Mistletoes as keystone species in pine woodlands: exploring the ecological consequences of a new interaction cocktail

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Mistletoes as keystone species in pine woodlands: exploring the ecological consequences of a new interaction cocktail

Memoria presentada por la licenciada Alba Lázaro–González para optar al Grado de Doctora con Mención Internacional en Ciencias Biológicas por la Universidad de Granada. Esta memoria ha sido realizada bajo la dirección de Dr. Regino Zamora Rodríguez, Catedrático de Ecología de la Universidad de Granada, y el Dr. José Antonio Hódar Correa, Profesor Titular de Ecología de la Universidad de Granada

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Declaran que los trabajos de investigación desarrollados en la Memoria de Tesis Doctoral: "Mistletoes as keystone species in pine woodlands: exploring the ecological consequences of a new interaction cocktail", son aptos para ser presentados por la Lda. Alba Lázaro González ante el Tribunal que en su día se designe, para aspirar al Grado de Doctora con Mención Internacional en Ciencias Biológicas por la Universidad de Granada. Y para que así conste, en cumplimiento de las disposiciones vigentes, extiendo el presente certificado a 12 de junio de 2020.

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Resumen general

En esta tesis doctoral estudiamos los diferentes papeles que juega simultáneamente el muérdago europeo (*Viscum album* subsp. *austriacum*) y sus consecuencias ecológicas en un pinar mediterráneo, mediante las múltiples interacciones planta–planta y planta–animal que genera en su ecosistema. Dada su naturaleza de planta hemiparásita, el muérdago ha sido tradicionalmente estudiado como patógeno del hospedador, causándole daños en el vigor vegetativo, morfológicos y reproductivos. Sin embargo, recientemente se han comenzado a poner en valor las interacciones ecológicas que el muérdago establece en el ecosistema forestal, no sólo con su hospedador, sino también con el resto de la comunidad donde habitan. Como consecuencia de estas nuevas interacciones, la presencia de muérdago en el dosel forestal de un bosque, puede provocar efectos directos e indirectos en su ecosistema mediante relaciones tróficas y no tróficas, favoreciendo la reestructuración de la composición de la comunidad. Así pues, dividimos esta tesis en tres grandes partes, donde desarrollamos diferentes capítulos estudiando el papel del muérdago I) como recurso clave para sus artrópodos asociados (Capítulos 1–3), II) como competidor directo con su hospedador (Capítulos 4–5) y III) como competidor indirecto con herbívoros del hospedador (Capítulo 6) y facilitador para la comunidad vegetal (Capítulo 7).

En el Capítulo 1 caracterizamos la comunidad de artrópodos asociada a *V. album* y la comparamos con la comunidad de artrópodos que habitan en su hospedador principal en nuestra zona de estudio, *Pinus nigra*, y su segundo hospedador, *Pinus sylvestris*. Los resultados obtenidos muestran cómo, a pesar de que las comunidades asociadas a los pinos difieren entre la especie de hospedador, *Viscum album* alberga una artropodofauna estable, simple, e independiente a la de sus hospedadores. Esta comunidad

está compuesta por organismos altamente especialistas: un herbívoro principal, *Cacopsylla visci*, un herbívoro secundario, *Pinalitus viscicola*, y un depredador especialista, *Anthocoris visci.*

En el Capítulo 2 identificamos las reglas ecológicas de ensamblaje que siguen las comunidades de artrópodos asociadas al muérdago bajo diferentes factores ecológicos a nivel forestal (gradiente altitudinal) y de planta (tamaño, distancia a otras plantas, y temporalidad). Nuestros resultados indican que los muérdagos son habitados por una comunidad especialista, descrita en el Capítulo 1, y una comunidad turista, procedente del pino hospedador, que siguen reglas de ensamblaje contrastadas. Descubrimos que la comunidad especialista mantiene una regla de ensamblaje constante bajo cualquier factor ecológico, consistente en una secuencia trófica determinista de colonización, mientras que la comunidad turista actúa de forma independiente a la presencia de muérdago.

En el Capítulo 3 nos centramos en otro recurso que ofrece el muérdago durante un periodo corto del año, la floración. En este periodo, el muérdago ofrece no sólo tejido vegetativo, que estará siendo consumido por la comunidad especialista, sino también flores con recompensas en forma de néctar y polen. Este nuevo recurso, es único en el espacio y en el tiempo, ya que se encuentra en la copa de los pinos y, dada su temprana floración, en un momento del año en que el resto de recursos florales aún no están disponibles. A lo largo de este capítulo, exploramos las relaciones funcionales y tróficas de toda la comunidad de artrópodos visitantes de muérdagos durante su periodo de floración, así como el papel que juegan en la formación de frutos. Los resultados demuestran que el muérdago no sólo atrae a una comunidad especialista, sino también a una comunidad diversa de polinizadores, tanto diurnos como nocturnos, siendo determinantes en la formación de frutos. Así pues, el muérdago fomenta nuevas y mutualistas interacciones planta–animal, convirtiéndose en una especie de fundación secundaria en el dosel forestal.

En la segunda parte de la tesis, estudiamos la interacción parásito–hospedador y los efectos directos que el muérdago ejerce como competidor sobre su hospedador. En el Capítulo 4 analizamos el perfil químico de las hojas del muérdago y acículas del hospedador, así como las respuestas químicas del pino en función de su carga parasítica. Los resultados indican que, a pesar de que el muérdago se encuentra continuamente obteniendo agua y nutrientes de su hospedador, el perfil químico de los tejidos vegetativos de parásito y de hospedador son completamente distintos. Por un lado, el muérdago almacena gran cantidad de nitrógeno en sus hojas, pero no se beneficia del pino adquiriendo compuestos secundarios de defensa. Por otro lado, en general, el pino reacciona frente a la parasitación por muérdago incrementando sus compuestos de defensa acorde con el grado de parasitación. Además, los pinos altamente parasitados presentan menos contenido en nitrógeno, reduciendo la calidad como alimento para sus herbívoros, y tienen menos capacidad para sintetizar otros compuestos de defensa pesados y/o difíciles de movilizar.

En el Capítulo 5 profundizamos hasta el nivel más fino del perfil químico del sistema parásito–hospedador, el metaboloma. A lo largo de este capítulo analizaremos, mediante técnicas ecometabolómicas, por primera vez el perfil metabolómico de la interacción parásito–hospedador y las respuestas espaciotemporales del metaboloma del pino hospedador. Acorde con el Capítulo 4, el muérdago adquiere fundamentalmente compuestos del metabolismo primario del hospedador, siendo capaz de sintetizar sus propios compuestos de defensa, y actuando como un sumidero constante de recursos para el hospedador. Además, el pino hospedador sufre cambios en una cuarta parte de su metaboloma de forma sistémica y permanente, lo que convierte la copa del pino parasitado en una nueva identidad metabólica disponible en el dosel forestal.

La tercera parte de la presente tesis se centra en las interacciones indirectas mediadas por el muérdago. En el Capítulo 6 estudiamos el papel como competidor indirecto de herbívoros del pino hospedador. A raíz de los cambios químicos descritos en la segunda parte de la tesis, donde el muérdago provoca un incremento de compuestos de defensa de manera sistémica y permanente en el pino, en este capítulo analizamos cómo esos cambios se trasladan a lo largo de la cadena trófica causando efectos tritróficos en fluidófagos y folívoros del pino. Los resultados muestran que el muérdago es capaz de inducir interacciones indirectas de competencia reduciendo la abundancia de herbívoros que comparten el mismo pino hospedador. Por tanto, el muérdago actúa como control biológico diluyendo la presión por herbivoría del pino hospedador, haciendo que la relación parásito–hospedador se acerque a una relación más mutualista que parasítica.

En el Capítulo 7 estudiamos el rol que desempeña el muérdago como facilitador indirecto, enfocándonos en la comunidad herbácea bajo la copa de los pinos parasitados y un herbívoro mamífero. A través de su hojarasca rica en nutrientes, el muérdago es capaz de enriquecer localmente el suelo, modificando sus propiedades químicas y biológicas, favoreciendo la comunidad microbiana. A lo largo de este capítulo, analizamos cómo ese enriquecimiento local del suelo afecta a la cobertura y diversidad de la comunidad herbácea, y a su vez, atraen al conejo como consumidor. Los resultados muestran que el muérdago promueve efectos indirectos más allá del pino hospedador, incrementando localmente la cobertura, abundancia y diversidad de especies herbáceas, así como las visitas del conejo como consumidor. Esto refuerza la idea del muérdago como facilitador y generador de heterogeneidad ambiental, expandiendo su impacto a toda la comunidad forestal.

Teniendo una visión holística, concluimos que los muérdagos son especies claves que desencadenan una serie de interacciones con importantes consecuencias ecológicas al nivel de comunidad, provocando efectos directos e indirectos en diferentes niveles tróficos. Esto tiene profundas implicaciones en la dinámica del ecosistema forestal, reestructurando toda la comunidad, desde la dinámica de nutrientes y plantas herbáceas hasta consumidores primarios y secundarios. Así pues, jugando simultáneamente los roles como nuevo recurso, competidor y facilitador, los muérdagos se convierten en ingenieros de ecosistemas, que añaden un nivel adicional de heterogeneidad al dosel forestal, promoviendo el incremento de la biodiversidad y complejidad del ecosistema.

Summary

In this thesis, a study is made of the different roles that the European mistletoe (*Viscum album* subsp. *austriacum*) can play simultaneously in a Mediterranean pine forest, and their ecological consequences generating multiple plant–plant and plant–animal interactions in their ecosystem. Due to their hemiparasitic nature, the mistletoe has been traditionally regarded as a host pathogen, causing detrimental effects on growth, morphology, and reproduction. However, recently other ecological interactions that mistletoe establishes in the forest ecosystem have been found to be noteworthy, not only with its host but also with the rest of the community where they live. Consequently, the presence of mistletoe in the forest canopy can cause direct and indirect effects in their ecosystem through trophic and non–trophic relationships, favoring the restructure of community composition. Therefore, this thesis has been split into three main parts examining the role of mistletoe: I) as a keystone resource for its associated arthropods (Chapters 1–3); II) as direct competitor with its host (Chapters 4–5); and III) as indirect competitor with host–feeding herbivores (Chapter 6) and facilitator for the herbaceous community (Chapter 7).

In Chapter 1, characterizations and comparisons are made regarding the arthropod community inhabiting *V. album*, their main host at the study site, *Pinus nigra*, and their secondary host, *Pinus sylvestris*. Although the arthropod communities associated with pine trees differ between host species, the results show that *V. album* harbors a stable, simple, and independent arthropofauna from that of its hosts. This community is composed by highly specialized organisms: the main insect herbivore, *Cacopsylla visci*, a secondary insect herbivore, *Pinalitus viscicola*, and a specialist predatory insect, *Anthocoris visci*.

In Chapter 2, the ecological assembly rules followed by the arthropod

communities associated with mistletoe are identified under different ecological factors at forest (elevational gradient) and plant level (plant size, distance to other plants, and temporal variation). The results indicate that this mistletoe is inhabited by a specialist community, described in Chapter 1, and a tourist community coming from the host pine, which follow contrasting assembly rules. It was discovered that the specialist community followed a constant assemblage rule under practically any environmental factor, consisting in a deterministic trophic sequence of colonization, while tourist community acted independently of mistletoe presence.

Chapter 3 focuses on the mistletoe flowering as a new resource available for a short period. During this period, mistletoe offers vegetative tissues to be consumed by the specialist herbivores, and also floral rewards such as pollen and nectar. This new resource is unique in space because it is found in the pine canopy, and in time because it occurs during early flowering, when the rest of the floral market is still unavailable. Throughout this chapter, the functional and trophic relationships of the entire arthropod community visiting mistletoes during their flowering period are explored, as well as the role determining the mistletoe fruit set. The results show that mistletoe attracts not only the specialist community but also a diverse community of floral visitors, both diurnal and nocturnal, which are essential to mistletoe fruit set. Thus, mistletoe fosters new and mutualist plant–animal interactions, becoming a secondary foundation species in the forest canopy.

The second part of this thesis deals with the host–parasite interaction and the direct effects exerted by mistletoe as a competitor on its host. In Chapter 4, the chemical profiles of mistletoe leaves and pine host needles are analyzed, as well as the chemical responses of pines according to mistletoe parasitic load. Despite continuous mistletoe uptake of water and nutrients from its host pine, the results indicate sharply different chemical profiles of mistletoe leaves with respect to pine needles. On the one hand, mistletoe leaves store large concentrations of nitrogen, but the plant does not benefit by acquiring secondary defense compounds from the host pine. On the other hand, overall, the host pine reacts to mistletoe parasitism by increasing its defense compounds according to mistletoe parasitic load. In addition, highly parasitized pines present lower N concentrations, reducing the quality as food for their herbivores, and have less capacity to synthesize costly and difficult to mobilize defense compounds.

In Chapter 5, the chemical profile of host–parasite system is analyzed in depth to the finest level, the metabolome. Throughout this chapter,

ecometabolomic techniques are used for the first time to analyze the metabolomic profile of host–parasite interaction and the spatiotemporal metabolome responses of host pine. In agreement with the results of Chapter 4, mistletoe was found to derive basic compounds from the host's primary metabolism, and to synthesize its own defense compounds, thus acting as a permanent sink of host resources. Furthermore, the host pine undergoes changes in a quarter of its metabolome in a systemic and permanent way, converting the pine canopy into a new plant metabolic identity in the forest canopy.

The third part of this thesis is focused on indirect interactions triggered by mistletoe. In Chapter 6, the role of mistletoe as an indirect competitor of pine–feeding herbivores is explored. As a result of chemical changes described in the second part of the thesis, where mistletoe is shown to induce the production of defense compounds systemically and permanently in host pine, this chapter describes how these changes, via tritrophic phenomena, affect pine–feedings herbivores such as sap–suckers and folivores. The results show that mistletoe is able to induce indirect interactions of competition by reducing the abundance of herbivores sharing the same host pine. Thus, it is shown that mistletoe can act as an agent of biological control, reducing the herbivore pressure on the host pine and thereby turning the parasite–host interaction a more mutualist rather than parasitic relationship.

In Chapter 7, mistletoe is studied as an indirect facilitator focusing on the herbaceous community under the canopy of parasitized pines and the presence of a mammalian herbivore. Through its nutrient–rich leaf litter, mistletoe is found to be able to enrich the soil locally, modifying its chemical and biological properties, and favoring the microbial community. Throughout this chapter, it is analyzed how this local soil enrichment influences the cover and diversity of the herbaceous community, and in turn attracts the rabbit as a consumer. The results demonstrate that mistletoe promotes indirect effects beyond the parasitized pines, locally increasing the cover, abundance and diversity of plant species, as well as the presence of rabbits as consumers. This reinforces the idea that mistletoe is a facilitator and generator of environmental heterogeneity, expanding its impact to the entire forest community.

From a holistic view, it is concluded that mistletoes are keystone species that trigger a series of interactions with important ecological consequences at the community level, causing direct and indirect effects at different trophic levels. This has profound implications for the dynamics of the forest

ecosystem, restructuring the entire community, from nutrient dynamics and herbaceous community to primary and secondary consumers. Thus, by simultaneously providing new resources while acting as a competitor and facilitator, mistletoes become ecosystem engineers, building an additional level of heterogeneity to the forest canopy and amplifying biodiversity and complexity in their ecosystem.

Introducción general

Introducción general

A lo largo de la historia, todo aquello inusual o anómalo en la naturaleza ha despertado siempre nuestro interés. Las plantas parásitas son un claro ejemplo de rareza en el ecosistema, con aproximadamente unas 4550 especies (1,2 % de todas las angiospermas) pertenecientes a 280 géneros y 20 familias (Rubiales & Heide-Jørgensen, 2011). Son un grupo de plantas ampliamente distribuido, presentes en todas las zonas climáticas y ecosistemas terrestres, desde zonas tropicales hasta desiertos y la tundra (Kuijt, 1969; Press & Graves, 1995). Muchos aspectos sobre su ecología siguen siendo un misterio, y los pocos estudios realizados sobre ellas se han centrado en su taxonomía, anatomía y fisiología (Kuijt, 1969; Press & Graves, 1995), así como en intereses medicinales en numerosas culturas. Paralelamente, es usual que el término "parásito" se ligue a connotaciones negativas como agentes dañinos y patógenos. Por este motivo, tradicionalmente, las plantas parásitas y en especial los muérdagos, también han sido estudiados desde el punto de vista del hospedador y sus efectos negativos en el crecimiento y supervivencia. Los estudios sobre estos efectos perjudiciales se magnifican especialmente en sistemas agrícolas (Parker & Riches, 1993; Riches & Parker, 1995; Watson *et al.*, 2020) y plantaciones forestales, donde se trata a los muérdagos como plagas (Pérez-Laorga *et al.*, 1999; Sallé & Frochot, 2002). El enfoque de todos estos estudios tiene como denominador común una visión antropocéntrica del ecosistema, definiendo las plantas parásitas como patógenas cuando éstas causan pérdidas económicas por su impacto negativo sobre hospedantes utilizados en actividades agrícolas y plantaciones comerciales (López-Sáez *et al.*, 2002; Geils & Hawksworth, 2002; Reid & Shamoun, 2009). Sin embargo, es imperativo disociar los conceptos de parásito y patógeno, dejando de lado la visión antropocéntrica para tener una visión holística al nivel de ecosistema.

Es por ello que, recientemente, se han comenzado a poner en valor las interacciones ecológicas en las que las plantas parásitas están implicadas, identificando los diferentes roles simultáneos que pueden jugar y evaluando su impacto al nivel de comunidad y ecosistema. El enfoque tradicionalmente estudiado ha sido como enemigo natural del hospedador, especialmente en agricultura y plantaciones forestales como hemos mencionado. Además, debido a su íntima relación, las plantas parásitas pueden tener un efecto desproporcionado en relación a su biomasa y abundancia (Watson, 2001; Fadini *et al.*, 2020), causando profundos cambios ecofisiológicos en la planta huésped, como la pérdida en el crecimiento y reproducción, y finalmente la muerte (Pennings & Callaway, 2002). El segundo papel es como recurso clave en su ecosistema, proveyendo de alimento y/o refugio a diferentes especies animales (Pennings & Callaway, 1992; Watson, 2001; Cooney *et al.*, 2006). Ambos roles causan efectos directos a lo largo de la cadena trófica, mediante efectos de arriba hacia abajo (*top-down*) sobre su hospedador y de abajo hacia arriba (*bottom-up*) sobre sus herbívoros. Sin embargo, el último y más reciente rol estudiado, y por tanto el menos conocido, es como facilitador de otras especies provocando efectos indirectos sobre el ecosistema. Por un lado, las plantas parásitas mediante la caída de hojarasca promueven el enriquecimiento y reciclaje de nutrientes del suelo (Ndagurwa *et al.*, 2013; Muvengwi *et al.*, 2015; Mellado *et al.*, 2016), lo cual beneficia a las comunidades microbianas, herbáceas y de artrópodos del suelo (Bardgett *et al.*, 2006; Mellado *et al.*, 2016, 2019). Por otro, parásitas de suelo también juegan un papel facilitador incrementando indirectamente la competitividad de especies herbáceas vecinas y aumentando la diversidad en estas comunidades (Pennings & Callaway, 1996; Bardgett *et al.*, 2006; Hartley *et al.*, 2015).

Por todo esto, las plantas parásitas se han considerado como especies clave en el ecosistema (Press & Phoenix, 2005), y los muérdagos en particular (Watson, 2001), capaces de desencadenar multitud de interacciones con consecuencias ecológicas al nivel de comunidad (Watson & Herring, 2012; Hartley *et al.*, 2015; Hartley & Gange, 2009; Mellado, 2016). Esto nos ofrece la posibilidad de ver a las plantas parásitas como organismos con un papel determinante en la organización de las comunidades, y como un excelente caso de estudio para explorar y poner a prueba los paradigmas actuales en el ámbito de las interacciones ecológicas planta-herbívoro y parásito-hospedador.

Los muérdagos: Un cóctel de interacciones

Los muérdagos son un grupo funcional de plantas hemiparásitas aéreas, comunes en el dosel de bosques alrededor del mundo. Son un grupo polifilético del orden de las Santalales, con más de 1500 especies pertenecientes a 5 familias y 84 géneros (Těšitel, 2016). La más ancestral, Misodendraceae, con sólo 8 especies, se originó hace 80 millones de años, seguida de Viscaceae (72 Ma), Eremolepidaceae en la familia Santalaceae (53 Ma) y Amphorogynaceae (46 Ma). Finalmente se originó la familia Loranthaceae (28 Ma), la más numerosa (940 especies), distribuida por zonas tropicales y subtropicales (Vidal-Russell & Nickrent, 2008; Nickrent *et al.*, 2010).

Los muérdagos tienen en común la forma de vida hemiparásita, comportándose a la vez como plantas autotróficas, produciendo sus propios hidratos de carbono a través de la fotosíntesis, y al mismo tiempo con un componente parcialmente heterotrófico. Como competidores directos, los muérdagos consiguen extraer agua y nutrientes de su hospedador a través del haustorio, que es una estructura en forma de gancho que penetra en la planta hospedera (Ehleringer *et al.*, 1985; López-Sáez *et al.*, 2002). La retirada de nutrientes puede debilitar y disminuir en el crecimiento, desarrollo y reproducción del hospedador (Geils & Hawksworth, 2002; Koenig *et al.*, 2018; Zamora & Mellado, 2019), llegando incluso a provocar su muerte (Sallé & Frochot, 2002; Sangüesa-Barreda *et al.*, 2012). Sin embargo, la presencia de muérdagos en un ecosistema puede ser el detonante de una serie de interacciones directas e indirectas con multitud de especies de la comunidad situadas en diferentes niveles tróficos (Pennings & Callaway, 1996; Press & Phoenix, 2005). Por un lado, estas plantas pueden representar un nuevo nicho en el dosel forestal jugando el rol de un nuevo recurso disponible. De esta manera atraerían a una nueva comunidad de consumidores como polinizadores, herbívoros, aves frugívoras y mamíferos herbívoros (Burns, 2009; Burns *et al.*, 2014; Umucalilar *et al.*, 2007), generando así nuevas interacciones planta-animal. Por el otro, mediante el impacto desproporcionado en sus hospedadores, estas plantas podrían generar interacciones no-tróficas indirectas de facilitación, mediadas por la planta hospedera, con especies que usan el hospedador como hábitat y/o alimento (Werner & Peacor, 2003; Mooney *et al.*, 2006). Por ejemplo, los cambios ecofisiológicos que sufre el hospedador provocan patrones de crecimiento anómalo (Schulze *et al.*, 1984; Marshall & Ehleringer, 1990; Marshall *et al.*, 1994), cambiando su morfología y arquitectura, generando así un nuevo refugio para aves e insectos (Stevens & Hawksworth, 1970; Bennetts *et al.*, 1996; Watson, 2001; Mathiasen *et al.*, 2008). Al mismo tiempo, pueden darse interacciones tritróficas antagónicas de competencia con consumidores del hospedador, ya que representan un recurso compartido potencialmente limitante (Gómez, 1994; Puustinen & Mutikainen, 2001; Ohgushi, 2005).

Finalmente, pueden intervenir de manera indirecta en la actividad y dinámica de nutrientes del suelo (March & Watson, 2007, 2010). Gracias a la caída de la hojarasca, de baja reabsorción y por tanto rica en nutrientes (Lamont, 1983; Quested *et al.*, 2002; López-Sáez *et al.*, 2002), y la llegada de nuevas semillas mediante la deposición de aves frugívoras atraídas por los muérdagos (Watson, 2009; Mellado & Zamora, 2014a), éstos son capaces de generar una "isla de fertilidad" bajo la copa de sus hospedadores (Mellado *et al.*, 2016). Esto mejora las condiciones del establecimiento de nuevas semillas, actuando así como facilitadores y favoreciendo la dinámica de sucesión al nivel de comunidad (Mellado & Zamora, 2017). Así pues, los muérdagos son una importante fuente de nutrientes, especialmente en suelos pobres (March & Watson, 2007; Ndagurwa *et al.*, 2016), facilitando la actividad de la comunidad arbustiva y microbiana del suelo (Mellado *et al.*, 2016).

Por todo esto, los muérdagos, además de ser considerados como especies clave, pueden ejercer un papel fundamental como ingenieras del ecosistema (Watson, 2001; Watson & Herring, 2012). Así pues, una visión ecosistémica actualizada debe considerar a los muérdagos como una parte integral de la comunidad forestal, lo que nos obliga a cambiar nuestra visión antropocéntrica, basada exclusivamente en la eliminación y control de plagas, hacia un nuevo sistema de gestión que debe buscar el mantener un balance entre plantas parásitas, sus hospedadores, y las restantes especies con las que ambos interaccionan en la comunidad forestal (Stanton, 2006; Watson, 2001).

Viscum album subsp. *austriacum*

La segunda familia más numerosa de muérdagos es la familia monofilética Viscaceae, con 7 géneros y aproximadamente 350 especies que se distribuyen por zonas templadas y tropicales (López-Sáez *et al.*, 2002; Nickrent, 2002; Nickrent *et al.*, 2010). Esta familia contiene el género *Viscum*, donde se encuentra el muérdago europeo *Viscum album* (*V. a.*), nativo de muchas regiones de Europa, limitando al sur con el mar Mediterráneo, al oeste con el océano Atlántico, y llegando por el norte hasta Suecia y al este hasta el Mar Negro (Zuber, 2004). Está compuesto por 4 subespecies, *V. a. album, V. a. abietis, V. a. creticum* (restringido a la isla de Creta), y finalmente

V. a. austriacum, subespecie en la cual se centra esta tesis.

La especie de estudio, *V. a.* subsp. *austriacum*, es un arbusto leñoso y perenne con un crecimiento anual dicotómico que le confiere un aspecto globoso, llegando a tener una envergadura de hasta 1,5 m de diámetro. El proceso de parasitación se da mediante la fijación en ramas (generalmente) y tronco (menos común) de su hospedante. Esta fijación se produce a través del haustorio, una estructura compuesta por cordones corticales que no sólo penetran, sino que se fusionan con el xilema del hospedante, a través del cual adquiere agua y macronutrientes (Zuber, 2004). Es una planta dioica, con flores inconspicuas, sésiles, pequeñas y de color amarillo verdoso, y forma unos frutos carnosos blanquecinos presentes la mayor parte del año (Zuber, 2004; López-Sáez *et al.*, 2002). *Viscum album* subsp. *austriacum* está especializado en la parasitación de coníferas del género *Pinus*, concretamente en la península ibérica parasita a 5 especies: *P. nigra, P. sylvestris, P. halepensis, P. pinaster y P. uncinata* (López-Sáez *et al.*, 1992).

El sistema parásito-hospedador formado por *Viscum album* y *Pinus* sp. nos ofrece la particularidad de una considerable longevidad frente a otros sistemas similares, donde tanto el pino hospedador (hasta 300 años) como el muérdago *V. album* (hasta 40 años) son dos plantas de vida larga. Además, el muérdago europeo presenta un mecanismo de dispersión con una alta frecuencia de reinfección sobre el mismo hospedante (Mellado & Zamora, 2016). Así pues, nos encontramos con un sistema parásito-hospedador de vida larga, en el que el pino hospedador sufre una parasitación crónica incrementando gradualmente su carga parasítica. Es por eso que el muérdago europeo constituye una plaga de ciertos bosques de coníferas, provocando incluso la muerte de los hospedadores altamente parasitados (Pérez-Laorga *et al.*, 1999; Sallé & Frochot, 2002; Dobbertin & Rigling, 2006; Mellado & Zamora, 2016). Sin embargo, aunque pueda llegar a ser un agente de mortalidad, está por determinar el efecto neto en todo el ecosistema forestal.

A pesar de que *V. album* es el muérdago más abundante y ampliamente distribuido de toda Europa, han sido pocos los estudios dedicados a la evaluación de su efecto neto, poniendo en valor su importancia al nivel de ecosistema a largo plazo. Recientemente se ha comenzado a explorar en *V. album* parte de las interacciones descritas en otras especies de muérdago presentes en diferentes ecosistemas (Mellado, 2016). El efecto neto de la presencia de muérdago europeo en un bosque se podría valorar construyendo el puzle ecosistémico en el que está inmerso. Las piezas de este puzle estarían formadas por cada una de las interacciones directas e indirectas que desencadena la presencia de *V. album* en el ecosistema, incluyendo interacciones planta-animal, planta, suelo y microorganismo. Hasta la fecha, sólo algunas de estas piezas han sido descritas (Mellado, 2016), como la interacción directa planta-animal entre el muérdago y frugívoros (Mellado & Zamora, 2014b,a), planta-planta evaluando el impacto en el crecimiento y reproducción del hospedador (Mellado & Zamora, 2020), así como sus efectos indirectos planta-suelo-microorganismos afectando la dinámica de nutrientes del suelo y la comunidad arbustiva bajo la copa de los pinos parasitados (Mellado *et al.*, 2016; Mellado & Zamora, 2017). Conociendo todo el cóctel de interacciones que se pueden desencadenar a partir de diferentes especies de muérdago en distintos ecosistemas, creemos que *V. album* podría también estar ejerciendo un papel fundamental en los bosques europeos, engranando distintas interacciones que forman el puzle ecosistémico.

Por tanto, en esta tesis queremos descubrir nuevas piezas para completar parte de este puzle, desarrollando una visión integradora de su impacto al nivel de comunidad y sus consecuencias ecológicas. Para ello tendremos en cuenta no sólo los efectos antagónicos y negativos directos en su hospedador, sino también los efectos positivos y sinergias en las nuevas interacciones. Este nuevo cóctel de interacciones podría provocar una cascada de efectos indirectos aún por descubrir en múltiples niveles tróficos, desde la atracción de una nueva comunidad de polinizadores e insectos herbívoros y depredadores, hasta la comunidad herbácea y sus herbívoros asociados.

Objetivos generales

Como se ha indicado anteriormente, la presencia de muérdago en el dosel forestal de un bosque puede conllevar multitud de nuevas interacciones ecológicas planta-planta y planta-animal, dejando una huella ecológica aparentemente sutil pero muy patente en la red trófica. En el presente proyecto de tesis vamos a evaluar las consecuencias ecológicas derivadas en la comunidad y el ecosistema forestal de la presencia del muérdago europeo (*Viscum album* subsp. *austriacum*) y, fundamentalmente, de la interacción con su hospedador principal, el pino salgareño (*Pinus nigra* subsp. *salzmannii*). Nuestra hipótesis general es que el muérdago europeo es el detonante de una serie de interacciones, directas e indirectas, que alcanzan diferentes niveles tróficos y cuyas consecuencias ecológicas contribuyen al aumento de la biodiversidad y heterogeneidad espacio-temporal de las comunidades que integran el ecosistema. Para ello, esta tesis se ha dividido en tres grandes partes, cada una de ellas con un objetivo general que se desgrana en varios objetivos específicos o capítulos (Figura 1). En la primera parte estudiamos el rol del muérdago como un nuevo recurso disponible para la comunidad de artrópodos (Capítulos 1, 2 y 3). En la segunda, nos centramos en los efectos directos y el rol del muérdago como competidor de su hospedador, analizando la reacción del hospedador a nivel químico (Capítulos 4 y 5). Finalmente, en la tercera parte nos centramos en las interacciones indirectas de competencia con los herbívoros del pino (Capítulo 6) y de facilitación con la comunidad herbácea y herbívoros asociados (Capítulo 7).

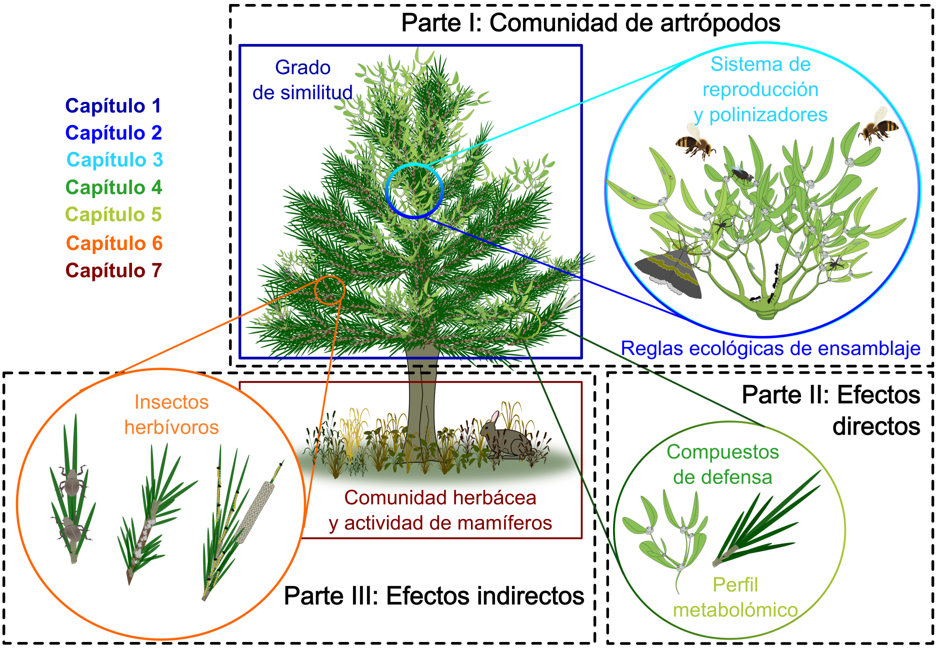

Figura 1: Esquema conceptual de las nuevas interacciones y efectos ecológicos planta–planta y planta–animal desencadenadas por la presencia de *Viscum album* subsp. *austriacum* al nivel de comunidad en los pinares de montaña.

Parte I: Explorar la nueva comunidad de artrópodos asociada al muérdago

La presencia de muérdago europeo en el dosel forestal de un pinar supone el aporte de un punto de heterogeneidad en el mosaico ecológico, ofreciendo un nuevo hábitat y recurso alimenticio. Así pues, el primer objetivo específico (Capítulo 1) que nos planteamos es la caracterización de la comunidad de artrópodos asociada al muérdago y a dos especies de pino hospedador (*P. nigra* y *P. sylvestris*), evaluando el grado de similitud o disimilitud entre todas ellas. El segundo objetivo específico (Capítulo 2) es determinar las reglas ecológicas de ensamblaje que sigue la comunidad de artrópodos asociada al muérdago, y cómo los factores ecológicos operando a diferentes escalas espaciales (a lo largo de un nivel de gradiente altitudinal y a escala de planta hospedadora) definen su estructura y composición. Para ello se analizó la influencia de la altitud, el tamaño de la planta, la distancia a otras plantas y la variación temporal. En último lugar (Capítulo 3), nos proponemos caracterizar taxonómica y funcionalmente la comunidad de artrópodos que interactúa con el muérdago durante su floración, así como identificar la importancia de los diferentes vectores polínicos con un papel preponderante en la formación de frutos del muérdago.

De esta manera tendremos una visión integral de toda la red trófica de artrópodos asociados al muérdago y de su especificidad, desde herbívoros y depredadores (Capítulo 1), así como las reglas de ensamblaje que siguen (Capítulo 2), hasta polinizadores (Capítulo 3).

Parte II: Investigar los efectos directos del muérdago sobre el pino hospedador

A pesar de que los cambios morfológicos y físicos del muérdago en su hospedador han sido bien estudiados, las respuestas a nivel químico del pino frente a una parasitación por muérdago han sido menos investigadas. Es por eso que, en un primer objetivo específico (Capítulo 4), nos proponemos determinar el perfil químico de grandes grupos de compuestos (fenoles, taninos, terpenos) así como del contenido en nitrógeno, tanto de las hojas del muérdago como de las acículas de su principal pino hospedador *P. nigra*. Además, debido a las diferentes cargas parasíticas que pueden sufrir los hospedadores (desde pinos sin muérdago, hasta pinos con más de la mitad de su copa cubierta por muérdago), queremos investigar si los cambios producidos en sus hospedadores son proporcionales al nivel de carga parasítica que soportan. Por otro lado, en el segundo de los objetivos específicos (Capítulo 5) se profundiza en el perfil químico hasta niveles más finos de identificación de compuestos. Para ello, se utiliza una reciente técnica de identificación llamada ecometabolómica (Rivas-Ubach *et al.*, 2012), integrando el estado de la planta en un momento determinado y su perfil metabolómico. Así pues, estudiamos el efecto directo que ejerce el muérdago en su pino hospedador, analizando el metaboloma completo de las acículas de pinos parasitados y no parasitados, en dos estaciones biológicamente importantes (verano y otoño) considerando, a su vez, distintas alturas dentro de la copa del pino para analizar un posible efecto de estratificación. También hemos analizado el metaboloma de las hojas del muérdago con el objetivo de dilucidar si la fusión del muérdago con el xilema del hospedador causa intercambio de fluidos y, por tanto, también de compuestos metabólicos.

Estos análisis nos permitirán evaluar la reacción del pino hospedador frente a una parasitación por muérdago considerando los grandes grupos químicos (Capítulo 4) y el metaboloma completo, englobando metabolitos primarios y secundarios (Capítulo 5), así como el grado de similitud entre el perfil químico del sistema parásito-hospedador.

Parte III: Investigar los efectos indirectos del muérdago al nivel de comunidad

Debido a los posibles cambios en el perfil químico de las acículas del pino a causa de la parasitación por muérdago, nos planteamos si estos cambios se podrían traducir en una pérdida de la calidad como alimento para los herbívoros del pino. Así pues, nuestro objetivo específico (Capítulo 6) es determinar, mediante aproximaciones experimentales de campo y laboratorio, los efectos indirectos del muérdago en relación a su carga parasítica, a través de su pino hospedador en las poblaciones de tres especies focales herbívoros del pino: la procesionaria del pino (*Thaumetopoea pityocampa*), un curculiónido (*Brachyderes* sp.) y un áfido (*Cinara pini*). Como último objetivo específico (Capítulo 7) queremos determinar las consecuencias del enriquecimiento del suelo, gracias a la caída de las hojas del muérdago ricas en nutrientes ("isla de fertilidad"), bajo la copa de los pinos parasitados sobre la comunidad herbácea, así como de mamíferos herbívoros asociados.

Así pues, podemos determinar los efectos indirectos de la presencia de muérdago al nivel de comunidad de insectos herbívoros (Capítulo 6), plantas herbáceas y la actividad de herbívoros mamíferos (Capítulo 7).

Los resultados obtenidos en esta tesis nos permitirán adquirir una visión global y a largo plazo de las consecuencias ecológicas que desencadena la presencia de muérdago en un pinar mediterráneo. Desde un punto de vista aplicado, esto será especialmente valioso para mejorar la gestión integral de las plagas forestales, fomentar la diversidad de interacciones ecológicas, y la conservación forestal en lugares de especial interés.

Materiales y métodos generales

Zona de estudio

Todos los muestreos y experimentos de esta tesis se han desarrollado en el Parque Natural de la Sierra de Baza (Granada, 2°51' 48"W, 37°22'57"N), una montaña mediterránea de la Subregión de las Sierras Penibéticas Septentrionales al sudeste de la península ibérica. El macizo de Baza, con una extensión aproximada de 52337 Ha (Medina & Chirosa, 1992), tiene continuidad hacia el este con la Sierra de los Filabres, componiendo así la Cordillera Baza-Filabres (Gómez-Mercado & Valle, 1988). A su vez, el macizo de Baza está rodeado por llanuras y altiplanicies del Surco Intrabético, constituido por la Hoya de Guadix (al noroeste) y Baza (al norte), los Llanos del Marquesado y el pasillo de Fiñana (al sur, Gómez-Mercado & Valle, 1988; Olmedo-Cobo, 2011). Así pues, el contexto en el que se encuentra la Sierra de Baza la convierte en una isla climática y biogeográfica que favorece la especiación y actúa como refugio de especies (Olmedo-Cobo, 2011). Consta de un rango altitudinal que va desde los 847 m de altitud en Baza hasta los 2269 m en el Calar de Santa Bárbara, y se compone de dos de los tres grandes complejos geológicos de las Sierras Béticas: el Nevado–Filábride al sureste, y el Alpujárride, el más representado. Climatológicamente, se da una amplia gama pisos bioclimáticos, donde se suceden cambios en la vegetación acorde con la altitud: el Mesomediterráneo (∼900–1450 m), el Supramediterráneo (∼1450–1800 m) y el Oromediterráneo (∼>1800 m, Gómez-Mercado & Valle, 1988). En general, la Sierra de Baza presenta un clima Mediterráneo continental, con precipitaciones concentradas en primavera y otoño, y con veranos calurosos y secos (junio–septiembre) seguidos de inviernos fríos y húmedos (diciembre–marzo). Como en muchos sistemas montañosos, las temperaturas y precipitaciones varían acorde al gradiente altitudinal; así pues, encontramos en la Sierra de Baza unas temperaturas y precipitaciones medias anuales desde 12–14℃ y 300–400 mm en la zona basal hasta 5–7℃ y 600–800 mm en las cumbres, respectivamente (Olmedo-Cobo, 2011).

La zona de estudio se compone de un mosaico de especies vegetales, donde la vegetación forestal dominante pertenece a coníferas, con un 43 % de la superficie total cubierta (CMAOT 2008). Entre las distintas especies del género *Pinus* spp., destaca el pino salgareño (*Pinus nigra*), siendo el dominante en toda la sierra y con una distribución altitudinal desde los 1200 m hasta los 1900 m. *Pinus nigra* es el principal hospedador de nuestra especie de estudio, *V. album* subsp. *austriacum*, que se distribuye ampliamente desde los 1300 m hasta los 1900 m en las laderas norte y oeste, representado a su vez, el límite meridional de su distribución geográfica. Encontramos también otras coníferas como el pino carrasco (*P. halepensis*) y el marítimo (*P. pinaster*) desde 1200 m hasta 1400 m, y el pino albar (*P. sylvestris*) a partir de 1750 m. Mezclado con la vegetación dominante se encuentra también quercíneas (*Quercus ilex, Q. coccifera*) con un 9 % de la superficie total cubierta, matorrales y pastizales (23 %), y finalmente, espacios abiertos con escasa cobertura vegetal (9 %, CMAOT 2008).

Diseño experimental general

Los objetivos marcados en esta tesis se llevaron a cabo en distintas zonas de la sierra según los requerimientos de cada uno de ellos. Al trabajar a distintas escalas espaciales, se realizaron 3 diseños experimentales espacialmente bien diferenciados:

a) Escala individual

Con el objetivo de muestrear la artropodofauna asociada a los muérdagos y a sus hospedadores principales, se localizó una cota altitudinal situada a 1700 m, donde coexisten *Pinus nigra* y *Pinus sylvestris.* En este punto de muestreo se llevó a cabo el Capítulo 1, seleccionando 10 pinos de cada especie hospedera, junto con una mata de muérdago en cada uno de ellos. Así pues, se realizó un muestreo de su artropodofauna al nivel de individuo de pino y muérdago.

b) Escala poblacional

En una única cota altitudinal a 1400m, situamos el siguiente diseño experimental. Aquí, encontramos un pinar de repoblación del principal hospedador, *P. nigra*, con individuos coetáneos (∼40 años de edad) y de similar arquitectura. Además, en esta población encontramos un rango de parasitación y tamaños de muérdago muy amplio, desde pinos no parasitados hasta pinos altamente parasitados. La principal ventaja de este punto de muestreo es que nos situamos frente a unas condiciones ecológicas (suelo, altitud, exposición) y ambientales similares en toda la población de pinos, pero con un amplio rango en el grado de parasitación, lo que nos permite evaluar el impacto gradual del parásito. En este punto de muestreo se llevó a cabo parte del Capítulo 2, muestreando muérdagos durante el periodo estival, incluyendo un rango amplio de edades y distancias entre plantas. También se llevaron a cabo los muestreos de los Capítulos 3, 4 y 5 en su totalidad, así como parte del Capítulo 6 mediante la selección de pinos con distintos niveles de parasitación. Para estos capítulos, se seleccionan diferentes pinos de cada categoría de parasitación (pinos focales): pinos no parasitados, pinos con un nivel de carga parasítica bajo (<20 % de la copa del pino ocupada por muérdago), medio (20–50 %) y alto (>50 %).

c) Gradiente altitudinal

Este diseño cubre gran parte de la heterogeneidad forestal, incluyendo zonas de pinar denso alternando con zonas de pinar aclarado. Por tanto, engloba una amplia gama de las condiciones ecológicas típicas de los pinares en las montañas mediterráneas, y nos permite abordar un gradiente ecológico que recoge buena parte de la variabilidad natural representada por la altitud. Así pues, parte del Capítulo 2 se llevó a cabo desde 1300 m hasta 2000 m de altitud seleccionando 10 pinos parasitados de similares características y grado de parasitación, en 8 cotas diferentes separadas cada 100 m. Por otro lado, los Capítulos 6 y 7 se llevaron a cabo en tres cotas altitudinales: baja (1350 m), media (1650 m) y alta (1850 m). En dichas cotas se georreferenciaron parejas de pinos parasitados y no parasitados (20 en 1300 m y 1650 m, y 15 en 1850 m), asociados espacialmente en su proximidad (separados 40–80 m entre sí, para poder analizar efectos de vecindad independientes espacialmente), y de similares características ecológicas (edad, tamaño, microhábitat de crecimiento), haciendo un total de 110 pinos.

Metodología general

Debido a la distinta naturaleza de cada uno de los objetivos, cada capítulo fue desarrollado mediante una metodología distinta. A continuación, daremos una visión general de ellas, mientras que la metodología más detallada se describe en cada uno de los capítulos.

Parte I: Explorar la nueva comunidad de artrópodos asociada al muérdago

Las comunidades de artrópodos asociadas tanto al muérdago como a sus hospedadores (Capítulo 1), se caracterizaron realizando embolsamientos de plantas de muérdagos enteras y partes apicales de las ramas del pino a escala individual (a). Inmediatamente después, tanto las matas de muérdago como las ramas de pino se cortaron y rociaron con spray insecticida dentro de las bolsas para evitar que los artrópodos pudieran escapar. Una vez en el laboratorio se identificaron todos los artrópodos hasta el nivel taxonómico más fino posible.

Por otro lado, para determinar las reglas de ensamblaje y los factores ecológicos que determinan la estructura y composición de la artropodofauna asociada (Capítulo 2), se procedió con dos metodologías y escalas distintas. En primer lugar, a lo largo del gradiende altitudinal (c) se procedió al embolsamiento del mismo modo que en el capítulo 1, seleccionando un muérdago de similares características (tamaño, forma) por cada pino marcado (80 en total). Al mismo tiempo, pero a escala poblacional (b), se seleccionaron 98 muérdagos de diferentes tamaños situados en 24 pinos con distintas cargas parasíticas, para testar el efecto de la variación temporal, tamaño y distancia entre muérdagos. La metodología empleada en estos muérdagos fue la realización de censos visuales durante toda la temporada de máxima actividad de la entomofauna (desde finales de primavera hasta finales de verano), anotando todos los artrópodos presentes en ellos.

En último lugar, para caracterizar la comunidad de artrópodos completa durante la floración del muérdago y la implicación de esta comunidad en la formación de sus frutos (Capítulo 3) se llevaron a cabo, a escala poblacional (b), varias metodologías de muestreo. En ellas se engloban censos circadianos mediante trampas de caída, censos diurnos mediante observación directa, censos nocturnos mediante trampas de luz, y un experimento de exclusión de los diferentes vectores polínicos (artropodofauna, viento y mecanismo de apomixis).

Parte II: Investigar los efectos directos del muérdago sobre el pino hospedador

Los estudios sobre ecología química requieren un mayor control de las condiciones ecológicas, es por eso que el Capítulo 4 se llevó a cabo a escala poblacional en el pinar de repoblación (b). Se recolectaron, en 40 pinos focales pertenecientes a distintas categorías de parasitación, tanto hojas de muérdago como acículas de pino de las dos últimas cohortes. Las muestras se congelaron en nitrógeno líquido inmediatamente después de ser cortadas, conservando así su composición química hasta el procesado en el laboratorio. Mediante diferentes técnicas de extracción de compuestos químicos, se analizaron fenoles, taninos, terpenos y concentración de nitrógeno de cada muestra. Por otro lado, se seleccionaron otras diez parejas de pinos (con una carga media-alta de parasitación y no parasitado) en la misma zona Capítulo 5, para analizar mediante técnicas ecometabolómicas los metabolitos primarios y secundarios de las acículas de pino y hojas de muérdago. Las muestras se recolectan en dos estaciones (verano y otoño), y en tres módulos diferenciados del pino en sentido vertical (basal, medio y apical).

Parte III: Investigar los efectos indirectos del muérdago a nivel de comunidad

Finalmente, el Capítulo 6 se dividió en dos partes bien diferenciadas, la primera se llevó a cabo a lo largo del gradiente altitudinal (c) donde se estimó la abundancia global de las tres especies de herbívoros seleccionados para el estudio (*Thaumetopoea pityocampa*, *Brachyderes* sp. y *Cinara pini*). Para ello se realizaron vareos en los 110 pinos seleccionados a lo largo del gradiente altitudinal, determinando así la abundancia total de áfidos (*C. pini*) y curculiónidos (*Brachyderes* sp.). Por otro lado, se contabilizaron el total de bolsones de procesionaria encontrados en todos los pinos adicionales presentes dentro de una parcela (2 Ha.) a 1650 m. En segundo lugar, se realizaron bioensayos con las tres especies focales con el fin de determinar, de una manera más controlada, el efecto que el muérdago ejerce sobre la calidad del alimento en las acículas del pino, que serán consumidas por estos herbívoros. Por un lado, se realizaron dos bioensayos experimentales de campo con grupos de áfidos y puestas de procesionaria, emplazados en cada uno de los pinos focales de diferentes categorías de parasitación en el pinar de repoblación a escala poblacional (b). Por otro lado, se llevaron a cabo bioensayos en condiciones controladas de laboratorio (cámara de cultivo con temperatura y horas de luz controladas) con larvas de procesionaria y curculiónidos, siendo alimentados con acículas procedentes de los mismos pinos focales. De esta manera podremos determinar el impacto indirecto que ejerce el muérdago sobre las poblaciones de estas tres especies focales a través de su planta hospedera.

En último lugar, para el Capítulo 7, se llevó a cabo a lo largo del gradiente altitudinal (c), donde se caracterizó la comunidad herbácea presente bajo la copa de los 110 pinos focales. La metodología empleada fue la determinación directa en un área uniforme del porcentaje de roca, arbustos y cobertura herbácea, además de la identificación hasta nivel de especie de todas las plantas herbáceas presentes. Paralelamente, se recolectaron todos los excrementos de conejo (*Oryctolagus cuniculus*) en la misma área muestreada para utilizarlos como indicador de su actividad.

Part I

Arthropod communities associated

Chapter 1

Do the arthropod communities on a parasitic plant and its hosts differ?

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Abstract

Parasitic plants growing on tree branches may be a novel niche and phytoresource for arthropods. The spatial continuity between hosts and their parasites in canopies might provide a homogeneous environment for arthropod communities, but differences in mistletoe leaves and host needles could be exploited by different species of arthropods. Therefore, it is important to determine insect–community assemblages in order to discover the role of parasitic plants as a different habitat for arthropods. Thus, we aim to evaluate the level of similarity between the arthropod community on mistletoe (*Viscum album*) and that on its two principal pine hosts, black pine (*Pinus nigra*) and Scots pine (*P. sylvestris*), on a Mediterranean mountain. Our results reveal that, irrespective of the species of pine host, *V. album* has a stable, independent and simple arthropod trophic web, composed mainly of two specialist hemipteran herbivores, *Cacopsylla visci* (Psyllidae) and *Pinalitus viscicola* (Miridae), and a hemipteran predator, *Anthocoris visci* (Anthocoridae). Despite this, the composition of the arthropod communities differed significantly on both host species, with a greater richness and diversity on Scots than black pine and these differences are not reflected in the community on mistletoe. Overall, we conclude that, although *Viscum album* is considered to be a pest of pine, its presence increases the heterogeneity of the forest canopy by providing a novel habitat for a new and specific community of arthropods. In addition, this is a new record for and most probably an extension of the southernmost limit of the known geographical distribution of the arthropod community inhabiting *V. album*.

Key words – mistletoe; *Viscum album*; hemiparasitic plant; pine host; *Pinus*; arthropod communities; specialist insect; *Cacopsylla visci*; *Pinalitus viscicola*; *Anthocoris visci*; hemiptera

Introduction

Forest tree canopies are a heterogeneous environment, in which many traits (nitrogen content; leaf size, shape and toughness; and concentration of secondary compounds) vary widely and determine the foraging and behaviour of the resident arthropod community (Schultz, 1983). Taking advantage of this environmental heterogeneity a great diversity of organisms inhabit forest canopies, including thousands of species of plants, arthropods, vertebrates and microorganisms.

Mistletoes, a diverse group of hemiparasitic plants, inhabit forest canopies and take water as well as mineral resources from host plants, and are considered keystone species in forest ecosystems around the world (Watson, 2001; Watson & Herring, 2012). By consuming plant resources, mistletoes are effectively functioning as herbivores (Pennings & Callaway, 2002), with the important functional distinction that they can also compete with host plants for light. By doing so, mistletoes provide new pathways for plant–plant (Fisher *et al.*, 2013; Graffis & Kneitel, 2015) and plant–animal interactions in forest ecosystems (Watson, 2015; Mellado, 2016). For example, the presence of mistletoes growing on the branches of trees may introduce a novel niche within the canopy and increase the diversity of phytoresources for herbivores. In this respect, several herbivorous insects (Burns, 2009; Burns *et al.*, 2014) and some mammals are reported as consumers of mistletoe foliage (Cooney *et al.*, 2006; Umucalilar *et al.*, 2007).

Because mistletoes are in intimate spatial association with the branches of their host, the spatial continuity of their photosynthetic tissues in canopies might foster a homogeneous distribution and abundance of the arthropod communities inhabiting both the host and the parasite. An alternative possibility is that the presence of mistletoes introduces an additional level of heterogeneity in a forest canopy, since both the physiology and morphology of mistletoe leaves and stems differ from those of its host plant (Těšitel *et al.*, 2010). Consequently, on the one hand, host–dwelling arthropods might not be able to use the parasite as a new resource because of strong biochemical differences, which could nevertheless be exploited by a different arthropod species. This could result in different arthropod assemblages occurring on the host and the parasite. This is potentially a key scenario, since from the standpoint of the arthropods living on the parasite, the hemiparasitic plant may be an “ecological island” surrounded by an “unsuitable sea” of host canopy.

