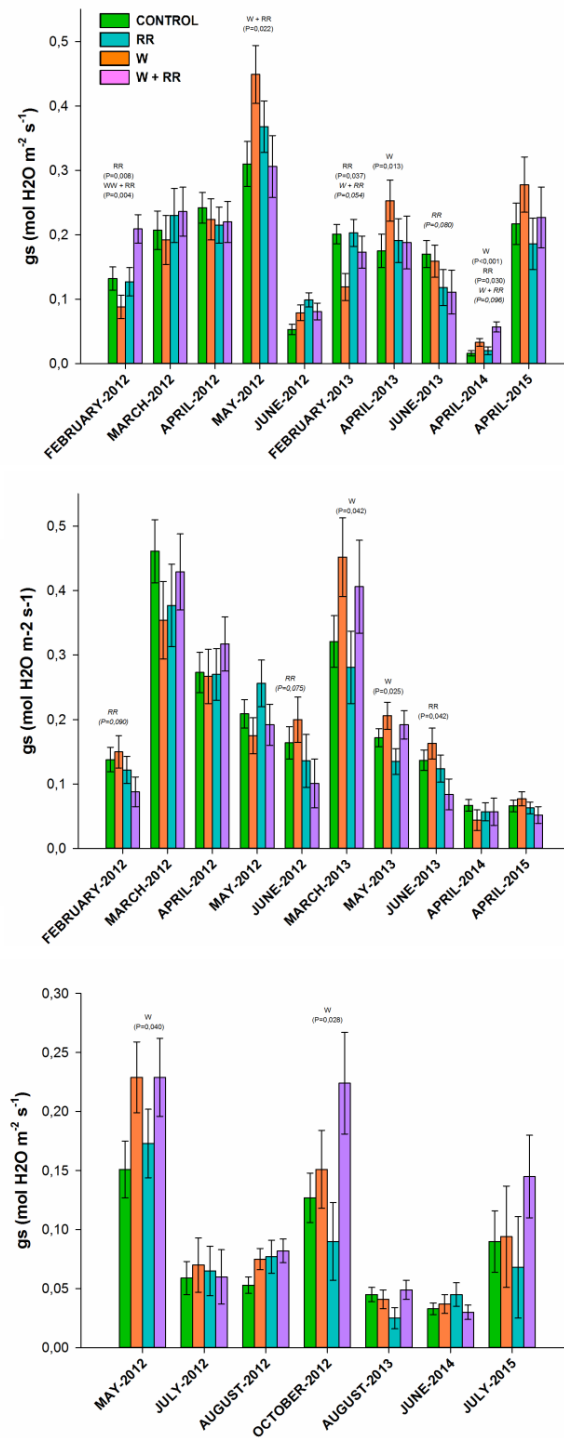
Figure 10 a, b, c. Stomatal conductance of *Helianthemum squamatum* (n= 30-35), *Helianthemum syriacum* (n= 30-40) and *Gypsophila struthium* (n= 20-25) throughout the four years of the study. W: warming; RR: rainfall reduction; W+RR: warming plus rainfall reduction. Significant factors are shown above columns.
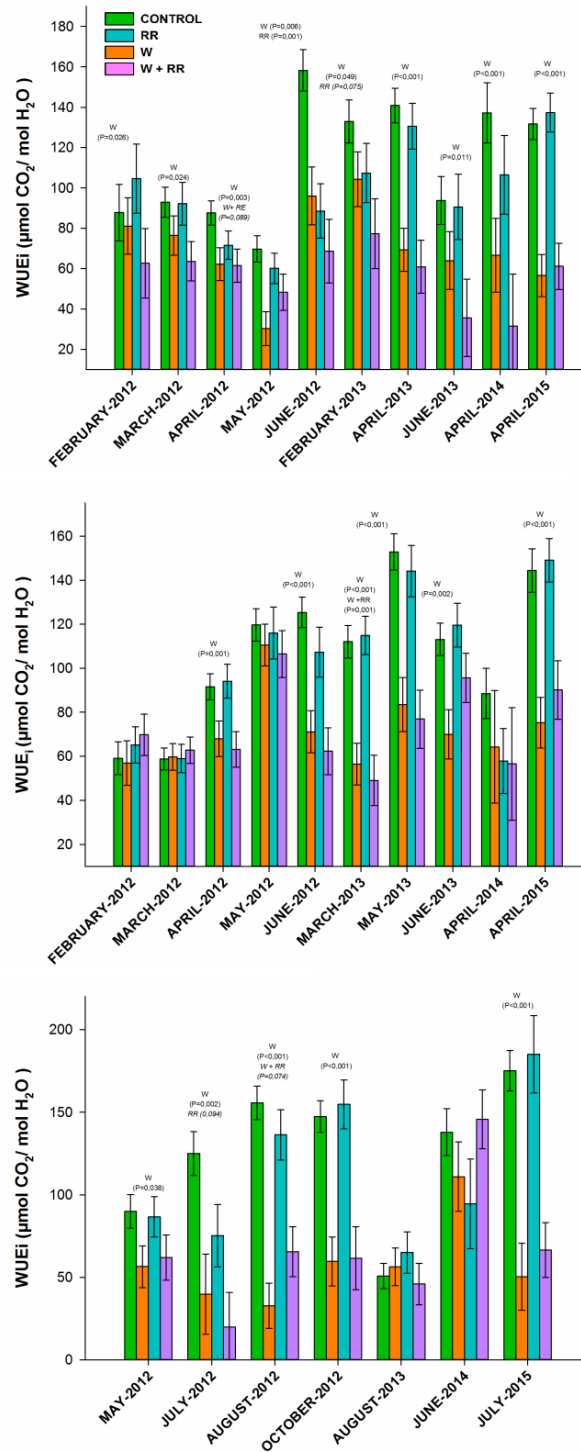
Figure 11 a, b, c. Intrinsic water use efficiency of *Helianthemum squamatum* (n= 30-35)*, Helianthemum syriacum* (n= 30-40) and *Gypsophila struthium* (n= 20-25) throughout the four years of the study. W: warming; RR: rainfall reduction; W+RR: warming plus rainfall reduction. Significant factors are shown above columns.
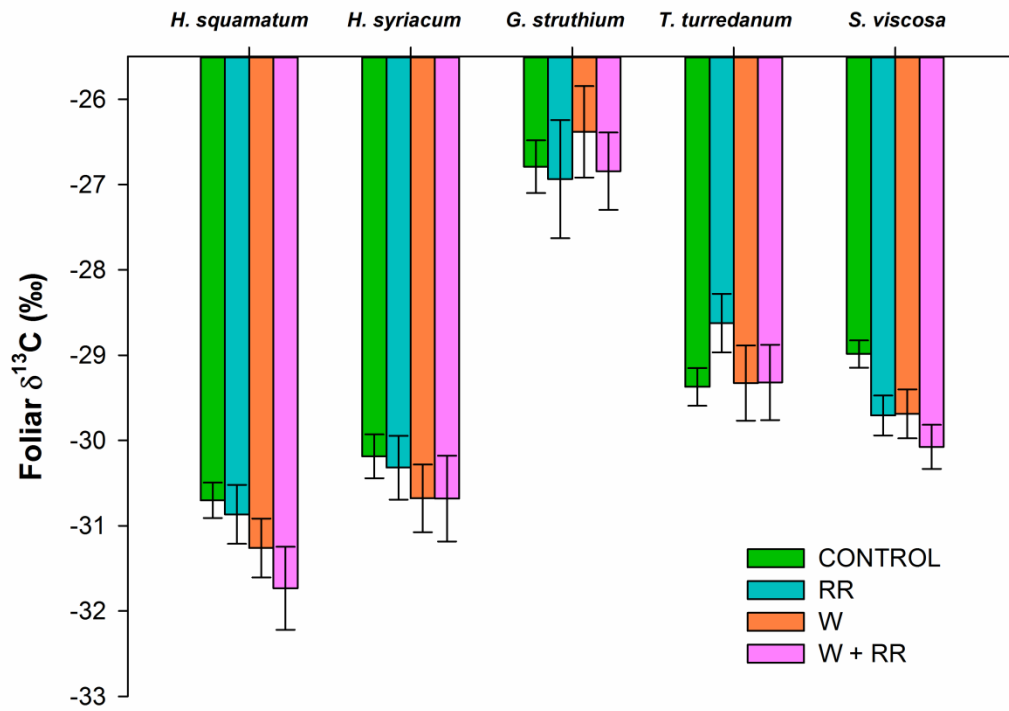
Figure 12. Mean leaf carbon isotope ratios (δ^{13}C) in *Helianthemum squamatum* (n= 32), *Helianthemum syriacum* (n= 41), *Gypsophila struthium* (n= 31), *Teucrium turredanum* (n= 24) and *Santolina viscosa* (n= 30) at the end of the 4-year study period (October 2015). W: warming; RR: rainfall reduction; W+RR: warming plus rainfall reduction.
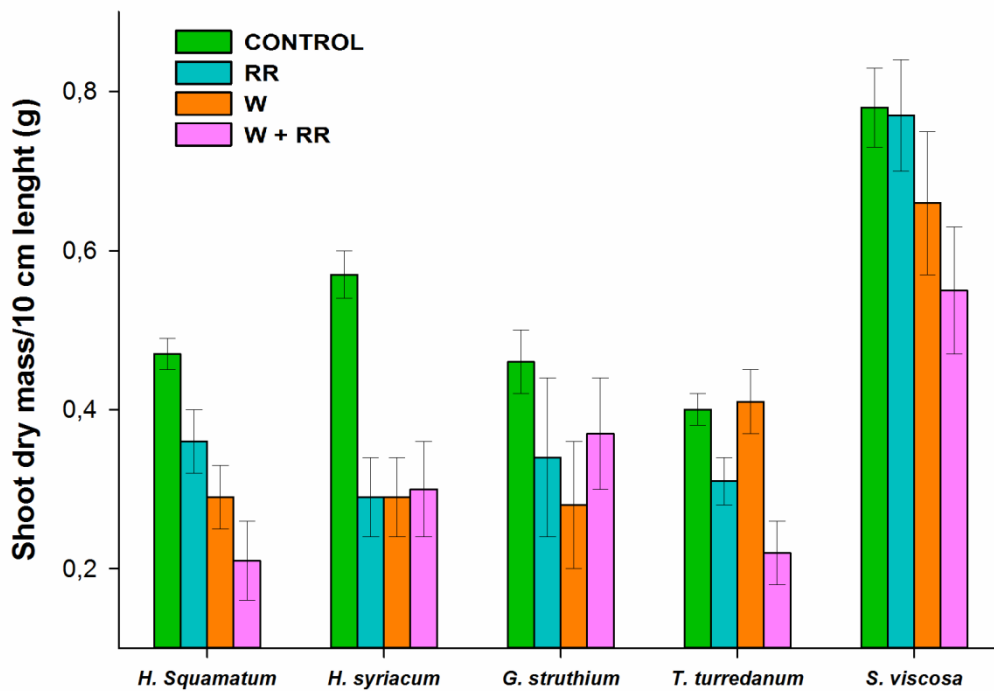
Figure 13. Mean dry biomass production (leaves plus stems) per unit shoot length in *Helianthemum squamatum* (n= 32), *Helianthemum syriacum* (n= 41), *Gypsophila struthium* (n= 31), *Teucrium turredanum* (n= 24) and *Santolina viscosa* (n= 30) at the end of the four year study period (October 2015). W: warming; RR: rainfall reduction; W+RR: warming plus rainfall reduction.
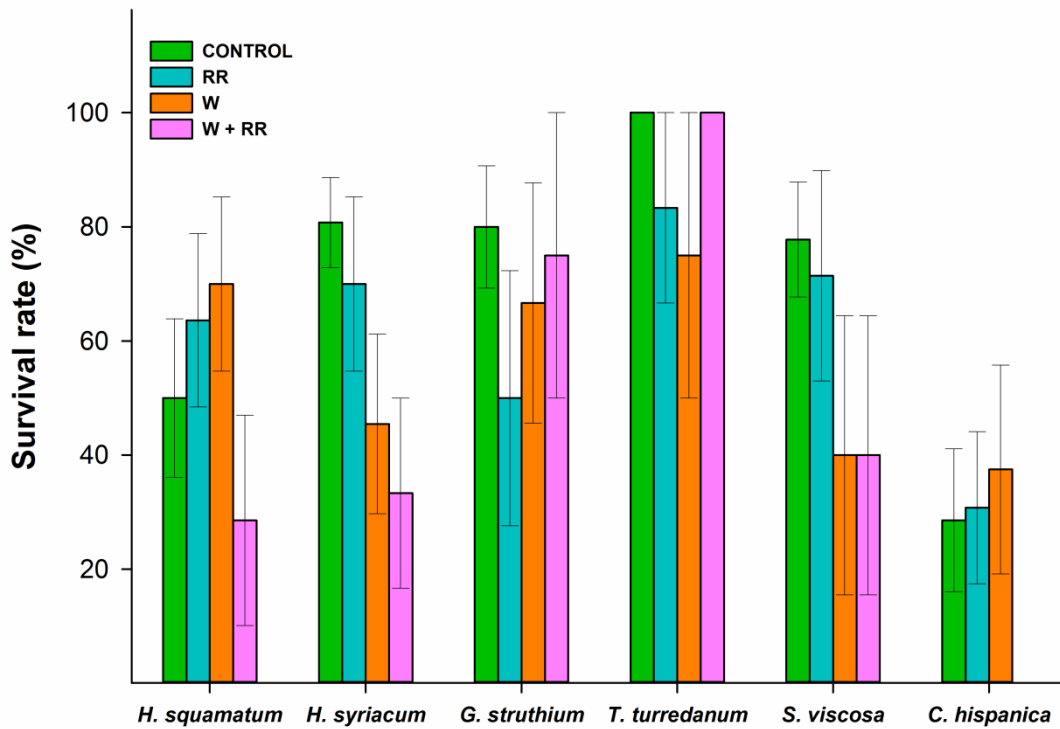
Figure 14. Mean plant survival rate at the end of the 4-year experimental period (October 2015) in *Helianthemum squamatum* (n= 42), *Helianthemum syriacum* (n= 56), *Gypsophila struthium* (n= 31), *Teucrium turredanum,* (n= 26), *Santolina viscosa* (n= 35) and *Coris hispanica* (n= 41). W: warming; RR: rainfall reduction; W+RR: warming plus rainfall reduction. No *Coris hispanica* individuals survived in the W+RR treatment.

