UNIVERSIDAD DE EXTREMADURA

TESIS DOCTORAL

Manejo innovador del ganado y enfoques metodológicos para el control de plagas en ecosistemas agroforestales

Tara Canelo Hernández

Doctorado en Biología Molecular y Celular, Biomedicina y Biotecnología

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La conformidad por el director de la Tesis, el Dr. Raúl Bonal Andrés, consta en el original en papel de esta Tesis Doctoral

Manejo innovador del ganado y enfoques metodológicos para el control de plagas en ecosistemas agroforestales

Innovative livestock management and methodological approaches for pest control in agroforestry ecosystems

A mi familia

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RESUMEN

Los agroecosistemas tradicionales están aumentando su relevancia por producir alimentos al mismo tiempo que conservan la biodiversidad. Sin embargo, al igual que los sistemas intensivos, su productividad se enfrenta a problemas, como las plagas, que deben ser abordados. En este sentido, la intensificación ecológica propone aprovechar los servicios ecosistémicos que determinados organismos del propio sistema pueden aportar. Esta Tesis Doctoral aborda esta cuestión en el contexto del control de plagas en las dehesas de encina *Quercus ilex*, el sistema silvopastoral más extenso de Europa.

Se demostró experimentalmente por primera vez que las orugas defoliadoras (Lepidoptera) reducen la producción de bellotas. Asimismo, se cuantificaron las pérdidas provocadas por los depredadores pre-dispersivos de bellotas: en las dehesas de estudio estos insectos fueron *Curculio elephas* (Coleoptera) y, en menor medida, *Cydia fagiglandana* (Lepidoptera). A nivel de bellota no hubo diferencias ni en peso ni en riesgo de depredación del embrión; el menor volumen de las bellotas atacadas por *Cydia* fue compensado por el mayor consumo de cotiledón por parte de las larvas de *Curculio*. A nivel de cosecha, si bien *Curculio elephas* fue la especie dominante, determinados cambios en el clima (sequías estivales más largas) pueden reducir su incidencia y aumentar la de *Cydia*. Las bellotas atacadas por insectos caen prematuramente. Tras ello, las larvas de *Curculio elephas* pasan 20 días dentro de las bellotas. En ese periodo muchas son depredadas por el ganado, que se alimenta de bellotas infestadas igual que de sanas.

Los resultados de la Tesis demuestran a través de estudios correlaciónales y experimentales/manipulativos, que un correcto manejo del ganado puede servir para controlar las plagas de bellotas. Dentro de las fincas, habría que concentrar el ganado en determinadas cercas cuando las larvas de *C. elephas* son vulnerables (septiembre-octubre). En paralelo, estimaciones de la biodiversidad de artrópodos realizadas mediante delimitación molecular de especies (usando Secuenciación de Próxima Generación, NGS siglas en inglés), indican que la riqueza de especies baja cuando el ganado está presente, pero se recupera al poco tiempo de excluirlo. Por tanto, la estrategia óptima consistiría en combinar y rotar dentro de fincas zonas de intensificación del pastoreo con pequeñas exclusiones de ganado a corto plazo. De esta manera, se compatibilizaría el aumento de la productividad con la conservación de la biodiversidad en un agroecosistema del valor natural de la dehesa.

SUMMARY

Traditional agroecosystems are increasing their relevance as they produce food at the same time that preserve biodiversity. However, likewise intensive systems, their productivity faces problems, such as pests, which need to be approached. In this sense, ecological intensification proposes profiting from the ecosystem services that some organisms within the system may provide. This Doctoral Thesis addresses this subject within the context of pest control in Holm oak *Quercus ilex*. dehesas, the most widespread silvopastoral system in Europe.

It was experimentally demonstrated for the first time that leaf-feeding caterpillars (Lepidoptera) reduce acorn production. In addition, acorn losses by pre-dispersal predators were quantified: in the study dehesas these insects were *Curculio elephas* (Coleoptera) and, in a lesser extent, *Cydia fagiglandana* (Lepidoptera). At the acorn level there were not differences either in weight or embryo predation likelihood; the smaller volume of the acorns attacked by *Cydia* was compensated by the higher consumption of cotyledon by *Curculio* larvae. At the crop level, although *Curculio elephas* was the dominant species, certain changes in the climate (longer summer droughts) may reduce its incidence and increase that of *Cydia*. Acorns attacked by insects are prematurely dropped. After that, *Curculio elephas* larvae spend 20 days within the acorns. In that period many are killed by livestock, which feeds on sound and infested acorns alike.

The results of the Thesis show, by means of correlational and experimental/manipulative studies, that a correct livestock management may serve to control oak pests. Within the farms, livestock should be concentrated in certain plots when *C. elephas* larvae are more vulnerable (September-October). In parallel, arthropod biodiversity assessments performed by means of molecular species delimitation (using Next Generation Sequencing), show that species richness decreases when livestock is present, but recovers soon after excluding it. Hence, an optimal strategy would consist in combining and rotating within the farms plots of intensified grazing with short-term ungulate exclosures. By doing this, the increase of productivity and biodiversity conservation would become compatible in an agroecosystem with a high natural value like is the case of dehesas.

INTRODUCCIÓN GENERAL

Uno de los grandes retos que debe afrontar la humanidad en el siglo XXI es el aumento de la producción de alimento para una población creciente (FAO 2016; IPBES 2018) y, además, hacerlo compatible con la conservación del medio ambiente. Las estrategias que se agrupan dentro de la comúnmente conocida como intensificación ecológica (Naylor y Ehrlich 1997; Crowder y Jabbour 2014; Vanlauwe et al. 2014) persiguen ese fin. Este tipo de intensificación se basa en aprovechar al máximo los servicios ecosistémicos que ciertos organismos pueden ofrecer a la sociedad, pero para ello, es necesario tener un conocimiento profundo de las interacciones ecológicas (Bommarco et al. 2013; Gaba et al. 2014; Goedhart et al. 2018). Un ejemplo de ello es el control biológico de las plagas (Chaplin-Kramer et al. 2011; Thies et al. 2011; Crowder y Jabbour 2014), que consiste en favorecer a las poblaciones de enemigos naturales de las mismas para tratar de reducirlas (Landis et al. 2000; Greenop et al. 2018). Un buen conocimiento de las interacciones entre las plagas y sus depredadores nos permite optimizar el manejo de estos organismos e incrementar la productividad en los sistemas agropecuarios. Esta Tesis Doctoral se enmarca en este contexto, y aborda cómo favorecer el control biológico de algunas de las principales plagas del arbolado de las dehesas mediante un manejo innovador del ganado. El fin último es poder compatibilizar el incremento de la productividad de la dehesa con la conservación de estos sistemas silvopastorales ibéricos de altísimo valor natural.

Las dehesas (llamadas "montados" en Portugal) son el sistema agroforestal más extenso de Europa (Joffre et al. 1999; Moreno et al 2007) con aproximadamente 1 millón de hectáreas en Portugal y 3.5 millones en España (Burgalho et al. 2018). De éstas, entre 900.000 y 1.200.000 ha. se encuentran en Extremadura (las cifras varían dependiendo de la fuente que se consulte debido a las diferencias en los criterios para hacer los cálculos). Esto supone aproximadamente el 51% de la superficie agraria útil extremeña (López Sáez et al. 2007) y uno de los principales motores económicos, especialmente en las zonas rurales. Las dehesas constituyen un ejemplo del equilibrio entre la explotación de los recursos naturales del bosque y su conservación. De hecho, la importancia de la diversidad biológica de las dehesas se ve reflejada en la tipificación de las Dehesas de Encina y Alcornoque como Hábitat de Interés Comunitario (código 53510 de la Directiva 92/42/CEE de 21 de mayo de 1992). La principal actividad económica que se realiza en la dehesa es la ganadería (Moreno y Pulido 2009; Pulido et al. 2007), que en el caso del sector del cerdo ibérico llega a generar unos ingresos de alrededor de 1.88 billones de euros anuales (Vilches et al. 2014). No obstante, actualmente existe una gran dependencia de la compra de alimentos procedentes de fuera del ecosistema para la alimentación del ganado; hecho que hace vulnerables los márgenes comerciales de este sector (Pulido y Picardo 2010).

La dehesa es un sistema complejo formado por numerosos elementos y sus interacciones. El arbolado está compuesto principalmente por encinas (*Quercus ilex*, Linnaeus 1735) y alcornoques (*Quercus suber*, Linnaeus 1735) que se encuentran distribuidos en una matriz de pastos con diferentes densidades. La encina (*Q. ilex*) es el árbol más abundante en la dehesa y presenta una amplia variabilidad en la producción interanual de frutos, denominados comúnmente bellotas (Bonal y Muñoz 2007; Espelta et al. 2008; Fernández-Martínez et al. 2015). Las bellotas maduran en un año: la floración ocurre en primavera, las flores polinizadas desarrollan los frutos en el verano hasta que maduran completamente y caen durante el otoño y el invierno (Bonal y Muñoz 2008). Esta época es conocida en las dehesas como la montanera, período durante el cual la bellota se aprovecha para la alimentación del ganado, especialmente del cerdo ibérico (Rodriguez-Estévez 2009a).

La bellota es un fruto compuesto por dos partes fácilmente distinguibles: el fruto y el involucro, una cúpula de escamas que proviene del cáliz de las flores. El fruto, a su vez, se constituye por una capa externa, dura, llamada pericarpio que protege la semilla, en la que se integran dos cotiledones y el embrión, localizado la parte apical, el extremo más puntiagudo (Bonal et al. 2007). Su masa varía poco entre las bellotas de un mismo árbol, pero puede ser muy diferente si se comparan bellotas de diferentes madres: se han demostrado rangos de variación dentro de una misma población de hasta 8 gramos dependiendo del árbol de procedencia (Bonal et al. 2007). Esta masa también se ve afectada por la altitud y latitud, las poblaciones de encinas situadas a menores altitudes y latitudes producen bellotas de mayor tamaño (Bogdziewicz et al. 2018). Estos frutos son ricos en lípidos y proteínas, puesto que contienen el material necesario para el desarrollo de las plántulas (Tejerina 2011), por lo que son una fuente de alimento fundamental para la fauna de los ecosistemas mediterráneos durante el otoño e invierno (Gómez et al. 2003; Muñoz y Bonal 2008).

La producción total de bellotas está determinada por diversos factores tanto ambientales como biológicos que pueden afectar a la floración (Bogdziewicz et al. 2020), polinización (Pearse et al. 2015) y al desarrollo del fruto en el árbol (Espelta et al. 2008). Durante las primeras fases del desarrollo del fruto, la producción puede verse mermada por el efecto de los defoliadores en la planta madre. Diferentes especies del orden Lepidoptera, que engloba mariposas diurnas y nocturnas, pasan su estado larvario en forma de oruga alimentándose de plantas. La defoliación provoca una disminución de la superficie foliar (menor tejido fotosintético) lo que reduce la productividad, el crecimiento y la supervivencia de la planta (Piper y Fajardo 2014; Camarero et al. 2015). Los árboles del género *Quercus* pueden sufrir defoliaciones muy severas por algunas especies de lepidópteros como *Tortrix viridana* (Linnaeus, 1758), *Lymantria dispar* (Linnaeus, 1758) o *Malacosoma neustria* (Linnaeus, 1758) entre otras (Tiberi et al. 2016).

A medida que el fruto se desarrolla, puede ser atacado por insectos depredadores pre-dispersivos (atacan a la bellota antes de que se desprenda del árbol), cuyas larvas se alimentan de los cotiledones de la semilla. Los principales predadores pre-dispersivos de las bellotas son los coleópteros del género *Curculio* y los lepidópteros del género *Cydia*. En el caso de las dehesas de encinas, las especies dominantes dentro de estos grupos son *Curculio elephas* (Gyllenhal, 1836) (el más abundante) y *Cydia fagiglandana* (Zeller, 1841) (Rupérez 1957; Vázquez 1998; Bonal et al. 2007; Torres-Vila et al. 2008). *Cydia fagiglandana* es un lepidóptero que pertenece a la familia *Tortricidae*, mientras que *C. elephas* es un coleóptero perteneciente a la familia *Curculionidae*.

En el caso de *Cydia fagiglandana* los adultos vuelan a partir de junio y hacen las puestas en las ramas del árbol (Torres-Vila et al. 2008). Los huevos eclosionan y las orugas recién nacidas entran a partir de mediados de agosto en las bellotas que están aún creciendo y madurando. En el caso de *Curculio elephas*, los adultos emergen a partir de finales de agosto con las primeras lluvias de las tormentas de final de verano (Bonal et al. 2010a; Bonal et al. 2015; Bogdziewicz et al. 2019). Las hembras disponen de un rostro muy alargado que utilizan para perforar la cubierta de la bellota. A través del mismo introducen el oviscapto para realizar la puesta dentro de la bellota mientras ésta aún está en fase de crecimiento y maduración. En muchas ocasiones sólo ponen un huevo por semilla, aunque a veces pueden ser dos o tres. Además, varias hembras pueden realizar la puesta en la misma bellota (Desouhant et al. 2000; Bonal y Muñoz 2008). El huevo eclosiona al cabo de 5 días y la larva se alimenta de los cotiledones durante aproximadamente un mes.

Tanto en el caso de *Curculio* como el de *Cydia,* las bellotas atacadas son prematuramente abortadas por la encina. Caen al suelo con larvas aún en su interior, que deben completar su desarrollo antes de poder abandonar la bellota. En *C. elephas* ese periodo de tiempo es de 20 días en promedio (Bonal y Muñoz 2007; Bonal et al. 2010a; Canelo et al. 2020). Una vez que han terminado su desarrollo, las larvas de *Cydia fagiglandana* quedan enterradas entre la hojarasca, donde puparán (Ruíz-Carbayo et al. 2018). En cambio, las larvas de *Curculio elephas* se entierran nada más salir de la bellota a una profundidad de unos 20 cm. Donde permanecerán enterradas y en estado de diapausa; la mayoría puparán el verano siguiente y emergerán como adultos a los pocos días (Menu 1993; Bonal et al. 2015); un pequeño porcentaje, sin embargo, puede tener diapausas extendidas y pasar bajo tierra 2 y hasta 3 años (Menu and Debouzie 1993; Venner et al. 2011).

Los insectos defoliadores y los depredadores de semillas reducen la producción de bellotas en las dehesas (Rupérez 1957). Este descenso del número total de bellotas o de la proporción de bellotas no infestadas se traduce en importantes pérdidas económicas, ya que los frutos de las encinas son un recurso clave para la alimentación del ganado y la producción de carne de calidad (especialmente en el caso del cerdo ibérico) (Rodríguez-Estévez et al. 2009b; Tejerina et al. 2010). En el caso de los defoliadores se han documentado casos de daños muy cuantiosos, que han reducido enormemente la masa foliar (p. ej. Soria y Notario 1990). Sin embargo, aún faltan estudios que cuantifiquen de manera más precisa el efecto de la defoliación en la producción de bellotas. Este tipo de estudios, además, deberían incluir aproximaciones experimentales de reducción de la defoliación y cuantificación de los resultados en comparación con controles (ver Crawley 1985, para un trabajo similar en *Quercus robur*).

En el caso de los perforadores de bellotas, las pérdidas por *Curculio elephas* se han cuantificado en más de un 50% en muchas ocasiones, pudiendo llegar en algunos casos al 80% de la cosecha (Bonal et al. 2010b). No obstante, estos trabajos se han centrado más en la infestación por *Curculio* que por *Cydia* (aunque hay estudios que han abarcado ambos, p. ej. Torres-Vila et al. 2008). En

Curculio elephas, además, se ha encontrado que el tamaño de sus poblaciones varía mucho de año a año debido a su dependencia de la meteorología. Aquellos años en que las lluvias de finales de verano son copiosas, la cantidad de adultos que emergen de sus refugios subterráneos es mayor (ya que, de no ser así, la dureza del suelo impide que muchos puedan hacerlo). Por eso, las cuantificaciones de las tasas de infestación deberían incluir este tipo de variables meteorológicas que, además, permitirían predecir hasta cierto punto las pérdidas potenciales de bellotas. Queda también por saber, cuál es el efecto de la precipitación en *Cydia* y en las tasas de infestación global por el conjunto de insectos perforadores de bellotas. Todo esto es más relevante aún si se tiene en cuenta que las proyecciones de los modelos de Cambio Climático predicen alteraciones sustanciales en los patrones de precipitación de final de verano en el área Mediterránea (Giorgi y Lionello 2008; Serrano et al. 2020).

Las bellotas son el alimento de insectos especialistas (como *Cydia* o *Curculio*) pero también de muchos otros animales, entre los que destacan los mamíferos: desde los roedores (Sunyer et al. 2016) a los grandes herbívoros salvajes (ciervo, *Cervus elaphus* y jabalí *Sus scrofa*) (Linnaeus, 1758) (Bonal y Muñoz 2007) o domésticos (cerdos, ovejas, cabras y vacas de las dehesas). Esta gran variedad de depredadores compite por un mismo recurso: las bellotas, lo que propicia la interacción entre vertebrados e invertebrados (Gómez et al. 2003). Dentro de la lógica de la "intensificación ecológica", un buen conocimiento de esas interacciones nos podría permitir "manipularlas" para reducir el impacto negativo de los insectos.

En esta competición por el recurso (las bellotas), los depredadores que acceden a las bellotas cuando aún están en el árbol (insectos pre-dispersivos) tienen una ventaja temporal respecto a los post-dispersivos (mamíferos post-dispersivos). Éstos últimos sólo tendrán disponible aquella cantidad de recurso que hayan dejado los primeros, el tipo de competencia sería, por tanto, lo que en inglés se denomina "exploitative competition". Sin embargo, los grandes herbívoros también pueden afectar negativamente a las poblaciones de los pequeños artrópodos por otras vías. De manera general, se sabe que los grandes herbívoros, como el ganado, tienen un gran impacto en el funcionamiento de los ecosistemas terrestres (Foster et al. 2014), consumen una gran fracción de la producción primaria, modifican la estructura de la vegetación y la fisicoquímica del suelo y de las plantas (Asner et al. 2004; Bakker et al. 2004; Peco et al. 2006; Wolf et al. 2013). Por lo tanto, sus efectos sobre las poblaciones de artrópodos son muy significativas, aunque difíciles de predecir debido a los efectos en cascada que generan a través de las redes tróficas (Kruess y Tscharntke 2002; Sankaran y Augustine 2004). En el caso de los insectos depredadores de bellotas, sin embargo, el mayor efecto negativo que podrían sufrir no sería debido a la competencia por el alimento, sino por depredación directa.

La predación de herbívoros por otros herbívoros, depredación entre animales en el mismo nivel trófico, un tipo de "intraguild predation" en inglés; (Polis et al. 1989) está favorecida por las grandes diferencias de tamaño corporal (Zamora y Gómez 1993; Gómez y González-Megías 2002; Rooney y Waller 2003; Gómez y González-Megías, 2007; Gish et al., 2010). Los grandes herbívoros ingieren de forma accidental a otros herbívoros de menor tamaño cuando se alimentan de las plantas. Este hecho es más frecuente cuando ambos comparten la misma fuente de alimento, como es el caso de insectos depredadores de bellotas y el ganado de las dehesas. Se ha demostrado que los ungulados silvestres disminuyen las poblaciones de *Curculio elephas* con la ingestión de las bellotas infestadas (Bonal y Muñoz 2007). Estos insectos, desde que la bellota cae al suelo hasta que completan su desarrollo, tienen que pasar más de dos semanas dentro de la bellota; y en ese periodo son muy vulnerables a la depredación. Ciervos y jabalíes se comen las bellotas con las larvas dentro y reducen las tasas de infestación (Bonal y Muñoz 2007). Así pues, en dehesas ganaderas, favorecer e intensificar la depredación sobre las larvas de *Curculio* podría contribuir a reducir el impacto negativo de esta especie plaga. Es más, es el propio ganado el que podría manejarse para llevarlo a cabo. Para ello, no obstante, primero hay que saber si, al igual que los ungulados silvestres, el ganado también come bellotas infestadas. En segundo lugar, habría que saber en qué época y en qué medida habría que intensificar la depredación sobre las larvas de *Curculio*. Si el control de los insectos se hiciese efectivo, el ganado podría ofrecer un servicio ecosistémico muy importante en las dehesas de encinas.

El control biológico de insectos plaga mediante la intensificación del pastoreo podría tener, no obstante, algunos efectos colaterales no deseados, sobre todo en un agroecosistema con el gran valor natural que tiene la dehesa. Al mismo tiempo que podría hacer disminuir las poblaciones de *Curculio elephas*, otros artrópodos del suelo y el estrato herbáceo de la dehesa correrían el riesgo de verse afectados negativamente. Los grandes herbívoros modifican la estructura y composición del suelo (Armas-Herrera et al. 2019; Zhang et al. 2020) y generan grandes cambios en la funcionalidad de los ecosistemas (Eldridge et al. 2016, 2017). Se ha comprobado que la presencia de ganado afecta a la comunidad de artrópodos que habitan en el suelo. No obstante, si bien elevada carga ganadera puede tener efectos negativos, los mayores niveles de biodiversidad de artrópodos no se dan en ausencia de ganado, sino a niveles moderados de intensidad de pastoreo (Qin et al. 2017; Winck et al. 2019). Este hecho se explica mediante la hipótesis de la perturbación intermedia ("intermediate disturbance hypothesis" en inglés; Connell 1978) la cual postula que los grados intermedios de intensidad de una perturbación (como puede ser el pastoreo) sostienen una mayor diversidad de especies que los niveles extremos.

El mecanismo detrás de la hipótesis de la perturbación intermedia está basado en un equilibrio dependiente de la competencia entre organismos. Cuando el nivel de perturbación es muy fuerte, existe una selección constante que favorece a unas pocas especies. Por otro lado, si la perturbación es muy débil, se benefician los competidores más fuertes; por lo que también puede disminuir la biodiversidad. Este hecho está ampliamente demostrado comparando entre diferentes grados de densidades ganaderas (Joubert et al. 2016; Lázaro et al. 2016a, 2016b; Qin et al. 2017; Winck et al. 2019). Sin embargo, existen muchos menos estudios sobre los efectos del abandono del pastoreo en dehesas. En esta Tesis se pretende conocer a qué velocidad se recupera la biodiversidad de artrópodos de suelo y hierba una vez que el ganado es excluido. Esta información es muy importante para saber la capacidad de recuperación tras una intensificación del pastoreo encaminada a reducir el impacto de las plagas de bellotas. Para conocer la biodiversidad global de artrópodos (delimitación de especies), se ha utilizado una técnica de genética molecular de vanguardia: "Next-generation sequencing DNA metabarcoding" (NGS metabarcoding).

El NGS metabarcoding permite evaluar todas las categorías taxonómicas en menos tiempo que lo haría la taxonomía clásica. La determinación de especies es un trabajo que requiere la especialización profesional de quien realiza las determinaciones; por lo que, de forma habitual, los estudios se centran en comprobar sólo algunos grupos taxonómicos de los cuales el equipo de trabajo es experto. Así pues, en aquellos que no se centran en un determinado grupo de artrópodos, se hacen determinaciones a niveles taxonómicos muy generales (Didham et al. 2009; Jackson et al. 2015), en muchas ocasiones Orden y, raramente, Familia (Andrés et al. 1999; Sadaka y Ponge 2003; Rota et al. 2015). El NGS DNA metabarcoding, sin embargo, nos permite evaluar todos los grupos taxonómicos de artrópodos en un tiempo récord y hasta niveles taxonómicos más bajos. Hay que destacar, no obstante, que su capacidad para la identificación a nivel de especie se basa en la existencia de trabajos previos de taxonomía clásica. Estas especies determinadas morfológicamente deben, además, haber sido secuenciadas para poder disponer de una gran base de datos mundial con secuencias de referencias de multitud de taxones: The Barcode of Life DataSystems (BOLD).

El objetivo general de esta Tesis es cuantificar, de manera observacional y experimental, los efectos negativos de los insectos defoliadores y de los depredadores de semillas sobre la eficacia biológica de la encina y la producción de bellotas. Asimismo, se estudiará si la intensificación del pastoreo puede servir para controlar las poblaciones del principal insecto perforador de bellotas: *Curculio elephas*. Por último, mediante técnicas moleculares punteras, se analizarán las consecuencias negativas que este manejo ganadero podría tener sobre las comunidades de artrópodos de la dehesa en general. El fin último es proponer un manejo innovador de la dehesa (intensidad de pastoreo y calendario) que compatibilice el control biológico de las plagas del arbolado con la preservación de la biodiversidad de artrópodos.

Respecto a los objetivos concretos por capítulos, el desarrollo será el siguiente:

-Capítulo 1: Se cuantificará el efecto de los insectos defoliadores (orugas de Lepidópteros) en la pérdida de biomasa foliar y la producción de bellotas. Para ello se utilizará una aproximación experimental que implica la reducción artificial de la defoliación.

-Capítulo 2: Se analizará, a nivel detallado de bellota, el efecto de los dos tipos de insectos defoliadores (*Curculio* y *Cydia*) en la probabilidad de depredación del embrión y la pérdida de masa de los cotiledones. Aparte, con series de datos temporales, se estudiará hasta qué punto dependen de la meteorología las tasas de infestación de bellotas (por *Cydia*, *Curculio* y por ambos a la vez). Se discutirá cual puede ser la evolución de las mismas en el contexto de las predicciones de los modelos de Cambio Climático para la Cuenca Mediterránea.

-Capítulo 3: Se estudiará si el ganado de las dehesas depreda bellotas infestadas por *Curculio* y su grado de preferencia/rechazo de las mismas. En caso de que el ganado las consuma, se analizará cual es el momento adecuado en el que intensificar el pastoreo para maximizar la mortalidad de las larvas.

-Capítulo 4: Mediante un estudio experimental con un control y dos tratamientos opuestos (exclusión del ganado y eliminación manual de todas las bellotas infestadas), se cuantificará el efecto del ganado en las tasas de infestación por *Curculio elephas*. Esta aproximación permitirá conocer no sólo su efecto respecto a la ausencia de depredación de larvas, sino la reducción máxima de las tasas de infestación que podría llegar a alcanzarse mediante la intensificación del pastoreo.

-Capítulo 5: Utilizando técnicas moleculares punteras (NGS DNA metabarcoding) para la delimitación de especies, se analizará el efecto que la intensificación ganadera puede tener sobre la biodiversidad general de artrópodos del suelo y del estrato herbáceo de la dehesa.

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Chapter 1

Seed loss before seed predation: Experimental evidence of the negative effects of leaf feeding insects on acorn production

Abstract

Insect herbivory decreases plant fitness by constraining plant growth, survival and reproductive output. Most studies on the effects of herbivory in trees rely on correlational inter-individual comparisons and could, thus, be affected by confounding factors linked to both herbivory and plant performance. Using the Mediterranean Holm oak (*Quercus ilex* Linnaeus, 1753) as a study model, we followed an experimental approach in which leaf-feeding insects (mainly Lepidoptera caterpillars) were excluded from some shoots in all study trees. Shoots subjected to herbivore exclusion exhibited lower defoliation rates and produced more acorns than control shoots. Defoliation constrained shoot growth throughout the study period, but had no effect on the number of female flowers produced per shoot. Acorn production was, however, lower in control shoots due to their higher abortion rates, and also to their greater mortality risk during summer drought, as shoots with fewer leaves were less likely to survive. Plant reaction to herbivory inhibits certain physiological pathways involved in plant growth, which, together with the effects of physical damage, reduces the amount and efficiency of the photosynthetic tissue. This increases their vulnerability to environmental stresses, such as water deficit, which limit resource assimilation. Defoliation is likely a key factor affecting oak regeneration, as it may be a significant source of seed loss prior to pre-dispersal acorn predation. Further experimental studies could help to elucidate its effects in contrasting environments. In Mediterranean regions, the harsher droughts predicted by climate change models could worsen the effects of insect herbivory on oak reproductive output.

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1. INTRODUCTION

Herbivory constitutes one of the main antagonistic interactions between plants and insects, being the trigger of numerous coevolutionary processes (see review in Strauss and Zangerl 2002). The loss of photosynthetic tissue reduces plant productivity (Nabity et al. 2009), constraining their growth and threatening their survival (Rausher and Feeny 1980; Davidson et al. 1999; Piper and Fajardo 2014; Camarero et al. 2015). Herbivory may also reduce plant fecundity directly or indirectly. Direct effects include the consumption of flowers by herbivorous insects (review in Crawley 1989), whereas indirect effects result from the lower number of flowers produced due to the reduction of shoot growth (Samson and Werk, 1986). In addition, seed set and growth is costly, and a decrease of available resources increases seed abortion rates (Rausher and Feeny 1980; Marquis 1992; Niesenbaum 1996; Espelta et al. 2008; Sánchez-Humanes and Espelta 2011). The negative effects of defoliation on plant reproductive output may be extended in time and persist for many years (Morrow and LaMarche 1978; McConnel 1988).