In this study, we analyse this scenario using the mistletoe *Viscum album* subsp. *austriacum* as a case study. At the study site, a natural park in south–eastern Spain, mistletoe parasitizes almost exclusively two species of pine (López-Sáez *et al.*, 1992), black and Scots pine (*Pinus nigra* subsp. salzmannii and *P. sylvestris* subsp. *nevadensis*, respectively). Black pine is a Mediterranean species and thus at our study site it should be at an optimum, while Scots pine has a Eurasian distribution and therefore at the site studied is a relict element from glaciations. Due to their distinct nature, these two host species could present different characteristics as resources for insect herbivores and therefore to have different arthropod communities.

Arthropod communities' on mistletoes are described in previous studies (Burns *et al.*, 2011; Anderson & Braby, 2009; Tassone & Majer, 1997; Room, 1972). Of these, (Burns *et al.*, 2011) and (Tassone & Majer, 1997) compare the arthropod communities' on mistletoes and their hosts in Australia at an ordinal level and restricted to the genus Psylloidea (Burns *et al.*, 2014). In addition, the community assemblage on *Viscum album* is described at the species level in Central (Zuber, 2004; Hellrigl, 2006; Briggs, 2011; Varga *et al.*, 2012) and Northern Europe (Struwe *et al.*, 2009; Hansen & Hodkinson, 2006) but parallel studies on the arthropod communities on their hosts are lacking. Thus, this is the first comparative study of a community assemblage at a higher taxonomic resolution in terms of the composition of arthropods inhabiting *Viscum album* and its host, pine trees.

In this respect, the question we seek to answer is which of these two forces, the "spatial intimacy" or "mistletoes–pine taxonomic differentiation in terms of vegetal tissues", is the stronger. Our hypothesis is that tissue differentiation should be more important than the spatial privacy settings of arthropod communities, so we expect mistletoes to have a singular arthropod community of specialist insects, irrespective of the host species. In this study, we explore the arthropod community associated with the two host species and the parasite, focusing on the abundance, species richness, the herbivore–predator ratio (H:P ratio), Shannon Index (H') and composition of arthropod communities.

Materials and Methods

Study site

Mistletoe *Viscum album* subsp. *austriacum* (Wiesb.) Vollman is a dioecious parasitic plant native to the European region, which throughout its distribution specializes in parasitizing conifers, mainly species of *Pinus*

(Zuber, 2004). This study was conducted in the Natural Park of Sierra de Baza, Granada (a subregion of Sierras Penibéticas Septentrionales, southeastern Spain, 2° 51' 48" W, 37° 22' 57" N, 1650 m asl), which is at the southernmost limit of the geographical distribution of mistletoe (Zuber, 2004). The climate at the site is typically Mediterranean, with an annual mean temperature of 15.5℃ and annual mean rainfall of 307 mm (CMAOT, 2015), concentrated in autumn and spring, and with hot and dry summers (June–September) followed by cold winters (December–March). Conifers are the dominant forest vegetation (43% of the total surface coverage) and black (*Pinus nigra* Arn. subsp. *salzmannii*) and Scots (*P. sylvestris* L.) pine are the main host species of mistletoe although there are other species of pine present, such as Aleppo (*P. halepensis* Mill.) and Maritime (*P. pinaster* Ait.) pine, as well as oaks (*Quercus ilex* L., 9%) and shrubland (23%; CMAOT, 2008).

Sampling

Plants were sampled in August 2012. Since our interest was focused on the differences between the arthropods living on mistletoe and their hosts, we selected midsummer because it is when arthropods are most active. Furthermore, at this time the new leaves on both mistletoe and pine are fully developed, and both plants are metabolically active (Lázaro–González, unpublished results).

We selected 10 black and 10 Scots pines with similar parasitic loads (30–50% of the tree canopy consisted of mistletoe). For each pine selected, we recorded tree height and DBH, and chose an adult mistletoe plant (35–90 cm in diameter, corresponding to individuals >9 years old), which was placed in a bag in situ. Quickly, we cut and sprayed the mistletoe inside the bag with pyrethroid insecticide (Coopermatic ® with pure natural pyrethrins: 1.67% and piperonyl butoxide: 11.10%). On the opposite side of the tree, we chose and sampled the apical portion of a live pine branch, including needles and shoots, which was not infected with mistletoe but of a similar size to the mistletoe sample, following the same procedure.

Pine branches and mistletoe plants were then quickly taken to a laboratory and processed. Bags with samples inside were carefully shaken above a tray to separate the arthropods. Arthropods were identified to the lowest taxonomic level, counted to determine the total abundance for each taxonomic level, classified according to trophic level (herbivores and predators), and stored in vials with Scheerpeltz. Then, pine branches and mistletoes were measured, recording their wet weight, basal diameter, length, width, age and sex for mistletoes, and height and DBH for pine trees (Appendix Table S1). Hereafter, abundance refers to the number of insects divided by either the wet weight of pine branches or mistletoe plants, and thus abundance is a corrected sample–size value and comparable between samples. The H:P ratio was calculated as the herbivore abundance divided by predator abundance. Species richness is the total number of different taxonomic groups identified.

Statistical analyses

Generalized Linear Mixed Models (GLMM's) were used to test host species (black or Scots pine) and vegetal–tissue effects (mistletoe leaves or pine needles) on species richness and the H:P ratio of the arthropod community, assuming a Poisson distribution of data and tree as a random factor.

Linear Mixed Models (LMM's) were used to test the host species and tissue effects (fixed factors) on abundance and Shannon index, assuming tree as a random factor. Abundance and the Shannon Index were log transformed, and all model assumptions were tested.

All statistical analyses were done using R software, Version 3.2.1 (R development Core Team, 2018). GLMM and LMM's were implemented in the *glmer* and *lmer* function of the *lmerTest* package. Shannon index was calculated using the *diversity* function in the *vegan* package.

Multivariate analyses were used to test differences in arthropod composition between mistletoe plants parasitizing black and Scots pine, and between the branches of both these hosts. Firstly, samples were ordered using Non–metric MultiDimensional Scaling (nMDS) based on the Bray–Curtis similarity distances using the *vegdist* function. Then, an analysis of similarity (ANOSIM), based on 10,000 permutations, was used to establish significant differences in arthropod abundance depending on host species (*P. nigra* or *P. sylvestris*) and plant tissue (pine or mistletoe) using the *anosim* function. ANOSIM returns R values that can vary from 0 (similar communities between groups) to 1 (big differences in the communities between groups), and P–values that are significance levels. Finally, the taxonomic groups that most contributed to differences in assemblage composition were identified by similarity percentages (SIMPER). *Vegdist*, *anosim* and *simper* functions are included in the *vegan* package. NMDS was used to determine the composition of the assemblages from databases, using the last matrix distance and *isoMDS* function in the *MASS* package.

Throughout the paper, means are expressed ± 1SE.

Results

Abundance, species richness, H:P ratio, and Shannon index of the arthropod communities

A total of 1271 arthropods were collected, which included individuals belonging to 12 orders and 33 taxonomic groups (Appendix Table S2). The arthropod communities on mistletoes and their host pine differed in terms of all the aspects tested (Table 1.1), although no significant interactions were found between host species and plant tissue. Although a significantly greater species richness and diversity was recorded on Scots pines (29 taxonomic groups and H' = 1.82 ± 0.1) than on black pines (18 taxonomic groups and H' = 1.56 ± 0.1), mistletoes consistently had the same richness on both hosts, with 18 and 16 taxonomic groups, H' = 1.31 ± 0.1 and H' = 1.33 ± 0.1, respectively. The average abundance of arthropods and their H:P ratio were significantly greater for the communities inhabiting pine trees (72.0 ± 11.1 arthropods/kg and 2.5 ± 0.4) than mistletoe (51.2 ± 7.1 arthropods/kg and 1.6 ± 0.2). Overall, pines had higher richness, abundance, H:P ratio and diversity than mistletoe (Figure 1.1).

Table 1.1: The effect of host species (*P. nigra* and *P. sylvestris*), vegetal tissue (mistletoe leaves and pines needles) and their interaction on the abundance, richness of species, herbivore–predator ratio and diversity of arthropod communities. χ^2, *F–values*, significance (P) and degrees of freedom (df) come from the GLMM and LMM_{ANOVA} tables. Bold type indicate significant results.

	Abundance			**Richness**			**H:P ratio**			**Shannon Index**		
	df	F	P	df	χ^2	P	df	χ^2	P	df	F	P
Host species	1,18	0.53	0.473	1	5.34	**0.021**	1	0.01	0.917	1,18	0.49	0.491
Vegetal tissue	1,18	3.29	**0.086**	1	10.95	<**0.001**	1	3.99	**0.046**	1,18	25.46	<**0.001**
Interaction	1,18	2.99	0.101	1	1.05	0.306	1	0.20	0.635	1,18	3.51	0.077

All these results imply that Scots pine has a richer and more diverse arthropod community than black pine but similar abundance and H:P ratio; however, these differences are not reflected in the arthropod community on mistletoe, which is composed of fewer species with a lower abundance and diversity than that recorded on pines.

Composition of the arthropod communities

The compositions of the arthropod communities recorded on mistletoe and its hosts (Figure 1.2) were highly significantly different for both host

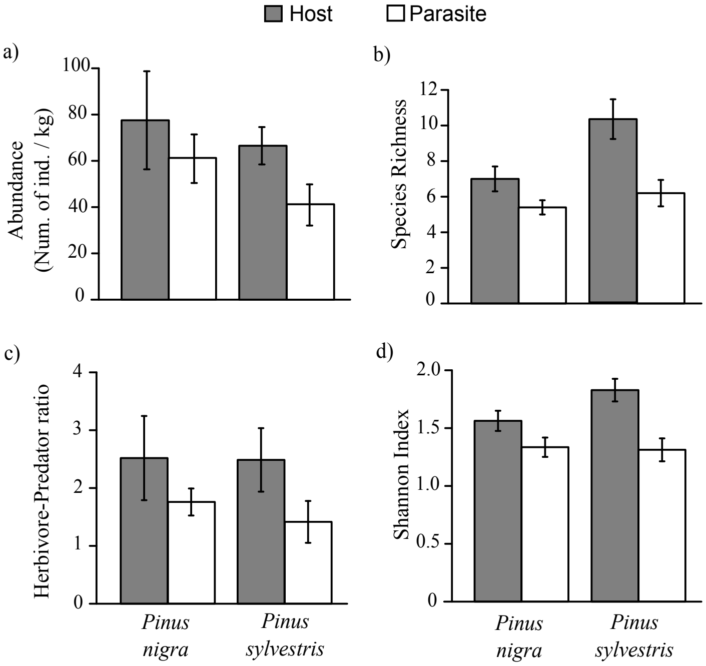

Figure 1.1: Mean (± SE) of a) abundance, b) species richness, c) H:P ratio and d) Shannon index of arthropod communities associated with two species of pine (grey bars) and their parasite *Viscum album* (white bars).

species, based on the *R* and *P–value*, respectively ($\text{ANOSIM}_{P.\ nigra}$: R = 0.885, $P < 0.001$; $\text{ANOSIM}_{P.\ sylvestris}$: R = 0.999, $P < 0.001$).

Although the only significant differences between hosts were species richness and diversity, these analyses reveal that there were also fewer differences in the arthropod communities on black and Scots pine ($\text{ANOSIM}_{\text{Host}}$, R = 0.212, $P < 0.001$). The SIMPER analysis revealed that these differences are attributable principally to psyllids, which were three times more abundant on Scots pine (20.46 ± 3.04 individuals/kg) than on black pine (7.21 ± 1.90 individuals/kg). Spiders and thrips were also twice as abundant on black (21.56 ± 7.29 and 15.46 ± 9.98 individuals/kg, respectively) than on Scots pine (12.36 ± 1.97 and 6.24 ± 2.61 individuals/kg, respectively) and aphids were abundant on black (14.82 ± 8.13 individuals/kg) but practically absent on Scots pine (0.91 ± 0.50 individuals/kg). The aphids were *Cinara pini* and *Eulachnus sp.* (Hemiptera: Sternorrhyncha).

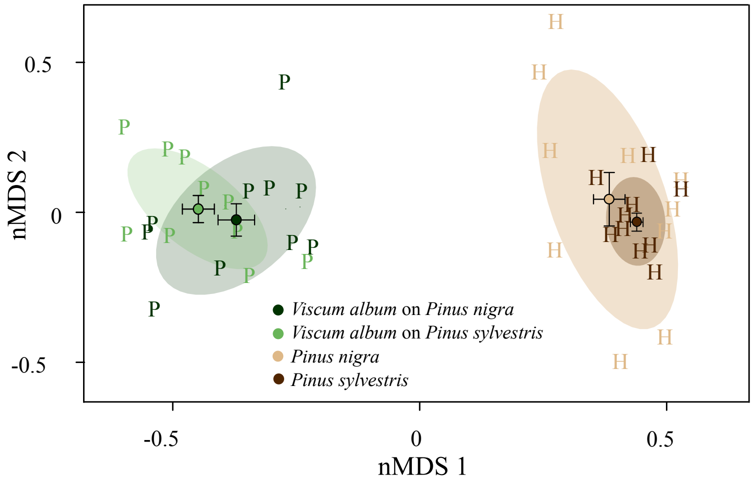

Figure 1.2: Non–Metric MultiDimensional Scaling (nMDS) ordination of arthropod communities recorded on mistletoe (P = dark green on *P. nigra* and light green on *P. sylvestris*) and their hosts (H = light brown on *P. nigra* and dark brown on *P. sylvestris*). Points are means (± SE) of the distributions of the compositions of arthropods inhabiting mistletoe and its hosts.

The composition of the arthropod community on mistletoe plants parasitizing both host plants was similar ANOSIM$_{\mathrm{Parasites}}$, R = 0.105, P = 0.069). These similarities were due to the mistletoe bug, *Pinalitus* cf. *viscicola*, the sap–sucking bug, *Cacopsylla* cf. *visci*, and their predator, *Anthocoris* cf. *visci*, making up 79% and spiders 8% of the arthropod assemblage on mistletoes. These results closely fit the preceding results, since the host and parasite differed in all variables tested as well as in the composition of the arthropod communities, while the arthropod community on mistletoe remained constant despite those on Scots and black pines differing in richness, diversity and relative abundance.

The Global SIMPER analysis also revealed that the main differences recorded between mistletoe and its hosts were attributable to three main taxa: *Cacopsylla visci* (19% of total contribution), *Anthocoris visci* (15%) and *Pinalitus viscicola* (5%), which clearly accounted for more of the arthropods on mistletoe (19.45 ± 3.84, 15.66 ± 2.90 and 5.21 ± 1.60 individuals/kg, respectively) than on pine trees (0.0 ± 0.0, 0.29 ± 0.25 and 0.07 ± 0.07 individuals/kg, respectively both pine species merged). Other arthropods such as Psyllidae, Araneae and Thysanoptera were also responsible for these differences (contributing 14%, 12% and 8%, respectively), but there were more psyllids, spiders and thrips on pine trees

(13.84 ± 2.31,16.96 ± 3.83 and 10.65 ± 5.13 individuals/kg, respectively) than on mistletoe (0.0 ± 0.0, 4.25 ± 1.07 and 0.27 ± 0.22 individuals/kg, respectively) where they were practically absent.

Discussion

The arthropod community on *V. album* is well studied and there are several host specific insects associated with this plant in Central Europe (Hellrigl, 2006; Briggs, 2011; Varga *et al.*, 2012; Schumacher, 1918 in Zuber, 2004). In addition, some studies on arthropod assemblages support the contention of a high degree of host specificity of the psyllids on European mistletoe (Struwe *et al.*, 2009; Hansen & Hodkinson, 2006) and other species of mistletoes (Burns & Watson, 2013; Whittaker, 1982). However, there are a few direct comparisons of the arthropod communities on mistletoes and their hosts that are restricted to the Psylloidea (Burns *et al.*, 2014) or an ordinal level of taxonomic resolution, which record finding the same arthropod orders on both mistletoes and their hosts (Tassone & Majer, 1997; Burns *et al.*, 2011), but that the total abundance is greater on the foliage of the host (Burns *et al.*, 2011).

By contrast, our study provides detailed information on all the arthropod species at a fine taxonomic resolution living on both mistletoe and its host plants; the results reveal clear differences between the arthropod communities associated with two species of pines (*Pinus nigra* and *P. sylvestris*) and their shared parasitic plant (*Viscum album* subsp. *austriacum*).

The arthropod community on *P. sylvestris* is significantly richer, more diverse and different in composition than that on *P. nigra* (relative abundances of psyllids on Scots pine and spiders, thrips and aphids on black pine), probably due to the distinct nature of these two species. Despite these differences the mistletoe parasitizing them harbour a simpler and specific arthropod community, consisting of two herbivores and their predator, and a lower abundance itinerant spiders (Burns & Watson, 2013). The herbivores are *Cacopsylla visci* and *Pinalitus viscicola*, which are recorded inhabiting *V. album* across Europe (Zuber, 2004; Hansen & Hodkinson, 2006; Struwe *et al.*, 2009; Varga *et al.*, 2012) and their main predator *Anthocoris visci* (Briggs, 2011; Hellrigl, 2006). In line with this, the arthropod community recorded on *V. album* in these studies there are 8 host specific insects inhabiting mistletoes in Hungary (Varga *et al.*, 2012) and northern Italy (Hellrigl, 2006), 6 in England (Briggs, 2011), 5 in France (Schumacher, 1918 in (Zuber, 2004) and a few less (minimum 3) in Sweden (Struwe *et al.*, 2009). Thus, the results of our study indicates that *V. album* in Southern Europe has a simpler community than mistletoe in the rest of Europe (Zuber, 2004; Hellrigl, 2006; Briggs, 2011; Varga *et al.*, 2012), given that we recorded only three insect mistletoe specialists. In addition, the present study enlarges the distribution of these insects, with most probably a new southern limit to the distribution of *C. visci*, *P. viscicola* and *A. visci.* The most important difference in the composition (Fig. 2) of arthropod assemblages between hosts and parasite was due to the high specificity of these three species for mistletoe; while the arthropods restricted to pine are pine–feeding herbivores (e.g. thrips, psyllids, leafhoppers, aphids) that are unable to use mistletoe as a resource. In addition, other arthropods are canopy generalists, and opportunistically occur on mistletoe when seeking food and/or shelter (Burns & Watson, 2013). In line with this, the arthropod assemblage on mistletoe is similar in abundance, species richness, H:P ratio and diversity, irrespective of the host species. Our results are consistent with the general pattern (Pimm, 1991) in which communities with many predators and prey species, as those on pine trees have high H:P ratios.

Even though the "spatial intimacy" in terms of the close contact between mistletoe and the branches of their host may seem to provide spatial continuity in tree canopy, the arthropods associated with mistletoe avoid pine needles and branches and vice versa. This supports the hypothesis that the mistletoe–pine taxonomic differentiation in vegetal tissue is a stronger force than is spatial intimacy.

Despite the fact that *Viscum album* is traditionally considered to be a pest of its pine hosts (Sangüesa-Barreda *et al.*, 2012; Muñoz *et al.*, 2007) it increases the heterogeneity of the forest canopy by providing a novel habitat for a new and specific community of arthropods. This additional level of heterogeneity in a forest canopy increases the arthropod diversity and spatial range for some insects, by providing mistletoes a new ecological value. Arthropods are the most diverse and numerous of all living animals, a major part of the global biodiversity and important components of ecosystems (New, 1998; Weisser & Siemann, 2008). By contrast, arthropods are usually overlooked by policy makers and the conservation community (Cardoso *et al.*, 2011). Only 1268 insect species are included in the 2016 IUCN "Red List of Threatened Species", which includes a total of 12630 animals (65% of them vertebrates; (IUCN, 2016)). In Spain, a total of 617 species or subspecies are catalogued as threatened, of which only 116 are arthropods (IUCN, 2016), undoubtedly mainly due to a lack of information. This work clearly exemplifies the mismatch between considering mistletoe as a pest that needs to be controlled vs. a keystone species providing new opportunities for novel species (Mellado & Zamora, 2016; Mellado *et al.*, 2016).

In summary, *Viscum album* in Southern Europe has a stable, independent and the simplest arthropod community in Europe. For this parasitic plant, *Cacopsylla visci* and *Pinalitus viscicola* are the only two specialist herbivores, and *Anthocoris visci* the only specialist predator, which probably at this location are at the southernmost limit of their geographical distribution. Thus, mistletoe parasitizing pine is not in spatial continuity in terms of the distribution and abundance of arthropod communities between parasite and host. Therefore, this parasitic plant represents isolated patches surrounded by the foliage of its host, inhabited by only a few insects specific to mistletoe. In short, *Viscum album* determines the presence of particular specific insects by providing an ecological island in a sea of host foliage and as such is a keystone species for a unique and specialized trophic web.

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Chapter 2

Ecological assembly rules on arthropod community inhabiting mistletoes

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Abstract

The colonisation of a new habitat by a community is led by deterministic and stochastic processes at different spatio–temporal scales. Parasitic plants, such as mistletoe, represent a new habitat within forest canopy that is free to be colonised by many organisms. This study investigates how ecological factors operating at forest and plant scales drive changes in both specialist (mistletoe–dwelling) and tourist (transient visitors) arthropod communities inhabiting European mistletoe, *Viscum album* subsp. *austriacum*, in a Mediterranean pine forest. The influence of elevation along a broad elevational gradient was tested by sampling arthropod communities dwelling in mistletoe plants and host pine branches and the effects of mistletoe plant size, distances to other mistletoes, and temporal variation in arthropod assemblages inhabiting mistletoes. The diversity of the specialist community remained constant along the elevational gradient and over the summer period, while the tourist and pine–dwelling arthropod communities showed species turnover. Larger mistletoes were occupied by more species and individuals, whereas more isolated mistletoes presented the same equilibrium point as the more aggregated ones. Thus, mistletoe size is key to the composition of the arthropod community. In conclusion, this study's findings indicate contrasting assembly rules for specialised and tourist arthropod communities associated with mistletoe. The specialist community was highly stable and followed a deterministic trophic sequence of colonisation as the assemblage rule: first, colonisation by the main specialist herbivore, *Cacopsylla visci*, and, second, by its predator *Anthocoris visci*. Meanwhile, the tourist community, being a subset of the arthropod assemblage of the pine, acts independent of mistletoe presence.

Key words – alpha– and beta–diversity; arthropod communities; mistletoe; size and distance effects; species–elevation relationship; species–temporal relationship

Introduction

The mechanisms driving species colonization and coexistence in a given habitat is one of the main puzzles in community ecology. Community assembly is the process by which species from a regional pool colonize and interact to form local communities (HilleRisLambers *et al.*, 2012). Historically, community assembly has been interpreted through longstanding niche–differentiation theory (MacArthur & Levins, 1967; Chase & Leibold, 2003; Tilman, 2004; Leibold & McPeek, 2006), which explains community structure as the result of deterministic processes such as trophic habit. Since the late 20th century, this interpretation has been challenged by neutral theory, which emphasizes the importance of stochastic processes such as dispersion (Hubbell, 2001; Rosindell *et al.*, 2011). Currently, the predominate theory concerns the complementarity and simultaneity of both stochastic and deterministic processes in explaining community assembly rules (Tilman, 2004; Gravel *et al.*, 2006; Vergnon *et al.*, 2012).

In terms of species attributes, dispersal ability in space and time determines the home range of species in nature (Tscharntke *et al.*, 2002). For example, changes in habitat connectivity commonly affect diversity and abundance of specialist insects (Thomas *et al.*, 2001b; Kruess, 2003), with the poorest dispersers exhibiting colonization success in recent habitats (Heiniger *et al.*, 2014). Therefore, changes in the spatio–temporal patterns of habitat patches might lead to insect community shifts according to species attributes such as specialization level, trophic habit, or dispersal ability (Thomas *et al.*, 2001a; Menéndez, 2007; Hendrickx *et al.*, 2009).

Parasitic plants such as mistletoes, on establishing themselves, constitute a new habitat within forest canopy that is free to be colonized by many organisms such as birds, mammals (Watson, 2001; Mathiasen *et al.*, 2008), and invertebrates (Burns & Watson, 2013). Several studies have shown how mistletoes are inhabited by highly specialized insects (Hansen & Hodkinson, 2006; Hellrigl, 2006; Struwe *et al.*, 2009; Briggs, 2011; Burns *et al.*, 2011; Varga *et al.*, 2012). This specialized community differs from the host arthropod community, representing isolated patches surrounded by the foliage of its host (Anderson & Braby, 2009; Burns, 2009; Burns *et al.*, 2011; Lázaro-González *et al.*, 2017). However, mistletoes are also visited by generalist tourist fauna, which is a subset of host arthropod assemblages (Burns *et al.*, 2011; Lázaro-González *et al.*, 2017). Thus, the arthropod community is composed of specialized insects exclusively inhabiting mistletoes (specialist community), and a second set of species that visit mistletoe leaves from the host pine foliage (tourist community;

Lázaro-González *et al.*, 2017). The resulting assembly rules of mistletoe arthropod communities have not been investigated in any previous study available. Only a limited set of studies has highlighted the importance of the interplay between landscape attributes (e.g. size, distance, habitat age) and species traits (e.g. dispersal ability, trophic habit) in the assembly of insect communities in new habitat patches (Hunter, 2002).

Despite of ample literature identifying arthropods associated with mistletoes (Room, 1972; Tassone & Majer, 1997; Anderson & Braby, 2009; Burns *et al.*, 2011), general diversity patterns remain undetermined. Along environmental gradients, strong abiotic variation occurs (e.g. falling temperatures with rising elevation). This environmental variation is accompanied by changes in the number, identity, and abundance of species. Mistletoe is sensitive to cold temperatures as its geographic and elevational distribution in Europe is strictly limited by winter temperatures (Zuber, 2004; Dobbertin *et al.*, 2005). Within a global warming scenario, a general pattern of rising temperatures in the second half of the 20th century has been recorded in the southern Mediterranean mountains (Herrero *et al.*, 2013; Zamora *et al.*, 2016). The consequence is that mistletoe is expanding its elevational distribution towards the summits due to warming temperatures and to suitable vectors for parasite dispersion along the gradient (Zamora & Mellado, 2019). This recent elevational colonization by mistletoes raises the question of whether the arthropod community has accompanied mistletoes to the top elevational distribution, shaping the same stable specialist and tourist mistletoe community. Conversely, mistletoes located at their top distribution limit harbour unsaturated communities because mistletoe expansion is too recent for a complete arthropod colonization and establishment.

In addition, mistletoes show a markedly patchy pattern throughout their geographical distribution, providing a wide range of degrees of plant isolation, from an isolated mistletoe to high densities in the same host (Overton, 1994; Aukema, 2004; March & Watson, 2007; Mellado & Zamora, 2014a). For this patchiness, mistletoe can be considered “ecological islands” surrounded by a hostile matrix (Burns & Watson, 2013), susceptible to colonisation by a new trophic web. In this context, the Theory of Island Biogeography (MacArthur & Wilson, 1963, 1967) provides a conceptual framework for understanding how communities can be modelled by key ecological factors. These factors could be the island size at the plant scale (species–area relationship) and distance to other islands within and between pine canopies at the pine tree scale (species–isolation relationship; Rosenzweig, 1995).

In this study, we explore the entire arthropod community inhabiting European mistletoe, *Viscum album* subsp. *austriacum* (Wiesb.) Vollmann, and how ecological factors operating at different spatial scales drive changes on specialists and tourist communities. Therefore, this is the first study on mistletoe plants that combines taxonomy and ecology at the community level by analysing the specific composition (α– and β–diversity) and abundance across a wide range of environmental conditions, including the following factors: (i) a broad elevational gradient recently colonised by mistletoe, (ii) a fine–scale approach considering the size of the mistletoe plants and (iii) their degree of isolation (distance between plants within the canopy of a parasitized pine and within the nearest parasitized pines), and (iv) a temporal gradient through the summer season. Specialist insects are among the first organisms to colonize new habitats (Lawton, 1983). Thus, we hypothesised that the community subset of specialist species would be more affected by "internal" ecological factors related to the size and degree of isolation of mistletoe plants, whereas the tourist assemblage composition and abundance would depend more on "external factors" such as the arthropod community of the pine canopy. Specifically, we test the following specific predictions: (i) Elevation will be negatively related to species richness and abundance; mistletoes that recently colonised high elevations will particularly be inhabited by unsaturated communities (more evident in the specialist subset); (ii) larger mistletoes will be occupied by more species and individuals; (iii) more isolated mistletoes will contain fewer species and individuals (more evident in the specialist subset); and (iv) finally, differences will appear in composition through the summer season driven by a general turnover species process (more evident in the tourist subset). Thus, the aim of the present study is to identify the ecological assembly rules for arthropod communities inhabiting mistletoes.

Materials and Methods

Study site

The study was located in a Mediterranean pine forest on Sierra de Baza (37° 24' N, 2° 50' W, Granada, Spain), with an elevational gradient from 1200 to 2269 m asl. This area (south–eastern Iberian Peninsula) has a typical Mediterranean climate, with hot and dry summers (June–September) and cold winters (December–March), with precipitation concentrated in spring and autumn. The historical annual mean temperature and precipitation are 15.5 ± 1.3℃ (CMAOT, 2017) and 495 ± 33 mm, respectively

(1991–2006 period; Cortijo Narváez weather station, 1360 m asl).

Viscum album subsp. *austriacum* (hereafter *V. album*), a parasitic plant native to European forests, specialises in parasitising conifers (Zuber, 2004). At our study site, 43% of the total surface is covered by conifers, where black pine (*Pinus nigra* Arn. subsp. *salzmannii*, hereafter *P. nigra*) serves as the main host for *V. album* from 1300 m to 2000 m (Mellado & Zamora, 2014b). Other pine species present include Scots (*P. sylvestris* L.), Aleppo (*P. halepensis* Mill.), and Maritime pine (*P. pinaster* Ait.), mixed with Holm oaks (*Quercus ilex* L., 9%) and shrublands (23%, CMAOT, 2008).

Sampling

Two experiments were conducted to determine the effects of four ecological factors on the diversity and abundance of arthropod communities associated with mistletoe plants parasitising black pines. The first examined the changes of these arthropod communities at the forest scale, in relation to shifts in their host, over the entire elevational gradient of *V. album* range distribution in the Natural Park of Sierra de Baza (1300 – 2000 m asl). The second experiment, at plant scale, was conducted at 1400m asl to investigate the temporal changes in arthropod communities during their period of maximum activity (early July to end of September). Concurrently, this second experiment focused on changes in the arthropod community of the mistletoe according to plant size and distance.

Forest scale: elevational gradient

In July 2015, the first experiment was conducted across an elevational gradient where *V. album* parasitises *Pinus nigra*. Eight elevations were selected at 100–m intervals, from 1300 m to 2000 m. For each sampling plot, we analysed 10 mistletoes of similar weight (267.3 ± 15.3 g of wet weight) and size (0.55 ± 0.03 m^3) that were parasitising 10 different pine trees of similar height (6.1 ± 0.2 m), similar diameter at breast height (DBH; 73.4 ± 2.8 cm), and similar parasitic load (30–50% of canopy cover by mistletoe). The height and DBH of the host pine trees were measured *in situ*. Mistletoe plants and an apical section of a pine branch of similar dimensions were independently bagged *in situ*, cut off at the base, and the inside of the bag was quickly sprayed with pyrethroid insecticide (Coopermatic® with pure natural pyrethrins: 1.67% and piperonyl butoxide: 11.10%; see Lázaro-González *et al.*, 2017, for a similar procedure). A total of 160 bags (10 mistletoe plants and 10 section of pine branch per elevation level) were processed at the laboratory, where all samples were removed from the bags and shaken above a tray to separate the arthropods. All arthropods were identified to the lowest taxonomic level possible, determining the total abundance of each taxonomic group, and were stored in vials with Scheerpeltz solution (70% ethanol 96°, 28% distilled water, 1% acetic acid, and 1% glycerine). Then, mistletoe plants and pine branches were measured for wet weight and size (height, width, length; Appendix Table S3).

All samplings were performed on sunny and windless days. Arthropod communities of both experiments were characterised by local species richness (number of different taxonomic groups identified according to elevation, α–diversity), assemblage composition (β–diversity), and density (number of total arthropods per plant volume).

Plant scale: size, distance, and temporal variation

The second experiment was conducted in Cortijo Casimiro (1400 m asl), in a *P. nigra* forest of similar age and under similar ecological conditions, where pine trees bear diverse mistletoe loads. We selected 98 mistletoe individuals (hereafter focal mistletoes), 1–30 years old (see counting details in Appendix S1), parasitizing 24 different black pine trees (hereafter focal pines) of similar height (7.7 ± 0.4 m) and DBH (83.7 ± 3.1 cm), but with different numbers of mistletoe plants. The height and DBH of the host pine trees were measured *in situ*.

Samplings were performed from early July to end of September 2013 in order to test the temporal variation in arthropod community structure and composition. This period coincides with the maximum activity of herbivores specialising in mistletoe (Hansen & Hodkinson, 2006). The samplings consisted of 5 min of mistletoe observation performed biweekly for 6 sampling days, with a total of 2940 min of observation (98 mistletoe plants × 5 min of observation × 6 sampling days). Two technicians performed the observations simultaneously on different mistletoes between 9h and 16h, in arbitrary order to avoid any bias of the results. Arthropod counts were conducted by detailed inspections of mistletoe branch, with binoculars being used when necessary. The individuals making contact with the observed mistletoe surfaces were counted and identified *in situ* to the lowest taxonomic level. Younger mistletoes (1–6 years old) were observed in their entirety due to their small size. Meanwhile, older mistletoes (>7 years old), because of their large size, were observed only partially, with three of the most visible branches selected. We estimated the number of branches (see details Appendix S1 Fig. S1) and calculated arthropod density as total abundance per mistletoe plant. Similarly, the plant volume of all focal mistletoes was predicted according to plant age (see details Appendix S1 Fig. S2), and used as a plant–size variable.

Second, isolation degree was considered to be the distance between mistletoes within the same pine canopy (distance–within) and the distance to other parasitised pines accompanied by their parasite load (neighbourhood index). For distances–within, 10 mistletoes were arbitrarily assigned per focal pine, and we measured distances to the three nearest mistletoe plants located on the same pine canopy. Then, we calculated their average distance–within by each focal pine. For the neighbourhood index, we measured distance (D) from each focal pine to the three nearest parasitised pines ($n = 3$), and we counted the number of mistletoe plants parasitising them (M). Then, the neighbourhood index was calculated using the following equation:

$$Neighbourhood\ index = \frac{\sum_{i=1}^{n=3}(\frac{M_i}{D_i})}{n}$$

Therefore, we compared arthropod communities associated with *V. album* over a wide range of plant size, isolation degree considering distance–within canopy and the neighbourhood index, and throughout the summer season.

Statistical analyses

Forest scale: elevational gradient

We calculated a dissimilarity matrix for community–assemblage composition (β–diversity) using the Bray–Curtis index. In addition, we calculated a spatial matrix between plots and a distance matrix for volume of each sample, both using Euclidean distance. We performed a Partial Mantel Test based on Spearman correlation to test spatial correlation between community assemblages across the elevational gradient, controlling for the effect of sample volume. We also used a Partial Mantel Correlogram to test significant differences of paired “distance classes” using 999 permutations and Bonferroni correction for multiple testing.

The Generalized Linear Model (GLM), assuming a Poisson distribution, and Linear Model (LM) was used to test differences in species richness (α–diversity) and densities, respectively, of the arthropod communities associated with mistletoe plants and the host pine along the elevational gradient. Densities of arthropod communities associated with mistletoes and pine hosts were measured using square root and were log–transformed, respectively, in order to meet assumptions of normality and homoscedasticity. Tukey's honestly significant difference (HSD) test (95% confidence level) was used for comparisons between elevational gradient levels.

Plant scale: size, distance, and temporal variation

We calculated a dissimilarity matrix using the Bray–Curtis index for each community assemblage composition (β–diversity) according to the four ecological factors evaluated (mistletoe plant size, distance–within, neighbourhood index, and time sampling). Concurrently, one distance matrix, using Euclidean distance, was calculated per factor tested: a volume matrix based on different mistletoe plant sizes, a spatial matrix based on different distances–within, a neighbourhood matrix based on different neighbourhood indices, and a temporal matrix based on different sampling days. Finally, dissimilarity in species composition was also tested using the Mantel Test, based on Spearman correlation, and the Mantel Correlogram in order to determine whether β–diversity was autocorrelated with "distance classes" of each tested variable.

Similarly, due to a high number of zeros, zero–inflated models (ZINB), assuming a negative binomial distribution, were used to test the effects of mistletoe size, distance to other mistletoes within pine canopy and between infested trees, and sampling period on species richness (α–diversity) and abundance. Each focal pine and mistletoe plant was used as a nested random factor. Second, Tukey's pairwise comparisons were used to identify differences between time sampling weeks (J1= early July, J2= mid–July, A1= early August, A2= mid–August, S1=early September, S2= mid–September) and plant size categories (<0.001, 0.001–0.01, 0.01–0.05, 0.05–0.15, >0.15 m^3).

All statistical analyses were conducted using R software, Version 3.6.0 (R Core Team, 2019). ZINB's models were implemented with the *glmmadmb* function in the *glmmADMB* package (Fournier *et al.*, 2012; Skaug *et al.*, 2016). Tukey tests were conducted using *glht* of the *multcomp* package (Hothorn *et al.*, 2008). GLMs, LMMs and LMs were running with *glm, lm* and *lmer* function of the *stats* and *lme4* packages, respectively (Bates *et al.*, 2015). Distance matrices were calculated using *vegdist* function, and Partial Mantel Test and Mantel Test were calculated using *mantel* and *mantel.partial* functions, all using the *vegan* package (Oksanen *et al.*, 2019). Finally, Partial Mantel Correlogram and Mantel Correlogram was carried out with *mpmcorrelogram* function of the same package (Matesanz *et al.*, 2011).

Results

Forest scale: elevational gradient

A total of 967 arthropods were captured in mistletoe plants and 1147 in host pine trees. These arthropods belonged to 12 orders and 38 taxonomic groups (Appendix Table S5). The Partial Mantel Test showed no differences in the specialist community composition along the elevational gradient. However, tourist species inhabiting mistletoes and arthropod communities living on pine host presented an isolation by distance throughout the elevational gradient, where distant plant hosts (more than 600 m of elevational gradient) harboured a statistically different community (Fig. 2.1a, Table 2.1a). Nevertheless, specialists and arthropod assemblages inhabiting pine host presented a constant α–diversity (Fig. 2.1b, Table 2.1a) along elevational gradient, whereas α–diversity of tourist community increased at mid–elevational levels (1500 m asl). Arthropod communities dwelling in mistletoes, both specialists and tourists, registered peak densities at medium elevations (1500 m asl), while arthropod assemblages on pine hosts increased their density at high elevations (1900 m asl, Fig. 2.1c, Table 2.1a).

Community assemblages inhabiting mistletoes were composed mainly of the specialist herbivores *Cacopsylla visci* Curtis, 1835 (63.1% relative abundance of total arthropod community over the entire elevational gradient) and *Pinalitus viscicola* Puton, 1888 (2%) and their common specialist predator *Anthocoris visci* visci Douglas, 1889 (4.1%), with tourists such as spiders (8.3%) and parasitic wasps (7%) coming from the pine host. Host pine branches bore a more diverse community composed of spiders (22.2% of total pine–dwelling arthropod community), thrips (19.5%), aphids (12.3%), ants (8%), psyllids (*C. visci*; 7.8%), parasitic wasps (3.7%) and leafhoppers (3.5%; Appendix Table S5).

Plant scale: size, distance, and temporal variation

A total of 525 arthropods inhabiting mistletoe plants were counted during the observations, belonging to 14 orders and 27 taxonomic groups (Appendix Table S6).

Mantel Correlation revealed that the composition of the specialist community (β–diversity) was identical in all mistletoe plant sizes and had

Table 2.1: The effects at forest scale of a) elevational gradient on dissimilarities in composition (β–diversity), local species richness (α–diversity), and the density of specialist and tourist communities associated with mistletoe plants and arthropods inhabiting pine host. In addition, the effects at plant scale of b) mistletoe plant size, distance to other mistletoes, and the neighbourhood index, and c) temporal variation on β–diversity, α–diversity and, the density of specialist and tourist communities inhabiting mistletoes. Results on density and α–diversity come from ZINB_{ANOVA} tables and β–diversity from Mantel and Partial Mantel Correlation. All significant values (P<0.05) appear in bold type.

(a) Elevational gradient

	β–diversity		α–diversity		Density	
Community	rM	P	T	P	T	P
Specialists	−0.04	0.907	−0.54	0.588	−3.28	**0.002**
Tourists	0.08	**0.015**	−2.30	**0.021**	−2.35	**0.021**
Arthropods on pine	0.11	**0.002**	−0.02	0.987	4.92	<**0.001**

(b) Size and distances

		β–diversity		α–diversity			Density		
	Factor	rM	P	df	χ^2	P	df	χ^2	P
Specialists	Size	0.333	<**0.001**	1	6.52	**0.011**	1	62.86	<**0.001**
	Distance–within	0.037	0.159	1	0.21	0.646	1	1.51	0.220
	Neighbourhood	−0.059	0.930	1	0.01	0.978	1	0.02	0.902
Tourists	Size	0.067	0.296	1	23.13	<**0.001**	1	49.18	<**0.001**
	Distance–within	−0.033	0.888	1	0.74	0.388	1	0.02	0.881
	Neighbourhood	−0.019	0.747	1	0.50	0.479	1	0.18	0.673

(c) Temporal variation

	β–diversity		α–diversity			Density		
Community	rM	P	df	χ^2	P	df	χ^2	P
Specialists	0.004	0.402	5	28.93	<**0.001**	5	47.97	<**0.001**
Tourists	0.043	**0.008**	5	7.78	0.168	5	34.71	<**0.001**

T = T statistic value
P = Significance value
rM = Mantel correlation
df = Degrees of freedom
χ^2 = Chi square index

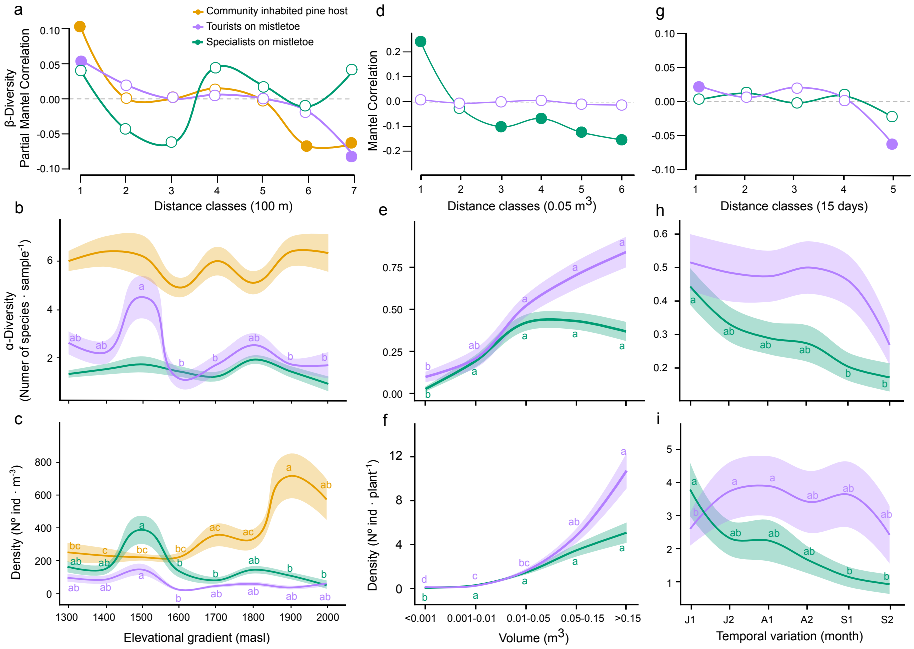

Figure 2.1: β–diversity (first row: a, d, g), α–diversity (second row: b, e, h), and arthropod densities (third row: c, f, i) of specialist (green) and tourist (purple) communities dwelling in mistletoes and the arthropod community inhabiting the pine host (orange) throughout the elevational gradient (first column: a, b, c), at different mistletoe plant size (second column: d, e, f), and over the summer (third column: g, h, i). Solid points on β–diversity figures indicate statistically significant ($P<0.05$) similarities in positive and dissimilarities in negatives values of the Partial Mantel Correlogram and Mantel Correlogram. Different letters on α–diversity and density figures indicate significant differences. Shadows signify 1 standard error.

constant α–diversity, except in the smallest mistletoe (Fig. 2.1d–e, Table 2.1b). This was because the smallest mistletoes received few visits, and these were exclusively by *C. visci*, while the other mistletoes were more frequently visited by the three specialists (Fig. 2.2). Regarding the size–species relationship, the density of specialists increased according to mistletoe size, where the largest mistletoes registered four–fold more specialist density than did the smallest mistletoes (Fig. 2.1f). Similarly, α–diversity (Fig. 2.1e) and density (Fig. 2.1f) of tourist species increased 10–fold from the smallest mistletoes to the largest ones but showed no differences on assemblage composition (β–diversity, Table 2.1b). Meanwhile, the arthropod community inhabiting mistletoes were not affected by distance to other mistletoe plants (considered islands) within the same pine canopy (considered the archipelago) nor between neighbouring infested trees (Table 2.1b).

With respect to temporal variation, the specialist community showed the same composition throughout the sampling period (Fig. 2.1g, Table 2.1c). However, the results showed a significant and progressive decline in their α–diversity (Fig. 2.1h) and density (Fig. 2.1i) from early to late summer (Table 2.1c). The Mantel Test indicated that, unlike specialists, the tourist community composition of early summer statistically differed from those of late summer (Figs. 2.1g, 2.3, Table 2.1c). Finally, the tourist community showed a constant α–diversity (Fig. 2.1h) over the entire summer, with greater density in early summer (Fig. 2.1i).

Overall, the mistletoe arthropod community was composed basically of the mistletoe specialist *C. visci* (37.9% relative abundance of entire arthropod community), its specialist predator *A. visci* (4.0%), and generalists such as spiders (17.9%), flies (12.2%), and ants (9.0%).

Discussion

Our study analyses for the first time the interplay between host plant traits (e.g. plant age and size), mistletoe patch configuration (e.g. distance from other host plants), and species traits (e.g. degree of specialisation, trophic habit, dispersal ability) in arthropod communities inhabiting mistletoes in a Mediterranean pine forest. By acting as ecological islands for mistletoe specialists and having a patchy distribution, *V. album* are colonised and inhabited by specialists and tourists following contrasting assemblage rules. Therefore, the specialist community was highly stable and followed similar assemblage rules under practically all environmental conditions, underlying a deterministic trophic sequence of colonisation. In contrast, the tourist community, being a subset of the pine host arthropod assemblage, acts independent of mistletoe presence.

Forest scale: elevational gradient

Viscum album presents a wide and patchy distribution along a broad elevational gradient. Thus, the arthropod community associated with this plant is exposed to the same environmental gradient. In Sierra de Baza, the oldest mistletoe infestation site is located at intermediate elevations (1400 – 1600 m asl), where *P. nigra* is the only host available—here, mistletoe prevalence (proportion of parasitized trees) and intensity (number of mistletoes per tree) show the highest values (Zamora & Mellado, 2019). In recent decades, *V. album* has been expanding its elevational distribution towards the summits due to warming temperatures, as well as to the presence of suitable vectors for parasite dispersion along the gradient (Zamora & Mellado, 2019). This mistletoe elevational shift offers opportunities for arthropod colonization at the upper range limit.

Overall, our results show that the insect specialist community inhabiting mistletoes remained constant and simple along the entire elevational distribution. Basically, the specialist community was composed of its main herbivore *Cacopsylla visci*, which had the highest relative abundance in all mistletoe plants (more than 57%, 8.2 ± 1.1 individuals/plant); their specialist predator *Anthocoris visci* (2–7%); and the rare herbivore *Pinalitus viscicola*, which had very low relative abundance (less than 6%; see Appendix Table S5a). However, at the highest elevation (2000 m asl), the most recent area occupied by mistletoe plants, *C. visci* presented the lowest abundance (2.9 ± 1.2 individuals/plant, three–fold less than at the other elevational levels), and the predator *A. visci* was absent. This finding suggests that the specialist community at the highest elevations is still being established and, in addition, follows an assemblage rule driven by a deterministic trophic sequence of colonisation. Thus, according to the present results, the likely sequence is the following: a) mistletoes firstly expand their distribution; b) then, herbivore specialists quickly colonise mistletoe plants in new areas, c) followed by predators when herbivores reach higher abundance. In addition, the highest density of the specialist community was located at elevational levels where the mistletoe population registered the greatest prevalence and abundance. This site corresponds to the oldest infestation site (Zamora & Mellado, 2019), suggesting that mistletoe plants and their arthropod specialists could present an optimal point at the mid–elevational level (reflected by a bell–shaped distribution, Fig. 2.1c).

On the other hand, the tourist community showed a composition pattern (α– and β–diversity) different from that of specialists, but similar to that of the pine arthropod community because tourists are a subset of the arthropod community dwelling in pine trees. This pattern results from an isolation by distance driven by a turnover process from a low to a high elevational distribution. We found common taxonomic groups along the elevational gradient such as aphids, thrips, parasitic wasps, and spiders, while ground pearls (Fam. Margarodidae), some beetle species, and some ant species underwent an upward replacement by curculionids and certain ant species (Appendix Table S5). The tourist community also reached its maximum α–diversity and density at mid–elevation levels. This finding suggests that historical events may trigger current diversity patterns, not only for host plant, but for specialists and tourist arthropods associated with mistletoes.

In summary, the specialist community is more stable than the tourist community, maintaining their composition and structure stable along the elevational gradient. In addition, the assemblages of the specialist community are driven by a trophic sequence of colonisation as a deterministic assemblage rule. Conversely, tourists form a simple subset of the pine arthropod community, and thus, both present a similar β–diversity pattern, which is driven by a species–turnover process along the elevational gradient.

Plant scale: size, distance, and temporal variation

Due to the long–lived nature of mistletoe and its patchy distribution (Mellado & Zamora, 2014a), we found a wide range of plant sizes and degrees of isolation. For specialist arthropods, mistletoes have been considered ecological islands, representing isolated patches of resources, surrounded by an "unsuitable sea" of host canopy (Lázaro-González *et al.*, 2017). In line with this, our results reveal that the mistletoe size is an important factor conditioning the composition and abundance of the specialist community, but the degree of isolation does not affect the specialist subset within or between pine canopies.

Regarding plant size, the smallest mistletoes practically lacked visits (87.4% of arthropod–free mistletoes), perhaps for being typically the youngest ones (<5 years old) and thus for having only internodes and decussate leaves (Kahle-Zuber, 2008). This makes the plant unattractive to herbivores, while larger and older mistletoes had been exposed to herbivore colonisation for several years. As an example, considering that specialists are multivoltine organisms with at least 2–3 generations per year (Bin, 1970; Briggs, 2011; Hodkinson, 2009), a typical mistletoe that is 20 years old would be exposed to around 40–60 generations of specialists, increasing the probability of the plant becoming colonised. Concurrently, this ecological size range shows the same assemblage pattern as with the elevation gradient, where the smallest mistletoes receive few visits and exclusively by the main herbivore *C. visci*, whereas large mistletoes are colonized by *A. visci* and *P. viscicola*. Finally, specialist and tourist communities follow an abundance–area relationship, increasing their densities logarithmically in relation to plant size (Fig. 2.1f). A similar pattern has been found in

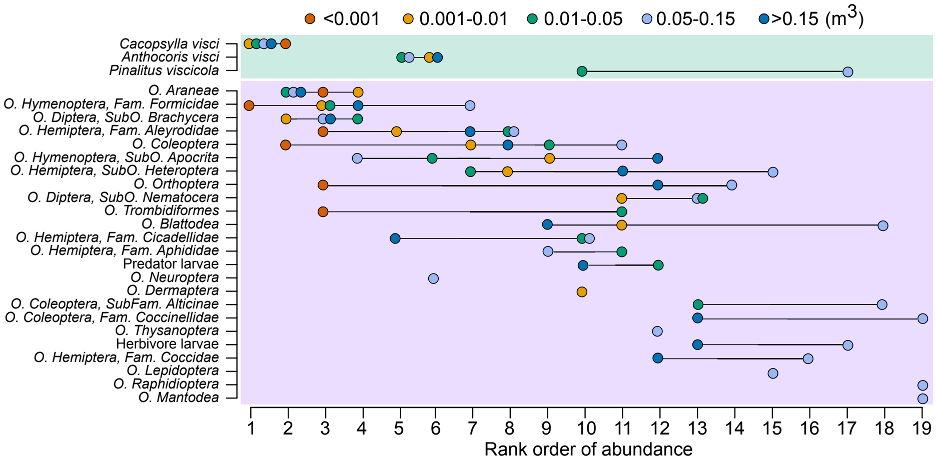

Figure 2.2: Arthropod community variation ordered by rank abundance of assemblage composition inhabiting mistletoes' plant size (orange: $<0.001\text{m}^3$, yellow: 0.001–0.01m^3, green: 0.01–0.05m^3, light blue: 0.05–0.15m^3, and dark blue: $>0.15\ \text{m}^3$). Horizontal lines connect different rank position occupied by each taxonomic group considering specialist (green rectangle) and tourist communities (purple rectangle) present during summer.

other plants colonising forest canopies, such as invertebrate communities inhabiting tank bromeliads in a neotropical forest canopy (e.g. Richardson, 1999). Bromeliad size, like mistletoe size, is the only factor explaining the species assemblage composition. However, the composition of the tourist community remains constant because this community is a subset of arthropod assemblages dwelling in pine hosts, and therefore, tourists are independent of mistletoe presence and insularity trait.