		N	P	K	Fe	Cu	Zn	Ca	Mg	S
W	F	8.910	6.670	0.304	20.659	7.259	15.549	0.533	0.006	0.044
	P	**0.003**	**0.010**	0.582	**< 0.001**	**0.008**	**< 0.001**	0.466	0.938	0.834
RR	F	1.054	0.221	5.641	3.006	7.147	0.062	1.917	0.009	1.632
	P	0.306	0.638	**0.018**	0.084	**0.008**	0.803	0.167	0.924	0.203
W*RR	F	0.198	1.922	3.044	4.511	1.148	3.941	0.407	0.185	0.184
	P	0.657	0.167	0.082	**0.035**	0.285	**0.048**	0.524	0.668	0.668
Species	F	21.864	43.592	48.171	130.610	71.196	38.482	232.404	148.735	318.614
	P	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
W*Species	F	0.364	2.388	0.450	3.885	0.629	2.881	1.039	0.600	0.944
	P	0.834	0.051	0.772	**0.004**	0.642	**0.023**	0.387	0.663	0.439
RR*Species	F	0.290	0.399	1.889	5.136	4.133	1.469	1.939	0.712	0.356
	P	0.885	0.809	0.113	**0.001**	**0.003**	0.212	0.104	0.584	0.840
Year	F	140.091	43.592	11.846	14.064	1.075	38.702	0.735	56.911	1.203
	P	**< 0.001**	**< 0.001**	**0.001**	**< 0.001**	0.301	**< 0.001**	0.392	**< 0.001**	0.274
W*Year	F	0.178	0.887	1.447	0.328	0.784	0.106	0.897	0.744	1.142
	P	0.673	0.347	0.230	0.567	0.377	0.745	0.344	0.389	0.286
RR*Year	F	0.617	1.420	1.554	0.194	0.126	0.192	0.008	0.182	< 0.001
	P	0.433	0.234	0.214	0.660	0.722	0.662	0.927	0.670	0.991

Table 1. Statistical significance and F-values of the effects of the experimental factors and their interactions on foliar nutrient concentrations across the target plant species. W: warming; RR: rainfall reduction; Species: plant species (*Helianthemum squamatum, Helianthemum syriacum, Gypsophila struthium, Santolina viscosa, Teucrium turredanum*); Year: year of measurement (2012, 2013).