Most studies on the effects of insect herbivory on plant fitness have followed a correlational approach, especially in the case of woody species (Landsber and Ohmart 1989; Simmons et al. 2014; Camarero et al. 2015). Those including manipulation (i.e. removal or reduction of herbivory) have been mostly carried out on herbaceous plants (but see Crawley 1985) due to the ease of performing such experimental treatments (Hawkes and Sullivan 2001; see review in Massad 2013). However, correlational studies may sometimes mislead our conclusions when other factors related to herbivory and acorn production are involved. For example, recent reports have shown that, within the same population, genetic diversity and relatedness between trees affect caterpillar herbivory rates in oaks (see Fernandez-Conradi et al. 2017) and these factors might, also, be related to plant reproductive output.

Oaks *Quercus* spp. are among the most prevalent tree species in the temperate forests of the northern hemisphere and may suffer severe defoliations (Schultz and Baldwin 1982; Davidson et al. 1999), especially with the periodic outbreaks of the gypsy moth (*Lymantria dispar* Linnaeus, 1758) among the most dramatic ever recorded in forest ecosystems (Gottschalk et al. 1998; Liebhold et al. 2000; Asaro and Chamberlin 2015). Defoliation, however, has not usually been included among the main factors impacting the decrease of natural regeneration, which is one of the main problems of oak forests worldwide. Rather, studies on recruitment failure have focused their attention on other issues, such as pre-dispersal and post-dispersal acorn predation, seed dispersal or the lack of favorable microhabitats for seedling establishment (e.g. Callaway 1992; Gómez 2004; Bonal et al. 2007; Zhang et al. 2008; Bonal and Muñoz 2009; Pulido et al. 2010; Xia et al. 2016).

Pre-dispersal seed predation reduces the number of sound seeds available for recruitment. In some oak species the proportion of acorns infested by insects averages 50% (Bonal et al. 2007), but in certain trees and years the losses may be much higher (Bonal and Muñoz 2007; Espelta et al. 2008; Xia et al. 2016). Post-dispersal acorn consumption may also be noteworthy and reduce the number of sound seeds available for dispersal (Bonal and Muñoz 2007, Zhang et al. 2008; González-Rodríguez and Villar 2012). Nonetheless, and in spite of the relevance of all these factors and bottlenecks along the regeneration cycle, constraints on acorn production may start before pre-dispersal predation.

Some factors related with early acorn losses are abiotic, especially those related with pollination failure (Pearse et al. 2016). In addition, in areas with severe droughts, like the evergreen Mediterranean forests, seed abortion during the summer may provoke sharp decreases in the number of acorns produced (Espelta et al. 2008). At this early stage, insect defoliation can also reduce the size of the acorn crop significantly. Different studies have shown strong decreases after massive defoliation events (Davidson et al. 1999; Nakajima 2015). Nonetheless, reports on the effects of the average defoliation rates are less common and mostly correlational. Moreover, the few experimental studies that have approached this subject on individual trees have quantified the effects once the acorns have matured (e.g. Crawley 1985). They have analyzed neither the stage of flower or seed development at which defoliation has a stronger effect, nor the consequences of the interactions between defoliation and the environmental conditions on acorn production.

We carried out an experimental study to determine the effects of insect defoliation on acorn production using the Holm oak (*Quercus ilex* Linnaeus, 1753) as a model. The Holm oak is the most widespread tree in the Mediterranean forests of the Iberian Peninsula, where it inhabits areas with strong summer droughts and suffers defoliation mainly by moth caterpillars (Lepidoptera) (Ruiz-Carbayo et al. 2016). Contrary to previous studies with other oak species, in which defoliators were excluded from certain trees and compared with control ones (Crawley 1985), we randomly excluded caterpillars from a number of branches of all trees and compared them with control branches in the same oaks, thus controlling for potential individual effects. Moreover, we followed the effects of defoliation along the whole season: from early shoot elongation to the final stage of acorn growth after summer drought. In addition, analysing whether the effects of defoliation differed through the season, we applied 2 different exclusion treatments: in some branches caterpillars were excluded from early new shoot elongation and in others, exclusion experiments were carried out 1 month after the caterpillars had started feeding.

Our concrete objectives were: (i) to analyse whether the exclusion of caterpillars increased the final acorn production; (ii) to assess whether defoliation reduces oak growth, shoot length and/or the number of leaves per shoot; and (iii) to investigate the mechanisms by which defoliation may reduce acorn production, analysing the effect of shoot length and the number of leaves on female flower production and acorn set success.

2. MATERIALS AND METHODS

2.1 Study area

The study was performed at a Holm oak *Q. ilex* L. savannah located in Malpartida de Plasencia, in the province of Cáceres, western Spain (39°56′N, 5°58′W) between April and September 2016. Oak savannahs (so called dehesas in Spain) are agroecosystems resulting from the human use of Mediterranean forests over centuries. Tree clearing has produced landscapes with oaks interspersed within a grassland matrix, in which livestock raising is the main use of land (Moreno and Pulido 2009). Our study was carried out in a dehesa of 50 ha with a mean density of 14 trees/ha.

2.2 Study species

The broadleaved evergreen Holm oak (*Q. ilex* L.) spreads over the western Mediterranean basin, being the most widely distributed tree species in the Iberian Peninsula (Blanco et al. 1997). It inhabits a wide range of habitats but predominates in dry Mediterranean regions with strong summer droughts, which will presumably suffer the consequences of global warming more severely (Petit et al. 2005). In these areas, July and August are the warmest months, with temperatures that can reach 40°C and almost no precipitation except for some storms later in the season with an average rainfall of just 6 mm in July and 7 mm in August (AEMET, Spanish Meteorological Agency).

Holm oak acorns mature in 1 year and budburst takes place in early April. Flowering occurs in April–May and acorns grow throughout the summer until early autumn. Summer drought, with its extreme temperatures and water deficit, constitutes the main cause of premature acorn abortion (Espelta et al. 2008).

Fresh new oak shoots are defoliated by a number of insect species, mainly moth caterpillars (Lepidoptera) (Ruiz-Carbayo et al. 2016). According to a previous sampling at our study site the more abundant species feeding on *Q. ilex* are: *Catocala nymphagoga* (Esper, 1787), *Dryobota labecula* (Esper, 1788), *Dryobotodes eremita* (Fabricius, 1775), *Dryobotodes monochroma* (Esper, 1790), *Phycita torrenti* (Agenjo, 1972), *Tortricodes alternella* (Denis and Schiffermüller, 1775) and *Tortrix viridana* (Linnaeus, 1785) (unpublished results). Caterpillars start feeding from early budburst in early April and continue throughout the spring until early June, when the sclerified leaves become too tough to be eaten (Elkinton et al. 1996). This phenology is, however, subjected to variability due to the stochasticity of the Mediterranean climate (Gilioli et al. 2013). The species composition of caterpillar guilds also changes during the spring, with the family *Tortricidae* the first to feed on the incipient new shots and the *Geometridae* species the last (Soria 1987). Their reproductive strategies also differ and go from short-cycle species, which oviposit before summer, to long-cycle ones that overwinter as pupae. Caterpillar damage in the Holm oak also exhibits inter-annual variability and severe defoliations, especially by *Tortrix viridana* L. (Lepidoptera: *Tortricidae*) (Schroeder and Degen 2008).

2.3 Experimental design and sampling methodology

Fifteen adult Holm oaks of similar size were selected for the experiment, excluding those with evident symptoms of decay. At each tree, 12 branches were assigned randomly to 4 different categories: Control (C, no treatment), Treatment 1 (T1, caterpillars excluded from the onset of new shoot elongation onwards), Treatment 2 (T2, excluded 1 month after caterpillars started feeding) and Negative Control (NC, treated with water). At each tree, 1 branch was assigned randomly to 1 treatment and, starting from it, the rest were allocated to the other treatments following a clockwise design (see Fig. 1A).

Ten shoots per branch were arbitrarily selected on April 1st (a total of 1800 shoots initially marked including all trees: 15 trees, ×12 branches/tree, ×10 shoots per branch). All shoots were individually identified by surrounding their base with a soft wire with a numbered label. The soft wire was adjusted just below the axillary bud, and was wide enough not to damage the shoot. Caterpillars were excluded by spraying insecticide dissolved in water (cypermethrin 0.005% vol.) over the treated branches 2 times per week. When it was raining, the spraying was repeated the day after the rain stopped. Shoots subjected to Treatment 1 (T1) were sprayed from the onset of shoot elongation (April 1st) to June 1st. In the case of Treatment 2 (T2), spraying started 1 month later (May 1st) and ended also on June 1st. In the Control group (C) shoots were not treated at all and suffered natural defoliation levels throughout the study period. We also established a Negative-Control (CN) in which branches were sprayed from the beginning just with regular water containing no insecticide at all (see Fig. 1B).

Cypermethrin is a pyrethroid insecticide ($C_{22}H_{19}Cl_2NO_3$) with an environmental persistence lower than 90 days. The European Commission and the United States Environmental Protection Agency (EPA) allow its use on plants (Pesticide Properties DataBase: http://sitem.herts.ac.uk/aeru/footprint/index2.htm). Its bioaccumulation has been detected on aquatic animals such as freshwater fish when used in high concentrations (Corcellas et al. 2015) but there were no rivers in our study area. This insecticide can be extensively used in croplands (Al-Sarar et al. 2006; Varikou et al. 2015) and is also recommended for gardening and horticulture, thus having no adverse effect on plant growth or productivity (what excludes a potential bias of insecticide application on our results). We used the dose provided by the commercial product Cythrin spray (0.005% vol.) approved by the Spanish Ministry of Agriculture (license number 25581). This product is aimed at phytophagous insects and our results showed that that concentration was, indeed, enough to reduce defoliation rates.

Control, T1 and T2 shoots were monitored 3 times: on May 1st, June 1st and September 5th. The Negative-Control shoots were measured at the end of the experiment on September 5th, to assess whether the application of the experimental treatment (water spraying) might have had any independent effect on the variables of interest, and so rule out any potential confounding effects (Fig. 1). At each revision we recorded whether the shoot was still present or it had been prematurely abscised, registered shoot length to the nearest millimetre and the number of leaves. The number of female flowers was recorded in June to compare it with the number of grown acorns in September, just after the summer drought. In September, we harvested all shoots and took them to the laboratory to record their length, the number of leaves and the number of acorns produced. We also calculated the final defoliation rates by dividing at each shoot the number of leaves with signs of herbivory by the total number of leaves.

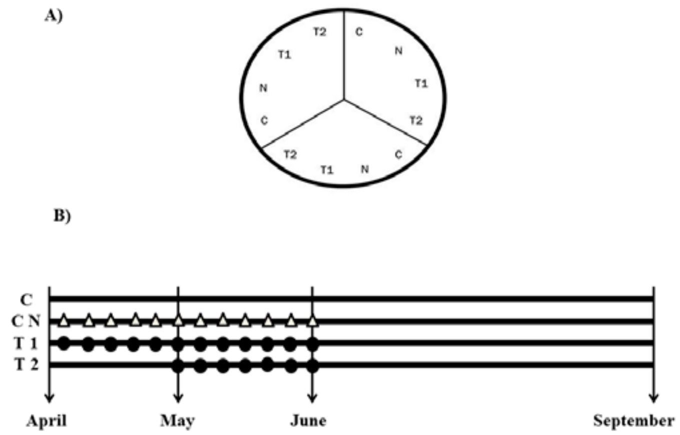

Figure 1. Experimental design. **A**) Scheme depicting the distribution of all the experimental treatments and controls at the canopy of each tree (T1, T2, C and CN) corresponding to Treatment 1, Treatment 2, Control and Negative Control, respectively. **B**) Chronology of the experimental treatments: filled black dots show the timing of insecticide spraying in T1 and T2. Empty triangles show the duration of the water spraying (no insecticide) on the Negative Controls. Control shoots received no treatment throughout the study period.

2.4 Statistical analyses

We used the Tukey test of additivity to assess whether the experimental design adjusted to a complete randomized block, and so the effects of the experimental treatments on defoliation rates and acorn production per shoot in September did not differ among trees. The differences between treatments (C, T1, T2 and N-C) were analysed using an ANOVA to assess the differences in defoliation rates and in the number of acorns produced. Post-hoc analyses for pairwise treatment comparisons were performed with Tukey tests.

We analysed whether the temporal changes in shoot length differed between treatments using a linear mixed model (LMM) in which shoot identity was included as a random factor. For the analysis on the number of leaves per shoot, a generalized linear mixed model (GLMM) with a Poisson distribution of errors was conducted, including shoot length as a continuous covariate and shoot identity as a random factor. Pairwise post-hoc Tukey tests were performed in both cases (shoot length and number of leaves) to assess differences between treatments at different times of the study period.

The differences between treatments (C, T1 and T2) in the number of female flowers in June were evaluated using an ANOVA. To test whether acorn set success differed between treatments (C, T1 and T2) we used 2 GLMMs with a binomial distribution of errors and shoot identity as a random factor. In the first test, we compared the number of female flowers in June versus the number of grown acorns in September in all shoots that had flowers in June. In those that were not present in September (shoots prematurely abscised during the summer) acorn set success was zero. In the second test, we did the same but only including those shoots that had flowers in June and were alive in September. Doing this it is possible to estimate the relative impact of shoot mortality during the summer drought on acorn set success.

Shoot survival throughout the study period was analysed using a Kaplan–Meier analysis. In addition, we investigated the factors affecting shoot mortality during summer drought with a generalized linear model (GLM). The dependent variable was shoot mortality risk (binomial distribution: variable takes the value 1 when the shoot died and zero when it did not); independent variables included the shoot length, the number of leaves and the number of flowers.

All data analyses were performed with the statistical analysis program R Core Team (2016) and using the following libraries: rJava (Urbanek 2016), xlsxjars (Dragulescu 2014a), xlsx (Dragulescu 2014b), asbio (Aho 2016), multcomp (Hothorn et al. 2008), nlme (Pinheiro et al. 2016), lme4 (Bates et al. 2015), Matrix (Bates and Maechler 2016), survival (Therneau 2015) and survMisc (Dardis 2016).

3. RESULTS

The Tukey test of additivity showed that the effect of the experimental treatments (C, T1, T2 and CN) did not differ between trees (block factor) either in the defoliation rates (F = 0.029; df = 1461; P = 0.87), nor in the number of acorns produced (F = 0.122; df = 1461; P = 0.72). We could, thus, perform an ANOVA to assess the effect of the experimental treatment on both defoliation rates and the number of leaves per shoot. Caterpillar exclusion (i.e. insecticide spraying) reduced the percentage of leaves defoliated (ANOVA $F_{3,1462} = 47.595$; $P < 0.001$; Fig 2A). In both exclusion treatments (T1 and T2) the final defoliation rates recorded in September were significantly lower than in C shoots (T1 estimate: −16.098; t = −7.697; P < 0.001 and T2 estimate: −10.879; t = −5.178; P = 0.001) (Fig. 2A). There were not significant differences between C and NC, whereas defoliation rates were lower (marginally significant) in T1 compared to T2 shoots (in the latter caterpillars were excluded 1 month later) (estimate: 5.219; t = 2.575; P = 0.05) (Fig. 2A). Defoliation decreased acorn production, as there were differences between treatments (ANOVA $F_{3,1462} = 5.106$; $P = 0.002$) (Fig. 2B). The number of acorns in September was higher in shoots subjected to T1 than either in the C (estimate = 0.065; t = 2.689; P = 0.036) or the NC shoots (estimate = 0.072; t = 3.156; P = 0.009). The rest of the pairwise comparisons were not significant (P > 0.05 in all cases) (Fig. 2B).

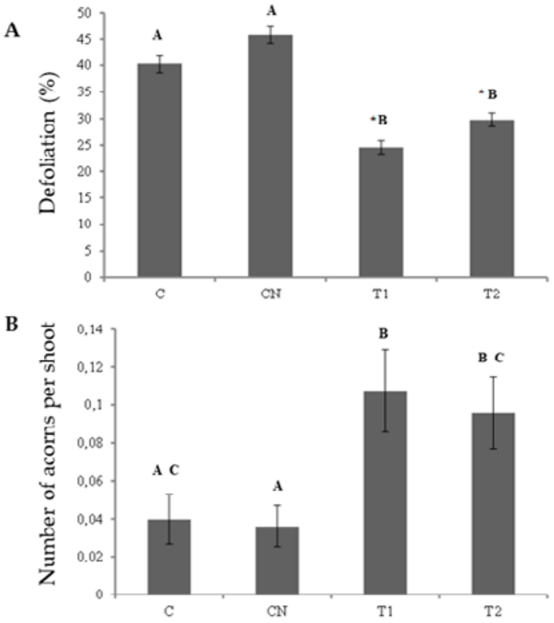

Figure 2. Bar plot showing **A**) the defoliation rates (number of leaves with signs of herbivory/total number of leaves; mean ± SE) and **B**) the number of acorns per shoot (mean ± SE) measured at oak shoots subjected to all the different treatments and controls (T1, T2, C and NG) at the end of the study period (September).

Different letters on the bars indicate statistically significant differences between groups ($P < 0.05$). The asterisks show the groups with the same letters among which the differences are marginally significant ($0.10 > P > 0.05$).

Caterpillar defoliation constrained shoot development. The LMM showed a significant interaction between time and treatments in shoot length ($F_{4,2333} = 9.467$; $P < 0.001$; Fig. 3A) as T1 shoots grew more than the others throughout the study period. In June only a marginal significant result (comparison between T1 and C) was found (estimate = 5.726; $Z = 2.804$; $P = 0.092$), but in September, T1 shoots were significantly longer than C shoots (estimate = 7.325; $Z = 3.426$; $P = 0.014$; Fig. 3A). Differences in the number of leaves per shoot were found between treatments as well. Longer shoots had more leaves (estimate = 0.0086; $Z = 32.64$; $P < 0.001$) but, irrespective of their length, the number of leaves was higher in T1 compared with the C shoots in June (estimate = 0.1625; $Z = 4.919$; $P < 0.001$; Fig. 3B). The number of leaves was also higher in T2 compared to C shoots, but the result was only marginally significant (estimate = 0.10; $Z = 2.979$; $P = 0.067$). In September the gap between treatments decreased, as even the differences between C and T1 did not reach significance (estimate = 0.097; $Z = 3.008$; $P = 0.062$; Fig. 3B).

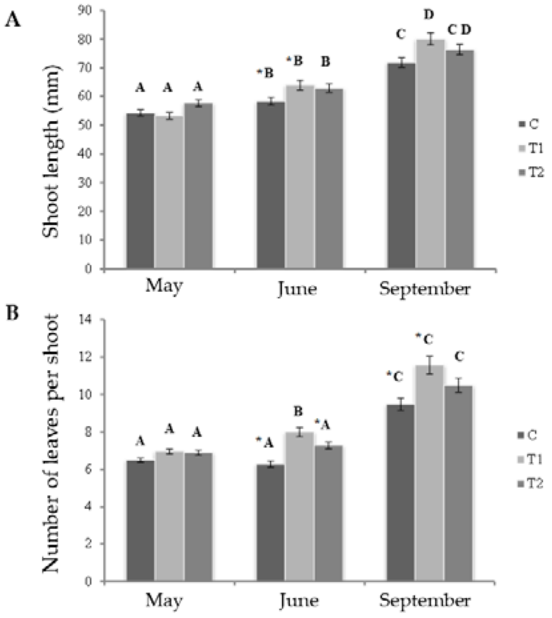

Figure 3. Temporal changes in shoot length (mean ± SE) and number of leaves per shoot (mean ± SE) registered along the study period in shoots subjected to Treatment 1, Treatment 2 and Control (T1, T2 and C, respectively). Different letters on the bars indicate statistically significant differences between groups ($P < 0.05$). The asterisks show the groups with the same letters among which the differences are marginally significant ($0.10 > P > 0.05$).

Caterpillar feeding had no effect on the number of female flowers in June, which did not differ between treatments (C, T1 and T2) (ANOVA $F_{2,1273} = 2.107$; $P = 0.122$). The number of acorns in September, however, did vary between them (Fig. 2B). The first GLMM including all the shoots bearing female flowers in June ($N = 137$) and setting to zero the number of acorns in those shoots that died and were abscised during the summer drought (i.e. not present in September), showed that acorn set success was significantly higher in T1 than in C (estimate = 1.380; $Z = 2.478$; $P = 0.013$; Table 1A). Acorn set rates were also higher in T2 compared to C, although the result was marginally significant (estimate = 1.055; $Z = 1.945$; $P = 0.052$). To figure out the effect on acorn abortion, a second GLMM test was analysed only with those shoots that had flowers in June and had survived the dry season ($N = 112$). In this case, the differences between treatments decreased (Table 1A); only the comparison between T1 and C was significant (estimate = 1.095; $Z = 1.990$; $P = 0.045$; Table 1B).

A

Treatment	Number of shoots	Flowers	Acorns	Set rate
Control	34	57	13	0.23
Treatment 1	49	88	39	0.44
Treatment 2	54	92	35	0.38

B

Treatment	Number of shoots	Flowers	Acorns	Set rate
Control	25	40	13	0.33
Treatment 1	39	76	39	0.51
Treatment 2	46	81	35	0.43

Table 1. Acorn set rates (number of female flowers in June divided by the number of acorns in September) in C, T1 and T2: **A**) set rates calculated including all shoots bearing flowers in June and setting to zero the number of acorns in those shoots that were prematurely abscised (i.e. died) during the summer and **B**) set rates including only those shoots that were alive both in June and September and, thus, reflecting only acorn losses due to abortion, not to shoot mortality.

Shoot survival probability changed throughout the study period (Fig. 4). The Kaplan–Meier analyses showed that summer survival (from June to September) decreased less in those shoots sprayed with insecticide (T1 and T2) compared to C shoots subjected to natural defoliation rates (Chi^2 = 26.1; df = 2; P < 0.001; Fig. 4). A further general lineal model of the causes underpinning this higher mortality risk showed that those shoots with a lower number of leaves in June had a higher chance of dying (i.e. being prematurely abscised) during the summer drought period (Fig. 5) (estimate = −0.06; Z = −2.875; P = 0.004), whereas neither shoot length (estimate = 0.004; Z = 1.259; P = 0.208) nor the number of flowers (estimate = −0.015; Z =−0.128; P = 0.898) had any significant effect.

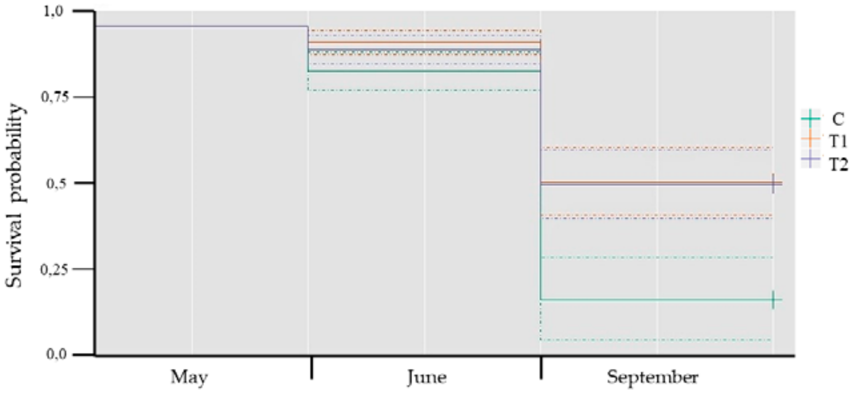

Figure 4. Graph of the results of the Kaplan–Meier analysis showing the temporal changes in mean survival probability of the oak shoots subjected to the 2 treatments, T1 (orange line) and T2 (purple line) and the Control (green line) during the study period. Dashed lines represent the standard errors of the mean of each group.

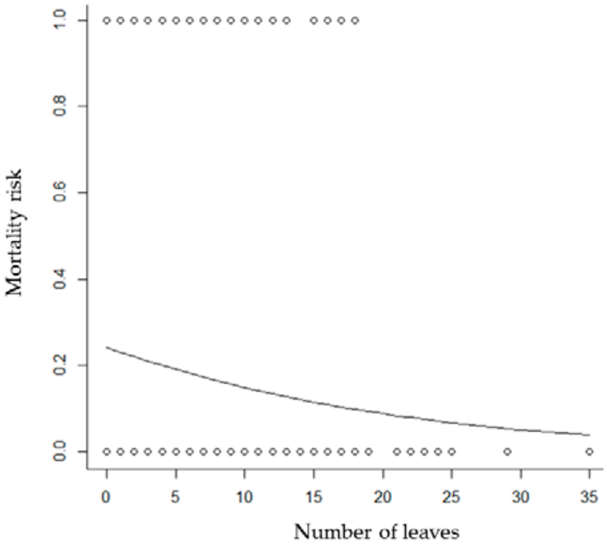

Figure 5. Relationship between the number of leaves per shoot and shoot mortality risk during the summer drought. Shoot mortality risk is coded as 1 when the shoot died from June to September and as zero when it did not. The function shows that shoots with more leaves were less likely to die.

4. DISCUSSION

Defoliation significantly reduces acorn production in the Holm oak *Q. ilex*; that is, experimental shoots sprayed with insecticide exhibited lower defoliation rates and produced more acorns. Defoliation decreased shoot growth throughout the study period, but the number of female flowers at the end of the spring did not differ between treatments. However, acorn abortion rates and premature shoot abscission during the summer drought were higher in the smaller and heavily defoliated control shoots than in the experimental shoots, which provoked a sharp reduction of the acorn crop.

With respect to the few previous experimental approaches that have used trees as study models to assess the effects of defoliation on seed production (e.g. Crawley 1985), ours comprises a number of novelties. We included a negative control group in the analyses, which ruled out any effect of the experimental procedure itself (i.e. liquid spraying) on the variables of interest, thus confirming the negative relationship of caterpillar defoliation with shoot length, number of leaves and acorn production. In addition, we monitored the effects of defoliation throughout the study period, which evidenced the progressive negative effects on shoot growth. More importantly, this temporal monitoring showed the impact of summer drought on shoot premature abscission (i.e. mortality). To the best of our knowledge, this negative effect had gone previously unnoticed in oaks, because measurements in previous studies of acorn production were made just once in autumn comparing control and treated trees (e.g. Crawley, 1985). That sort of approach attributes acorn losses from defoliation just to seed abortion, while this work has shown that they are the consequence of both seed abortion and summer shoot abscission (i.e. mortality).

There are a number of mechanisms involved in plant responses to defoliation that may be underpinning the decreased shoot growth observed in our study. Plants are able to recognize herbivory damage thanks to fine-tuned sensors of the elicitors of the saliva (Kessler and Baldwin 2002; Darwish et al. 2008). The reaction may start even before the damage occurs, as oviposition can also be detected (Balbyshev and Lorenzen 1997; Doss et al. 2000; Meiners and Hilker 2000; Meiners et al. 2000). When non-lethal herbivory stress occurs, compounds like reactive oxygen species (ROS) trigger plant responses by modifying the transport of phytohormones and/or plant metabolites (Potters et al. 2007).

The physiological response to insect damage may reduce plant growth. Insect defoliation activates the synthesis of jasmonates (Filella et al. 2006; Nabity et al. 2009; Erb et al. 2012), which interact with the pathways of other compounds such as abscisic acid, cytokinins, brassinosteroids, gibberellins and auxins (Erb 2012). Auxins (indole-3-acetic acid, IAA) deserve special attention, due to their importance for plant growth and development (Geldner et al. 2003; Teale et al. 2006). Jasmonates reduce auxin activity, which, in turn, decreases photosynthetic PSII efficiency and CO_2 fixation rates (Agtuca et al. 2014); in angiosperms auxin inhibition may also reduce vessel size (Uggla et al. 1996). Our results suggest, however, that the physiological pathways that constrain plant growth may be reverted if defoliation disappears or decreases its intensity. The shoots subjected to Treatment 2 suffered defoliation at the beginning of the study period, as insecticide spraying started 1 month later; however, these shoots could partially recover their normal growth rates and finally produced more acorns than control shoots (which suffered defoliation throughout the spring).

In the case of our study model, *Q. ilex*, experimental increase of jasmonates on leaves has been shown to decrease photosynthetic rates and stomata conductance, thus reducing CO_2 assimilation rates (Filella et al. 2006). These changes that alter photosynthetic efficiency and water balance make plants especially vulnerable to environmental stresses, such as water deficit (Quentin et al. 2011; Sánchez-Humanes and Espelta 2011). During drought periods, such as occurs in Mediterranean summers, plants face a trade-off between carbon gain and water loss (Cowan and Farquhar 1977) so that an optimal stomata functioning is essential to overcome severe water-stress conditions (Xu and Baldocchi 2003). The Holm oak *Q. ilex* has evolved physical and physiological adaptations to the Mediterranean climate, such as sclerotic leaves and the capability of closing its stomata to avoid excessive evapotranspiration. Stomatal closure limits the number of hours in which CO_2 may be assimilated during summer drought (Chaves et al. 2002), which may explain the high mortality rates observed in the more heavily defoliated shoots. To the natural environmental limitation for CO2 assimilation during the summer, defoliated shoots would add a reduced photosynthetic tissue (i.e. fewer leaves), a diminished photosynthetic efficiency and a reduced stomata conductance. Moreover, the direct mechanical damage would increase their vulnerability to drought, as dehydration risk is higher in damaged leaves (Aldea et al. 2005). This higher risk could also explain the lower number of leaves in the control shoots (not only explained by their shorter length) as dehydration could lead to premature leaf abscission.