On the other hand, our results show that more isolated mistletoes present the same equilibrium point as more aggregated ones, at least within the same parasitized patch. This may be because the potential dispersion distances of specialist community are longer than distances between mistletoes located in the same or neighbouring pine hosts. In our case, the dispersion distances of psyllids such as *C. visci*, the main specialist of *V. album*, are highly variable, that is, from few meters to kilometres (Van den Berg & Deacon, 1988; Boina *et al.*, 2009; Henne *et al.*, 2010; Kobori *et al.*, 2011; Lewis-Rosenblum *et al.*, 2015). Similarly, species of the family Anthocoridae, such as the main specialist predator inhabiting *V. album*, has a dispersal distance of several meters per day (Madeira & Pons, 2015; Prasifka *et al.*, 2004). Thus, the distance between mistletoes does not limit the colonisation ability of the arthropod community associated

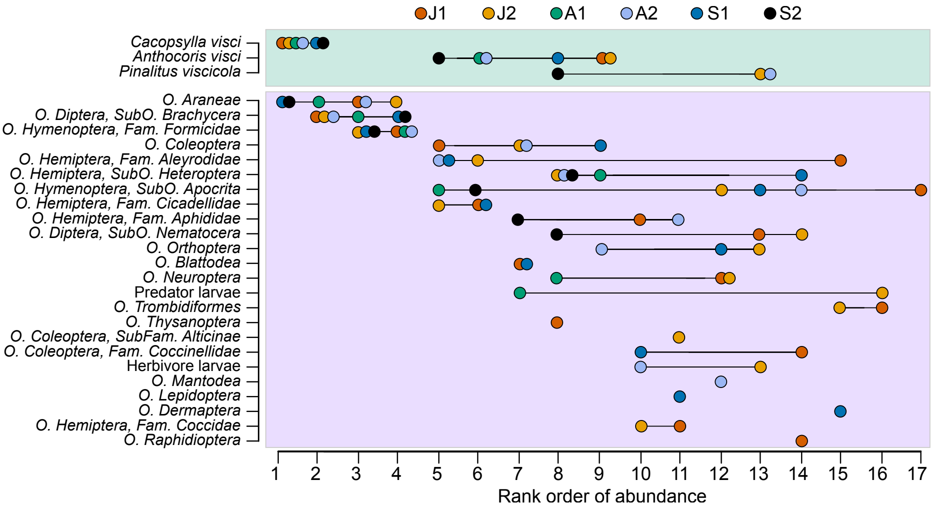

Figure 2.3: Temporal variation ordered by rank abundance of specialist (green rectangle) and tourist (purple rectangle) arthropods inhabiting mistletoes over the summer (J1 orange: early July, J2 yellow: mid–July, A1 green: early August, A2 light blue: mid–August, S1 dark blue: early September, and S2 black: mid–September). Horizontal lines connect different rank position occupied by each taxonomic group in each of the six sampling periods.

with mistletoes.

Our study also reveals temporal changes more accentuated for the tourist than for the specialist community. Whereas the specialist community was stable over the summer season, the tourist community showed turnover and nestedness processes throughout summer (Fig. 2.1g, Fig. 2.3, Table 2.1c). The specialists maintained a constant composition (β–diversity) and a progressive decline of α–diversity and density (Fig. 2.1h–i, Table 2.1c) in late summer, probably because their three specialists hibernate outside mistletoe leaves (Goula *et al.*, 2008; Hodkinson, 2009; Briggs, 2011). In line with this, we found more arthropod–free plants at the end of summer (80%) than in early summer (58%, GLMM, d.f.=5, χ^2=20.88, P<0.001). On the contrary, the tourist community had a markedly different structure and composition pattern through the time. Transient herbivores and generalist predators, which come from the pine host, colonise mistletoe plants and assemble different composition from early to late summer. In addition, species richness slightly decreased at the end of summer due to the absence of rare species (Fig. 2.3, Appendix Table S6). Therefore, the tourist community presented an isolation–by–time throughout the summer season, merging a turnover and nestedness process.

In conclusion, our empirical results indicate contrasting assembly rules for specialized and tourist arthropod communities associated with mistletoe. The specialist community strongly depends on plant size, that is, the larger mistletoe plant, the more diverse and abundance the community of arthropods inhabiting it. It also depends on species trophic habit, with a deterministic trophic rule: the first to arrive is the dominant herbivore species, then the predator, and the subordinate herbivore species. Therefore, the specialist community was highly stable and followed a similar assemblage rule under practically all environmental conditions, underlying a deterministic trophic sequence of colonisation, while the tourist community acted independently of mistletoe presence, being only a subsample of the pine canopy arthropod community. Thus, in agreement with our predictions: (i) overall, the specialist community was more stable along elevational and temporal gradients than was the tourist community, maintaining their identity, structure, and composition stable; (ii) mistletoes with a recent expansion at the mountaintop were inhabited by unsaturated communities of specialized herbivorous arthropods; (iii) larger mistletoes were occupied by more species and individuals (equivalent to the species–area relationships); and (iv) more isolated mistletoes presented the same equilibrium point as the more aggregated ones. Thus, mistletoe size is key to the composition of the arthropod community (supporting deterministic assembly rules), whereas interplant distance is not (no dispersal limitation) within the same parasitized patch. These results strongly support the complementarity and simultaneity of both deterministic (more evident in the specialized community) and stochastic processes (more evident in the tourist community) in explaining community assembly rules of the arthropod community living on mistletoe.

Data availability – The field–collected data that support the findings of this study are openly available in Zenodo repository at http://doi.org/10.5281/zenodo.3782443.

Acknowledgments

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Chapter 3

Secondary foundation species foster novel plant–animal interactions in the forest canopy: evidence from mistletoe

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Abstract

Forest canopies provide the initial physical and biological framework to secondary, dependent species, such as parasitic plants. In a Mediterranean pine forest, we have taxonomically and functionally characterised the entire arthropod community that interacts with mistletoe during its flowering period. We hypothesise that a secondary foundation species such as mistletoe enhances the arthropod diversity and abundance, fostering novel plant–animal interactions in the canopy. Our results clearly show contrasting guilds of herbivores (highly specialised) and floral visitors (highly generalist) with markedly different taxonomic and ecological profiles, the latter determining the fruit set of the mistletoe.

By acting as a secondary foundation species, mistletoe, during flowering, increases the diversity and abundance of newcomers in the pine canopy. New species attracted to the canopy include a specialised herbivore, *Cacopsylla visci*, and a diverse guild of floral visitors, including the orders Hymenoptera, Diptera, Hemiptera, and Lepidoptera. In conclusion, mistletoe creates conditions that support the co–occurrence of functionally distinct organisms in the canopies, fostering pine forest biodiversity and complexity of ecological interactions.

Key words – arthropod biodiversity; forest canopies; herbivores and pollinators; novel mutualistic interactions; parasitic plants; secondary foundation species

Introduction

The forest canopy has been termed “the last biotic frontier” (Erwin, 1983; Nadkarni, 1994). Forest canopies are biogenic incubators for multitudes of species of microorganisms, invertebrates, epiphytes, birds, and mammals that rarely or never appear on the forest floor (Erwin, 1982; Nakamura *et al.*, 2017). Canopies represent major hot spots of biological diversity in terrestrial ecosystems, providing many resources such as leaves, stems, flowers, fruits, and seeds to a diverse group of consumers (Basset *et al.*, 2003, 2012). The importance of canopy organisms and processes is becoming increasingly recognised in relation to understanding biodiversity and ecosystem processes in a global–change context (Nakamura *et al.*, 2017).

Forest canopies provide the physical and biological setting for secondary, dependent species, such as epiphytes and parasitic plants. Secondary foundation species develop structurally complex biogenic habitats that alter the environmental matrix of the primary host (Jones *et al.*, 1997; Ellison *et al.*, 2005; Thomsen *et al.*, 2018). By providing a further variety of resources and biota, these secondary species add additional levels of habitat complexity and biodiversity to the primary, dominant species forming forest canopies (Altieri *et al.*, 2007). In this respect, ecologists have documented higher abundance and richness of species in marine, coastal, and terrestrial ecosystems where a primary foundation species facilitates a secondary foundation one (Altieri *et al.*, 2007; Yakovis *et al.*, 2008; Bishop *et al.*, 2012). Recent research in plant communities of mangroves, savannahs, tropical forests, and temperate woodlands has shown that 20–80% of the diversity in these ecosystems can be attributed to the presence of secondary foundation species (Ellwood & Foster, 2004; Angelini & Silliman, 2014; Watson & Herring, 2012). Facilitation has been proposed as the dominant interaction in these multiple–foundation–species assemblages (Angelini *et al.*, 2011). That is, the structure of a primary foundation species creates a new buffered habitat in which other, obligate, secondary foundation species can proliferate. Collectively, these species increase habitat complexity to enhance biodiversity and food–web complexity (e.g. supporting novel feeding guilds; Angelini & Silliman, 2014), irrespective of the net effect of the secondary species on inhabitants (Thomsen *et al.*, 2018).

Conifer trees, as an example of primary foundation species in terrestrial ecosystems, are spatially dominant, long–lived plants whose canopy structure promotes new possibilities for species coexistence through the amelioration of physical stress and the creation of fine–scale, novel biogenic habitats. In addition, the canopies of conifer trees also provide habitats to parasitic plants, which exert a negative effect in their host (primary foundation species, Sangüesa-Barreda *et al.*, 2012; Lázaro-González *et al.*, 2019b). Yet, these parasites also provide consumers with new resources other than those of the host and therefore boost the overall diversity of species and ecological interactions (Lázaro-González *et al.*, 2017). The resulting association between trees and parasitic plants is thus a useful system in which to assess the contribution of secondary foundation species in fostering plant–animal interactions and assembly rules of communities inhabiting the canopies (Watson, 2001, 2002; Thomsen *et al.*, 2018).

In the present study, we characterise, both taxonomically and functionally, the entire community of arthropods associated with a secondary foundation species (mistletoe) strongly associated with a primary foundation tree species (the pine tree). In this characterisation, we determine the degree of specialisation/generalisation of insect herbivore guilds and floral visitors of the mistletoe. We interpret these results in the ecological context (the pine canopy), the phenological period (early spring flowering), and the trophic habit (specialisation/ generalisation) of the insects interacting with mistletoe. Mistletoe constitutes a suitable study system to explore this question, being a long–lived plant with multiple long–term biotic interactions. Mistletoes comprise a diverse group of aerial hemiparasitic plants that derive water and minerals from host plants and are considered keystone species in forest ecosystems worldwide (Watson, 2001). In addition to being parasites, mistletoes establish mutualistic interactions with animal pollinators and seed dispersers (e.g. Watson, 2001; Mellado & Zamora, 2014a), while attracting herbivorous insects and mammals (e.g. Umucalilar *et al.*, 2007; Hódar *et al.*, 2018).

In a previous study, Lázaro-González *et al.* (2017) determined that *Viscum album* subsp. *austriacum*, an evergreen, perennial, epiphytic hemiparasite parasite, has a stable, independent, and simple arthropod community during summer. For this parasitic plant, *Cacopsylla visci* and *Pinalitus viscicola* are the only two specialist herbivores, and *Anthocoris visci* the only specialist predator. Mistletoes are also visited by generalist tourist fauna, which is a subset of the host-pine arthropod assemblages (Lázaro-González *et al.*, 2020). Nevertheless, mistletoe is also a flowering plant, and thus can potentially attract flower consumers (Zuber, 2004). Here, we provide the first exploration of the functional and trophic relationships of the entire arthropod community visiting flowering mistletoes in a pine forest.

Our general hypothesis is that parasitised pines should attract more consumers than non–parasitised ones during the flowering period, when mistletoe offers both foliar and floral resources, fostering novel plant–

animal interactions in the canopy. Thus, mistletoe can alter: (i) the invertebrate abundance and richness in the pine canopy and (ii) the diversity, complexity, and functional relationships of plant–animal interactions in the pine canopy. In this way, mistletoe behaves as a secondary foundation species such as epiphytes layered on trees (Ellwood & Foster, 2004; Angelini *et al.*, 2015; Thomsen *et al.*, 2018) and can modify an ecosystem's multifunctionality through canopy engineering and by boosting the diversity of ecological interactions. These ecological consequences have far–reaching implications for biodiversity assessment, conservation, and management in the pine forest.

Materials and Methods

Study site

This study was made in a pine afforestation located at 1400 m asl in Sierra de Baza, south–eastern Iberian Peninsula (2° 51' W, 37° 22' N, Granada, Spain). The climate in this area is typically Mediterranean, with cold winters (December to March) and hot, dry summers (June to September), with an annual mean temperature of 15.5°C and annual mean rainfall of 495 ± 33 mm (CMAOT, 2017). Temperatures were measured in the study area throughout the sampling period in both 2013 and 2014 with HOBO data loggers (TidbiT v2 Water Temperature Data Logger- UTBI-001) directly located on pine branches bearing tagged mistletoe plants (see Appendix S2, Fig. S3).

The European mistletoe [*Viscum album* subsp. *austriacum* (Wiesb.) Vollman], a dioecious parasitic plant native of most European regions, specialises in conifer parasitism. In Sierra de Baza, we studied a pine forest where the 43% of the total surface area is covered by conifers, among which black pine (*Pinus nigra* Arn. subsp. *salzmannii*) is the main host for *V. album.* From March to May for three consecutive years (2013–2014–2015), we sampled a population of coetaneous *P. nigra* parasitised by mistletoe widely ranging in sizes.

Arthropods during the *Viscum album* flowering period

To assess the entire arthropod community inhabiting the pine canopy throughout the flowering period of the mistletoe, we placed a sampling station in each focal pine trees (15 *P. nigra* individuals parasitised by mistletoe and 15 that were unparasitised), consisting of two pan traps hanging from opposite branches. Each pan trap (25 × 15 cm) was painted yellow with UV–reflecting paint (Sparvar ®) simulating mistletoe flowers. Pan traps constitute the most efficient, unbiased, and cost–effective method to sample arthropod communities, especially pollinators (Westphal *et al.*, 2008). This sampling method is particularly effective in early spring when flowers are scarce (Roulston *et al.*, 2007; Cane *et al.*, 2000; Baum & Wallen, 2011). We conducted 5 weekly surveys from early April to early May 2013, totaling 300 pan traps (30 pines x 2 pan traps x 5 samplings), in which pan traps were active 9:00–11:00 h and collected 24 hours later, following the same order of activation. Each pan trap was filled with water and a small amount of odourless detergent, in order to break the water surface tension. The two pan traps were mixed in only one sample per pine, and all captured specimens were identified in the laboratory. The entire community was evaluated for species richness (total number of different taxa), abundance (total number of individuals), and composition (abundance of each taxon) in each sample per pine and day.

Statistical analyses were conducted with a Generalised Linear Mixed Model (GLMM) and a Linear Mixed Model (LMM) to test the effects of mistletoe presence on species richness and abundance of arthropod community around pine trees, respectively. Species richness was tested assuming a Poisson distribution of data, and abundance was log–transformed, in order to meet the model assumptions. Parasite status (parasitised and unparasitised pines) was a fixed factor, while focal pine and sample week were used as random independent factors. On the other hand, differences in community composition captured on parasitised and unparasitised focal pines were tested using an Analysis of Similarities (ANOSIM), based on 999 permutations and a Bray–Curtis similarity distance. Finally, the taxonomic groups that contributed to these differences were detected by similarity percentages (SIMPER).

Mistletoe arthropod visitors

Diurnal visitors

We selected 120 (67 females and 53 males) and 129 (68 females and 61 males) mistletoe plants in 2013 and 2014, respectively, which presented similar sizes (20–30 years old), parasitising different host pine trees in the study area. Each sampling, performed weekly from early March to early May, consisted in 5 min of direct observation per plant and day, using binoculars when needed. The total observation time was 8070 min, distributed among 13 surveys (7 in 2013 and 6 in 2014). Observations were conducted on sunny days when insects were most active. All arthropods making contact with leaves and/or stems of a mistletoe plant were recorded as foliar visitors, while arthropods making contact any part of mistletoe flowers were recorded as flower visitors. The arthropods were identified in situ to the lowest taxonomic level possible. Surveys were conducted by two observers simultaneously monitoring different mistletoe plants, at 9:00–16:00 h, randomly ordered in each survey in order to avoid any bias. Additionally, we estimated the floral market available during the mistletoe flowering period. For this, we carried out samplings on the same days as the diurnal visitor surveys (see Appendix S2, Fig. S4 for methodological details).

GLMM was used to test differences between visitor communities (foliar and floral), plant sex (female and male) and sampling year (2013 and 2014) on abundance (total number of arthropods making contact with any part of a mistletoe plant) and species richness (total number of different taxonomic groups). A data Poisson distribution was assumed in both models, adding the sampling day and mistletoe plant as random factors. Community composition was also analysed using a ANOSIM and SIMPER test.

Nocturnal flower visitors

In April 2015, a light trap was installed weekly in an open area surrounded by parasitised pines, close to mistletoe plants where diurnal surveys were conducted. Light trapping is the most widely used tool for investigating communities of nocturnal Lepidoptera (Fayle *et al.*, 2007; Infusino *et al.*, 2017). The light trap was activated from 22:00 h to 2:00 h, and all the moths attracted were captured. We collected the pollen grains from the body of each moth by cutting small cubes of fuchsine–stained gelatin and rubbing it over the body of the insect. Afterwards, the samples were placed on glass slides, and by burning it below, the gelatin was melted and fixed by a cover glass. The fuchsine stains of the pollen grains were studied under a light microscope (x40) in the laboratory to find the presence or absence of mistletoe pollen grains. Finally, all captured moths were photographed, in order to identify them to species level, and were released at the end of the sampling.

Visitation rate and quantity component of the interaction

For diurnal flower visitors recorded, the visitation rate refers to the average number of flowers visited per hour by each species. This measurement was determined for each foraging bout by following a floral visitor and counting the number of flowers visited as well as the time spent. The quantity component of the interaction was calculated by multiplying the abundance of each pollinator species, expressed as the number of individuals per hour, by its flower visitation rate (NFV, *sensu* Herrera, 1989). Thus, the quantity component was expressed as the total number of flowers of an individual plant visited by the population of each pollinator species in 1 hour. This estimated the quantity of interactions of a mistletoe plant with each pollinator species.

Experimental determination of mistletoe breeding system

To identify the potential role of floral visitors as pollinators, we performed an exclusion experiment on February 2015, before mistletoe flowering. We labelled 20 reproductive female mistletoe plants (around 20 years old) located on 10 different host trees. Three branches per mistletoe were selected to carry out three types of exclusion in each mistletoe. The first branch was bagged with a mesh (0.5 mm mesh opening) to exclude the arthropods and test the net effect of wind in the mistletoe pollination. The second branch was bagged with cellophane bags, avoiding arthropod pollinators and wind, to test apomixis mechanism. Finally, the third branch was used as a control to estimate the total effects of pollinators, wind, and apomixis. We frequently reviewed the exclusion experiment to ensure its proper performance. The total number of flowers during the flowering period, and fruits (in August 2015) were counted per branch. Therefore, we calculated the fruit:flower ratio in order to standardise the results by treatment and mistletoe plant. To test the effects of each treatment on fruit:flower ratio, we used a GLMM, where the mistletoe plant and host pine tree were nested random factors, and a binomial distribution was assumed.

All statistical analyses were conducted the open source software Statistical R version 3.6.0 (R Core Team, 2019). GLMM and LMM was implemented with *glmer* and *lmer* functions respectively of the *lme4* package (Bates *et al.*, 2015), whereas ANOSIM and SIMPER was run using *anosim* and *simper* functions of the *vegan* package (Oksanen *et al.*, 2019).

Results

Arthropods during *Viscum album* flowering period

A total of 5615 arthropods (4011 and 1604 individuals on parasitised pines and unparasitised pines, respectively) caught in pan traps belonged to 23 taxonomic groups and 12 orders (Appendix Table S7). The arthropods caught represent a broad sample of diverse trophic profiles, including predators (e.g. Cantharidae, Araneae), herbivores (e.g. Hemiptera), floral visitors (e.g. Hymenoptera and Diptera), and generalist consumers such as ants (Formicidae). Mistletoe presence had a positive effect on total abundance of arthropods (LMM: df = 27.7, $T = 6.14$, $P < 0.001$), showing more than two–fold the number of individuals in pan traps set in parasitised pine canopies (57.30 ± 7.01 individuals$\cdot$pine$^{-1}\cdot$day^{-1}) than in unparasitised ones (22.91 ± 2.50 ind.$\cdot$pine$^{-1}\cdot$day^{-1}). Although species richness did not statistically differ (GLMM: df = 1, $\chi^2 = 0.34$, $P = 0.559$), the community composition around parasitised and unparasitised pine canopies differed (ANOSIM, R = 0.17, $P < 0.001$). These differences were due basically to a high abundance of the main specialist on mistletoes, the herbivore *C. visci* (SIMPER: 44% of total dissimilarities), gnats (mainly Simuliidae, SIMPER: 17%) and flies (mainly Calliphoridae, SIMPER: 13%) in parasitised pines in comparison to unparasitised ones (see Appendix Table S7). These three taxonomic groups represented the 82.7% of all the arthropod community detected.

Mistletoe arthropod visitors

Diurnal visitors

We detected 1194 specimens during diurnal surveys (244 in 2013 and 950 in 2014) corresponding to 26 taxonomic groups and 7 orders (Fig. 3.1, see Appendix Table S8). Overall, the species richness in both communities, plant sex, and sampling year did not differ (Table 3.1, Fig. 3.1). However, the community of foliar visitors was greater (2.18 ± 0.08 individuals $\cdot$observation^{-1}, hereafter IO) than that of the floral visitors (1.42 ± 0.07 IO, Table 3.1). More importantly, both communities cleary differed in composition (ANOSIM, R = 0.49, $P < 0.001$), due to presence of the mistletoe specialist *C. visci*, which was the most abundant foliar visitor (72.4% of the total community of foliar visitors), and generalist pollinators such as bees (*Apis mellifera*), flies (mainly Calliphoridae) and gnats (mainly Simuliidae) as the main floral visitors (38%, 25.7%, and 12.3%

of the total community of floral visitors, respectively). These taxonomic groups represent a 41.9%, 15.1%, 13.2%, and 7.6%, respectively, of dissimilarities between foliar and floral communities (Fig. 3.1).

Table 3.1: Differences between communities (foliar and floral), sex (female and male plants) and sampling year (2013 and 2014) on species richness and abundance of mistletoe visitors. Degrees of freedon (df), chi–square values (χ^2), and significance (P) come from GLMM$_{ANOVA}$ tables

	Abundance			Species richness		
Factor	df	χ^2	P	df	χ^2	P
Community	1	25.71	<**0.001**	1	2.23	0.14
Sex	1	5.76	**0.016**	1	0.45	0.50
Year	1	16.16	<**0.001**	1	0.44	0.51
Community×Sex	1	0.53	0.468	1	0.01	0.91
Community×Year	1	3.93	**0.048**	1	0.01	0.94
Sex×Year	1	2.46	0.117	1	0.24	0.63
Community×Sex×Year	1	0.33	0.556	1	0.44	0.51

In addition, female mistletoe plants showed more foliar (2.31 ± 0.11 IO) and floral visitors (1.50 ± 0.10 IO) than did male plants (2.00 ± 0.10 IO and 1.24 ± 0.08 IO, respectively, Table 3.1). Nevertheless, assemblage composition of foliar community did not differ between plant sex (ANOSIM, R = -0.007, P = 0.761), whereas floral visitors presented different assemblage composition (ANOSIM, R = 0.05, P = 0.002) where female flowers were more visited by A. mellifera (0.71 ± 0.11 IO, 47.8% of total female–flower visitors) and male flowers by flies (0.46 ± 0.08, 37.3%) and gnats (0.28 ± 0.09 IO, 22.4% of total male–flower visitors).

Finally, the results also show differences between samplings years, where both foliar and floral communities were more abundant in 2014 (2.34 ± 0.09 IO and 1.49 ± 0.12 IO of foliar and floral visitors, respectively) than in 2013 (1.57 ± 0.11 IO and 1.33 ± 0.07 IO of foliar and floral visitors, Table 3.1). These differences in abundance imply dissimilarities in foliar community composition (ANOSIM, R = 0.37, P < 0.001), for which *C. visci*, the main foliar visitor, was the responsible for increasing their relative abundance from 36.4% (2013) to 78.8% (2014, Fig. 3.1: Foliar visitors). Floral visitors also showed different assemblage composition in both years (ANOSIM, R = 0.03, P = 0.007), corresponding to an increase of *Apis* *mellifera* in 2014 (0.38 ± 0.07 IO, 28.7% of total floral visitors in 2013 and 0.66 ± 0.13 IO, 44.4% in 2014) and a decrease of flies (from 43.6% in 2013 to 13.2%, in 2014, see Fig. 3.1: Foliar visitors).

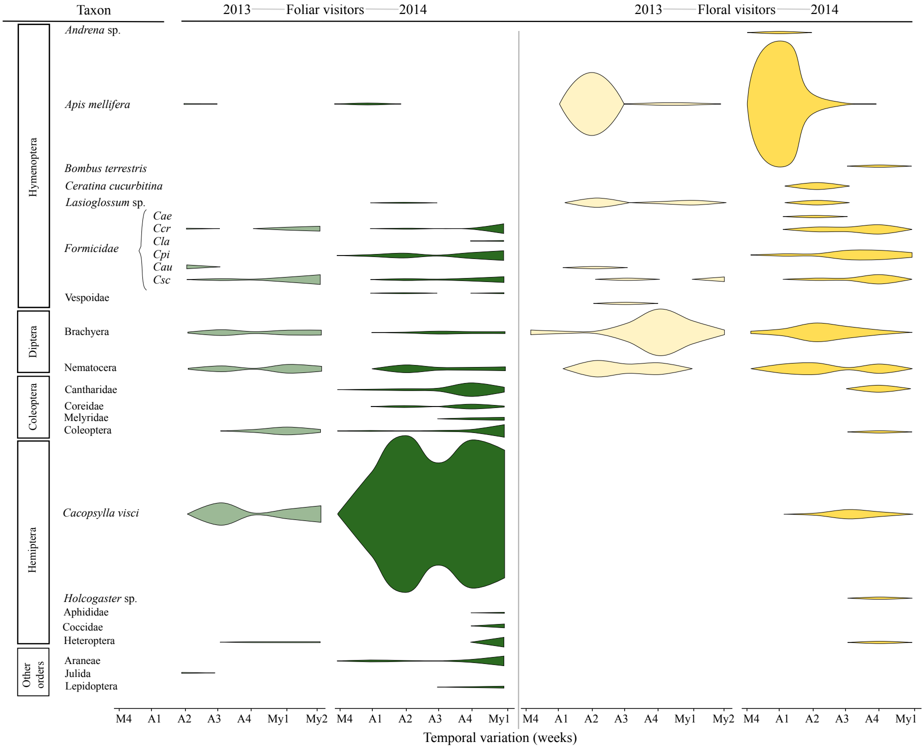

Figure 3.1: Abundance of mistletoe visitors by taxonomic group in contact with leaves or branches (green) and flowers (yellow) during sampling period in 2013 (light colours) and 2014 (dark colours). Temporal variation corresponds to each sampling week (M4, last week of March; A1, A2, A3, and A4, first, second, third, and fourth week of April, respectively; My1 and My2, first and second week of May). Abbreviation of species from Formicidae are: Cae, *Camponotus aethiops*; Ccr, *Camponotus cruentatus*; Cla, *Camponotus lateralis*; Cpi, *Camponotus piceus*; Cau, *Crematogaster auberti*; Csc, *Crematogaster scutellaris*.

Nocturnal visitors

The light traps attracted 127 moth specimens belonging to 24 different species, of which the 40.9% (52 moths, corresponding to 17 species) carried mistletoe pollen. The most abundant species carrying pollen on their body were *Panolis flammea* (17.3% of total moths with pollen), *Caradrina noctivaga* and *C. flavirena* (15.4%), *Rheumaptera andalusica* (9.6%), *Spudaeae ruticilla* (9.6%), *Valeria jaspidea* (9.6%), *Mythimna sicula* (5.8%), and *Hadena sancta* (5.8%) (Fig. 3.2: Nocturnal visitors).

Visitation rate and quantity component of the interaction

We observed a total of 1461 flower–pollinator interactions involving 245 flower visitors, corresponding to 18 taxonomic groups and 4 orders (Fig. 3.2: Flower–pollinator interactions). The honeybee was the main flower visitor (67.5% of total flower visits), followed by flies (SubO. Brachycera: 17.8%), ants (4.8%), and gnats (SubO. Nematocera: 4.5%, Fig. 3.2: Flower–pollinator interactions).

Table 3.2: Effects of sex plant (female vs. male) and sampling year (2013 vs. 2014) on species richness of pollinators guilds and number of flower visited. Degrees of freedon (df), chi–square values (χ^2), and significance (P) come from GLMM$_{ANOVA}$ tables

	Abundance			Species richness		
Factor	df	χ^2	P	df	χ^2	P
Sex	1	98.36	<**0.001**	1	0.01	0.92
Year	1	83.83	<**0.001**	1	0.22	0.64
Sex×Year	1	8.48	**0.004**	1	0.68	0.41

The pollinator guild presented the same species richness in both sampling years (2013 and 2014) and plant sex (female and male, Table 3.2). However, the number of flowers visited by arthropods was greater during 2014 (104.5 ± 19.1 number of flowers visited per hour, hereafter NFV) than 2013 (97.3 ± 13.2 NFV, Table 3.2). Also, community composition differed between years (ANOSIM, R = 0.03, P = 0.004), due to a surge in honeybee visits in 2014 (from 56.1 ± 14 in 2013 to 78.1 ± 19.7 NFV in 2014), as opposed to a decline in fly visits (from 31.1 ± 6.0 to 7.8 ± 2.8 NFV).

On the other hand, pollinators visited female flowers more (128.1 ± 16.9 NFV) than male ones (42.4 ± 5.4 NFV, Table 3.2), and their assemblage community also differed (ANOSIM, R = 0.06, $P < 0.001$). The honeybees and other bees showed a clear preference for female over male flowers (Table 3.3). On the contrary, dipterans, both flies and gnats, interacted more with male plants than female plants (Table 3.3).

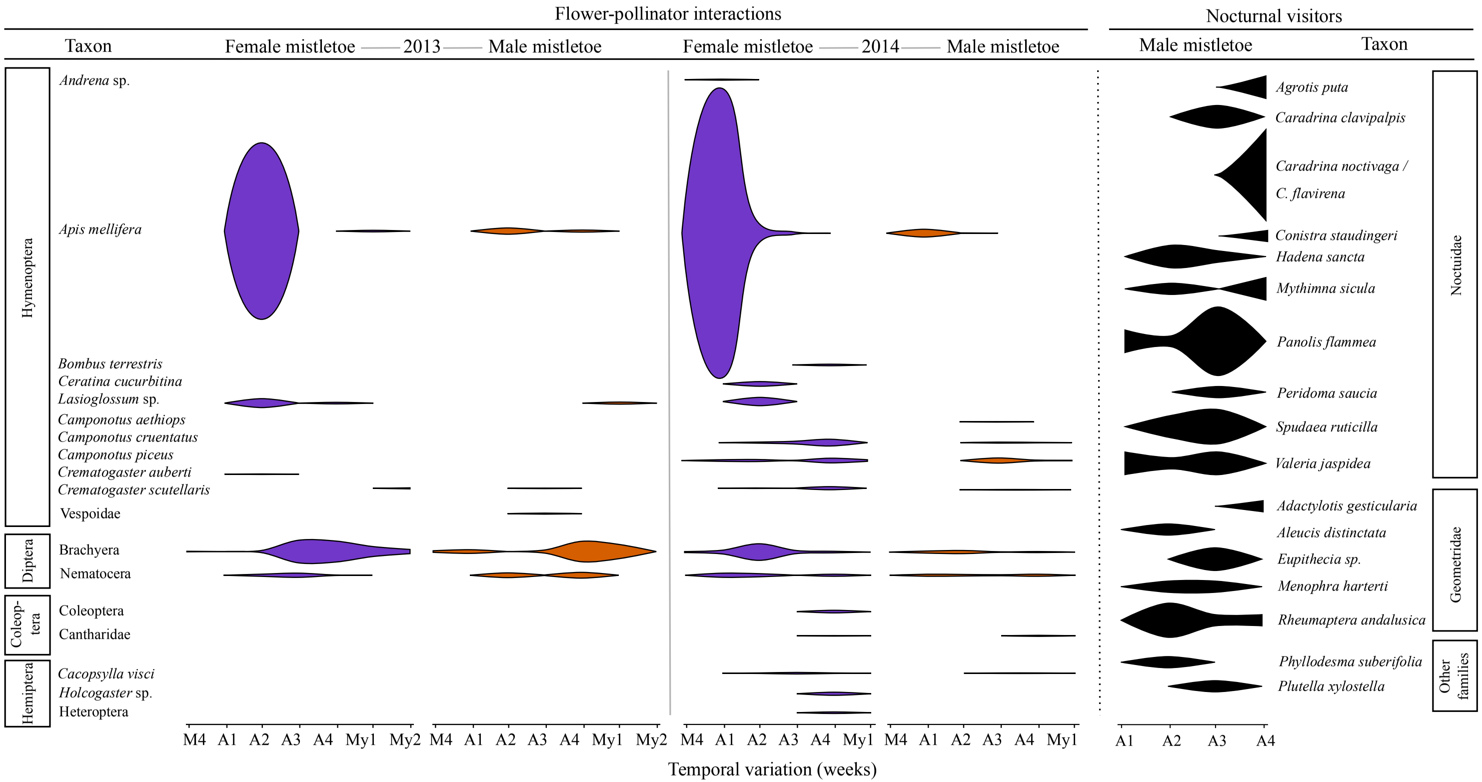

Figure 3.2: Abundance of flower-pollinator interactions by taxonomic group in female (purple) and male (orange) mistletoe plants in both years of sampling (2013 and 2014), followed by abundance of nocturnal visitors carrying mistletoe pollen. Temporal variation corresponds to each sampling week (M4, last week of March; A1, A2, A3, and A4, first, second, third, and fourth week of April, respectively; My1 and My2, first and second week of May).

Table 3.3: Number of female and male flowers visited (mean ± SE) by hour in each pollinator guild

Taxon	Female flowers	Male flowers
Honeybees	96.2±17.7	7.1±3.2
Other bees	5.0±1.8	0.9±0.9
Flies	16.6±4.1	21.1±4.7
Gnats	3.1±1.0	7.6±2.6
Ants	5.0±0.3	4.4±1.8
Beetles	0.6±0.5	0.4±0.3
Bugs	1.4±0.7	0.4±0.3

Experimental determination of mistletoe breeding system

The exclusion experiment showed a significant effect on fruit set (GLMM: df $= 2$, $\chi^2 = 87.54$, $P < 0.001$). Under natural conditions (control treatment), a 54.6% of the flowers produced fruit, but only a 3.5% when all pollinators were excluded. On the contrary, no flowers produced fruit when insects and wind were both excluded. Thus, the breeding system of *V. album* was basically entomophilous and incidentally anemophylous.

Discussion

In this study, we taxonomically and functionally characterised the entire arthropod community interacting with mistletoe during its flowering period, and we identified the guilds of specialist herbivorous, floral visitors, and the tourist species coming from the pine foliage. We interpreted these results in the ecological context of the pine canopy and of the early spring date in which the mistletoe flowering occurs. Our results clearly show that mistletoe adds two new contrasting guilds to the pine canopy: a specialised herbivore guild composed of a single but abundant species, *Cacopsylla visci,* and a diverse floral guild with diverse taxonomic and ecological profiles, the latter being determinants of the mistletoe fruit set. This is the first estimate available concerning the contribution of a parasitic plant to the arthropod taxonomic and trophic biodiversity of a Mediterranean pine forest (see also Lázaro-González *et al.*, 2017, 2020).

Does flowering mistletoe attract consumers to the pine canopy?

The presence of flowering mistletoes causes a concentration of arthropods in the canopy of infected pines. The abundance of these arthropods doubles those that visit the canopy of the nearby pines without mistletoe (Appendix Table S7). In this situation, *C. visci*, the herbivorous specialist of mistletoe together with some generalist floral visitors such as bees, flies, and gnats, become the main elements accounting for these qualitative and quantitative differences (Lázaro-González *et al.*, 2020). At a single–tree scale, *V. album* determines the presence of particular species of insect herbivores and floral visitors. At the forest–stand scale, infected pines act as attractors of many insect species associated with mistletoe in comparison with uninfected pines, increasing the spatial heterogeneity of canopy biodiversity. Therefore, the canopy of the parasitised pines during the flowering period of the mistletoe had a greater amount and diversity of arthropods than did the canopy of the nearby unparasitised pines.

Which taxonomic and functional characteristics have the community of arthropods associated with mistletoes?

The arthropods restricted to the host pine are pine–feeding herbivores (e.g. thrips, psyllids, leafhoppers, aphids), whereas other arthropods are canopy generalists, and opportunistically occur on mistletoe when seeking food and/or shelter ("tourist" subset of the pine arthropod community, *sensu* Lázaro-González *et al.*, 2017). On the other hand, the fauna strictly associated with mistletoe shows outstanding differences between the guilds of herbivorous and florivorous insects. The herbivore species recorded during the mistletoe flowering season is *C. visci* (Fig. 3.1: Foliar visitors), reportedly inhabits *V. album* across Europe (Zuber, 2004; Hansen & Hodkinson, 2006; Struwe *et al.*, 2009; Varga *et al.*, 2012). *Cacopsylla visci* appeared both years (Fig. 3.1: Foliar visitors), although with significant quantitative differences between years, probably related to annual meteorological differences (Appendix S2, Fig. S3). Mistletoe completes its specific fauna adding the second specialist herbivore, *Pinalitus viscicola*, together with its main predator *Anthocoris visci* during summer (Lázaro-González *et al.*, 2017, 2020).

On the contrary, the fauna that visits mistletoe flowers is much more diverse in species and heterogeneous in higher taxonomic groups (Family and Order level, see Fig. 3.1). The floral visitors of mistletoe include the most common floral generalists known, such as bees, flies, ants (Fig.

3.1: Floral visitors), and a select group of nocturnal moths (Fig. 3.2: Nocturnal visitors), for which little information on their trophic habit and degree of specialisation is available. Although the guild of floral visitors that visit the male and female flowers is also overall the same, some species (e.g. bees) preferably visit female flowers, while others (dipterans) prefer to visit male flowers (Table 3.3). These differences may be associated with the different rewards offered by male and female flowers—that is, female mistletoe flowers have more floral reward in the form of nectar, while males offer residual nectar but also pollen and a more intensive odour than do females (Zuber, 2004).

Comparing the community of diurnal flower visitors with available plant visitors captured by traps, we found that all the taxonomic groups available (e.g. Hymenoptera and Diptera) captured by pan traps were also found visiting mistletoe flowers (see Appendix Table S7 and S8), including groups that pan traps usually do not catch (e.g. bumble bees) or catch less frequently than expected (e.g. honey bees, Cane *et al.*, 2000; Roulston *et al.*, 2007; Wilson *et al.*, 2008). On the other hand, comparing the community of nocturnal flower visitors with available moths, we estimated that almost 70% of total moth species available (24) in our pine forest study system visited the flowers of mistletoe (17, see Fig. 3.2: Nocturnal visitors). In this sense, it bears mentioning that light–trap sampling is a relatively unbiased and highly efficient nocturnal sampling method (Raimondo *et al.*, 2004; Hirao *et al.*, 2008). In this context, being the first species to bloom, mistletoe must endure harsh weather conditions, but it has the advantage that all the available floral consumers need to visit mistletoe flowers, the only floral resource available at this time (Appendix S2, Fig. S4).

These contrasting ecological profiles between the species assemblages that mistletoe adds to the pine canopy: herbivores (very few, highly specialised species) and flowers visitors (many species of disparate taxonomic origin, diurnal and nocturnal species), suggest that the ecological and evolutionary processes underlying these interaction networks might differ between pollination and herbivory communities (Fontaine *et al.*, 2009). Such ecological pressure towards specialisation in our plant–herbivore case might be less strong for pollinators since the actual flower handling time by a pollinator is brief compared with that of herbivores that live on a plant for a long period (Fontaine *et al.*, 2009). As an example, given that herbivore specialists of mistletoe are multivoltine organisms with at least two to three generations per year (Hodkinson, 2009), a typical mistletoe 20 years old would be exposed to around 40–60 generations of specialists, ensuring a reliable resource for herbivores over the long term. On the contrary, the generation time of the generalist pollinators of the mistletoe (particularly honeybees) is much longer than the flowering time of the mistletoe (1 month), so flower visitors of mistletoe need other flowering plant species in order to complete their life cycle.

To what extent do floral visitors act as true pollinators, generating a novel mutualistic interaction in the pine canopy?

The results of the bagging experiments confirm that insects are necessary for reproductive success in *V. album*. Previous studies indicate that the genus *Viscum* can use both wind and insects for pollination, although wind pollination appears to play a minor role in *V. album* (Kuijt, 1969). Aparicio *et al.* (1995) found that *V. cruciatum*, a closely related mistletoe species, is entomophilous. Additionally, mistletoe flowers show insect–pollination features, such as barbed pollen and the intense odour from the male flowers (Zuber, 2004). Thus, disparate floral visitors such as honeybees, flies, gnats, ants, and nocturnal moths can act as mistletoe pollinators, with wind as a complementary pollen vector. All this points to a generalist pollinator system, where the fruit set of the mistletoe depends essentially on insect pollination (Zuber, 2004). Honeybees (Aparicio *et al.*, 1995; Sipes *et al.*, 2014), flies (Player, 1979; Aparicio *et al.*, 1995; Wiesenborn, 2016), and ants (de Vega *et al.*, 2009) have been identified as major pollinator species from other hemi– and holoparasitic plants from environments as different as deserts, scrublands, and forests.

The generalist pollination system of mistletoe, combining both diurnal and nocturnal pollinators, could help this species to sustain viable populations in mountains habitats where it is the only flowering species in early spring. In addition, because of their long–lived habit, constant and predictable reproduction due to its hemiparasite habit, mistletoe constitutes a reliable food resource, being the only source of flowers (Appendix S2, Fig. S4) and fruit available (Mellado & Zamora, 2016) in the pine forest during some periods. In this sense, mistletoe behaves as a secondary foundation species in the organisation of the pine forest ecosystem that it inhabits, inducing changes that are disproportionate to the plant's abundance and biomass, thereby favouring local and regional wildlife diversity (Watson, 2001, 2002; Watson & Herring, 2012; Mellado *et al.*, 2016; Mellado & Zamora, 2017). Although *Viscum album* has traditionally been considered a pest of its pine hosts, it increases the heterogeneity of the forest canopy by providing resources for a new community of consumers. This work clearly exemplifies the mismatch outlined by Watson (2001) between considering mistletoe as a pest that needs to be controlled vs. considering it a keystone species that provides new opportunities for species and ecological interactions. Now widespread evidence characterises mistletoe as a keystone resource, boosting diversity in many ecosystems, such as eucalypt forests of southeastern Australia (Watson, 2001, 2002; Watson & Herring, 2012), mesquite woodland of the southwestern USA (Anderson & Anderson, 1973), tropical forests in India (Davidar, 1985; Ali & Ripley, 1999) and South America (Fadini *et al.*, 2018), and cactus–dominated deserts of Chile (Martínez del Rio *et al.*, 1996; Medel, 2000).

Concluding comments: Mistletoe as an attractor of biodiversity in pine canopies

From these results, we conclude that mistletoe significantly enhances biodiversity in pine forests. By acting as a secondary foundation species, mistletoe introduces greater diversity and abundance of newcomers to the pine canopy. Qualitatively, mistletoe brings to the forest stand new species, mostly belonging to the orders Hymenoptera, Diptera, Lepidoptera (floral visitors) and Hemiptera (herbivores and predator), while quantitatively it provides twice as many arthropods as appear in the canopy of the unparasitised pines. While mistletoe attracts a new community of specialists, different from host pine (Lázaro-González *et al.*, 2017), mistletoe parasitism causes detrimental indirect effects on pine-feeding herbivores across its shared host, suggesting a worsening of host quality as food (Lázaro-González *et al.*, 2019a,b). These changes (i.e. detrimental effects on pine herbivores and novel niche for specialised mistletoe fauna) reorganise the canopy herbivore community of the pine forest (Lázaro-González *et al.*, 2019a,b, 2020). Additionally, mistletoe increases the diversity of consumers in the forest canopy by providing floral resources at a time of the year (early spring) when there are still no other flowers in the floral market. In this way, mistletoe extends the period of activity of floral visitors in a new habitat, the forest canopy. In other words, mistletoe expands the spatial niche of floral visitors by attracting them to the pine canopy, while expanding the time frame of insect activity. In short, it offers resources at a time of year where it is the only species in bloom available because of the harsh thermal environment in mountain pine forest.

In functional terms, mistletoe adds to the pine canopy two plant–animal interactions with mutualistic consequences that do not occur in the pine forest without mistletoe: the pollination derived from floral visitor's activities during the early spring, and the dispersion of seeds derived from the frugivorous bird activity during autumn and winter (Mellado & Zamora, 2014a, 2016, 2017). Thus, with mistletoe, a relatively simple pine canopy, dominated by antagonistic plant–herbivorous interactions with a limited number of species involved (Lázaro-González *et al.*, 2017, 2019a), becomes a complex bank (*sensu* Darwin, 1859) of plant–animal interactions of different signs, with a marked role of mutualistic interactions such as pollination and zoochory. In this way, mistletoe creates conditions that support the co–occurrence of functionally distinct organisms in the canopies, fostering pine forest biodiversity and complexity. This has profound implications for the dynamics of the entire forest ecosystem, including the nutrients dynamics of the forest floor, the associated herbaceous community, and the bank of woody recruits (Mellado & Zamora, 2016, 2017; Mellado *et al.*, 2016; Hódar *et al.*, 2018; Zamora & Mellado, 2019).

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Part II

Direct effects caused by mistletoes

Chapter 4

Mistletoe versus host pine: Does increased parasite load alter the host chemical profile?

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Abstract

Stress caused by parasitic plants, e.g. mistletoes, alters certain host–plant traits as a response. While several physical implications of the parasite–host relation have been well studied, shifts in the host chemical profile remain poorly understood. Here we compare the chemical profiles of mistletoe (*Viscum album* subsp. *austriacum*) leaves and host pine (*Pinus nigra* subsp. *salzmannii*) needles and we investigate chemical changes in host needles of trees with different parasite loads (control, low, medium, and high). Our results reveal that despite the intimate contact between mistletoe and host pine, their chemical profiles differed significantly, revealing extremely low concentrations of defense compounds (including a complete lack of terpenes) and high levels of N concentrations in mistletoe leaves. On the other hand, parasitized pines showed unique chemical responses depending on parasite loads. Overall, the content in monoterpenes increased with parasitism. Higher parasitized pines produced higher amounts of defense compounds (phenols and condensed tannins) than less parasitized trees, but amounts in samples of the same year did not significantly differ between parasitized and unparasitized pines. Highly parasitized pines accumulated less N than pines with other parasite loads. The strongest response was found in sesqui– and diterpenes, which were at lower levels in pines under medium and high parasitism. Chemical responses of pines to mistletoe parasitism resembled reactions to other kinds of stress. Low levels induced reactions resembling those against drought stress, while medium and high parasitism elicited responses comparable to those against burning and defoliation.

Key words – hemiparasite; defense compounds; terpenes; chemical responses; stressors; *Viscum album*; plant–plant interactions; parasitic plants

Introduction

Plants are continuously exposed to different stress factors, biotic (herbivores, pests, parasites) as well as abiotic (e.g. drought, heat, salinity, cold, fire). Because these stressors can devastate plant growth and yield (Suzuki *et al.*, 2014), plants have developed strategies to chemically respond (e.g. defense compounds; (Karban & Baldwin, 1997; Keeling & Bohlmann, 2006) and to adapt (Orcutt & Nilsen, 2000; Atkinson & Urwin, 2012). Most abiotic or biotic stressors are generally episodic, eventually increasing their intensity over short time periods (e.g. droughts, herbivore outbreaks). However, parasitic plants constitute chronic, long–term attack stressors by sequestering water and macronutrients continuously from the host (Ehleringer *et al.*, 1985; Schulze *et al.*, 1984), becoming a singular biotic stressing factor.

The effects of a wide variety of mistletoes on their hosts have been well studied (Aukema, 2003; Kuijt, 1955; Pennings & Callaway, 2002; Press & Phoenix, 2005; Shaw *et al.*, 2004). Water stress and sequestration of macronutrients (Ehleringer *et al.*, 1986), growth reduction (Mutlu *et al.*, 2016; Sangüesa-Barreda *et al.*, 2012), host morpho–physical traits (Scalon *et al.*, 2017), and reduction of stomatal aperture (Zweifel *et al.*, 2012) are some of these effects, which can eventually kill the host (Dobbertin & Rigling, 2006; Roth, 2001). The increased water stress due to parasite transpiration, especially under water–limited conditions (Fisher, 1983), exacerbates host–tree susceptibility to insect and fungal attacks (Aukema, 2003; Parker & Riches, 1993). Parasitic plants can in turn gain antiherbivore properties by taking up secondary metabolites from their host (e.g. (Marko & Stermitz, 1997; Schneider & Stermitz, 1990; Stermitz *et al.*, 1989). Thus, parasitic plants give rise to new ecological interactions that modify biological communities (Hartley *et al.*, 2015; Pennings & Callaway, 1996; Press & Phoenix, 2005; Watson, 2002) and are thus recognized as keystone species (Watson, 2001; Watson & Herring, 2012). However, despite the ample information concerning mistletoe effects on host physiology, morphology, and growth patterns, the changes in the host chemical profile while parasitized and the cascading effects of this response on other organisms, remain unclear.

To help elucidate this process, we focus on the European mistletoe *Viscum album* subsp. *austriacum* (Wiesb.) Vollman (hereafter, *V. a. austriacum*), a long–lived parasite (30–35 years; (Mellado & Zamora, 2017; Nierhaus-Wunderwald & Lawrenz, 1997) that specializes in *Pinus* spp. (Mellado & Zamora, 2014b; Zuber, 2004). The dispersal mechanisms of mistletoe prompt a continual deposition of seeds onto already parasitized trees, increasing re–infestation probabilities (Aukema, 2003; Mellado & Zamora, 2017). Thus the host may harbour a large number of parasitic plants of different ages. Therefore, the impact of mistletoe on host performance may last several years or decades before the host–parasite system ends in the death of both plants (Dobbertin & Rigling, 2006; Roth, 2001).

In this study, we hypothesized that 1) mistletoes, due to their fusion with host–xylem and continuous uptake of sap and fluids (Zuber, 2004), can take up and store secondary chemical compounds from the host, similar to other hemiparasite–host systems (e.g. (Marko & Stermitz, 1997; Schneider & Stermitz, 1990; Stermitz *et al.*, 1989); and 2) mistletoe parasitism causes varying qualitative and/or quantitative changes in the host–pine chemical profile, depending on the parasite load. To test these hypotheses, we first investigated the chemical profile of mistletoe leaves and pine needles on current–year (new) and previous–year (old) cohorts. Then, we tested host chemical responses by determining the chemical profile of host needles in both cohorts, from pines with different parasite loads (Control, Low, Medium, High). We analysed how these chemical profiles of pine needles vary according to parasite load densities and characterized the chemical profile by quantifying the N content, phenols, condensed tannins, and terpenes. Finally, we discuss the effects of mistletoe on the host chemical profile tested in this study in comparison to other common stressors on Mediterranean pine forests. Responses of pines to herbivory by the pine processionary moth (PPC, Thaumetopoea pityocampa, Lepidoptera; (Carrillo-Gavilán *et al.*, 2012; Hódar *et al.*, 2015; Moreira *et al.*, 2013)), drought (Camarero *et al.*, 2017; Kainulainen *et al.*, 1992), and wildfires (Cannac *et al.*, 2009a; Lavoir *et al.*, 2013) are factors that have been thoroughly covered in the literature.

Materials and Methods

Study site and experimental design

This study was conducted in the Natural Park of Sierra de Baza, Granada (south–eastern Spain). European mistletoe (*V. a. austriacum*) is a dioecious parasitic plant, native to the European region (Zuber, 2004), and our site study represents the southernmost limit of its geographical distribution. Here the black pine (*Pinus nigra* Arn. subsp. *salzmannii*), which is the main host of mistletoe (López-Sáez *et al.*, 1992), was selected because it shows a wide range of mistletoe infestations over the elevational gradient. The climate at the site is typically Mediterranean, with an annual historic mean temperature of 15.5°C (CMAOT, 2016) and annual mean rainfall of 495±33 mm (1991–2006 period; Cortijo Narváez meteorological station, 1360 m asl) concentrated in autumn and spring, with hot, dry summers (June–September) and cold winters (December–March). Conifers are the dominant forest vegetation (43% of the total surface coverage), where black pine (*P. nigra*) is the main mistletoe host species. Other pine species such as Scots pine (*P. sylvestris* L.) occupy higher elevations (1700–2100 m asl) while Aleppo pine (*P. halepensis* Mill.) and Maritime pine (*P. pinaster* Ait.) occur at lower levels (1200–1400 m asl), with interspersed oaks (*Quercus ilex* L., 9%) and shrublands (23%) (CMAOT, 2008).

Two sets of samples were collected during the summer of 2012. The first set was collected at Cortijo Narváez (2° 51' 25" W, 37° 25' 11" N, 1360 m asl), in a pine forest with low tree density (33.42±5.79 trees $\cdot$ ha^{-1}), where black pine is a dominant tree and the main host of *V. a. austriacum*. We selected 10 parasitized black pines ("first focal pines" hereafter), spaced 40 to 80 m in order to avoid direct contact (overlapping), but remaining under the same environmental conditions. The trees selected presented similar architecture, trunk diameter at breast height (DBH, 119.23±10.98 cm), and height (8.00±0.63 m), and had moderate to intense parasite loads (more than 20% of canopy foliage occupied by mistletoe). This set of samples was collected to test the first hypothesis by comparing chemical profiles of mistletoe leaves and pine needles.