		A	ØPSII	gs	WUEi
W	**F**	61.968	22.768	5.438	307.264
	P	**< 0.001**	**< 0.001**	**0.020**	**< 0.001**
RR	**F**	2.105	0.560	0.937	2.657
	P	0.147	0.454	0.333	0.103
W*RR	**F**	0.034	3.297	0.826	0.994
	P	0.854	0.070	0.425	0.319
Species	**F**	9.700	9.710	5.408	2.291
	P	**< 0.001**	**< 0.001**	**0.005**	0.102
W*Species	**F**	2.267	1.364	1.090	4.124
	P	0.104	0.256	0.337	**0.017**
RR*Species	**F**	0.646	0.843	0.666	2.336
	P	0.524	0.431	0.514	0.097
Time	**F**	21.809	12.455	26.843	10.236
	P	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
W*Time	**F**	1.366	0.806	1.701	8.959
	P	0.051	0.663	**0.038**	**< 0.001**
RR*Time	**F**	0.943	1.668	0.523	1.589
	P	0.522	0.058	0.943	0.061

Table 2. Statistical significance and F-values of the effects of the experimental factors and their interactions on leaf-gas exchange parameters across the target plant species. W: warming; RR: rainfall reduction; Species: plant species (*Helianthemum squamatum, Helianthemum syriacum* and *Gypsophila struthium*; Time: time of measurement (2012, 2013).

		Shoot dry mass	Leaf dry mass	Stem dry mass	Leaf δ^{13}C
W	F	20.891	17.597	8.403	4.405
	P	**< 0.001**	**< 0.001**	**0.004**	**0.038**
RR	F	11.598	12.499	1.151	0.500
	P	**0.001**	**0.001**	0.285	0.481
W*RR	F	1.443	0.866	0.864	0.178
	P	0.232	0.354	0.354	0.674
Species	F	28.401	22.143	11.960	71.370
	P	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
W*Species	F	0.931	0.864	0.735	0.908
	P	0.448	0.488	0.570	0.461
RR*Species	F	0.756	0.760	0.243	0.716
	P	0.556	0.553	0.913	0.583
W*RR*Species	F	2.635	2.478	2.109	0.372
	P	**0.037**	**0.047**	0.083	0.828

Table 3. Effects of the experimental treatments on shoot biomass production and leaf δ^{13}C across the target plant species in 2015. W: warming; RR: rainfall reduction; Species: plant species (*Helianthemum squamatum, Helianthemum syriacum, Gypsophila struthium, Santolina viscosa, Teucrium turredanum*).

		CONTROL	RR	W	W + RR
H. squamatum	Leaf Dry Mass (mg)	0.37 ± 0.02	0.29 ± 0.04	0.24 ± 0.04	0.12 ± 0.05
	Stem Dry Mass (mg)	0.11 ± 0.02	0.07 ± 0.03	0.05 ± 0.03	0.09 ± 0.04
H. syriacum	Leaf Dry Mass (mg)	0.44 ± 0.03	0.21 ± 0.04	0.21 ± 0.04	0.23 ± 0.05
	Stem Dry Mass (mg)	0.12 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.08 ± 0.01
G. struthium	Leaf Dry Mass (mg)	0.36 ± 0.04	0.26 ± 0.09	0.21 ± 0.07	0.29 ± 0.06
	Stem Dry Mass (mg)	0.11 ± 0.01	0.09 ± 0.02	0.07 ± 0.01	0.08 ± 0.01
S. viscosa	Leaf Dry Mass (mg)	0.62 ± 0.04	0.59 ± 0.06	0.51 ± 0.07	0.42 ± 0.07
	Stem Dry Mass (mg)	0.17 ± 0.02	0.18 ± 0.02	0.15 ± 0.03	0.13 ± 0.03
T. turredanum	Leaf Dry Mass (mg)	0.33 ± 0.02	0.24 ± 0.03	0.34 ± 0.04	0.16 ± 0.03
	Stem Dry Mass (mg)	0.08 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.06 ± 0.01

Table 4. Leaf dry mass and stem dry mass per unit shoot length of *Helianthemum squamatum* (n= 32), *Helianthemum syriacum* (n= 41), *Gypsophila struthium* (n= 31), *Teucrium turredanum* (n= 24) and *Santolina viscosa* (n= 30) at the end of the study period. W: warming; RR: rainfall reduction; W+RR: warming plus rainfall reduction.

	Likelihood ratio χ^2	Df	P_value
SPECIES	11.466	4	**0.022**
W	6.6413	1	**0.009**
RR	1.8169	1	0.178
W*RR	0.0009	1	0.976
SPECIES*W	4.5131	4	0.341
SPECIES*RR	0.2953	4	0.990
SPECIES*W*RR	7.6976	4	0.103

Table 5. Deviance table for effects of species, warming (W), rainfall reduction (RR) and their combination (W+RR) along with all the interactions on plant survival. Model was a binary logistic regression and shown are the model χ^2, degrees of freedom (df) and P-values for each effect. Significant effects (P<0.05) are shown in bold.

GENERAL DISCUSSION

"Whatever it is you're seeking won't come in the form you're expecting."

Haruki murakami, *Kafka on the Shore*

GENERAL DISCUSSION

Strong common patterns in the response of *H. squamatum* to warming across three study sites

H. squamatum was the only plant species present in the three experimental sites of this thesis, and it showed strongly consistent patterns in response to experimental climate warming across all the study locations, despite the differences in average temperature and precipitation among sites. Simulated climate warming sharply reduced the carbon assimilation rate of *H. squamatum* in all the study sites throughout the experimental period, as evidenced by a 35% average decrease in A (33.25% in plants exposed only to warming treatment) and 43% average decrease in WUE_i (44% in plants exposed only to warming treatment) across the three study locations. The only exceptions to this robust pattern were found on just two measurement dates: one during a very cold period in Aranjuez (February 2012) when warmed plants showed significantly higher net photosynthetic rates than control plants, and the other during an unusually rainy period in Sax (November 2012) when warmed and control plants had the same net photosynthetic rates. Moreover, *H. squamatum* did not show any evidence of photosynthetic acclimation to warming over the four years of measurements (Gunderson et al. 2010) at any of the experimental sites.Warmed plants of *H. squamatum* showed a significant downregulation of photosystem II (Sage and Kubien 2007) in the three study sites throughout the study period, likely as a consequence of a reduced carboxylation capacity linked to decreased leaf nutrient (N, P) status.

These large decreases in A and WUE_i in response to warming are indicating a potential detrimental feedback mechanism whereby plants assimilate far less carbon than plants exposed to current climate conditions despite same (Sax) or higher (Aranjuez and Sorbas) stomatal conductance and transpiration, which appears to be a rather maladaptive response to climate warming and aridification in the mid to long terms. Across the three study sites and throughout the study period, the leaf $\delta^{13}C$ values of *H. squamatum* shrubs were consistently lower in plants exposed to warming (with or without rainfall reduction) than in non-warmed plants. This consistent general trend is in agreement with leaf-gas exchange data and highlights that warming strongly decreased time-integrated WUEi throughout the study (Cernusak et al., 2013). Stable isotopes thus provided accurate time-integrated information on *H. squamatum*

ecophysiological responses to a changing environment (Dawson et al 2002), thus demonstrating their validity as a useful tool in ecophysiological studies investigating vegetation responses to climate change in dryland ecosystems. *H. squamatum* showed slightly different behaviors in response to warming in terms of stomatal conductance and transpiration rate (which consistently followed the same trend as stomatal conductance) across the three study sites. In the most humid site, Aranjuez, plants significantly increased their stomatal conductance and transpiration rate in response to warming (by 32% on average). Increased stomatal conductance and transpiration under warming may help prevent leaf overheating and photosynthetic machinery damage through enhanced evaporative leaf cooling. In Sorbas, the hottest and driest site, there were no significant differences in stomatal conductance between warmed and control plants, although there was a non-significant trend towards higher stomatal conductance in warmed plants. This somewhat different stomatal response between the two sites is likely due to the much lower soil water availability in Sorbas, which strongly constrains the plant's ability to increase stomatal conductance and transpiration in response to warming (which could lead to greater plant vulnerability to leaf overheating and damage of the photosynthetic machinery in Sorbas). The experimental site of Sax has quite different environmental conditions from the other two sites, due to the fact that vegetation is dominated by a *Pinus halepensis* overstory that provides shade to understory *H. squamatum* shrubs and thus moderates temperature and vapor pressure deficit extremes, which may explain why plants in Sax did not increase their stomatal conductance significantly in response to warming.