The negative effects of defoliation on resource assimilation may also underlie the lower final reproductive output in control shoots. The initial investment in reproduction (number of flowers in June) did not differ between treatments, suggesting that, contrary to other cases (Crawley 1989), caterpillars did not decrease oak reproductive output by eating flowers. However, the final production of acorns per shoot did differ. Seed abortion was higher in control shoots (not sprayed with insecticide), probably because the investment in reproduction was programmed before spring and they could not adapt their inversion to the level of damage (Marquis 1992). Acorn growth is based on the photosynthates produced during the seeding season (Hoch et al. 2013, Fernández-Martínez et al. 2015) and the loss of leaf tissue would reduce the capability of storing resources available for acorn production. The lower resource availability may also account for the higher summer mortality (premature abscission) recorded in control shoots, which reduced acorn production significantly. Even though adult Holm oaks are well adapted to the dry Mediterranean climate and rarely die (Lopez-Iglesias et al. 2014), shoot mortality during the summer may be very significant.

In accordance with previous studies, our results show that, due to the modular architecture of trees (Takenaka 1994), oaks did not mobilize resources from healthier shoots to more damaged shoots (Sánchez-Humanes et al. 2011; Hoch et al. 2013). Acorn abortion or shoot mortality could, thus, be barely counterbalanced by the shoots nearby. Treated and control shoots were in the same trees, but their mortality and reproductive output differed greatly. In addition, irrespective of the treatment, those shoots with a higher number of leaves in June had a higher probability of surviving summer drought. In September there were no differences in the number of leaves between control and experimental shoots, as the control shoots that were more heavily defoliated were prematurely abscised during the summer.

From a conservation perspective, these results stress the importance of defoliation as a limitation of natural oak recruitment. Defoliation may reduce the number of acorns available for regeneration even more than pre-dispersal acorn predators. In the Holm oak, infestation rates by acorn-boring insects average 50% of the crop, but a percentage of the infested acorns may still germinate and produce viable seedlings (Bonal et al. 2007). In comparison, acorn production in control shoots was almost half of that of the experimental shoots, and the difference could be wider considering that more severe defoliations rates have been reported in oaks (Schroeder and Degen 2008).

Within the global change context, the negative effects of defoliation on oak recruitment may worsen. Climate models predict an increase of air temperatures and water deficit in the Mediterranean areas, which will presumably favour leaf feeding insects (Hódar and Zamora 2004; Dymond et al. 2010; Carnicer et al. 2011; Jacquet et al. 2012). Considering that defoliated shoots are more vulnerable to die during the summer drought, such a climatic scenario will seriously hamper oak natural regeneration. The economic losses would also increase in that context, especially in the Spanish oak savannahs (dehesas), where acorns constitute a key food source for livestock in autumn (Moreno and Pulido 2009). However, the natural value of such agroecosystems, included in the Directive Habitats of the European Union, advise against any massive insecticide spraying to control caterpillar populations. Rather, management measures should promote their natural enemies, such as insectivorous birds (Sanz 2001) or parasitoids.

In summary, these results put forward that seed loss may start before pre-dispersal acorn predation. Defoliation should be included in future studies on oak natural regeneration, preferentially in contrasting ecological contexts, as its negative effects could vary depending on the environmental conditions. At least in Mediterranean oak forests, subjected to strong summer droughts, defoliation poses an important threat to oak reproductive output.

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Chapter 2

Effects of longer droughts on acorn pest species prevalence: consequences for Holm oak, *Quercus ilex* fitness and seed production.

Abstract

Climate change is expected to worsen the effects of forest pests. It may modify the species composition and/or relative prevalence in insect trophic guilds, what will have further consequences for the host plants. The effects of Climate Change on oak *Quercus* spp. forests are a main environmental concern, as they cover large regions of the Northern Hemisphere. In this context, we assessed the consequences that longer droughts could have on the interactions between the Holm oak *Quercus ilex* and its main acorn pests. Holm oak acorns were mainly attacked by *Curculio elephas* (Coleoptera) and in a lesser extent by *Cydia fagiglandana* (Lepidoptera). Infested acorns were abscised before reaching their potential size. Acorns attacked by *Cydia* were smaller than those attacked by *Curculio*, however, their weight did not differ because *Curculio* larvae consumed more cotyledon. For the same reason, embryo survival likelihood was not lower in *Cydia* acorns despite their smaller size. When late summer rains were delayed (longer droughts) infestation rates by *Curculio* decreased, as adult weevils need the rain to emerge from their underground cells. By contrast, *Cydia* was negatively affected by early and more abundant rainfall, which might hamper adult flight and eggs/L1 larvae survival. There was not a "zero-sum" effect, because the decrease of *Curculio* infestation rates was not compensated by the increase of *Cydia* prevalence. Climatic models predict longer droughts for the western Mediterranean Basin, where the Holm oak is the most widespread tree species. Under these conditions, our results predict a slight decrease of total infestation by insects. At the acorn level the effects of *Cydia* and *Curculio* on final acorn weight and embryo survival do not differ. Hence, a decrease of total infestation rates might be translated into increased tree recruitment and sound acorn production in oak silvopastoral systems. Yet, we must be cautious interpreting the net effects, as there are also negative factors linked to drought (e. g. increased acorn abortion or seedling mortality). However, our results show environmental changes do not necessarily have to be detrimental for oak fitness. This perspective should be considered in further research on this topic.

1. INTRODUCTION

The forecasted worsening of pest impacts due to Climate Change is a main worry for forest ecologists and managers (Logan et al. 2003; Pureswaran et al. 2018). It may affect insect physiology, individual fitness and population growth, what in turn may provoke changes in insect community composition, ecological interactions and ultimately in ecosystem functioning (McKone et al. 1998; Voight et al. 2003; Zhu et al. 2014; Solbrek and Knape 2017; review in Lewis and Gripenberg 2008). In this context, we analyse the potential consequences of the harsher droughts predicted for southern Europe (Giorgi and Lionello 2008; Serrano et al. 2020) on Holm oak *Quercus ilex* acorn pests.

Climate change effects on phytophagous insects can be direct or indirect. The firsts result from the consequences of environmental variables (e. g. rainfall, temperature) on insect fitness (e. g. insect development speed, reproduction rate, winter survival etc.) (Logan et al. 2003; Poncet et al. 2009; Lewis and Gripenberg 2008; Bonal et al. 2015; Pureswaran 2018; Bogdziewicz et al. 2019). Indirect ones are generally mediated by plants and include changes in food availability, plant community and/or structural composition, phenological mismatches between insects and their host plants etc. (McKone et al. 1998; Poncet et al. 2009; Zhu et al. 2014; Solbrek and Knape 2017; Renner and Zohner 2018). Trophic specialists are specially vulnerable to indirect effects. This is the case of many phytophagous insects, which are strongly affected by any change in their host plants. These insects usually form assemblies of species that feed on the same class of resources (Simberloff and Dayan 1991). In these assemblies, some species are more susceptible than others regarding environmental changes (Solbrek and Knape 2017; Renner and Zohner 2018).

In turn, changes in insect trophic guilds affect their host plants. In the case of antagonistic interactions, the negative effects on plant fitness differ between species (Lewis and Gripenberg 2008). In seed-feeding insects, for example, some species systematically predate more seeds than others in the same guild. A decrease in their populations translates into lower proportions of seeds destroyed when its place is not occupied by another species (Solbrek and Knape 2017). In other cases, population fluctuations are compensated leading to a "zero-sum effect" in terms of seed predation: the species composition changes, but infestation rates remain unchanged (Ruiz-Carbayo et al. 2018).

How will climate change affect oak *Quercus* spp. forests has attracted a lot of research attention in the last two decades. These trees are widespread over the Northern Hemisphere, and support rich and diverse ecosystems. A large number of studies have analysed what will be the consequences of harsher droughts on oak growth, mortality or natural recruitment (see Conrad et al. 2020 for a review). However, we know very little about its potential effects on oak pests (but see Galko et al. 2018). In the case of acorn borer insects, only one (Bonal et al. 2015) has addressed this subject and just for one insect species.

The communities of acorn borer insects include species from different orders. The larvae of *Curculio* spp. (Coleoptera) weevils and *Cydia* spp. moths (Lepidoptera) complete their development within acorns feeding on the cotyledons. The identity at the species level may change, but the tandem *Curculio*-*Cydia* has been reported in oak forests worldwide (e. g. Lewis 1992; Maeto 1995; Csóka and Hirka 2006; Bonal et al. 2007; Xiao et al. 2007; Ruíz-Carbayo et al. 2018). *Curculio* larvae are in most cases more prevalent than *Cydia* caterpillars; occurrence of other insect taxa is rare, with the exception of *Conotrachelus* spp. in North America (Gibson 1964).

Acorn-boring insects have received a lot of attention by pest researchers because they hamper natural regeneration (Leiva and Fernández-Alés 2005; Bonal et al. 2007; Xiao et al. 2007). Also, in silvopastoral systems like the Iberian oak savannahs, these pests provoke economic losses, as acorns are a key food source for free-range livestock (Moreno and Pulido 2009). Previous studies have shown that the lower summer rainfall predicted by climatic models may reduce weevil population sizes (Bonal et al. 2010; Bonal et al. 2015, Espelta et al. 2017, Bogdziewicz et al. 2019). However, it remains unknown whether it will change the species composition of acorn-feeding guilds (i. e. relative prevalence of *Curculio* and *Cydia*).

In addition, if species composition changed, we still know little about what would be the consequences for oak fitness. Such consequences would depend not only infestation rates but also on the fate of the infested acorns. *Curculio* larvae do not always kill the acorns; they can germinate if the embryo is not eaten, although the loss of cotyledon will reduce seedling size (Branco et al. 2002; Bonal et al. 2007). This might differ in the case of *Cydia* depending on their way of feeding within the acorn. However, to the best of our knowledge, only two studies have compared embryo predation likelihood between acorn pests (Maeto 1995; Ruíz-Carbayo et al. 2018). Moreover, it remains unknown whether the final acorn weight differs between those infested by *Curculio* or *Cydia*. This is key in oak silvopastoral systems like Iberian dehesas, as acorn biomass loss translates into lower productivity (i. e. meat production decrease) (Tejerina et al. 2011).

We carried out a multi-year monitoring of Holm oak (*Quercus ilex*) acorn crops and pest insect community composition in three localities of Central and Western Spain. In addition, we carefully measured infestation rates and recorded the fate of each acorn. Relevant meteorological variables (i. e. late summer rainfall) were registered as well. Our general aim was to know whether a reduction and delay of rainfall could change the relative prevalence of acorn pests and have further consequences on infestation rates. Our specific objectives were: i) to test the differences between sound acorns and those infested by *Curculio* spp. and *Cydia* spp. in terms of: timing of acorn drop, acorn volume, final weight, cotyledon mass and embryo predation likelihood, ii) to assess the effects of late summer rainfall (timing and total amount) on acorn infestation rates by *Curculio* and *Cydia*.

2. **MATERIAL AND METHODS**

2.1 Study areas

The data used in this study came from three localities of Central and Western Spain. The first one was Huecas, Toledo Province (39°59′N, 4°13′W), a flat agricultural land in which Holm oaks are interspersed within cereal fields as isolated trees or in small plots (see Bonal et al. 2012 for a detailed description). In Huecas, acorn production and infestation rates by insects were monitored for 6 years (from 2008 to 2013) in 24 oaks. In three of those years every single acorn was measured and opened to assess infestation, biomass loss and embryo predation. Besides, in two localities of the province of Cáceres: Guijo de Granadilla (40°8'N, 6°7'W) and Malpartida de Plasencia

(39°56'N, 5°58'W), acorn production, infestation rates and infested acorns fate were measured for three years (2016 to 2018) in 16 and 8 trees, respectively. These last two places are human-made oak savannahs (so called *dehesas* in Spain and *montados* in Portugal), traditional agroforestry systems in which free range livestock rearing is the main human use (Moreno and Pulido 2009).

2.2 Study species

The Holm oak *Quercus ilex* is an evergreen oak distributed all over the Western Mediterranean Basin, being the most widespread tree species in the Iberian Peninsula (Blanco et al. 1997). Flowering occurs in spring and acorns grow throughout the summer and early autumn until mid-October. Sound acorns are dropped from then on until late December; those infested by insects are prematurely abscised and fall on average earlier (Bonal and Muñoz 2008). Insect larvae feed on the cotyledons, which contain the reserves for seedling development (Bonal et al. 2007; Bartlow et al. 2018). Infested acorns can germinate and produce viable seedlings if the larvae do not predate the small embryo located at the pointed end of the acorn (Branco et al. 2002; Bonal et al. 2007; Bartlow et al. 2018). The nutrient-rich acorns constitute an important food source in autumn and winter for free-range livestock in traditional silvopastoral systems (i. e. dehesas) (Moreno and Pulido 2009).

The main insects that feed on acorns are the specialist *Curculio* spp. weevils (Coleoptera: *Curculionidae*) and *Cydia* spp. moths (Lepidoptera: *Tortricidae*) (Torres-Vila et al. 2006; Bonal et al. 2007). In both cases the larvae grow and complete their development within acorns feeding on the cotyledons, but other traits of their life histories differ. In the case of weevils, females drill the acorn shell with their long rostrum and introduce the oviscapt through the oviposition hole. Usually a single egg is laid at each acorn, but larger clutches per seed may occur (Desouhant et al. 2000; Bonal and Muñoz 2009). By contrast, adult moths do not drill the pericarp, but lay their eggs in the shoots close to the acorns. The newly hatched caterpillars get into the acorn by drilling the softer pericarp under the acorn cap.

Curculio weevils are strictly univoltine. Larvae bury underground just after finishing their development in October-November and remain in diapause within individual earthen cells until the following year. Pupation takes place in underground refuges shortly before emerging as adults. The timing of adult emergence differs among species. In some it occurs in April-May, whereas in others from August onwards, once acorns are available in the canopies. Species with both types of strategies co-exist in the same oak forests (Espelta et al. 2009; Bonal et al. 2011). In our study areas we only recorded one species, namely *Curculio elephas*. *C. elephas* distribution is circum-Mediterranean, and it is very common in evergreen oak forests located in dry areas. Adults emerge from late August onwards favoured by late summer storms that soften the soil surface (Menu 1993; Bonal et al. 2015, Bogdziewicz et al. 2019).

Among *Cydia* moths, *Cydia fagiglandana* is the most prevalent species in evergreen Mediterranean oak woodlands (Torres-Vila et al. 2006, 2008). In our study area, we used DNA barcoding to identify a sample of 10 caterpillars and only recorded that species (unpublished data). *C. fagiglandana* is generally univoltine, but a small proportion of individuals may develop to adults and lay eggs in the same year (Torres-Vila et al. 2008). *Cydia* caterpillars can walk, and their mobility is much higher than that of legless weevil larvae. They may leave the acorn within which they started their development and walk to get into another one through the exit hole drilled by a conspecific or weevil larvae (Ito and Higashi 1991). Pupae overwinter slightly buried among the oak leaves on the ground (Ruíz-Carbayo et al. 2018) or in small crevices in the trunk (R. Bonal, pers. observ.). Adult flight starts in early summer (June-July) of the following year and oviposition takes place from August onwards, when grown acorns are available (Torres-Vila et al. 2008).

2.3 Field sampling and acorn classification

Seed traps were randomly placed under the canopies of all the study trees (hung from lower branches). The number of traps per tree was proportional to the canopy surface to cover a similar proportion in all trees (1 to 2%); canopy surface was calculated on an aerial picture using a Geographical Information System: ArcGIS (ESRI, 2011). This seed-trap based methodology provides very reliable estimates of acorn crops (see Bonal et al. 2007). Trap monitoring started in September and ended when the last acorns fell in late December. The content of the seed traps was collected every two weeks and taken to the laboratory, where acorns were classified as aborted or grown. In turn, all grown acorns were classified as sound or infested by insects (*Curculio* larvae or *Cydia* caterpillars).

Acorn classification was carefully carried out and confirmed following different procedures. In first place, all grown acorns received a unique code, were placed individually in plastic vials opened above and revised daily to record the number of larvae emerging from each one. Weevil larvae are legless and could not escape from the vials, what allowed an easy classification of the acorns attacked by *Curculio*. *Cydia* caterpillars were also frequently found in the vials, but sometimes they had left at the time of the daily monitoring. When we found an exit hole but no larvae, the acorn was tentatively classified as infested by *Cydia*, however, all acorns were subjected to further checking. *Cydia* infested acorns can be also told apart by the shape of the exit hole drilled through the pericarp (irregular shape compared to the round ones chewed by *Curculio* larvae). Moreover, when the acorn was opened, we could inspect the size and shape of the excrements (dust-like in the case of *Curculio* larvae and small granules in *Cydia*) (Pulido 1999).

One month after the last larva had emerged all acorns were oven dried for 48 hours at 80ºC. In order to calculate the volume, we measured acorn length and width with a digital calliper to the nearest 0.01 mm (Espelta et al. 2009). After that, to confirm a correct classification, we opened all of them to check whether there were dead larvae inside, excrements or any other sign of insect infestation. We also checked whether the embryo (located at the pointed end of the acorn) had been predated or not. By last, all acorns were weighted to the nearest 0.01 g. In the case of those infested by insects, we weighted only the acorn shell and the remains of cotyledons (if any) after removing the excrements carefully.

2.4 Acorn crop and infestation rates

Infestation rates by *Curculio* and *Cydia* were computed for each tree as the ratio between the number of acorns of each class divided by the total number of grown acorns (sound plus infested by both types of insects). We also calculated the total number of grown acorns produced by each tree by multiplying the number of acorns produced per square metre by the surface of the canopy.

2.5 Meteorological data

We used the daily precipitation records from the closest meteorological station for each of the three study sites. For Huecas, province of Toledo, the data came from the meteorological station of Toledo (39º 53′ N, 04º 02′ W). For the two study sites at the province of Cáceres, Guijo de Granadilla and Malpartida de Plasencia, the data came from the stations of Guijo de Granadilla (40º 11.5′ N, 06º 09.9′ W) and Plasencia (39º 58.7′ N, 06º 09.0′ W), respectively.

We considered the same variables than in previous studies on the effects of meteorology on *C. elephas* phenology (Bonal et al. 2015; Espelta et al. 2017; Bogdziewicz et al. 2019), namely total rainfall during emergence period and the date of the first late summer rain. Total rainfall corresponds to the summed rainfall (litres/m^2) during the period in which most adult *Curculio* emergence concentrates, from late August to October. This is also the period in which *Cydia* females are ovipositing, hence, we considered appropriate to use the same variable for both. The first late summer rain is the date from August 4th onwards in which at least a daily precipitation of 1 litre/m^2 was recorded (please see Bonal et al. 2015 for a detailed explanation of the two variables).

2.6 Statistical analyses

We used Linear Mixed Models (LMMs) to test the differences between sound acorns and those infested by *Curculio* spp. and *Cydia* spp. in terms of: timing of acorn drop, volume and final weight. The fixed independent factor had three levels (sound, *Cydia*, *Curculio*). We also tested the differences in cotyledon mass with another LMM including the same fixed factor but adding a continuous covariate (acorn volume). In all LMMs the tree and site identities were the random factors (the former nested within the latter). The likelihood of embryo predation was tested only between acorns infested by *Cydia* and *Curculio*. To do so we performed a Generalized Linear Mixed Model (GLMM) with binomial family (dependent variable: embryo predated/not predated). The fixed independent factor had two levels (*Cydia* – *Curculio*). Tree and site identities were the random factors (the former nested within the latter).

To assess the effects of late summer rainfall (timing and total amount) on acorn infestation rates we performed three Generalized Linear Mixed Models (GLMMs) (binomial family). In all of them, tree and site identities were the random effects (the former nested within the latter). The dependent variables in each of the three GLMMs were the proportion of grown acorns infested by: i) *Cydia*, ii) *Curculio*, and iii) pest insects as a whole (*Curculio* plus *Cydia*). The independent variables were in all GLMMs the total rainfall during insect emergence period, the date of the first late summer rain and the total number of acorns produced by the tree (log. transformed). In addition, to test for any interference between pest insects, we included *Cydia* infestation rates as a predictor variable of *Curculio* infestation rates and vice versa.

All models were validated testing homogeneity and normality of the residuals. Also, all post hoc test were made with Tukey test. Additionally, all data analyses were performed with the statistical analysis program R Core Team (2019) and the following libraries were used: ggplot2 (Wickham 2016), plyr (Wickham 2011), nlme (Pinheiro et al. 2018), lme4 (Bates et al. 2015) and multcomp (Hothorn et al. 2008).

3. RESULTS

Using a sample of more than 6 000 acorns we assessed that acorns attacked by insects were prematurely abscised and fell, on average, more than one month earlier than sound ones (mean dates: November 26th for sound acorns; October 21st and 20th for those infested by *Curculio* and *Cydia*, respectively) ($F_{2, 6285} = 1135.939$; $P < 0.001$). Post-hoc pairwise tests showed significant differences between sound acorns and those infested by *Cydia* ($\text{estimate}_{Cydia} = 30.077$; $Z_{Cydia} = 35.245$; $P_{Cydia} < 0.001$) or *Curculio* ($\text{estimate}_{Curculio} = 28.940$; $Z_{Curculio} = 41.813$; $P_{Curculio} < 0.001$). By contrast, the *Curculio*-*Cydia* comparison was not significant ($P=0.418$).

Acorn volume varied significantly between the three categories of acorns ($F2, 6285 = 254.719$; $P < 0.001$). Sound acorns were the largest, followed by those infested by Curculio; acorns attacked by Cydia were the smallest (Fig. 1). All post-hoc pairwise comparisons were significant (see Table 1).

Type of acorn	estimate	Z	P
Cydia - Curculio	-0.318	-8.266	< 0.001
Sound - Curculio	0.440	14.932	< 0.001
Sound -Cydia	0.758	20.899	< 0.001

Table 1. Results of the Tukey post hoc pairwise comparison test in acorn volume analysis.

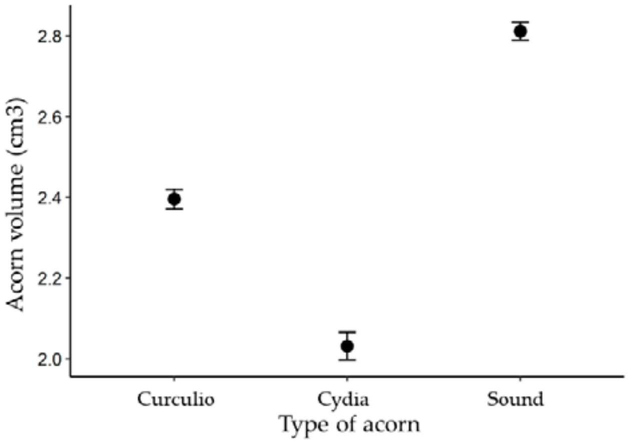


Figure 1. Acorn volume (cm^3) of the three different types of grown acorns. Dots show the mean per category and error bars the standard deviation of the mean.

Acorn total dry weight (shell plus cotyledon) also differed significantly among categories ($F_{2,6276}$ = 1462.573; $P < 0.001$). Sound acorns were twice as heavy as those infested by acorn borer insects (mean±SE: 2.43±0.02 grams *vs* 1.16±0.02 grams -*Curculio*- and 1.17±0.03 grams -*Cydia*-). All pairwise post-hoc comparisons between sound acorns and the rest were significant ($\text{estimate}_{\text{Curculio}}$ = 1.194; Z_{Curculio} = 48.896; $P_{\text{Curculio}} < 0.001$; $\text{estimate}_{\text{Cydia}}$ = 1.139; Z_{Cydia} = 37.894; $P_{\text{Cydia}} < 0.001$). On the contrary, there were not significant differences between acorns infested by *Curculio* and *Cydia* (estimate = 0.055; Z = 1.727; P = 0.193).

In spite of their larger size, *Curculio* infested acorns were not heavier than those attacked by *Cydia* because weevil larvae consumed more cotyledon. Cotyledon mass increased with acorn volume ($F_{1,4495}$ = 6196.162; $P < 0.001$), but it differed between acorn types ($F_{2,4495}$ = 2926.622; $P < 0.001$). For any given acorn volume, cotyledon mass was higher in sound acorns, in which nothing had been consumed by insects. Moreover, there were differences between those infested by insects as well. For a given volume, the weight of the cotyledon remains once the larvae had finished feeding was larger in acorns infested by *Cydia* (Fig. 2). The interaction between the fixed factor and the covariate was significant too ($F_{2,4495}$ = 330.031; $P < 0.001$; Fig. 2).

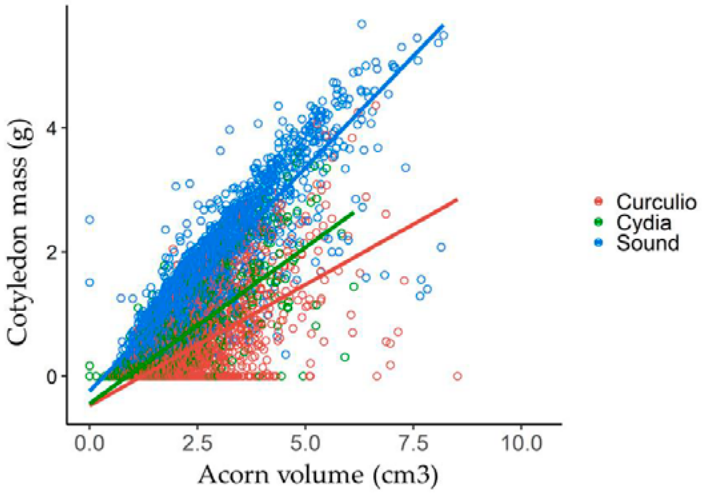


Figure 2. Relationship between acorn volume (cm^3) and cotyledon mass (grams) in the different types of acorns. Red dots represent *Curculio* infested acorns, blue and green dots correspond to sound acorns and those attacked by *Cydia*.

In 68.24% of *Curculio* infested acorns the embryo was killed (N = 1851); this percentage was lower in those attacked by Cydia 54.89% (N = 891). Embryo survival likelihood differed between type of acorn (Chi^2 = 81.933; $P < 0.001$) and was lower in smaller acorns (estimate = 0.0007; Z = 10.483; $P < 0.001$). The interaction between the fixed factor and the covariate was significant (Chi^2 = 18.621; $P < 0.001$). In the acorns infested by *Cydia* acorn survival likelihood increased with acorn size more quickly (Fig. 3).

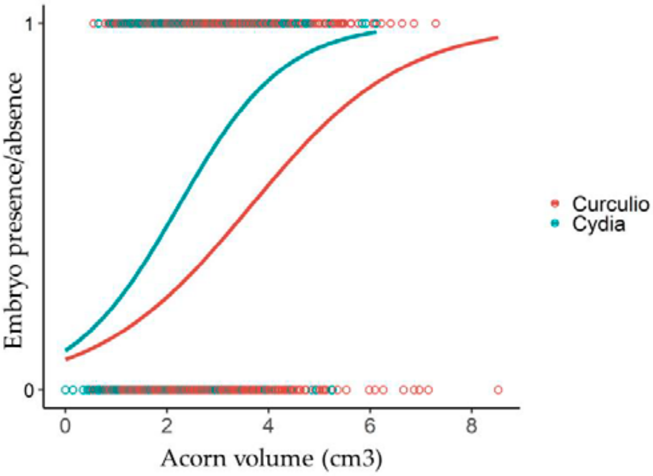


Figure 3. Relationship between acorn volume and embryo survival in acorns infested by *Curculio* and *Cydia*. The X-axis represents the acorn volume in cm^3. The Y-axis depicts embryo survival: (0- embryo killed. 1- embryo not killed). Red dots correspond to *Curculio* acorns and blue ones to those infested by *Cydia*.

3.1 Effects of meteorology

Infestation rates by *Curculio* were lower when late summer storms were delayed. Infestation rates depended on the first rain (Chi^2 = 169.65; P < 0.001) and acorn production (Chi^2 = 216.07; P < 0.001). It was negatively related with acorn crop size (estimate = -0.470; Z = -14.700; P < 0.001) and the date of the first late summer rains (estimate = -0.033; Z = -13.030; P < 0.001; Fig. 4). The total amount of rainfall during the weevil emergence period and the proportion of acorns attacked by *Cydia* did not have any significant effect (all P > 0.05). In the case of *Cydia*, infestation rates depended on the first rain (Chi^2 = 27.232; P < 0.001), the amount of rain (Chi^2 = 8.160; P = 0.004) and acorn production (Chi^2 = 93.625; P < 0.001). *Cydia* acorn infestation rates were also negatively related with acorn crop size (estimate = -0.358; Z = -9.676; P < 0.001). However, contrary to *Curculio*, they were positively related with the date of first late summer rainfall (estimate = 0.020; Z = 5.218; P < 0.001; Fig. 4) and negatively related with the total amount of rain during the main *Cydia* activity period (estimate = -0.003; Z = -2.857; P = 0.004). In addition, *Curculio* infestation rates had no effect on *Cydia* infestation rates (P = 0.355).