The second set of samples was collected, at Cortijo Casimiro (2° 51' 47" W, 37° 24' 59" N, 1400 m asl), in a black pine afforestation (57.33±3.18 trees ha^{-1}) of the same age and similar DBH (48.43±2.55 cm) and height (6.05±0.29 m). Many of these pine trees were parasitized by mistletoes spanning a wide range of ages and had different mistletoe parasite loads. We selected 40 pines ("second focal pines" hereafter) according to four levels (10 trees each) of parasite load: control, trees free of mistletoe; low, trees <20% of canopy foliage occupied by mistletoe; medium, trees 20–50% of canopy foliage occupied by mistletoe; and high, trees >50% of canopy foliage occupied by mistletoe. This sampling was used to test the second hypothesis by comparing pine–needle chemical profiles of pines with different parasite loads.

Both samplings were conducted using the following procedure. For all pine samples, four terminal twigs were cut from each focal pine (one per cardinal point) including current–year (2012) and previous–year (2011) needle cohorts. Similarly, four mistletoes were taken randomly from each parasitized pine from Cortijo Narváez (first sample set), where leaf cohorts

(2011 and 2012) were collected. All samples of the same pine or mistletoe were pooled, but cohorts were differentiated in order to collect a sample for pine or mistletoe and cohort year. Thus, a total of 40 (10 current–year and 10 previous–year pine needles, 10 current–year and 10 previous–year mistletoe leaves) and 80 samples (10 current–year and 10 previous–year pine needles from each parasite load), for the first and second set, respectively, were transported in an ice chest at 4°C and within 2 hr after collection were stored in the laboratory at -18°C until analysis.

N content, phenols, and condensed tannins

All fresh samples were dried at 40°C for 72 hr in darkness and then ground with a ball mill (Mixer Mill MM400, Retsch Technology, Haan, Germany) at 1800 rpm for 2 min to produce a fine powder. The total N content of the dry powder was analysed by combustion at 850°C in a Leco TruSpec auto-analyzer (LECO Corp., St Joseph, MI, USA). The N content is expressed as a percentage of the dry weight.

Total phenolic compounds were analysed by the Folin–Ciocalteu method (Waterman & Mole, 1994). Briefly, phenols were extracted from 500 mg dry powder with 10 ml of aqueous methanol (50%) in an ultrasonic bath for 15 min, and ultracentrifuged (Unicen 21, Ortoalresa, Madrid, Spain) at 2500 rpm for another 15 min. Part of this extract was diluted with water 50–fold, from 50 mg/ml to 1 mg/ml final concentration, because pine needles are low in phenolic compounds. Then, diluted extracts were assayed with 10% of Folin–Ciocalteu phenol reagent, and 5 min later, 20% of sodium carbonate (CO_3Na_2) was added to block the excess of the reagent. After 2 hr, absorbance was measured at 740 nm (Hódar & Palo, 1997) by spectrophotometry (Infinite 200 PRO series, Tecan Group Ltd., Männedorf, Switzerland). Amounts of phenolic compounds are expressed as mg of tannic acid equivalents per g of dry weight of the sample.

Condensed tannins were analysed using the proanthocyanidin assay (see (Waterman & Mole, 1994), for more details). Briefly, the same extract was assayed with butanol–hydrochloric acid reagent (0.7 g of ferrous sulphate heptahydrate in 50 ml conc. HCl with n–butanol added up to 1 l of volume). Under an extractor hood, 2.8 ml of reagent and 0.2 ml of extract were mixed in a tube assay. All samples were warmed to 95°C for 1 hr, and absorbance was measured at 550 nm (Mole & Waterman, 1987; Waterman & Mole, 1994). Condensed tannin amounts are expressed as mg of quebracho equivalents per g of dry weight of the sample.

Terpenes

Samples of terpenes were analysed following the procedure described by Kainulainen *et al.* (1992), with some modifications. Briefly, 500 mg of fresh pine needles and mistletoe leaves of each sample were weighed, finely diced ($\leq$ 1 mm) and immersed in 4 ml of *n*–hexane containing 0,001% chlorooctane as the internal standard, in a tube assay for 15 hr at room temperature. All samples were filtered in vials to obtain the extracts, and then different terpenes were analysed by gas chromatography using an Agilent 7890A instrument (Agilent Technologies, CA, USA) equipped with a ZB–5MS capillary column (30 m × 0.25 mm ID × 0.25 μm film, Phenomenex, CA, USA) coupled to a mass spectrometer Quattro micro GC (Waters Chromatography, USA). Helium was the carrier gas at a 1 mL min^{-1} flow rate and injection with a split ratio of 100:1 in. The injector and transfer line were kept at 220℃. The temperature program began at 50℃ for 2.5 min and was ramped at 4℃ min^{-1} to 200℃, where it was held for 8 min. Terpenes were identified by comparing their Kováts indices (Table 4.1), calculated relative to the retention times of a series of *n*–alkanes (C_8–C_{20}, Sigma–Aldrich, Merck KGaA, Darmstadt, Germany) analysed under same chromatographic conditions, with those reported in the literature (Adams, 2007; Ioannou *et al.*, 2014; Zhao *et al.*, 2010). In addition, compounds were identified by comparing their relative retention times (Table 4.1) and mass spectra with those obtained from authentic standards (α–pinene, $\geq$99% purity; β–pinene, $\geq$98.5% purity and Myrcene, $\geq$90% purity, Sigma–Aldrich, Merck KGaA, Darmstadt, Germany), as well as the NIST/EPA/NIH Mass Spectral Library (NIST 08 v2.0f, National Institute of Standards and Technology). All compounds were quantified by using peak areas in comparison with the internal standard. Terpene amounts are expressed as mg · g^{-1} of fresh weight of pine needles or mistletoe leaves. Analyses were performed at the technical facilities of the Scientific Instrumentation Centre (CIC) at University of Granada.

Statistical analyses

The N content, phenols, and condensed tannins were analysed by running linear mixed models (LMM), using tissue (pine needles and mistletoe leaves) for the first sample set or parasite load (Control, Low, Medium and High) for the second sample set, and cohort year (2011 and 2012) as fixed factors and tree (1–10 for first and 1–40 for second sample set) as a random factor.

Also, all terpenes were analysed together by running a principal–component

Table 4.1: Terpenoids identified in *Pinus nigra* needles based on retention times (RT), retention index (RI), and Match Factor (MF), Reverse Match Factor (RMF) and Relative Probability (RP) provided by NIST Mass Spectral Library.

Compound	RT	RI^a	RI^b	RI^c	RI^d	MF	RMF	RP(%)
Tryciclene	7.34	913	918	919	918	566	785	6.97
α–Thujene	7.49	918	927	924	924	724	824	7.66
α–Pinene*	7.70	924	933	932	933	902	903	9.47
Camphene	8.28	941	945	946	943	824	902	28.70
Sabinene	9.10	964	964	969	967	575	812	4.86
β–Pinene*	9.20	967	973	974	971	877	877	53.10
Myrcene*	9.63	980	981	988	985	817	820	68.10
Limonene	11.09	1020	1019	1024	1030	847	854	13.70
β–Phellandrene	11.14	1022	1024	1025	1022	755	821	8.84
Ocimene	11.72	1037	1040	1044	-	822	863	13.20
Terpinolene	13.17	1077	1078	1086	1079	747	848	10.70
Linalool	13.70	1091	1093	1095	-	530	738	2.48
Bornyl acetate	20.26	1273	1275	1284	1268	752	808	8.07
Terpinyl acetate	22.43	1337	1338	1346	-	680	782	1.83
β–Caryophyllene	24.74	1407	1395	1408	1411	826	827	10.20
α–Caryophyllene	25.88	1443	1431	1452	-	803	811	19.40
Germacrene–D	26.68	1469	1468	1484	1473	857	873	45.80
Sesquiterpene 1	26.99	1479	-	-	-	-	-	-
Sesquiterpene 2	29.48	1561	-	-	-	-	-	-
Diterpene 1	34.98	1757	-	-	-	-	-	-
Diterpene 2	38.41	1889	-	-	-	-	-	-

* Confirmed identification by comparison with authentic standards
RI^a = Kovats indices calculated from the retention times of the compounds in relation to those of a series of n–alkanes (C_8–C_{20}) analysed under the same chromatographic conditions in a ZB–5MS column
RI^b = Kovats indices from literature data (Ioannou *et al.*, 2014)
RI^c = Kovats indices from literature data (Adams, 2007)
RI^d = Kovats indices from literature data (Zhao *et al.*, 2010)

analysis (PCA). The first step was to reduce the number of highly correlated terpenes by the variance inflation factor (VIF) method, removing all terpenes with VIF>5 from the analysis, to avoid multicollinearity. A PCA was performed with the principal function of psych package, applying varimax rotation and selecting components with eigenvalues>2. Finally, LMMs were used to test the effects of the parasite load (Control, Low, Medium and High) and cohort year on rotation components (scores) selected.

All model assumptions were tested and principal components were log transformed when needed in order to meet the requirements of normality and homoscedasticity, and Tukey's HSD test (95% confidence level) was used for comparisons between levels of factor parasite load. LMMs and Tukey's test were implemented in the *lmer* and *glht* functions of the *lmerTest* and *multcomp* packages, respectively. R software system Version 3.4.4 (R development Core Team, 2018) was used for all statistical analyses in this work. Throughout the paper, means are expressed ± 1SE.

Results

Pine host needles vs. mistletoe leaves

Current–year pine needles showed less N content than current–year mistletoe leaves, but both had similar concentrations of total phenolic compounds (Table 4.2a, Fig. 4.1a). In addition, tannins were highly abundant in pine needles and practically absent in mistletoe leaves (Fig. 4.1a). Also, a wide range of terpenes was found in pine needles (14 monoterpenes, 5 sesquiterpenes, and 2 diterpenes), while mistletoe leaves completely lacked terpenes (Appendix Fig. S5). Cohort pine needles and mistletoe leaves also significantly differed between years. Current–year mistletoe leaves had some 60% more N content than previous–year leaves (N_{2011}: 1.12±0.04%, N_{2012}: 1.78±0.05%). By contrast, the percentage of N in previous–year pine needles showed a greater accumulation than in current–year needles (N_{2011}: 1.04±0.03%, N_{2012}: 0.93±0.02%, Fig. 4.1a). Likewise, phenolic compounds and condensed tannins accumulated significantly more in previous–year pine needles and mistletoe leaves (Table 4.2a), reaching double the amounts of phenols and more than twice the tannin concentration in both cases (Fig. 4.1a).

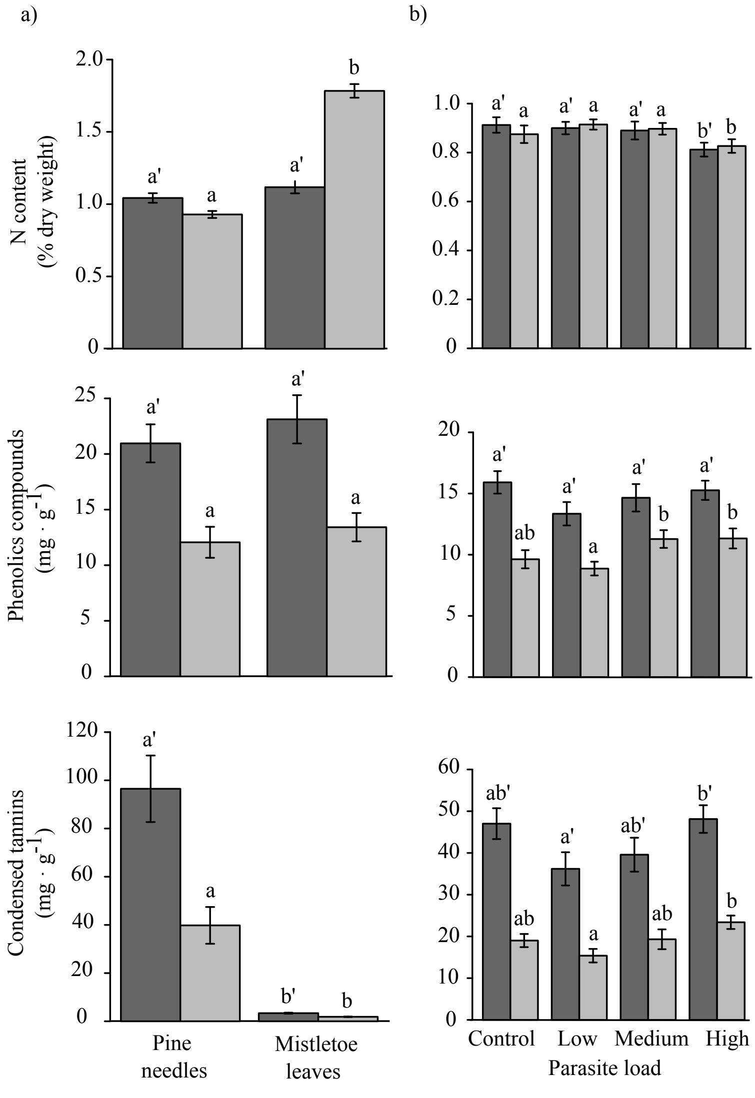

Figure 4.1: Means (± SE) of N content (%), phenolic compounds (mg · g^{-1} of dry tissue) and condensed tannins (mg · g^{-1} of dry tissue) in a) the needles of the pine *Pinus nigra* and the leaves of the mistletoe *Viscum album* subsp. *austriacum*, and b) pine needles with four different mistletoe parasite loads (Control, Low, Medium, and High). Light–grey bars indicate current–year while dark–grey bars indicate previous–year needles. Significant differences ($\alpha = 0.05$) based on multiple–comparison tests are indicated by different letters within previous– (prime lower case) and current–year needles.

Table 4.2: The effects of (a) tissue (*P. nigra* pine needles and *Viscum album* subsp. *austriacum* leaves) and (b–c) parasite load (Control, Low, Medium, and High), cohort year (2011 and 2012), and their interaction on (a–b) N content (%), concentration of phenolic compounds (mg · g^{-1} of dry tissue) and condensed tannins (mg · g^{-1} of dry tissue), and on (c) terpenes aggregate on the first three principal components. Statistical values generated come from the (a–b) LMM$_{\text{ANOVA}}$ (c) and PERMANOVA tables. Significant ($P<0.05$) and marginally significant ($0.05<P<0.1$) values are marked in bold type and italics, respectively.

(a) Tissue

	N content			**Phenolic compounds**			**Condensed tannins**		
Factor	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Tissue	1,27	212.82	<**0.001**	1,27	1.21	0.280	1,27	534.25	<**0.001**
Cohort	1,27	75.12	<**0.001**	1,27	33.90	<**0.001**	1,27	33.32	<**0.001**
Interaction	1,27	149.99	<**0.001**	1,27	0.06	0.803	1,27	1.80	0.191

(b) Parasite load

	N content			**Phenolic compounds**			**Condensed tannins**		
Factor	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Parasite load	3,36	2.39	*0.085*	3,36	1.68	0.188	3,36	2.97	**0.045**
Cohort	1,36	0.001	0.975	1,36	141.55	<**0.001**	1,36	222.02	<**0.001**
Interaction	3,36	0.84	0.480	3,36	2.77	*0.056*	3,36	1.33	0.280

(c) Parasite load

	PC 1			**PC 2**			**PC 3**		
Factor	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Parasite load	3,36	1.17	0.334	3,36	5.60	**0.003**	3,36	1.36	0.269
Cohort	1,36	72.70	<**0.001**	1,36	12.05	**0.001**	1,36	22.84	<**0.001**
Interaction	3,36	0.45	0.720	3,36	1.27	0.299	3,36	4.38	**0.010**

Chemical profile of pine needles in relation to parasite loads

Mistletoe parasitism only marginally affected the N content of host pine needles (Table 4.2b and Fig. 4.1b), while leaf–N values decreased in highly parasitized pines (Appendix Table S9). The results between current and previous years of needle cohorts presented the same N content (Table 4.2b and Fig. 4.1b).

Amounts of total phenols and tannins did not differ between parasitized and unparasitized pines. However, their concentrations followed a similar pattern for different parasite loads, with a significant increase, especially in current–year needles, in pines with medium (for phenolic compounds) and high (for phenols and tannins) parasitism as compared to low parasitism

(Table 4.2b and Fig. 4.1b). In addition, phenol concentrations in previous–year's needles showed a significant increase from 30% to 60% and more than twice the amounts of tannins in relation to those of current years, regardless of the mistletoe parasite load.

Table 4.3: Loadings, eigenvalues, and variance explained (%) of the three first principal components of PCA for all terpenes analysed in *P. nigra* pine needles with 4 different *Viscum album* subsp. *austriacum* parasite loads (Control, Low, Medium, and High). Bold type indicates the compound highly correlated with each principal component.

		Compounds	**PC1**	**PC2**	**PC3**
Terpenes (mg · g^{-1} of fresh samples)	Mono-	α–Pinene	**0.84**	−0.11	0.35
		Sabinene	**0.66**		0.38
		β–Pinene		−0.21	**0.67**
		Myrcene	0.44	0.46	−0.23
		Limonene	0.49	−0.18	**−0.52**
		β–Phellandrene		0.18	**0.76**
		Ocimene	**0.82**	−0.13	
		Linalool	0.12	0.39	−0.39
		Bornyl acetate	**0.76**	−0.16	0.14
		Terpenyl acetate	0.30		**0.53**
	Sesqui–	β–Caryophyllene		**0.69**	−0.47
		Germacrene–D	−0.13	**0.79**	
		Sesquiterpene 1	−0.11	**0.76**	
		Sesquiterpene 2	0.22	0.19	
	Di–	Diterpene 1	−0.42	**0.60**	
		Diterpene 2		**0.64**	
		Eigenvalues	4.10	2.41	2.00
		% Variance explained	20.00	18.80	14.50
		% Cumulative	20.00	38.80	53.20

Due to the lack of terpenes in mistletoe leaves, PCA was applied only to this second set of samples (focal pines with different parasite loads). The first three principal components (PC) accounted for 53.2% of the total variance (eigenvalues$\geq$2, Table 4.3). PC1 was strongly represented by several monoterpenes, such as bornyl acetate, α–pinene, ocimene, and sabinene (Fig. 4.2), and is also highly correlated with other monoterpenes (camphene, α–thujene, terpinolene, and tricyclene).

Similarly, PC2 was linked to sesquiterpenes (germacrene–D, β–caryophyllene and sesquiterpene 1), including α–caryophyllene by correlation, and diterpenes (diterpenes 1 and diterpenes 2). PC3 was positively linked to other monoterpenes, such as β–pinene, β–phellandrene, and terpinyl acetate, and negatively with limonene (Fig. 4.2). The LMM analysis revealed that monoterpenes added in the first principal component differed significantly between cohort years (Table 4.2c), being greater in current–year needles (Fig. 4.2). Overall, although mistletoe parasitism showed no statistical differences, these monoterpene concentrations increased proportionally to the mistletoe load (Appendix Table S9). The concentrations of sesqui– and diterpenes (compound aggregates on PC2) showed a significant decrease in needles of medium and highly parasitized pines (Table 4.2c) and an accumulation in all previous–year needles (Fig. 4.2). Finally, the concentration of monoterpene aggregates in PC3 showed a significant interaction, where previous–year needles did not differ between parasite loads. Meanwhile, current–year needles showed a significant increase in limonene and a decrease in β–pinene, β–phellandrene, and terpinyl acetate proportional to mistletoe densities.

Discussion

Pine host needles *vs.* mistletoe leaves

Despite the fusion of mistletoe with host xylem and continuous uptake of sap and fluids, mistletoe leaves and pine needles had completely different chemical profiles. On the one hand, mistletoe leaves contained a high N content, especially in current–year leaves (Fig. 4.1a). On the other hand, the same leaves showed low amounts of condensed tannins and lacked terpenes (Fig. 4.1a), which have antidigestive (Kraus *et al.*, 2003; Mole & Waterman, 1986), toxic and/or deterrent effects for herbivores (Keeling & Bohlmann, 2006; Reid *et al.*, 2017; Tiberi *et al.*, 1999). However, one similarity appeared in both cases: the concentration of phenolic compounds and their accumulation almost doubled that of the previous–year cohorts (Fig. 4.1a). Therefore, *V. a. austriacum* does not benefit from the pine host by acquiring antiherbivory properties.

The lack of defensive compounds and high N content make mistletoe an attractive food for herbivores (Mattson Jr, 1980). Therefore, mistletoe foliage appears to be more palatable and of better quality than pine needles and could, thus, be consumed by a large diversity of herbivores. Surprisingly, although *V. a. austriacum* can be consumed by some mammals (Deforce *et al.*, 2013; Hejcman *et al.*, 2014; Kühn *et al.*, 2013), mistletoe is inhabited and consumed by very few highly specialized arthropods under natural conditions (Briggs, 2011; Lázaro-González *et al.*, 2017; Struwe *et al.*, 2009). However, other mistletoe species in North America are consumed by arboreal mammalian herbivores, which control the density of the parasite (Shaw *et al.*, 2004), as occurs also in Australia and New Zealand (Norton & Reid, 1997). Basically, if terrestrial mammalian herbivores, have access to mistletoe, they could consume entire plants (Deforce *et al.*, 2013; Hejcman *et al.*, 2014; Kühn *et al.*, 2013), however, Mediterranean forests lack arboreal herbivore mammals that are able to feed on mistle-

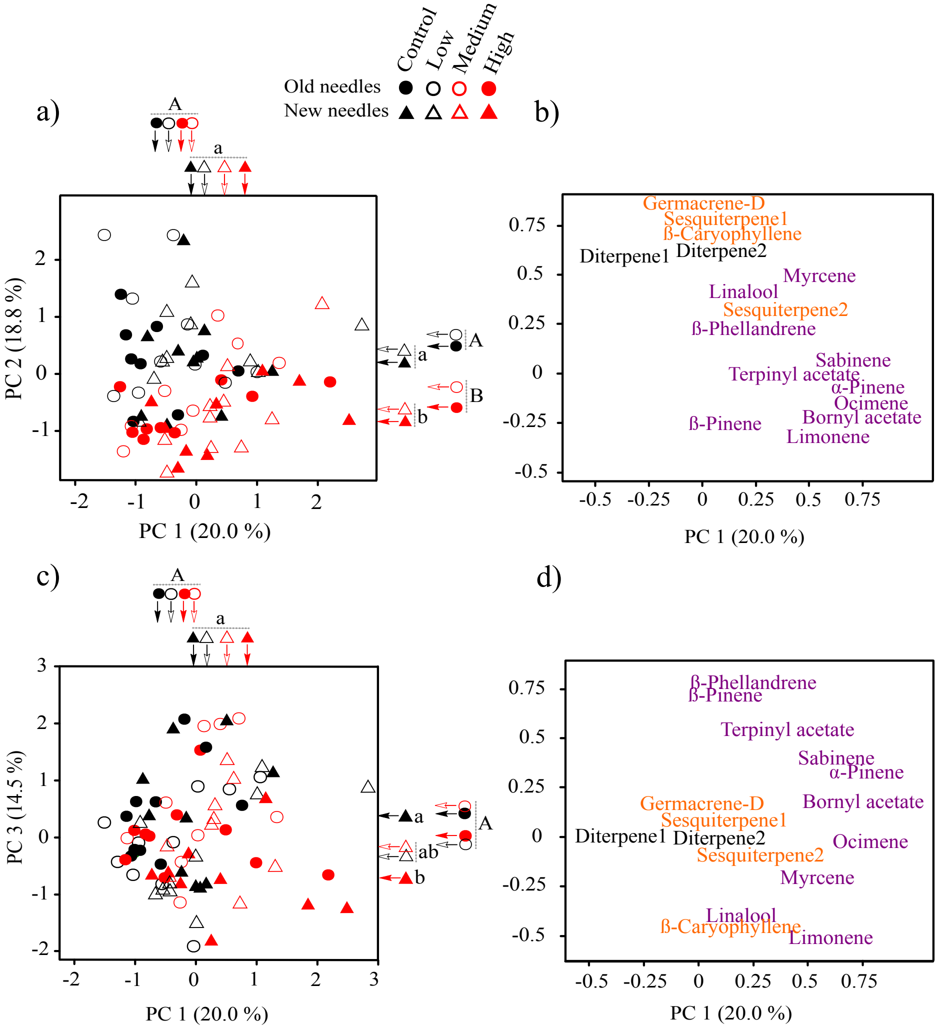

Figure 4.2: Principal–component analysis conducted by 16 non–correlated terpenes (mg/g of fresh weight), including 10 monoterpenes (purple), 4 sesquiterpenes (orange) and 2 diterpenes (black). The first three PC are represented (a–b: PC1 *vs.* PC2, c–d: PC1 *vs.* PC3) with terpenoid compounds (b–d) and case scores (a–c). Circles represent the old pine–needle cohort (from 2011) and triangles the new pine–needle cohort (from 2012). Different colours represent mistletoe parasite loads: black filled, unparasitized; black open, Low; red open, Medium; and red filled, High. Arrows indicate the coordinate averages of parasite load and cohorts on PCA axes. Different letters close to arrows indicate significance difference between parasite load levels and year cohorts detected by Tukey's HSD post hoc test ($P < 0.05$)

toe. The pine canopy, thus, represents a relatively enemy–free site for *V. a. austriacum*.

Chemical profile of pine needles in relation to the parasite loads

Host pines can recognize and respond to mistletoe parasitism by changing concentrations of their chemical compounds. Firstly, although parasitized and unparasitized pines showed similar concentrations of phenols and condensed tannins, pines reacted proportionally to mistletoe parasitism by increasing phenols (especially in current–year needles) and tannins in needles from low parasitism to high (Table 4.2b and Fig. 4.1b). In line with this, most monoterpenes (bornyl acetate, α–pinene, ocimene, sabinene, camphene, tricyclene, and limonene, Appendix Table S9, Fig. 4.2) increased in concentration according to the intensity of mistletoe parasitism. Finally, the most parasitized pines contained lower concentrations of N, and sesqui– and diterpenes than pines with low or no parasitism.

In general terms, host pines reacted by modifying their chemical profiles with two types of responses: I) proportional mistletoe load–dependent responses, and II) responses from the most parasitized pines. Therefore, the combination of these chemical changes according to mistletoe parasite loads reveals that host pines showed a different chemical profile at each parasitism level. Chemical compounds in parasitized and in unparasitized pines were qualitatively the same, but with quantitative changes in their concentrations, this reaction can be described as induced chemical defense (Karban & Baldwin, 1997).

Low–parasitized pines reacted against mistletoes by slightly reducing the phenol and condensed–tannin concentration, and increasing most terpenes relative to the control pine trees. On the other hand, the most parasitized pines accumulated more phenolic compounds and condensed tannins, and less N under high parasitism, than under low parasitism. Finally, although medium and high parasitism in pines greatly increased the amounts of monoterpenes, the total terpenes decreaseed due to a strong reduction in the amounts of sesqui– and diterpenes (Appendix Table S9). Terpenes are stored in specialized secretory structures that clearly require a substantial amount of resources (Gershenzon, 1994), and stressed pines with high parasitic load probably cannot afford such costs.

Does the pine respond equally to all types of stressors?

Chemical changes are induced in pines by many abiotic and biotic ecological factors including herbivory (Karban & Baldwin, 1997), drought (Camarero *et al.*, 2017; Kainulainen *et al.*, 1992), and fire (Lavoir *et al.*, 2013; Cannac *et al.*, 2009a). Here, we show that pines parasitized by mistletoe respond against stress by changing their chemical profiles. However, in contrast to herbivory, drought, and fire, which in general are episodic and short–term stressors, mistletoe represents a chronic long–term biotic stressor. Thus, the question is to what extent the pine's chemical response to mistletoe resembles (or contrasts with) reactions to other stressing factors, whether abiotic or biotic.

Pine reaction to PPC attack, the main defoliator on pine Mediterranean forest, has been well analysed in previous studies by the same procedure as in the present study (Hódar *et al.*, 2015), and therefore the results in the two cases may be compared. Mistletoes and PPC are biotic stressors for pines, but in contrast the parasitic damage does not mechanically harm pine needles. Needle damage, e.g. by defoliators, is a key factor because it promotes emissions of volatile compounds. For instance, after needle consumption by the PPC, pines show a weak chemical response (Hódar *et al.*, 2015) because the low concentrations of compounds in pine needles hardly change (Rivas-Ubach *et al.*, 2016b). This idea has been reinforced by many authors (Achotegui-Castells *et al.*, 2013; Hódar *et al.*, 2004, 2015; Moreira *et al.*, 2013) who report that pine needles attacked by the PPC have the same (or less) concentration in monoterpenes. Nevertheless, despite that the activity of monoterpene cyclase increases in attacked pine needles (Litvak & Monson, 1998), high monoterpene concentrations are not reflected because the mechanical action of PPC feeding by wounding the needles causes high emissions of volatile compounds. In our case, parasitized pines react against mistletoe parasitism with an increase in most monoterpenes, suggesting that mistletoe parasitism stimulates monoterpene synthase activity in pine, but, due to the lack of needle damage, the increase in monoterpenes is reflected in our results. Finally, pines with high parasite loads also showed higher concentrations of phenols and condensed tannins and lower N content, sesqui– and diterpenes, responses reported elsewhere for defoliations by the PPC (Carrillo-Gavilán *et al.*, 2012; Moreira *et al.*, 2013; Roitto *et al.*, 2003).

Abiotic stress factors such as drought and wildfire, could alter chemical profiles of pines undergoing non–mechanical harm in the same way as mistletoe parasitism. Despite that the effects of abiotic factors vary depending on severity, duration, and location, many pine responses discussed in the literature in these contexts resemble the reaction of our pines to mistletoe parasitism. Overall, drought episodes cause an overall increase in terpenes (Llusià & Peñuelas, 1998; Llusià *et al.*, 2006; Turtola *et al.*, 2003), especially monoterpenes α–pinene, camphene, tricyclene and limonene (Kainulainen *et al.*, 1992; Llusià & Peñuelas, 2000) and sesquiterpenes α–caryophyllene and β–caryophyllene (Llusià & Peñuelas, 1998, 2000). Curiously, these identical chemical changes (although not statistically significant) were found in the present study in host pines undergoing low mistletoe parasitism (Appendix Table S9). Also, a drought episode can stunt plant growth (Guada *et al.*, 2016; Camarero *et al.*, 2017) and reduce stomatal conductance (Klutsch *et al.*, 2017; Wellburn *et al.*, 1996), in the same way as mistletoe parasitism depresses host pine growth (Mellado *et al.*, 2016; Sangüesa-Barreda *et al.*, 2012) and causes water stress (Ehleringer *et al.*, 1986). According to Llusià & Peñuelas (1998), growth inhibition due to water limitation can cause carbon accumulation, triggering the reallocation of defense compounds such as terpenes.

In line with these findings, pines also react to non–lethal fires, e.g. prescribed burnings, changing their chemical profile, boosting total phenolic compounds (Cannac *et al.*, 2009b; Lavoir *et al.*, 2013) and limonene (Cannac *et al.*, 2009a), and reducing the pine–needle N content (McCullough & Kulman, 1991) and diterpene concentration (Cannac *et al.*, 2009a). In general, the effects of prescribed burning on the chemical profile of pine are quite similar to those of medium and high mistletoe parasite loads.

Therefore, the pine chemical response to mistletoe parasitism depends basically on the parasite load. Slight but prolonged parasitism (low mistletoe loads) induces a response similar to those of water stress and drought. Meanwhile, prolonged and heavy parasitism (medium and high parasite loads) affects pines more severely, diminishing their capacity to produce costly defense compounds, in agreement with responses to prescribed burnings and PPC herbivory.

In conclusion, despite that mistletoe takes most of its resources from the host plant, the chemical profile of the parasite, including volatile compounds and N content, differs from that of pine. The changes in the pine's chemical profile depend on the parasite load, which represents changes in the levels of the inducible chemical defense of pine. Despite that mistletoe parasitism is a permanent stressor, some pine responses are similar to those caused by other episodic events such as drought, fires, and PPC herbivory, but the exact response varies according to the parasite load.

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Chapter 5

Implications of mistletoe parasitism for the host metabolome: a new plant identity in the forest canopy

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Abstract

Mistletoe–host systems exemplify an intimate and chronic relationship where mistletoes represent protracted stress for hosts, causing long–lasting impact. Although host changes in morphological and reproductive traits due to parasitism are well known, shifts in their physiological system, altering metabolite concentrations, are less known due to the difficulty of quantification. Here we use ecometabolomic techniques for the first time in plant–plant interaction, comparing the complete metabolome of the leaves from mistletoe (*Viscum album*) and needles from their host (*Pinus nigra*), both parasitized and unparasitized, to elucidate host responses to plant parasitism. Our results show that mistletoe acquires metabolites basically from the primary metabolism of its host, and synthesises its own defence compounds. In response to mistletoe parasitism, pines modify a quarter of their metabolome over the year, making the pine canopy metabolome more homogeneous by reducing the seasonal shifts in top–down stratification. Overall, host pines increase antioxidant metabolites, suggesting oxidative stress, and also increase part of the metabolites required by mistletoe, which act as a permanent sink of host resources. In conclusion, by exerting biotic stress and thereby causing permanent systemic change, mistletoe parasitism generates a new host–plant metabolic identity available in forest canopy, which could have notable ecological consequences in the forest ecosystem.

Key words – ecometabolomic; mistletoe–host system; new plant identity; plant–plant interaction; oxidative stress; seasonality; permanent systemic effects

Introduction

Plants react to biotic and abiotic stress, causing a wide range of well–known biotic changes, e.g. by modifying plant ecophysiology, growth, reproduction, and phenology (Strauss & Zangerl, 2002; Pérez-Ramos *et al.*, 2019). These responses could be almost instantaneous in response to a pulse disturbance, or could cause a permanent reaction, leaving a long–lasting fingerprinting and, eventually, causing a generalized effect throughout the system over time (Sutherland, 1981; Bender *et al.*, 1984). Thus, while insect outbreaks and some abiotic disturbances (e.g. episodic drought events, wildfires, and strong storms) have short–term implications, the case of parasitic plants such as mistletoe represent a long–term host–parasite interaction that might cause a permanent host reaction (Lázaro-González *et al.*, 2019b). Mistletoe are long–lived plants with a perennial endophytic system called haustorium, which is embedded in the host xylem system and serves to parasitize by extracting water and minerals from the host (Ehleringer *et al.*, 1985; Marshall & Ehleringer, 1990; Hawksworth & Wiens, 1998). Vast literature is available on the visible changes that mistletoe cause to their host, such as growth and reproductive changes (Kuijt, 1955; Pennings & Callaway, 2002; Press & Phoenix, 2005), as well as to their neighbouring plants (Hartley *et al.*, 2015; Hódar *et al.*, 2018; Mellado & Zamora, 2017) and insect community (Hartley *et al.*, 2015; Lázaro-González *et al.*, 2019a; Mellado *et al.*, 2019). However, less evident effects such as chemical–profile alterations, have been less studied, with attention usually focused on a single compound or group of metabolites (e.g. Anselmo-Moreira *et al.*, 2019; Lázaro-González *et al.*, 2019b).

The first reaction of a plant in response to biotic or abiotic stress starts with their phenotypical response including physiological and metabolic acclimation. Therefore, these metabolite changes could be episodic or permanent according to the nature of the stress factor (e.g. Peters *et al.*, 2018, and references therein). The challenge of studying chemical and physiological plant responses to environmental stress is the extraordinary variety of traits that can be altered, as well as the range of analytical methods that researchers need in order to disentangle the situation. A consequence of this complexity is that most of the research using traditional techniques focus on a single compound or a group of compounds, such as chemical defence which act as toxins and deterrents (e.g. Chen, 2008; Sampedro *et al.*, 2011). Plant metabolite profiles comprise a complex set of primary metabolites (sugars, amino acids, nucleotides, etc.) and secondary ones (terpenoids, phenolics, etc.), jointly called the metabolome, which is synthesised by the biologic system of plants and which shapes the real functionality of plants at a specific time (Fiehn, 2002; Weckwerth, 2003; Tomita & Nishioka, 2003). For this reason, ecometabolomic techniques which have great sensitivity have been developed, allowing us to combine ecological and biochemical studies on plants and to capture these ecophysiological and functional changes in a dynamic way at the finest metabolite level (Peñuelas & Sardans, 2009; Bundy *et al.*, 2009; Allwood *et al.*, 2010; Lima *et al.*, 2010; Sardans *et al.*, 2011; Gargallo-Garriga *et al.*, 2017).

Biotic stress exerted by mistletoe parasitism could alter pine metabolomic identity in different ways. As modular organisms, pine trees could have a high phenotypic plasticity, adjusting the response of the entire module population against environmental conditions. Also, tree canopies offer a stratified top–down trait because they are exposed under a vertical gradient of different microclimatic conditions (e.g. light availability, wind speed, air temperature), generating top–down differences in ecophysiological properties (Brooks *et al.*, 1997; Lewis *et al.*, 2000; Parker & Brown, 2000). On the temporal scale, the metabolome of any organism is dynamic and highly susceptible to change under variable conditions. For example, in spring, new shoots start a burst of growth, and therefore their metabolic requirements differ from those of more mature needles, which contain compounds from other pathways (Gargallo-Garriga *et al.*, 2015; Meijón *et al.*, 2016). In this case, two crucial and metabolically different periods for current pine needles could be early summer, after the first elongation, and early autumn, after a stress period of hot temperatures and drought. For these reasons, pine stratification and the time period become essential for researchers to analyse correctly the diversity and spatio–temporal consistency of metabolic profile on the whole host–parasite system.

Here, we focus on the European mistletoe (*Viscum album* subsp. *austriacum* Wiesb. Vollman, hereafter *V. album*), an evergreen, epiphytic, and dioecious parasitic plant native of most regions of Europe, which specializes on conifers (Zuber, 2004). Part of changes in the chemical profile of the main host, the black pine, *Pinus nigra* subsp. *salzmannii* (hereafter *P. nigra*), caused by *V. album*, have recently been studied (Lázaro-González *et al.*, 2019b). This prior study shows how highly parasitized pines react against mistletoe parasitism, provoking changes in the concentrations of nitrogen and defence compounds in pine needles. However, the overall metabolic profile (the complete set of metabolites) of the plant host–mistletoe interaction has not yet been examined. Thus, a higher–level resolution in the analyses of host metabolic profile, could help to elucidate the diversity and spatio–temporal consistency of metabolic profile of the host–parasite system. In addition, ecometabolomic approaches have been specially applied in plant–animal, plant–fungus, and plant–microbe interactions, but this is the first time such an approach has been used in a plant–plant interaction, which are involved a host–parasite system with two long–lived plants (Peters *et al.*, 2018).

Our general hypothesis is that parasitized pines react permanently to mistletoe due to the chronic parasitism, changing their metabolome over the year. Thus, we expect the following: a) parasitized pines will increase the concentration of metabolites according to mistletoe requirements, and therefore, the metabolome of parasitized pines and mistletoe would be more similar than the metabolome of non–parasitized pines and mistletoe; and b) parasitized pines will promote their secondary metabolism to bolster the production of defence compounds against mistletoe parasitism. In addition, due to the intimate connection of the haustorium with the vascular vessels of the pine and the long–lasting attack of the mistletoe, we expect these changes in metabolomics to manifest themselves systemically throughout the parasitized pine canopy. This study advances our understanding of plant–parasitism ecology and the plant–host responses at the finest metabolic level in two long–lived plants, a relationship that in turn can promote far–reaching ecological consequences in forest ecosystems.

Materials and Methods

Study site

This study was conducted in a Mediterranean pine forest in Sierra de Baza (Granada, south–eastern Spain, 2° 51' 48" W – 37° 22' 57" N), which has an altitudinal gradient of 850–2269 m asl and represents the southernmost limit of the *V. album* geographical distribution. The climate is typically Mediterranean with mean annual temperature of 15.5°C (CMAOT, 2017) and annual mean precipitation of 495±33 mm (1991–2006 period; Cortijo Narváez meteorological station, 1360 m asl) concentrated in spring and autumn, hot and dry summers (June–September) and cold winters (December–March). This site is dominated by conifers (43%), especially *Pinus nigra* Arn., which is the main host and frequently parasitized by *V. album* (Mellado & Zamora, 2020). There are other species of pines, such as Aleppo (*P. halepensis* Mill.), maritime (*P. pinaster* Ait.), and Scots pine (*P. sylvestris* L.), as well as oaks (*Quercus ilex*, *Q. coccifera*, 9%) and an ensemble of shrubs and herbaceous areas (23% CMAOT, 2008).

Experimental design

The study was conducted during 2015 in a stand of afforested *P. nigra* (57.3±3.2 trees ha^{-1}) located at 1450 m asl in Sierra de Baza. These trees have the same age (~40 years old) and similar architecture (DBH: 48.4±2.6 cm, height: 6.1±0.3 m). In addition, due to the self–reinfection system of mistletoe (Mellado & Zamora, 2014a), pine hosts have a wide range of mistletoe parasite loads, since mistletoe–free pines to heavily parasitized ones. We selected 10 unparasitized pines and 10 highly parasitized ones (>50% of canopy foliage occupied by mistletoe), paired by their structural similarities (i.e. canopy configuration and size) and spatial proximity. The pines were selected from within a maximum distance of 30 m and minimum of 10 m in order to ensure that the trees constituted independent sampling units. We collected 3 samples of current–year needles in each pine at three different strata within pine canopy (upper, medium, and bottom third of canopy) and two seasons (summer and autumn). Each sample was formed by mixing different terminal twigs from the same canopy stratum. Therefore, the experimental design contained a total of 120 pine–needles samples: 10 pine trees per treatment (parasitized and unparasitized), two sampling seasons (summer and autumn), and three strata for pine–needle samples due to their vertical gradient (upper, medium, and bottom third of the canopy). In addition, current–year mistletoe leaves from the upper part of the canopy were also collected from parasitized pines in both seasons.

Collection and preparation of tissue samples

The sample preparation is described in detail by Rivas-Ubach *et al.* (2013). Briefly, the samples were frozen immediately in liquid nitrogen and then lyophilized and stored in plastic cans at -80℃. The samples were then ground with a ball mill (Mikrodismembrator–U, B. Braun Biotech International, Melsungen, Germany) at 1700 rpm for 4 min, producing a fine powder that was stored at -80℃. See the supplementary material of Gargallo-Garriga *et al.* (2014) for details.

Analysis by liquid chromatography–mass spectrometry (LC–MS)

The LC–MS platform (all from ThermoFisher Scientific, San Jose, CA, USA unless otherwise noted) consisted of an Accela U–HPLC system with quaternary pumps, an HTC PAL autosampler (CTC Analytics AG, Zwingen, Switzerland), a Keystone hot pocket column heater, and an Exactive

Orbitrap mass spectrometer controlled by Xcalibur 2.1. Reversed–phase LC separation used a Synergy Hydro–RP column (100 × 2 mm, 2.5 μm particle size, Phenomenex, Torrance, CA, USA) with the ion–pairing agent tributylamine in the aqueous mobile phase to enhance retention and separation. The LC used a column with a small particle size (2.5 μm instead of 4 μm) to reduce peak widths and expedite analysis. The total run time was 25 min, and the flow rate was 200 μL/min. Solvent A was 97:3 water:methanol with 10 mM tributylamine and 15 mM acetic acid; solvent B was methanol. The gradient was 0 min, 0% B; 2.5 min, 0% B; 5 min, 20% B; 7.5 min, 20% B; 13 min, 55% B; 15.5 min, 95% B; 18.5 min, 95% B; 19 min, 0% B; 25 min, 0% B. Afterwards the column was washed and stabilized for 5 min before the next sample was injected. Other LC parameters were: autosampler temperature, 4℃; injection volume, 10 μL; and column temperature, 25℃. HESI (heated electrospray ionization) was used for MS detection. All samples were injected twice, once with the ESI operating in negative ionisation mode (-H) and once in positive ionisation mode (+H). The Orbitrap mass spectrometer was operated in FTMS (Fourier Transform Mass Spectrometry) full–scan mode with a mass range of 50–1000 m/z and high–mass resolution (60,000). The resolution and sensitivity of the spectrometer were monitored by injecting a caffeine standard after every 10 samples, and the resolution was further monitored with lock masses (phthalates). Blank samples were also analysed during the sequence. The assignment of the metabolites was based on standards, with the retention time and mass of the assigned metabolites in both positive and negative ionisation modes.

Statistical analyses

Firstly, the normality of each metabolite signal–intensity data was tested by Kolmogorov–Smirnov tests. The data for all metabolites followed a normal distribution, except 5 unidentified compounds (0.25%), which were removed from the data set. Then, a permutational multivariate analyses of variance (PERMANOVA) was performed to test differences between pine needles, from parasitized (PPN) and unparasitized pines (UPN), and mistletoe leaves (ML) in both seasons. Thus, treatment (PPN, UPN, and ML) and season (summer and autumn) were included as fixed factors and pine tree individual as random factor. In the same way, a partial least squares discriminant analysis (PLS–DA) was also performed to determine general trends on a sample ordination, and a Linear Mixed Model and Tukey *post hoc* test with score coordinates of the two first PLS–DA components were used to test differences among metabolomes of ML, PPN, and UPN for summer and autumn. Finally, one–way ANOVAs were performed for each individual metabolic compound to identify any statistical differences between ML, PPN, and UPN metabolomes.

Secondly, the whole metabolomic profile of *P. nigra* needles (1991 metabolites), including 55 identified from our metabolite library, was analysed in order to test global effects of mistletoe parasitism (parasitized and unparasitized pines), canopy modularity (upper, middle, and bottom third of the pine canopy), and season (summer and autumn). These three factors were run on a PERMANOVA using the Euclidean distance, with 10000 permutations, as fixed independent factors and each pine tree as random factors. One–way ANOVAs between treatment and season were also performed for each individual metabolic compound. Multivariate ordination PLS–DAs were also performed to detect general patterns of sample ordination in the metabolomes. The PLS–DAs allowed us to reduce the dimensionality of the entire data set of identified and unidentified metabolites and to project our samples and variables on a biplot. Therefore, we were able to identify metabolomic trends of parasitized and unparasitized *P. nigra*, seasons, and canopy modularity. To test differences among the metabolome of different groups across the scores coordinates of two first components of the PLS–DAs, we used a LMM for each component and a Tukey *post hoc* test, with three factors as fixed and pine tree as the random factor.

All statistical analyses were conducted with R software (R Core Team, 2019) and were performed to detect shifts in both the metabolomes and individual metabolites as well as the variables controlling them. The PERMANOVA was conducted with the *adonis* functions in *vegan* package (Oksanen *et al.*, 2019). One–way ANOVAs and the Kolmogorov–Smirnov test were performed by *aov* and *ks.test* functions in *stats* package (R Core Team, 2019). PLS–DA was conducted with the *plsda* function in the *mixOmics* package (Rohart *et al.*, 2017). All data were scaled for the PLS–DA by setting the parameter “scale=TRUE” in the function. Finally, LMM and Tukey *post hoc* tests were performed with the *lme* and *lsmeans* functions of the *nlme* (Pinheiro *et al.*, 2019) and *lsmeans* (Russell, 2016) packages, respectively.

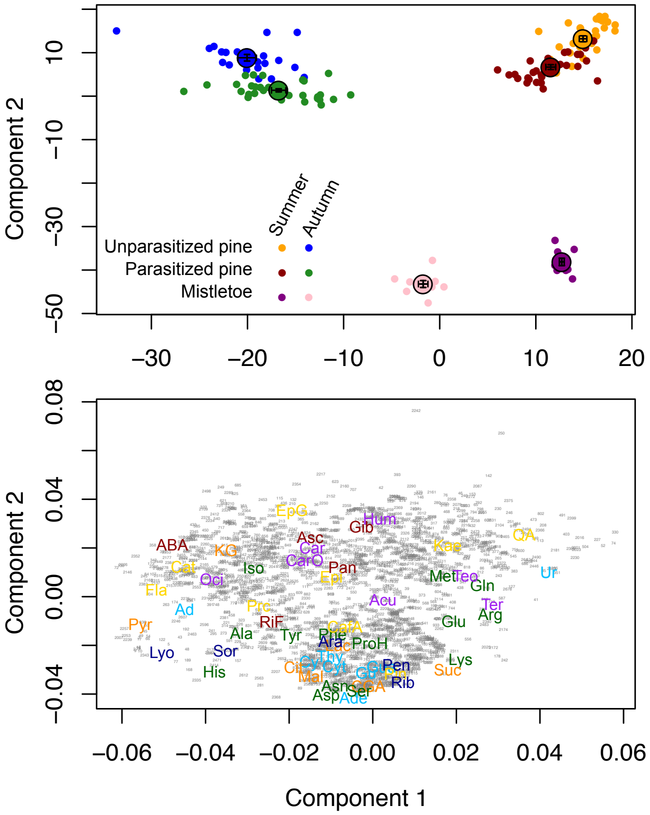

Figure 5.1: Component 1 *vs.* Component 2 of the partial least squares discriminant analysis (PLS–DA) conducted with all metabolome of parasitized and unparasitized pine needles, and mistletoe leaves. Biplots of the two first components of the PLS–DA of metabolomic data presenting the scores (mean ± SE) of the *Pinus nigra* needles (dark red and green, parasitized pines; orange and blue, unparasitized pines) and mistletoe leaves (purple and pink), and summer and autumn season, respectively. The various metabolomic families are represented by colours: green, amino acids; cyan, nucleotides; orange, organic acids related to the tricarboxylic acid cycle; red, other secondary metabolites; dark blue, sugars; yellow, phenolics; purple, terpenes; and grey, unknown metabolites. Aspartic acid (Asp), serin (Ser), lysin (Lys), asparagine (Asn), arginine (Arg), tyrosine (Tyr), methionine (Met), histidin (His), glutamine (Gln), glutamic acid (Glu), isoleucin (Iso), phenilalanin (Phe), hydroxyproline (ProH), alanine (Ala), adenine (Ad), uracil (Ur), guanosine (Gua), guanine (Gu), cytidine (Cy), cytosine (Cyt), adenosine (Ade), thymine (Thy), α–ketoglutaric acid (KG),), chlorogenic acid (CGA), citric acid (anhydrous) (Cit), L–(-)–malic acid (Mal), lactic acid (Lac), pyruvic acid (Pyr), succinic acid (Suc), pantothenic acid hemicalcium salt (Pan), abscisic acid (ABA), ascorbic acid (Asc), gibberellic acid (GA3) (Gib), riboflavin (RiF), (+)–catechin hydrate (anhydrous) (Cat), 5,7–dihydroxy–3,4,5–trimethoxyflavone (Fla), epicatechin (Epi), epigallocatechin (EpG), protocatechuic acid (Prc), D–pinitol (Pin), caffeic acid (CafA), quinic acid (QA), 2–deoxy–D–ribose (Rib), D–(-)–lyxose (Lyo), pentose (Pen), D–(+)–sorbose (Sor), D–(+)–arabitol (Ara), aucubin (Acu), α–humulene (Hum), α–terpinene (Ter), caryophyllene oxide (CarO), kaempferol (Kae), ocimene (Oci), α–terpineol (Teo), carvone (Car).

Results

Metabolomic profile differences between pine host and its hemiparasite

All metabolites detected in pine needles (1991 compounds) were found in PPN and UPN. However, the metabolic profile of ML lacked 17 and 15 of metabolites with respect to pine needles in summer and autumn season, respectively, 5 of these being absent in all cases. The PERMANOVA of the entire data set indicated differences in the overall metabolomes among treatments (F_1=32.21; P<0.001), seasons (F_1=43.39; P<0.001), and their interaction (F_1=4.73; P<0.001).

Table 5.1: *Post hoc* results from LMMs of two first components from PLS–DA between treatment (ML= mistletoe leaves, PPN=parasitized pine needles, UPN=unparasitized pine needles) and season (S=summer, A=autumn). Bold type indicates significant effects (P<0.05).

	Component 1					Component 2				
	ML–S	ML–A	PPN–S	PPN–A	UPN–S	ML–S	ML–A	PPN–S	PPN–A	UPN–S
ML–A	**<0.001**	–	–	–	–	**<0.001**	–	–	–	–
PPN–S	0.898	**<0.001**	–	–	–	**<0.001**	**<0.001**	–	–	–
PPN–A	**<0.001**	**<0.001**	**<0.001**	–	–	**<0.001**	**<0.001**	**<0.001**	–	–
UPN–S	0.444	**<0.001**	**0.013**	**<0.001**	–	**<0.001**	**<0.001**	**<0.001**	**<0.001**	–
UPN–A	**<0.001**	**<0.001**	**<0.001**	**0.038**	**<0.001**	**<0.001**	**<0.001**	0.677	**<0.001**	**<0.001**

Overall, the ML metabolome differed markedly from that of pine needles, and although PPN metabolome was displayed close to UPN, their metabolic profile was statistically different, being PPN more similar to ML. When all the data were analysed at once, these differences were displayed on component 2 of PLS–DA (Table 5.1, Fig. 5.1). The one–way ANOVAs of all metabolic compounds showed that the ML metabolome differed from UPN in 80% of the compounds (1542 out of 1991), while the metabolic profile of PPN showed fewer differences with regard to ML (72.5%, 1444 compounds). The concentration of 933 and 842 metabolites were higher in UPN and PPN, respectively, than ML, and the rest (609 and 602 compounds) proved higher in ML (see Fig. 5.2 and Appendix Table S10 for identified compounds). Thus, the metabolic profile of the ML showed a higher proportion of most amino acids, most sugars, organic acids associated with the Krebs cycle, and a higher proportion of most of the nitrogenous bases (Fig. 5.2 , Appendix Table S10). The metabolic profile of pine needles showed higher concentrations of most of the defence compounds such as phenols and terpenes, some amino acids, and other secondary metabolites (Fig. 5.2, Appendix Table S10).

Metabolomic responses of pine host to mistletoe

All of the metabolites detected in pine needles were found in both treatments (parasitized and unparasitized *P. nigra*) and seasons (summer and autumn), but with different concentrations and ratios. The PERMANOVA of the entire data set revealed significant differences in the overall metabolomic profile between parasitized and non–parasitized pines. Moreover, seasonality and the interaction between treatments (parasitized *vs.* non–parasitized pines) x season were also significant (Table 5.2).