Significant reductions in leaf N and P concentrations were found in plants exposed to experimental warming relative to control plants across study sites over the 4-years study period. Foliar N and P concentrations were on average 7.3% and 10.2% lower, respectively, in plants exposed to warming (W treatment in Sax, average values of W and W+RR treatments for Aranjuez and Sorbas,) than in control plants across the three locations. Average reductions in leaf nutrient concentrations were similar for plants exposed to warming alone (W treatment: 7.5% and 11.1% decreases in leaf N and P concentrations, respectively, relative to controls across the three sites). These reductions in leaf N and P status could be the result of a hindered soil nutrient mineralization, solubilization, diffusion and/or uptake by roots under warming (Sardans and Peñuelas 2013), owing to a more rapid and severe drying of the upper soil layers where the

majority of nutrients available for plants are located. Leaf N and P are essential macronutrients for primary producers in terrestrial ecosystems (Dijkstra et al., 2012), since both play a key role in photosynthesis/plant physiology (Wright et al 2004; Thomas et al 2006), and their deficiency is well known to cause inhibition of photosynthesis and plant growth (Chandler & Dale 1995, Huang et al., 2004; Wright et al 2004, Thomas, Montagu & Conroy 2006, Reich, Oleksyn & Wright 2009). The positive correlations found between foliar nutrient concentrations and net photosynthetic rates, as well as between foliar nutrients and shoot dry biomass production further suggest nutrient limitation of carbon assimilation and plant growth under climate change conditions. Therefore, we found compelling evidence that the simultaneous reductions of leaf N and P status found in this study across study sites may have contributed to (and intensified) the strong detrimental effects of climate warming on *H. squamatum* photosynthetic performance. However, other non-stomatal limitations (i.e. biochemical or metabolic limitations aside from nutritional) may have further contributed to reduce the net photosynthetic rates of *H. squamatum* shrubs under warming (Sharkey 2005), although disentangling the relative roles of nutritional and non-nutritional limitations is beyond the scope of this thesis, and will be the subject of future research efforts.

Shoot biomass growth in *H. squamatum* also responded to warming in a rather consistent way across the three study sites. At the end of the study period (4 years), it was found that warming reduced shoot dry biomass production by 33% on average across sites, relative to control plants (32% average reduction in plants exposed to warming alone, W treatment). This sharp reduction in shoot biomass growth was mainly due to a decreased leaf dry biomass production in warmed plants, more than to a reduction of stem dry biomass. Shoot growth phenology (based on shoot elongation data) was also altered by experimental warming in a consistent manner across study sites, as warmed plants showed an advanced phenology concentrating vegetative growth in the earlier part of Spring (coupled with an earlier cessation of growth in late Spring), which is a widely reported pattern in the literature (Badeck et al 2004; Menzel et al 2006; Parmesan 2007).

In Sax, post-summer survival rate remained unchanged under warming throughout the study period, presumably due to the combination of the adaptive physiological

mechanisms mentioned above, and the presence of an Aleppo pine overstory that promotes an environment of higher relative humidity and shade for understory vegetation during the stressful hot and dry summer. In Aranjuez, all treatments had similarly high post-summer survival rates (>85%) in years with above- or near-average rainfall (2013, 2014 and 2015), despite the large reductions of A, WUE$_i$, ØPSII and foliar nutrient concentrations in warmed plants (W and W+RR treatments). This could be due to the fact that plants from Mediterranean-type semiarid ecosystems have evolved a wide range of adaptive mechanisms that enable them to adjust their physiology in response to warming and drought stress to ensure survival, such as an advanced shoot growth phenology (Parmesan 2007), reductions in leaf dry mass and LMA (Poorter 2009), enhanced stomatal conductance and transpiration (Crawford et al 2012) and reduced shoot biomass production and total leaf area. However, in a dry year (2012), warmed plants (W and W+RR plants) exceeded their phenotypic plasticity capacity, leading to sharp declines in post-summer survival rate (which was on average 35% lower than that of control plants). This sharp increase in plant summer mortality with warming during a drought year in Aranjuez (2012) may have been the consequence of plant hydraulic impairment caused by xylem embolism (favored by faster soil moisture depletion under warming), carbon starvation, or the synergistic or additive detrimental effects of both factors (McDowell et al., 2011). In Sorbas, the hottest and driest site of the study, *H. squamatum* survival rate at the end of the 4-year study period was only 52.5% across treatments. The survival rate of *H. squamatum* shrubs was sharply reduced by experimental warming in Sorbas, especially in plants exposed to the combination of warming and rainfall reduction (46.5% reduction in the W+RR treatment relative to the controls).

**

H. squamatum **responses to warming, rainfall reduction and their combination at Aranjuez and Sorbas.**

The Aranjuez and Sorbas sites are located in contrasting Mediterranean ecosystems with important differences in their climate, since the Aranjuez site is more humid (349 mm annual average precipitation) and has a lower average annual temperature (15ºC), whereas the Sorbas site is drier (275 mm annual average precipitation) and warmer (17ºC average temperature). Moreover, the Aranjuez site is located in central Spain and

hence has a continental Mediterranean climate, whereas the Sorbas site is located near the sea (<30 km), and therefore its Mediterranean climate shows narrower daily, seasonal and annual thermal amplitudes. Mean annual temperature in Sorbas increased throughout the 4 year study period, reaching an average temperature of 19ºC in the last year of the study (2015), which further contributed to harsher climatic conditions (warmer and drier) at this site. *Helianthemum squamatum* shrubs at the warmest and driest site (Sorbas) are smaller in size, and they generally show lower photosynthetic and stomatal conductance rates and leaf nutrient (N, P) concentrations (and higher intrinsic water use efficiency values) than those at the cooler and wetter site (Aranjuez).

Despite these differences in climatic conditions, we found rather consistent responses of *H. squamatum* to climate warming and/or rainfall reduction in these two contrasting Mediterranean ecosystems. Across years and sites, warming (W and W+RR treatments) caused large average reductions in A and WUEi (35% and a 45%, respectively) relative to the control treatment. Furthermore, consistent decreases in *H. squamatum* leaf $\delta^{13}C$ values under warming over the entire experiment and across sites indicate a decreased time-integrated WUEi in both the W and W+RR treatments, which agrees well with, and supports, leaf-gas exchange data (Cernusak et al., 2013). In Aranjuez, stomatal conductance and transpiration rate were on average 32% and 34% higher (respectively) in warmed plants than in control plants, while in Sorbas there were no significant differences in stomatal conductance or transpiration rate among treatments, as described above, albeit there was a trend towards higher g_s and E values in warmed plants (especially in the W treatment).

The quantum efficiency of photosystem II (ØPSII) was negatively affected by warming across years (2012-2014) and sites, with an average 11.5% reduction in the average ØPSII values of W and W+RR plants. In contrast, the maximum efficiency of photosystem II under light conditions (Fv':Fm') remained unaffected by the climate manipulation treatments, thus suggesting that warming did not severely damage the photosynthetic machinery of photosystem II (Baker & Rosenqvist 2004). We only found two exceptional moments when Fv':Fm' was negatively affected by warming: one time in Aranjuez in 2012 (in a very hot summer period of the driest year at this study site) and another time in Sorbas in 2014 (an extremely dry spring and year). The combination of decreased ØPSII and unchanged Fv':Fm', in conjunction with the large

decreases in A, suggests photosystem II down-regulation to match reductions in the rates of downstream photosynthetic reactions under warming (Baker & Rosenqvist 2004; Sage & Kubien 2007).