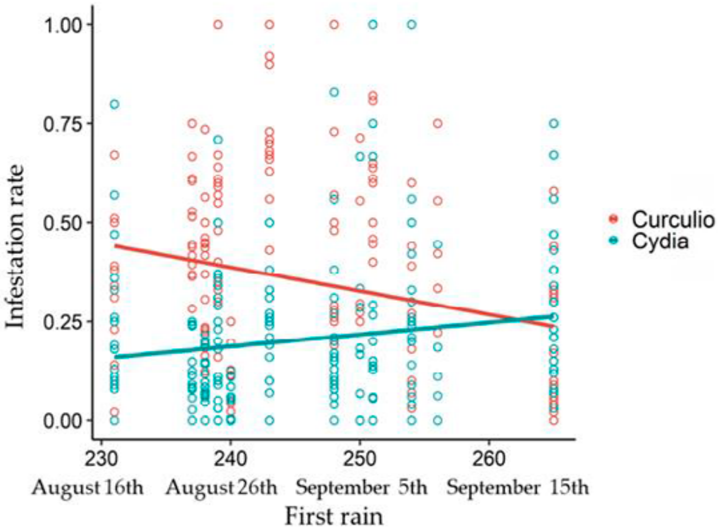
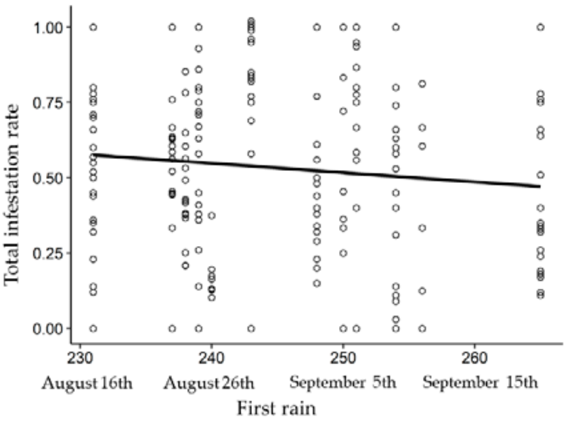


Figure 4. Relationship between the date of the first late summer rains and acorn infestation rates by *Curculio* and *Cydia*. First late summer rain is the date from August 4th onwards in which at least a daily precipitation of 1 litre/m^2 was recorded. Julian dates (day 1 = January 1st) are used.

When we pooled together all insect infested acorns (*Curculio* plus *Cydia*) infestation rates greatly depended on acorn production (Chi^2 = 395.014; $P < 0.001$) and first rain (Chi^2 = 31.466; $P < 0.001$). Rates were negatively related with crop size (estimate = -0.711131; Z = -21.743; $P < 0.001$) and the date of the first late summer storms (estimate = -0.020890; Z = -9.022; $P < 0.001$; Fig. 5). The effect of the total amount of rain during the insect activity period had a marginal significant effect (estimate = -0.002; Z = -1.920; P =0.055).

Figure 5. Relationship between the date of the first late summer rains and acorn infestation rates by pest insects as a whole (*Curculio* plus *Cydia*). First late summer rain is the date from August 4th onwards in which at least a daily precipitation of 1 litre/m^2 was recorded. Julian dates (day 1 = 1 January) are used.



4. DISCUSSION

Infested acorns were prematurely abscised before reaching their potential size. Acorns attacked by *Cydia* were smaller than those infested by *Curculio,* despite their overlapping drop dates. However, their weight once the larvae left did not differ, because *Curculio* larvae consumed more cotyledon mass than *Cydia* caterpillars. Accordingly, embryo survival likelihood did not change significantly between the two types of acorns. Meteorology had an effect on insect infestation rates. The total amount of rainfall significantly reduced acorn attack by *Cydia*, but had no effect on *Curculio*. By contrast, the delay of late summer rainfall reduced infestation rates by *Curculio*, whereas it increased acorn infestation by *Cydia*. However, *Cydia* increase did not compensate *Curculio* decrease, and total infestation rates (pooling together all insect infested acorns) decreased in those years in which late summer storms started later.

Acorns infested by *Curculio* or *Cydia* were prematurely abscised and, consequently, their volume was smaller than that of sound ones (dropped one month later). Strikingly, acorn size also differed between acorns attacked by *Curculio* and *Cydia*, although they were dropped in the same period. Numerous studies have reported the premature abscission of infested acorns compared to sound ones (Yu et al. 2003; Pulido and Díaz 2005; Csóka and Hirka 2006; Bonal and Muñoz 2007). Insects oviposit on/into acorns that stop their growth before completing their development, what explains their smaller size (Fukumoto and Kajimura 2001; Bonal and Muñoz 2008). Thus, the smaller volume of the acorns infested by *Cydia* could suggest an earlier reproductive phenology compared to *Curculio*. However, their largely similar abscission dates do not match with this hypothesis, unless the period from infestation to abscission were shorter the case of *Cydia*. The mechanisms behind this differential response, if exist, remain unknown.

The differences in size between infested acorns could be a consequence of differences in acorn selection. Reports from both chestnut tree orchards (*Cydia splendana* and *Curculio elephas*) (Debouzie et al. 1996) and Holm oak forests (*Cydia fagiglandana* and *Curculio elephas*) (Torres-Vila et al. 2008) have shown an earlier emergence of adult moths but an overlapping acorn use by the larvae of the two types of insects. *Cydia* moths oviposit earlier, but it is the first instar caterpillar which enters into the acorn, and egg hatching takes aprox. 10 days. In the case of *Curculio elephas*, females oviposit into the acorns as soon as they emerge and eggs hatch in 5 days (Debouzie et al. 1996). *Cydia* caterpillars depend on acorn cotyledons for completing their development, however, they may move between acorns during the growing period (Ito and Higashi 1991). By contrast, the legless weevil larvae have to complete their development within a single acorn. *Curculio* females should thus be more selective and choose bigger acorns, as seed size could constrain the growth of their offspring and future fitness (Bonal and Muñoz 2009; Desouhant et al. 2000). Nonetheless, further acorn selection tests are necessary to confirm this hypothesis.

In spite of their smaller volume, the mass of the acorns infested by *Cydia* was not lower compared to those attacked by *Curculio*. Weevil larvae consumed larger amounts of cotyledons and the differences disappeared. Multi-infestation is frequent in *Curculio* weevils, specially when seed availability is lower (Maeto 1995; Desouhant et al. 2000; Bonal and Muñoz 2008). When more than one weevil larvae share the same acorn, cotyledon depletion is not rare and larval weight can be constrained by acorn size (Maeto 1995; Bonal and Muñoz 2009). By contrast, *Cydia* caterpillars develop singly, being multi-infestation extremely rare (Maeto 1995; Debouzie et al. 1996; Torres-Vila et al. 2006). Conspecific aggressions exclude other caterpillars (see Torres-Vila et al. 2006 and references therein), and *Curculio* females avoid *Cydia* infested acorns for oviposition (Debouzie et al. 1996). Single *Cydia* caterpillars rarely deplete the cotyledons, and their weight is not constrained by acorn size (Maeto 1995).

Embryo predation risk was higher the smaller the acorn but, surprisingly, it did not differ between acorns infested by *Curculio* and *Cydia*. The lower amount of cotyledon eaten by *Cydia* caterpillars compensated the smaller acorn size. *Curculio* weevils start boring the acorn from the bottom and, in large acorns, they are less likely to predate the embryo on the opposite side (Maeto 1995; Bonal et al. 2007). *Cydia* caterpillars start feeding from the same side, but they are satiated earlier. In small acorns, the embryo is more likely to survive if it is infested by *Cydia* than if it is attacked by *Curculio*. Accordingly, in forests in which acorns are smaller than in our study site, total embryo survival likelihood is significantly higher in acorns infested by *Cydia* (Ruiz-Carbayo et al. 2018).

The delay of late summer rainfall decreased infestation rates by *Curculio*, as it hampers adult emergence. Adult *Curculio elephas* need the rain to soften the soil and facilitate emergence from their underground earthen cells (Menu 1993; Bonal et al. 2015). A previous study, based on a single site and a shorter time series, showed that infestation rates are positively related the total rainfall throughout the emergence period (Espelta et al. 2017). The present results show that it is the rainfall timing what has a stronger effect. Moreover, after including it in the model, the effect total rainfall on infestation rates was not significant. Total rainfall is, however, positively correlated with the total number of *C. elephas* adults that emerge (Bonal et al. 2015; Bogdziewicz et al. 2019). Hence, a plentiful late rain may trigger an abundant adult emergence, but not necessarily massive oviposition. The onset of adult emergence may change a lot between years (up to 1 month.) (Espelta et al. 2017) and, later in the season, the suitability of acorns for oviposition might decrease (e. g. increased hardness, see Guidone et al. 2007 for the case of *Curculio nucum* and the hazelnuts of *Corylus avellana*).

Contrary to *Curculio*, infestation rates by *Cydia* increased when late summer rainfall was lower and started later; however, the underlying mechanisms differ. *Cydia* pupae are not buried underground, but among the litter beneath oak canopies (Ruíz-Carbayo et al. 2018). They do not need the rain to soften the soil. On the contrary, storms could have negative effects on adults, eggs and newly hatched caterpillars. Rain hinders small insect flight (Dickerson et al. 2014) and reduces adult activity. In the case of eggs and first instar caterpillars, storms may provoke high mortality rates (see Chen et al. 2019 and references therein.). *Cydia* oviposition takes place mainly in September, and there is a period of two weeks in which eggs and L1 larvae are unprotected (on the oak shoots). This might explain the higher acorn infestation rates by *Cydia* in dry years.

Acorn infestation rates by borer insects as a whole (*Curculio* plus *Cydia*) decreased significantly but slightly when late summer storms were delayed. The increase of infestation rates by *Cydia* did not compensate the decrease of those by *Curculio*; hence, the effect was not "zero-sum" (*sensu* Ruiz-Carbayo et al. 2018). According with data on other seed feeding insects (Solbrek and Knape 2017), the population of one species did not "fill the gap" left by the other. Under strong inter-specific competition such effects could have appeared, however, this does not seem to be the case of acorn borer insects. Reports on the same species in chestnut trees *Castanea sativa*, have not found inter-specific interference at the tree level (Debouzie et al. 1996). In oaks, although infestation rates may be high, rarely get to 100% (Bonal et al. 2007; Bogdziewicz et al. 2018). The present results contrasts with a previous study, in which the low *Curculio* numbers in fragmented oak forests was compensated by the increase of *Cydia* moths (Ruiz-Carbayo et al. 2018). The lower total infestation rates in those oak plots (20%) probably facilitated the species substitution.

In our study sites, as in most oak woodlands worldwide, *Curculio* weevils were the main acorn borer insects (Lewis 1992; Maetô 1995; Csóka and Hirka 2006; Torres-Vila et al. 2006). However, our results show that such dominance may change if late summer rainfalls arrive late. In the Mediterranean region, current projections forecast longer and drier summers (Giorgi and Lionello 2008; Serrano et al. 2020). Under this scenario, more acorns might escape predation, moreover, the substitution of *Curculio* by *Cydia* would not reduce embryo survival likelihood or the mass of infested acorns. Oak recruitment might be favoured and, in the case of Iberian oak savannahs, meat production might increase along with a decrease in acorn infestation (Tejerina et al. 2011). However, there are a number of things to explore before making such statement.

In first place, it remains unknown whether the effect of rainfall can be extrapolated to other climates. Acorn predation by insects occurs in oak forests worldwide, including tropical and subtropical areas with very different climatic conditions (Xiao et al. 2007; Peguero et al. 2017). In addition, although rain delay can reduce insect infestation, harsh summer drought increases acorn abortion (Espelta et al. 2008) and hampers seedling recruitment (Pulido and Díaz 2005). The net effect of environmental changes on ecological interactions are difficult to predict due to the number of co-occurring factors. However, according with other studies on acorn production in temperate forests (Caignard et al. 2017), our results show that not all the consequences might be detrimental for oak fitness. Further research on the outcome of climate change should contemplate this double perspective.

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Chapter 3

Intraguild predation of weevils by livestock reduces acorn pests in oak silvopastoral systems

Abstract

Iberian oak savannahs are traditional silvopastoral systems in which acorns constitute a key food source for livestock. Acorn feeding insects provoke significant economic losses, however, the high natural value of Iberian oak savannahs precludes any chemical treatment. This paper shows a novel way of biological pest control based on promoting livestock predation on these insects. Female *Curculio elephas* (Coleoptera: *Curculionidae*) oviposit into developing acorns, which are prematurely abscised before reaching their potential size. Larvae still spend 20 days feeding on the cotyledons after acorn drop, being during this period very vulnerable to predation by livestock. We experimentally assessed that cows, pigs and sheep ate sound and infested acorns in the same proportion, so that infestation rates were lower in those Iberian oak savannahs in which livestock density was higher. An effective biological control of *Curculio elephas* weevils should involve an early predation of infested acorns by livestock, preferentially within 10 days after falling. Doing this, most larvae will be predated before completing their development and, in addition, the nutritional value of infested acorns will still be high (cotyledons not yet depleted by weevils). We encourage landowners to increase livestock densities during September-October, when the infested acorn dropping peaks. These increased densities should rotate over the farm, and be maintained at the same plot for a maximum of three years in a row. Otherwise, a prolonged and concentrated livestock predation on sound acorns and seedlings would hamper long-term oak regeneration.

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1. INTRODUCTION

Ecological intensification states that a deep knowledge of ecological functions is essential to improve performance in agricultural systems. Sound information of ecological interactions optimizes the management of service-providing organisms that contribute to increase productivity (Kremen 2005; Bianchi et al. 2006; Kremen et al. 2007; Bommarco et al. 2013; Gaba et al. 2014; Goedhart et al. 2018). Natural pest control is one of the main ecosystem services (Naylor and Ehrlich 1997; Chaplin-Kramer et al. 2011; Thies et al. 2011; Crowder and Jabbour 2014; Vanlauwe et al. 2014) and can be incorporated to agroecosystem management by increasing predatory species richness and abundance (Landis et al. 2000; Greenop et al. 2018). The present article studies a novel way to control acorn pests in oak agroforestry systems based on promoting intraguild predation on insects by livestock.

Nowadays, extensive livestock rearing prevails in agroforestry systems worldwide. Trees do not only provide shelter for livestock, but also important complementary food (Aganga and Mosase 2001; Khair et al. 2001). In Iberian oak savannahs, free-range livestock feed profusely on acorns during autumn-winter after these fall on the ground (Moreno and Pulido 2009). The nutritive oak acorns contain all the resources necessary for early seedling growth (Shimada 2001; Tejerina et al. 2011; Bartlow et al. 2018) and are a key food resource for animals ranging from insects to large herbivores (Steele et al. 1996; Gómez et al. 2003; Muñoz and Bonal 2008).

The Holm oak (*Quercus ilex.*) is the most common tree species in many Iberian oak savannahs and its acorns are very rich in lipids and proteins (Tejerina et al. 2011). In this silvopastoral system acorns contribute to natural regeneration (Plieninger et al. 2004) and are a main food for livestock. Their role in natural regeneration has been widely studied before and, moreover, pre-dispersal insect seed predators are not the main bottleneck for natural recruitment (Pulido et al. 2010). In this study we focused on their role as food for livestock, as acorns are essential for producing high quality meat, specially in the case of the valued Iberian pigs (Rodríguez-Estévez et al. 2009a; Tejerina et al. 2010).

Acorn production is subjected to marked inter-annual variability (Bonal and Muñoz 2007, Espelta et al. 2008, Bogdziewicz et al. 2019), but every year acorn availability for livestock is reduced by pre-dispersal insect seed predators (Vázquez 1998; Bonal et al. 2007; Pulido et al. 2010; Bogdziewicz et al. 2018). *Curculio elephas* (Coleoptera: *Curculionidae*) is the main predator of oak *Quercus ilex* seeds, these insects feed on acorn cotyledons during their larval period (Bonal et al. 2007). *C. elephas* females oviposit into the acorns when still in the tree canopy (Bonal and Muñoz 2007, 2008) and this earlier access to the common resource (acorns) reduces food availability for livestock (i. e. inter-specific exploitation competition) (Petren and Case 1996; Richards et al. 2000; Lach 2005). Oaks reduce to some extent insect advantage over livestock due to the premature abscission of a large proportion of insect infested acorns. Larval development is not yet completed before acorn abscission (Bonal and Muñoz 2008). Therefore, partially predated seeds become available for large post-dispersal predators (livestock, wild ungulates) days before fully-grown larvae deplete the cotyledons and leave the acorn (Bonal and Muñoz 2007).

Premature abscission favours intraguild predation on weevil larvae by large herbivores. Intraguild predation is a combination of competition and predation favoured by body size asymmetry: competitors for the same food source kill or eat the other decreasing exploitative competition and influencing population dynamics (Polis et al. 1989; Gómez and González-Megías 2002, 2007; Finke and Denno 2005; Holt and Huxel 2007; Gish et al. 2010; Sunyer et al. 2016). In Holm oak savannahs, predation on *Curculio elephas* larvae by wild ungulates has shown to reduce acorn infestation rates (Bonal and Muñoz 2007) and contribute to the effects of other natural enemies of oak pests such as birds (Ceia and Ramos 2014).

Promoting intraguild predation on weevil larvae by livestock could thus reduce infestation rates in Iberian oak savannahs too. However, in first place it is necessary to know whether livestock eats acorns with larvae. Small rodents can differentiate infested acorns from sound ones and select the latter (Muñoz and Bonal 2008) and so do birds like blue jays (Dixon et al. 1997). By contrast, squirrels (Weckerly et al. 1989) or ungulates (red deers and wildboars) (Bonal and Muñoz 2007) do not discriminate between both classes. In the case of free-range livestock in Iberian oak savannahs, only in the case of Iberian pigs detailed studies have analysed preferences according to acorn size, chemical composition, etc. (Rodríguez-Estévez et al. 2009a, 2009b), but no experiments testing discrimination between sound and insect infested acorns have been performed.

To implement successfully intraguild predation by livestock as biological control it is also necessary to assess larval development speed. Early consumption of abscised infested acorns could benefit livestock because, as time goes by, larvae would progressively deplete cotyledons. On the other side, predation risk would decrease, as larvae would have more time to finish their growth and leave the acorn before being eaten. So far, most studies on the effects of weevils on acorns have focused on the consequences for germination, seedling establishment and recruitment (Espelta et al. 2009a, 2009b; Hou et al. 2010; Perea et al. 2011; Bartlow et al. 2018). However, we lack data of the speed of cotyledon consumption by larvae or the total amount of acorn biomass lost.

We studied how livestock management can contribute to reduce the negative effects of pests on sound acorn production. Our general objective was to obtain sound information to develop a grazing scheme (intensity and calendar) to reduce acorn infestation rates. Our specific objectives were: i) to assess the effects of premature abscission on the timing of acorn drop, acorn size and mass, ii) to estimate the speed of larval growth and infested acorn mass loss after being dropped, iii) to assess livestock preferences between infested and sound acorns, and iv) to measure the effects of livestock density on acorn infestation rates by weevils.

2. MATERIAL AND METHODS

2.1 Study species

Holm oak flowering occurs in spring (April-May) and by mid-autumn (October) the seeds (i. e. acorns) are fully grown (Bonal and Muñoz 2008). Acorns are bullet-shaped nuts with their wider side covered by a cupule with bracteoles. The pericarp surrounds two large cotyledons that contain a small embryo located at the pointed end (Bonal et al. 2007). Holm oak acorns are attacked by *Curculio* weevils (Coleoptera) and, in a lesser extent, by *Cydia* spp. moths (Lepidoptera) (Bonal et al. 2007). Acorn infestation rates by *Curculio* weevils usually average 50% of the total crop (Bonal et al. 2007), if well there is variability among localities, among years within the same localities, and among Holm oaks at the same site (reported values for individual trees may range from 0% to 100%) (Vázquez 1998; Bonal and Muñoz 2007; Bogdziewicz et al. 2019). The most common *Curculio* species in Holm oak savannahs is *Curculio elephas* Gyll. (Coleoptera: *Curculionidae*) and this is the only species we have found in our study area (104 trees monitored with adult emergence traps for three years, unpublished results) and in previous studies in similar environments (Bonal et al. 2007; Bonal et al. 2012). However, there are other species (e. g. *Curculio glandium* and *Curculio venosus*) that may occur in a lesser extent in Holm oak forests with milder summer droughts (Espelta et al. 2009a; Pélisson et al. 2012).

2.2 Intensive monitoring of acorn mass losses due to weevil infestation

In Huecas (Toledo Province, Central Spain), tree productivity was monitored for three years to assess the effects of weevil predation on acorn losses (see Fig. 1). We monitored 24 trees for three years (2011, 2012 and 2013) placing seed traps (plastic buckets, area $0.12m^2$) randomly under tree canopies (hung from lower branches) and collecting the content biweekly. Trap number per tree ranged between 3 and 9, and was related to its surface to cover a similar proportion of the canopy (between 1.5% and 2%). Canopy surfaces were calculated from the average of three random diameter measures considering the canopy as roughly circular (see Bonal et al. 2007 for a detailed description of the methodology). After each revision acorns were taken to the laboratory, placed individually in plastic vials opened above and reviewed every day to record the number of emerging larvae.

One month after the emergence of the last larva, all acorns were dried for 48 hours at 80ºC and their maximum widths and lengths measured to the nearest 0.01 millimetre with a digital calliper. We did so to estimate weight based on their volume (using the formula from Bonal et al. 2007) and assess in which extent premature abscission reduces the size of infested acorns. All acorns were classified as infested by insects or sound by visual evaluation. During the insect rearing we recorded the type of larvae (*Curculio* or *Cydia*) emerged from each infested acorn. Moreover, to confirm the correct classification as sound or infested, all acorns were halved at the end to look for dead larvae, excrements or any other sign of insect infestation. We weighted all acorns to the nearest milligram; in those infested by insects we only weighted the seed shell and the remains of cotyledons after cleaning the frass.

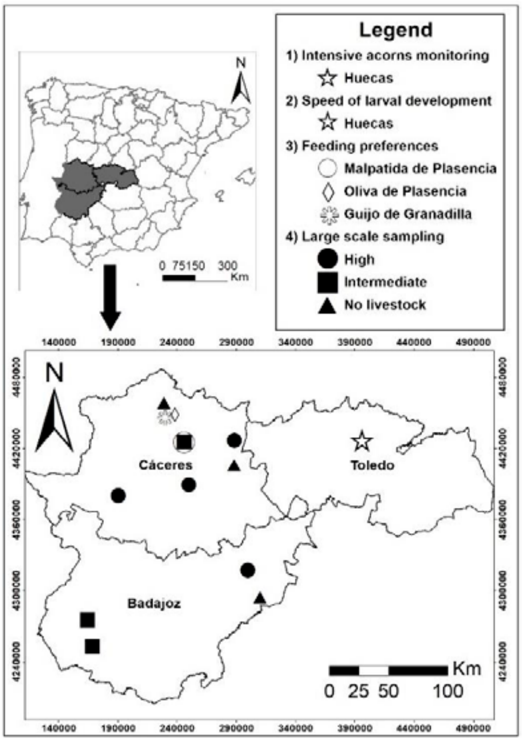

Figure 1. Location of all study sites (Iberian oak savannahs) showing where each experiment/sampling was carried out. Filled shapes (circle, square and triangle) represent livestock density at each study site: high, intermediate or abandoned (no livestock present), respectively.

2.3 Measurement of the speed of larval development and infested acorn mass decrease

In Huecas, we assessed the speed of mass loss of infested acorns after dropping (see Fig. 1). We randomly chose one tree and cleaned a section of the floor under the canopy on which we placed a 7 m^2 insect mesh on September 10th, 2015. Five days later, we came back to collect the acorns dropped in that short period of time and took them to the laboratory. We collected 170 acorns in total, all with weevil oviposition scars visible, as early in the season only infested acorns are prematurely dropped (Bonal and Muñoz 2008). We randomly divided them in 5 groups of 34 acorns each. We left all acorns outdoors in open trays covered with insect mesh. In order to stop larval development at different stages, in regular intervals we randomly chose one of the groups of acorns and dried them at 80ºC during 48 hours. We dried the first group on September 15th (we considered that date the Day 0) and, from then on, one group was dried every ten days. Dried acorns were measured (length and width) to the nearest millimetre and then, opened in order to look for any larva inside large enough to be visible at naked eye and/or the presence of excrements; we also recorded the number of exit holes chewed by larvae. Once opened, all acorns were weighted (seed shell and remains of cotyledons separately) to the nearest 0.01 gram after cleaning the insect frass. This methodological approach, drying acorns and killing larvae at different moments, allowed us to assess both the speed of larval development and of acorn mass loss.

2.4 Feeding preferences experiment

In three sites of the Province of Cáceres (Western Spain) we performed experiments to measure feeding preferences by livestock (see Fig. 1). The three of them are Holm oak savannahs dedicated to free-range livestock rearing: Iberian pigs, cows and sheep. The experiment started on November 15th 2017 supplying three types of acorns to livestock: sound (without any sign of infestation), infested (with oviposition scar and the larva still inside the acorn) and with larval exit holes (infested acorns from which larvae had left). We placed 10 acorns of each of the three categories (sound, infested or with exit holes) across a surface a surface of 1 m^2 under the canopies of the 7 study oaks at each farm. Experimental acorns were intermixed with the natural crop, which averaged 133 ± 17 acorns/m^2 (mean ± EE), to make the test more realistic. To recognise the experimental acorns, all of them were marked with a tiny incision at their basal part. Fourteen days after the start of the experiment, we counted how many acorns of each type remained under the study trees. After abscission, larvae spend inside the acorn 20 days (Bonal and Muñoz 2007), thus, in those acorns eaten within 14 days, most of the larvae would have been predated before finishing their development. Acorn removal reflects livestock preferences, because wild ungulates rarely enter the study farms and the same happens with birds like the European Jay (*Garrulus glandarius* L.) (Pons and Pausas 2007). In the case of rodents, our own rodent samplings showed extremely low abundances: 0.005 rodents/trap night (unpublished results), similar to those reported for other Iberian oak savannahs (Muñoz and Bonal 2007).

2.5 Livestock density and acorn infestation rates: large scale sampling

We carried out a large geographical scale sampling over two years (2018 and 2019) in ten localities widely spread over Extremadura Region, Western Spain (see Fig. 1) to assess the effects of livestock density on acorn infestation rates. We randomly started a 750 m transect at each site and every 50 metres we stopped and sampled the nearest tree (15 trees per Iberian oak savannah, 150 trees in total). At each one, we collected in early October 30 acorns from the lower branches to avoid any potential effect of branch height on infestation rates. We could not assess infestation rates in all the study oaks (150) due to the lower production of some; we only analysed those trees in which we could collect at least 10 acorns (111 in 2018 and 129 in 2019, we indicate in Table 1 how many trees from each locality). The acorns of each oak were placed in different plastic pots and left open air to allow larval development and emergence from the seeds. One month after the last larva had emerged, all acorns were opened to check whether there was any dead larva inside. Infestation rates were calculated as the number of weevil infested acorns divided by the total number of grown acorns (sound plus infested), what correlates well with estimations from seed traps (Pérez-Izquierdo et al. in prep). In both years we also measured seed production in all study oaks by making 15-seconds acorn counts at three different canopy orientations (see Koenig et al. 1994 for a detailed description).

In 2018, the ten study sites were classified into three categories with respect to livestock density: i) with high livestock density ii) intermediate and iii) no livestock. We did so using grazing intensity as a proxy of livestock density. At each of the 15 study trees of each Iberian oak savannah we estimated, at the time of acorn collection, the cover of grass higher than 10 centimetres. Below each canopy we sampled two circles of 1-meter diameter (north and south of the trunk). Each circle was divided in 4 quarters and in each quarter we registered whether there was any grass higher than 10 centimetres. The percentage of high grass cover per study site was given as the number of quarters with presence of grass over 10 centimetres divided by the total number of quarters sampled (120 per site -4 quarters per circle, 2 circles per tree, 15 trees-). The limits (high grass cover percentage) to segregate by categories were: high livestock density (lower than 0.25); intermediate density (from 0.25 to 0.75) and no livestock (higher than 0.75). The three categories differed dramatically in grass cover percentages (see Table 1). Moreover, besides these quantitative measures, differences in livestock density were visually obvious. In areas with high livestock density not only high grass cover was low, large surfaces had no grass at all (bare soil). By contrast, in no livestock Iberian oak savannahs (abandoned at least 4 years before the beginning of the study) there was shrub resprouting.