Differences between UPN and PPN were displayed in the Component 2 of PLS–DA (Fig. 5.3). One–way ANOVAs show that mistletoe presence was related to a shift in the concentrations of a 26% of metabolites detected in *P. nigra* needles (518 out of 1991). Approximately half of these metabolites (239) presented higher concentrations in parasitized pines, while concentrations of the rest (279) was lower (see Fig. 5.4a for identified compounds). Therefore, the metabolic profiles of the PPN had higher concentrations of most amino acids, a higher proportion of the guanine nitrogenous bases, some sugars such as arabitol, and some secondary metabolites such as phenols (Fig. 5.4a). The UPN had higher concentrations of Vit. B5 (pantothenic acid) and some phenols (Fig. 5.4a).

Table 5.2: Effects of treatment (parasitized and unparasitized pines), and stratification level of canopy (upper, medium, and bottom third part), and season (summer and autumn) in a complete set of the metabolome of pine needles. Results come from the PERMANOVA model, including all metabolomic variables (1991 compounds). Bold type indicates significant effects (P<0.05).

Factors	Df	F–value	R^2	P–value
Treatment	1	4.62	0.031	<**0.001**
Stratification	2	0.45	0.006	0.596
Season	1	39.32	0.263	<**0.001**
Treatment×Stratification	2	0.18	0.003	0.979
Treatment×Season	1	4.03	0.027	**0.048**
Season×Stratification	2	0.25	0.003	0.892

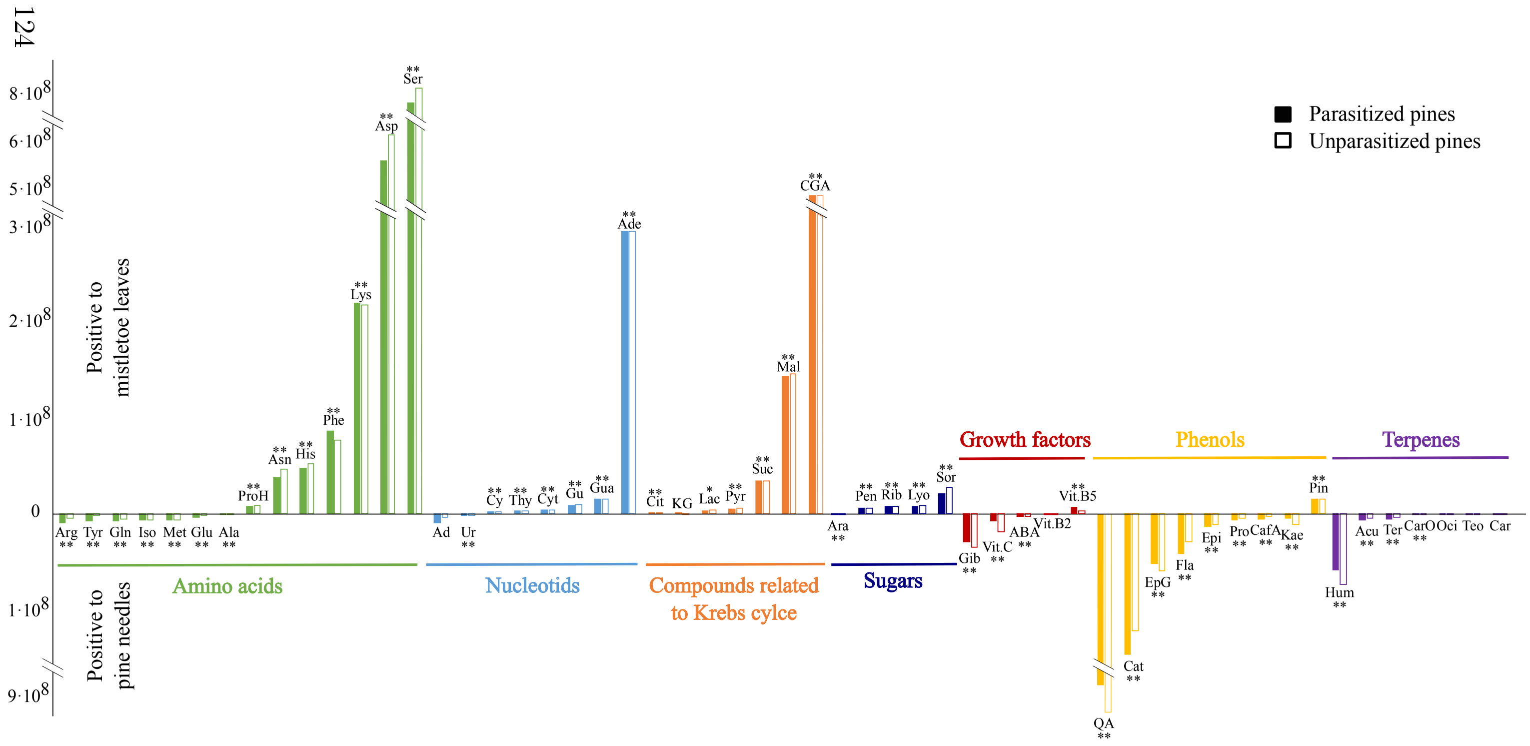

Figure 5.2: Differences of total concentration of parasitized (filled bars) and unparasitized pine needles (open bars) of all identified compounds with respect to mistletoe-–leaf concentrations. Bars above zero correspond to greater metabolite concentrations in mistletoe leaves, while bars under zero correspond to greater metabolite concentrations in pine needles. Asterisks indicate significant results from the one–way ANOVA ($P<0.05$ **; $P<0.1$ *). Different metabolomic families are coloured and described in the caption of Fig. 5.1.

Seasonality of the host–mistletoe system and the vertical within–canopy gradient

The concentrations of 38.3% (761 out of 1986) of the total detected metabolites in the mistletoe–leaf metabolome changed between seasons, while the overall metabolic profile of the pine needles showed a difference of 65.4% (1303 out of 1991). The differences in seasonality between parasite–host metabolome were displayed on component 1 of PLS–DA (Fig. 5.1, Table 5.1). For mistletoe leaves, the one–way ANOVA identified a trend in which the concentration of 276 metabolites (13.9% of the total detected metabolites) was higher during summer, including few amino acids, sugars and defence compounds. Conversely, another 485 compounds (24.4%), including most amino acids, some nucleotides, compounds associated with the Krebs cycle, and growth factors such as Vit. B5 and gibberellic acid, increased their concentrations in autumn.

On the other hand, the PERMANOVA of the entire data set of PPN and UPN also reflected a significant interaction between seasonality and parasitism status (Table 5.2, Fig. 5.3). The PLS–DAs of entire data clearly separated their component according with PERMANOVA results, where the Component 1 separated the cases by seasons (Fig. 5.3). The concentration of one third part of seasonally altered metabolites of pine needles (420 out of 1303) had higher concentrations in summer and two–thirds (883 of 1303) had higher values in autumn (see Fig. 5.4b and Appendix Table S10 for identified metabolites). Overall, pine needles in autumn had lower relative concentrations in some amino acids, nucleotides, and terpenes, but higher relative concentrations of other nucleotides, organic acids typically related to the Krebs cycle, sugars, phenols, terpenes, and growth factors such as abscisic acid (Fig. 5.4b).

In addition, the *post hoc* test from LMM, realized with score coordinates of the two first PLS–DA components, showed an interaction between treatment, season, and canopy strata (Table 5.3). This interaction showed that the metabolome from both parasitized and unparasitized pines was homogeneous within the pine canopy during summer, while the chemical profile of pine needles differed from the bottom to the upper part of pine canopy, especially in unparasitized pines in autumn (Table 5.3, Fig. 5.3).

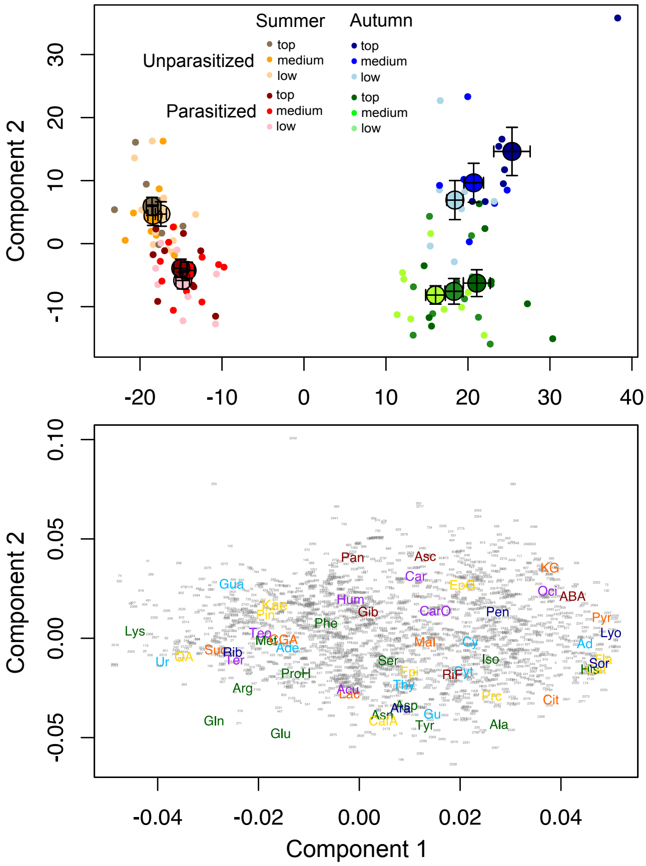

Figure 5.3: Component 1 *vs.* Component 2 of the partial least squares discriminant analysis (PLS–DA) of the changes of the metabolomes of pine–needle samples. Biplots of the two first components of the PLS–DA of metabolomic data presenting the scores (mean±S.E.) of the different *Pinus nigra* treatments (red and green, parasitized *P. nigra*; brown and blue, *P. nigra* uninfected) and different season (red and brown, summer; green and blue; autumn). The different intensity of the colour indicates the height (high intensity indicated the top, the medium indicated in medium, and the lowest indicated the bottom). Different metabolomic families are coloured and described in the caption of Fig. 5.1.

Discussion

This study provides for the first time an integral view of overall shifts in the metabolic profile caused by European mistletoe, *Viscum album* subsp. *austriacum*, on its main host black pine, *Pinus nigra* subsp. *salzmannii*, in a Mediterranean forest. Mistletoe parasitism has a systemic effect, making the pine host a more unitary rather than modular organism in space and time. Overall, by causing shifts in host metabolism, mistletoe is able to convert its host into a new plant metabolomic identity available in the forest canopy. In addition, our results strongly suggest that mistletoe acquires resources, derived from primary metabolism, directly from their host, and changes in the metabolic profile of parasitized pines closely fits the hemiparasite metabolome. This indicate that pine host works for mistletoe, constituting a sink of host–resources.

Table 5.3: *Post hoc* results from LMMs of two first components from PLSDA between parasitism status (PPN=parasitized pine needles, UPN=unparasitized pine needles), seasons (S=summer, A=autumn) and stratification level (T= the treetop, M= the middle third, L= the bottom third of the tree). Bold type indicates significant effects (P<0.05).

(a) LMM of Component 1

	PPN–SL	PPN–SM	PPN–ST	PPN–AL	PPN–AM	PPN–AT	UPN–SL	UPN–SM	UPN–ST	UPN–AL	UPN–AM
PPN–SM	1	–	–	–	–	–	–	–	–	–	–
PPN–ST	1	1	–	–	–	–	–	–	–	–	–
PPN–AL	<**0.001**	<**0.001**	<**0.001**	–	–	–	–	–	–	–	–
PPN–AM	<**0.001**	<**0.001**	<**0.001**	0.887	–	–	–	–	–	–	–
PPN–AT	<**0.001**	<**0.001**	<**0.001**	**0.021**	0.671	–	–	–	–	–	–
UPN–SL	0.808	0.604	0.878	<**0.001**	<**0.001**	<**0.001**	–	–	–	–	–
UPN–SM	0.433	0.260	0.523	<**0.001**	<**0.001**	<**0.001**	0.999	–	–	–	–
UPN–ST	0.392	0.231	0.479	<**0.001**	<**0.001**	<**0.001**	0.999	1	–	–	–
UPN–AL	<**0.001**	<**0.001**	<**0.001**	0.944	1	0.864	<**0.001**	<**0.001**	<**0.001**	–	–
UPN–AM	<**0.001**	<**0.001**	<**0.001**	0.289	0.948	1	<**0.001**	<**0.001**	<**0.001**	0.968	–
UPN–AT	<**0.001**	<**0.001**	<**0.001**	<**0.001**	**0.014**	0.310	<**0.001**	<**0.001**	<**0.001**	**0.003**	0.210

(b) LMM of Component 2

	PPN–SL	PPN–SM	PPN–ST	PPN–AL	PPN–AM	PPN–AT	UPN–SL	UPN–SM	UPN–ST	UPN–AL	UPN–AM
PPN–SM	0.992	–	–	–	–	–	–	–	–	–	–
PPN–ST	0.965	1	–	–	–	–	–	–	–	–	–
PPN–AL	0.885	0.207	0.121	–	–	–	–	–	–	–	–
PPN–AM	0.985	0.440	0.294	1	–	–	–	–	–	–	–
PPN–AT	1	0.951	0.872	0.971	0.999	–	–	–	–	–	–
UPN–SL	**0.031**	0.098	0.123	**0.005**	**0.008**	**0.023**	–	–	–	–	–
UPN–SM	**0.036**	0.111	0.139	**0.006**	**0.010**	**0.026**	1	–	–	–	–
UPN–ST	**0.012**	**0.041**	**0.052**	**0.002**	**0.003**	**0.009**	0.999	0.997	–	–	–
UPN–AL	**0.010**	**0.031**	**0.039**	**0.002**	**0.003**	**0.007**	0.981	0.963	1	–	–
UPN–AM	**0.003**	**0.009**	**0.011**	<**0.001**	<**0.001**	**0.002**	0.446	0.374	0.894	0.994	–
UPN–AT	<**0.001**	<**0.001**	<**0.001**	<**0.001**	<**0.001**	<**0.001**	<**0.001**	<**0.001**	<**0.001**	**0.003**	0.114

Metabolomic profile differences between the pine host and its hemiparasite

Previous studies have shown that the concentration of functional chemical groups (basically defence compounds) in the European mistletoe leaves and black pine needles sharply differ (Lázaro-González *et al.*, 2019b). According to this, our study shows that these differences are evident not only at the level of chemical groups, but also at the finest metabolic level. Overall, the main metabolome differences between mistletoe leaves and pine needles, concern a high concentration of amino acids, nucleotides, compounds related to Krebs cycle, and sugars, and therefore involve a higher up–regulation of primary metabolism (Fig. 5.2). All these changes suggest, on the one hand, that the hemiparasite requires high amounts of metabolic resources to invest in their development in comparison with their plant host. Interestingly, mistletoe acquires at least a part of these resources (Stewart & Press, 1990; Pate *et al.*, 1991b), derived from primary metabolism, directly from their host, which accumulates extra amounts of these compounds, benefitting mistletoe rather than itself. In addition, mistletoes have high transpiration rates and low hydric potential (Schulze *et al.*, 1984; Schulze & Ehleringer, 1984; Ehleringer *et al.*, 1985), guaranteeing the unidirectional flow from host to hemiparasite plant, especially for carbohydrates and amino acids (Glatzel & Geils, 2009; Lamont, 1983; López-Sáez *et al.*, 2002). Our results reinforce the idea that the mistletoe–host relationship is a one–way flow system—an asymmetrical relationship where the pine host is forced to work for mistletoe becoming an irreversible sink of resources and water, this being consistent with results of previous non–ecometabolomic studies (López-Sáez *et al.*, 2002; Glatzel & Geils, 2009; Schulze *et al.*, 2019).

On the other hand, our results show that mistletoe has a weak secondary metabolism relative to the pine host, where the concentration of mostly secondary metabolites, especially defence compounds (e.g. flavonoids, tannins, and terpenes), are practically absent in mistletoe (Fig. 5.2, Appendix Table S10, Lázaro-González *et al.*, 2019b). According to Lázaro-González *et al.* (2019b), these results reinforce the idea that *V. album* does not benefit from the pine host by acquiring antiherbivory properties. However, the higher concentrations of free amino acids in mistletoe coming from plant host are consistent with the higher concentrations of N–rich molecular herbivore deterrent substances in this parasite. These amino acids correspond to a series of compounds necessary to synthesise the sequence of their own toxic proteins for animal cells, such as viscotoxin (Samuelsson & Pettersson, 1971; Olson & Samuelsson, 1972; Samuelsson, 1973) and lectins (Soler *et al.*, 1996, 1998). Thus, by generating a net flux of primary metabolites from the host related mainly to a source of matter (C and nutrients) an energy, but not to secondary plant compounds, mistletoe lead its efforts to synthetize their own anti–herbivore defences rather than anti–abiotic stress.

Metabolomic responses of pine host to mistletoe

Mistletoe can modify the metabolic profile of their pine host by altering the concentration of a quarter of their metabolome (26% of the metabolites analysed). Overall, parasitized pines increase the concentration of most of the metabolites intercepted by the mistletoe such as amino acids, nucleobases, compounds related to the Krebs cycle, and carbohydrates, while decreasing the concentration of secondary metabolites such as vitamins and certain phenols (Fig. 5.4a, Appendix Table S10). Therefore, as a consequence of mistletoe requirements and their inability to take up essential resources from soil, parasitized pines respond enhancing the concentrations of metabolites especially rich in nitrogen.

In addition, the pine reaction against mistletoe shows common responses to other biotic stressors such as the specialist and more abundant pine–feeding herbivore the pine processionary moth (PPM), which also induces greater concentrations of amino acids, compounds related to Krebs cycle, and carbohydrates (Rivas-Ubach *et al.*, 2016a,b). Besides, PPM generate oxidative stress on pine Rivas-Ubach *et al.* (2016a,b), a response commonly induced by folivory in attacked plants (Bi & Felton, 1995). Our results suggest that mistletoe parasitism also provokes oxidative stress, since parasitized pines raise the concentration of some phenols such as flavonoids with antioxidant properties (Fig. 5.4, Appendix Table S10). Despite the similarities of pine responses to PPM attack and mistletoe parasitism, the folivory of PPM causes a slighter effect, with only 12.9% of host metabolome altered (Rivas-Ubach *et al.*, 2016b). Meanwhile, mistletoe parasitism has a greater impact on the host pine, modifying 26% of the pine metabolome due presumably to the chronic parasitism and intimate host–parasite relationship.

Parasitized pines also increase the relative concentration of the aucubin (Fig. 5.4a), an iridoid glycoside known as a secondary defence compound against generalist insect herbivory (Bowers & Puttick, 1988; Nieminen *et al.*, 2003), but it also attracts specialist lepidopteran oviposition and feeding (Nieminen *et al.*, 2003; Harvey *et al.*, 2005; Peñuelas *et al.*, 2006).

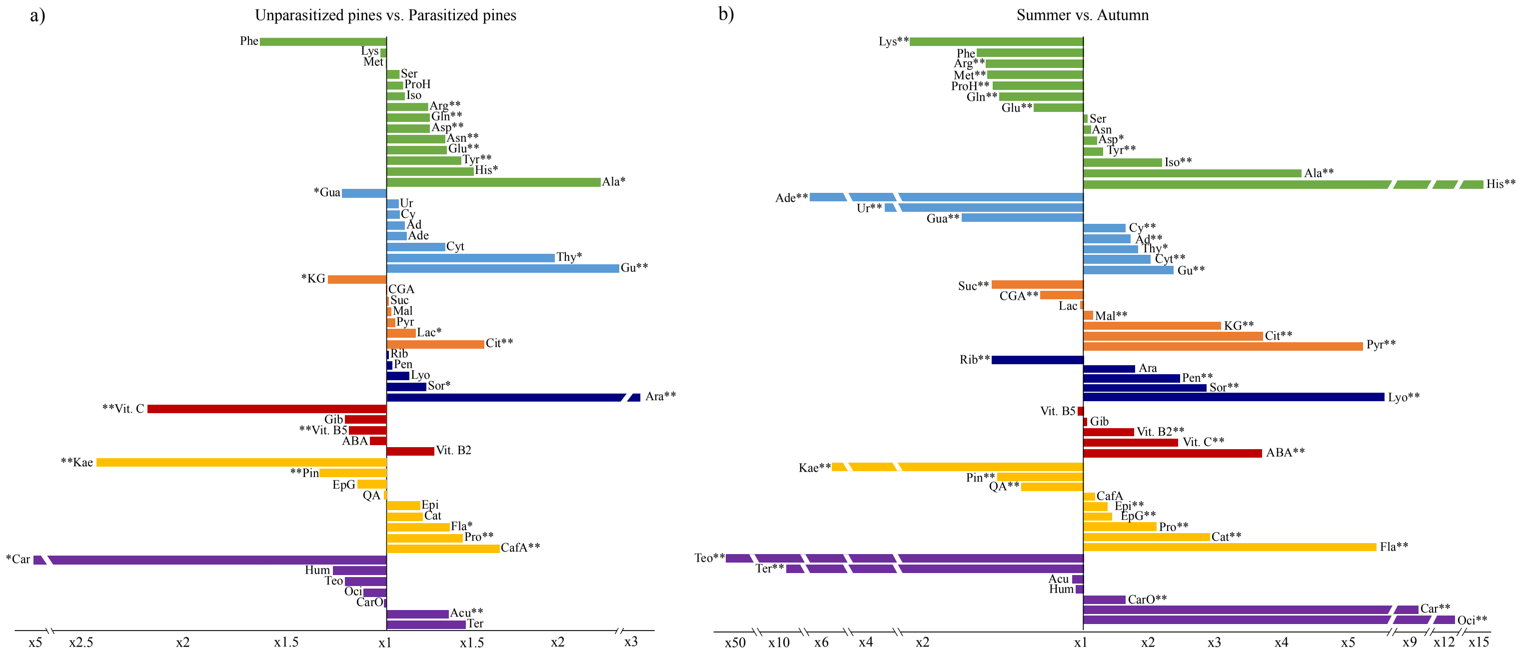

Figure 5.4: Differences between a) treatments (parasitized and unparasitized pines) and b) season (summer and autumn) of identified compounds. Asterisks indicated significant results from one–way ANOVA (P<0.05 **; P<0.01 *). Different metabolomic families are colored and described on caption of Fig. 5.1.

Therefore, parasitized pines could attract the oviposition of the main pine–feeding specialist, the PPM, and at the same time, a low–quality food for caterpillar (Lázaro-González *et al.*, 2019a). As a consequence, pine woodland with mistletoe presence would make pine processionary outbreaks less prevalent. Further studies are needed to assess whether PPM, or other specialist lepidopteran, lead their oviposition to parasitized pines, and whether hatched larvae are able to sequester any defensive compound of pine host for their own defence (Bowers & Collinge, 1992).

Seasonality of the host–mistletoe system and the vertical within–canopy gradient

Mistletoe leaves and pine needles undergo metabolomic changes from summer to autumn, although mistletoe leaves are more stable, showing less seasonal variance in their metabolome (38% of metabolites change their concentration) than pines (65.4%). This indicates that *V. album* functionality (metabolome) depends less on the environment than on the functionality (metabolome) of the host, as expected from the hemiparasite habit. Thus, both mistletoe–leaf and pine–needle metabolomes increase their concentration of amino acids associated with chlorophyll synthesis and nutrient assimilation (such as lysine, arginine, etc.) in summer, while other amino acids, nucleotides, compounds associated with the Krebs cycle, and vitamins increase in autumn (Appendix Table S10). This suggests than the host–parasite system, as well as mistletoe–free pines, begin to accumulate metabolites for the growth period several months before the resources are needed, showing similar responses to seasonality.

At the canopy scale, the pine needles respond permanently to mistletoe parasitism over the year (Fig. 5.3). New needles sprout with a common metabolic profile and shift to a vertical within–canopy gradient in autumn, with changes being more intense from the crown to the bottom part of canopy (Fig. 5.3). However, the vertical gradient in parasitized pines is less accentuated than in unparasitized ones, and therefore mistletoe parasitism makes the metabolome of parasitized pine needles more homogeneous by softening the stratification during autumn. This suggests that metabolic responses of pines are systemic at the canopy scale, turning a modular pine tree into an organism with unitary responses. Thus, mistletoe is an agent of systemic changes (see also Cocoletzi *et al.*, 2016), able to generate a new plant metabolic identity in the host pine with respect to mistletoe–free pines, prompting ecological consequences. Notably, this systemic reaction appears to be mistletoe–specific in *Pinus nigra*, given that pines attacked by other biotic stressors such as PPM react to folivory more at local level rather than at the systemic level (Rivas-Ubach *et al.*, 2016a,b).

The ecological consequences of mistletoe parasitism

Mistletoe has simultaneously a permanent and systemic effect on metabolic profile of pine hosts needles. On the one hand, mistletoe parasitism causes damage in the form of permanent oxidative stress and more solubilisation of compounds rich in N over the year. On the other hand, the pine host has a systemic reaction, which prevents minimizing the effects of parasitism by discarding a part of their canopy and acting as a more unitary rather than modular organism. As a result, the pine–feeding herbivores cannot find safe sites in different parts of pine canopy free of the mistletoe impact, triggering tritrophic mediated indirect interactions. For instance, changes in chemical profile in response to mistletoe have direct detrimental effects, including death, on many pine–feedings herbivores such as the PPM (Lázaro-González *et al.*, 2019a), one of the most severe and widespread pests in the Mediterranean forests (Hódar *et al.*, 2002, 2003). Thus, mistletoe generates non–trophic links with pine–feeding herbivores, where the systemic reaction of pine host has indirect effects on arthropod herbivores via changes in the host quality as food (Lázaro-González *et al.*, 2019a,b). Therefore, mistletoe parasitism indirectly benefits the pine host by reducing herbivore pressure (Lázaro-González *et al.*, 2019a), making the host–mistletoe system a mutualistic more than parasitic relationship.

In conclusion, by exerting a press disturbance, mistletoes cause a permanent and long–lasting systemic effect, making the pine host a more unitary rather than modular organism in space and time. By causing shifts throughout the host metabolism, mistletoe is able to generate a new metabolomic identity in the host, available on the forest canopy, which increases the complexity and heterogeneity of the forest canopy. This in turn triggers an ecological cascade of consequences, causing detrimental changes in pine herbivores (Lázaro-González *et al.*, 2019a). Nevertheless, the new identity could mean a novel niche and new opportunities for tolerant and adapted herbivores, promoting the local and regional forest biodiversity at ecosystem level, which can have valuable implications for conservation and management of pine forests.

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Part III

Indirect effects caused by mistletoes

Chapter 6

Mistletoe generates non–trophic and trait–mediated indirect interactions through a shared host of herbivore consumers

LÁZARO–GONZÁLEZ A, HÓDAR JA & ZAMORA R (2019)
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Abstract

Indirect interactions emerge among a wide range of herbivores sharing the same plant resource. Consumers usually belong to different trophic guilds, from folivores and sapsuckers to parasitic plants. We propose that mistletoes parasitizing pines could play a key role acting as herbivores on host pines and coming indirectly into competition with other herbivores feeding on the same host. Changes caused by mistletoes on its host have been well studied, but its effects running across trophic webs remain unrevealed. In this study, we investigate the effect of European mistletoe (*Viscum album* subsp. *austriacum*) on the host–feeding herbivores via trait–mediated indirect interactions (TMIIs) across their shared pine host (*Pinus nigra* subsp. *salzmannii*). We performed field and laboratory experiments, and analyzed the net effect of different mistletoe parasite loads on three host–phytophagous species: the sapsucker *Cinara pini* (Aphididae), the winter folivore *Thaumetopoea pityocampa* (Thaumetopoeidae), and the summer folivore *Brachyderes* sp. (Curculionidae), all being members of different functional feeding groups (FFGs). We summarize the mistletoe–host–herbivore interactions by means of a TMII, where mistletoe parasitism causes non–trophic links and detrimental indirect interactions on pine–feeding herbivores across its shared host, suggesting a worsening of host quality as food. These indirect interactions vary according to three parameters. First, the intensity has a non–proportional relation with parasite load, showing an impact threshold on highly parasitized pines. Second, the movement capacity of insect herbivores determines their response, by decreasing the abundance of herbivores with low movement ability (aphids and pine processionary caterpillars) while altering the behavior (plant selection) of more mobile herbivores (pine weevils). Finally, FFG determines the intensity of mistletoe parasitism effects, folivores being more responsive than sapsuckers. Overall, mistletoe generates non–trophic interaction linkages in the forest able to modify community structure by becoming a nexus of the entire herbivore community of the pine canopy.

Key words – aphids; folivores; hemiparasite; host pine; insect herbivores; Mediterranean forest; pine processionary; pine weevil; plant–animal interaction; sapsuckers; *Viscum album*

Introduction

Plants are simultaneously consumed by organisms as diverse such as insects, vertebrates, fungi, nematodes or parasitic plants. The resulting interactions between phylogenetically disparate organisms sharing a common resource appear to be a common phenomenon, but little known (Hochberg & Lawton, 1990; Wardle *et al.*, 2004; Bass *et al.*, 2010). In fact, studies on interactions between invertebrate consumers of the same plant have documented both competitive and positive interactions (Denno *et al.*, 1995; Ohgushi, 2005), and the consequences of the participating species for the population dynamics have been examined (Denno *et al.*, 2000; Ohgushi, 2005). These consumers induce plant responses (Karban & Baldwin, 1997) and generate interaction linkages caused by non–trophic indirect effects, able to modify the food–web structure (Ohgushi, 2005, 2008). Indirect effects can result from changes in the density of a species involved in a food web, triggering interactions known as density–mediated indirect interactions (DMIIs) (Peacor & Werner, 1997; Werner & Peacor, 2003) or, alternatively, changes in the phenotypes of any species (morphological, physiological, phenological, and behavioral) involved in a trophic web, thus causing the trait–mediated indirect interactions (TMIIs) (Werner & Peacor, 2003; Schmitz *et al.*, 2004; Gómez & González-Megías, 2007). Despite recent advances in the knowledge of indirect interactions, there are hardly any studies on the magnitude and ecological importance of the interactions between parasitic plants as initiators and insects as receptors, that use the same plant as the intermediary, and in turn as a trophic resource (Hartley & Gange 2009; but see Bass *et al.* 2010 and Ewald *et al.* 2011)

The direct impact of parasitic plants on their hosts, unlike that of herbivores, is often disproportionately strong in relation to their abundance (Watson, 2001, 2009; Hartley *et al.*, 2015) and able to restructure the plant (Pennings & Callaway, 1996; Davies *et al.*, 1997; Hódar *et al.*, 2018) and invertebrate community (Stevens *et al.*, 1970; Hartley *et al.*, 2015). Parasitic plants are also the trigger for a series of indirect interactions with many other species in the community located at different trophic levels. Parasitic plants can compete with other species that consume the host plant, such as herbivores, which represent a potentially limiting shared resource (Gómez, 1994; Puustinen & Mutikainen, 2001). Additionally, as parasitic plants can change the morphology and architecture of their host, they can also affect other species that use the host as a habitat (Mooney *et al.*, 2006). This is the case of mistletoes, hemiparasitic plants able to play a prominent role in the forest canopy acting as an herbivore (Ehleringer *et al.*, 1985; Pennings & Callaway, 2002; Zuber, 2004), taking up minerals and nutrients through the haustorium embedded in the host xylem (Kuijt, 1977; Zuber, 2004). All mistletoe–induced changes on host species could affect other host consumers in several ways, running across the trophic web to primary (herbivores) and secondary consumers (predators and insectivorous birds). Therefore, mistletoe parasitism could be the starting point for a novel case of TMIIs, where the initiator of these indirect interaction is a plant, resembling an herbivore but with effects disproportionately strong with respect to their size (Hartley *et al.*, 2015; Watson, 2001). These indirect interactions link mistletoes with host–feeding herbivores mediated by changes in traits of its shared plant–host.

In this study, we aim to investigate the indirect effects of the European mistletoe (*Viscum album* subsp. *austriacum*) on population responses of herbivores, through mistletoe–induced changes on their shared black pine host (*Pinus nigra* subsp. *salzmannii*). Using field and laboratory manipulation experiments, we tested the hypothesis that the presence of mistletoe would influence a range of insect herbivores and that this impact would be proportional to mistletoe abundance. We focused the study site in a Mediterranean mountain (Natural Park of Sierra de Baza) in southeastern Spain, where *V. album* parasitizes mainly *P. nigra*. Our study system represents a particular case because, while many plant–parasite interactions have been studied in short–lived organisms (Bass *et al.*, 2010; Ewald *et al.*, 2011), here we work with two long–lived plants (>300 years for *P. nigra*, until 40 years for mistletoe). Furthermore, pines parasitized by mistletoe show a high rate of re–infestation (Mellado & Zamora, 2016), which increases the host parasite load, leading eventually to death (Mellado & Zamora, 2017). Thus, while herbivory is generally episodic, increasing in intensity over short time periods (outbreaks of defoliators), mistletoe constitutes a chronic, long–term stressor, sequestering water and macronutrients continuously from the host (Ehleringer *et al.*, 1985; Schulze *et al.*, 1984). In a previous study, we show how the pine host responds to different mistletoe parasite loads by altering the chemical profile of pine needles, decreasing their quality as food and synthesizing defense compounds in moderate and highly parasitized pines (Lázaro-González *et al.*, 2019b). Thus, from the standpoint of a pine–eating arthropod, there are three potential food sources: unparasitized pines, parasitized pines, and the mistletoe itself. We know now that the last possibility is not an option, because the arthropods feeding on mistletoe are narrow specialists (Lázaro-González *et al.*, 2017).

Herbivore insects are more likely to respond quickly to mistletoe parasitism in relation to changes in the host, due to their short life cycles and sensitivity to food quality throughout the first instars (Hódar *et al.*, 2002; Zalucki *et al.*, 2002). In addition, considering that mistletoes could affect not only pine needles (Lázaro-González *et al.*, 2019b), but different pines tissues (e.g. phloem), we used three phytophagous species as a case study, according to different functional feeding groups (FFG): the summer sap–sucking aphid *Cinara pini* (Hemiptera: Aphididae), the winter folivore pine processionary moth *Thaumetopoea pityocampa* (Lepidoptera: Thaumetopoeidae), and the summer folivore beetle *Brachyderes* sp. (Coleoptera: Curculionidae). These three target species have a high impact as phloem and needle consumers, respectively, being common in the pine forests of our study site (Lázaro-González et al., *unpublished data*).

To elucidate the TMIIs initiated by mistletoes, firstly we explore how a gradient of mistletoe parasitism affects the presence and abundance of the three target species on wild populations on its shared host. Because prior evidence of mistletoe–induced chemical changes in medium and high degrees of parasitism in pines (Lázaro-González *et al.*, 2019b), we expect to find lower populations at higher levels of mistletoe loads, either by site selection (actively choosing the preferred tissue) or by differences in survival or reproduction. Secondly, we test how the parasite load determines the population responses under field and laboratory experimental conditions. We expect stronger responses on insect populations fed on pines undergoing high rather than low parasitism, according to the severity of the chemical changes (Lázaro-González *et al.*, 2019b). Finally, we asked how the functional feeding group (sap–sucking or folivore) determines the indirect effects according to gradient of mistletoe parasitism. Because pine tissues, (e.g. phloem and needles), could be differently affected by mistletoe parasitism, responses of our focal herbivore species could also differ between functional feeding groups, since they are sapsuckers and folivores, respectively.

Materials and Methods

Study site

Mistletoe, *Viscum album* subsp. *austriacum* (Wiesb. Vollman), is a dioecious parasitic plant native to the European region that specializes in parasitizing conifers, mainly *Pinus* species, across its distribution range (Zuber, 2004). This study was conducted in the Natural Park of Sierra de Baza, Granada (south–eastern Spain, 2° 51’ 48” W, 37° 22’ 57” N), representing the southernmost limit of its geographical distribution. The climate at the site is typically Mediterranean, with historic annual mean temperature of 15.5°C (CMAOT, 2015) and annual mean rainfall of 495±33 mm (1991–2006 period; Cortijo Narváez meteorological station, 1360 m asl) concentrated in autumn and spring, hot and dry summers (June–September) and cold winters (December–March). Conifers are the dominant forest vegetation (43% of the total surface coverage), where black pine (*Pinus nigra* Arn. spp. *salzmannii*) is the most main species that hosts mistletoe. The site has other pine species, such as Scots (*P. sylvestris* L.), Aleppo (*P. halepensis* Mill.), and maritime (*P. pinaster* Ait.) pine, as well as oaks (*Quercus ilex* L., 9%) and shrublands (23%) (CMAOT, 2008).

To characterize the wild populations of the target species, we selected 55 unparasitized black pines and 55 parasitized ones, according to these mistletoe loads: control (C), trees free of mistletoe; low (L), trees < 20% of canopy occupied by mistletoe; medium (M), trees 20–50% of canopy occupy by mistletoe; and high (H), trees > 50% of canopy occupy by mistletoe. Sample sizes for control, low, medium, and high were 55, 17, 27, and 11, respectively. Trees were selected along an elevational gradient (from 1300 to 1850 m asl) on the Natural Park of Sierra de Baza, considering within–forest heterogeneity such as areas with different tree densities. Trees were randomly selected and spatially paired (one parasitized and one unparasitized). Paired trees were of similar architecture, size (diameter at breast height: 25.55±1.31 cm), age (90–110 yr. old; see Herrero *et al.* 2013) and height (6.87±0.23 m). These were located 40 to 80 m apart to control the environmental variability (e.g. climatic factors and composition of neighboring vegetation).

The experimental study was performed at Cortijo Casimiro (Natural Park of Sierra de Baza, 1400 m asl), in a coetaneous afforestation of *P. nigra*, where trees bear various mistletoe loads. Two field bioassays were conducted in situ while two laboratory bioassays were performed with pine needles collected at the site. We selected 40 pines according to the aforementioned four levels of parasite loads (control, low, medium, and high; 10 trees each).

Sampling of wild populations of target species

Brachyderes sp. and *Cinara pini* were sampled on July 2013 by the beating technique, in which a heavy stick was used for 10 sec to beat a branch on three randomly chosen branches per pine. All non–sessile and non–flying arthropods inhabiting pine branches, including these two species, dropped onto a cloth collector (1 m^2) under the pine branches. All curculionids and aphids were sorted, identified and counted. Additionally, we examined all pines present on a large marked plot ($\sim$ 2 Ha) located at 1650 m asl during spring 2009. The plot included 230 black pines, parasitized and unparasitized ones, where the parasite load of each pine was recorded. At this area, the population of *Thaumetopoea pityocampa* was estimated by counting the number of winter nests per tree. Since the larvae are gregarious from hatching and congregate in a silk cocoon (hereafter nest) and develop during winter, the beating technique is not a reliable method for this species, while counting winter nests is a widely used procedure in most countries in which this pest is present (Battisti *et al.*, 2015).

All data were tested in two different ways: on the one hand, we used a binomial data of presence/absence; and the other hand, we tested their abundance. Due to lack of our target species in many samples, we used a negative binomial error distribution for abundance data. For *Brachyderes* sp. and *Cinara pini* data, we used a Generalized Linear Mixed Model (GLMM) where mistletoe loads (C, L, M and H) was a fixed factor, and elevation (from 1300 to 1850 m asl) and paired trees (from 1 to 55) were random spatial factors. *Thaumetopoea pityocampa* data were run with a Generalized Linear Model (GLM) where mistletoe load was a fixed factor. The models were run by *glm*, *glm.nb*, *glmer* and *glmer.nb* functions of the *stats*, *MASS* and *lmer4*, packages, respectively. Finally, Tukey's pairwise comparisons (95% confidence level) were conducted using *lsmeans* function of *lsmeans* package. R software system Version 3.4.3 (R development Core Team, 2018) were used to all statistical analyses of this work. Throughout the paper, means are expressed as ± 1SE.

Field bioassay with *Cinara pini*

Bioassay was conducted in situ from early July to mid–September 2012. Firstly, we selected four healthy pine branches of each focal pine, and wrapped the growth of the last two years with lycra bags (totaling 160 bags, 40 for each level of mistletoe load). Afterwards, the bags were sprayed with pyrethroid insecticide (Coopermatic®: pure natural pyrethrins (1.67%) and piperonyl butoxide (11.10%)) and closed. The insecticide was left to work for two weeks, to kill any arthropod inside the bags. Once the activity of the insecticide totally decayed, we set two parthenogenetic females of *Cinara pini* (Hemiptera: Aphididae) collected on healthy, mistletoe–free black pines nearby. All bags were checked biweekly, without introduction of new aphids. In mid–September we collected all bags and transported them to the laboratory, where all aphids per bag were counted under a magnifying glass.

Just as above, we used data to test the survival and abundance per bag, considering survival to be two or more live aphids inside a bag, and abundance the number of aphids by bag. We used a GLMM with binomial distribution for presence data, while due to high mortality, we used a GLMM with negative binomial error distribution to test the abundance. In both cases, mistletoe load (C, L, M and H) was a fixed factor and individual tree (1–40) was random factor.

To test for a microclimatic effect of the bags, we set three pairs (A, B and C) of data logger (HOBO UTBI–001, TidbiT®v2 Temp Logger, Onset Comp. Corp., Bourne, MA, USA), inside and outside bags on randomly selected pines. Data loggers recorded the temperature every 30 min throughout the bioassay (72 days). We analyzed four different variables of temperature: mean, minimum, maximum, and coefficient of variation. Linear mixed models (LMMs) were used to test bag effects on these four variables. Bag position (inside and outside) were fixed factor, time (days) was a temporal random factor, and pair (A, B and C) and data logger (1 to 6) were spatially hierarchical random factors. Coefficient of variation was calculated by standard deviation/mean temperature of daily temperature, and log transformed to meet all model assumptions. The results show that bagging did not affect the microclimatic conditions on pine twigs. Although bagging marginally increased the maximum ($F_{1,2}$=11.35, P=0.078), mean ($F_{1,2}$=8.05, P=0.105), minimum ($F_{1,2}$=2.49, P=0.342) temperatures, and coefficient of variation ($F_{1,2}$=7.03, P=0.118), the temperature inside and outside the bags did not differ (Appendix Fig. S6). Thus, any effect on survival and abundance of aphids during field bioassay can be ruled out.

Field bioassay with *Thaumetopoea pityocampa*

Egg batches of pine processionary moth Thaumetopoea pityocampa were collected in a nearby (15 km apart) pine forest with similar moth phenology than Cortijo Casimiro (Hernán Valle, Granada, 3°03'W, 37°23'N, 1130 m asl) during August 2013. We fixed five egg batches on the pine needles of different apical branches of the 40 focal pines (see above). Egg batches depredated by birds or bush crickets (Hódar *et al.*, 2013) were replaced until egg batches hatched. After the larvae hatched we labelled the nest and removed the egg batch. In the laboratory, we analyzed the empty egg batch to record the number of larvae hatched. Nests in the field were monitored weekly, and specimens were collected once they moulted to the second instar. In laboratory, we counted the number of larvae that reached the second instar.

Unhatched egg batches were removed from statistical analyses. We considered survival as all nests with at least one 2^{nd} instar pine processionary caterpillar, and abundance as the proportion of larvae per nest and the number of eggs that hatched per batch (live vs. hatched caterpillars). Survival was tested by running a GLMM assuming a binomial distribution data, where mistletoe load (C, L, M and H) was a fixed factor and individual trees (1–40) constituted the random factor. Due to high caterpillars mortality, we tested differences in abundance using a GLMM with negative binomial error distribution and the same model structure than survival.

Laboratory bioassay with *Thaumetopoea pityocampa*

To complement the previous bioassay under controlled conditions, we performed a laboratory bioassay with pine processionary caterpillars. In August 2012, we collected 25 egg batches in a pine forest on Cortijo Quemado, Lanjarón (Granada, 3° 29' 41" W, 36° 56' 39" N, 1300 m asl). These egg batches were placed inside an incubator at laboratory, under controlled conditions of temperature (15°C–22°C) and humidity (40%), until eggs were hatched. Newly emerged caterpillars were placed in groups of 20 individuals in Petri dishes, with a moist base filter paper (see Hódar *et al.* 2002 for a similar procedure). A total of 2960 caterpillars hatched, and 148 Petri dishes were completed (ten focal pines x four mistletoe loads x three replicates, and another incomplete replicate of seven focal pines x four mistletoe loads). Each group of caterpillars was fed on daily with fresh needles of the same focal pine, until larvae had molted to the 2^{nd} instar, as in the previous field bioassay.

When bioassay ended, we considered survival as Petri dishes with two or more live larvae, and abundance as the number of larvae on 2^{nd} instar by Petri dish. Once again, we tested survival and abundance separately running a GLMM with binomial and negative binomial error distribution, due to high mortality, respectively. Mistletoe load (C, L, M and H) was fixed factor, and individual tree (1–40) and reply (1–4) were random factor.

Cafeteria test with *Brachyderes* sp.

In August 2013, we collected 40 individuals of *Brachyderes* sp. (O. Coleoptera, Fam. Curculionidae) in a pine forest on Lanjarón (Granada, 3°30'W, 36°57'N, 1350 m asl). All beetles were transported to the laboratory and placed in a plastic box inside the incubator during 24 h, under controlled condition of temperature and humidity (see above), and healthy mistletoe–free black pine needles and water *ad libitum*, to establish the same conditions in all individuals. Then, beetles were sorted into ten containers with four individuals each, and left 24 h without food. Afterwards, we placed fresh needles from four focal pines (one pine per mistletoe load: C, L, M and H) in each container. Needles were measured before the trial (length, in mm), ensuring a similar total needle length between mistletoe loads and between containers. Pine weevils were left to feed for 24 h. Afterwards, we measured the needles again, and recorded the difference between initial and final length per mistletoe loads as consumption by beetles. The trials were replicated three times, randomly sorting the beetles and focal trees per container for each replicate, and the beetles were placed together at the same container between trials with healthy mistletoe–free black pine needles and water provided ad libitum for 24 h.

To test the effect of the mistletoe load (C, L, M and H) on feeding preferences, we considered two different variables: nibbled needles (yes–no) and length of consumed pine needles (mm). Both variables were tested using GLMM, with binomial and negative binomial error distribution, where mistletoe load was a fixed factor, and focal pines (1–40), containers (1–10) and reply (1–3) were random factors.

Results

Target species on wild population

A total of 550 aphids and 97 curculionids were found on 79 and 50 pines, respectively, from 110 total sampled pines, during beating sampling, while 220 pine processionary nests were counted on 121 from 230 total pines trees. Presence (χ^2=1.90, df=3, P=0.593; Fig. 6.1a) and abundance (χ^2=2.16, df=3, P=0.539; Fig. 6.1b) of sap–sucking *Cinara pini* in the wild population showed no significant differences between mistletoe loads (Appendix Table S11a). Conversely, folivores are affected by mistletoe parasitism, decreasing their presence (*Thaumetopoea pityocampa*, χ^2=6.91, df=3, P=0.075; *Brachyderes* sp., χ^2=10.17, df=3, P=0.017; Fig. 6.1c–e) and abundance (*Thaumetopoea pityocampa*, χ^2=8.46, df=3, P=0.038, and *Brachyderes* sp., χ^2=12.48, df=3, P=0.006; Fig. 6.1d–f) on pines parasitized by high and medium–high parasite loads of mistletoes, respectively (Appendix Table S11b–c).

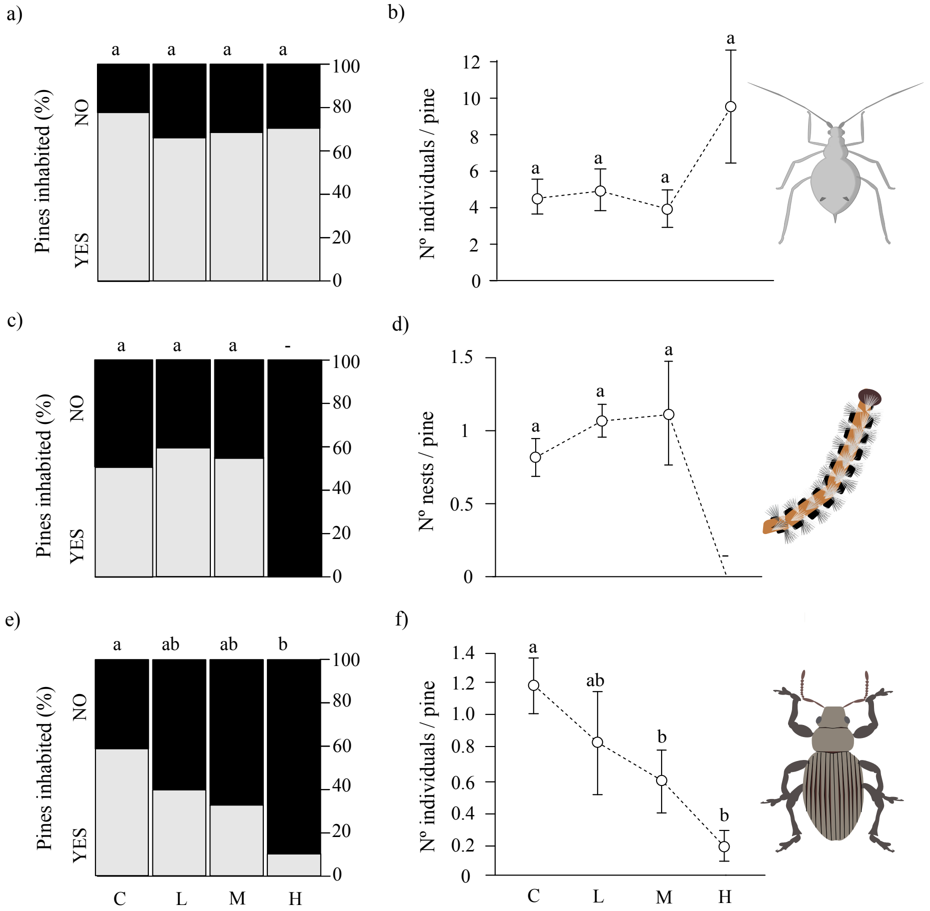

Figure 6.1: Pine–feeding herbivores in natural conditions: presence/absence (left column, grey and black bars, respectively) and abundance (right column, mean ± SE) of a–b) aphids (n=110), c–d) nests of pine processionary moth (n=230) and e–f) pine weevils (n=110) per pine according to the mistletoe loads on the pine host (C=control, L=low, M=medium and H=high). Differences among parasitism levels are indicated by different lower–case letters.

Field bioassay with *Cinara pini*

A total of 123 bags out of from 157 contained at least two live aphids at the end of the bioassay, totaling 1290 aphids. Multiple comparisons did not reveal any difference on survival of aphids between levels of mistletoe loads (χ^2=4.76, df=3, P=0.190; Table C1, Fig. 6.2a), but their abundance decreased significantly from unparasitized pines to those with high parasite loads (χ^2=14.98, df=3, P=0.002; Fig. 6.2b), with the differences between these two levels of mistletoe loads being significant (C: 15.4±3.7 individuals, H: 1.9±0.3 individuals), and marginal between pines of low (L: 10.3±3.0 individuals) and high parasitism (Appendix Table S12a).

Field bioassay with *Thaumetopoea pityocampa*

A total of 127 hatched egg batches were analyzed; from these, 78 bore at least one 2^{nd} instar larvae, for a total of 4614 larvae molted to 2^{nd} instar. Survival per nest was not significantly affected by mistletoe load (χ^2=3.11, df=3, P=0.374; Appendix Table S12a, Fig. 6.2c), but there was a slight trend in mistletoe–free pines to show a higher rate of nest survival (C: 74%, n=34) than in parasitized pines, regardless of their parasite load (L: 59%, n=32; M: 59%, n=29, H: 53%, n=32). However, the pattern was much clearer when considering abundance of caterpillars inside survivor nests (χ^2=91.75, df=3, P<0.001; Appendix Table S12a, Fig. 6.2d), reflecting a significant and progressive decline from unparasitized (44.2±6.4 individuals per nest) and low parasitized pines (43.3±7.1 individuals per nest), to those with to medium (39.9±6.9 individuals per nest) followed by high parasitism (30.5±6.5 individuals per nest).

Laboratory bioassay with *Thaumetopoea pityocampa*

In contrast to field bioassay, the results in laboratory bioassay showed clear–cut differences on survival (χ^2=8.99, df=3, P=0.029; Fig. 6.2e) and abundance (χ^2=15.62, df=3, P=0.001; Fig. 6.2f) of caterpillars between unparasitized pines and parasitized ones (Appendix Table S12b). Thus, survival and abundance on Petri dishes of caterpillars fed on pine needles of control (C: 32%, 4.1±1.1 individuals, n=37) or low mistletoe load pines (L: 49%, 6.3±1.3 individuals, n=37) significantly differed in survival and abundance of caterpillars fed on needles of pines with medium (M: 5%, 0.8±0.6 individuals, n=37) and high parasitism (H: any alive caterpillar, n=37).