In Aranjuez, rainfall reduction alone moderately decreased net photosynthetic rate (12% average reduction in RR plants relative to control plants) and stomatal conductance (20% reduction in RR plants relative to control plants), and enhanced intrinsic water use efficiency (6% increase in RR plants relative to control plants). In Sorbas, rainfall reduction did not have statistically significant effects on photosynthesis rate and stomatal conductance, but had a negative effect on WUEi (11.4% average reduction in RR plants relative to control plants). In general, the largest reductions in A and WUE_i were found in plants exposed to the combination of warming and rainfall reduction (W+RR treatment) across sites, with 37.5% and 49% average reductions in A and WUE_i, respectively. These reductions were largest at the site with the warmest and driest climate conditions, Sorbas. Nevertheless, no statistically significant interactions between warming and rainfall reduction were found for any leaf gas exchange parameter at any site, suggesting that the combined detrimental effects of these key climate change drivers on *H. squamatum* shrubs are more additive than multiplicative (Wu et al., 2011; Rodgers et al., 2012).

Leaf N and P concentrations in *H. squamatum* shrubs were significantly reduced by warming across years and sites (8.3% average foliar N reduction and 9% average foliar P reduction in W and W+RR plants). The rainfall reduction alone treatment (RR) also moderately reduced foliar N by 5% on average across both sites. Given the key importance of N and P for plant ecophysiology, these concurrent decreases of leaf N and P in plants exposed to warming (with or without rainfall reduction) likely caused interactive and mutually reinforcing detrimental effects on the photosynthetic capacity of *H. squamatum* (Reich, Oleksyn & Wright 2009). However, as it has been already mentioned, there might be other concurrent non-nutritional limitations (biochemical or metabolic) that further contribute to reduce the net photosynthetic rates of *H. squamatum* under simulated warming, whose potential role is beyond the scope of this thesis.

Shoot dry biomass production in *H. squamatum* was strongly reduced by warming across both sites, as discussed above. Rainfall reduction (RR and W+RR treatments)

also reduced the shoot dry biomass of *H. squamatum* by 25.6% and 40% in Aranjuez and Sorbas, respectively (13.5% and 23.5% average reductions in plants exposed to RR alone in Aranjuez and Sorbas, respectively). Moreover, the combination of warming and rainfall reduction (W+ RR treatment) had the most detrimental effect on the shoot biomass production of *H. squamatum*, with average reductions of 38% and 47% in Aranjuez and Sorbas, respectively. The most damaging effects of simulated climate change on plant growth were found in the driest and warmest site (Sorbas), with sharp reductions of shoot biomass production in all the climate manipulation treatments (W, RR and W+RR) relative to control plants, especially in plants exposed to warming (with or without rainfall reduction). Post-summer survival was significantly reduced by warming in Aranjuez in a dry year only (2012), and in Sorbas over the entire study period. However, the post-summer survival rate of *H. squamatum* remained unaffected by the climate manipulation treatments in Aranjuez in the years with average or near-average precipitation, (2013, 2014 and 2015). *H. squamatum* was able to maintain a high post-summer survival rate in the wetter and cooler experimental site (Aranjuez) despite large reductions in key parameters of plant performance, such as A, WUEi, ØPSII, foliar nutrient concentrations and shoot dry biomass,, likely thanks to the wide range of phenotypic plasticity mechanisms evolved by native plants to cope with the stressful climate conditions characteristic of Mediterranean semiarid ecosystems (Nicotra et al., 2010; Bussotti et al., 2014; Nardini et al., 2014). Nevertheless, in the warmest and driest site of this study (Sorbas), the combination of warming and rainfall reduction led to an exceedance of the adaptive phenotypic plasticity capacity of *H. squamatum*, so that plants in the W+RR treatment had only 28.5% survival rate at the end of the 4 year study period (versus 50% survival rate in the control treatment, thus indicating a 43% reduction relative to plants exposed to current climate conditions). Interestingly, however, the post-summer survival rate of *H. squamatum* in Sorbas was not significantly affected by warming or rainfall reduction when these treatments were applied separately.

Community-level responses to experimental climate change at Sorbas

Plant community-level responses to simulated climate change were assessed throughout 4 years at the Sorbas site. Among these 4 hydrological years, there was an extremely

dry year, with less than 100 mm of annual rainfall (2013-2014), followed by a below-average year, with 180 mm of annual rainfall (2014-2015). Moreover, mean annual temperature was increasing steadily over the study period (from 17.6 to 19.1ºC), thus gradually exacerbating heat and water stress at this experimental site, which was already the warmest and driest of the 3 experimental sites of this thesis.

Across 5 native shrub species (*H. squamatum*, *H. syriacum*, *G. struthium*, *S. viscosa* and *T. turredanum*), leaf macro- and micronutrient (N, P, K, Fe, Cu and Zn) concentrations were significantly decreased by warming (W and W+RR treatments) during years 2012 and 2013. Consequently, C/N and C/P ratios also increased significantly in warmed plants across species. In addition, leaf K and Cu concentrations were also significantly reduced by rainfall reduction across species, with all the climate manipulation treatments showing lower values than control plants according to LSD post-hoc test. Given the key importance of these essential macro- and micronutrients for the correct functioning of the leaf photosynthetic machinery, these consistent reductions of foliar nutrient concentrations across species may have strongly exacerbated non-stomatal (nutritional) limitations of photosynthesis in native vegetation under a climate change scenario (Reich, Oleksyn & Wright 2009).

Leaf-gas exchange parameters were also strongly affected by experimental warming (W and W+RR treatments) across species. Consistent reductions in A, WUEi and ØPSII were found in warmed plants across the 3 measured species (*H. squamatum*, *H. syriacum* and *G. struthium*) and throughout the study period. In contrast, stomatal conductance and transpiration rate were significantly enhanced by warming across species and years. Large reductions in A together with enhanced gs led to nearly halved WUE_i in warmed plants across the 3 measured species along the study. Concurrent large decreases in ØPSII values across species under warming suggest photosystem II down-regulation to match a reduced carboxylation capacity across species (Sage and Kubien 2007). Leaf $\delta^{13}C$ data measured at the end of the study period in the 5 target species revealed a consistent reduction of time-integrated water use efficiency across species (Scheidegger et al., 2000; Cernusak et al., 2013). Interestingly, the 5 coexisting shrub species show large differences in their mean leaf $\delta^{13}C$ values, indicating that they have sharply contrasting water-use strategies ranging from the conservative water use pattern of *G. struthium* to the profligate water use pattern of *H. squamatum* (Moreno-Gutiérrez

et al., 2012). The consistent decrease of leaf δ^{13}C values under warming across species (with no significant *Species x Warming* interaction) thus demonstrates that stable isotope techniques provide a useful tool for assessing the time-integrated ecophysiological responses of plants to a changing environment (Dawson et al., 2002; Ferrio et al., 2005). Despite the different phylogenetic origins (families), plant size, life history traits, mycorrhizal association type and water use strategies of the native shrub species integrating the community, they all showed strong common patterns of response to simulated climate change, with decreases in leaf nutrient concentrations, net photosynthetic rates and intrinsic and time-integrated water use efficiency. Moreover, climate change consistently reduced shoot dry biomass production across the five target species, by simultaneously reducing leaf and stem biomass in all the climate manipulation treatments (W, RR and W+RR) relative to the control treatment). These detrimental effects were staggered, with average reductions in shoot dry biomass production of 22% in RR plants, 27% in W plants and 39% in plants exposed to the combination of warming and rainfall reduction (W+RR) across species. Once again, the combined detrimental effects of warming and rainfall reduction (W+RR treatment) on shoot biomass production tended to be additive (or not even that) rather than synergistic across species.