Site	Grass Cover (mean ± SE)	Category	N 2018	N 2019	N total
Arroyo de la Luz	0.000 ± 0.000	High	10	14	24
Puebla de Alcocer	0.125 ± 0.027	High	12	12	24
Rosalejo	0.058 ± 0.032	High	11	12	23
Trujillo	0.167 ± 0.026	High	10	14	24
Barcarrota	0.508 ± 0.066	Intermediate	14	15	29
Jerez de los Caballeros	0.625 ± 0.068	Intermediate	6	13	19
Malpartida de Plasencia	0.517 ± 0.072	Intermediate	15	13	28
Bonal de Ibor	1.000 ± 0.000	No livestock	13	12	25
Mohedas de Granadilla	0.792 ± 0.074	No livestock	7	9	16
Zarza-Capilla	0.892 ± 0.050	No livestock	13	15	28

Table 1. Cover grass and number of tress analysed per site. Table showing the percentage of grass cover (mean ± EE, N=15) at each of the study sites classified according to livestock density. The number of trees in which acorn infestation rates could be calculated each year are also provided.

2.6 Statistical analysis

The differences between sound and weevil infested acorns in terms of: dropping date, acorn size estimated from linear dimensions (length and width), final acorn mass and cotyledon mass were analysed in four separate Linear Mixed Models (LMMs). The type of acorn was the independent factor (two levels, sound/weevil infested) and there were two random factors: tree identity and year (2011, 2012 and 2013). In the case of the differences in cotyledon mass we additionally included the acorn size estimated from its length and width as an independent covariate, as the amount of cotyledon left would depend on the size of the acorn.

To assess the speed of larval development and infested acorn mass decrease, we tested the differences among the groups of acorns dried at different times (i. e. experimental acorns in which larval development was stopped at different stages). We compared total acorn mass, cotyledon mass, the number of visible larvae per acorn and the number of exit holes (dependent variables). The normality distribution and homogeneity of variance were assessed and all groups showed lack of normality, therefore, we performed four Kruskal-Wallis tests (non-parametric) and multiple comparison tests with kruskalmc (library pgirmess) to assess pairwise differences among the experimental groups.

Feeding preferences by livestock among sound acorns, infested acorns with the larvae still inside and acorns with larval exit holes were analysed using Generalized Linear Mixed Models (GLMMs) with a binomial distribution of errors (dependent variable: eaten/not eaten). The type of acorn was the independent factor and the tree identity the random factor. As the type of livestock differed among Iberian oak savannahs, we carried out one separate analysis for each one. In the case of the farm with free-range Iberian pigs no analysis was performed as all the experimental acorns were consumed, none was left.

Finally, to test whether there were differences in infestation rates among oaks located in Iberian oak savannahs with different livestock density, we performed a Generalised Linear Mixed Model (GLMM) with a binomial distribution of errors. The dependent variable was the number of infested on the total number of acorns and there were two random factors (tree and site identity), the first nested within the second. The model included two independent factors, livestock density (high, intermediate, no livestock) and year (2018 and 2019). We tested the interaction between them but it was not significant as well as the covariate acorn production (log. average acorn count + 1). Tukey post-hoc tests were executed to assess pairwise differences between livestock density categories.

All data analyses were performed with the statistical analysis program R Core Team (2019) and the following libraries were used: ggplot2 (Wickham 2016), plotrix (Lemon 2006), plyr (Wickham 2011), nlme (Pinheiro et al. 2018), lme4 (Bates et al. 2015), multcomp (Hothorn et al. 2008) and pgirmess (Giraudoux 2018).

3. RESULTS

3.1 Effects of weevil infestation on acorn dropping, size and cotyledon mass

The infested acorns were prematurely abscised and fell significantly earlier than sound ones ($F_{1,\ 2594}$ = 960.56; P < 0.001), concretely 33.43 ± 1.25 (mean ± SE) days before. Premature abscission stopped infested acorn growth before they reached their final size. Therefore, their size estimated from their linear dimensions (length and width), was also significantly lower (6.43%) than that of sound acorns (1.99 ± 0.02 (mean ± SE) grams *vs* 2.12 ± 0.02 (mean ± SE) grams; $F_{1,\ 2594}$ = 141.79; P < 0.001). The final dry mass of the infested acorns (weight once larvae had finished their development) was much lower, just half of that of sound ones (1.25 ± 0.02 grams *vs* 2.48 ± 0.02 grams (means ± SE); $F_{1,\,2591} = 1484.89$; $P < 0.001$).

The weight of the cotyledons also differed between the two types of acorns. Acorn size (estimated from linear dimensions) had an effect on cotyledon dry mass ($F_{1,\,2589} = 3192.59$; $P < 0.001$; Fig. 2), because larger acorns had heavier cotyledons. However, in acorns of the same size (estimated from their length and width), the dry mass of the cotyledons was lower in infested acorns due to larval feeding ($F_{1,\,2589} = 3934.69$; $P < 0.001$; Fig. 2). The interaction between the factor and the covariate was also significant ($F_{1,\,2589} = 209.15$; $P < 0.001$; Fig. 2).

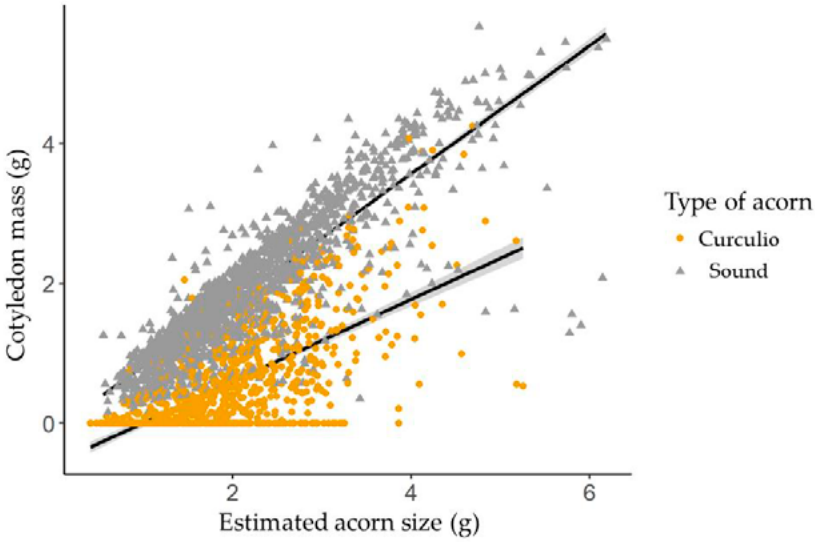

Figure 2. Relationship between cotyledon dry mass and acorn size estimated from acorn linear dimensions (length and width). Yellow circles represent Curculio elephas infested acorns. Grey triangles correspond to sound acorns.

3.2 Speed of larval development and acorn mass decrease

When infested acorns were dropped, larvae were still in a very early stage of development and in many cases too small to be visible at naked eye. Hence, when these acorns were opened, the number of larvae found inside differed among experimental groups and increased as time passed by and larvae grew ($Chi^2 = 21.44$; $df = 4$; $P < 0.001$). Differences were especially marked among the first group (acorns dried on September 15th, larval development thus stopped shortly after acorn abscission) and the rest of the groups (all post-hoc tests $P < 0.001$). Similarly, acorn mass decreased with time too ($Chi^2 = 68.21$; $df = 4$; $P < 0.001$). In the first 20 days after dropping there was a steady decrease of infested acorn mass due to cotyledon consumption by larvae. The same trend was observed in the amount of cotyledon left, which decreased from September to early October and remained constant from then on ($Chi^2 = 74.70$; $df = 4$; $P < 0.001$; Fig. 3). Acorn and cotyledon masses (acorn shell excluded) did not differ significantly from October 5th onwards because by that time larvae start to complete their development and leave the acorn. In fact, the number of exit holes drilled by larvae was minimal in the first 20 days after infested acorn falling and increased from then on ($Chi^2 = 21.442$; $df = 4$; $P < 0.001$).

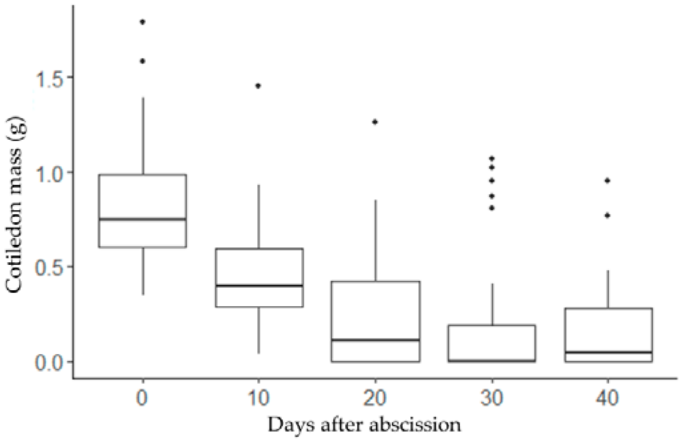

Figure 3. Dry cotyledon mass of infested acorns (grams) at different times after acorn drop. The number of days after acorn abscission are shown in the X axis. The boxes represent the median and interquartile range. Lines correspond to ranges and dots show potential outliers.

3.3 Feeding preferences experiment

Livestock did not show any preference between sound and infested acorns (either with the larvae still inside or not). The GLMMs showed no differences in removal rates among the three types of acorns, neither in the Iberian oak savannah with sheep ($F_2 = 1.64$; $P = 1.00$) nor in the one with cows ($F_2 = 6.06$; $P = 0.35$). Iberian pigs did not show any preferences either, and consumed all the acorns offered in the cafeteria experiment. As the dependent variable showed no variability we did not perform any analysis.

3.4 Large geographical scale sampling: infestation rates and livestock density

Acorn infestation rates by weevils differed among categories of livestock densities ($F_2 = 11.002$; $P < 0.001$; Fig. 4) and between years ($F_1 = 78.960$; $P < 0.001$). Post-hoc analyses showed that infestation rates in areas with high livestock density were significantly lower than those in areas with intermediate and no livestock (intermediate: $Z = 3.920$; $P < 0.001$ and no livestock: $Z = 4.143$; $P < 0.001$). Nevertheless, infestation rates between Iberian oak savannahs with intermediate livestock densities and with no livestock did not differ significantly ($Z = 0.246$; $P = 0.967$).

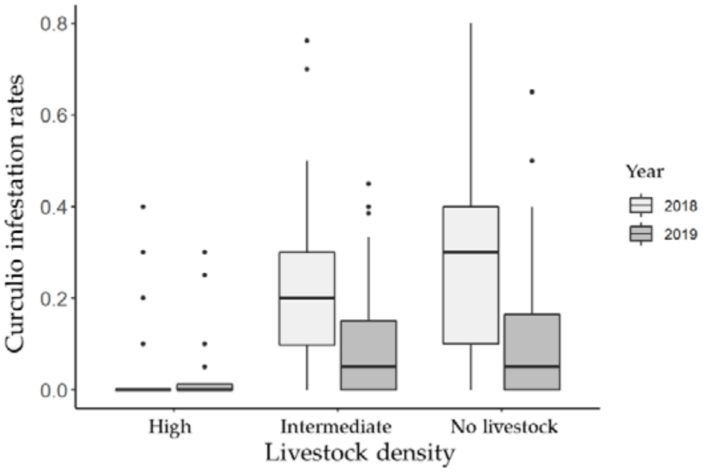

Figure 4. Acorn infestation rates by *Curculio elephas* in Iberian oak savannahs with different livestock densities (high, intermediate and no livestock) over two years (2018 and 2019). Boxes represent the median and interquartile range. Lines show the data ranges and dots correspond to potential outliers.

4. DISCUSSION

Curculio elephas attacked developing acorns in late summer and early autumn provoking the premature abscission. These acorns containing larvae in very early growth stages were thus dropped before reaching their maximum potential size. Larvae still spent three weeks developing within the fallen infested acorns. Their growth progressed quickly, as just after acorn abscission cotyledons were almost intact but 20 days later, larvae had depleted them in most cases. During that period, they were vulnerable to intraguild predation by all types of livestock, which consumed sound and infested acorns alike. Thanks to premature abscission, livestock could feed on infested acorns with a higher nutritive value (cotyledons not yet fully depleted), moreover, it allowed predation on weevil larvae before they left the acorn. Accordingly, infestation rates were lower in Holm oak savannahs with high livestock density during the period of infested acorn drop.

As reported in other *Quercus* spp. (Oliver and Chapin 1984; Csóka and Hirka 2006; Yi and Yang 2010), Holm oak infested acorns fall on average one month before sound ones without reaching their potential size. Nonetheless, our data showed that acorn mass loss is mostly due to larval feeding on cotyledons rather than a consequence of growth suppression. According with previous reports in field conditions (Bonal and Muñoz 2007), our results exhibited that most larvae spend 20 days inside infested acorns after abscission. The development speed may differ depending on the temperature (Bonal et al. 2010), but this duration (approx. 30 days after egg hatch) matches with previous studies (Manel and Debouzie 1995; Bonal et al. 2010). However, we showed for the first time that cotyledon mass does not decrease in a linear fashion throughout larval growth. Just after abscission, cotyledons of infested acorns were almost intact; ten days later, acorns had lost half of their weight and after twenty days had been almost depleted. The reason for the acceleration of acorn mass loss may be the successive larval moults (Desouhant et al. 2000), with feeding rates sharply increasing in the last development stage/s.

Livestock did not show any preferences for either infested or sound acorns, contrary to small mammals and birds (Semel and

Anderson 1988; Steele et al. 1996; Dixon et al. 1997; Muñoz and Bonal 2008; Sunyer et al. 2014). Within fourteen days most infested acorns had been predated before larvae had time to leave the acorn. In general, rodents reject infested acorns (Semel and Anderson 1988; Muñoz and Bonal 2008; Sunyer et al. 2014), especially in the case of those with insect excrements and little cotyledon left (Muñoz and Bonal 2008). Nonetheless, some small mammals feed on them and even eat weevil larvae as a dietary supplement (Steele et al. 1996). Livestock (sheep, cows and pigs) foraging resembled that of large sized ungulates (red deer and wildboars) in Mediterranean forests, which also ate both sound and infested acorns (Bonal and Muñoz 2007). Body size may be important in this context, as for a rodent each acorn supposes a time-taking foraging decision involving whether to consume or transport/cache it (Muñoz and Bonal 2008; Sunyer et al. 2014). Previous reports have shown that pigs can be very selective regarding acorn size (Rodríguez-Estévez et al. 2009b), but they consumed all the infested acorns offered in the cafeteria experiment, even those mostly depleted by larvae.

In small plants, ungulates can prey on pre-dispersal insect predators before seed dropping (Gómez and González-Megías 2002). In the case of *Curculio elephas* acorns are out of reach of livestock at oviposition up in the oak canopy, but the majority of infested seeds are early abscised, which increases dramatically intraguild predation risk. Premature abscission also makes infested acorns available for livestock before cotyledon depletion by larvae. In trophic guilds, those species with an earlier access to the common food source or better at exploiting it have important advantages over the rest (Colegrave 1995; Petren and Case 1996; Lach 2005). Premature abscission reduces such advantage, as livestock may feed on acorns almost uneaten if they do so soon after dropping. This is also common in many Mediterranean oak forests, in which many infested acorns are predated by wild boars and deers in less than 10 days after abscission (Bonal and Muñoz 2007).

Previous studies have reported the role of birds as oak pest predators in Iberian oak savannahs (Ceia and Ramos 2014). Interestingly, our results show that ungulate intraguild predation on weevil larvae can contribute to pest control provided a well-planned management plan (i. e. grazing calendar). Some types of livestock live free-range all year round, but others, like the valued Iberian pigs, are commonly released in the fields for fattening on November 1st (Rodríguez-Estévez et al. 2009b). By that time infested acorns will have lost most of their biomass and larvae will have finished their development and buried themselves underground avoiding livestock predation. Accordingly, our large-scale samplings over two years showed that infestation rates peaked in no-livestock Iberian oak savannahs and were lower in those with high livestock density. The use of high livestock densities to control pests should be carried out with caution though, as these animals consume large quantities of sound acorns and browse on seedlings, thus hampering oak natural regeneration (Leiva and Fernández-Alés 2005; Bonal and Muñoz 2007; Hou et al. 2010; Otvos et al. 2012; Arosa et al. 2015, 2017).

Iberian savannah farmers should promote density intensification in September-October, when the drop of acorns infested by *Curculio elephas* peaks. This management could potentially be extrapolated to other oak silvopastoral systems but, before that, there are a number of things that deserve further research. In the case of *C. elephas* acorns stop growing after being attacked (Bonal and Muñoz 2008), moreover, at least 80% of the infested acorns are dropped before the larvae finish their development (only 20% of the infested acorns collected in the biweekly seed trap revisions had exit holes). However, other *Curculio* species show an earlier phenology (Pélisson et al. 2012, 2013), the infested acorns may keep growing and more larvae may leave the seed before it is abscised from the tree. Thus, further studies should explore in depth acorn abscission in other *Curculio* spp. and, specially, larval development time after acorn drop to assess whether they are as vulnerable as *C. elephas* to intraguild predation by livestock. Differences between species would also determine the number of years that an increased livestock density should be maintained at a site to decrease weevil numbers. In the case of *C. elephas* it should be at least two (and ideally three) autumns in a row, because most adults of the same cohort emerge after two years, but average diapause length is longer in other species (Menu and Debouzie 1993; Venner et al. 2011).

In conclusion, ecological intensification aims at generating “actionable” knowledge to be implemented in daily management (Geertsema et al. 2016) and this was the purpose of this study. We demonstrated that high livestock densities can reduce acorn infestation rates in Iberian oak savannahs. Our results show that land owners should concentrate their livestock during September-October in those areas of their farms in which they want to reduce acorn pests. After that, this treatment should be rotated to other sectors of the farm to avoid major negative effects on oak regeneration. We encourage further research on trophic webs in agroecosystems to learn about other unexpected ecosystem services that some organisms may provide.

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Chapter 4

Experimental study for implementing acorn weevil predation by livestock as biological pest control

Abstract

Large herbivores can have strong impacts on terrestrial ecosystems. They can modify vegetation structure and soil properties, what has further consequences on many organisms. The effects on phytophagous insects are particularly remarkable. These do not only include competition for food, but also intraguild predation. This antagonistic interaction may be more usual than expected and strong enough to reduce insect numbers. In fact, the intensification of predation on acorn weevils (*Curculio* spp.: Coleoptera) by livestock has been proposed to reduce the economic losses provoked by these pests. For the first time, we used an experimental approach to address the practical implementation of this novel way of pest control in the Iberian oak savannahs (dehesas). Livestock were the main predators of infested acorns (with larvae inside). When they were artificially excluded, infested acorn removal rates decreased sharply. The manual removal of infested acorns reduced the number of adult weevils and infestation rates in the following year. Under natural conditions (control trees) acorn infestation rates were also negatively related with infested acorn removal by post-dispersal predators (mainly livestock) in the previous year. To be used as biological control, focalized increased grazing has to be promoted just on some farm plots during September-October, when the weevils are vulnerable (larvae inside the acorns on the floor). Moreover, increased livestock density should be rotatory; no more than three years on the same plot to avoid hampering oak natural regeneration. In this sense, our results show that infestation rates can be reduced even at the spatial scale of single trees in oak savannahs. This finding points at the poor dispersal abilities of *Curculio elephas*, what is further supported by the significant relationship between infestation rates and adult weevil numbers at the tree level. Iberian oak savannahs are traditional silvopastoral systems with not only high socioeconomic importance, but also outstanding naural values. Livestock can thus provide a key ecosystem service as predators of acorn pests. Moreover, similar approaches could be potentially exported to other silvopastoral systems of the world, in which livestock predation on pest insects has been reported.

1. INTRODUCTION

Large herbivores are keystone species in ecosystems worldwide (Brody et al. 2010; Coverdale et al. 2016). They play a very important role as ecosystem engineers due to their strong impact on their functioning (Fleischner 1994; Asner et al. 2004; Foster et al. 2014). Large herbivores consume a vast fraction of the primary production, shaping vegetation structure and modifying physicochemical composition of soil and plants (Asner et al. 2004; Bakker et al. 2004; Peco et al. 2006; Wolf et al. 2013). Thus, directly or indirectly, they affect many organisms of different trophic levels. Their effects on small-sized animal species are specially outstanding, although not always easy to predict because they result from complex cascade effects (Kruess and Tscharntke 2002; Sankaran and Augustine 2004).

In this study, we analysed the effects of intraguild predation on acorn-borer weevils (Coleoptera: Curculionidae) by free-range livestock in Iberian oak savannahs. Acorns are a key food source for free-range livestock, specially in the case of pigs (Rodriguez-Estevez et al. 2009; Tejerina et al. 2010); only Iberian ham sales suppose incomes of 1.55 billion euros for local producers (Vilches 2014). For the first time, we used multiple experimental exclosures to disentangle the factors that condition weevil predation risk and assessed the consequences on their population size/acorn infestation rates.

The number of studies reporting antagonistic interactions between large herbivores and phytophagous insects is not high (but see Zamora and Gómez 1993; Gómez and González-Megías 2002; Canelo et al. 2020). Among them, those showing evidences of intraguild predation of insects by ungulates have attracted the attention of both animal and plant ecologists. Intraguild predation (Polis et al. 1989) combines competition and predation: two competitors for the same resource get engaged in a direct predator-prey interaction. Intraguild predation is quite usual between carnivores (Omori et al. 2006; Périquet et al. 2014; Robinson et al. 2014; Losinger et al. 2017; Potter et al. 2018; Nájera et al. 2019; Pudiatkmoto 2019), but less obvious (or expected) between herbivores. Nevertheless, intraguild predation occurs frequently between herbivores favoured by body size asymmetry, like in the case of insects and vertebrates (Gómez and González-Megías 2002; Rooney and Waller 2003; Bonal and Muñoz 2007; Gómez and González-Megías 2007; Gish et al. 2010). Intraguild predation is usually unintentional, and large herbivores consume plants/seeds at the same time that eat the insects feeding on them (Gómez and González-Megías 2002; Canelo et al. 2020). Thus, predation risk for insects would be higher when the abundance of large herbivores is higher and/or the availability of the shared plant source lower.

Intraguild predation on *Curculio* spp. weevils by wild ungulates (Bonal and Muñoz 2007) and free-range livestock (Canelo et al. 2020) has been recorded in Iberian oak savannahs. These insects are the most important pre-dispersal acorn predators (Torres-Vila 2006; Bonal et al. 2007; Bogdziewicz et al. 2018a). Females oviposit into the acorns when still in the tree canopy (Bonal and Muñoz 2007; Bonal and Muñoz 2008). Oak trees abort infested acorns, which are dropped on average one month before sound acorns fall (Bonal and Muñoz 2008; Canelo et al. 2020). Larvae continue their development within dropped acorns for 20 more days. In this period, they are very susceptible to predation by large wild and domestic herbivores (Bonal and Muñoz 2007; Canelo et al. 2020).

In Iberian oak savannahs, acorn predation by weevils hampers natural oak regeneration and provokes significant economic losses (Vázquez 1998; Pulido et al. 2010; Canelo et al. 2020). In these silvopastoral systems (which spread over more than 4 million hectares), weevils may predate more than half of the acorn crop (Vázquez 1998; Bonal et al. 2007; Bogdziewicz et al. 2018a), thus reducing meat productivity (Tejerina 2010). Nonetheless, the high environmental value of this traditional silvopastoral system (included in the EU Habitat Directive) precludes any chemical treatment against weevils. Hence, some studies (Canelo et al. 2020) have recently proposed intraguild predation on weevils by livestock as a novel method of biological control. Moreover, this new approach has the potential to be exported to other silvopastoral systems, like Central American dry tropical woodlands, where predation on seed beetles by livestock has been reported as well (Peguero and Espelta 2013)

All the types of livestock kept in oak savannahs predate acorns with larvae inside. Moreover, large geographical scale comparisons among dehesa farms showed acorn infestation rates were negatively related to free-range livestock density (Canelo et al. 2020). This result, albeit correlational, stresses that intraguild predation on weevils may effectively reduce their negative effects. However, correlation cannot prove causation, and experiments are now required before intraguild predation can be widely recommended and implemented nationwide as pest control strategy. The set-up of small livestock exclosures, paired with control areas within the same farm, would exclude any potential confounding variable linked to large-scale comparisons.

Small livestock exclosures could also provide important information for the practical field implementation of locally increased predation on weevils. Canelo et al. (2020) propose to concentrate livestock in certain sectors of the farms temporarily, and rotate it to other plots in different years. Nevertheless, the success of this focalized treatment relies on weevil dispersal abilities. If mobility were high, immigrant weevils would readily recolonize those trees previously subjected to increased predation on *Curculio* spp. Wind tunnel experiments have shown maximum flight distances of hundreds of metres (Pélisson et al. 2013). However, the spatial distribution of infestation rates suggests much shorter movements in field conditions, specially when the distance between trees is high (Bonal and Muñoz 2007; Bogdziewicz et al. 2018a). Hence, we could expect differences in infestation rates between nearby trees in which predation on weevils by livestock differs.

We carried out a landscape scale experimental study to assess the effects of livestock predation on acorn weevils. We chose three different Holm oak (*Quercus ilex*) savannahs, and at each one we built eight individual livestock exclosures around 8 individual trees. Nearby each excluded oak we chose another two. In one of them (control tree) infested acorns could be removed both by livestock and other small post-dispersal seed predators (i. e. rodents). In the other one, all infested acorns were removed by hand weekly before larvae could emerge (predation intensification treatment). Infestation rates in predation intensification treatment will show the maximum decrease of infestation rates achievable by local elimination of weevil larvae at a small spatial (i. e. tree) scale.

The trios of study oaks were randomly distributed over the dehesa farms. For three years we monitored acorn crops, infestation rates, removal of infested acorns by post-dispersal seed predators and adult weevil numbers. We specifically assessed whether: i) infested acorns removal was lower beneath livestock excluded oaks than at control ones, ii) infestation rates by acorn weevils were higher at livestock excluded oaks compared to those trees subjected to predation intensification (i. e. manual removal of all infested acorns), iii) adult weevil numbers increased at ungulate excluded oaks compared to predation intensification trees, iv) acorn infestation rates were correlated with adult weevil numbers at the tree level, and v) in control trees, infestation rates by weevils were negatively related to infested acorn removal rates by post-dispersal predators in the previous year. We predict that, when infested acorn removal is experimentally increased, adult numbers and acorn infestation by weevils decrease in the following years.

2. MATERIAL AND METHODS

2.1 Study area

The study was carried out in the province of Cáceres, Region of Extremadura, western Spain. It was conducted at three Holm oak (*Quercus ilex* L.) savannahs placed in Malpartida de Plasencia (Site 1: 39°56'N, 5°58'W), Oliva de Plasencia (Site 2: 40°9'N, 6°5'W) and Guijo de Granadilla (Site 3: 40°8'N, 6°7'W). Iberian oak savannahs (so called “dehesas” in Spain and “montados” in Portugal) are agroecosystems resulting from the clearing of shrubs and trees in Mediterranean forests over centuries (Moreno and Pulido 2009; Pulido et al. 2010). The remaining trees, mainly Holm oaks *Quercus ilex* and Cork oaks *Quercus suber*, are interspersed within a grassland matrix. Livestock rearing is the main use in dehesas, and free-range sheep, cows and Iberian pigs feed profusely on acorns when these are dropped in autumn-winter (Moreno and Pulido 2009).

2.2 Study species

The Holm oak (*Quercus ilex* L.) is the most widely distributed tree species in the Iberian Peninsula (Blanco et al. 1997). It is an evergreen oak with a strong inter-annual variability in seed production (Bonal and Muñoz 2007; Espelta et al. 2008). Acorns mature in one year: flowering occurs in spring and acorns grow throughout the summer and early autumn. Sound acorns are dropped from mid-October until late December, but those infested by insects are abscised before (see below).

Acorn weevils (*Curculio* spp., Coleoptera, *Curculionidae*) are the main pre-dispersal acorn predators in oak forests worldwide (Dalgleish et al. 2012; Elwood et al. 2017; Williams and Hawkins 2020). In Iberian oak dehesas *Curculio elephas* (Coleoptera: *Curculionidae*) is the most prevalent species. *C. elephas* females oviposit into the acorns when still in the tree canopy (Bonal and Muñoz 2007; Bonal and Muñoz 2008). Infested acorns are prematurely aborted 15 days after oviposition, one month before sound acorns fall (Bonal and Muñoz 2008; Canelo et al. 2020). Larvae still spend 20 days on average within fallen infested acorns feeding on the cotyledons (Bonal and Muñoz 2007; Canelo et al. 2020). Larvae complete their growth within a single acorn and, once it is finished, they perforate the seed coat to leave and bury underground (Menu and Debouzie 1993; Bonal and Muñoz 2007). They will remain within underground earthen cells in a diapause stage. More than 60% of the individuals go through a one-year diapause, but it may last up to two or three years in 30% and 4% of the larvae, respectively (Menu and Debouzie 1993; Soula and Menu 2005; Venner et al. 2011).