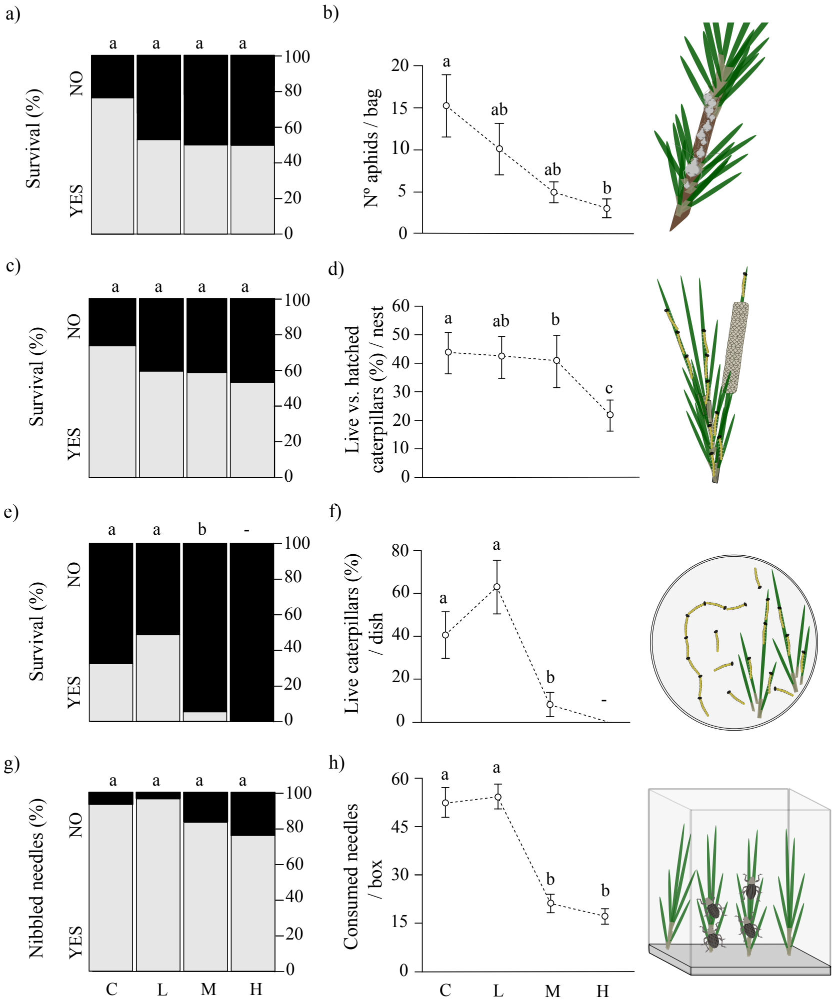

Figure 6.2: Pine–feeding herbivores in experimental conditions: survival (left column, grey and black bars, respectively) and abundance (right column, mean ± SE) of the three target herbivore species, according to the mistletoe loads on the pine host (C=control, L=low, M=medium and H=high). Survival rates show the percentage of a) bags inhabited by at least two live aphids (n=157), c) nests and e) Petri dish (n=148) with any live pine processionary caterpillars (n=127), during field and laboratory bioassays, respectively, and g) pine needles per box (n=120) nibbled by pine weevils during cafeteria test. Abundance column shows b) number of aphids inside the bags, d) percentage of live vs. hatched pine processionary caterpillars inside the nests, f) percentage of live out of 20 pine processionary caterpillars per Petri dish, and h) pine needles consumed (mm) by the pine weevils. Differences among parasitism levels are indicated by different lower–case letters.

Cafeteria test with *Brachyderes* sp.

Although the cafeteria test with the curculionid beetles showed no significant differences in the nibbled needle (χ^2=5.76, df=3, P=0.124; Appendix Table S12b), samples from Control (93%, n=30) and low (97%, n=30) mistletoe loads were more nibbled than medium (83%, n=30) and highly (77%, n=30) parasitized pines (Fig. 6.2g). In agreement with the results found in the pine processionary bioassay described above, the results of the cafeteria test showed strong and significant differences (χ^2=29.95, df=3, P<0.001; Appendix Table S12b) on consumed needles (Fig. 6.2h), where unparasitized (51.6±6.7 mm) and pines with low mistletoe load (55.3±5.6 mm) were preferred over pines with medium (23.4±3.9 mm) and high parasite loads (17.4±3.1 mm).

Discussion

Here, we demonstrate, by sampling wild populations and by field and laboratory experiments, that mistletoe–induced indirect interactions reduced abundance of several insect herbivores sharing the same host. In addition, the intensity of this detrimental effect has a non–proportional relation to parasite loads, insect herbivores feeding on medium and highly parasitized pines being more affected by decreasing their presence and abundance. Thus, the impact of mistletoe on the herbivores sharing the host plant is more evident when hemiparasite biomass is at a maximum (see Ewald *et al.* 2011 for a similar result). Therefore, consequences for insect herbivore performance depended basically on the parasite load, because of prolonged and heavy parasitism (medium and high parasite loads) affects pines more severely, diminishing their needle N content and increasing defense compounds (Lázaro-González *et al.*, 2019b).

We identified the indirect effects of mistletoe on arthropod herbivores as a TMII, via changes in the quality (trait) of the pine as food. The changes caused by mistletoe parasitism in pine (Zweifel *et al.*, 2012; Scalon & Wright, 2015) transform pine tissues into a worse food for the insect due to stronger induced defenses (Lázaro-González *et al.*, 2019b) and a reduction of mineral nutrients (Mutlu *et al.*, 2016). These negative effects of mistletoe on pines prove especially stressful at a site such as Sierra de Baza, with poor soils (Mellado *et al.*, 2016) and a severe summer drought, where high mistletoe load inevitably leads to the death of the pine (Mellado & Zamora, 2017). When the parasitism is not yet massive, although the pine remains alive, it already shows changes that negatively affect the herbivores that feed on the pine. Consequently, we confirm that mistletoe parasitism generates non–trophic links by producing trait–mediated indirect interactions and non–proportional effects on pine–feeding herbivores of a shared host.

The effect of mistletoe on pine herbivores across its shared host (Fig. 6.3) includes non–trophic connections and three different links. The first interaction (link 1 in Fig. 6.3), widely reported in the literature, is a direct and top–down effect, or in parallel effects, depending on whether mistletoe is considered a herbivore (Pennings & Callaway, 2002) or a plant, respectively, caused by a hemiparasitic plant (*Viscum album*) on a host plant (*Pinus nigra*) removing nutrients (Mutlu *et al.*, 2016) and altering their concentrations of chemical defenses (Lázaro-González *et al.*, 2019b). These effects suggest that mistletoe reduces pine quality as food, regardless of the tissue considered (phloem or needles). This idea is confirmed by the fact that all herbivores used in our trials, both in field and lab experiments, proved consistently detrimental. This is noteworthy because sometimes a benefit has been suggested for herbivores when the nutrient plant undergoes some type of stress, for example due to parasitism (Schwartz *et al.*, 2003). In our case, the pine processionary moth and pine weevil appeared to benefit from low levels of mistletoe load (Fig. 6.2e–f and 6.2g–h), although without significant differences with the unparasitized pines, and by contrast they are clearly harmed by medium or high parasitism. Also noteworthy is the marked increase in the natural populations of aphids in highly parasitized pines (though without significant differences with others level of mistletoe load, Fig. 6.1a–b), although this appears rather to be an effect of the reduction in natural enemies. It is known that parasitic plants interfere with the emission of volatile defense compounds produced by the plant against herbivory (Runyon *et al.*, 2008). The contrast of the censuses of natural populations with those of the bioassay, in which natural enemies are totally excluded, points in this direction.

The second link (link 2 in Fig. 6.3) is the next direct and bottom–up effect above host–feeding herbivores (*Cinara pini*, *Thaumetopoea pityocampa* and *Brachyderes* sp.), represented by different guilds, where their responses vary according to the mobility of each herbivore species. On the one hand, we found lower survival rates in the aphid and caterpillar populations due to their scant ability to switch from parasitized to unparasitized pines. On the other hand, pine weevils changed their selection, on detecting unpalatable food in parasitized trees and leaving it in order to find and choose a mistletoe–free tree. Therefore, the last link corresponding to TMIIs (link 3 in Fig. 6.3) appears when *V. album* competes indirectly with host–feeding

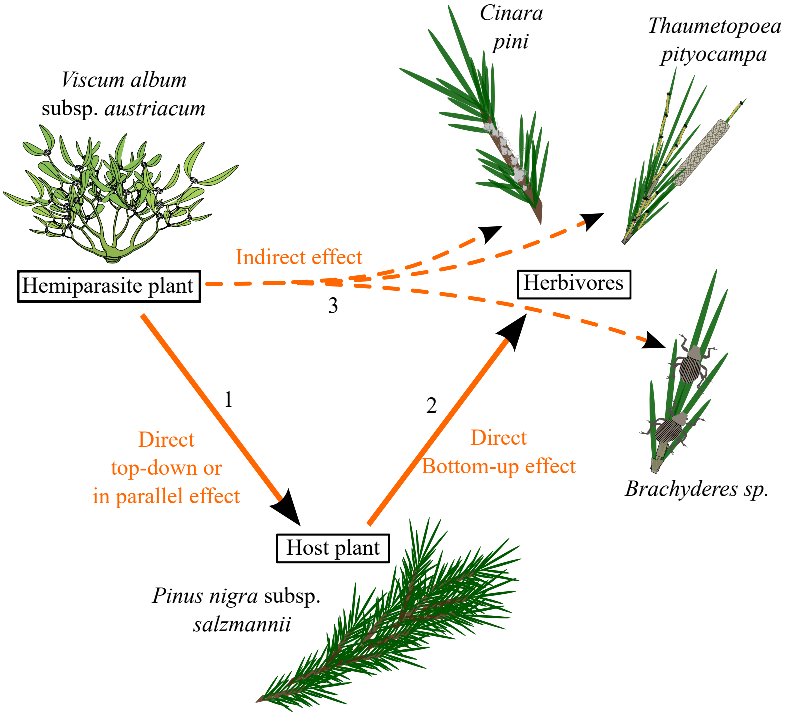

Figure 6.3: Scheme of effects of mistletoes on pine–feeding herbivores across its shared host.

herbivores for a shared resource (Puustinen & Mutikainen, 2001; Press & Phoenix, 2005).

Mistletoes triggered negative TMIIs through pines, but only with medium and high parasite loads, given that in all cases pines with low parasitism acted as unparasitized pines. For pine–feeding folivores, a medium parasite load means a threshold where their survival or abundance will decrease significantly, while for sap–sucking herbivores, this decrease will be gradual as the parasite load increases, being affected significantly only at high parasitism levels. In other words, the relationship between parasite load and their intensity is non–proportional. Despite the general pattern of more detrimental effects for herbivores as mistletoe parasitism intensifies, there are some response differences between folivores and sap-suckers. *Cinara pini* is less sensitive to the parasitism threshold, being affected only by high parasitism levels. By contrast, pine folivores, represented by *T. pityocampa* and *Brachyderes* sp., show lower tolerance to mistletoe parasitism than did sap–sucking herbivores, being affected by the medium parasite load. Thus, in all cases the insect population diminished in parasitized trees, although, in agreement to our predictions, the response differed between functional feeding groups, being stronger on folivorous herbivores than on sap–sucking ones. Previous studies have stated that sharing a host plant with a hemiparasite may be strongly detrimental (Bass *et al.*, 2010; Hartley *et al.*, 2007) and, on the contrary, can generate some benefits for insect herbivores from different functional feeding groups (Ewald *et al.*, 2011; Hartley *et al.*, 2015). In all cases, the effects of these indirect interactions are context dependent and frequently difficult to explain. Therefore, in our case, it is essential to determine and consider the parasite load, the functional feeding group, and the mobility of the host–feeding herbivore in order to describe accurately the TMIIs effects on mistletoe–host–herbivores system.

Overall, our results suggest that folivores are more responsive than sapsuckers, or mistletoes are causing more severe changes in pine needles than in phloem. We suggest that needles become a low quality resource, due to changes in chemical defense of pine needles to mistletoe parasitism (Lázaro-González *et al.*, 2019b), resembling a folivore attack. Meanwhile, although phloem may contain less nutrients on parasitized pines, could be lacking defense compounds. For these reasons, more studies delving into the chemical–profile change in different pine tissues are required in order to understand the mechanisms leading to herbivore–feeding responses.

In summary, the present study shows that mistletoe competes against arthropod herbivores and causes a decrease on their populations via a TMII mediated by shared pine resource. In this way, the global impact of consumers on pines is not the addition of negative partial effects of mistletoe and insect herbivores attack, but rather an interaction between biotic stressors. Thus, mistletoe becomes a determinant of the abundance of some species of host–feeding herbivores, relieving the host pines of many of them. This event become critical since the mistletoe acts indirectly as a regulator of one of the most severe defoliating plagues in the Mediterranean pine forests, the pine processionary moth. In fact, we show that a pine parasitized by mistletoe is unlikely to be attacked by the processionary. Consequently, indirect mistletoe interaction diminishes host stress and consumption intensity by herbivores, and thus lowers the probability of tree death, thereby helping to lengthen the parasite's own lifespan.

While mistletoe attracts a new community of specialists, different from host pine (Lázaro-González *et al.*, 2017), the pine–feeding herbivore insect populations undergoes dramatic and lasting impacts by mistletoe parasitism. These changed assemblages (i.e. detrimental effects on pine herbivores and novel niche for specialized mistletoe fauna) generate a pine canopy, which offers a new combination within the herbivore community to high trophic levels on parasitized pines, with impact on predators such as insectivorous birds, parasitoids, pathogens and mutualists associated as ant–tended aphids. All these mechanisms and processes make mistletoe a key species able to reorganize the canopy community of the pine forest, providing a clear demonstration of the importance of indirect interactions as major structuring forces in plant–animal interactions.

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Chapter 7

Beneath the mistletoe: parasitized trees host have more diverse herbaceous vegetation and are more visited by rabbits

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Abstract

Mistletoes are a diverse group of aerial hemiparasitic plants and are considered keystone species in forest ecosystems around the world. They produce nutrient–enriched litter, which exerts a substantial effect on soil–nutrient concentration, and the enriched nutrient patch alters the vegetation at the site as well as the associated fauna. Our goal is to ascertain whether mistletoe (*Viscum album*) parasitism of pine forest of a Mediterranean mountain favors herbaceous vegetation and attracts mammalian herbivores. We recorded in Sierra de Baza (SE Spain) the composition of the herbaceous vegetation under pines with and without mistletoe parasitism, and estimated the rabbit activity at the same sites by collecting their excrements. An effect on herbaceous vegetation, especially in grasses belonging to the family Poaceae, was reflected in a notable increase in soil cover, species richness, and species diversity beneath parasitized pines with respect to unparasitized ones. As a consequence, parasitized pines attract the activity of rabbits, as shown by a fivefold quantity of excrement with respect to control ones. We conclude that parasitism by mistletoe, by creating patches of greater nutrient availability under the host canopy, extends its effects beyond the host tree to other members of the forest community, such as herbaceous plants and associated herbivorous animals, which in turn contribute to environmental heterogeneity with their activity.

Key words – fertility island; herbaceous plant diversity; herbivore occupancy; mistletoe litterfall; nutrient concentration; pine woodlands; Poaceae; rabbit

Introduction

Plants and animals live in habitats that are heterogeneous, both in space and time (Wiens, 2000). Field research has focused on the effect of heterogeneity in habitat quality on the distribution and abundance of organisms (Stewart *et al.*, 2000); however, plants and animals are also able to initiate, maintain, and re–inforce heterogeneous spatial patterns (Wiens, 2000). Many processes of resource redistribution and concentration — some being abiotic such as changes in light patchiness, water runoff, and water availability, and others biotic such as litterfall, excrement accumulation in latrines, or food hoarding — create non–random patches of heterogeneous nutrient availability (Pickett *et al.*, 2000). Among the biotic processes, plants can promote spatial heterogeneity by concentrating nutrients around themselves (Moro *et al.*, 1997; Watson, 2009) or emitting particular secondary metabolites (Iason *et al.*, 2005). Similarly, animals can do so by depositing excrement and/or foraging selectively in preferred patches (Willott *et al.*, 2000; Bokdam, 2001). Thus, the activity of plants and animals could modify spatial heterogeneity in nature.

A notable case of nutrient redistribution and concentration with strong effects on plant quantity and quality as food are parasitic plants (Watson, 2009). Among these, mistletoes, a diverse group of aerial hemiparasitic plants, are considered keystone species in forest ecosystems around the world ((Watson, 2001)). Recent studies with parasitic plants have shown their important role in regulating belowground processes by enhancing soil–nutrient availability and bolstering soil spatial heterogeneity (Quested *et al.*, 2002; Fisher *et al.*, 2013; Mellado *et al.*, 2016). Mistletoes draw water and mineral resources from host plants, attaining higher concentrations of foliar nutrients than their hosts (Ehleringer & Marshall, 1995; Bowie & Ward, 2004), and they produce nutrient–enriched litter due to scant reabsorption prior to abscission (Pate *et al.*, 1991a; Quested *et al.*, 2002) and the input of bird–derived debris (Mellado *et al.*, 2016). Mistletoe litter often contributes large amounts of biomass to the forest floor, including leaves, flowers, and fruits (March & Watson, 2007), which are rich in nutrients and have a high decomposition rate, enhancing soil–nutrient cycling (March & Watson, 2010; Ndagurwa *et al.*, 2016; Muvengwi *et al.*, 2015). It has been shown that the volume of litter beneath a tree crown amasses with mistletoe infestation, increasing the overall litterfall (March & Watson, 2007, 2010; Mellado *et al.*, 2016), and thus the parasite may contribute to higher nutrient returns beneath host trees. Mistletoe litter contains lower C/N ratios (Mellado *et al.*, 2016) and therefore decomposes faster and releases nutrients more rapidly than does the litter of co–occurring species, and may also stimulate the decomposition of more recalcitrant litter when mixed, for instance with those from the host plant (Quested *et al.*, 2002). In this way, mistletoes can have a profound effect on soil–nutrient concentrations in terrestrial habitats, which may in turn affect the growth of neighboring plants (Quested *et al.*, 2003; March & Watson, 2010). In addition, mistletoes attract animal associates from a wide range of groups including mammals, birds, and insects, thereby augmenting diversity in environments where they occur (Watson, 2009). According to the Dryad hypothesis (Watson, 2009), mistletoe and parasitic plants are generally regarded as facilitators in low–productivity habitats, boosting heterogeneity in nutrient availability and productivity by shedding large quantities of enriched leaf litter (Watson, 2015). The enriched nutrient patch alters the vegetation at the site as well as the associated fauna, and animal visitors may reinforce this nutrient increase by providing excrement, urine, or other animal remains (Van Der Wal *et al.*, 2004; Watson, 2009; Mellado *et al.*, 2016).

In this study, we address these questions by focusing on mistletoe (*Viscum album* subsp. *austriacum* (Wiesb.)) parasitizing Black pines (*Pinus nigra* Arn.) on a Mediterranean mountain (SE Spain). Through its nutrient–rich litter and the accumulation of excrement from seed–dispersing birds, mistletoe enhances soil–nutrient availability beneath parasitized pines (Mellado *et al.*, 2016), which could favor the growth of other plant species. Moreover, this parasite has a constant and uniform annual fruit production that makes parasitized trees constant food resources for frugivorous birds, thereby providing consistent sites for organic matter deposition in space and time (Mellado & Zamora, 2016; Mellado *et al.*, 2016).

In previous studies, we have measured the abundance of seeds moved by zoochory under the canopy of unparasitized and parasitized trees (Mellado & Zamora, 2016) and soil–nutrient availability beneath the canopy of these same trees (Mellado *et al.*, 2016). To fully understand the consequences of mistletoe parasitism in the whole forest community, here, we focus on herbaceous vegetation and a mammalian herbivore. Specifically, we test whether (1) plant communities beneath parasitized pines show a different cover and diversity of herbaceous vegetation with respect to control, and (2) whether parasitized pines promoted more occupancy by rabbits, in comparison to unparasitized pines.

Materials and Methods

Study site

The study was performed from autumn 2012 to summer 2013 in the Natural Park of Sierra de Baza (south–eastern Spain; 2° 51' W, 37° 22' N). The site is a mountain range with limestone rocks and calcareous soils, ranging between 1200 and 2269 m asl (Calar de Santa Bárbara, the highest peak). Climate is typically Mediterranean, with cold winters (snow cover above 1700 m lasts 2–4 months per year), precipitation concentrated in spring and autumn, and a pronounced summer drought (June–August). The main vegetation cover is pine woodlands, Aleppo (*Pinus halepensis* Mill.) and maritime pine (*P. pinaster* Ait.) at lower elevations, and Black (*Pinus nigra*) and Scots pine (*P. sylvestris* L.) from middle to high elevations. Pine woodlands are intermingled with oaks (*Quercus ilex* L.), maples (*Acer opalus* L. subsp. *granatense* Boiss.), and several fleshy–fruited shrubs, lianas, and trees, forming part of the plant community throughout the mountain (see (Blanca & Morales, 1991) for a detailed description of the Sierra de Baza vegetation). The mistletoe *Viscum album* subsp. *austriacum* is a hemiparasitic, dioecious epiphyte widely distributed across European coniferous forests. *Pinus nigra* and *P. sylvestris* constitute the most common host species in southern Spain and at the study site (Mellado, 2016), where individual *Viscum album* subsp. *austriacum* can live for more than 30 years (Zuber, 2004, *pers. obs.*).

For this study, we selected 110 Black pines (hereafter focal trees), half of them parasitized by mistletoe and the other half unparasitized. Parasitized trees presented moderate to intense parasitic loads (20–40 and 50–80% of the host canopy covered by mistletoe, respectively), bearing at least one mistletoe $>$ 30 years old (Mellado *et al.*, 2016). Pines were selected to cover a large range of the mountain's heterogeneity, including stands with low (open) and high (closed) tree densities and three elevation levels (1350, 1650, and 1850 m). Trees were randomly selected and spatially paired (one parasitized and one unparasitized). Paired trees were of similar architecture, size (diameter at breast height [mean±SE] 25.55±1.31 cm), age (90–110 years old; see (Herrero *et al.*, 2013), and height (6.87±0.23 m). Paired trees were located 40 to 80 m apart to keep environmental contexts as similar as possible (e.g., climatic factors and composition of neighboring vegetation). The shrub cover beneath the tree canopy was similar between control and parasitized pines (Mellado & Zamora, 2016; Mellado, 2016).

Sampling herbaceous vegetation

The study took place between autumn of 2012 and summer of 2013 for three reasons. First, we were interested in herbaceous vegetation, which in Mediterranean ecosystems shows its growing peak during early spring, becoming dry later due to summer drought (Archibold, 1995). Second, litterfall (leaves and fruits) from mistletoe falls to the soil mostly during winter, thus being available for consumption by herbivores and contributing to the nutrient return (Mellado *et al.*, 2016; Ndagurwa *et al.*, 2016). Finally, as a consequence of the previous points, most mammalian herbivores graze on herbs and grasses while available, shifting to a shrub–based diet when dry (summer) or unavailable (mid–winter) (Martínez, 2009).

In spring 2013, we checked the soil under the pine canopy of all tagged trees. A square of 1 m^2 made with PVC tube was used to delimit the plots. The square was placed three times randomly beneath each pine canopy. We first recorded the percentage of surface area of bare soil, covered by rocks and covered by herbaceous vegetation within the plots. Then, we estimated the cover of every plant species in the plots according to a semi–quantitative scale (0 to 5, following the method proposed by (Braun-Blanquet, 1979), excepting shrubs (already studied in (Mellado & Zamora, 2016)). Later, we converted the scores of the semi–quantitative scale of cover to percentage of plant cover in each plot (+ = 0.3%, 1 = 3%, 2 = 15%, 3 = 35%, 4 = 65%, 5 = 85%). All plants were identified to the lowest taxonomic level to test differences beneath parasitized and unparasitized pines in species richness, Shannon Index (as diversity), families cover (Poaceae, Fabaceae, and other families), and composition of herbaceous species. Plant nomenclature follows (Blanca *et al.*, 2011).

Estimating the occupancy under trees by rabbits

Several ungulate species live in Sierra de Baza, both wild (red deer *Cervus elaphus*, Spanish ibex *Capra pyrenaica*, wild boar *Sus scrofa*) and domestic (mixed herds of sheep *Ovis aries* and domestic goat *Capra hircus*) (CMAOT, 2016). These species increase its browsing character during winter (Garin *et al.*, 2001; Ferreira *et al.*, 2013), and in reason of its body size, they sample the habitat on a coarse scale (Senft *et al.*, 1987; Hódar & Palo, 1997; Schaefer *et al.*, 2008). Thus, we discarded them as a focus for this study. Conversely, we focus our attention on rabbit (*Oryctolagus cuniculus*). Rabbit is a small mammal with a restricted home range size (Soriguer, 1981; Lombardi *et al.*, 2007); thus, we consider it as an adequate species model to discriminate between individual trees depending on its parasitism by mistletoe. To examine the differential occupancy, we counted excrements beneath trees with and without mistletoe. Pellet count has been repeatedly used as an appropriated method to estimate density and/or habitat selection by rabbits (Lombardi *et al.*, 2003; Santilli & Bagliacca, 2010; Mutze *et al.*, 2014).

At the beginning of autumn 2012, we checked the soil beneath the pine canopy of all tagged trees and cleaned away most of the recent excrement, whatever its source. Old excrement was in general easily recognizable, but in this way, we minimized the possibility of mistakes. At the end of winter (March–April 2013), we randomly delimited three 1 m^2 plots with the PVC square (as describe above) beneath each pine and collected all the excrement found. Clearly old excrements moved by abiotic factors (e.g., rain) were discarded. Initially, we distinguished whenever was possible between rabbit and hare (*Lepus granatensis*); however, hare was conspicuously scarce and restricted to the higher of the mountain, so hereafter we considered together as rabbit. We kept the excrement specimens in cellophane bags, dried them in the laboratory for 48 h at 70°C, and then weighed them.

Statistical analyses

All analyses were performed under the same structure, using tree condition (parasitized and unparasitized pines) as fixed factor and elevation (1350, 1650, and 1850 m asl), paired trees (1–55), and individual pines (1–110) as nested random ones. We used generalized linear mixed models (GLMM) assuming binomial error distribution to analyze the effects of tree conditions (parasitized or not) on percentage cover beneath pine canopies (bare soil, rock, and herbaceous vegetation) and Poisson error distribution and log–link function for total species richness of herbaceous vegetation. Herbaceous diversity (H') was tested using linear mixed model (LMM). Permutational multivariate analysis of variance (PERMANOVA), using the Bray–Curtis distance and 9999 permutations, was used to analyze significant differences on plant species assemblages beneath parasitized and unparasitized pine trees. Individual significance by plant species on herbaceous community was analyzed using GLMMs with negative binomial error distribution, and square root transformation was required on community matrix.

For Fabaceae family cover, we applied a GLMM with negative binomial distribution and the log–link due to its absence from several samples, while Poaceae and other families cover were square–transformed, in order to meet assumptions of normality and homoscedasticity, and tested using LMM. The species richness by families was tested using GLMM with Poisson error distribution and log–link function.

To analyze preference of occupancy under parasitized or unparasitized pine trees across excrement records, we used a GLMM with negative binomial distribution due to high number of zeros. Data of rabbit presence/absence as well as amount of excrement recorded were averaged for the three plots of each pine tree, in order to reduce the number of empty samples, and therefore individual pine was removed from random factors.

All statistical analyses were conducted with the R software system Version 3.4.1 (R development Core Team, 2018). GLMMs and LMMs were carried out under *glmer*, *lmer*, and *glmer.nb* functions of *lmer4* packages, and the Shannon diversity index was calculated by *diversity* function. For PERMANOVA, we used only species above 0.5% of total cover on each plot and was performed under *adonis* function. *Diversity* and *adonis* functions were included in the *vegan* package.

Throughout the paper, means are expressed ± 1SE.

Results

Herbaceous vegetation

A total of 167 plant taxa were identified within the plots, most specimens being identified to the species level (Appendix Table S13). The herbaceous vegetation (GLMM, $\chi^2 = 11.13$, *d.f.* $= 1$, $P < 0.001$) and rock cover (GLMM, $\chi^2 = 4.02$, *d.f.* $= 1$, $P = 0.045$) were significantly greater under parasitized pines in detriment to bare soil (GLMM, $\chi^2 = 16.08$, *d.f.* $= 1$, $P < 0.001$; Fig 7.1a). Herbaceous plant assemblages beneath parasitized pines showed more species richness (GLMM, $\chi^2 = 14.56$, *d.f.* $= 1$, $P < 0.001$, Fig 7.1b) and diversity (LMM, $\chi^2 = 10.18$, *d.f.* $= 1$, $P = 0.002$, 7.1c) than unparasitized ones. According to these results, their species composition was also significantly different between herbaceous communities under parasitized and unparasitized pine trees (PERMANOVA, *pseudo–F* $= 4.32$, *d.f.* $= 1$, $P < 0.001$). Individual species analysis reveals that these differences were due to 14 species, 13 of them more abundant beneath parasitized pines (four Poaceae, two Brassicaceae, three Caryophyllaceae, two Rubiaceae, and two Asteraceae), while only one specie (Fabaceae) was more abundant under unparasitized pines (Fig 7.2).

Overall, plant species from Poaceae and Other families presented fuller cover ($\text{LMM}_{\text{Poaceae}}$, $\chi^2 = 13.66$, *d.f.* $= 1$, $P < 0.001$; $\text{LMM}_{\text{Other}}$, $\chi^2 =$

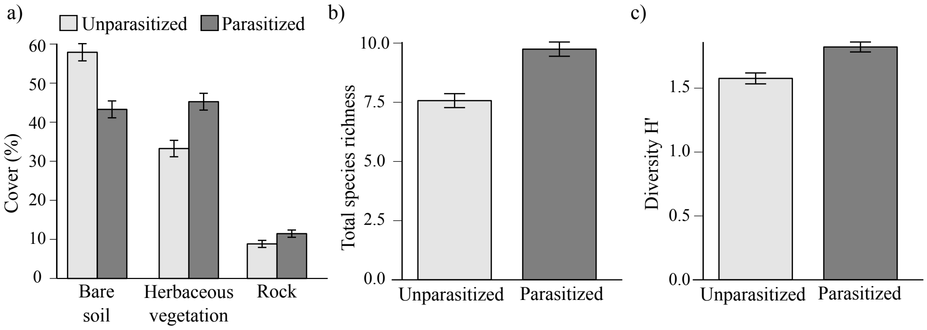

Figure 7.1: Mean (± SE) of a) percentage cover (bare soil, herbaceous vegetation, and rock cover), b) species richness, and c) Shannon diversity Index by square meter, beneath parasitized (dark–gray bars) and unparasitized (light–gray bars) pines

6.35, *d.f.* = 1, $P = 0.012$) and species richness (GLMM$_{\text{Poaceae}}$, $\chi^2 = 23.12$, *d.f.* = 1, $P < 0.001$; GLMM$_{\text{Other}}$, $\chi^2 = 8.31$, *d.f.* = 1, $P = 0.004$) under parasitized pines (Fig. 7.3), while Fabaceae showed no significant differences (GLMM$_{\text{Cover}}$, $\chi^2 = 0.57$, *d.f.* = 1, $P = 0.452$; GLMM$_{\text{Richness}}$, $\chi^2 = 0.31$, *d.f.* = 1, $P = 0.581$).

Estimating the occupancy under trees by rabbit

Rabbit clearly prefers parasitized trees: The presence of rabbits under parasitized pines is much more frequent than under control ones (69 vs 25%, respectively; GLMM, *z*–value = 3.705, *d.f.* = 1, $P < 0.001$). Accordingly, the amount of excrement under parasitized pines is fivefold than those found under control ones (0.40±0.03 vs 0.08±0.07 g/m^2, respectively; GLMM, $\chi^2 = 10.89$, *d.f.* = 1, $P < 0.001$).

Discussion

Mistletoe clearly determines the cover and botanical composition of herbaceous vegetation beneath parasitized pines in comparison to unparasitized ones. The most noteworthy results are a general increase in plant cover, species richness (especially in Fam. Poaceae), and diversity. In line with these, plant species composition shows also differences, where several grass species clearly responded to the presence of mistletoe in trees by increasing its abundance with respect to control trees, as revealed by individual species analysis (Fig 7.2).

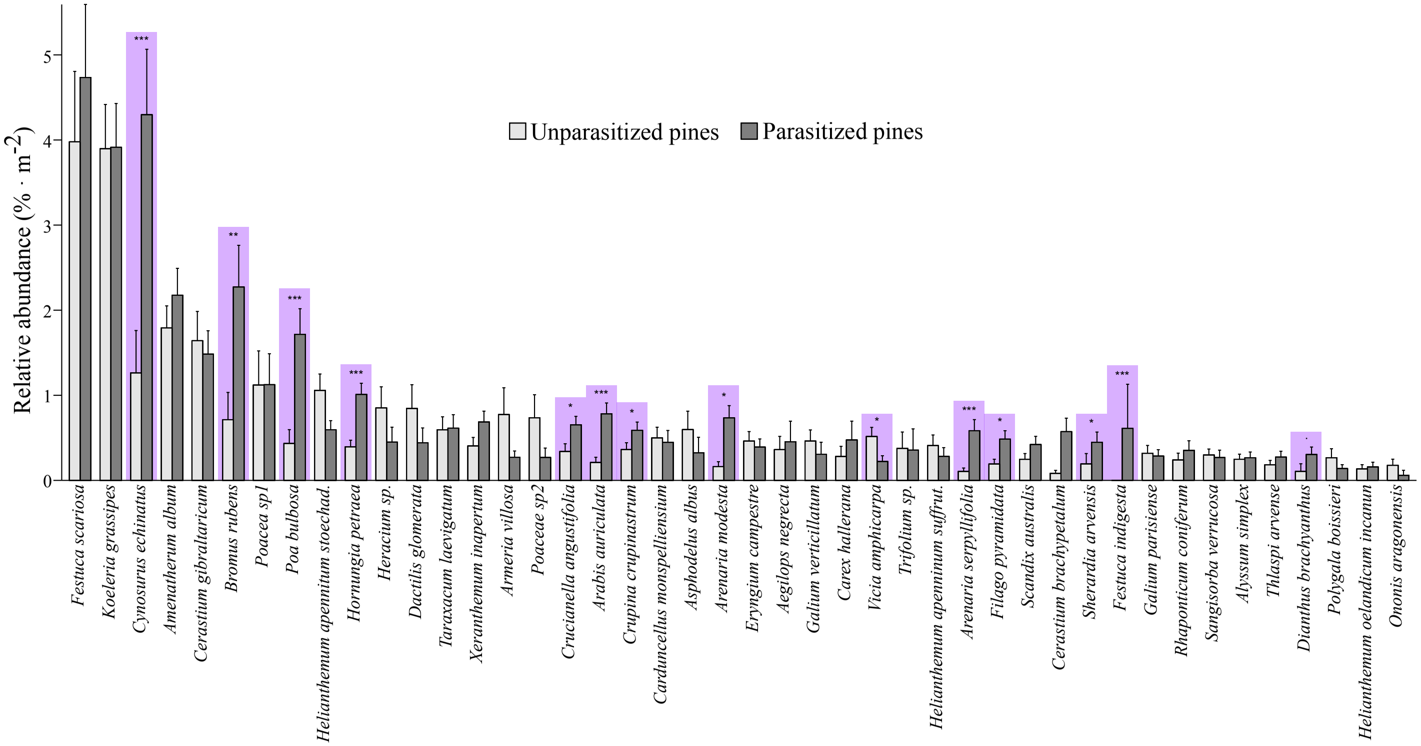

Figure 7.2: Relative abundance by square meter of plant species (above 0.5% of total cover) presents on each plot sampled beneath parasitized (dark–gray bars) and unparasitized (light–gray bars) pines. Significant differences between parasitized and unparasitized trees are indicated as $P<0.1$ (·), $P<0.05$ (*), $P<0.01$ (**), $P<0.001$ (***) and highlighted by purple rectangle

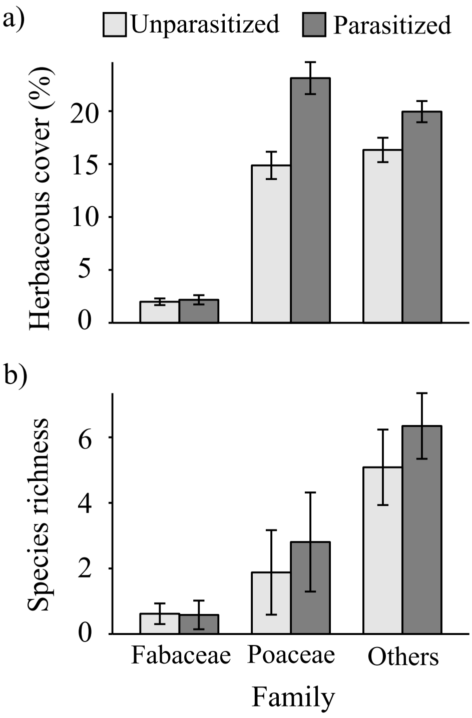

Figure 7.3: Mean (± SE) of a) herbaceous cover and b) species richness by square meter of Fabaceae, Poaceae, and other families of herbs, beneath parasitized (dark–gray bars) and unparasitized (light–gray bars) pine

There are at least two different, non–mutually exclusive, possibilities to explain why grasses are especially favored. First, they are highly responsive to fertilization. A recent work (Mellado *et al.*, 2016) performed exactly in the same pines used by us clearly demonstrated that mistletoe increased the amount, quality, and diversity of organic matter input beneath the host canopy, directly through its nutrient–rich litter and indirectly through a reduction in host litterfall and an increase in bird–derived debris. As a result, the spaces beneath parasitized pines became an enriched hotspot. Parasitized pines show a higher NPK soil availability than did control pines (Mellado *et al.*, 2016); see also (Ndagurwa *et al.*, 2016), and this fertilization stimulates the development of Poaceae more than, for example, Fabaceae (Grünzweig & Körner, 2003). Moreover, N–fixing species such as Fabaceae are more responsive to an exclusive increase in P (Stöcklin & Körner, 1999; Stöcklin *et al.*, 1998) than NPK together (Grünzweig & Körner, 2003), and even a unique increase of N does not benefit their growth (Xia & Wan, 2008; Huang *et al.*, 2015) or can lower abundance and richness of N_2–fixing species in a herbaceous community (Suding *et al.*, 2005). Thus, legumes can lose their advantage with respect to other non–N_2–fixing species under N fertilization (Suding *et al.*, 2005). Second, rabbits, by showing a preference for grazing under parasitized pines, can spread seeds in their excrement during their foraging activity (Malo & Suárez, 1995; Dellafiore *et al.*, 2010). Poaceae is the staple food for rabbits, especially during winter (see below), and consequently seeds from this family will be preferentially dispersed. Furthermore, Poaceae are in general more resistant to grazing than are Fabaceae or other forbs, thus reinforcing the pattern.

The greater herbaceous cover, richness, and diversity under parasitized pines were notable in afforested pine woodlands. Pine plantations are widespread in Mediterranean environments, and due to the characteristics of most of them (low tree diversity, high density, lack of vegetation heterogeneity) sometimes have been considered "green deserts" unuseful to restore the natural biodiversity (Chirino *et al.*, 2006; Bremer & Farley, 2010). A study in the nearby Sierra Nevada mountain range (Gómez-Aparicio *et al.*, 2009) showed that pine plantations in fact bore lower total plant richness and H' index values than native stands, especially when compared with deciduous broadleaf forests, but that this negative effect appeared only for herbaceous species, and not for woody species. This result was attributed to the high tree density of plantations in comparison to native fragments, which in turn implies dimmer understory light, usually responsible of low herbaceous richness and cover. Mistletoe parasitism, by increasing nutrient availability and heterogeneity (Mellado *et al.*, 2016) as well as light penetration to the understory (Mellado, 2016), ameliorates the environment for the growth of herbaceous vegetation. In this context, the role of the mistletoe facilitating diversity and heterogeneity should be considered as a positive factor for the naturalization of the artificial stands of pine plantations.

The mistletoe footprint translates to herbivorous animals, by increasing growth and diversity of understory herbaceous plants (March & Watson, 2007). Rabbits are small–bodied mammals with a limited home range (ca. 1 Ha in (Soriguer, 1981); 0.5–2 Ha in (Lombardi *et al.*, 2007)), for which an intense use of understory of parasitized trees is easy to record. Rabbits might prefer grazing under parasitized pines because grasses, mainly Poaceae, are more abundant in these pines with respect to control, as a result of increased amounts of litter and excrement and thus more vigorous microbial mineralization activity (Mellado, 2016). Poaceae respond better to this kind of fertilization than do other groups of forbs (Grünzweig & Körner, 2003), increasing its biomass and presumably its nutrient content, while rabbits clearly prefer foods having higher nutrient contents (Somers *et al.*, 2008). Furthermore, Poaceae are the staple food for rabbits during winter (Soriguer, 1988; Martins *et al.*, 2002; Kontsiotis *et al.*, 2015). Another reason why rabbits could be attracted to parasitized pines is the mistletoe litterfall. Mistletoe is a nutritive plant for herbivores: (Rodrigáñez, 1949) and (González-González & González-Doncel, 1999) refer to mistletoe as an important livestock food source, searched for by shepherds, who cut them down to feed to the animals. Thus, for small mammals with a restricted home range, such as rabbits, it should be easy to track the natural fall of mistletoe leaves from the parasitized pines within its home range, quickly consuming them.

The role of mistletoe as a generator of environmental heterogeneity has previously been identified by shaping the spatial deposition pattern of zoochorous seeds in the forest, which may be reflected in the future configuration of the woody–plant community (Mellado & Zamora, 2016), as well as in greater soil availability and spatial heterogeneity (Mellado *et al.*, 2016). The present study, however, is the first to show that the impact of parasite litter on the understory extends to other members of the forest community such as herbaceous plants and associated herbivorous animals, which in turn can contribute to reinforce the environmental heterogeneity with their activity. This effect is especially important in Mediterranean environments, where, due to afforestation trends during recent decades, large areas are covered by pine plantations characterized by a homogeneous spatial structure and monospecific composition. In this context, mistletoe can provide more habitat and resources for other herbaceous plant species to become established, fostering plant diversity in the pine forest.

Conclusion

In conclusion, the effects promoted by mistletoe go beyond the host tree, affecting the whole herbaceous plant community beneath the parasitized tree and their herbivorous consumers. The increase of diversity and heterogeneity could even be considered as a positive factor for the naturalization of the artificial stands of pine plantations, widespread in Mediterranean environments. These findings strengthen the idea of mistletoes as ecosystem engineers alter the microenvironment of soil and create patches of increased nutrient availability under the host canopy, while as keystone species, they alter species composition of the herbaceous community and the activity of grass–eating mammals such as rabbits (Jones *et al.*, 1994; Watson, 2001), expanding their long–lasting effects to other organisms of the forest community.

Data availability – The datasets generated and/or analyzed during the current study are available in the Zenodo repository.
https://doi.org/10.5281/zenodo.1311757 (Hódar *et al.*, 2018).

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Part IV

Final

Discusión general

Discusión general

En esta tesis se han explorado las interacciones y consecuencias ecológicas que conlleva la presencia del muérdago europeo, *Viscum album* subsp. *austriacum*, en un pinar mediterráneo, donde su principal hospedador es el pino salgareño *Pinus nigra* subsp. *salzmannii*. El primer lugar, encontramos nuevas interacciones planta–animal, identificando toda la comunidad de artrópodos asociada al muérdago. Descubrimos que el muérdago representa un nuevo recurso en el dosel forestal que es habitado por una nueva, y a la vez simple, red trófica de artrópodos especialistas (Capítulo 1), que siguen las mismas reglas de ensamblaje en diferentes escalas espacio–temporales (Capítulo 2). Además, la floración del muérdago atrae a un conjunto de polinizadores que no estarían presentes en un dosel forestal sin muérdago (Capítulo 3). En segundo lugar, analizamos los efectos directos del muérdago en el perfil químico de su principal hospedador, *Pinus nigra* subsp. *salzmannii*. Encontramos que el pino hospedador reacciona frente a la parasitación por muérdago tanto al nivel de grandes compuestos químicos (Capítulo 4) como al nivel metabolómico (Capítulo 5), generando así una nueva identidad de planta en el dosel forestal. Finalmente, analizamos los efectos indirectos que el muérdago promueve en el ecosistema. Por un lado, el muérdago jugando el rol de competidor indirecto, provoca un efecto perjudicial en cascada a través de su pino hospedador hasta sus consumidores (Capítulo 6). A su vez, el muérdago, tras un proceso de reciclaje de nutrientes creando una isla de fertilidad bajo la copa de los pinos parasitados (Mellado et al. 2016), actúa como facilitador generando parches de una nueva comunidad herbácea y un incremento de la presencia de mamíferos herbívoros (Capítulo 7). En general esta tesis resalta la importancia del muérdago como especie clave en un pinar, siendo el desencadenante de multitud de interacciones con importantes consecuencias ecológicas directas e indirectas, incrementando la heterogeneidad, complejidad y biodiversidad en el bosque, y redefiniendo así la estructura y dinámica de toda la comunidad al nivel de ecosistema. Así pues, mediante una visión holística, vemos cómo el impacto de los muérdagos en un ecosistema tiene un balance global positivo.

La presencia de muérdagos en el dosel de un pinar representa la adición de un nuevo recurso en el ecosistema, disponible para la fauna como alimento y/o refugio. Gracias a este nuevo recurso, una entomofauna diferente a la del pino hospedador, es capaz de colonizar y establecerse en bosques parasitados por muérdago. Se trata de una nueva comunidad compuesta por dos herbívoros especialistas, *Cacopsylla visci* y *Pinalitus viscicola*, y su depredador también especialista, *Anthocoris visci* (Capítulo 1). Comunidades especialistas con mayor riqueza de especies se han descrito habitando *V. album* a lo largo de Europa (Zuber, 2004; Briggs, 2011; Varga *et al.*, 2012). Sin embargo, esta tesis revela que la comunidad especialista que habita los muérdagos de Sierra de Baza es la más simple y la más meridional de toda Europa, agrandando por tanto su área de distribución conocida hasta hoy. Además, esta nueva comunidad es altamente estable, ya que se rige por una regla de ensamblaje determinista y constante bajo diferentes factores ecológicos. Esta regla consiste en una secuencia trófica de colonización, donde el especialista más abundante, *C. visci*, coloniza en primer lugar la planta de muérdago, seguido del segundo herbívoro especialista, *P. viscicola*, y de su depredador *A. visci* (Capítulo 2).

Al mismo tiempo, el muérdago no sólo representa un recurso para los herbívoros, sino que su potencial como recurso alcanza a otros gremios como los visitantes florales. Durante la floración del muérdago, muy temprana con respecto otros recursos florales, el dosel forestal de un pinar parasitado funciona como un atrayente para este gremio (Capítulo 3). Se amplía así, tanto el rango temporal en el que los polinizadores (himenópteros, dípteros y lepidópteros) están activos, como el rango espacial ampliando su área de forrajeo desde el sotobosque hasta el dosel forestal. Por tanto, la presencia del muérdago reestructura la comunidad entomológica del ecosistema, generando nuevas interacciones mutualistas planta–animal e incrementando espaciotemporalmente la biodiversidad local y regional. Así pues, los muérdagos son parches de recursos aislados que representan islas ecológicas para la fauna especialista y generalista que atrae, rodeadas de un mar hostil compuesto por el follaje del hospedador (Burns & Watson, 2013). El muérdago actúa, por tanto, como especie fundadora secundaria, introduciendo mayor diversidad y abundancia de consumidores de forma directa, no sólo herbívoros, depredadores y polinizadores como hemos descrito en esta tesis, sino también aves frugívoras atraídas por sus frutos carnosos (Mellado & Zamora, 2014a, 2016, Fig. 8.1).

Además de las interacciones planta–animal y sus efectos directos sobre la biodiversidad de artrópodos en el dosel forestal, el muérdago tiene una relación planta–planta muy íntima con su hospedador. En esta relación, el pino hospedador reacciona a nivel químico frente a la parasitación como lo puede hacer frente a otros estreses abióticos y bióticos. Sin embargo, esta relación planta–planta tiene dos particularidades, la primera de ellas es que se trata de dos plantas de vida larga con una parasitación crónica, en la que el hospedador permanece siempre parasitado, y la segunda es que esta parasitación incrementa su carga con el tiempo debido al patrón de reinfección dado por el muérdago y sus principales dispersores (Mellado & Zamora, 2014a). De este modo, se genera una reacción permanente en el pino hospedador que se va intensificando acorde a su grado de parasitación. Los pinos con una baja carga parasítica apenas modifican su perfil químico; sin embargo, a partir de cargas parasíticas medias ($>50\,\%$ de la copa ocupada por muérdago), el pino reconoce y responde a la parasitación modificando las concentraciones de diferentes compuestos. Grandes grupos químicos de defensa como fenoles, taninos y monoterpenos incrementan sus concentraciones proporcionalmente a su grado de parasitación, mientras que el contenido de nitrógeno y compuestos costosos de sintetizar y movilizar como sesqui– y diterpenos (Gershenzon, 1994), disminuyen su concentración en pinos altamente parasitados (Capítulo 4). Además de reorganizar los compuestos de defensa de su hospedador, el muérdago actúa como un sumidero constante de recursos derivados del metabolismo primario del pino. Mediante esta extracción de recursos, el muérdago obtiene los nutrientes necesarios para su desarrollo, así como aquellos componentes esenciales para la síntesis de sus propios compuestos tóxicos contra la herbivoría. Esto hace que el pino tenga que reestructurar diferentes rutas metabólicas para reponer y acumular estos recursos, forzando al hospedador a trabajar en beneficio del muérdago constantemente. Además de tener una reacción permanente, el pino responde sistémicamente a la parasitación por muérdago, modificando el perfil metabolómico de las acículas en toda la copa (Capítulo 5). Esto hace que el pino parasitado se convierta en un nuevo organismo con una nueva identidad metabólica en el dosel forestal, añadiendo un nivel adicional de complejidad y heterogeneidad en el ecosistema.

Muchas de las respuestas que hemos observado en los hospedadores presentan similitudes con reacciones a otros agentes bióticos, como la herbivoría, y abióticos, como la sequía. Sin embargo, dado que la relación parasito–hospedador es muy íntima, crónica y duradera, los efectos que el muérdago causa en su pino hospedador son mucho más profundos, e inician una cascada de consecuencias ecológicas que se trasladan a otros niveles tróficos. La primera consecuencia directa es que la nueva identidad metabólica del pino hospedador le convierte en un recurso de menor calidad como alimento para sus herbívoros, de tal manera que el muérdago y los consumidores del pino entran en competencia por un mismo recurso. Sin embargo, los efectos desproporcionados del muérdago y la reacción sistémica hacen del pino un organismo unitario, convirtiendo toda la copa en un lugar hostil para sus consumidores habituales, impidiéndoles evitar el impacto del muérdago. Así pues, el muérdago genera interacciones indirectas no tróficas mediadas por el hospedador, causando una disminución en la supervivencia de los herbívoros del pino (Capítulo 6). Por tanto, de manera indirecta el muérdago consigue beneficiar al pino hospedador atenuando la presión por herbivoría de sus consumidores habituales. Entre estos herbívoros se encuentra la procesionaria del pino, la plaga más severa en los pinares mediterráneos (Hódar *et al.*, 2002, 2003). En consecuencia, podríamos considerar al muérdago como un agente de control biótico de la procesionaria del pino, disminuyendo su supervivencia en pinos parasitados y, por tanto, reduciendo así su incidencia. Sin embargo, la nueva identidad metabólica del hospedador podría configurar un nuevo nicho ecológico, donde por un lado los herbívoros habituales se ven perjudicados, pero por otro, organismos tolerantes podrían adaptarse a esta nueva identidad encontrando un nuevo recurso y/o refugio.

Otro de los beneficios de la presencia de muérdago en un pinar, es la creación de una isla de fertilidad bajo la copa de los pinos parasitados, modificando las propiedades químicas, biológicas y microambientales del suelo (Mellado *et al.*, 2016; Mellado & Zamora, 2017). Este enriquecimiento local favorece la comunidad microbiana del suelo (Mellado *et al.*, 2016) y la comunidad arbustiva (Mellado & Zamora, 2017, Fig. 8.1). Ahora, además, sabemos que esta facilitación se extiende también a la comunidad herbácea, la cual incrementa su diversidad y riqueza de especies. Este incremento de diversidad actúa como atrayente para mamíferos herbívoros como el conejo, que a su vez podría reforzar la heterogeneidad ambiental con su actividad (Capítulo 7).

En general, esta tesis nos ayuda a completar parte del puzle ecosistémico en el que está implicado *V. album*, añadiendo conocimiento sobre los efectos de las interacciones planta–planta y planta–animal (Fig. 8.1 en color) a las recientemente descritas en Mellado (2016) (Fig. 8.1 en negro).

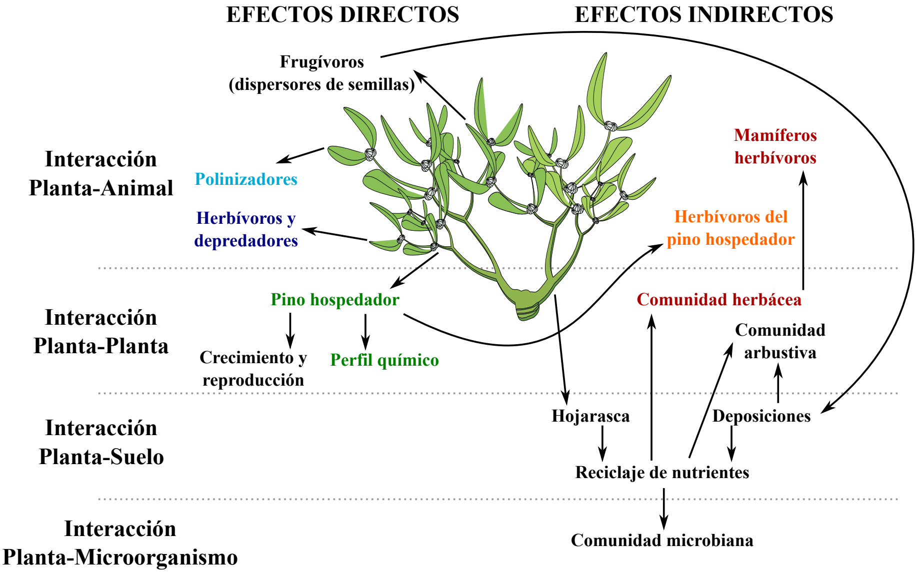

Figura 8.1: Esquema conceptual del conjunto de interacciones desencadenadas por la presencia de *Viscum album* subsp. *austriacum* al nivel de comunidad en los pinares de montaña descritas (negro) en (Mellado, 2016) y en la presente tesis doctoral (coloreado según capítulos de la Fig. 1 en la Introducción General).