Plant survival rate after four years since the beginning of the experiment was strongly negatively affected by simulated climate change at the warmest and driest site (Sorbas), with gradual, staggered reductions across treatments (C>RR>W>W+RR). Plant survival rate plummeted in the W+RR treatment across target species (46% average reduction relative to the control treatment), whereas the warming alone (W) treatment reduced survival rate by 23.2% on average across species, and the rainfall reduction alone treatment (RR) reduced plant survival by 17.6% on average across species, relative to control plants. Despite the wide range of phenotypic plasticity mechanisms with a presumably adaptive value to cope with the new climatic conditions, (such as phenological advancement, stoichiometric and physiological flexibility, reduced biomass production and growth), plant survival was dramatically reduced across coexisting plant species in the mid-term (after four years of exposure to experimental climate change). Across species, the detrimental effects of warming on plant performance were generally stronger than those of rainfall reduction, likely because native plant species are well preadapted to nearly chronic drought stress. In contrast, the

supraoptimal temperatures achieved by experimental warming overwhelmed and exceeded their phenotypic adaptation capacity, particularly during dry years.

In conclusion, warming consistently reduced foliar nutrient concentrations, net photosynthesis rate and water use efficiency, and enhanced stomatal conductance and transpiration, across coexisting shrub species throughout the study period. The detrimental effects of simulated climate change on soil water availability (i.e., reduced rainfall combined with heat-induced faster drying of the nutrient-rich surface soil layer) lead to an exacerbation of drought stress and to an impaired plant nutrient status, which, in turn, contributed to reducing plant photosynthetic capacity, water use efficiency, shoot biomass production and survival rates, thus reducing ecosystem primary productivity through multiple detrimental feedback loops. Moreover, the reduction of plant nutritional quality under climate change conditions may have cascading negative effects on herbivores and decomposers, and could slow down nutrient cycling rates. Sharply reduced primary productivity under climate change conditions will also decrease plant litter inputs to soil, thus potentially decreasing soil organic matter content and soil quality in the long-term, which might eventually lead to a degradation pathway of enhanced erosion and desertification. In light of these results, we suggest that the response of this shrubland plant community to simulated climate change is largely mediated by shifts in plant stoichiometry and nutrient status, whereby plants need to spend more water in order to gain less carbon, with important consequences for ecosystem functioning. Overall, the results of this study highlight the potential vulnerability of Mediterranean-type native semiarid shrublands to forecasted climate change.

Mycorrhizal mediation of *H. squamatum* responses to climate change

Mycorrhizal fungi are key players and drivers of terrestrial ecosystem processes due to their fundamental role in nutrient uptake by plants, and because their symbiotic association with host plants provides multiple other benefits for both partners (Bolan, 1991; Van der Heijden et al., 1998; Leake et al., 2004; Smith & Read 2008). However, their role in mediating ecosystem responses to environmental change has been little studied in drylands (Mohan et al 2014). The symbiotic association between mycorrhizal fungi and their hots plants is evolutionarily stable since both partners obtain mutual rewards (by exchanging plant carbon for nutrients taken up by the mycorrhizal fungi;

General Discussion

Kiers et al., 2011). However, climate change could lead to a decoupling in the symbiotic relationship between mycorrhizal fungi and their host plants in dryland ecosystems (Bardgett et al., 2013; Bardgett & van der Putten, 2014) by unbalancing the stability of this mutual rewards mechanism. In the site of Aranjuez, simulated climate change (warming, rainfall reduction and their combination) sharply reduced the diversity and relative abundance of ectomycorrhizal fungal (EMF) communities in the rhizosphere of *H. squamatum*. The strong negative effects of climate change conditions on ectomycorrhizal fungi are likely the consequence of the combination of the low tolerance of EMF to heat and drought stress (references) and of the large drop in net photosynthesis rate of *H. squamatum* caused by experimental climate change. The reduced carbon assimilation capacity of plants under heat and drought stress probably led to a decreased belowground carbon allocation to their mycorrhizal fungal partners, thus impairing the exchange of mutual rewards between ectomycorrhizal fungi and *H. squamatum*. The presumably reduced C input to mycorrhizal fungal partners negatively affected plant nutrient status, due to the key role of EMF in plant N and P uptake (Leake et al., 2004; Smith & Read 2008). Reduced relative abundance of EMF within the soil fungal community under warming and/or rainfall reduction likely decreased their ability to take up nutrients and to compete for soil resources with coexisting saprotrophic fungi in soil. Moreover, decreased EMF operational taxonomic unit (OTU) richness per plot under warming and/or rainfall reduction likely reduced the multiple benefits to the host plants derived from functional complementarity among coexisting EMF (Jones et al. 2010).

Climate change apparently leads to an EMF-mediated detrimental feedback loop on the primary productivity of semiarid shrublands, by which supraoptimal temperatures for native plants during the growing season negatively affect net photosynthetic rates through multiple non-stomatal mechanisms, while at the same time enhancing evapotranspiration and soil moisture depletion as a consequence of warming-induced increases in VPD and rainfall reduction. Owing to the likely reductions in belowground carbon allocation by plants and also due to the direct detrimental effects of soil warming and drying, EMF richness and relative abundance are drastically reduced, thus resulting in large decreases in EMF carbon-sink strength (Wright et al., 2000) and nutrient-uptake capacity (Leake et al., 2004). This decreased plant N and P status, together with reduced carbon-sink stimulation of photosynthesis by EMF, further reduce carbon assimilation

General Discussion

rates, WUE and biomass production (Guehl & Garbaye 1990), thus closing the detrimental feedback loop on plant productivity in ectomycorrhizal dryland ecosystems. This detrimental feedback loop may have strong impacts on ecosystem functioning in a climate change scenario. For example, reduced foliar nutrient concentrations could have potentially cascading detrimental effects on ecosystem nutrient retention and cycling (van der Heijden 2010) and could decrease plant nutritional quality for herbivores and slow down litter decomposition and nutrient recycling. Overall, these results suggest that forecasted climate change will have harmful consequences for both plants and their mycorrhizal fungal partners, due to their interdependent and mutually reinforcing responses to heat and drought stress, especially to warming. These interdependent responses of both symbiotic partners may amplify the strong detrimental impacts of climate change on plant photosynthetic capacity and on the nutrient uptake capacity of mycorrhizal fungi, by reducing the mutual rewards in which this symbiotic relationship is based. This detrimental feedback loop triggered by climate change could thus lead to an alternative state of decreased vegetation productivity and might push these ecosystems to a degradation and desertification pathway.

In this thesis, climate change impacts on mycorrhizal fungi were investigated only on *H. squamatum* (a mixed EMF/AMF species) in a single site (Aranjuez). However, the large decreases in leaf nutrient status and plant biomass production with warming found in Sorbas were consistent across plant species with contrasting mycorrhizal association types (including purely AMF species like *G. sthrutium, S. viscosa, T. turredanum* and *C. hispanica*), which suggests that an impaired mycorrhizal functioning under climate change conditions might also occur in plant communities dominated by the arbuscular mycorrhizal symbiosis. Further research is thus warranted to investigate the potential role of arbuscular mycorrhizal fungi (AMF) in shaping vegetation responses to climate change in AMF-dominated vegetation types.

General Discussion

References

Badeck FW, Bondeau A, Böttcher K, Doktor D, Lucht W, Schaber J, Sitch S (2004) Responses of spring phenology to climate change. *New Phytologist*, **162**, 295-309.

Baker NR, Rosenqvist E (2004) Applications of chlorophyll fluorescence can improve crop production strategies: an examination of future possibilities. *Journal of Experimental Botany,* **55**, 1607–1621.

Bardgett RD, Manning P, Morrien E, Vries FT (2013) Hierarchical responses of plant–soil interactions to climate change: consequences for the global carbon cycle. Journal of Ecology, 101(2), 334-343.

Bardgett RD, & van der Putten WH (2014) Belowground biodiversity and ecosystem functioning. *Nature*, **515**, 505-511.

Bolan NS (1991) A critical review on the role of mycorrhizal fungi in the uptake of phosphorus by plants. *Plant and soil*, **134**, 189-207.

Bussotti F, Ferrini F, Pollastrini M, Fini A (2014) The challenge of Mediterranean sclerophyllous vegetation under climate change: From acclimation to adaptation. Environmental and Experimental Botany, 103, 80-98

Cernusak LA, Ubierna N, Winter K, Holtum JAM, Marshall JD, Farquhar GD (2013) Environmental and physiological determinants of carbon isotope discrimination in terrestrial plants. *New Phytologist*, **200**, 950–965.

Chandler JW, Dale JE (1995) Nitrogen deficiency and fertilization effects on needle growth and photosynthesis in Sitka spruce (*Picea sitchensis*). *Tree Physiology*, **15**, 813-817.

