TESIS DOCTORAL

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From flower to fruit: Ecological and evolutionary implications of abiotic and biotic factors affecting *Juniperus thurifera* L. cone development.

De la flor al gálbulo: Implicaciones biológicas y evolutivas de los factores abióticos y bióticos que controlan la producción de gálbulos en *Juniperus thurifera* L.

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From flower to fruit: Ecological and evolutionary implications of abiotic and biotic factors affecting *Juniperus thurifera* L. cone development.

Presentada por Erik Rodríguez García para optar al grado de doctor con Mención Internacional por la Universidad de Valladolid

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Capítulo 1: Conos femeninos de sabina albar
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“Creo que la naturaleza une a las culturas del
mundo. Un árbol tiene raíces en el suelo y ramas que
rozan el cielo, y nos recuerda que para prosperar
tenemos que saber de dónde venimos.”

Wangari Muta Maathai

A la memoria de “Takuni”

CONTENTS

RESUMEN

El desarrollo de frutos y semillas es un factor crítico en la demografía de las plantas. La cantidad de frutos y semillas que se desarrollan correctamente depende de múltiples factores: disponibilidad y uso de los recursos para la reproducción, éxito en la polinización y fertilización y depredación predispersiva. Estos factores actúan a escala individual o poblacional conjuntamente con otros factores como las condiciones climáticas, la densidad de individuos o la composición específica de los bosques.

La sabina albar (*Juniperus thurifera* L.) muestra una alta variabilidad interanual en la producción de gálbulos (denominados indistintamente frutos, aunque desde un punto de vista botánico sean pseudofrutos). Por otra parte, las semillas muestran una viabilidad baja lo que puede estar relacionado tanto con la disponibilidad de recursos, como con la eficiencia de la polinización. Comprender los mecanismos que determinan el éxito reproductivo de la sabina albar y cómo se ven afectados por diferentes motores del cambio global es crítico para conocer el futuro de esta especie.

La presente tesis se estructura en cinco capítulos en los que se exploran los diferentes factores que limitan la producción de gálbulos y semillas viables en la sabina albar a diferentes escalas temporales y espaciales. En el capítulo 1 se expone el marco teórico de la tesis, se muestra su diseño, así como los principales resultados y conclusiones obtenidos en la misma.

En el capítulo 2 se monitoreó el proceso de maduración de los gálbulos de sabina albar y la incidencia y selección de gálbulos de sus depredadores predispersivos durante tres cohortes de frutos entre los años 2013 y 2016. La mayor pérdida de gálbulos ocurre durante los cinco primeros meses de su desarrollo. Esta pérdida ocurre simultáneamente con la entrada de los principales depredadores predispersivos (ácaros, polillas, cochinillas y avispas). La identificación de los criterios de selección de gálbulos por parte de los artrópodos fue más eficiente si se realizaba en frutos inmaduros, que en maduros tal y como suele realizarse.

En el capítulo 3 se evaluó el papel que juegan los niveles de recursos, el éxito en la polinización y la depredación predispersiva en el éxito reproductivo. Se realizó un experimento con un diseño factorial completo que incluía dos tratamientos aporte extra de polen y de pes-

ticidas que fueron aplicados en la cohorte de frutos de 2014. El experimento mostró cómo los niveles de recursos y la exclusión de los artrópodos depredadores fueron los principales factores que determinan el éxito reproductivo, afectando tanto a la cantidad de gálbulos que finalizan su desarrollo, como a la calidad de sus semillas. En este contexto de un sabinar denso, la polinización no resultó ser un factor limitante, y de hecho la adición de polen tuvo efectos negativos en la cantidad de gálbulos.

En el capítulo 4 se estudió a una escala geográfica amplia el impacto combinado de las condiciones climáticas y de la depredación predispersiva sobre la producción de gálbulos y la calidad de las semillas. Se analizaron muestras recogidas en los años 2007 y 2008 procedentes de catorce sabinares ibéricos. La producción de gálbulos y su calidad estuvo favorecida por condiciones de humedad superiores a la media durante el primer año de formación del gálbulo. A nivel de árbol una producción elevada tuvo un efecto de saciado en ácaros, pero resultó en una atracción para polillas y avispas.

En el capítulo 5 se evaluó de qué modo la configuración específica de los sabinares puede afectar a la producción de gálbulos y niveles de depredación predispersiva. Se realizó un diseño pareado con sabinares puros y mixtos en nueve localidades del centro de la península Ibérica. Los sabinares puros tuvieron una mayor producción de gálbulos, que pudo estar ligado tanto a mayores niveles de recursos, un mayor éxito polinizador (como indica la menor tasa de aborción) y a la reducción global de la predación predispersiva en sabinares mixtos.

Los resultados de la tesis analizan los principales factores bióticos y abióticos el éxito reproductivo de la sabina albar. Los niveles de recursos medidos como el esfuerzo inicial en floración tienen un gran efecto en la producción final de frutos y en la calidad de las semillas. El papel de la polinización es complejo, y sugiere un efecto contexto dependiente ya que, si bien el diseño experimental en una masa pura y densa, mostró una ausencia de efecto en la adición de polen, es posible que en masas mixtas el polen sea limitante. Por último, los artrópodos tienen un efecto clave en el desarrollo de los frutos con un gran impacto oculto debido a la aborción selectiva de frutos. Por otra, esfuerzo reproductivo y predación predispersiva interaccionan ya que una mayor cantidad de frutos satura a algunos artrópodos, pero actúa como atractivo de otros a nivel de árbol. La inclusión de escalas espacio-temporales mayores indica que las respuestas varían en función de la región geográfica y de la variabilidad climática.