En conclusión, los efectos promovidos por el muérdago europeo en un dosel forestal mediterráneo van mucho más allá de un simple efecto perjudicial sobre sus hospedadores, al perder vigor vegetativo, reproductivo o incluso provocándoles la muerte (Dobbertin *et al.*, 2005; Sangüesa-Barreda *et al.*, 2012). Es por eso que consideramos al muérdago como el detonante de un cóctel de interacciones que provoca una cascada de efectos, dejando una huella ecológica muy patente en todas las direcciones posibles, combinando efectos directos e indirectos tanto en su red trófica (*top–down* y *bottom–up* effects) como en relaciones no tróficas (Fig. 8.1). Por tanto, ejerce un papel fundamental en el ecosistema, desde la escala más fina en términos metabolómicos del hospedador, hasta la escala de paisaje modificando la estructura y composición de toda la comunidad forestal (Mellado & Zamora, 2017), aumentando el número de interacciones mutualistas, diversidad y heterogeneidad en el ecosistema. En definitiva, pasamos de percibir a las plantas parásitas en general, y a los muérdagos en particular, como agentes patógenos causantes de plagas, a desarrollar una visión integradora y definirlos como ingenieros de ecosistemas. De esta manera, ponemos en valor su impacto global neto a largo plazo, el cual inclina la balanza hacia efectos positivos para el conjunto del ecosistema. Así pues, nuestros resultados resaltan el papel de los muérdagos como facilitadores y especie clave en el ecosistema forestal (Watson, 2016, 2017), lo que plantea importantes implicaciones en la conservación y gestión forestal en los pinares de montaña.

Sistemas parásito–hospedador en el mundo

Son muchos los sistemas parásito–hospedador en el mundo estudiados hasta el momento en los que se describen al menos uno de los roles mencionados a lo largo de esta tesis. Sin embargo, nuestro sistema muérdago–pino habitando en un bosque mediterráneo se convierte en uno de los puzles ecosistémicos más completos y mejor estudiados. Según la literatura sobre los sistemas parásito–hospedador mejor conocidos, encontramos ejemplos en una gran variedad de ecosistemas, como el desierto semiárido en Chile, la sabana amazónica en Brasil, los bosques húmedos en México, los bosques de eucalipto en Australia, y los pastizales en las islas británicas. Teniendo como referencia el esquema conceptual (Fig. 8.1) donde describimos todas las interacciones directas e indirectas que *Viscum album* desencadena en los pinares de montaña, comparamos el conocimiento actual de varios sistemas parásito–hospedador habitando diferentes ecosistemas del mundo.

Muérdago endofítico – Cactos: Ecosistema semiárido

En el ecosistema semiárido en Chile, se encuentra el sistema endémico parásito–hospedador formado por el muérdago endofítico *Tristerix aphyllus* (Fam. Loranthaceae), y dos especies de cactos como hospedadores, *Echinopsis chilensis* y *E. acida*. Además de en su anatomía y desarrollo (Mauseth *et al.*, 1984, 1985), los estudios del muérdago *T. aphyllus* se han enfocado únicamente en las interacciones directas a) planta–planta con su hospedador, y b) planta–animal con polinizadores y dispersores de semillas.

En primer lugar, se han estudiado las respuestas coevolutivas de la carrera armamentística dada en la interacción parásito–hospedador (Medel *et al.*, 2010) y los efectos directos que la infección por *T. aphyllus* causa en el vigor reproductivo de su hospedero. En general, *T. aphyllus* actúa como enemigo natural, afectando negativamente la producción de flores, frutos y semillas (Silva & Martínez del Rio, 1996; Medel, 2000). Además, tal y como hipotetizan Silva & Martínez del Rio (1996), del mismo modo que los muérdagos endofíticos pueden tener efectos negativos en la reproducción del hospedador más intensos que en los sistemas de plantas hemiparásitas, podríamos pensar que los efectos en el sistema fisiológico, aún por estudiar, también podrían serlo.

Por otro lado, se han realizado varios estudios analizando la comunidad de visitantes florales, actuando como polinizadores legítimos y no legítimos (Medel *et al.*, 2002; Caballero *et al.*, 2013). Sin embargo, la interacción más estudiada en este sistema es la de planta–animal formada por el muérdago y sus frugívoros y aves dispersoras de semillas (Martínez del Rio *et al.*, 1995; Martínez del Rio *et al.*, 1996; Medel *et al.*, 2002), así como los patrones de dispersión de éstas (Botto-Mahan *et al.*, 2000; Medel *et al.*, 2004). Al tratarse de un muérdago endofítico, donde sólo las flores son externas, es lógico pensar que el sistema de polinización y dispersión de semillas haya sido el más estudiado (Fig. 8.2). Sin embargo, mediante la caída de la flor, restos de frutos no consumidos y las deposiciones de las aves consumidoras, podría estar dándose también un efecto en el enriquecimiento del suelo que promoverían el reciclaje de nutrientes, con efectos encima y debajo del suelo como el descrito en otros sistemas parásito–hospedador como en el mediterráneo (Mellado *et al.*, 2016), en la sabana semiárida africana (Ndagurwa *et al.*, 2016) y en los bosques de eucalipto australianos (March & Watson, 2007).

Muérdago hemiparásito – Árbol: Sabana amazónica y bosque húmedo

El muérdago hemiparásito del género *Psittacanthus* (Loranthaceae) se distribuye ampliamente por el continente americano, desde la baja California hasta el norte de Argentina (Kuijt, 2009). Este género ha sido estudiado mayoritariamente en dos ecosistemas diferenciados: la sabana amazónica de Brasil y los bosques húmedos de México Central. La anatomía vegetativa y biología reproductiva de esta hemiparásita se conoce detalladamente (Kuijt, 1969; Gómez-Sánchez *et al.*, 2011; Pérez-Crespo *et al.*, 2016), así como el aporte de biomasa local que representa su presencia en los ecosistemas que habita (Fadini *et al.*, 2020).

El estudio de la interacción de *Psittacanthus* con su hospedador se ha centrado fundamentalmente en el grado de especificidad del sistema parásito–hospedador (Monteiro *et al.*, 1992; López de Buen & Ornelas, 2002; Fadini *et al.*, 2014) y los determinantes de la distribución, abundancia y prevalencia del muérdago (López de Buen & Ornelas, 1999; Fadini *et al.*, 2010; Fadini & Lima, 2012). En relación a la distribución del muérdago, el patrón de dispersión y deposición de semillas, así como la supervivencia y el establecimiento de éstas, han tenido un papel muy importante en muchos de los estudios (Monteiro *et al.*, 1992; López de Buen & Ornelas, 1999, 2002; Ramírez & Ornelas, 2012). Como consecuencia del estudio de dispersión de semillas, uno de los roles mejor estudiados en este sistema parásito–hospedador ha sido el de recurso alimenticio para, en primer lugar, la comunidad de visitantes florales y, en segundo lugar, frugívoros y dispersores de semillas (Fig. 8.2). Se trata de una comunidad de polinizadores formada por colibríes, y menos comúnmente por mariposas, abejorros y murciélagos (Pérez-Crespo *et al.*, 2016; Fadini *et al.*, 2018; Ornelas, 2019), mientras que frugívoros y dispersores de semillas son mayoritariamente aves (López de Buen & Ornelas, 2001; Fadini & Lima, 2012; Ramírez & Ornelas, 2012).

Por tanto, nos encontramos frente a un sistema en el que se desconocen casi por completo los efectos directos del muérdago sobre su hospedador (Arruda *et al.,* 2012, ver también Cocoletzi *et al.*, 2016). Los efectos directos negativos que las parásitas tienen sobre sus hospedadores han sido más estudiados en sistemas forestales, donde los muérdagos causan pérdidas económicas importantes, como lo son en plantaciones de coníferas en EEUU y Europa y, por tanto, es posible que este sea uno de los motivos por el cual las regiones Neotropicales han sido menos estudiadas en ese sentido (Arruda *et al.*, 2012; Watson, 2017). Por otro lado, también se desconocen las consecuencias que podría tener la biomasa local que representan los muérdagos, dado que a pesar de ser irrelevante en comparación a toda la biomasa regional (Fadini *et al.*, 2020), podrían favorecer el proceso de reciclaje de nutrientes en el suelo, teniendo consecuencias ecológicas importantes mediante efectos indirectos tritróficos en toda la comunidad.

Muérdago hemiparásito – Eucalipto: Bosque templado

Sin duda uno de los sistemas muérdago–hospedador mejor estudiados hasta el momento es el formado por el muérdago *Amyema miquelii* (Loranthaceae) y su hospedador *Eucalyptus* sp. en los bosques templados de Australia (Shaw *et al.*, 2004, y referencias allí incluidas). Como es común, los primeros estudios dedicados a esta hemiparásita se enfocaron en su morfología, anatomía y distribución (Shaw *et al.*, 2004; Ward, 2005). También se han explorado las relaciones hídricas, de gases y nutricionales que se dan en el intercambio de flujo a través del haustorio en esta interacción parásito-hospedador (Küppers, 1992; Küppers *et al.*, 1992, 1993), e incluso se ha explorado el perfil químico de los aceites esenciales en diferentes especies del género *Amyema* (Preston *et al.*, 2010). Sin embargo, aunque *A. miquelii* causa una reducción en el crecimiento, biomasa del follaje y supervivencia del hospedador actuando como competidor directo (Reid *et al.*, 1994), las respuestas de éste desde un punto de vista fenotípico a nivel químico siguen siendo un misterio.

Más recientemente, la mayoría de investigaciones sobre la ecología de *A. miquelii* se han centrado en el rol como recurso clave (Watson, 2009, Fig. 8.2). *Amyema miquelii* provee tejido vegetativo a insectos especialistas y mamíferos arborícolas (Watson, 2009; Burns *et al.*, 2011, 2014), néctar y polen a aves nectarívoras generalistas (Watson, 2009), frutos y semillas a aves frugívoras especialistas (Watson, 2009; Napier *et al.*, 2014), así como como refugio o lugar de anidación a aves y mamíferos (Cooney *et al.*, 2006; Watson, 2009). Derivado de los estudios enfocados en aves dispersoras de semillas, se han analizado también los patrones de dispersión, germinación y establecimiento de semillas (Yan & Reid, 1995; Ward & Paton, 2007; Hume & Schmidt, 2018).

También se ha analizado la interacción muérdago–suelo, encontrando un enriquecimiento local del suelo y reciclaje de nutrientes a través de la caída de la hojarasca (March & Watson, 2007, 2010). Como consecuencia, *A. miquelii* causa un efecto indirecto como facilitador en la comunidad herbácea bajo la copa de los árboles parasitados (March, 2007; March & Watson, 2007), así como un incremento en la abundancia y diversidad de artrópodos asociados a esta hojarasca (Mellado *et al.*, 2019). Sin embargo, se desconoce en qué medida se ven modificadas las propiedades químicas y biológicas del suelo gracias a la caída, no solo de la hojarasca, flores, frutos y ramas del sistema muérdago–hospedador, sino también por el aporte de excrementos y restos por parte de aves visitantes. Finalmente, también se ha descrito una interacción tritrófica entre el muérdago *A. miquelii* y la comunidad de aves insectívoras (Watson, 2009, 2015), probablemente atraídas por el incremento de la comunidad de artrópodos tanto en el dosel forestal como en el suelo.

Así pues, esta hemiparásita genera multitud de nuevas interacciones planta–animal, directas e indirectas, en el dosel forestal que han sido ampliamente exploradas, favoreciendo la heterogeneidad y diversidad del ecosistema donde habitan (Watson, 2001, 2009; Griebel *et al.*, 2017). Este sistema es uno de los mejores estudiados (Fig. 8.2); sin embargo, si lo comparamos con la Fig. 8.1, vemos cómo las respuestas del hospedador a nivel químico y las interacciones tritróficas, mediadas por el hospedador, con los consumidores primarios, permanecen aún sin explorar, siendo un campo disponible para futuras investigaciones.

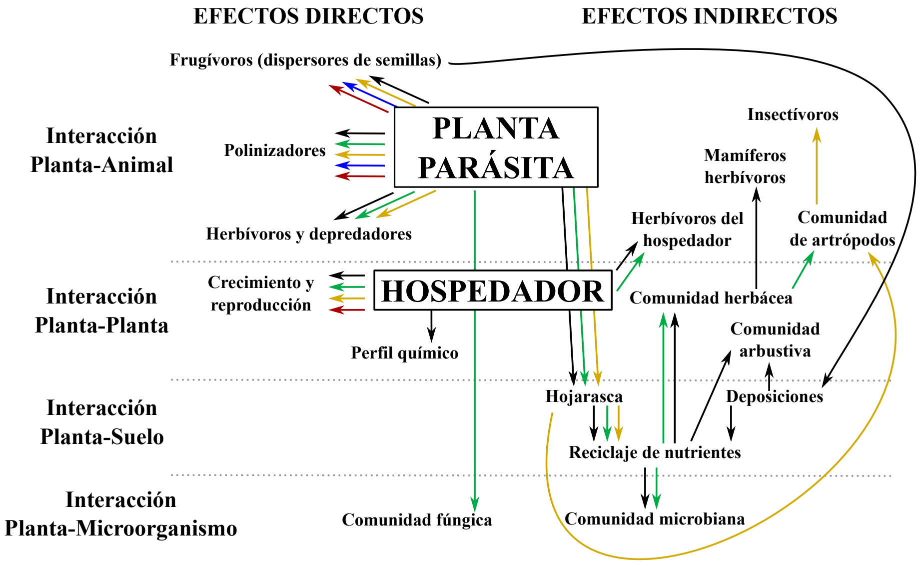

Figura 8.2: Esquema conceptual del conjunto de interacciones descritas en sistemas parásito-hospedador alrededor del mundo donde las plantas parásitas son *Viscum album* subsp. *austriacum* (flechas negras), *Trysterix aphyllus* (flechas rojas), *Psittacanthus* sp. (flechas azules), *Amyema miquelii* (flechas amarillas) y *Rhinanthus minor* (flechas verdes).

Hemiparásita de raíz – Herbáceas: Pastizales

Además de hemiparásitas aéreas como los muérdagos, encontramos hemiparásitas de raíz como el caso de *Rhinanthus minor* (Scrophulariaceae), una herbácea estival anual que parasita a varias especies de hospedadores, generalmente gramíneas y leguminosas, y ha sido especialmente estudiado en los pastizales de las islas británicas. Su morfología, fenología y distribución se han estudiado exhaustivamente, así como las respuestas a factores bióticos y abióticos (Westbury, 2004, y referencias allí incluidas), parte del perfil químico (e.g. contenido en N) y efectos en su reproducción (e.g. número de semillas y frutos), que varían en función de su planta hospedera (Westbury, 2004, y referencias allí incluidas).

Rhinanthus minor juega también el papel de recurso alimenticio (efectos *bottom–up*) para una comunidad fúngica y de artrópodos herbívoros que se alimentan en diferentes partes de la planta (Fig. 8.2), así como para el pastoreo del ganado (Westbury, 2004, y referencias allí incluidas). También se han estudiado las relaciones directas planta-animal en el sistema de polinización, siendo abejas y abejorros sus principales visitantes florales (Westbury, 2004; Natalis & Wesselingh, 2012). Por otro lado, a pesar de que se ha analizado la dispersión de semillas, generalmente distancias cortas mediadas por viento y con un banco de semillas temporal (Bullock *et al.*, 2003; Westbury, 2004), se desconoce si existen relaciones de depredación y/o transporte de semillas mediada por granívoros como hormigas.

También se han estudiado las relaciones fisiológicas que se dan a través del haustorio mediante una conexión xilema–xilema del parásito con el hospedador, donde se produce un intercambio de agua y compuestos orgánicos en beneficio del parásito (Westbury, 2004, y referencias allí incluidas). Esto causa un efecto directo *top-down* de *R. minor* en sus hospedadores, siendo capaz de reducir su productividad, biomasa y contenido hídrico actuando como enemigo natural (Press & Phoenix, 2005). Esto, a su vez, tiene efectos indirectos sobre los herbívoros que comparten una misma planta hospedadora, generando así una interacción tritrófica positiva para algunos consumidores y negativa para otros (Bass *et al.*, 2010; Ewald *et al.*, 2011, Fig. 8.2). Sin embargo, se desconoce el motivo por el cual los herbívoros que comparten la planta hospedadora se ven afectados. Para las futuras investigaciones que pueden dirigirse en este sentido, resultaría muy útil el estudio del set completo de compuestos químicos del sistema parásito–hospedador y de plantas hospedadoras no parasitadas, para dilucidar los posibles efectos directos que *R. minor* causa en sus hospedadores a nivel químico, y qué compuestos son los que acabarían afectando a sus consumidores primarios.

En este sistema se ha explorado también la relación planta–suelo y los efectos indirectos de *R. minor* en el proceso ecosistémico de reciclaje de nutrientes (Fig. 8.2), donde se demuestra un incremento de la disponibilidad de N en el suelo (Quested *et al.*, 2003; Ameloot *et al.*, 2008; Bardgett *et al.*, 2006), afectando a su vez a la abundancia de micorrizas arbusculares de algunos hospedadores (Davies & Graves, 1998), la descomposición de la hojarasca y la comunidad microbiana del suelo (Bardgett *et al.*, 2006). Sin embargo, una de las relaciones más estudiadas en este sistema radica en la interacción tritrófica planta–planta–animal, abarcando el impacto de *R. minor* no sólo en sus hospedadores y herbívoros sino en toda la comunidad herbácea y de invertebrados en la que habita, describiendo interacciones de hasta cuarto nivel en la cadena trófica (Hartley *et al.*, 2015). Estos estudios demuestran cómo *R. minor* ejerce un papel fundamental en la estructura y dinámica de toda la comunidad herbácea (Ameloot *et al.*, 2005; Bardgett *et al.*, 2006), teniendo fuertes efectos indirectos y positivos en toda la comunidad de invertebrados (e.g. detritívoros, herbívoros y depredadores, Hartley *et al.*, 2015).

El muérdago en el ecosistema: ¿elemento colateral o artífice protagonista?

Los sistemas muérdago–hospedador se han estudiado mayoritariamente enfocándose en la ecología de la polinización, frugivoría y dispersión de semillas del parásito, seguido de los efectos directos y negativos del parásito sobre el desarrollo y crecimiento de su hospedador (Fig. 8.2). Como hemos visto, también se han explorado en algunos de los ecosistemas, aunque en menor medida, el rol de las plantas parásitas como facilitadoras en la dinámica de nutrientes del suelo y su impacto en la comunidad herbácea. Por el contrario, las respuestas de los hospedadores desde el punto de vista químico son prácticamente desconocidas en todos los sistemas, así como sus consecuencias en consumidores primarios y secundarios de la planta hospedera (Fig. 8.2). Por tanto, mientras que otros sistemas parásito–hospedador se encuentran parcialmente estudiados, el sistema formado por el muérdago europeo (*Viscum album*) y el pino (*Pinus nigra*) en un ecosistema mediterráneo se convierte en el más completamente explorado, abarcando el mayor número de interacciones directas e indirectas, tanto horizontal como verticalmente en la cadena trófica. No sólo hemos avanzado en el conocimiento de las interacciones directas del sistema muérdago–pino, sino que hemos dado un paso más llegando a interacciones de tercer y cuarto grado que afectan al nivel de comunidad. Sin embargo, no podemos decir que este sistema esté estudiado por completo, ya que podrían existir otras interacciones indirectas impactando, por ejemplo, en la comunidad de artrópodos del suelo, aves insectívoras que dependen de los artrópodos que habitan en el suelo y/o en el dosel forestal, y aves granívoras que se alimentan de semillas del pino.

La visión global del conjunto de roles que las plantas parásitas en general, y los muérdagos en particular pueden presentar, nos permite afirmar que no sólo los papeles que juegan son comunes en todos los sistemas parásito–hospedador, sino también los procesos y patrones ecosistémicos que desencadenan. Lejos de pensar en las plantas parásitas como simples patógenos para sus hospedadores, éstas se convierten en verdaderas ingenieras de ecosistemas, generando un balance positivo en su impacto neto, al promover el incremento de la biodiversidad y complejidad del ecosistema.

Conclusiones / Conclusions

Conclusiones generales

1. El muérdago europeo, *Viscum album* subsp. *austriacum*, en un pinar mediterráneo, es capaz de jugar diferentes roles simultáneamente generando nuevas interacciones en el ecosistema forestal. El muérdago representa un nuevo recurso en el dosel forestal que alberga una comunidad de artrópodos especialista y estable, independiente de la del pino hospedador, compuesta por dos herbívoros (*Cacopsylla visci* y *Pinalitus viscicola*) y un depredador (*Anthocoris visci*), siendo a su vez la más simple y meridional de toda Europa.

2. Esta comunidad especialista se rige por una regla de ensamblaje determinista y constante bajo diferentes factores ecológicos, como el gradiente altitudinal, el tamaño de la planta donde habitan y la variación temporal. Así pues, siguiendo una secuencia trófica de colonización, el herbívoro principal y más abundante, *C. visci*, es el primero en colonizar la planta de muérdago, seguido de *P. viscicola* y el depredador *A. visci*.

3. Además de representar un nuevo recurso alimenticio para esta nueva comunidad de herbívoros y depredadores, el muérdago ofrece recompensas florales y ejerce como atrayente de un gremio de visitantes florales generalistas tanto diurnos como nocturnos. La floración del muérdago se convierte en un recurso espacio–temporalmente único, dado que se sitúa en el dosel forestal y florece en un periodo muy temprano, donde y cuando no se encuentran otros recursos florales.

4. Así pues, el muérdago representa una isla ecológica para la nueva entomofauna especialista y generalista, actuando como una especie fundadora secundaria, con efectos directos en el incremento de la diversidad y abundancia de consumidores primarios y secundarios.

5. Los pinos hospedadores reaccionan desde el punto de vista químico frente al parasitismo del muérdago, poniendo así de manifiesto su papel como competidor directo. Estas respuestas son proporcionales al grado de parasitación, aumentando compuestos de defensa como fenoles, taninos y monoterpenos, y disminuyendo el contenido en N, sesqui– y diterpenos (compuestos costosos) en pinos altamente parasitados.

6. Las respuestas químicas del pino no sólo se dan en compuestos de alto peso molecular, sino al nivel metabolómico, modificando una cuarta parte de todo su metaboloma. El muérdago, además de representar un sumidero constante de metabolitos para el hospedador, tiene un impacto sistémico y permanente en toda la copa del pino, generando así una nueva identidad metabólica en el dosel forestal.

7. Los efectos directos que el muérdago causa desde el punto de vista químico en el pino convierten sus acículas en un recurso alimenticio de menor calidad para los artrópodos herbívoros asociados, entrando en competencia indirecta con éstos y disminuyendo su supervivencia. Así pues, el muérdago actuaría como control biótico generando interacciones indirectas no tróficas mediadas por el pino hospedador, que benefician al pino al atenuar la presión por herbivoría de sus consumidores.

8. Los muérdagos inician un nuevo proceso de facilitación en el establecimiento de especies herbáceas mediante la creación de una isla de fertilidad bajo la copa de los pinos parasitados, a través de la caída de la hojarasca y restos orgánicos. Se genera así un incremento en la diversidad y riqueza de especies de la comunidad herbácea local, atrayendo a su vez a mamíferos herbívoros, como el conejo, que podrían reforzar la heterogeneidad ambiental con su actividad.

9. En conclusión, el muérdago es el detonante de un cóctel de interacciones con importantes consecuencias, dejando una huella ecológica muy patente, con un balance neto positivo. Dejamos de percibir los muérdagos como simples patógenos y empezamos a desarrollar una visión integradora de todas sus interacciones, erigiéndose en ingenieros de ecosistemas capaces de promover el incremento en la heterogeneidad, biodiversidad y complejidad del ecosistema forestal.

General conclusions

1. The European mistletoe, *Viscum album* subsp. *austriacum*, inhabiting a Mediterranean pine forest, plays simultaneously different roles, generating new interactions in the forest ecosystem. The mistletoe represents a new resource in the canopy forest, harboring a stable and specialist arthropod community independent of the host pine, composed mainly of two herbivores (*Cacopsylla visci* and *Pinalitus viscicola*) and a predator (*Anthocoris visci*), this community being the simplest and southernmost in Europe.

2. This specialist community follows a deterministic and constant assemblage rule under different ecological factors, such as elevational gradient, mistletoe plant size, and temporal variation. Therefore, following a trophic sequence of colonization, the main and the most abundant herbivore, *C. visci*, is the first specialist colonizer of the mistletoe plant, and is followed by *P. viscicola* and the specialist predator *A. visci*.

3. In addition to representing a new food resource for this new specialist community, mistletoe offers floral rewards and serves as an attractor for a guild of generalist floral visitors both diurnal and nocturnal. The mistletoe flowering period provides a spatiotemporally unique resource, given that it is located in the forest canopy and blooms in an early period, when the floral market is absent there.

4. In this sense, the mistletoe represents an ecological island for the new arthropofauna, both specialist and generalist, acting as a secondary foundation species boosting the diversity and abundance of primary and secondary consumers.

5. Host pines react chemically to mistletoe parasitism, thus highlighting their role as a direct competitor. This response is proportional to parasitic load, increasing defense compounds such as phenols, tannins, and monoterpenes, and decreasing the N–content as well as sesqui– and diterpenes (costly compounds) in highly parasitized pines.

6. Chemical responses of host pines occur not only in high–molecular–weight compounds, but at the metabolomic level, modifying a quarter of its entire metabolome. Mistletoe represents a constant sink of metabolites for the host pine and has a permanent and systemic impact in the pine canopy, thus generating a new plant metabolic identity in the forest canopy.

7. The direct effects that mistletoe exerts on the pine chemical profile convert their needles into a lower–quality resource for their arthropod herbivores. Thus, mistletoe indirectly competes with pine–feeding herbivores and reduces their survival. Therefore, the mistletoe acts as an agent of biotic control generating non–trophic and trait–mediated indirect interactions, which benefit the host pine by softening the herbivory pressure of its consumers.

8. Mistletoe triggers a new facilitation process in the establishment of herbaceous species by creating an island of fertility under parasitized pines through the fall of leaf litter and organic matter. This process boosts diversity and species richness in the local herbaceous community, which in turn attracts mammal herbivores such as rabbits that could reinforce the environmental heterogeneity with their activity.

9. In conclusion, the mistletoe triggers interactions that have far–reaching consequences, leaving a major ecological footprint with a positive net balance. Thus, rather than a perception of mistletoe as a simple parasite, an integral view develops of overall range of mistletoe interactions, making this plant an ecosystem engineer capable of augmenting the heterogeneity, biodiversity, and complexity of forest ecosystem.

Appendices

Supporting Information Chapter 1

Table S1: Details of the measurements of a) mistletoe plants and b) pine tree host branches collected from Black pine (n=10) and Scots pine (n=10).

(a) Mistletoe plants

Host species	Wet weight (g)	Basal diameter (mm)	Lenght (cm)	Width (cm)	Sex	Age (years)
Pinus nigra	0.30	14.90 x 16.70	32	45	Female	10
	0.28	20.60 x 19.00	35	35	Female	9
	1.10	23.40 x 28.60	48	80	Male	16
	0.30	15.40 x 14.50	34	37	Female	9
	0.17	15.80 x 20.42	30	33	Female	10
	0.27	14.54 x 15.62	29	46	Male	10
	0.22	17.75 x 15.17	37	35	Female	9
	0.23	13.57 x 15.72	42	51	Female	11
	0.59	27.98 x 23.91	49	56	Male	14
	0.31	26.98 x 21.71	51	47	Female	23
Pinus sylvestris	3.45	52.10 x 42.40	98	65	Female	24
	0.58	31.98 x 24.62	43	63	Female	14
	3.04	31.40 x 44.20	120	67	Female	30
	0.90	19.40 x 23.60	58	60	Male	19
	0.87	25.00 x 30.00	49	55	Female	20
	0.14	15.48 x 16.19	30	42	Female	11
	0.23	19.85 x 17.85	49	50	Female	14
	0.37	20.54 x 19.30	32	65	Female	15
	1.39	39.64 x 32.34	102	88	Female	21
	0.55	17.21 x 17.01	70	67	Female	9

(b) Pine tree host branches

Host species	Wet weight (g)	Basal diameter (mm)	Lenght (cm)	Width (cm)	Height tree (cm)	DBH tree (cm)
Pinus nigra	0.40	16.00 x 16.80	75	43	8.1	117
	0.47	18.90 x 19.80	70	40	8.1	155
	0.71	22.70 x 21.70	150	60	7.7	95
	0.31	16.22 x 16.06	52	57	5.9	92
	0.26	17.40 x 17.81	66	54	8.5	89
	0.24	15.71 x 14.42	50	33	6.8	71
	0.18	12.41 x 12.33	66	37	4.7	50
	0.25	14.46 x 15.20	58	41	7.2	56
	0.59	21.59 x 21.54	96	87	7.2	115
	0.47	16.67 x 15.45	100	58	9.4	138
Pinus sylvestris	1.15	21.20 x 21.40	85	60	7.2	125
	0.48	19.04 x 19.29	59	40	9.9	124
	1.33	25.70 x 26.10	98	63	9.0	105
	0.71	19.00 x 19.00	70	35	6.3	118
	0.76	21.08 x 20.01	85	60	7.2	112
	0.19	17.47 x 14.67	48	25	7.2	120
	0.64	20.80 x 20.55	96	53	7.2	145
	0.34	16.19 x 15.73	73	51	8.5	149
	1.10	25.86 x 5.64	99	51	8.9	138
	0.55	18.87 x 18.57	91	51	8.9	138

Table S2: Taxonomic composition (at the ordinal, subordinal, family, subfamily, genus and species level), trophic level and relative abundance of the arthropods communities on mistletoe and its hosts (n=10 for each host species).

Order	Taxonomic group	Trophic level	Community inhabiting mistletoe		Community inhabiting pines	
			P. nigra	*P. sylvestris*	*P. nigra*	*P. sylvestris*
Coleoptera	SubFam. Alticinae	Herbivour	3.5	3.3	0.0	1.4
Hemiptera	*Cacopsylla visci*		42.1	32.0	0.0	0.0
Hemiptera	*Cinara pini*		0.5	0.0	10.2	0.3
Hemiptera	Fam. Coreidae		0.0	0.0	0.7	0.4
Coleoptera	Fam. Curculionidae		0.4	0.0	0.0	0.5
Hemiptera	*Eulachnus* sp.		0.0	0.0	8.9	0.5
Hemiptera	*Holcogaster* sp.		0.0	0.0	2.4	0.8
Hemiptera	Fam. Cicadellidae		1.7	0.9	6.2	12.1
Lepidoptera	Lepidoptera larvae		0.5	0.0	0.5	0.1
Hemiptera	*Ligaeus militaris*		0.0	0.0	0.7	0.8
Hemiptera	Fam. Margarodidae		0.0	0.0	0.0	0.2
Hemiptera	SuperFam. Miroidea		0.0	0.0	1.2	2.2
Hemiptera	*Pinalitus viscicola*		8.7	12.4	0.0	0.2
Hemiptera	Fam. Psyllidae		0.0	0.0	9.3	30.8
Thysanoptera	Fam. Thripidae		0.0	1.3	20.0	9.4
Hemiptera	*Anthocoris visci*	Predator	27.6	35.2	0.6	0.1
Araneae	O. Araneae		9.1	7.1	27.8	18.6
Coleoptera	Fam. Carabidae		0.9	0.3	0.0	0.4
Coleoptera	Fam. Coccinelidae		0.7	0.4	2.4	5.4
Dermaptera	Fam. Forficulidae		0.0	0.0	0.0	0.0
Hemiptera	SubO. Heteroptera		0.0	0.0	0.0	0.3
Neuroptera	Neuroptera larvae		0.0	0.5	0.0	0.6
Pseudoscorpionida	O. Pseudoscorpionida		0.0	3.2	4.4	3.5
Hemiptera	Fam. Reduviidae		0.0	0.1	0.0	1.3
Dictioptera	SubO. Blattodea	Omnivour	0.0	0.0	0.0	0.1
Diptera	SubO. Brachycera		0.0	1.2	0.0	0.4
Hymenoptera	SuperFam. Chalcidoidea		1.1	0.1	0.6	0.3
Hymenoptera	Fam. Formicidae		0.6	0.2	0.5	0.2
Orthoptera	Fam. Gryllidae		0.0	0.0	0.3	0.0
Hymenoptera	SuperFam. Ichneumonoidea		1.3	1.7	3.2	7.9
Lepidoptera	O. Lepidoptera		0.5	0.0	0.0	0.0
Diptera	SubO. Nematocera		0.7	0.0	0.0	0.3
Psocoptera	O. Psocoptera		0.0	0.0	0.0	0.1
SPECIES RICHNESS			16	18	18	29

Supporting Information Chapter 2

Table S3: Details of the measures associated with mistletoes plants and branches of pine tree hosts across the altitudinal gradient.

	Mistletoe plants				Branch of pine tree hosts					
Altitude	Wet weight (g)	Height (cm)	Width (cm)	Length (cm)	Wet weight (g)	Height (cm)	Width (cm)	Length (cm)	Pine DBH (cm)	Pine height (m)
1300	277.2	51	34	37	282.7	62	50	26	76	6.4
	240.2	38	37	31	319.9	64	35	38	71	7.1
	143.7	37	24	30	309.1	68	43	36	90	6.8
	296.9	47	37	35	270.4	56	36	31	56	7.1
	183.8	32	31	40	300.6	49	47	31	96	8.2
	126.4	39	42	32	184.8	57	35	24	101	8.5
	151.8	46	33	30	135.1	49	33	18	98	7.0
	183.2	34	42	25	294.0	57	44	30	80	7.3
	152.6	35	26	31	249.7	57	49	22	94	7.8
	159.3	35	27	22	241.9	58	33	35	90	7.7
1400	166.4	43	25	44	360.5	61	51	40	123	7.7
	141.4	47	28	32	224.7	56	30	19	78	6.8
	193.2	42	36	26	318.5	59	41	26	103	6.6
	278.1	49	46	36	243.3	67	28	27	124	6.8
	197.3	39	36	25	348.0	64	44	23	92	8.2
	264.5	45	47	28	213.4	64	37	33	100	6.0
	216.3	35	41	32	316.0	65	49	32	117	7.0
	239.8	39	35	18	398.8	62	40	19	60	5.8
	208.8	50	43	27	198.7	54	42	19	83	6.5
	176.8	42	24	28	260.8	57	49	34	113	8.0
1500	376.5	53	45	40	371.6	64	43	30	116	10.0
	746.6	56	52	55	287.0	48	22	25	49	6.3
	305.9	43	32	23	259.0	58	36	21	73	5.6
	160.2	38	31	29	266.1	63	37	18	73	7.3
	313.7	43	28	37	314.9	82	26	22	96	8.5
	178.8	44	25	32	444.4	61	43	23	85	7.7
	257.6	50	37	29	280.6	87	35	31	81	7.7
	247.3	50	42	28	358.9	65	42	33	67	8.5
	229.9	43	27	37	317.6	62	39	28	53	7.1
	381.3	73	45	28	477.3	60	57	38	89	7.1
1600	238.3	57	33	29	253.0	58	40	21	65	4.3
	237.4	61	27	42	207.1	57	38	19	94	5.3
	221.3	47	35	32	370.6	57	43	29	79	5.3
	111.0	52	31	20	179.9	58	30	18	94	5.1
	230.4	60	34	43	238.1	62	26	24	76	5.4
	282.0	52	41	35	229.2	50	41	17	66	5.3
	234.9	41	37	26	328.5	55	36	30	53	5.1
	187.8	56	44	19	222.6	60	21	23	47	4.8
	128.8	20	42	37	241.7	37	62	30	53	5.8
	256.3	44	47	23	338.4	47	41	26	83	6.3
1700	269.7	47	43	25	215.4	49	29	17	49	4.9

Continued on next page

Table S3 – *Continued from previous page*

	Mistletoe plants				Branch of pine tree hosts					
Altitude	Wet weight (g)	Height (cm)	Width (cm)	Length (cm)	Wet weight (g)	Height (cm)	Width (cm)	Length (cm)	Pine DBH (cm)	Pine height (m)
1700	504.4	51	35	39	235.2	41	29	18	60	4.9
	170.7	44	28	21	363.3	56	47	27	60	6.3
	115.9	45	31	26	428.5	45	35	28	79	6.6
	180.3	52	37	24	280.2	54	37	27	44	5.3
	274.9	52	34	27	409.0	53	48	34	54	5.4
	144.9	43	46	20	269.4	51	33	24	53	5.1
	563.8	56	50	31	184.3	46	36	33	51	4.6
	245.8	52	38	33	173.4	47	35	19	56	4.8
	229.5	44	29	26	143.1	35	23	25	63	5.4
1800	218.4	35	51	27	84.2	32	40	17	126	9.4
	1041.3	65	40	54	206.1	54	39	30	67	6.8
	226.7	53	36	20	217.8	34	51	28	63	6.8
	495.0	59	47	43	211.8	61	36	15	119	7.3
	226.3	45	41	36	247.8	52	38	18	80	7.7
	336.7	51	37	38	245.5	56	30	17	74	7.7
	159.0	43	31	41	258.6	56	48	21	59	6.1
	213.5	45	47	18	182.1	37	43	19	37	5.3
	604.2	64	60	43	232.0	49	24	28	61	5.4
	454.5	58	36	42	322.1	51	29	25	72	7.1
1900	490.3	46	34	40	395.2	36	37	24	55	5.1
	249.3	62	37	30	303.1	43	35	19	43	4.9
	320.6	62	43	32	217.1	39	19	47	135	7.7
	487.7	62	29	40	292.1	54	32	18	59	6.0
	405.7	56	39	32	223.6	52	38	19	59	6.3
	154.1	42	34	30	195.1	45	28	21	135	6.8
	202.7	52	35	28	299.5	44	47	21	45	3.7
	368.9	60	42	27	315.9	51	19	24	48	4.9
	534.6	59	56	47	258.4	42	31	18	46	4.3
	632.8	59	40	46	221.6	44	31	13	44	5.1
2000	444.1	51	37	30	368.2	51	32	24	53	4.1
	107.2	25	16	36	246.4	52	41	21	32	2.4
	555.1	57	46	32	280.1	51	23	22	54	3.9
	627.9	56	41	39	202.3	58	23	18	44	4.4
	272.5	55	47	28	268.8	49	29	16	57	3.4
	136.4	39	36	26	247.5	39	48	22	50	3.6
	256.1	46	29	31	263.7	48	30	19	62	4.6
	129.2	31	28	22	261.2	52	37	18	57	5.3
	102.6	44	16	26	289.8	16	45	21	56	3.7

Appendix S1. Predicting procedure for number of branches (NB) and volume (PV) by mistletoe plant.

Mistletoe plants, especially large ones, are usually tangled among the branches of the pine host. This hampers the observation of the entire mistletoe plant, and thus the counting of the total number of branches (NB) and the calculation of the plant volume (PV). However, plant age is easily determined, and at the same time, it is highly correlated with NB and PV because of their annual dichasial branching pattern (Zuber, 2004). *Viscum album* branches firstly form a fan shape and then grows one short and one long internode and one pair of scale and foliage leaves per year. In this way, mistletoes annually increase their size, forming a globe (Zuber, 2004). Thus, in order to estimate the NB and PV of our focal mistletoes in this study, we selected 51 mistletoes, aged 1–30 years old, in nearby pine hosts and were cut off at the base. Then, we recorded their age by counting their internodes (one per year, Zuber, 2004), the NB, and measured the PV (height, width, length; Table S4).

Firstly, we found the best model adjustment for NB–age correlation. The analyses revealed that a second–degree polynomial, with constant 0, was the model most highly correlated with plant age (R^2=0.88, ANOVA: F_{age}= 352.62, P_{age}<0.001; F_{age^2}= 352.62, P_{age^2}<0.001, Fig. S1). Therefore, NB of focal mistletoe was predicted using the following equation:

$$\hat{y}_i = 0.2128x_i + 0.0360x_i^2 + \hat{\varepsilon}_i$$

$$where\ \varepsilon \sim N(0, 3.93^2)$$

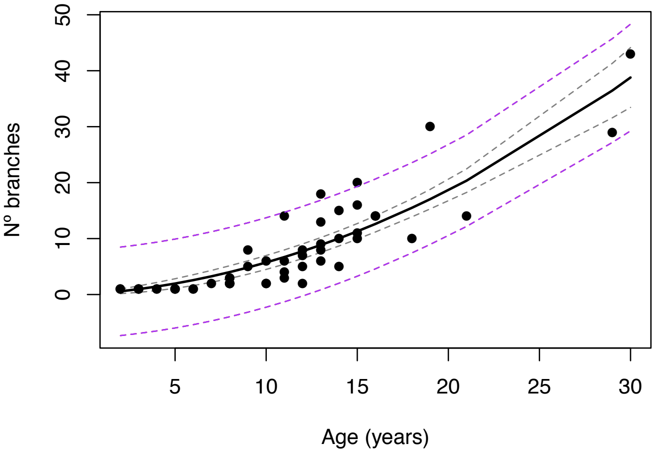

Figure S1: Observed values (points) of number of branches and plant age (years old) of each cut mistletoe plant. The broken lines indicate 95% confidence interval (purple for individual and grey for population) and solid line indicates the regression line according to the polynomial model.

Next, NB-predicted values were assigned according to the plant age of the focal mistletoes selected for the study in order to calculate the total arthropod abundance per plant.

Afterwards, measurements of cut mistletoe plants were used to calculate plant size (height, width, length; see Table S4) and the radius of each plant based on the equation for the volume of a sphere ($\frac{3}{4}\ \pi \mathrm{r}^3$). Then, a lineal model with constant 0 proved to be the better model to correlate the radius and age of the plant (ANOVA: F= 1147.6, P<0.001, R^2=0.96, Figure S2), and the radius of focal mistletoe was predicted using the equation:

$$\hat{y}_i = 0.0145x_i + \hat{\varepsilon}_i$$

$$where\ \varepsilon \sim N(0, 0.038^2)$$

Finally, we assigned one measurement–predicted radius to each focal mistletoe according to their age. Thus, plant size of our focal mistletoes was calculated by applying the equation for calculating the volume of a sphere, and this variable was used in statistical analyses of the study.

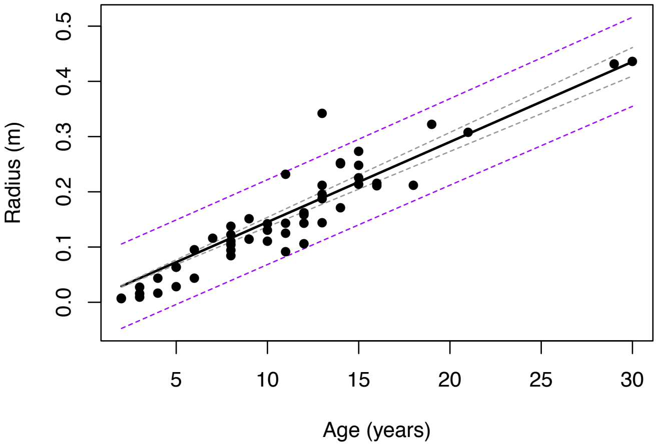

Figure S2: Observed values (points) of the plant radius and age (years old) of each cut mistletoe plant. The broken lines indicate 95% confidence interval (purple for individual and grey for population) and solid line indicates the regression line according to the lineal model.

Table S4: Details of measurements of mistletoe plants selected from outside our focal pines.

Sex	Age (years old)	NB	Height (cm)	Width (cm)	Length (cm)	Size (dm^3)
Male	18	10	43	42	22	39.732
Female	10	6	27	28	16	12.096
Female	14	5	40	25	21	21.000
Female	29	29	85	65	61	337.025
Female	9	8	25	19	13	6.175
Female	12	8	43	23	17	16.813
Female	30	43	72	72	67	347.328
Female	9	5	43	24	14	14.448
Male	8	2	18	14	10	2.520
Female	13	18	59	57	50	168.150
Female	15	11	43	37	40	63.640
Female	13	9	34	34	27	31.212
Female	13	8	35	30	38	39.900
Female	13	13	30	33	28	27.720
-	3	1	3	2,5	2	0.015
Female	15	20	50	44	39	85.800
Male	7	2	25	13	20	6.500
Male	8	3	22	20	13	5.720
Female	21	14	53	46	50	121.900
Male	11	3	21	14	11	3.234
-	3	1	7	4	3	0.084
Female	12	7	25	26	19	12.350
-	6	1	19	16	12	3.648
-	5	1	6	4	4	0.096
Male	8	2	25	14	22	7.700
-	8	3	26	22	19	10.868
-	5	1	12	10	9	1.080
Male	11	4	25	22	15	8.250
Female	10	2	19	20	15	5.700
Male	8	2	20	16	15	4.800
Male	13	6	33	20	19	12.540
Female	11	14	45	40	29	52.200
Male	10	2	26	20	18	9.360
Male	14	15	46	48	30	66.240
Female	14	10	50	41	33	67.650
Male	15	10	34	37	38	47.804
Male	8	2	22	20	8	3.520
Male	12	5	34	24	22	17.952
Female	11	6	26	25	19	12.350
-	2	1	2	1	0,5	0.001
Female	15	16	49	26	32	40.768
Male	16	14	45	29	32	41.760
Male	12	2	20	18	14	5.040
Female	19	30	56	50	50	140.000
Female	16	14	35	33	34	39.270
Male	5	1	12	8	11	1.056
-	4	1	9	8	5	0.360
-	6	1	9	8	5	0.360
-	2	1	2	1,5	0,5	0.002
-	3	1	3,8	2	0,5	0.004
-	4	1	6	2	1,5	0.018

Table S5: Relative abundance of specialist and tourist communities associated with a) mistletoes plants and b) their pine host throughout the elevational gradient in the Natural Park of Sierra de Baza.

(a) Mistletoe plants

Community		Orders	Taxonomic group	1300	1400	1500	1600	1700	1800	1900	2000
Specialists		Hemiptera	*Cacopsylla visci*	59.1	57.7	66.2	80.3	57.6	63.9	67.5	40.5
		Hemiptera	*Pinalitus viscicola*	0.0	0.7	3.1	0.7	0.0	5.2	0.6	4.3
		Hemiptera	*Anthocoris visci*	3.9	5.3	3.4	5.0	6.6	2.2	7.4	0.0
Tourists	Herbivores	Coleoptera	*Alticinae*	0.0	0.0	0.2	0.0	0.0	5.3	0.0	0.0
		Coleoptera	*Anobidae*	0.0	0.0	0.0	1.4	0.0	0.0	0.0	0.0
		Coleoptera	*Curculionidae sp1*	0.0	0.0	0.0	0.0	0.0	0.4	0.0	8.3
		Coleoptera	*Curculionidae sp2*	0.0	0.0	0.0	0.0	0.0	0.0	1.6	8.5
		Hemiptera	*Aphididae*	2.1	0.0	0.2	2.4	1.2	0.0	0.0	1.1
		Hemiptera	*Heteroptera*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		Hemiptera	*Holcogaster*	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0
		Hemiptera	*Leafhopper*	1.1	4.0	0.6	1.3	1.6	2.2	0.0	0.0
		Hemiptera	*Margarodidae*	0.0	0.0	0.4	1.3	0.0	0.0	0.0	0.0
		Hemiptera	*Miroidea*	0.0	0.0	0.0	0.0	0.0	1.3	0.0	0.0
		Lepidoptera	*Larvae*	0.0	0.9	0.0	0.0	0.0	0.0	0.0	0.0
		Thysanoptera	*Thysanoptera*	1.6	0.7	0.0	0.7	5.5	0.4	0.0	5.3
	Predators	Araneae	*Araneae*	14.4	6.4	10.1	1.9	9.9	5.6	4.9	6.5
		Coleoptera	*Cantharidae*	0.0	0.0	1.0	0.0	0.0	0.0	1.0	0.0
		Coleoptera	*Carabidae*	1.2	0.0	1.0	1.6	0.0	0.0	0.0	0.0
		Coleoptera	*Coccinelidae*	0.6	1.5	0.0	0.0	0.0	0.0	0.0	0.0
		Coleoptera	*Coleoptera*	5.1	0.9	1.8	0.0	2.2	0.8	0.0	0.0
		Different orders	Predator larvae	0.6	0.0	1.1	0.0	0.0	1.3	0.6	0.0
		Neuroptera	*Neuroptera*	0.0	0.0	0.3	0.0	1.2	2.1	0.0	0.0
		Pseudoscorpionida	*Pseudoscorpionida*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		Raphidioptera	*Raphidioptera*	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0
	Omnivores	Diptera	*Brachycera*	2.5	1.5	1.1	0.0	0.0	0.9	0.0	0.0
		Diptera	*Nematocera*	0.0	3.4	1.6	0.0	0.0	2.2	0.0	3.0
		Hymenoptera	*Camponotus cruentatus*	2.3	0.0	0.4	0.0	3.1	0.0	0.0	0.0
		Hymenoptera	*Camponotus piceus*	0.0	0.0	0.1	0.0	0.0	0.0	0.0	5.3
		Hymenoptera	*Camponotus pilicornis*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		Hymenoptera	*Crematogaster auberti*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		Hymenoptera	*Formica fusca*	0.0	0.0	0.0	0.0	0.0	0.0	1.1	0.0
		Hymenoptera	*Iberoformica subrufa*	1.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		Hymenoptera	*Lasius cinereus*	0.0	0.0	0.0	0.0	0.0	0.0	11.8	7.7
		Hymenoptera	*Plagiolepis pygmea*	0.0	0.0	0.0	0.0	0.9	0.0	0.0	0.0
		Hymenoptera	*Parasite*	4.2	15.2	6.2	3.4	10.3	5.6	3.4	9.5
		Lepidoptera	*Lepidoptera*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		Orthoptera	*Orthoptera*	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0
		Trombidiformes	*Acarina*	0.0	0.9	0.1	0.0	0.0	0.4	0.0	0.0
		SPECIES RICHNESS		14	14	21	11	11	16	10	11

(b) Pine host

Community		Orders	Taxonomic group	1300	1400	1500	1600	1700	1800	1900	2000
Specialists		Hemiptera	*Cacopsylla visci*	0.0	1.4	8.3	7.1	4.5	28.8	3.9	9.0
		Hemiptera	*Pinalitus viscicola*	0.0	0.0	0.7	0.0	0.0	0.0	0.3	0.0
		Hemiptera	*Anthocoris visci*	2.5	0.0	0.5	3.9	0.0	1.4	0.0	0.0
Tourists	Herbivores	Coleoptera	*Alticinae*	0.0	0.5	0.7	0.0	0.0	0.0	0.0	0.0
		Coleoptera	*Anobidae*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		Coleoptera	*Curculionidae sp1*	0.7	1.4	0.5	3.3	1.3	3.4	1.7	1.3
		Coleoptera	*Curculionidae sp2*	0.0	0.0	0.0	1.3	0.0	2.2	3.0	4.2
		Hemiptera	*Aphididae*	20.3	23.4	3.4	23.9	11.6	4.0	10.7	10.3
		Hemiptera	*Heteroptera*	2.6	2.2	0.0	0.0	0.0	0.0	0.9	0.5
		Hemiptera	*Holcogaster*	2.7	4.5	1.6	2.4	4.5	0.0	1.8	3.4
		Hemiptera	*Leafhopper*	0.0	5.3	10.2	9.6	1.2	1.9	1.6	4.3
		Hemiptera	*Margarodidae*	3.2	3.4	1.2	0.0	2.0	0.0	0.0	0.0
		Hemiptera	*Miroidea*	0.6	1.6	1.3	0.0	6.3	3.4	5.5	6.1
		Lepidoptera	*Larvae*	0.7	0.0	0.4	0.0	0.0	0.0	0.0	0.0
		Thysanoptera	*Thysanoptera*	26.2	1.4	5.1	16.4	10.0	12.1	26.7	33.1
	Predators	Araneae	*Araneae*	18.4	19.9	41.3	19.4	31.0	28.9	19.5	11.8
		Coleoptera	*Cantharidae*	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.0
		Coleoptera	*Carabidae*	0.5	3.8	0.0	0.0	0.0	0.0	0.0	0.0
		Coleoptera	*Coccinelidae*	1.3	1.0	1.7	0.6	1.8	4.9	2.1	5.2
		Coleoptera	*Coleoptera*	3.5	1.6	7.2	1.1	0.5	1.5	0.9	0.0
		Different orders	Predator larvae	7.8	4.4	0.7	6.8	6.5	0.0	0.9	0.0
		Neuroptera	*Neuroptera*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		Pseudoscorpionida	*Pseudoscorpionida*	0.6	4.0	1.3	0.0	0.0	0.0	0.4	0.0
		Raphidioptera	*Raphidioptera*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Omnivores	Diptera	*Brachycera*	0.0	1.2	1.0	0.9	0.0	1.5	0.9	0.0
		Diptera	*Nematocera*	0.0	0.0	3.7	0.9	0.0	0.0	0.0	0.0
		Hymenoptera	*Camponotus cruentatus*	2.8	8.6	0.0	1.6	7.5	0.0	0.0	0.0
		Hymenoptera	*Camponotus piceus*	0.0	0.9	0.0	0.0	0.7	0.0	0.0	4.3
		Hymenoptera	*Camponotus pilicornis*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5
		Hymenoptera	*Crematogaster auberti*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.3
		Hymenoptera	*Formica fusca*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		Hymenoptera	*Iberoformica subrufa*	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		Hymenoptera	*Lasius cinereus*	0.0	5.5	0.0	0.0	2.3	0.0	14.2	0.6
		Hymenoptera	*Plagiolepis pygmea*	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0
		Hymenoptera	*Parasite*	0.5	1.4	8.1	0.9	5.2	4.5	4.1	3.3
		Lepidoptera	*Lepidoptera*	0.0	1.4	0.0	0.0	0.0	0.0	0.4	0.0
		Orthoptera	*Orthoptera*	0.0	0.7	1.0	0.0	2.2	0.0	0.0	0.0
		Trombidiformes	*Acarina*	5.0	0.7	0.0	0.0	0.5	1.2	0.4	0.0
SPECIES RICHNESS				19	24	22	16	19	14	20	16

Table S6: Relative biweekly abundance of specialist and tourist community on mistletoe plant over summer (J1: early July, J2: mid–July, A1: early August, A2: mid–August, S1: early September, and S2: mid–September) and a range of mistletoe sizes.