Crawford AJ, McLachlan DH, Hetherington AM, Franklin KA (2012) High temperature exposure increases plant cooling capacity. *Current Biology*, **22**, 396–397.

Dawson TE, Mambelli S, Plamboeck AH, Templer PH, Tu KP. 2002. Stable isotopes in plant ecology. *Annual Review of Ecology and Systematics*, **33**, 507–559.

General Discussion

Dijkstra FA, Pendall E, Morgan JA, Blumenthal DM, Carrillo Y, LeCain DR, Follett RF, Williams DG. 2012. Climate change alters stoichiometry of phosphorus and nitrogen in a semiarid grassland. *New Phytologist*, **196**, 807–815.

Ferrio Díaz JP, Resco de Dios V, Williams DG, Serrano Endolz L, Voltas Velasco J (2005) Stable isotopes in arid and semi-arid forest systems. *Investigación Agraria. Sistemas y Recursos Forestales*, 2005, vol. 14, núm. 3, p. 371-382.

Guehl JM, Garbaye J (1990) The effects of ectomycorrhizal status on carbon dioxide assimilation capacity, water-use efficiency and response of transplanting in seedlings of Pseudotsuga menziesii (Mirb) Franco. *Annales des Sciences Forestières*, **21**, 551–553.

Gunderson CA, O'hara KH, Campion CM, Walker AV, Edwards NT (2010) Thermal plasticity of photosynthesis: the role of acclimation in forest responses to a warming climate. *Global Change Biology*, **16**, 2272-2286.

van der Heijden MG, Klironomos JN, Ursic M, Moutoglis P, Streitwolf-Engel R, Boller T *et al.* (1998) Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. *Nature*, **396**, 69-72.

van der Heijden MGA (2010) Mycorrhizal fungi reduce nutrient loss from model grassland ecosystems. *Ecology*, **91**, 1163–1171.

Huang ZA, Jiang DA, Yang Y, Sun JW, Jin SH (2004) Effects of nitrogen deficiency on gas exchange, chlorophyll fluorescence, and antioxidant enzymes in leaves of rice plants. *Photosynthetica*, **42**, 357-364.

Jones MD, Twieg BD, Ward V, Barker J, Durall DM, Simard SW (2010) Functional complementarity of Douglas-fir ectomycorrhizas for extracellular enzyme activity after wildfire or clearcut logging. *Functional Ecology*, **24**, 1139–1151.

Kiers ET, Duhamel M, Beesetty Y, Mensah JA, Franken O, Verbruggen E *et al.* (2011). Reciprocal rewards stabilize cooperation in the mycorrhizal symbiosis. *Science*, **333**, 880-882.

Leake JR, Johnson D, Donnelly DP, Muckle GE, Boddy L, Read DJ (2004) Networks of power and influence: the role of mycorrhizal mycelium in controlling plant

communities and agroecosystem functioning. *Canadian Journal of Botany*, **82**, 1016–1045.

McDowell NG, Beerling DJ, Breshears DD, Fisher RA, Raffa KF, Stitt M (2011) The interdependence of mechanisms underlying climate-driven vegetation mortality. *Trends in Ecology & Evolution*, **26**, 523-532.

Menzel A, Sparks TH, Estrella N *et al.* (2006) European phenological response to climate change matches the warming pattern. *Global Change Biology*, **12**, 1969-1976.

Mohan JE, Cowden CC, Baas P *et al.* (2014) Mycorrhizal fungi mediation of terrestrial ecosystem responses to global change: mini-review. *Fungal Ecology*, **10**, 3–19.

Moreno-Gutiérrez C, Dawson TE, Nicolás E, Querejeta JI (2012) Isotopes reveal contrasting water use strategies among coexisting plant species in a Mediterranean ecosystem. *New Phytologist*, **196**, 489-496.

Nardini A, Lo Gullo MA, Trifilò P, Salleo S (2014) The challenge of the Mediterranean type climate to plant hydraulics: responses and adaptations. *Environmental and Experimental Botany,* **103**, 68–79.

Nicotra AB, Atkin OK, Bonser SP, Davidson AM, Finnegan EJ, Mathesius U. *et al.* (2010) Plant phenotypic plasticity in a changing climate. *Trends in Plant Science*, **15**, 684–692.

Parmesan C (2007) Influences of species, latitudes and methodologies on estimates of phenological response to global warming. *Global Change Biology*, **13**, 1860-1872.

Poorter H, Niinemets Ü, Poorter L, Wright IJ, Villar R (2009) Causes and consequences of variation in leaf mass per area (LMA): a meta-analysis. *New Phytologist*, **182**, 565–588.

Reich PB, Oleksyn J, Wright IJ (2009) Leaf phosphorus influences the photosynthesis–nitrogen relation: a cross-biome analysis of 314 species. *Oecologia*, **160**, 207–212.

Rodgers VL, Hoeppner SS, Daley MJ, Dukes JS (2012) Leaf-level gas exchange and foliar chemistry of common old-field species responding to warming and precipitation treatments. *International Journal of Plant Sciences*, **173**, 957-970.

Sage RF, Kubien DS (2007) The temperature response of C3 and C4 photosynthesis. *Plant, Cell & Environment*, **30**, 1086–1106.

Sardans J, Peñuelas J (2013) Plant-soil interactions in Mediterranean forest and shrublands: impacts of climatic change. *Plant and Soil*, **365**, 1-33.

Scheidegger Y, Saurer M, Bahn M, Siegwolf R (2000) Linking stable oxygen and carbon isotopes with stomatal conductance and photosynthetic capacity: a conceptual model. *Oecologia*, **125**, 350-357.

Sharkey TD (2005) Effects of moderate heat stress on photosynthesis: importance of thylakoid reactions, rubisco deactivation, reactive oxygen species, and thermotolerance provided by isoprene. *Plant, Cell & Environment*, **28**, 269-277.

Smith SE, Read DJ (2008) Mycorrhizal symbiosis. Edn. 3rd., Academic Press.

Thomas DS, Montagu KD, Conroy JP (2006) Leaf inorganic phosphorus as a potential indicator of phosphorus status, photosynthesis and growth of Eucalyptus grandis seedlings. *Forest Ecology Management*, **223**, 267–274

Wright DP, Scholes JD, Read DJ, Rolfe SA (2000) Changes in carbon allocation and expression of carbon transporter genes in *Betula pendula* Roth. colonized by the ectomycorrhizal fungus *Paxillus involutus* (Batsch) Fr.. *Plant, Cell & Environment*, **23**, 39–49.

Wright IJ, Reich PB, Westoby M, Ackerly DD, Baruch Z, Bongers F, Cavender-Bares J, Chapin T, Cornelissen JHC *et al.* (2004) The worldwide leaf economics spectrum. *Nature*, **428**, 821–827.

Wu Z, Dijkstra P, Koch GW, Peñuelas J, Hungate BA (2011) Responses of terrestrial ecosystems to temperature and precipitation change: a meta-analysis of experimental manipulation. Global Change Biology, 17, 927-942.