2.3 Experimental design

We selected three Holm oak savannahs with free-range livestock. These areas differed in size, tree density and management (see Table 1). We followed the same experimental approach in the three study sites. At each one we set 8 oak trios so that, in total, 72 *Q. ilex* (3 trees X 8 groups X 3 farms) were monitored over three crop seasons: 2016, 2017 and 2018. Eight *Q. ilex* per site were randomly selected in February 2016, without any aprioristic information about acorn productivity and/or infestation rates. Only oaks with evident symptoms of decay were discarded. Around each of those trees we built a fence that excluded livestock from all the surface beneath the canopy (24 exclosures in total, 8 at each site). Then, we selected the nearest two trees to each excluded oak. One of them was left as control (livestock and other smaller post-dispersal predators, i. e. rodents, could feed on their acorns). The other one was subjected to experimentally intensified predation on weevil larvae. This treatment consisted in the weekly manual removal of all acorns (including infested ones) from the floor beneath their canopies along the study period. Larvae spend an average of 20 days within the acorn after it is dropped, hence, the weekly collection of infested acorns assured that most larvae were removed.

Fences were built in spring 2016 and the exclosures maintained throughout the three study years. The manual removal of infested acorns was performed in 2016 and 2017. The effects of the different treatments were expected one year after the onset of the experiment.

Study site	Area (ha)	Tree density (trees/ha)	Livestock density
Site 1	50	17	100 sheep and 30 goats
Site 2	20	22	20 cows
Site 3	12	17	12 pigs from October to January 10 cows from February to September

Table 1. Characteristics of the three study sites: area, tree density and livestock density and type.

2.4 Field monitoring

In order to assess whether livestock exclosure had any effect, we measured weekly predation rates on infested acorns during the crop seasons of 2016 and 2017 (year 2016: from October 12th to December 19th; and year 2017: from October 16th to December 13th). We did so only beneath the canopies of livestock excluded and control oaks, as in intensification predation oaks the treatment consisted in removing all infested acorns. We collected 20 newly dropped infested acorns under the canopy of each oak. These could be easily recognised due to the tiny oviposition scar in the seed coat

(Bonal and Muñoz 2007). We marked all these acorns with a tiny incision at the basal part to identify them in the field. At each oak, we placed 10 acorns on the floor across 1m^2 beneath the northern part of the canopy and another 10 at the southern part. Experimental acorns were intermixed with natural crop, which in our study area averaged over 120 acorns/m^2. Thus, adding 10 experimental acorns did not increase their density on the floor significantly (see Canelo et al. 2020 for the same experimental procedure). Predation rates were monitored weekly, and all the remaining infested acorns were removed and changed by fresh ones after 2 weeks. Larvae spend on average 20 days within the infested acorns after these are dropped. Thus, in those removed by post-dispersal predators within a week, most larvae would have been eaten before having had time to leave the acorn (Bonal and Muñoz 2007).

In the three study years, we recorded the number of adult weevils at each oak using emergence traps (one per tree). Traps consisted of a mosquito net attached to the tree trunk with an inverted cone with a closed bottle on the top. After emerging from their underground refuges, weevils climbed up the trunk and walked into the net, which led them to the top bottle where they were caught (see Bonal et al. 2012 a detailed description). The traps partially covered the tree trunk so as not to interfere with the infestation rates; the number of adults caught was very low compared to the total number in each tree. Trap monitoring started on the last week of August, just before the onset of adult emergence, and continued until November, when it ceased. Each week all traps were checked and all weevils taken to the laboratory to confirm the species identity. As expected, all of them were *Curculio elephas*.

Acorn crop was measured in the three study years (2016, 2017 and 2018). We did so by hanging seed traps (buckets of 0.16 m^2) from the lower branches of each oak. The number of traps per tree was related to its surface to cover a similar proportion of the canopy in all of them (between 1 and 2%). This way of measuring acorn crops has shown to be effective, as differences between the seed traps of each oak are smaller than between-tree variability (Bonal et al. 2007; Pulido et al. 2010). Fine measurements of tree canopy surface were calculated on detailed RPAS drone images with a pixel size of 10 cm taken in 2016 (see Perez-Izquierdo et al. 2018 for details). Seed traps were checked every 15 days from September 15th until acorn fall ceased at the end of the year. At each revision traps were emptied and their content taken to the laboratory, where we placed the acorns individually in plastic vials opened above. Acorns were revised every day in order to know the number of larvae that emerged from each one. One month after the last larvae had emerged, all acorns were oven dried for 48 hours at 80ºC. Eventually, every acorn was halved to look for any dead *C. elephas* larvae that had died inside, excrements or any other sign of weevil infestation. We discarded empty (aborted) acorns and calculated infestation rates by dividing the number of acorns infested by weevils by the total number of grown acorns (sound plus insect-infested ones). Knowing the number of seed traps and their surface, we could also calculate the number of acorns produced by square metre. With this data we estimated the total acorn crop of each oak multiplying it by the canopy surface.

2.5 Statistical analysis

We fitted five different mixed effects models using the glmmTMB package (Brooks et al. 2019) in R (R Team 2019). In all of them, we tested for over- and underdispersion using the ratio of the sum of the squared Pearson residuals to the residual degrees of freedom (e.g., Zuur et al. 2009 p. 224) and also with the testDispersion function of DHARMa package (Hartig 2020). Zero-inflated was analysed as well as uniformity, outliers and residuals distribution. Temporal problems and spatial autocorrelation were also evaluated at each model.

All binomial family models were performed using "cbind" function to express the dependent variable with numerators and denominators of different units. Two trees of the predation intensification treatment (from Site 2) were not evaluated in 2018. In the previous year the soil beneath the canopies was ploughed and this could have affected the survival likelihood of adult weevils overwintering underground. In addition, the variable year was not included in any model due to the strong correlation with crop size.

Question 1: We analysed whether predation rates on infested acorns by posts-dispersal seed consumers were lower under excluded trees than under control ones. To do so we used a Generalized Linear Mixed Model (GLMM) with binomial family link. The dependent variable was the number of acorns removed with respect to the total number of experimental acorns. The Site and the Treatment were included as fixed factors and tree acorn production (log transformed) as the covariate. This covariate was incorporated to the analysis because removal rates may decrease when acorn availability is high. The interaction between treatments and crop size was included to detect whether the effect of crop size on acorn predation differed between excluded and control oaks. Besides, we evaluated the quadratic relationship between treatment and crop size as well as the interaction between them. The identity of the experimental trio of oaks and tree identity itself were the random factors (the latter nested within the former).

Question 2: We tested whether acorn infestation rates were higher at excluded oaks compared to predation intensification ones. We did not include control trees because we wanted to contrast the two experimental treatments representing extremes (very little or no acorn removal *vs* total removal). We used the data from 2017 and 2018; those from 2016 were excluded because the effects of the experimental treatments would only be seen in the following years. We used a GLMM with binomial family. Experimental trio and tree identity were the random effects (the latter nested within the former). The dependent variable (infestation rate), was included as the relative number of infested acorns compared to the total (sound plus infested). The Site and Treatment were included as fixed effects. Crop size was the covariate, as the total number of acorns produced could be negatively correlated with infestation rates at the tree level. We evaluated the linear relationship between acorn infestation rates and the log transformed crop production, besides, the quadratic relationship was evaluated but it was non-significant. The DHARMa test of overdispersion (Hartig 2020) was close to significance so that we included observation-level random effects in the model (Harrison 2014).

Question 3: We assessed whether adult weevil numbers increased at ungulate excluded oaks compared to predation intensification trees with a GLMM (generalized Poisson as family error term and group and tree identity as random effect). We used generalized Poisson family because we detected underdispersion. Generalized Poisson (GP) and Conway-Maxwell-Poisson (CMP) handle with data underdispersion (Brooks et al. 2019), therefore we tested both family distributions and selected GP due to its suitability for characterizing data that have a finite upper bound (Kendall and Wittman 2010) and its lower AIC versus CMP distribution. Site and treatment were included as fixed effects and the (log-transformed) acorn crop of the previous year was the covariate. This covariate was included because, when the acorn crop is high, the number of larvae may be higher and positively correlated with the number of adults in the following year. We also tested for a quadratic relationship in crop size effect but it was non-significant.

Question 4: We tested whether the number of adults trapped at each tree and acorn infestation rates were positively related. In this analysis we included all study trees (control, exclusion and intensification treatments) in 2016, 2017 and 2018. A generalized linear mixed model (GLMM) with binomial family was performed. The dependent variable was the number of infested acorns relative to the total. The Site was the fixed effect and the number of adults and acorn crop of the current year were the covariates (both were log-transformed). The number of acorns produced was included because it could be negatively related to acorn infestation rates. The DHARMa overdispersion test (Hartig 2019) was close to significance, so we included observation-level random effects (OLRE) in the model (Harrison 2014). The trio and tree IDs (the latter nested in the former) were included as random effect.

Question 5: We assessed whether, in control trees, infestation rates by weevils were negatively related to infested acorn removal rates by post-dispersal predators in the previous year. We used acorn infestation rates recorded in 2017 and 2018, as 2016 was the first year in which we recorded acorn removal rates by livestock. We performed a generalized linear mixed model (GLMM) with binomial family and tree ID as random effect. The dependent variable was the number of infested acorns relative to the total number of acorns (sound plus infested). Site was included as fixed factor. Crop size of the current year (log-transformed) and removal rates of infested acorns the year before were the covariates. We tested the quadratic relation for either co-variable, but only crop size was significant, not removal rates the previous year.

3. RESULTS

3.1 Infested acorn removal beneath the canopies of control and livestock excluded oaks

Livestock exclusion reduced significantly infested acorns removal rates (Chi^2 = 113.168; P < 0.001; Fig. 1). Total crop size reduced (non-linearly) acorn removal rates too, as fewer infested acorns were removed beneath more productive trees (Chi^2 = 5.763; P = 0.016; Fig. 1). Acorn removal by post-dispersal predators decreased as crop size increased, but it decreased more sharply beneath oaks from which livestock had been excluded (see Fig. 1). The interactions between the treatment and crop size were significant (linear relation interaction term: $Chi^2 = 6.865$; $P = 0.009$; quadratic relation interaction term: $Chi^2 = 16.643$; $P < 0.001$).

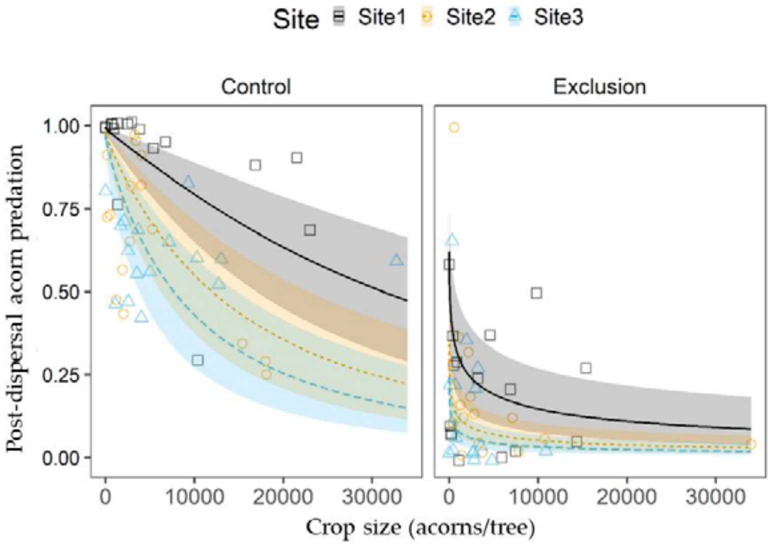

Figure 1. Relationship between post-dispersal acorn removal rates and crop size in control oaks and in oaks within livestock exclosures. The relationship is shown for the three study sites. Site 1: empty black squares, continuous black line; Site 2: empty orange circles, discontinuous orange line; Site 3: empty blue triangles, discontinuous blue line.

3.2 Infested acorn removal and infestation rates in the following year

As expected, when infested acorn removal increased, infestation rates by *C. elephas* decreased in the following year. Infestation rates were significantly lower in predation intensification oaks than in livestock excluded ones ($Chi^2 = 7.008$; $P = 0.001$; Fig. 2). Predator satiation occurred in the two treatments, i.e. infestation rates decreased as crop size increased ($Chi^2 = 13.151$; $P < 0.001$; Fig. 2).

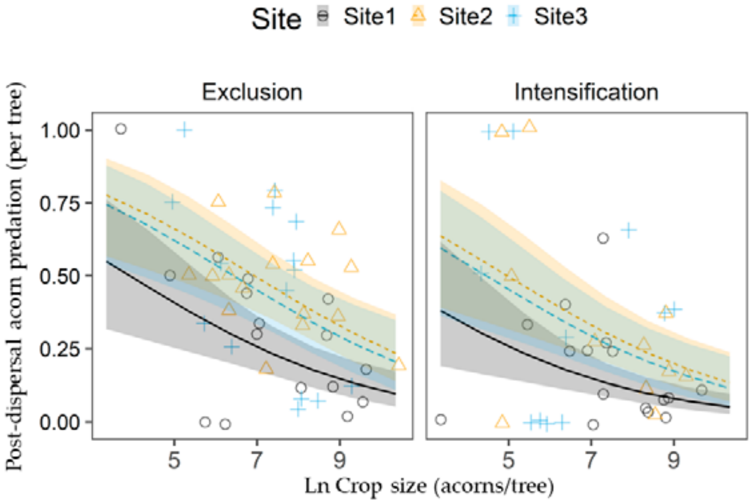

Figure 2. Relationship between pre-dispersal acorn predation rates by *Curculio elephas* and crop size in oaks within livestock exclosures and in oaks subjected to predation intensification. The relationship is shown for the three study sites. Site 1: empty black circles, continuous black line; Site 2: empty orange triangles, discontinuous orange line; Site 3: blue crosses, discontinuous blue line.

3.3 Infested acorn removal and adult abundance

As expected, increased infested acorn removal reduced adult weevil numbers in the following year as well. Fewer adult weevils were recorded at predation intensification oaks than at livestock excluded ones ($Chi^2 = 4.254$; $P = 0.039$; Fig. 3). The number of adults was also positively related with acorn production in the previous year in the two treatments ($Chi^2 = 5.615$; $P = 0.018$; Fig. 3).

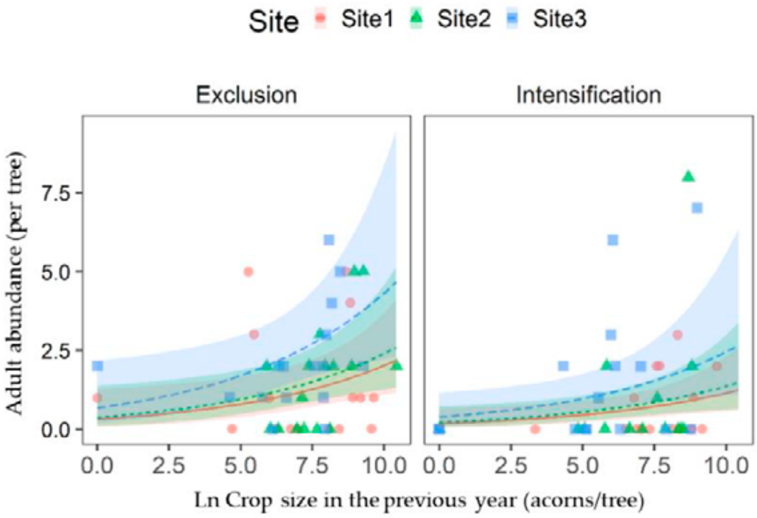

Figure 3. Relationship between the number of *Curculio elephas* adults and crop size in the previous year in oaks within livestock exclosures and in oaks subjected to predation intensification. The relationship is shown for the three study sites. Site 1: filled red dots, continuous red line; Site 2: filled green triangles, discontinuous green line; Site 3: filled blue squares, discontinuous blue line.

3.4 Relationship adult abundance and infestation rates

Pooling all the study trees (two treatments and controls), acorn infestation rates by *Curculio elephas* were directly related with the number of adult weevils trapped at each tree ($Chi^2 = 8.121$; $P = 0.004$; Fig. 4).

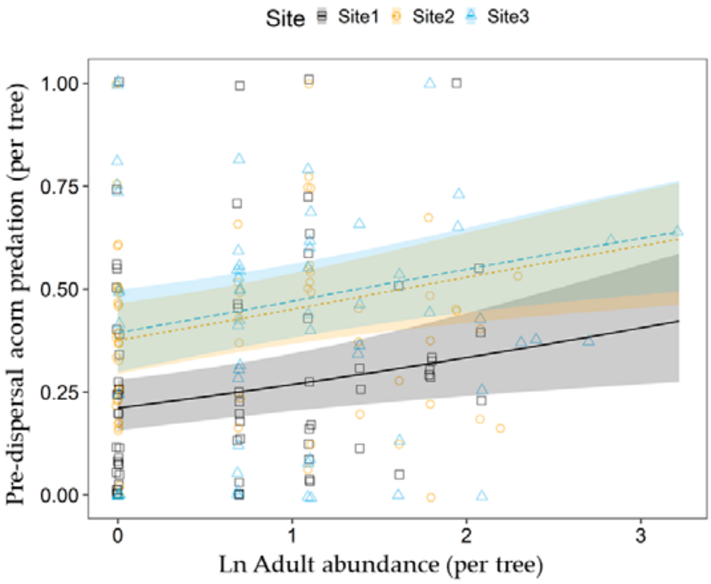

Figure 4. Relationship between the number of *Curculio elephas* adults and pre-dispersal infestation rates by weevils pooling together all study oaks. The relationship is shown for the three study sites. Site 1: empty black squares, continuous black line; Site 2: empty orange circles, discontinuous orange line; Site 3: empty blue triangles, discontinuous blue line.

3.5 Acorn infestation rates and infested acorn removal in control oaks

Finally, infested acorn removal and infestation rates were also negatively related in natural conditions. Removal rates by post-dispersal seed predators varied a lot among control trees, and went from 30% to 100% (Fig. 5). In all the study sites, infestation rates were lower in those oaks in which infested acorn removal by post-dispersal predators had been higher in the previous year ($Chi^2 = 15.652$; $P < 0.001$; Fig. 5).

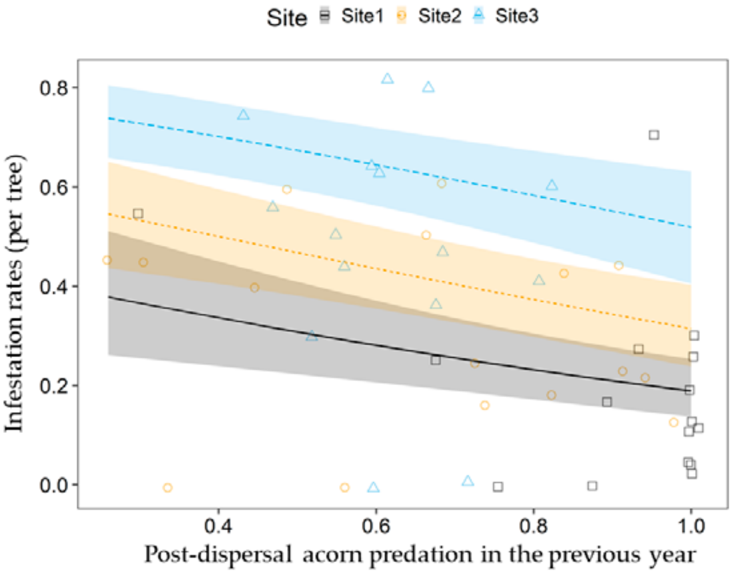

Figure 5. Relationship between acorn infestation rates by *Curculio elephas* and infested acorn removal rates by post-dispersal predators in the previous year in control oaks. The relationship is shown for the three study sites. Site 1: empty black squares, continuous black line; Site 2: empty orange circles, discontinuous orange line; Site 3: empty blue triangles, discontinuous blue line.

4. DISCUSSION

Free-range livestock is the main predator of infested acorns in Iberian oak savannahs. Within livestock exclosures, infested acorn removal decreased sharply. Predation on infested acorns had further consequences on weevil populations. The number of adult weevils was lower when acorn removal was experimentally maximised than in livestock excluded oaks. Accordingly, infestation rates decreased, as they were strongly correlated with the number of adults at the tree level. We found the same in oaks in which no experimental manipulation was made. In control oaks, infestation rates were negatively related to infested acorn removal rates beneath the canopy.

In accordance with previous studies (Bonal and Muñoz 2007), acorn removal rates decreased dramatically when large herbivores were removed. Wild ungulates and livestock are the main post-dispersal acorn predators in Mediterranean forests and oak savannahs (Muñoz and Bonal 2007; Pulido et al. 2010b; Castro et al. 2015; Sunyer et al. 2016) and they consume sound and infested acorns alike (Canelo et al. 2020). Small rodents also feed on acorns, however, their abundance decreases when the density of large herbivores is high (Muñoz and Bonal 2007; Muñoz et al. 2009). Although small mammals could find shelter within the exclosures and increase their numbers, the study period (3 years) is probably not enough. Actually, our own prospective rodent trapping showed that rodent density was low at livestock excluded trees too

(0.021 rodents per trap/night). Moreover, rodents can detect and reject infested acorns (Muñoz and Bonal 2008), what decreases predation on larvae as well.

Infested acorn removal was lower in more productive trees, especially when livestock was excluded. Some studies in North America have shown that predation on weevil larvae could be higher when acorns are abundant (Bogdziewicz et al. 2018b). In those forests crop size may remain extremely low for a few years in a row, and post-dispersal seed predators aggregate when acorn production rises, thus increasing predation on larvae. In Iberian oak savannahs, by contrast, acorn production fluctuates, but never falls so much and free-range livestock is always present. Thus, predation on infested acorns (and larvae) peaked beneath the canopy of control oaks (livestock present) producing fewer acorns. Larval predation risk decreased along with increasing crop size, but the decrease was sharper within exclosures. This result agrees with previous studies showing that rodents consume infested acorns only when food availability is very low (Bonal y Muñoz 2007).

This inverse relationship between larval predation risk and acorn availability provides a novel perspective for the consequences of masting in this system. As typical masting species, acorn productions in oaks *Quercus* spp. show a significant inter-annual variability (Kelly and Sork 2002; Espelta et al. 2008). Lean years would contribute to control the populations of specialised seed predators (like weevils) through decreased food availability (bottom-up force) (see Bogdziewicz et al. 2019 and references therein). Our results show that, in Iberian dehesas, top-down forces would also increase in these years, as predation on larvae would increase as well. Thanks to the premature acorn abscission, oaks facilitate intraguild predation between competing acorn predators, what would have a positive effect on oak fitness.

Infested acorn removal reduced the number of adults in the following year after controlling for the effect of past crop size. The number of adults was positively related with the number of acorns produced in the previous year. This shows that, as proposed, acorn availability may constrain weevil population size (Higaki 2016; Tong et al. 2017). However, irrespective of acorn production, the number of adults decreased significantly in those trees in which infested acorn removal was experimentally maximised (predation intensification oaks). Moreover, the effect appeared just one year after the experimental treatment. This quick response can be attributed to the life-history of the only species of acorn weevil recorded: *Curculio elephas*. Contrary to other species in which the bulk of adult emergence takes place two years after larvae bury, most *C. elephas* emerge after one year (Menu and Debouzie 1993; Venner et al. 2011). The length of the extended diapause has to be taken into account for the implementation of intraguild predation on *Curculio* spp. as biological control.

Infestation rates were significantly lower at predation intensification trees than at those within ungulate exclosures. This difference shows the maximum potential contribution of large herbivores as biological control agents of acorn weevils. Previous studies had not included an experimental removal of infested acorns throughout the acorn drop season (Bonal y Muñoz 2007). However, this treatment shows what would happen if livestock predated all infested acorns soon after falling. Infestation rates were almost reduced by half (≈40% depending on the site). Bonal y Muñoz (2007) report a slightly smaller reduction in two years (≈30%; 73% vs 59% and -61% vs 42%) comparing exclosures with control trees. Nonetheless, due to the extremely high density of ungulates in the control trees of that study, removal rates were close to those recorded in the experimental predation intensification oaks.

Our results show that nearby oaks subjected to different treatments showed differences in infestation rates. This short scale effects have important implications for the field implementation of intraguild predation as biological pest control. Canelo et al. (2020) propose experimentally increased densities on focalized areas. However, if weevil dispersal abilities were very high, such differences would disappear, what is not the case. Moreover, the relationship between the number of adults and infestation rates suggests limited dispersal by *Curculio elephas*. This proovigenic species oviposits soon after emerging (Pélisson et al. 2012) and has lower dispersal abilities than other *Curculio* spp. (Pélisson et al. 2013). Our results show that, although wind tunnel experiments recorded that they can fly up to 1 kilometre (Pélisson et al. 2013), their real dispersal in the field is much lower. In this sense, they agree with previous reports suggesting no or little dispersal among trees, especially when these are separated (Bonal et al. 2012; Bogdziewicz et al. 2018a). The explanation might be that competition for seeds has played an important role in the evolution of *Curculio* spp. life histories (Venner et al. 2011), and dispersal may deter time from oviposition, specially in a proovigenic species (Bonal et al. 2010).

Further studies should quantify weevil mobility by using molecular markers such as DNA microsatellites (SSR), which can detect dispersal limitations at short spatial scale in insects (Ortego et al. 2011). This information could be useful to know why infestation rates were not reduced even more when all infested acorns were removed. In addition, we started to record infestation rates one year after the onset of the experiment (in 2016). Thus, some of the weevils recorded in 2017 could be individuals going through a two-year diapause, and belong to the 2015 cohort. As in that year no experimental treatment had been applied yet, this could explain that, albeit significant, the differences between groups were a little smaller than expected.

Summarizing, the present study demonstrates that it is possible to implement intraguild predation on acorn weevils by livestock as biological control tool in oak dehesas. Such control could be carried out as proposed by Canelo et al. (2020): concentrating livestock in certain farm plots during the period of infested acorn drop (September-October). This treatment should be rotated to other plots after a maximum of three years; enough to reduce weevil numbers and not hamper oak natural regeneration. Iberian oak savannahs are the most extensive and diverse agroecosystem in the European Union (McCracken et al. 1995). It does not only have an outstanding natural value, but a key socio-economical importance in the centre and southwest of the Iberian Peninsula (Moreno and Pulido 2009). This novel way of pest control would increase the productivity of the system avoiding chemical treatments.

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Chapter 5

Using Next Generation Sequencing to assess the effects of livestock on arthropod biodiversity in Iberian Holm oak savannahs

Abstract

How to increase food production avoiding negative impacts on biodiversity constitutes one of the main challenges of our time. In this context, traditional silvopastoral systems such as the Iberian oak savannahs (dehesas) have gained visibility. Humans have reared free-range livestock in dehesas for centuries, but these agroecosystems have preserved their high natural values. However, there are factors that decrease the productivity of dehesas needing to be addressed, like the acorn losses provoked by pest insects (mainly *Curculio* weevils). A newly proposed strategy suggests using livestock to reduce infestation rates. An increased and focalized grazing on infested acorns would kill the larvae inside them and decrease weevil numbers. Nonetheless, increased livestock densities could have undesired side effects on soil and grass arthropod communities as a whole. We sampled arthropods beneath the canopies of 72 oaks in 6 different dehesas. Thanks to DNA metabarcoding aided by Next Generation Sequencing (NGS) we could delimit hundreds of arthropod Molecular Operational Taxonomic Units –MOTUs- (i. e. putative species) and largely overcome the taxonomic impediment. Our results show that using two mitochondrial markers (COI and 16S) increases the resolution of the sampling, as some taxa were detected by just one of them. The number of arthropod species and families was lower when livestock was present (control trees), but increased shortly after its exclusion. Arthropod diversity was higher beneath those trees within livestock exclosures for just one year. However, it was not significantly higher after 10 years of livestock exclusion than in control oaks. Yet, arthropod communities were very distinct in long-term exclosures compared to the rest. Despite the different context, our results support the Intermediate Disturbance Hypothesis. The higher arthropod taxonomic richness in short-term exclosures may be due to the higher microhabitat heterogeneity compared heavily grazed or long-term ungrazed areas. We propose a rotatory livestock management in dehesas: plots with increased grazing should co-exist with short-term livestock exclosures. If possible, a few long-term excluded areas could be also kept due to the singularity of the arthropod communities inhabiting them. This strategy would make possible the combination of pest control and arthropod conservation in Iberian oak savannahs.

1. INTRODUCTION

One of the great human challenges for the 21st century is to produce enough food for an increasing population in an environment-friendly way (FAO 2016; IPBES 2018). In this context, traditional agroecosystems play a very important role, as they usually combine natural values with economic activities. Iberian silvopastoral oak savannahs (so called "dehesas" in Spain and "montados" in Portugal) are a good example. Century-long shrub and tree clearing of Mediterranean forests has produced landscapes with oaks interspersed within a grassland matrix, in which livestock rearing is the main use (Moreno and Pulido 2009; Sá-Sousa 2014). Dehesas are included in the Habitats Directive (European Union) as they host a wide range of rare, threatened or endemic animal and plant species. Therefore, food production must go hand in hand with biodiversity conservation; the present study in framed within this context.