Community		Order	Taxonomic group	Sampling period (biweekly)						Mistletoe size (m^3)				
				J1	J2	A1	A2	S1	S2	<0.001	0.001 -0.01	0.01 -0.05	0.05 -0.15	>0.15
Specialists		Hemiptera	*Cacopsylla visci*	57.76	34.65	32.86	27.42	21.44	20.93	21.43	51.60	41.14	38.09	29.55
		Hemiptera	*Pinalitus viscicola*	0.00	0.98	0.00	0.56	0.00	0.87	0.00	0.00	1.26	0.56	0.00
		Hemiptera	*Anthocoris visci*	1.57	2.60	3.65	4.37	2.43	5.90	0.00	4.41	5.27	3.27	2.52
Toursits	Herbivores	Coleoptera	*Alticinae*	0.00	1.35	0.00	0.00	0.00	0.00	0.00	0.00	0.63	0.51	0.00
		Hemiptera	*Aleyrodidae*	0.19	4.19	0.00	4.55	5.07	0.00	7.14	5.18	2.28	2.10	2.30
		Hemiptera	*Aphididae*	1.28	0.00	0.00	1.75	0.00	2.35	0.00	0.00	1.02	1.89	0.00
		Hemiptera	*Coccidae*	1.01	1.68	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.61	0.65
		Hemiptera	*Leafhopper*	2.71	4.80	0.00	0.00	4.41	0.00	0.00	0.00	1.26	1.84	2.69
		Lepidoptera	*Larvae*	0.00	0.98	0.00	1.83	0.00	0.00	0.00	0.00	0.00	0.56	0.59
		Thysanoptera	*Thysanoptera*	2.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.22	0.00
	Predators	Araneae	*Araneae*	7.62	10.77	32.45	16.10	35.73	31.74	7.14	7.83	14.91	23.69	21.66
		Coleoptera	*Coccinelidae*	0.77	0.00	0.00	0.00	1.98	0.00	0.00	0.00	0.00	0.46	0.59
		Coleoptera	*Coleoptera*	2.90	3.18	0.00	2.59	2.00	0.00	21.43	3.46	1.50	1.74	1.90
		Dermaptera	*Dermaptera*	0.00	0.00	0.00	0.00	0.26	0.00	0.00	1.73	0.00	0.00	0.00
		Hemiptera	*Heteroptera*	0.00	2.66	0.62	2.17	0.49	0.87	0.00	3.23	2.51	0.71	0.96
		Mantodea	*Mantidae*	0.00	0.00	0.00	0.98	0.00	0.00	0.00	0.00	0.00	0.46	0.00
		Different orders	Predator larvaes	0.00	0.63	3.32	0.00	0.00	0.00	0.00	0.00	0.87	0.00	1.31
		Neuroptera	*Neuroptera*	0.93	1.25	3.17	0.00	0.00	0.00	0.00	0.00	0.00	3.11	0.00
		Raphidioptera	*Raphidioptera*	0.77	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.46	0.00
	Omnivores	Dictyoptera	*Blattodea*	2.46	0.00	0.00	0.00	3.24	0.00	0.00	0.96	0.00	0.51	1.58
		Diptera	*Brachycera*	11.66	14.57	9.09	26.22	9.48	13.81	0.00	10.33	10.63	9.35	18.28
		Diptera	*Nematocera*	0.87	0.90	0.00	0.00	0.00	0.87	0.00	0.96	0.63	0.97	0.00
		Hymenoptera	*Formicidae*	5.21	11.83	8.44	9.21	10.19	18.64	28.57	7.87	11.32	2.87	14.12
		Hymenoptera	*Parasite*	0.10	1.25	6.39	0.21	0.60	4.04	0.00	2.46	3.77	3.36	0.65
		Lepidoptera	*Lepidoptera*	0.00	0.00	0.00	0.00	1.63	0.00	0.00	0.00	0.00	0.71	0.00
		Orthoptera	*Orthoptera*	0.00	0.98	0.00	2.04	1.06	0.00	7.14	0.00	0.00	0.92	0.65
		Trombidiformes	*Acarina*	0.16	0.74	0.00	0.00	0.00	0.00	7.14	0.00	1.02	0.00	0.00
		SPECIES RICHNESS		16	16	7	11	13	7	6	10	13	21	14

Supporting Information Chapter 3

Appendix S2. Description of ecological context for thermal environment and floral market during the sampling period of the main study.

Thermal environment

Temperature was used as an estimator of the microclimatic environment conditioning mistletoe-arthropods interactions during the flowering period. Temperature was measured from March 21^{th} to May 7^{th} in both 2013 and 2014 with HOBO data loggers (TidbiT v2 Water Temperature Data Logger-UTBI-001) directly located on pine branches bearing labeled mistletoe plants. A total of 8 data loggers were placed, which recorded the temperature every 30 min. All data was used to test significant differences between sampling years with a Liner Mixed Model (LMM), where years was the fixed factor and data logger and day were random factors.

The results showed that mean temperature statistically differed between sampling years (LMM: T=2.87, P=0.005). In 2013, the mean day temperatures were colder (9.15±0.04℃) than in 2014 (12.07±0.05℃), as was the minimum (4.2±0.2℃ and 6.2±0.2℃, respectively) and maximum (16.8±0.3℃ and 21.8±0.4℃, respectively) temperatures registered during the sampling period (Fig. S3).

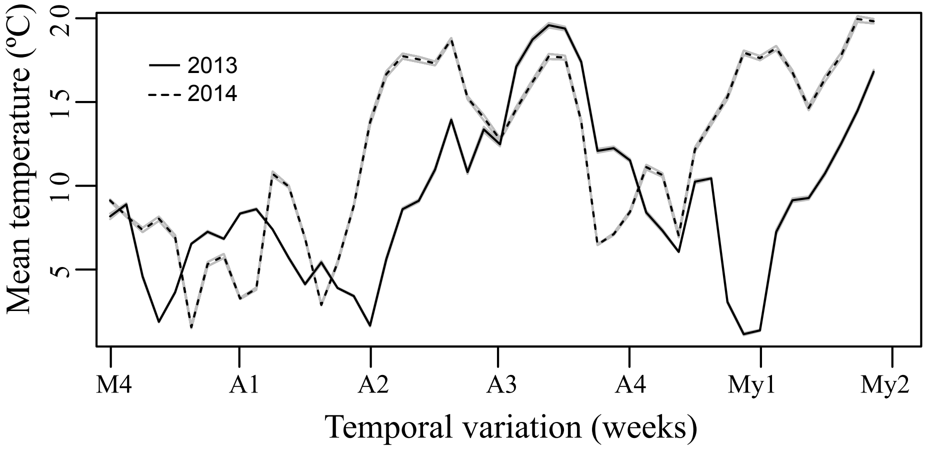

Figure S3: Mean temperature in both sampling years (2013 and 2014).

Floral market

We wished to determine whether mistletoe flowers were the only floral resource in the flowering market during the flowering period of the mistletoe, or on the contrary, whether the flowering of the mistletoe might overlap with other plant species with which it would potentially have to compete to attract pollinators. To estimate the floral market available during the mistletoe flowering period, we carried out samplings the same days as the diurnal visitor surveys (see section above). Then, we estimated the number of open flowers of each plant species, according to a semi-quantitative scale (0 = none, 1 = 1 flower, 2 = 2-5 flowers, 3 = 6-20 flowers, 4 = 15-50 flowers, and 5 = more than 50 flowers), present in a 4-m diameter around each parasitized pine harboring labeled focal mistletoes.

During the first half of mistletoe flowering period (up to mid-April) the floral market was practically limited to mistletoe flowers (Fig. S4). From the third week of April, the availability of flowers of other species increases exponentially. These new floral resources corresponded mainly to *Genista scorpius* (35.3%), *Bellis perennis* (21.8%) and *Erinacea anthyllis* (20.4%) in both sampling years. Therefore, the mistletoes were the first flower resource available for the floral visitors guild after the winter period, and the only one until mid-April. Afterwards, mistletoe flowering overlapped with the first species that begin flowering in the mountain pine forest.

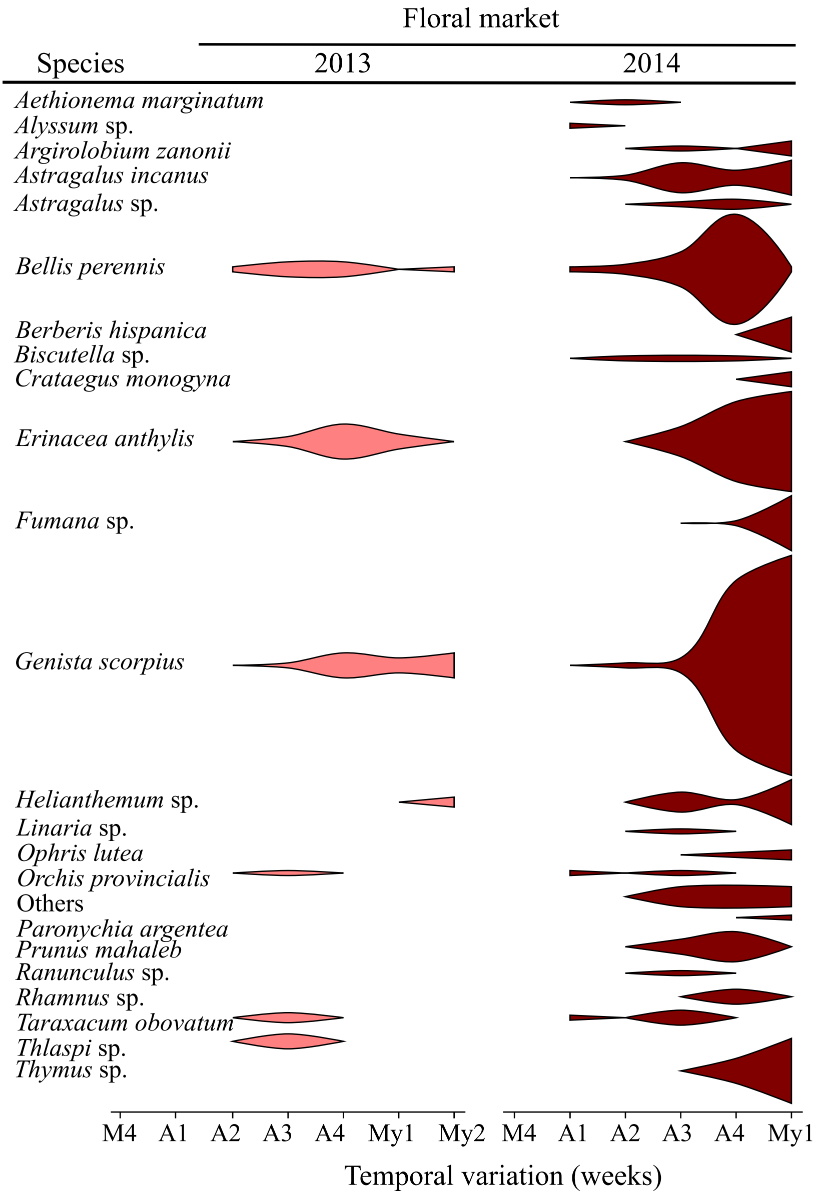

Figure S4: Abundance of open flowers, according to a semi-quantitative scale, of the entire floral market coexisting with mistletoe flowering.

Table S7: Abundance (mean ± SE) of each taxonomic group of the arthropod community collected on pairs of pan traps (n=280) hanging on parasitized and unparasitized pine branches.

Order	Taxonomic group	Unparasitized pines	Parasitized pines
Hymenoptera	*Andrena* sp.	0.24 ± 0.09	0.07 ± 0.04
	Apis mellifera	0.03 ± 0.02	0.07 ± 0.03
	Lasioglossum sp.	0.31 ± 0.11	0.36 ± 0.12
	Fam. Formicidae	0.64 ± 0.16	0.59 ± 0.14
	Fam. Vespoidae	1.17 ± 0.21	1.24 ± 0.20
Diptera	SubO. Brachycera	3.94 ± 0.63	7.16 ± 1.03
	SubO. Nematocera	6.49 ± 1.25	9.69 ± 1.15
Coleoptera	*Meligethes* sp.	0.28 ± 0.09	0.26 ± 0.12
	Fam. Melyridae	0.21 ± 0.07	0.51 ± 0.12
	O. Coleoptera	0.87 ± 0.17	0.77 ± 0.14
Hemiptera	*Cacopsylla visci*	5.19 ± 1.20	33.89 ± 5.38
	Fam. Aphididae	0.49 ± 0.11	0.61 ± 0.09
	Fam. Coccidae	-	0.04 ± 0.02
	Fam. Cicadellidae	0.09 ± 0.03	0.19 ± 0.06
	SubO. Heteroptera	0.03 ± 0.02	0.19 ± 0.07
Other orders	O. Araneae	0.70 ± 0.15	0.51 ± 0.11
	O. Collembola	0.33 ± 0.21	-
	O. Dermaptera	0.01 ± 0.01	-
	O. Julida	0.01 ± 0.01	-
	O. Lepidoptera	0.04 ± 0.03	0.01 ± 0.01
	O. Neuroptera	-	0.01 ± 0.01
	O. Opiliones	0.03 ± 0.02	-
	O. Thysanoptera	1.64 ± 0.34	1.07 ± 0.30

Table S8: Abundance (mean ± SE) of each taxonomic group of the arthropod communities visiting leaves and branches (foliar visitors) and flowers (floral visitors) on mistletoe plants (n =149 for each sampling) during their flowering period.

Order	Taxonomic group	Foliar visitors	Floral visitors
Hymenoptera	*Andrena* sp.	-	0.01 ± 0.01
	Apis mellifera	0.01 ± 0.01	0.54 ± 0.08
	Bombus terrestris	-	0.01 ± 0.01
	Ceratina cucurbitina	-	0.02 ± 0.01
	Lasioglossum sp.	$<$ 0.01 ± 0.01	0.05 ± 0.02
	Camponotus aethiops	-	0.01 ± 0.01
	Camponotus cruentatus	0.05 ± 0.01	0.05 ± 0.02
	Camponotus lateralis	$<$ 0.01 ± 0.01	-
	Camponotus piceus	0.06 ± 0.01	0.07 ± 0.02
	Crematogaster auberti	0.01 ± 0.01	0.01 ± 0.01
	Crematogaster scutellaris	0.07 ± 0.01	0.05 ± 0.02
	Fam. Vespoidae	$<$ 0.01 ± 0.01	0.01 ± 0.01
Diptera	SubO. Brachycera	0.06 ± 0.01	0.36 ± 0.04
	SubO. Nematocera	0.09 ± 0.02	0.17 ± 0.04
Coleoptera	Fam. Cantharidae	0.06 ± 0.01	0.02 ± 0.01
	Fam. Coreidae	0.02 ± 0.01	-
	Fam. Melyridae	0.01 ± 0.01	-
	O. Coleoptera	0.07 ± 0.02	0.01 ± 0.01
Hemiptera	*Cacopsylla visci*	1.58 ± 0.07	0.04 ± 0.02
	Holcogaster sp.	-	0.01 ± 0.01
	Fam. Aphididae	$<$ 0.01 ± 0.01	-
	Fam. Coccidae	0.01 ± 0.01	-
	SubO. Heteroptera	0.03 ± 0.01	0.01 ± 0.01
Other orders	O. Araneae	0.04 ± 0.01	-
	O. Julida	$<$ 0.01 ± 0.01	-
	O. Lepidoptera	0.01 ± 0.01	-

Supporting Information Chapter 4

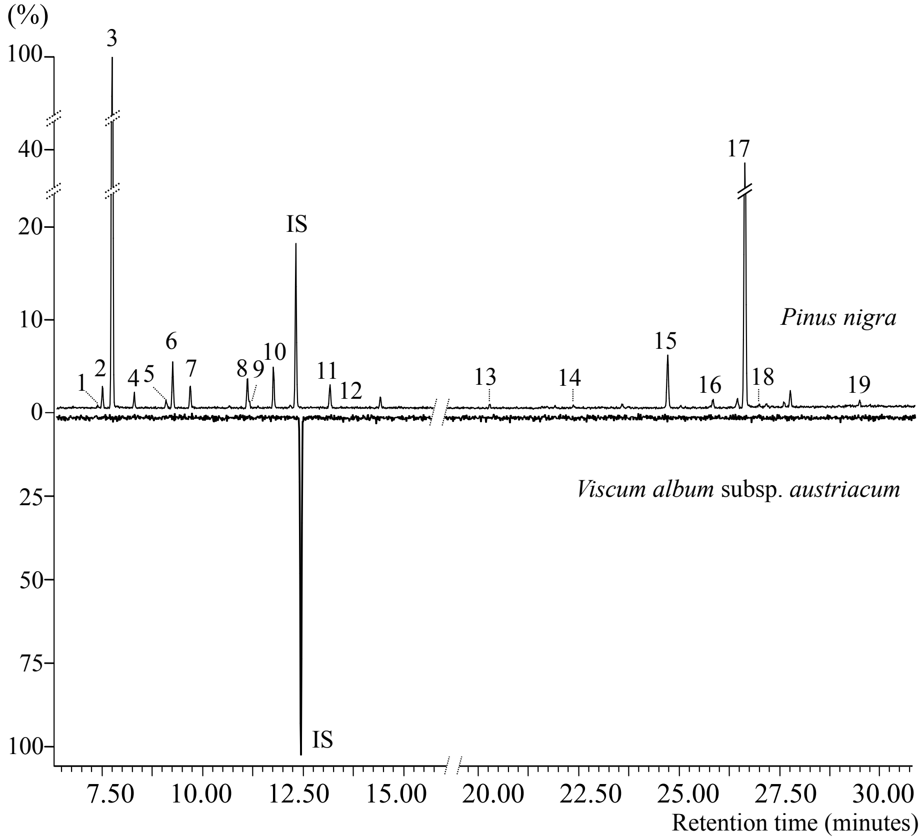

Figure S5: Representative gas chromatogram with numbered peaks according to compounds identified in pine needles (top) and mistletoe leaves extracts (bottom, inverted). Numbered peaks correspond to: 1) Tricyclene*, 2) α–Thujene, 3) α–Pinene, 4) Camphene, 5) Sabinene, 6) β–Pinene, 7) Myrcene, 8) Limonene, 9) β–Phellandrene*, 10) Ocimene, 11) Terpinolene, 12) Linalool*, 13) Bornyl acetate*, 14) Terpinyl acetate*, 15) β–Caryophyllene, 16) α–Caryophyllene, 17) Germacrene–D, 18) Sesquiterpene 1*, and 19) Sesquiterpene 2*. Peak labelled "IS" indicate internal standard, and * compounds detected in traces (<1% relative abundance)

Table S9: Mean ± SE chemical compound amounts in previous–year (2011) and current–year (2012) needle cohorts from pines with 4 parasite load levels (Control, Low, Medium, and High)

			Old needles (2011)				New needles (2012)			
		Compound	Control	Low	Medium	High	Control	Low	Medium	High
Phenols (mg · g^{-1})			15.91±0.92	13.35±0.96	14.65±1.12	15.26±0.79	9.63±0.74	8.87±0.56	11.28±0.73	11.33±0.82
Tannins (mg · g^{-1})			47.02±3.70	36.19±3.98	39.58±4.06	48.12±3.31	19.01±1.57	15.38±1.62	19.31±2.38	23.39±1.61
N (% on dry samples)			0.913±0.032	0.900±0.025	0.890±0.037	0.812±0.028	0.875±0.036	0.915±0.021	0.897±0.024	0.827±0.028
Terpenes (mg · g^{-1})	Mono–	Tricyclene	0.010±0.003	0.011±0.003	0.014±0.002	0.011±0.003	0.011±0.002	0.015±0.003	0.019±0.002	0.022±0.005
		α–Thujene	0.022±0.016	0.025±0.012	0.047±0.014	0.034±0.009	0.013±0.006	0.017±0.007	0.016±0.004	0.014±0.003
		α–Pinene	2.190±0.431	2.310±0.706	3.475±0.483	3.146±0.737	2.298±0.358	2.940±0.894	3.410±0.323	3.822±0.779
		Camphene	0.035±0.008	0.031±0.012	0.055±0.008	0.054±0.016	0.050±0.010	0.062±0.021	0.083±0.008	0.099±0.025
		Sabinene	0.015±0.006	0.018±0.005	0.029±0.007	0.026±0.005	0.014±0.005	0.019±0.004	0.020±0.003	0.020±0.003
		β–Pinene	0.514±0.195	0.355±0.143	0.468±0.193	0.518±0.171	0.614±0.222	0.430±0.171	0.334±0.069	0.414±0.117
		Myrcene	0.123±0.013	0.099±0.021	0.110±0.017	0.087±0.014	0.176±0.033	0.138±0.033	0.133±0.045	0.108±0.017
		Limonene	0.018±0.005	0.061±0.015	0.071±0.013	0.093±0.023	0.020±0.004	0.083±0.019	0.119±0.044	0.140±0.033
		β–Phellandrene	0.032±0.010	0.017±0.005	0.030±0.013	0.010±0.005	0.034±0.009	0.018±0.005	0.022±0.011	0.010±0.007
		Ocimene	0.044±0.008	0.040±0.007	0.054±0.009	0.073±0.021	0.086±0.013	0.081±0.015	0.114±0.011	0.169±0.043
		Terpinolene	0.028±0.014	0.031±0.013	0.055±0.017	0.048±0.012	0.019±0.008	0.026±0.009	0.024±0.006	0.022±0.004
		Linalool	0.005±0.001	0.007±0.003	0.004±0.001	0.002±0.001	0.004±0.001	0.005±0.002	0.002±0.001	0.006±0.001
		Bornyl acetate	0.011±0.003	0.013±0.005	0.026±0.007	0.020±0.006	0.033±0.009	0.058±0.023	0.086±0.013	0.100±0.035
		Terpinyl acetate	0.064±0.024	0.055±0.029	0.115±0.024	0.066±0.016	0.102±0.039	0.059±0.031	0.094±0.026	0.035±0.011
	Sesqui–	β–Caryophyllene	1.117±0.165	1.523±0.283	0.589±0.152	0.769±0.202	1.286±0.190	1.708±0.284	0.761±0.258	1.002±0.223
		α–Caryophyllene	0.194±0.026	0.272±0.050	0.109±0.024	0.128±0.033	0.230±0.037	0.314±0.056	0.132±0.037	0.187±0.038
		Germacrene–D	2.666±0.513	2.723±0.367	1.778±0.306	1.660±0.196	2.346±0.568	2.412±0.289	0.970±0.203	0.733±0.096
		Sesquiterpene 1	0.028±0.010	0.033±0.009	0.015±0.005	0.013±0.005	0.022±0.009	0.018±0.007	0.007±0.004	0.002±0.001
		Sesquiterpene 2	0.002±0.001	0.002±0.001	0.001±0.001	0.001±0.001	0.002±0.002	0.002±0.001	0.002±0.001	0.002±0.002
	Di–	Diterpene 1	0.018±0.005	0.018±0.006	0.013±0.002	0.006±0.002	0.006±0.002	0.011±0.001	0.005±0.001	0.006±0.002
		Diterpene 2	0.011±0.004	0.010±0.003	0.006±0.002	0.006±0.001	0.006±0.002	0.013±0.002	0.006±0.002	0.006±0.001
Total monoterpenes			3.111±0.631	3.073±0.865	4.554±0.613	4.190±0.847	3.476±0.549	3.951±1.120	4.477±0.366	4.982±0.934
Total sesqui– and diterpenes			4.034±0.657	4.580±0.548	2.510±0.384	2.582±0.346	3.896±0.700	4.476±0.520	1.881±0.473	1.937±0.344
Total terpenes			7.146±0.693	7.655±0.764	7.064±0.890	6.772±1.090	7.374±0.606	8.429±0.942	6.359±0.627	6.921±1.087

Supporting Information Chapter 5

Table S10: Identified compounds (mean ± SE) in the parasitized pine needles, unparasitized pine needles, and mistletoe leaves metabolome, on summer and autumn. Acronyms are described in the caption of Fig. 5.1 from the main text, and all values are expressed as 10^3.

Metabolomic family	Compound	Unparasitized pines needles		Parasitized pines needles		Mistletoe leaves	
		Summer	Autumn	Summer	Autumn	Summer	Autumn
Amino acids	Asp	184996±12142	204040±21628	223186±14677	257110±20255	715600±148894	941700±133521
	Ser	203530±10653	216750±14531	219200±10789	228616±14385	1016500±76044	1050400±118641
	Lys	84420±2111	46140±3631	94483±2464	39186±3682	387600±54929	174900±31243
	Asn	24876±1356	24646±2694	31526±2123	34439±3752	49590±10604	90610±15323
	Arg	22490±1762	13420±1049	29143±2656	17380±1303	17594±4221	10997±1471
	Tyr	11637±1026	13116±1356	14777±1291	19998±1925	1226±166	19211±6877
	Met	15416±1701	9048±1857	15648±1492	9918±1456	3558±941	9307±1715
	His	1029±196	23595±2086	3151±600	26930±2220	11859±3906	109700±17074
	Gln	8038±415	4522±413	9899±454	6623±473	876±125	1444±218
	Glu	6006±291	3364±447	7193±387	6088±701	3320±362	2131±406
	Iso	5187±888	9557±1127	4319±610	10978±1153	763±182	950±325
	Phe	34963±12808	8794±3871	12601±5145	17569±6752	11447±7575	187498±36783
	ProH	6514±839	1476±211	4855±580	4969±1332	3372±2276	22087±7134
	Ala	355±38	1064±227	496±69	2340±336	154±22	216±44
Nucleotids	Ad	39206±1092	75535±3441	46186±1441	72376±2754	33160±5590	66360±17320
	Ur	2966±244	747±115	3461±281	969±93	251±70	258±61
	Gua	1063±70	743±98	1046±81	485±74	16980±4266	13620±3346
	Gu	329±35	547±87	555±74	1385±338	11488±3425	7887±2008
	Cy	223±17	341±47	212±18	367±39	1072±441	3918±987
	Cyt	111±8.9	199±22	125±13	265±47	2718±1110	5208±1525
	Ade	346±171	40±11	423±116	75±13	263950±25866	310900±21184
	Thy	25.2±4.1	30.9±5.9	35.7±7.5	72.1±24.7	1631±563	4273±1502
Organic acids related to the Krebs cycle	KG	2535±283	10478±717	2610±418	6229±547	8287±1107	2344±634
	CGA	4196±278	3255±330	4256±349	3334±272	510500±51971	528900±75123
	Cit	22.8±2.5	93.7±12.1	36.8±4.5	122±10	246±49	841±256
	Mal	114900±7524	136350±5291	118736±9350	134133±5220	235600±13171	296000±19082
	Lac	10058±602	9867±1203	11865±1194	11340±796	8236±1516	19776±5214
	Pyr	2210±196	14149±1028	2683±240	11939±679	10404±1694	14220±860
	Suc	26270±1573	15693±907	27076±2189	17356±622	58210±6518	52420±4305
Sugars	Rib	1702±117	954±40	1693±169	1138±50	9685±1094	7811±848
	Lyo	2101±163	11364±744	1940±186	11134±524	14080±813	14500±969
	Pen	152±16	570±67	238±22	421±75	9398±1421	3233±743
	Sor	17276±617	51485±2945	20326±968	55490±2759	81540±8167	34850±2674
Growth factors	Pan	25033±1355	28960±3280	24406±969	20421±1785	21160±2916	36820±4362
	ABA	1374±130	6563±536	1583±187	4772±368	464±112	941±220
	Asc	9954±2900	36813±11526	7739±2720	11296±5106	3237±515	1926±298
	Gib	35428±3273	32270±4450	24785±3744	31830±4545	23±15	230±83
	RiF	91±13	157±25	109±15	191±25	76±13	153±45
Phenols	CafA	5129±550	3634±851	6174±807	8711±1486	471±98	3989±1504
	QA	1987000±38432	1326600±54890	1941804±102916	1449333±44633	1053300±43826	583700±42104
	Cat	72186±3673	188450±6937	68656±4797	217033±11258	0.79±0.53	1.65±0.71
	Fla	10913±827	65171±5443	14875±1163	73543±4155	279±277	7666±5095
	Epi	11049±2312	11025±1023	9785±1577	16459±2036	0.46±0.46	1.36±0.7
	Prc	3553±426	7538±1143	4908±534	9850±1049	1427±198	610±99
	EpG2	47178±5678	74870±4143	43177±4537	58630±3482	273±66	81±32
	Pin	321±34	141±12	193±21	180±21	19203±2731	10226±2133
Terpenes	Ara	198±20	355±53	657±253	1036±294	213±17	251±29
	Acu	5590±150	4973±209	7574±1060	6884±1234	282±104	852±176
	Hum	69165±12016	75781±14330	60757±6380	52770±4353	27±10	6.4±1.31
	Ter	5829±1435	647±204	10088±1810	822±274	22±6.39	7.58±1.4
	CarO	936±166	1984±382	1148±222	1517±236	1.71±0.48	3.63±0.31
	Kae	15876±5089	3335±2109	7745±1957	1210±458	39.97±7.35	31.37±6.99
	Oci	289±64	4052±742	171±21	3042±428	37.75±18.47	2420±1977
	Teo	747±173	13.25±3.75	737±290	13.71±6.05	7.259±1.68	2.95±1.51
	Car	74±18	2549±1342	177±71	240±111	6.67±0.47	7.06±0.47

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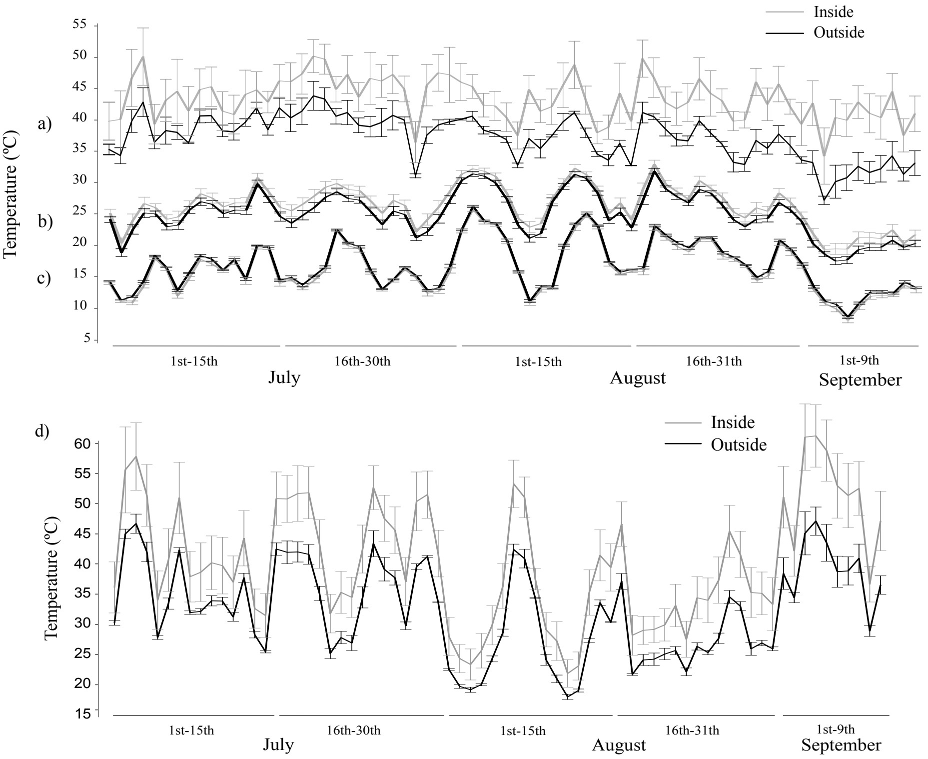

Figure S6: a) Maximum, b) mean, c) minimum and d) coefficient of variation (mean ±SE) of temperature (℃) inside (grey line) and outside bags (black line) during field bioassay of *Cinara pini*.

Table S11: Effects of mistletoe loads (Control, Low, Medium and High) on pines inhabited and abundance of a) *Cinara pini*, b) *Thaumetopoea pityocampa* and c) *Brachderes* sp. by pine tree on wild population. Estimates (E), standard errors (SE), Z-values (Z) and significance (P) derived from post hoc multiple comparisons. Significant (P<0.05) and marginally significant (0.05<P<0.1) values are marked in bold type and italics, respectively.

(a) *Cinara pini*

Mistletoe loads		Presence				Abundance (nº ind)			
Reference level	Contrast level	E	SE	Z	P	E	SE	Z	P
Control	Low	0.67	0.60	1.11	0.676	−0.36	0.39	−0.92	0.786
	Medium	0.58	0.52	1.12	0.673	0.11	0.31	0.35	0.984
	High	0.43	0.76	0.56	0.942	−0.42	0.47	−0.89	0.800
Low	Medium	−0.09	0.65	−0.13	0.999	0.47	0.43	1.09	0.684
	High	−0.24	0.86	−0.28	0.992	−0.05	0.59	−0.09	1.000
Medium	High	−0.15	0.80	−0.19	0.997	−0.53	0.51	−1.03	0.723

(b) *Thaumetopoea pityocampa*

Mistletoe loads		Pines inhabited				Abundance (nº nests)			
Reference level	Contrast level	E	SE	Z	P	E	SE	Z	P
Control	Low	−0.26	0.28	−0.92	0.616	−0.28	0.18	−1.50	0.279
	Medium	−0.12	0.53	−0.22	0.972	−0.32	0.34	−0.94	0.605
	High	-	-	-	-	-	-	-	-
Low	Medium	0.14	0.52	0.27	0.959	−0.04	0.32	−0.11	0.993
	High	-	-	-	-	-	-	-	-
Medium	High	-	-	-	-	-	-	-	-

(c) *Brachyderes* sp.

Mistletoe loads		Pines inhabited				Abundance (nº ind)			
Reference level	Contrast level	E	SE	Z	P	E	SE	Z	P
Control	Low	0.76	0.56	1.35	0.511	0.30	0.32	0.95	0.755
	Medium	1.10	0.49	2.23	0.105	0.73	0.29	2.51	**0.049**
	High	2.71	1.08	2.50	*0.055*	2.56	1.02	2.52	**0.048**
Low	Medium	0.34	0.64	0.53	0.949	0.43	0.40	1.05	0.689
	High	1.95	1.16	1.68	0.316	2.26	1.06	2.14	0.120
Medium	High	1.61	1.13	1.43	0.460	1.84	1.05	1.75	0.266

Table S12: Effects of mistletoe loads (Control, Low, Medium and High) on survival and abundance of a) *Cinara pini* and pine processionary caterpillars on field bioassay; and b) pine processionary caterpillars, and tasted and consumed pine needles by *Brachyderes* sp. on the laboratory bioassay and cafeteria test, respectively. Estimates (E), standard errors (SE), Z-values (*Z*) and significance (*P*) derived from post hoc multiple comparisons. Significant (*P*<0.05) and marginally significant (0.05<*P*<0.1) values are marked in bold type and italics, respectively.

(a) Field bioassays

		Cinara pini								*Thaumetopoea pityocampa*							
Mistletoe loads		Survival				Abundance (nº ind)				Survival				Abundance (%)			
Reference level	Contrast level	E	SE	*Z*	*P*	E	SE	*Z*	*P*	E	SE	*Z*	*P*	E	SE	*Z*	*P*
Control	Low	1.09	0.65	1.69	0.330	0.66	0.49	1.35	0.534	0.64	0.53	1.21	0.619	0.06	0.04	1.32	0.552
	Medium	1.21	0.65	1.87	0.240	1.10	0.49	2.25	0.110	0.67	0.54	1.24	0.599	0.13	0.05	2.96	**0.016**
	High	1.24	0.66	1.88	0.238	1.93	0.50	3.85	<**0.001**	0.90	0.53	1.71	0.321	0.43	0.05	9.05	<**0.001**
Low	Medium	0.12	0.61	0.19	0.997	0.44	0.49	0.90	0.808	0.03	0.52	0.06	1.000	0.08	0.04	1.75	0.294
	High	0.15	0.62	0.23	0.995	1.28	0.51	2.51	*0.057*	0.25	0.51	0.50	0.958	0.38	0.05	8.01	<**0.001**
Medium	High	0.03	0.62	0.04	1.000	0.83	0.51	1.64	0.356	0.22	0.52	0.43	0.973	0.30	0.05	6.11	<**0.001**

(b) Laboratory bioassays

		Thaumetopoea pityocampa								*Brachyderes* sp.							
Mistletoe loads		Survival				Abundance (%)				Tested needles				Consumed needles (mm)			
Reference level	Contrast level	E	SE	*Z*	*P*	E	SE	*Z*	*P*	E	SE	*Z*	*P*	E	SE	*Z*	*P*
Control	Low	−0.83	0.74	−1.13	0.492	−0.10	0.88	−0.12	0.992	−0.73	1.25	−0.58	0.935	−0.07	0.26	−0.27	0.993
	Medium	2.62	1.11	2.37	**0.045**	4.40	1.24	3.55	**0.001**	1.03	0.88	1.17	0.636	0.79	0.26	3.03	**0.013**
	High	-	-	-	-	-	-	-	-	1.45	0.85	1.71	0.310	1.09	0.26	4.15	<**0.001**
Low	Medium	3.45	1.15	3.00	**0.007**	4.50	1.22	3.68	<**0.001**	1.76	1.13	1.56	0.392	0.86	0.26	3.29	**0.005**
	High	-	-	-	-	-	-	-	-	2.18	1.11	1.97	0.191	1.16	0.26	4.41	<**0.001**
Medium	High	-	-	-	-	-	-	-	-	0.42	0.65	0.64	0.915	0.30	0.26	1.12	0.675

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Table S13: Mean cover (± SE) of all herbaceous species identified, as well as rock and bare-soil cover (%) behind unparasitized and parasitized pines by mistletoe, across an elevational gradient in Sierra de Baza.

	Unparasitized pines			**Parasitized pines**		
Species	1350 m	1650 m	1850 m	1350 m	1650 m	1850 m
Achillea millefolium	-	0.28±0.28	-	-	-	-
Acinos alpinus	-	-	0.09±0.07	-	0.07±0.07	0.05±0.05
Aegylops neglecta	-	0.97±0.58	-	-	0.14±0.11	1.46±0.92
Allium sp.	-	0.05±0.04	-	-	0.04±0.04	0.05±0.05
Alyssum alyssoides	-	-	0.09±0.07	-	-	0.20±0.14
Alyssum simplex	-	0.35±0.15	0.42±0.23	-	0.17±0.10	0.75±0.28
Anacyclus clavatus	-	0.03±0.03	0.19±0.19	0.03±0.02	-	0.57±0.41
Anagallis arvensis	-	-	-	0.11±0.10	0.14±0.14	-
Androsace maxima	-	0.05±0.04	-	-	0.01±0.01	-
Anthyllis vulneraria	0.17±0.09	0.14±0.11	-	0.19±0.10	0.14±0.09	-
Aphyllanthes monspeliensis	0.06±0.05	-	-	0.13±0.09	-	-
Apiaceae n.i. 1	-	-	-	-	0.01±0.01	-
Apiaceae n.i. 2	0.01±0.01	-	-	-	-	-
Apiaceae n.i. 3	0.03±0.02	0.17±0.11	-	-	0.47±0.31	0.09±0.09
Apiaceae n.i. 4	-	0.22±0.16	-	-	0.56±0.52	-
Apiaceae n.i. 5	-	0.15±0.10	-	-	0.35±0.25	-
Arabis auriculata	0.30±0.13	0.26±0.10	-	0.94±0.19	0.63±0.19	0.77±0.37
Arenaria grandiflora	-	0.29±0.29	0.10±0.10	-	0.26±0.16	-
Arenaria modesta	-	0.27±0.14	0.22±0.14	0.15±0.15	0.37±0.18	1.98±0.62
Arenaria serpyllifolia	0.21±0.11	0.07±0.07	-	1.55±0.39	0.04±0.04	-
Argyrolobium zannonii	0.07±0.04	-	-	0.04±0.04	-	-
Armeria villosa	0.27±0.27	1.70±1.23	0.15±0.10	0.07±0.07	0.53±0.19	0.19±0.10
Arrhenatherum album	1.98±0.52	2.30±0.51	0.71±0.43	3.63±0.90	1.69±0.40	0.84±0.29
Asphodelus macrocarpus	1.60±0.73	-	-	0.89±0.49	-	-

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Table S13 – *Continued from previous page*

	Unparasitized pines			**Parasitized pines**		
Species	1350 m	1650 m	1850 m	1350 m	1650 m	1850 m
Asterolinon linum-stellatum	0.03±0.02	-	-	0.24±0.13	0.01±0.01	-
Astragalus incanus	0.15±0.15	0.02±0.02	0.07±0.07	0.04±0.03	0.37±0.25	0.05±0.04
Astragalus stella	-	0.06±0.06	-	-	-	-
Astragalus vesicarius	-	0.39±0.39	-	-	0.04±0.04	-
Atractylis cancellata	0.09±0.09	0.05±0.05	0.14±0.14	0.16±0.10	-	-
Avena sterilis	0.01±0.01	-	-	0.03±0.02	0.09±0.07	-
Bellis perennis	0.05±0.05	-	-	-	-	-
Biscutella laxa	0.05±0.03	0.14±0.10	0.08±0.06	0.15±0.12	0.02±0.02	0.02±0.02
Bituminaria bituminosa	0.08±0.08	-	-	0.08±0.08	-	-
Brachypodium distachyon	0.01±0.01	0.05±0.05	0.19±0.19	0.49±0.46	0.25±0.14	0.26±0.26
Brachypodium retusum	-	-	-	0.37±0.26	-	-
Brachypodium sylvaticum	-	-	-	-	-	0.06±0.06
Briza media	-	-	0.21±0.10	-	-	0.61±0.28
Bromus rubens	0.41±0.27	0.54±0.54	1.27±0.99	3.50±1.40	0.72±0.41	2.64±1.09
Buglossoides arvensis	-	-	-	0.03±0.03	-	-
Bunium macuca	-	-	0.31±0.12	-	-	0.29±0.18
Campanula rotundifolia	-	-	0.05±0.05	-	-	-
Capsella bursa-pastoris	-	-	-	-	0.01±0.01	-
Carduncellus monspeliensis	-	1.02±0.39	0.42±0.19	-	0.65±0.33	0.76±0.35
Carex hallerana	0.75±0.43	-	-	0.46±0.20	0.84±0.82	-
Carlina hispanica	0.48±0.34	-	-	0.39±0.27	-	-
Centaurea melitensis	-	0.11±0.08	-	-	-	-
Centaurea triumfetti	0.11±0.10	0.03±0.02	0.07±0.07	0.41±0.20	0.04±0.04	0.01±0.01
Centranthus calcitrapae	-	0.09±0.09	-	-	-	-
Cerastium brachypetalum	0.03±0.02	0.19±0.12	-	1.49±0.46	0.07±0.06	-

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Table S13 – *Continued from previous page*

	Unparasitized pines			**Parasitized pines**		
Species	1350 m	1650 m	1850 m	1350 m	1650 m	1850 m
Cerastium gibraltaricum	0.75±0.70	1.98±0.70	2.23±0.55	0.22±0.16	1.56±0.41	3.02±0.91
Chaenorhinum villosum	-	-	-	0.03±0.03	-	-
Cirsium x nevadense	-	-	-	-	0.07±0.05	-
Compositae n.i. 1	0.04±0.04	-	0.43±0.24	0.04±0.04	-	0.18±0.08
Coronilla scorpioides	0.11±0.08	-	-	0.27±0.10	-	-
Crepis albida	0.05±0.05	-	-	-	0.41±0.30	-
Crocus nevadensis	0.01±0.01	0.02±0.02	-	0.01±0.01	0.04±0.04	-
Crucianella angustifolia	0.07±0.07	0.52±0.22	0.43±0.24	0.34±0.15	0.55±0.16	1.19±0.27
Crupina crupinastrum	0.46±0.23	0.44±0.18	0.10±0.05	0.82±0.26	0.46±0.17	0.44±0.15
Cynosurus echinatus	0.21±0.12	0.81±0.36	3.15±2.95	3.80±1.20	5.89±2.93	2.74±1.10
Dactilis glomerata	0.08±0.06	-	2.92±1.09	-	0.04±0.04	1.56±0.71
Dianthus brachyanthus	-	0.28±0.24	-	-	0.19±0.19	0.86±0.38
Echinaria capitata	-	0.05±0.05	0.04±0.04	-	-	0.04±0.03
Erodium cicutarium	-	-	-	0.34±0.18	-	-
Eryngium campestre	0.36±0.14	0.88±0.36	-	0.49±0.22	0.58±0.19	-
Erysimum baeticum	-	0.01±0.01	-	-	0.03±0.03	-
Euphorbia nicaeensis	-	0.17±0.09	0.71±0.43	-	-	0.30±0.17
Euphorbia squamigera	-	-	-	-	0.04±0.04	-
Festuca indigesta	-	-	-	-	-	2.23±1.87
Festuca scariosa	-	3.17±1.93	10.01±2.74	-	6.68±3.19	8.35±2.02
Filago pyramidata	0.25±0.13	0.27±0.11	-	0.89±0.23	0.14±0.07	0.40±0.26
Fritillaria lusitanica	-	-	0.02±0.02	-	-	-
Galium parisiense	0.39±0.19	-	0.62±0.28	0.57±0.28	-	0.28±0.19
Galium verticillatum	-	1.23±0.45	0.02±0.02	-	0.84±0.46	-
Geranium lucidum	-	-	-	0.22±0.14	-	-

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Table S13 – *Continued from previous page*

	Unparasitized pines			**Parasitized pines**		
Species	1350 m	1650 m	1850 m	1350 m	1650 m	1850 m
Geranium molle	-	0.32±0.16	-	0.31±0.22	0.18±0.11	-
Geum sylvaticum	-	-	-	-	0.12±0.12	-
Helianthemum apenninum std.	0.89±0.28	1.64±0.54	0.41±0.24	0.97±0.27	0.45±0.19	0.28±0.23
Helianthemum apenninum suf.	-	0.36±0.18	0.98±0.59	-	0.30±0.20	0.63±0.44
Helianthemum ledifolium	-	0.10±0.10	-	-	-	0.02±0.02
Helianthemum oelandicum	0.04±0.02	-	0.43±0.19	0.27±0.12	-	0.22±0.17
Hieracium sp.	0.08±0.06	1.61±0.83	0.80±0.37	0.20±0.20	0.87±0.51	0.21±0.15
Hordeum geniculatum	-	-	-	-	0.09±0.09	-
Hornungia petraea	0.33±0.12	0.38±0.11	0.47±0.23	1.02±0.30	0.92±0.23	1.09±0.30
Jurinea humilis	-	-	0.53±0.20	-	-	0.28±0.16
Koeleria crassipes	2.16±0.62	6.96±1.40	1.77±0.80	6.42±1.83	1.85±0.45	3.24±1.15
Lamium amplexicaule	-	0.08±0.08	-	0.11±0.11	-	-
Lathyrus cicera	-	-	-	-	1.38±1.31	-
Linaria micrantha	0.06±0.06	-	-	-	-	-
Linum narbonense	0.04±0.04	-	0.02±0.02	0.06±0.06	-	-
Linum strictum	-	0.11±0.11	-	0.06±0.06	-	-
Lomelosia simplex	-	-	0.43±0.24	0.24±0.14	-	0.37±0.18
Lotus corniculatus	-	-	0.35±0.25	-	-	0.11±0.09
Mantisalca salmantica	-	-	-	-	-	0.21±0.21
Medicago minima	0.04±0.04	-	-	0.16±0.11	-	-
Medicago rigidula	0.32±0.15	0.31±0.16	-	0.44±0.18	0.24±0.14	-
Minuartia funkii	-	0.14±0.14	-	-	0.04±0.04	0.20±0.11
Myosotis ramosissima	-	-	-	-	0.04±0.04	-
Myosotis stricta	0.05±0.05	-	-	0.15±0.11	0.08±0.07	-
n.i. 1	0.15±0.11	-	-	0.06±0.06	-	-

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Table S13 – *Continued from previous page*

	Unparasitized pines			Parasitized pines		
Species	1350 m	1650 m	1850 m	1350 m	1650 m	1850 m
n.i. 2	0.90±0.35	-	-	0.16±0.11	-	-
n.i. 3	-	0.17±0.10	-	-	0.17±0.10	-
n.i. 4	0.02±0.02	-	-	0.42±0.23	-	-
n.i. 5	0.06±0.06	0.57±0.27	0.27±0.17	-	0.14±0.10	-
Nonea micrantha	-	-	-	0.10±0.10	-	-
Ononis aragonensis	-	-	0.64±0.28	-	-	0.22±0.22
Ononis cristata	-	-	0.16±0.09	-	-	0.39±0.21
Ononis pusilla	-	-	0.05±0.05	-	-	0.13±0.13
Ononis reclinata	-	-	-	0.04±0.03	0.09±0.07	-
Papaver dubium	-	-	-	-	0.21±0.21	-
Phagnalon sordidum	0.08±0.08	-	-	-	-	-
Plantago lanceolata	0.08±0.08	0.23±0.19	-	0.45±0.23	0.11±0.08	-
Plantago sempervirens	0.22±0.22	-	-	0.05±0.05	-	-
Poa bulbosa	0.04±0.04	0.52±0.21	0.51±0.48	2.29±0.74	0.73±0.36	2.61±1.04
Poaceae n.i. 1	0.04±0.04	1.71±0.95	-	0.10±0.08	0.86±0.62	-
Poaceae n.i. 2	-	3.01±1.40	-	-	3.07±1.45	-
Poaceae n.i. 3	0.09±0.09	-	-	0.11±0.11	-	0.05±0.05
Polygala boissieri	-	0.42±0.26	0.39±0.16	-	0.13±0.07	0.33±0.15
Polygala rupestris	-	-	-	0.06±0.06	-	-
Potentilla reuteri	-	0.36±0.35	-	-	-	-
Ranunculus gramineus	0.27±0.27	-	-	0.09±0.09	-	-
Ranunculus paludosus	-	0.15±0.15	-	-	0.42±0.38	-
Rhaponticum coniferum	0.64±0.34	-	-	0.96±0.39	-	-
Rubia peregrina	0.72±0.27	0.17±0.12	-	0.24±0.13	0.01±0.01	0.23±0.23
Sanguisorba verrucosa	0.38±0.13	0.18±0.08	0.32±0.13	0.49±0.25	0.16±0.08	0.10±0.07

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Table S13 – *Continued from previous page*

	Unparasitized pines			**Parasitized pines**		
Species	1350 m	1650 m	1850 m	1350 m	1650 m	1850 m
Santolina rosmarinifolia	-	-	-	-	0.12±0.12	-
Saxifraga granulata/carpetana	-	-	-	0.01±0.01	0.08±0.08	-
Scandix australis	0.29±0.18	0.13±0.10	0.33±0.17	0.89±0.38	0.14±0.08	0.16±0.10
Sedum acre	-	-	0.03±0.03	-	-	0.05±0.05
Sherardia arvensis	-	-	0.69±0.54	-	-	1.63±0.43
Sideritis sp.	-	-	0.10±0.10	-	-	-
Silene colorata	-	-	-	0.01±0.01	-	-
Silene vulgaris	0.01±0.01	-	0.03±0.03	0.06±0.05	0.13±0.10	0.15±0.15
Sonchus tenerrimus	0.02±0.02	-	-	-	-	-
Stellaria media	-	-	-	0.25±0.25	0.14±0.14	-
Taraxacum laevigatum	0.22±0.13	0.93±0.50	0.59±0.26	0.34±0.18	1.28±0.50	0.08±0.08
Taraxacum obovatum	-	0.30±0.16	0.08±0.06	0.06±0.06	0.11±0.07	0.01±0.01
Teucrium capitatum	-	0.05±0.05	-	-	0.12±0.07	-
Teucrium webbianum	-	-	0.11±0.11	-	-	-
Thlaspi arvense	0.11±0.09	0.36±0.15	0.03±0.02	0.32±0.17	0.15±0.07	0.37±0.25
Thymelaea pubescens	0.02±0.02	0.05±0.05	-	0.14±0.10	-	-
Thymus sp.	-	-	-	-	0.72±0.49	-
Torilis sp.	-	-	0.01±0.01	0.07±0.06	0.04±0.04	0.22±0.14
Tragopogon angustifolius	0.02±0.01	0.05±0.05	-	0.08±0.06	0.08±0.08	0.10±0.07
Trifolium campestre	-	-	0.36±0.27	-	-	0.22±0.12
Trifolium sp.	-	0.97±0.72	0.05±0.05	-	0.97±0.97	-
Trifolium stellatum	-	-	-	-	-	0.05±0.05
Valerianella coronata	0.18±0.12	0.46±0.23	-	0.12±0.09	-	-
Velezia rigida	-	-	0.07±0.07	-	-	-
Veronica polita	0.02±0.02	0.17±0.10	-	0.07±0.07	0.50±0.20	0.05±0.04

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Table S13 – *Continued from previous page*

	Unparasitized pines			**Parasitized pines**		
Species	1350 m	1650 m	1850 m	1350 m	1650 m	1850 m
Vicia amphicarpa	0.18±0.16	0.90±0.32	0.41±0.24	-	0.41±0.18	0.25±0.21
Viola riviniana	0.07±0.07	0.01±0.01	0.02±0.02	-	-	0.01±0.01
Vulpia sp.	0.01±0.01	-	0.21±0.21	0.20±0.10	-	0.71±0.52
Xeranthemum inapertum	0.22±0.15	0.71±0.21	0.20±0.14	0.70±0.26	0.68±0.37	0.67±0.29
Xolantha guttata	-	-	-	0.01±0.01	-	-
Ziziphora hispanica	-	0.05±0.04	0.03±0.03	0.06±0.04	0.09±0.09	0.02±0.01
Stones	6.55±1.19	6.52±0.52	15.00±2.64	11.00±1.38	7.78±0.97	17.00±2.37
Bare soil	74.48±2.79	49.00±3.84	47.67±3.63	45.95±3.76	47.50±3.84	34.11±3.07
Total herbaceous cover	18.97±2.28	44.48±3.85	37.33±3.75	43.05±3.70	44.72±3.80	48.89±3.52

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