CONCLUSIONS
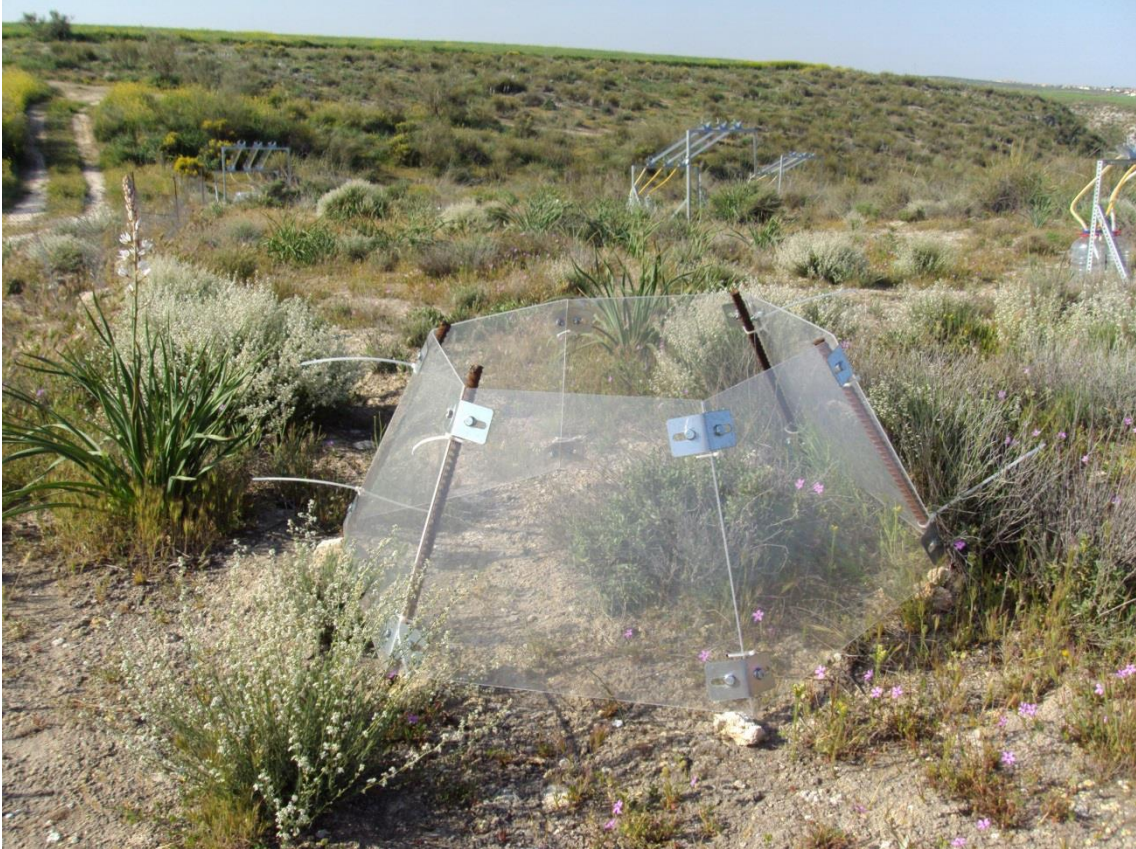
"We don't find out what's waiting for us around the next corner until we turn it."

Haruki Murakami, *1Q84*

CONCLUSIONS

1. Moderate increases in mean annual temperature led to large decreases in the net photosynthetic rates, quantum efficiency of photosystem II and water use efficiency of native *Helianthemum squamatum* shrubs throughout the 4-year study period, with no indications of photosynthetic acclimation to warming with time. These effects were rather consistent through time (within and across years) and were of roughly similar magnitude across three experimental sites with contrasting climatic conditions.

2. In contrast to photosynthetic rates, stomatal conductance and transpiration rates either increased (Aranjuez) or remained unchanged (Sax and Sorbas) under warming, which indicates enhanced non-stomatal limitation of photosynthesis with moderate increases of mean annual temperature in *H. squamatum*. The stomatal response to warming was constrained by soil water availability, as the increase in stomatal conductance and transpiration was much greater at the cooler and wetter site (Aranjuez) than at the drier and warmer sites (Sax and Sorbas).

3. Warming consistently decreased leaf N and P concentrations in *H. squamatum* shrubs across sites, which strongly suggests increased nutrient limitation of photosynthesis and water use efficiency under warming.

4. Lower leaf $\delta^{13}C$ values in warmed plants indicated decreased time-integrated water use efficiency under elevated temperature, which is in good agreement with leaf gas exchange data showing decreased leaf-level intrinsic water use efficiency under warming. This proves that leaf $\delta^{13}C$ measurements can be a valuable tool in manipulative field studies evaluating the integrative impact of climate change on plant ecophysiology.

5. Warming led to an advanced shoot growth phenology (i.e. greater shoot elongation during early spring, followed by earlier cessation of growth in late spring) in *H. squamatum* shrubs across sites, relative to control conditions. An advanced growth phenology under warming may represent an adaptive phenotypic response to escape increasing heat and drought stress during late Spring. Warming also led to large decreases in the mean leaf dry mass, LMA and total shoot biomass production of *H. squamatum* shrubs across sites, which is in good agreement with the large reductions in mean photosynthetic rates observed in warmed plants.

6. Warming had contrasting effects on the survival rate of *H. squamatum* shrubs among experimental sites. Plant survival rate was significantly decreased by the combination of warming and rainfall reduction at the driest and warmest site (Sorbas). The post-summer survival rate of *H. squamatum* was not significantly affected by warming at the wettest and coolest site (Aranjuez) during years with near- or above-average rainfall, but was strongly decreased by warming during a dry year. Post-summer survival rate was unaffected by warming at Sax, probably due to the presence of a pine overstory that moderated microclimatic conditions in the understory.

7. Rainfall reduction led to moderate decreases in the net photosynthetic rates and stomatal conductance of *H. squamatum* shrubs across two sites with contrasting climatic conditions (Aranjuez and Sorbas), particularly during wet periods. *Helianthemum squamatum* shrubs significantly increased their intrinsic water use efficiency in response to rainfall reduction at the coolest and wettest site (Aranjuez), but this potentially adaptive response to increased drought stress was not observed at the warmest and driest site (Sorbas). Rainfall reduction decreased shoot biomass production in *H. squamatum* across sites, with largest decreases found in Sorbas.

8. The combination of warming and rainfall reduction generally caused the largest decreases in net photosynthetic rates, stomatal conductance, leaf N and P status, shoot biomass production and plant survival rate. However, the combined detrimental impacts of warming and rainfall reduction on the performance of *H. squamatum* shrubs were generally additive (or not even that), rather than synergistic.

9. At the Aranjuez site, ectomycorrhizal fungal (EMF) diversity and relative abundance under *H. squamatum* shrubs were drastically reduced by warming, rainfall reduction and their combination. We suggest that the deeply interdependent responses of native plants and EMF to climate change may have decisively contributed to large decreases in plant nutrient status, growth and drought survival, through a detrimental feedback loop mediated by mycorrhizal fungi.

10. At the driest and hottest site (Sorbas), warming led to large decreases in leaf N, P, K, Fe, Cu and Zn concentrations across six coexisting plant species with contrasting mycorrhizal association types (*Helianthemum squamatum, Helianthemum syriacum, Gypsophila struthium, Santolina viscosa, Teucrium turredanum* and *Coris hispanica*. Rainfall reduction moderately reduced foliar K and Cu concentrations across species.

11. At the Sorbas site, warming consistently led to sharp decreases in the net photosynthetic rates, quantum efficiency of photosystem II and intrinsic water use efficiency of three coexisting plant species (*Helianthemum squamatum, Helianthemum syriacum, Gypsophila struthium*) across years. Additionally, warming moderately enhanced stomatal conductance and transpiration rate across the three target species, indicating increased non-stomatal limitation of photosynthesis under elevated temperature.

12. At the Sorbas site, warming strongly decreased shoot biomass production across coexisting shrub species. Rainfall reduction also decreased shoot biomass production significantly in these species, although to a lesser extent than warming. Additionally, warming significantly decreased leaf $\delta^{13}C$ values across shrub species with contrasting phylogenetic origins, plant size, life history traits and water use strategies, indicating decreased time-integrated water use efficiency under elevated temperature.

13. Plant survival rate at the end of the 4-year study period in Sorbas was drastically decreased by experimental warming across target species, with no significant *Warming x Species* interaction despite some species-specific differences in this respect.

14. The combination of warming and rainfall reduction (W+RR treatment) generally had the strongest detrimental effects on plant performance across shrub species at Sorbas, although there were no statistically significant differences between these plants and plants exposed only to climate warming (W treatment) according to post-hoc tests. The most detrimental effects of the combination of warming and rainfall reduction were found on shoot biomass production and, especially, survival rate.

15. Forecasted climate change will likely overwhelm the capacity for adaptive phenotypic plasticity across native shrub species in Mediterranean semiarid gypsum ecosystems. Climate change might thus lead to widespread declines in plant nutrient status, growth and survival through a strong detrimental feedback mechanism whereby plants will need to transpire more water in order to gain less carbon under warmer and drier conditions. The large and consistent cross-species decreases in plant nutrient status, photosynthetic activity and water use efficiency in response to experimental climate change will thus likely have strong negative impacts on the primary productivity and survival of these native semiarid plant communities in the

mid- to long-term, which might increase ecosystem vulnerability to land degradation and desertification.