Acorns are a key food resource for livestock, and a recent study has proposed temporary increased grazing for an effective biological control of acorn pests (Canelo et al. 2020). An increased grazing could, however, have undesired negative side effects on arthropod communities. To assess this potential problem, we performed an experimental study (building livestock exclosures) and measured arthropod diversity using cutting-edge genetic technology, namely Next Generation Sequencing (NGS) for species identification by DNA metabarcoding.

Livestock plays a major role in ecosystem functioning (Eldridge et al. 2016, 2017), but the influence of grazing on biodiversity remains controversial (Yuan et al. 2016; Filazzola et al. 2020). Grazing can change plant species richness, abundance and diversity altering plant/animal community composition and beta-diversity (Adler et al. 2001; Kirk et al. 2019; Magnano et al. 2019; Sims et al. 2019; Brambila et al. 2020; Song et al. 2020). In addition, livestock has substantial impacts on soil hardness and physicochemical properties (Armas-Herrera et al. 2019; Zhang et al. 2020) or water composition and quality (O'Callaghan et al. 2018; O'Sullivan et al. 2018; Kilgarriff et al. 2020). Livestock also reduces phytophagous insect numbers due to intraguild predation. Insects may be killed by large herbivores that feed on the same food resource, which consume them while eating (Gómez and González-Megías 2002, 2007; Retamosa et al. 2004; Bonal and Muñoz 2007). This is the mechanism underlying the recently proposed use of focalized grazing to control oak pests. Livestock consumes newly dropped infested acorns containing *Curculio elephas* larvae, hence, intraguild predation could reduce weevil numbers and infestation rates (Canelo et al. 2020).

Grazing effects are not always easy to predict because they result from complex cascade effects (Sankaran and Augustine 2004; Evans et al. 2015; Eldridge et al. 2016, 2017; Abdala-Roberts et al. 2019), which transcend ecosystem boundaries (Knight et al. 2005). The variability of direct and indirect effects, along with their interactions with environmental factors (Vojta et al. 2020), may make the consequences of grazing seem counterintuitive, as different organisms may respond differently to the same disturbance events (Didham et al. 2009; Jackson et al. 2015; Gossner et al. 2016). The intermediate disturbance hypothesis (IDH) (Connell 1978; Roxburgh et al. 2004; Svensson et al. 2007; Yang et al. 2015; Gao and Carmel 2020) states that species diversity peaks at intermediate levels of environmental stress. On the one hand, high disturbances result in constant selection favouring few species; on the other hand, the lack of disturbance benefits just a few strong competitors. However, in between, the competition between existing species decreases and new species can rapidly colonize. Consequently, species richness rises under intermediate disturbance regimes. This hypothesis has been proposed to explain the high arthropod diversity recorded in areas with moderate levels of grazing (Kati et al. 2012; Kaltsas et al. 2013; Joubert et al. 2016; Lazaro et al. 2016a, 2016b; Qin et al. 2017; Winck et al. 2019).

Livestock exclusion is seen as a good strategy to restore degraded ecosystems (Kröpfl et al. 2011; Prober et al. 2011; Su et al. 2015). At the same time, it is a very efficient way to evaluate the effects of grazing on other organisms (Adams 1975; Wassie et al. 2009; Trigo et al. 2020). Such effects usually appear over time, and not in a linear fasshion (Filazzola et al. 2020), hence, the use of exclosures of different age is recommendable. Moreover, these studies should take into account that the results may differ between trophic levels and taxonomic classifications.

In arthropod biodiversity studies, species identification is always a main challenge (Moretti et al. 2004). In this sense, identification by DNA metabarcoding aided by Next Generation Sequencing (NGS) has become a great support. Gaps in taxonomic identification are usually related to the nature of taxonomic knowledge. Due to the outstanding arthropod diversity, researchers need to focus on some taxonomic groups (Didham et al. 2009; Jackson et al. 2015). Next Generation Sequencing reduces this taxonomic impediment, allowing arthropod species delimitation at a community level. DNA barcoding delimits molecular operational taxonomic units, called MOTUs, which correspond to putative species (Gaytán et al. 2020). These MOTUs can be determined to the Order, Family, Genus or Species level depending the availability of identified reference barcodes. In this sense, using more than one marker for species delimitation enhances identification likelihood. This is why we selected two mitochondrial markers: COI (Cytochrome c oxidase I) and 16S (the mitochondrial rRNA gene 16S), which have shown to complement each other very well (Marquina et al. 2019a).

We performed a landscape scale sampling to assess the richness and species composition of arthropod communities after livestock exclusion. In three dehesa farms, we randomly chose some oaks and built livestock exclosures around them. Close to each experimental tree, another oak was selected as control. In both types of trees, arthropods were sampled one year after livestock exclusion. Apart from this, we monitored the same number of trees in three abandoned oak savannahs from which livestock had been absent for 10 years or more. We followed this approach to assess the potential negative effects of using intraguild predation by livestock as biological control of acorn pests. The differences between treatments would show what could be the consequences of increasing grazing pressure. At the same time, the results would reveal the speed of the changes in arthropod communities once livestock grazing stopped. According to the Intermediate Disturbance Hypothesis, we predict that species richness will peak shortly after livestock exclosure.

Our concrete objectives were: i) to compare the success of the two markers (genes COI and 16S) used for species delimitation by DNA metabarcoding, ii) to assess how much and how fast vegetation structure changes after livestock exclusion, and iii) to compare arthropod species richness and community species composition among control oaks and those in which livestock had been excluded for 1 year or more than 10.

2. MATERIAL AND METHODS

2.1 Study area and sampling design

This research was conducted at six Holm oak (*Quercus ilex* L.) savannahs located in the province of Cáceres, western Spain. Three of them were used for free-range livestock rearing (sites 1, 2 and 3); in the other three, livestock had been absent for at least ten years before the beginning of the study (sites 4, 5 and 6). At sites 1, 2 and 3 (dehesa farms with livestock), we randomly chose eight pairs of Holm oaks *Q. ilex* per site in February 2016. Within each pair, one oak was randomly chosen for experimental short-term livestock exclusion and the other was set as control. To sum up, 8 trees x 3 sites without livestock for 10 years or more ($N_{\text{(sites 4, 5 and 6)}}$ = 24 trees); and 8 trees x 2 treatments x 3 sites with livestock ($N_{\text{(sites 1, 2 and 3)}}$ = 48 trees). Therefore, 72 *Q. ilex* were monitored (N*(control)* = 24; N*(1-year excluded)* = 24 and $N_{\text{(10-years excluded)}}$ = 24). The following Table 1 shows the study design and location and characteristics per site.

Farm	Coordinate	Treatment	N trees
Site 1	39°56'N, 5°58'W	Control trees	8
		1-year excluded trees	8
Site 2	40°09'N, 6°05'W	Control trees	8
		1-year excluded trees	8
Site 3	40°08'N, 6°07'W	Control trees	8
		1-year excluded trees	8
Site 4	39°57′N, 5°58′W	10-years excluded trees	8
Site 5	40°09′N, 6°07′W	10-years excluded trees	8
Site 6	40°08′N, 6°02′W	10-years excluded trees	8

Table 1. Table showing the location of the study sites, treatments applied and number of trees.

In March 2016 (early spring), experimental trees of sites 1, 2 and 3 were fully enclosed by a fence to avoid livestock grazing beneath their canopies (24 trees in total). In April 2017 (spring), we sampled all study trees: 24 control trees (beneath which livestock had fed freely), 24 1-year excluded trees (within livestock exclosures for 1 year) and 24 10-years excluded trees (located in sites where livestock had been absent for, at least, 10 years). Arthropod sampling methodology was homogeneous in all study trees. We vacuumed $2m^2$ of the floor (2900w vacuum machine) beneath the canopy of each tree: a $1m^2$ plot at the northern half of the canopy and another one at the southern half. Each plot was vacuumed during 30 seconds to sample all arthropods on the soil surface and on the herbs. The samples of each tree were stored at cardboard boxes and brought to the laboratory. We kept the boxes in the fridge at 4ºC until insect sorting. By doing this we preserved the specimens and avoided them from flying during sample cleaning and classification. The content of each cardboard box was emptied on a tray and carefully inspected. Rests of herbs and soil were discarded, and all the insects taken and placed into a bottle filled with alcohol 96º. We applied the same criteria to conclude the inspection for all trees. After 5 minutes without finding any new specimen we decided that the sample revision was completed.

In addition, we measured vegetation density and height under tree canopy at the same time that sampled arthropods. At each tree, we randomly measured vegetation at 8 points with a tape measure fixed to a stick. We recorded if vegetation touched the stick at ground level, between 0-10 cm, 11-25 cm, 26-50 cm and more than 50cm. We also noted the maximum vegetation height at each sampling point (see Muñoz et al. 2009 for a similar sampling procedure).

2.2 Laboratory work

In the laboratory, the alcohol from the tubes was discarded and the samples were left to dry on large petri dishes with a sterile filter paper inside a fume hood for 3 h. Once dry, the arthropods were incubated in a lysis buffer (2 mL buffer ASL (Qiagen) + 250 μL proteinase K) for 14 h at 56 °C with a gentle rotation. Subsequently, the lysate was removed and the arthropods rinsed with distilled water, 90 % ethanol and stored in 80 % ethanol. DNA extraction was performed on 225 μL of the lysate on a KingFisher Duo (Thermo Fisher Scientific) robot with the Cell & Tissue kit (Thermo Fisher Scientific), with an elution volume of 100 μL. An empty vial was incubated along with the samples and extracted, and a blank just for the extraction was included.

As metabarcoding markers, two fragments of the mitochondrial cytochrome oxidase I (COI) and the 16S rRNA genes were amplified with the primers COIBF2-COIBR1 (Elbrecht and Leese 2017) and Chiar16SF-Chiar16SR (Marquina et al. 2019b) respectively. Forward and reverse primers for both markers had attached an 8 bp tag at the 5′ end for sample multiplexing (Binladen et al. 2007). The PCR reactions consisted of one Illustra Hot Start Taq Mix RTG bead (GE Healthcare Life Sciences), 1 μL of each primer (10 nM), 2 μL DNA template and 21 μL of biology-grade water; the temperature protocol was as follows: initial DNA denaturation and Taq activation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 30 s, annealing at 48/50 °C (COI/16S) for 45 s and extension at 68 °C for 45 s, and a final phase of extension at 72 °C for 10 min. All PCRs were run in duplicated, and a blank was included in the batches of both markers. PCR products were checked in an agarose gel, pooled by replicates and DNA concentration quantified using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific) with the broad range reagents. Lysis, extraction and PCR blanks were discarded after not producing a visible band in the gels and returning undetectable values of DNA concentration. The PCR products were pooled equimolarly and cleaned-up of primers and primer dimers using MinElute columns (Qiagen). Library preparation was carried out with the TrueSeq PCR-free kit (Illumina). Ligation products were visualized on a 2 % agarose gel and the fragments of the desired length with adapters were cut off and purified using the QIAquick gel extraction kit (Qiagen).

Different libraries were then pooled to equimolar concentrations and sequenced on a single Illumina MiSeq lane using 2 x 300 bp paired-end v3 chemistry run at SciLifeLab (Swedish Museum of Natural History, Stockholm).

2.3 Bioinformatics process

Raw sequencing data was processed using a pipeline based on OBITools (Boyer et al. 2015) in combination with other software (described below). A FastQC analysis (Andrews 2010) was conducted to check sequencing quality, and sequences were trimmed at the position where average phred score was lower than 28. Then, they were paired-end merged and alignments with a score lower than 30 were discarded. Sequences that passed the filtering were demultiplexed based on the 8 bp sample tag combinations and the primer sequences were trimmed away. Demultiplexed sequences were then filtered based on their length, selecting only those in the range of 310-330 bp long for COI and 260-375 bp long for 16S. Dereplicated reads were searched for chimeras using VSEARCH v2.7.1 (Rognes et al. 2016). Subsequently, the sequences were clustered into Molecular Operational Taxonomic Units (MOTUs) using SWARM v2.1.13 (Mahé et al. 2015), with maximum distance d = 9 and d = 5, for COI and 16S respectively, and combined with abundance information to generate molecular operational taxonomic unit (MOTU) occurrence tables. Each MOTU can be potentially considered a putative species (Pons et al. 2006) and as such be identified if reference sequences exist. These were curated with LULU v0.1.0 (Frøslev et al. 2017) to reassign false MOTUs to their parent MOTU. The representative or centroid sequences of each MOTU were assigned taxonomic identity with ecotag (OBITools) using a local reference database built with a combination of i) all the barcodes from all orders of Arthropoda with terrestrial representatives from BOLD (Ratnasingham and Hebert 2007) accessed in June 2018, and ii) the invertebrate and fungi sequences from EMBL's release r137 (Kulikova et al. 2004) (COI, ~ 4.1 M sequences), or just the invertebrate sequences from EMBL's release r137 (16S, ~ 60 k sequences). The final dataset was refined by collapsing all MOTUs with the same species identification, as well as removing those occurrences that represented less than 0.4 % of the total reads of the sample, and those MOTUs with less than 10 reads in total across all samples. The minimum abundance per sample threshold was defined after running an additional library with controlled empty sample tag combinations and calculating the proportion of reads recovered from those combinations from the total generated in that library.

2.4 Statistical analysis

Vegetation structure analysis

In order to assess the effects of livestock on vegetation structure we performed two Principal Component Analyses (PCA). We did so to summarize all the vegetation measurements made at the sampling points. PCA analysis is a method used to reduce the dimensionality of large data sets, by transforming a large set of variables into a smaller one that still contains most of the information of the original data. Both PCAs were based on standardized correlation matrix with "PCA" function from "FactoMineR" package. The first PCA was made using data from all study trees (N = 72) to assess the differences among the three categories of trees (control, 1-year excluded livestock and 10-years excluded livestock). In the second one, we only included control oaks (N = 24) to analyse the differences among the three oak savannahs with free-range livestock excluding the effect of the experimental treatment (Site 1, Site 2 and Site 3).

Specie, Family and Order abundance analysis

To assess the differences in species (i. e. MOTUs) richness we performed Generalised Linear Mixed Models (GLMMs) and Generalized Linear Models (GLMs) with binomial negative family links. We did separate analyses for the databases of MOTUs detected with the marker gene COI and 16S. We could not pool all MOTUs (i. e. putative species) in one single base because some of them could not be identified to the species level (I e. could not be assigned a Linnean name). If we had pooled them, we could have included some species twice.

Firstly, we performed Generalized Linear Mixed Models (GLMMs) with farms (i. e. Sites) as random factors to compare: control, 1-year and 10-years excluded trees (all study farms were included: Site 1, Site 2, Site 3, Site 4, Site 5 and Site 6; N = 72 oaks in total). All models were performed with binomial negative links. This distribution was chosen after comparing it with Poisson, Generalized Poisson and Conway-Maxwell Poisson distributions from package "glmmTMB" (Brooks et al. 2019). In addition, post-hoc analysis were made with Tukey tests.

In second place, we performed Generalized Linear Models (GLMs) including only the experimental and control trees of Sites 1, 2 and 3 (N=48 trees) (see Material and Methods study design Table 1, p. 105). Doing this, we had a full factorial design, in which the two categories (1-year excluded and control trees) were replicated in the three oak savannahs. The number of MOTUs was the dependent variable and Site and Treatment the Independent factors. All GLMs were performed with negative binomial family links due to the over-dispersion on Poisson models. Post-hoc analysis were made with Tukey tests. Interaction terms between treatments and sites were always non-significant, therefore, the effect of one factor was averaged across the levels of the other factor.

We repeated the same type of analyses (GLMMs and GLMs with negative binomial family link) but with the number of Families and the number of Orders as dependent variables. We counted the number of Families and Orders (detected by a single marker -16S or COI- or by both) recorded at each oak. We tested whether this number differed between treatments as in the case of MOTUs. Finally, all models were validated testing over- and under-dispersion, homogeneity and residual distribution.

MOTUs composition analysis

The main gradients of MOTUs (i. e. putative species) composition were described with nonmetric multidimensional scaling ordinations (NMDS) ordinations performed on matrices of sampling sites with the MOTUs detected by each marker (COI or 16S) in all study trees (N = 72) with the function metaMDS of R Package vegan (Oksanen et al. 2013).

Data were submitted to Wisconsin double standardization (species are first standardized by maxima and then sites by site totals). The Bray and Curtis method was used to measure the distance/similarity between plots.

The effect of treatments on MOTUs composition was explored by correlating them with the NMDS ordination using the "envfit" function of vegan (McCune and Grace 2002; Oksanen 2009). The strength of those relationships was evaluated through the squared correlation coefficient (r^2).

3. RESULTS

3.1 MOTUs delimitation and taxonomic identification

The number of different MOTUs (i. e. putative species) delimited by DNA metabarcoding differed between molecular markers: 398 using COI and 351 using 16S. After comparing the sequences with reference databases, we could assign species Linnean names to 40% of the MOTUs in the case COI and 37% in the case of 16S. Identification rates increased at higher taxonomic levels. More than 97% of the MOTUs could be classified to the Order level with either marker. The proportion was also very high at the Family level, but here the success was considerably higher using the marker COI: 89% vs 76% using 16S (Table 2).

The inclusion of two markers also increased the detection success. There were 22 different orders in total, all of them identified by COI but only 14 by 16S. The orders missed by the marker 16S were: Mantodea, Opiliones, Plecoptera, Poduromorpha, Sarcoptiformes, Symphypleona, Thysanoptera and Trombidiformes (see Supplementary Material, p. 119). At a lower taxonomic level, many families were detected by the two markers (N = 81), but some of them were only found by one: 36 in the case of COI and 8 in the case of 16S (Table 2). The Supplementary Material (p. 119) shows a table with all families and marker identification as well as the number of MOTUs classified into each family. Regarding determinations to the Genus level, roughly a 50% of the MOTUs were identified per marker, however the majority of the identified genera were different between markers: from a total of 266 genera, 117 were exclusively identified by COI and 149 by 16S (see Table 2); that is, only 58 genera were classified by both two markers.

	COI	16S
ARTHROPODS	398 MOTUs	351 MOTUs
ORDER **22** total orders detected	392 MOTUs identified 98.49% MOTUs classified into **22** different orders **8** orders were **detected exclusively** by COI	343 MOTUs identified 97.72% MOTUs classified into **14** different orders **Any** order was **detected exclusively** by 16S
FAMILY **125** total families detected	355 MOTUs identified 89.19% MOTUs classified into **117** families **36** families were **detected exclusively** by COI	269 MOTUs identified 76.63% MOTUs classified into **89** families **8** families were **detected exclusively** by 16S
GENUS **266** total genera detected	204 MOTUs identified 51.26% MOTUs classified into **175** genus **117** genera were **detected exclusively** by COI	180 MOTUs identified 51.28% MOTUs classified into **149** genus **91** genera were **detected exclusively** by 16S

Table 2. Taxonomic identifications summary.

3.2 Vegetation structure

The results of the first Principal Component Analysis (PCA) showed that vegetation structure starts to change soon after livestock is excluded. The analyses retrieved two dimensions, which together explained 78% of the variance (PC1 59.1% and PC2 16.7%). PC1 was significantly correlated with all variables (Table 3) and defined a gradient between sampling sites with high vegetation cover and height (mostly grass) versus those with a higher percentage of short-grass (i. e. Ground). PC2 was explained by 4 variables (Table 3), and separated areas with high cover of short and high grass from those with a prevalence of medium-sized grass.

Taken together, PC1 (horizontal axis) and PC2 (vertical axis) divided the plane in four quadrants. The position along PC1 showed that vegetation cover was higher beneath the canopies of oaks from which livestock had been excluded for 1 year or 10 years than in control trees. In turn, the position in PC2 shows that grass height was higher in long-term livestock excluded areas, whereas in 1-year exclosures medium-sized grass was more prevalent (see Fig. 1).

First PCA analysis (N = 72)		PC1		PC2	
Variables	**Meaning**	**correlation**	**P-value**	**correlation**	**P-value**
Max	Vegetation maximum height	0.937	< 0.001	0.288	0.014
X50_	Presence of vegetation upper 50cm	0.709	< 0.001	0.519	< 0.001
X26_50	Presence of vegetation between 26 and 50 cm	0.867	< 0.001	0.167	> 0.05
X11_25	Presence of vegetation between 11 and 25 cm	0.785	< 0.001	-0.112	> 0.05
X0_10	Presence of vegetation between 0 and 10 cm	0.609	< 0.001	-0.561	< 0.001
Ground	Presence of vegetation at ground level (0 cm)	-0.652	< 0.001	0.542	< 0.001

Table 3. Correlation coefficients and the P-values of the variables in the two PCA factors. All study trees are included (N = 72). Non-significant correlations are shown in red.

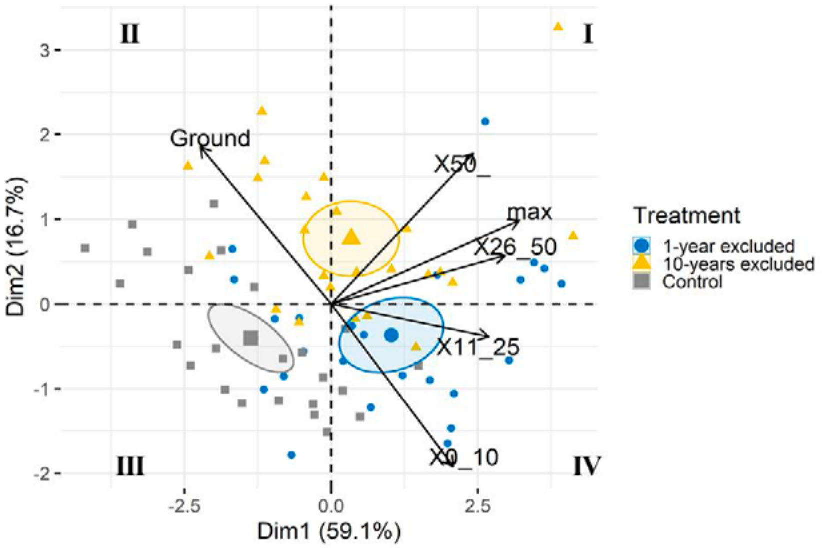

Figure 1. PCA biplot of all study trees (N = 72) classified per treatment. The ellipses show the 95 % confidence variance for the mean of each category (1-year livestock excluded, 10 year-excluded or control oaks). Quadrant numbers are given at the corresponding corners (I, II, III, IV).

The second Principal Component Analysis (PCA) was performed including only the control oaks (total N = 24 trees) from Sites 1, 2 and 3 (livestock present). The results showed that there were marked differences between farms in vegetation cover at their standard livestock densities. Two dimensions explained 75.4% of the variance (PC1 explained 59.6 % and PC2 15.8 %). PC1 was highly correlated with all variables and defined a gradient between areas with a higher proportion of short-grass and areas with high grass cover and height. PC2 was explained by only two variables, and differentiated areas with high herbs versus areas with a prevalence of medium-sized grass (Table 4).

PC1 (horizontal axis) and PC2 (vertical axis) defined four quadrats, which segregated very well the different study sites. In Site 1, the amount of vegetation beneath the oak canopies was lower and the proportion of short-grass much higher. There were differences, albeit smaller, between Sites 2 and 3 as well. Segregation was much lower along PC2, and mainly shows that grass height is higher in Site 3.

Second PCA analysis (N = 24)		PC1		PC2	
Variables	**Meaning**	**correlation**	**P-value**	**correlation**	**P-value**
Max	Vegetation maximum height	0.952	< 0.001	-0.019	> 0.05
X50_	Presence of vegetation upper 50cm	0.493	< 0.001	0.836	< 0.001
X26_50	Presence of vegetation between 26 and 50 cm	0.788	< 0.001	-0.456	< 0.001
X11_25	Presence of vegetation between 11 and 25 cm	0.827	< 0.001	0.076	> 0.05
X0_10	Presence of vegetation between 0 and 10 cm	0.762	< 0.001	-0.174	> 0.05
Ground	Presence of vegetation at ground level (0 cm)	-0.738	< 0.001	-0.049	> 0.05

Table 4. Correlation coefficients and the P-values of the variables in the two PCA factors. Control trees from Sites 1, 2 and 3 are included (N = 24). Non-significant correlations are shown in red.

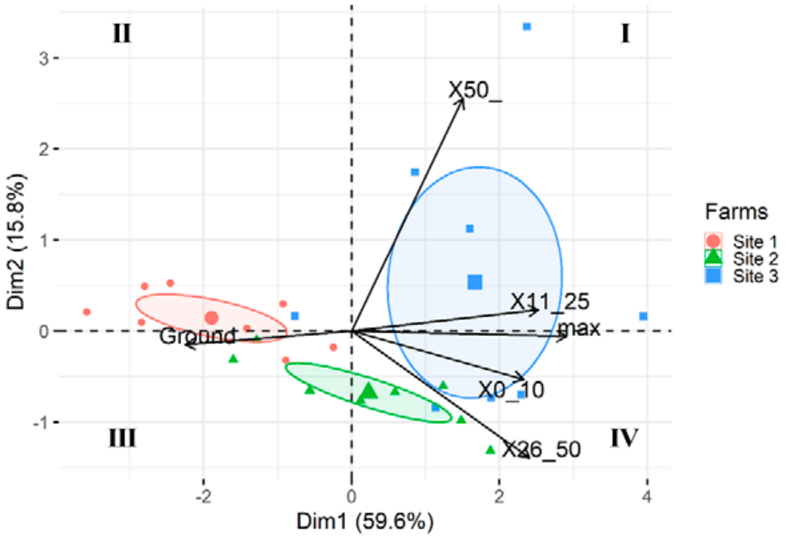

Figure 2. PCA biplot of control trees (N = 24) classified per study Site. The ellipses show the 95 % confidence variance for the mean of each category (Sites 1, 2 and 3). Quadrant numbers are given at the corresponding corners (I, II, III, IV).

3.3 Specie, Family and Order abundance

Number of COI MOTUs

The exclusion of livestock had no effect on the number of arthropod MOTUs (i. e. putative species) delimited using COI. The GLMM including all study sites and oaks (N = 72 trees) showed no differences between treatments ($Chi^2 = 4.051$; P = 0.132) (Fig. 3A).

When only the 3 sites with control and experimental 1-year exclosures were included (full factorial design model) there were marginal differences between treatments ($Chi^2 = 3.616$; P = 0.057), although differences among Sites were stronger ($Chi^2 = 8.260$; P = 0.016). The interaction term between both factors was non-significant ($Chi^2 = 0.574$; P = 0.750) (Fig. 3B). This GLM (N = 48) only explained 20% of the variance, however, post hoc analysis showed that the number of MOTUs (i. e. putative species) delimited using COI was slightly lower in control trees than in 1-year excluded ones (estimate = -0.221; Z = -1.901; P = 0.057). Moreover, the number of MOTUs was lower in Site 1 than in Site 2 (estimate = -0.4101; Z = -2.867; P = 0.0114); the rest of pairwise comparisons were not significant (all p > 0.05).

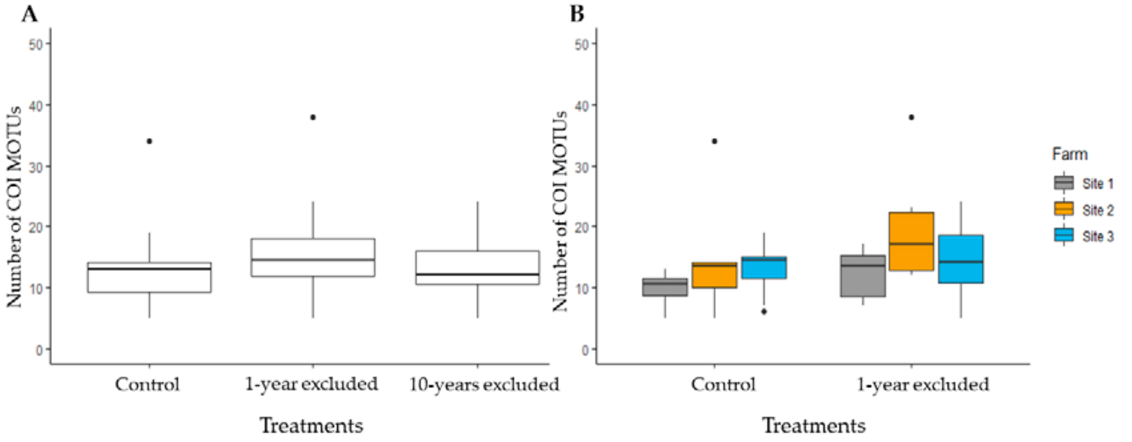

Figure 3. Number of MOTUs (i. e. putative species) delimited by the mitochondrial COI marker for different treatments and sites. Boxes represent the median and interquartile range. Lines show the data ranges and dots correspond to potential outliers. Left panel (**A**) shows the mean average number of COI MOTUs per oak (N = 72) in each treatment (1-year livestock excluded, 10-years livestock excluded and control trees). Right panel (**B**) depicts the differences in the number of COI MOTUs between control and 1-year excluded trees at Sites 1, 2 and 3 (N = 48).

Number of 16S MOTUs

The exclusion of livestock increased the number of arthropod MOTUs (putative species) delimited using 16S. The GLMM including all trees (N = 72) showed that there were differences between treatments (Chi^2 = 12.439; P = 0.002). Post hoc analysis showed that the number of MOTUs was lower in control trees than in 1-year exclusion ones (estimate = -0.366; Z = -2.271; P = 0.003) and also a marginal significant difference between control and 10-years excluded trees (estimate = -0.4207; Z = -2.271; P = 0.057) (Fig. 4A). The differences between 1-year excluded trees and those in which livestock had been excluded during, at least, 10 years, were not significant (estimate = 0.055; Z = 0.300; P = 0.950).

When only the 3 sites with control and experimental 1-year exclosures were included (full factorial design model) there were differences between treatments (Chi^2 = 16.412; P < 0.001) and between sites (Chi^2 = 26.453; P < 0.001), but the interaction term was non-significant (Chi^2 = 2.149; P = 0.342) (Fig. 4B). The GLM (N = 48) explained more than the 40% of the variability. Therefore, it explained twice the variance compared to the model performed with the MOTUs delimited by the other mitochondrial marker (COI). Post hoc analysis showed that the number of 16S MOTUs was greater in 1-year excluded trees than in controls (estimate = 0.3642; Z = 4.029; P < 0.001). In addition, Site 1 presented lower number of MOTUs than Site 2 (estimate = -0.519692; Z = -4.488; P < 0.001) and Site 3 (estimate = -0.516687; Z = -4.460; P < 0.001). Comparisons between Sites 2 and 3 were non-significant (P = 1).

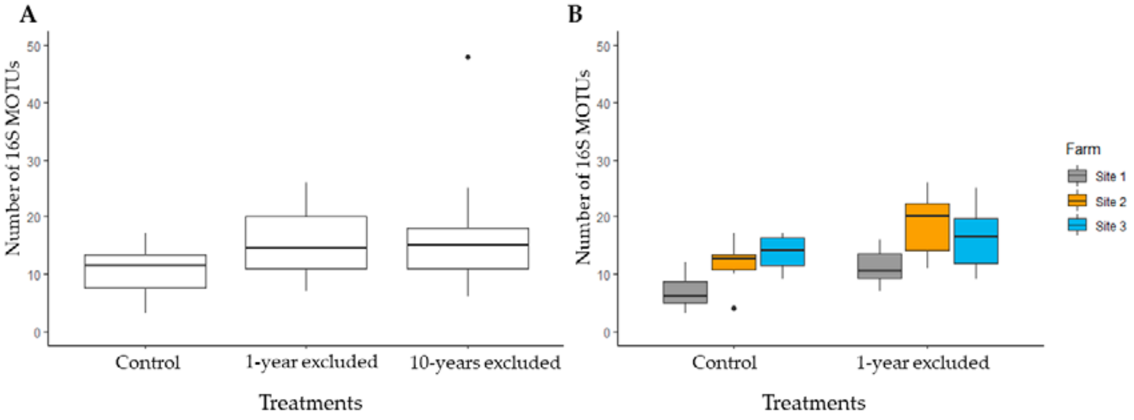

Figure 4. Number of MOTUs (i. e. putative species) delimited by the 16S mitochondrial marker for different treatments and sites. Boxes represent the median and interquartile range. Lines show the data ranges and dots correspond to potential outliers. Left panel (**A**) shows the mean number of 16S MOTUs per oak (N = 72) in each treatment (1-year livestock excluded, 10-years livestock excluded and control trees). Right panel (**B**) depicts the differences in the number of 16S MOTUs between control and 1-year excluded trees at Sites 1, 2 and 3 (N = 48).

Family abundance

Considering all study trees and categories (N = 72), the number of families per tree differed between treatments (Chi^2 = 12.439; P = 0.002). Post hoc analysis showed that it was higher in 1-year excluded trees than in control ones (estimate = 0.2438; Z = 1.335; p = 0.039). However, a greater abundance of families in 10-years excluded trees than in controls remain marginally significant (estimate = 0.421; Z = 2.271; p = 0.057) (Fig. 5A).

When only the 3 sites with control and experimental 1-year exclosures were included (full factorial design model), we could test differences between treatments and sites (GLM; N = 48). The interaction term was non-significant (Chi^2 = 0.282; P = 0.868) but there were differences between sites (Chi^2 = 15.848; P < 0.001) and treatments (Chi^2 = 4.856; P = 0.028). Post hoc analysis revealed that the number of families was higher in 1-year excluded trees than controls (estimate = 0.2445; Z = 2.201; P = 0.028). In addition, as in the case of the MOTUs, there were differences between sites; in Site 1 the number of families was lower than in Site 2 (estimate = -0.5340; Z = -3.838; P < 0.001) and Site 3 (estimate = -0.4138; Z = -2.934; P = 0.009) (Fig. 5B).

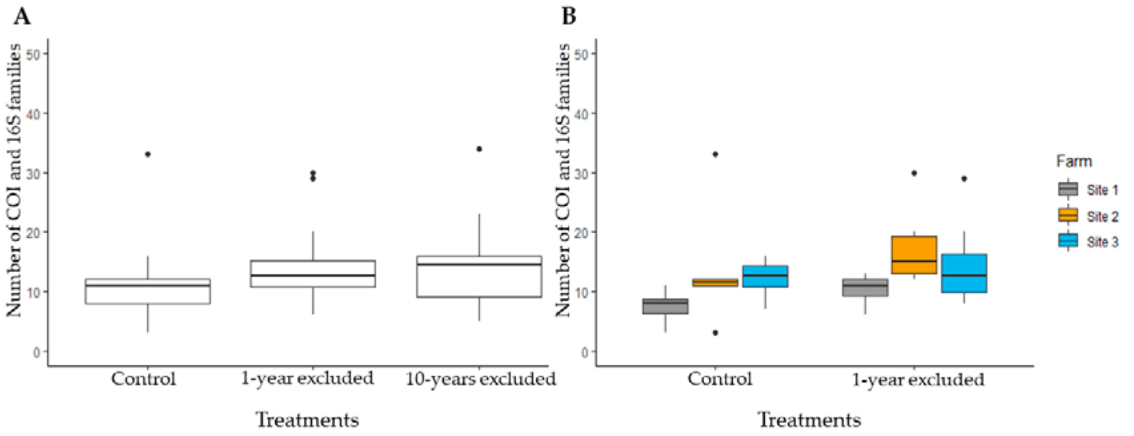

Figure 5. Differences between treatments and sites in the number of families detected by COI and/or 16S mitochondrial markers. Boxes represent the median and interquartile range. Lines show the data ranges and dots correspond to potential outliers. Left panel (**A**) shows the mean number arthropod families per oak (N = 72) in each treatment (1-year livestock excluded, 10-years livestock excluded and control trees). Right panel (**B**) depicts the differences in the number of arthropod families between control and 1-year excluded trees at Sites 1, 2 and 3 (N = 48).

Order abundance

The presence of livestock had no effect on the number of arthropod Orders. Taking all trees and treatments, the GLMM (N = 72) did not reveal any difference between treatments (Chi^2 = 0.777; P = 0.678). When only the 3 sites with control and experimental 1-year exclosures were included (full factorial design model; N = 48), the interaction between factors was non-significant (Chi^2 = 0.585; P = 0.746), neither was the treatment (Chi^2 = 0.399; P = 0.527). The only differences found were between sites (Chi^2 = 7.574; P = 0.023); the post hoc analysis showed that the number of arthropod Orders in Site 1 was lower than in Site 2 (estimate = 0.3887; Z = 2.684; P = 0.020).

MOTUs composition

The main gradients in MOTUs composition detected by each marker (COI or 16S) were described for the two axes of a non-metric multidimensional scaling ordination, with final stress values of 0.21 and 0.23 respectively (Fig. 6). For both markers, the first axis of the NMDS ordination (Axis 1; Fig. 6) identified a gradient between 10-year-excluded plots, presenting positive values, and control and 1-year-excluded sites. Correlation analyses showed that main factors conditioning the community differences for COI and 16S markers were Treatment ($r^2 = 0.36$; $P < 0.001$ and $r^2 = 0.32$; $P < 0.001$ respectively) and the study Site (dehesa farm) ($r^2 = 0.23$; $P < 0.001$ and $r^2 = 0.44$; $P < 0.001$ respectively) (Fig. 6).

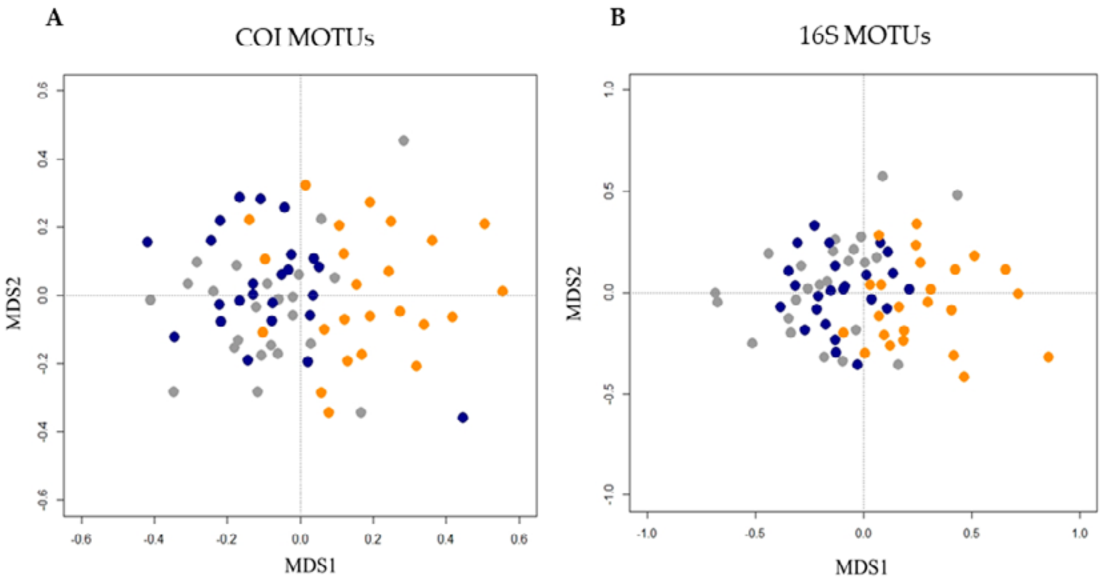

Figure 6. Axes 1 and 2 of the 2-dimensional nonmetric multidimensional scaling ordination (NMDS) of study trees. The left (**A**) panel shows the NMDS based on the presence and absence of MOTUs (i. e. putative species) delimited by the mitochondrial marker COI. The right (**B**) panel shows the results of the NMDS based on the presence/absence of 16S MOTUs. Filled dark orange dots represent trees from sites in which livestock had been absent for at least 10 years, grey dots are control trees and dark blue dots correspond to oaks within short-term livestock exclosures (1-year-excluded trees).

4. DISCUSION

The exclusion of livestock had significant effects on arthropod abundance and species composition. Thanks to Next Generation Sequencing (NGS) and DNA metabarcoding, we could detect hundreds of MOTUs (i. e. putative species). In control trees (livestock present) vegetation cover and height were lower, and the number of arthropod MOTUs and Families were lower as well. Taxonomic richness peaked in short term exclosures. However, long-term exclosures were the more distinct in terms of species composition.

NGS DNA metabarcoding has proved to be a very effective and powerful tool to evaluate arthropod communities. This useful genetic technic has overcome the taxonomic impediment, allowing us to assess the number of MOTUs (putative species) of very different arthropod taxa very efficiently. To the best of our knowledge, this is the first time that such a wide biodiversity analysis has been carried out at the species level in Mediterranean evergreen oak woodlands. Previous studies on soil arthropod communities had been performed grouping at higher taxonomic levels (Class, Order and, in rarer cases, Family) (Andrés et al. 1999; Sadaka and Ponge 2003; Rota et al. 2015). At a lower level, only checklists of species from certain Families have been reported (e. g. Zaballos 1983.). In no case molecular techniques such as New Generation Sequencing had been employed.

Identification success differed among taxonomic levels depending on the availability of reference sequences. Almost all arthropod MOTUs were identified to the Order level, and more than the 80 per cent to the Family level. We could thus assess the majority of the arthropod families coexisting in these Iberian oak savannahs. However, identification success at the species level was lower; we could assign Linnean species names to 40% of the MOTUs (putative species). This result highlights the importance of promoting classical taxonomy at the same time than DNA barcoding. Only extensive, reliable and geographically unbiased reference databases of DNA barcodes will allow identification to the species level of query sequences (Gaytán et al. 2020).

The use of two different genetic markers increased the sampling resolution. The use of 16S in addition to COI did not add up anything at the Order level. However, it did at the Family level. If well more Families were exclusively detected by COI, others were only retrieved by 16S. Moreover, these Families may have had an important role in the biodiversity analysis, as differences between treatments in the richness of MOTUs were always higher using the 16S database. These results agree with a previous study which concluded that the best markers to detect Hexapoda subphylum barcodes, are found in the mitochondrial ribosomal RNA (rRNA) genes, such as 12S and 16S (Marquina et al. 2019a). However, our results suggest that the conclusions of Marquina et al. (2019) may be applicable not just on Hexapoda, but also to all Arthropoda phylum.

Livestock reduced vegetation cover and height. Arthropod species richness was lower in control trees and at Site 1, the dehesa farm in which livestock density was higher. Vegetation height is a high-quality predictor of plant and insect diversity under contrasting grazing intensities (Kruess and Tscharntke 2002). Livestock and phytophagous arthropods compete for the same food resource: vegetation. Competition takes place through direct and indirect interactions. Big and small herbivores affect each other directly by decreasing food resource (density-mediated interaction; Werner and Peacor 2003; Feeley et al. 2006; Denis et al. 2008; Evans et al. 2015). This interaction is highly asymmetrical due to the fact that big herbivores affect arthropods but the converse rarely happens, owing to the large differences in body size (Zamora and Gómez 1993). Body size differences also favour other antagonistic interactions, such as the incidental intraguild predation of phytophagous arthropods by livestock (Gómez and González-Megías 2003; Canelo et al. 2020). Moreover, the negative effects of livestock are not restricted to phytophagous arthropods, as carnivores (e. g. active hunters, pasasitoids) may also incidentally killed by feeding livestock and/or suffer the scarcity of prey (i. e. phytopagous invertebrates) (Prieto-Benítez and Méndez 2011; King et al. 2014).

The number of arthropod taxa increased after livestock exclusure, but not in a linear fashion. Both the number of putative species (MOTUs) and arthropod families increased shortly after exclusion, but decreased as the years went by. After 1-year of livestock exclusion, vegetation cover gets higher and medium-sized grass prevails. Under this situation, there is a greater amount of food source for herbivore arthropods owing to the lack of competition with big herbivores. Herbivore arthropods may increase their population size and new species may arrive from neighbouring areas (Townsend et al. 1997; Catford et al 2012; Harvey et al. 2016). Therefore, the increase on primary production triggers a bottom-up trophic cascade at this initial period.

The intermediate disturbance hypothesis (IDH) predicts a peak of species richness at intermediate levels of disturbance. Within the context of our study, this would be the situation after 1 year of livestock exclosure. The sequence would be: high disturbance (livestock present), medium disturbance (1year-exclosure, the disturbance starts to disappear) and no disturbance (10-year exclosure). Shortly after exclusion grass cover increases and vegetation composition changes (Sims et al. 2019). In parallel, microhabitat heterogeneity increases (Song et al. 2020), what has positive effects on arthropods (Stephan et al. 2017).

Medium disturbance offers possibilities for species with different habitat and food requirements (for example, open habitat omnivore generalists and herbivore specialists). After 10 years of livestock exclusion, the competence between arthropods increases. Grass cover and height is higher, and the habitat becomes more homogeneous. According to the competitive exclusion principle (Hardin 1960; McPeek 2014), species which compete strongly for the same resource cannot coexist; one of them will overtake other. This is what may happen in long-term exclosures after 10 years without livestock. The lack of grazing homogenizes the habitat and promotes competition among herbivores, what decreases arthropod species richness compared to short time exclosures. However, the homogenization of the habitat may favour certain specialists. This may explain the markedly distinct species composition in long-term exclosures. With the pass of time the total number of species will decrease a little; some will disappear but others may increase their numbers. Further studies taking into account functional traits will help to understand the process underlying such temporal changes in arthropod communities.

In summary, the results show that intermediate-disturbance hypothesis (Connell 1978; Roxburgh et al. 2004; Svensson et al. 2007; Yang et al. 2015; Gao and Carmel 2020) not only explains the higher arthropod diversity under moderate grazing, but also the short-time recovery after livestock exclusion. Cessation of grazing for short-term periods increases richness and biodiversity of arthropod MOTUs (i. e. putative species) and Families. Canelo et al. (2020) proposed increasing livestock densities for decreasing the numbers of acorn pest insects. Increased grazing should be concentrated on certain plots of the farm and be maintained for a maximum of three years (to avoid negative effects on oak recruitment).

This article stresses that increased grazing would have negative effects not only on acorn pests, but on arthropod numbers as a whole. However, the results also put forward that the recovery after livestock exclusion is quick. Thus, an optimal management should combine, within the same dehesa, farm plots with temporary increased grazing and short-term livestock exclosures. This innovative livestock management would increase the productivity of Iberian oak savannahs (reduced acorn pests) and preserve its rich arthropod biodiversity at the same time.

5. SUPPLEMENTARY MATHERIAL

Nex table shows the number of MOTUs detected by COI and 16S markers. Red color represents those orders/families which has been detected only by COI. Blue color represents those orders/families which has been detected only by 16S.

ORDER	FAMILY	COI MOTUs	16S MOTUs
Araneae	Agelenidae	1	1
	Araneidae	2	0
	Dictynidae	3	0
	Gnaphosidae	1	2
	Linyphiidae	4	6
	Oxyopidae	3	1
	Philodromidae	2	2
	Pisauridae	1	1
	Salticidae	1	3
	Sparassidae	1	1
	Tetragnathidae	1	1
	Theridiidae	1	2
	Thomisidae	1	0
	Zodariidae	0	1
Archaeognatha	Machilidae	4	2
Blattodea	Ectobiidae	4	2
Coleoptera	Brentidae	5	4
	Buprestidae	2	0
	Cantharidae	2	1
	Carabidae	4	4
	Cerambycidae	1	1
	Chrysomelidae	12	10
	Coccinellidae	5	4
	Curculionidae	7	7
	Dermestidae	1	1
	Meloidae	1	0
	Melyridae	5	4
	Oedemeridae	1	2
	Phalacridae	0	3
	Prionoceridae	0	1
	Scarabaeidae	3	2
	Staphylinidae	2	0
	Tenebrionidae	2	3
Dermaptera	Forficulidae	4	1
Diptera	Acroceridae	1	2
	Agromyzidae	2	2

	Anthomyiidae	4	1
	Camillidae	1	0
	Cecidomyiidae	7	7
	Ceratopogonidae	1	2
	Chaoboridae	1	0
	Chironomidae	4	3
	Chloropidae	3	1
	Chyromyidae	1	0
	Culicidae	2	1
	Dolichopodidae	7	7
	Drosophilidae	3	3
	Ephydridae	2	2
	Heleomyzidae	4	0
	Hybotidae	1	0
	Keroplatidae	0	1
	Lauxaniidae	3	3
	Lonchopteridae	1	2
	Muscidae	6	4
	Opomyzidae	3	0
	Phoridae	9	9
	Pipunculidae	0	1
	Sciaridae	4	5
	Sepsidae	4	3
	Simuliidae	2	2
	Sphaeroceridae	6	1
	Stratiomyidae	1	1
	Tachinidae	1	0
	Tephritidae	1	1
	Tipulidae	3	1
	Ulidiidae	1	1
Entomobryomorpha	Entomobryidae	2	3
Hemiptera	Aphididae	2	7
	Aphrophoridae	2	2
	Berytidae	3	2
	Cicadellidae	19	17
	Cixiidae	1	0
	Coreidae	1	0
	Delphacidae	1	2
	Dictyopharidae	1	1
	Issidae	3	1
	Lygaeidae	2	0
	Malcidae	0	1
	Miridae	31	25
	Nabidae	2	2

	Pemphigidae	0	1
	Pentatomidae	2	1
	Psyllidae	1	0
	Rhyparochromidae	6	4
	Tingidae	2	2
	Triozidae	1	0
Hymenoptera	Andrenidae	1	0
	Aphelinidae	1	0
	Braconidae	15	10
	Encyrtidae	1	0
	Eurytomidae	0	2
	Formicidae	13	11
	Ichneumonidae	8	6
	Pteromalidae	1	3
	Tenthredinidae	2	1
Lepidoptera	Coleophoridae	1	0
	Elachistidae	5	1
	Erebidae	3	2
	Gelechiidae	4	1
	Geometridae	4	2
	Hesperiidae	1	1
	Lasiocampidae	1	2
	Lycaenidae	1	1
	Noctuidae	4	4
	Nymphalidae	1	2
	Tortricidae	3	0
Mantodea	Mantidae	1	0
Neuroptera	Chrysopidae	1	1
Opiliones	Leiobunidae	1	0
Orthoptera	Acrididae	2	3
	Gryllidae	1	1
	Tettigoniidae	4	3
Plecoptera	Perlodidae	1	0
Poduromorpha	Hypogastruridae	1	0
Psocoptera	Ectopsocidae	1	1
	Elipsocidae	1	1
	Liposcelidae	1	0
	Psocidae	1	0
Sarcoptiformes	Terpnacaridae	1	0
Symphypleona	Bourletiellidae	3	0
	Sminthuridae	1	0
Thysanoptera	Melanthripidae	1	0
Trombidiformes	Anystidae	1	0
	Erythraeidae	3	0

	Pygmephoridae	1	0

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GENERAL CONCLUSIONS

1.- Defoliation by Lepidoptera caterpillars reduced acorn production in the Holm oak *Quercus ilex*. The experimental exclusion of caterpillars (insecticide spraying) decreased defoliation rates, moreover, experimental shoots also grew longer than control ones. Caterpillars did not reduce acorn production by predating flowers, rather, they did so by increasing shoot abortion likelihood. Control shoots suffered more herbivory, were shorter and had less leaf tissue, what decreased their vigour. Thus, they were more likely to be abscised during the summer compared to experimental shoots, as shoot survival likelihood was positively related with the number of leaves. This Thesis is the first study using an experimental approach to demonstrate the negative effects of caterpillars on acorn production in Iberian oak savannahs. An early monitoring of caterpillars could aid dehesa managers to predict in advance the consequences on acorn production and act accordingly.

2) *Curculio elephas* (Coleoptera: *Curculionidae*) was the main pre-dispersal predator of Holm oak acorns in the study dehesas, followed by *Cydia fagiglandana* (Lepidoptera: *Tortricidae*). Acorns attacked by insects were prematurely abscised and smaller than sound ones. In turn, acorns infested by *Cydia* were smaller than those attacked by *Curculio*, although they were dropped in the same period. This size difference could be the consequence of differences in acorn selection between insects, but further studies should test this hypothesis explicitly. *Curculio elephas* larvae are usually larger than *Cydia* caterpillars. In addition, more than one larva may share the same acorn and compete for food, something that does not happen in *Cydia*. Hence, ovipositing *Curculio* females could be expected to be more selective regarding acorn size choice.

3) Despite their smaller size, the weight of *Cydia* infested acorns once the larvae left was not lower, because *Curculio* larvae consumed more cotyledon mass. Embryo predation risk did not differ either, what confirms that *Cydia fagiglandana* caterpillars need less cotyledon mass to be satiated at the seed level.

4) Late summer rainfall had contrasting effects in *Curculio* and *Cydia*. Adult weevils depend on rainfall to soften the soil and emerge from their underground earthen cells. Accordingly, *Curculio* weevil infestation rates were lower when late summer rains were delayed. By contrast, *Cydia* pupae are not buried underground but in the liter, hence, adults are not constrained by soil hardness. Acorn infestation rates by *Cydia* were lower when summer storms were early and precipitation abundant. Rain has negative effects on flying insects like *Cydia*. Moreover, these moths oviposit out of the acorn, so that the survival of eggs and early developing larvae (before entering the acorn) may be threatened by heavy rainfall.

5) Regarding oak fitness, there was not a “zero-sum” effect. In dry years, the decrease of acorn infestation rates by *Curculio* was not compensated by *Cydia*; total infestation rates were lower when late summer storms started later. Thus, under the scenario of longer summer droughts predicted by

climatic models for the Mediterranean Basin, the proportion of sound acorns might be higher. This might benefit oak recruitment and the productivity of silvopastoral systems like the Iberian dehesas. Yet, we must be cautious interpreting the net effects, as there are also negative factors linked to drought (e. g. increased acorn abortion or seedling mortality). However, our results show that environmental changes do not necessarily have to be detrimental for oak fitness. This perspective should be considered in further research on this topic.

6) *Curculio elephas* larvae spend almost three weeks feeding on the cotyledons after the acorns are dropped. Just after abscission larvae are at very early stages of development (hardly visible at naked eye) and cotyledons almost intact. After 10 days acorn mass has already decreased and larval size increased significantly. 20 days later, many larvae finish their development, by then, infested acorn mass has decreased dramatically.

7) The period spent by *Curculio* larvae within the acorns on the ground is very risky for their survival; livestock kills many of them. An experimental "cafeteria test" showed that cows, pigs and sheep ate sound and infested acorns in the same proportion. Accordingly, infestation rates were lower in those Iberian oak savannahs in which livestock density was higher. Our results show that intraguild predation by livestock could be used as an innovative pest control method. Dehesa managers should promote an early predation of infested acorns by livestock, preferentially within 10 days after falling. This should be mainly done in September-October, which is the period in which infested acorn drop is concentrated. By doing this, most larvae will be predated before completing their development and, in addition, the nutritional value of infested acorns will still be high (cotyledons not yet depleted by weevils).

8) A landscape scale experimental study corroborated that intraguild predation of *Curculio* larvae by livestock can be implemented to control acorn pests in Holm oak dehesas. The results showed that livestock are the main predators of acorn weevils. Within livestock exclosures, infested acorn removal decreased sharply, and the number of weevil/infestation rates increased in further years. The contrary happened in those oaks in which all infested acorns were manually removed after being dropped. The local elimination of weevil larvae resulted in lower infestation rates in the following years. Under natural conditions (control trees) acorn infestation rates were lower in those trees beneath which infested acorn removal by livestock had been higher in the previous year. Our results show that infestation rates can be reduced even at the spatial scale of single trees in oak savannahs, what can be explained by *Curculio elephas* poor dispersal abilities.

9) Along with the negative effects on pest insects, an increased and focalized grazing intensity may provoke undesired side effects on arthropod biodiversity. The results of a massive sampling and species delimitation using DNA metabarcoding (aided by Next Generation Sequencing, NGS) confirmed it. Next Generation Sequencing (based on two mitochondrial

markers: 16S and COI) allowed overcoming the taxonomic impediment and delimiting hundreds of arthropod Molecular Operational Taxonomic Units –MOTUs- (i. e. putative species).

10) Vegetation structure started to change and grass cover increased within a few months after livestock exclusion. The number of arthropod species and families collected from the soil and grass of different dehesas was lower when livestock was present (control trees). However, arthropod diversity recovered quickly, as it peaked in trees within livestock exclosures for just one year. Within the logic of the Intermediate Disturbance Hypothesis, the higher microhabitat heterogeneity in short term exclosures would allow the co-existence of species with different habitat or trophic needs. After 10 years or more without livestock (long-term exclosures) arthropod species and family richness decreased again, and were not significantly different from controls. However, their species composition differed significantly with respect to control trees or those within short-term exclosures. The selection for specialist taxa after years of continuous increase of grass cover may explain these differences.

11) The results of this Thesis follow the principles of ecological intensification for agroecosystem management. Concretely, those that promote a sound knowledge of ecological interactions to benefit from the ecosystems services that some organisms may provide. In first place, the effects of the main oak pests (defoliators, acorn borers) were carefully quantified. Moreover, their interactions with oaks and other organisms (i. e. livestock) were studied and delimited. On this basis, a novel way of biological control of the main Holm oak acorn pest (*Curculio elephas*) based is proposed.

12) Infestation rates by *Curculio elephas* can be reduced in Iberian oak dehesas by adjusting livestock grazing intensity and calendar. Given the low dispersal abilities of *Curculio elephas*, increased grazing can be concentrated on certain plots of the farm in September-October. To avoid major negative effects on oak recruitment, this treatment should be maintained for a maximum of three years and then rotated to a different plot. Each dehesa farm should combine plots with increased grazing with short-term livestock exclosures. This innovative livestock management would make possible the combination of pest control and arthropod conservation in Iberian oak savannahs.

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