CAPÍTULO 1

De la flor al gálbulo: Implicaciones biológicas y evolutivas de los factores abióticos y bióticos que controlan la producción de gálbulos en *Juniperus thurifera* L.

Una visión general de la tesis

"Es la hora
de mover lo soñado
de recurrir a aquello
que parecía imposible"

Ernestina de Champourcín

¿QUÉ OCURRE ENTRE LA FLORACIÓN Y LA DISPERSIÓN?: EL MARCO GENERAL DE LA TESIS

Las plantas necesitan desarrollar correctamente sus frutos y semillas para una dispersión de los mismos y obtener así una reproducción satisfactoria (Herrera 1991, Pías et al. 2007). Sin embargo, desde que se desarrollan las estructuras destinadas a la reproducción (flores en angiospermas, conos en gimnospermas) hasta que un fruto o semilla es dispersado existe una serie de factores, como el éxito de la polinización y fertilización o la depredación predispersiva, que controlan la cantidad de frutos que obtienen el nivel de desarrollo óptimo para su dispersión (Hainsworth 1984; Price et al. 2008; Boieiro et al 2012). Si bien, discernir la importancia relativa de cada uno de estos factores sobre el éxito reproductor es difícil debido a que varios componentes pueden interactuar simultáneamente y afectarse mutuamente (Hainsworth 1984; Herrera 1991; Gruwez et al. 2013; Walsh et al. 2014), las plantas deben adaptar su ciclo vital a las diferentes condiciones y presiones para optimizar su éxito reproductor. Por otra parte, los resultados de estos procesos dependen en gran medida de su fenología (Ehrlén 2015). De esta forma, la inclusión de la historia natural en la investigación juega un papel decisivo a la hora de comprender procesos ecológicos como las relaciones planta – artrópodo (Östergård et al. 2007; Espelta et al. 2009, Xia et al. 2016) o como afectan las condiciones climáticas al éxito reproductor (Gruwez et al. 2013).

Para lograr una correcta reproducción, las plantas deben invertir recursos en la formación de flores, frutos y semillas (Obeso; 2002, Hirayama et al. 2008; Teitel et al. 2016) de manera que a mayor inversión mayor éxito reproductivo. Sin embargo, la disponibilidad de recursos no es ilimitada, y su uso en reproducción promueve una competencia de recursos con otras funciones vegetativas, como el crecimiento (Obeso; 2002, Hirayama et al. 2008; Teitel et al. 2016), o incluso con los recursos disponibles para la reproducción futura (Karlsson et al 1990; Lyles et al. 2015; Pessendorfer et al. 2016).

El éxito en la polinización depende además de la inversión en la formación de flores (Kudo and Harder 2005), de la correcta sincronía entre las estructuras femeninas y masculinas (Albert et al. 2001; Obeso 2002; Koenig et al. 2015; Lyles et al. 2015) o la cantidad y calidad del polen (Knight 2003; Labouche et al. 2016). En el proceso de polinización también

Fig 1. Hembra de *Curculio elephas* sobre una bellota de *Quercus ilex* realizando el agujero con su rostro donde después depositará un huevo.

participan factores ambientales, por ejemplo: abundantes precipitaciones reducen el éxito de la polinización a través del viento o unas temperaturas cálidas que favorezcan la actividad de los insectos polinizadores (Knops et al. 2007; Rech et al. 2016).

Los frutos y semillas acumulan nutrientes durante su desarrollo. Este alto valor nutricional resulta atractivo para diversos depredadores especialistas, fundamentalmente artrópodos, que reducen la cantidad de frutos y semillas que llegan a la madurez (Fig 1.). Además, los artrópodos que se desarrollan en el interior de frutos y semillas también encuentran un refugio idóneo frente a sus enemigos e inclemencias climáticas (Sallabanks and Courtney 1992). Los artrópodos eligen los frutos por unas determinadas características, lo que genera una presión evolutiva sobre las plantas y su capacidad reproductora (Janzen 1971; Kolb et al. 2007). Como respuesta a esta presión, las plantas han desarrollado diferentes estrategias defensivas. Por un lado, presentan estrategias que actúan a corto plazo, como las defensas químicas y físicas (Janzen 1969; Schoonhoven et al. 2005; Boivin and Auger-Rozenberg 2016), la aborción selectiva (Bonal et al. 2010; Meyer et al. 2014; Boivin and Auger-Rozenberg 2016) o el retraso en la fertilización de óvulos hasta después la puesta de huevos para evitar la pérdida de embriones (Rouault et al. 2004; Aderkas et al. 2005). Por otro lado, presentan mecanismos que actúan a largo plazo entre los que se incluye la modificación de las características de los frutos para hacerlos menos atractivos a los depredadores (Janzen 1969; Espelta et al. 2009; Beckman y Muller-Landau 2011) o la alta variabilidad interanual en la producción de frutos y semillas, que obliga a los depredadores a ajustar sus ciclos demográficos a los ciclos de producción de frutos, aumentando las plantas su éxito reproductivo durante los años de alta producción (Turgeon 1994).

El éxito reproductivo es modulado, además, por factores que actúan a mayores escalas espaciales, como las condiciones climáticas (García et al. 2000; Obeso 2002), la fragmentación del hábitat (Sork et al. 2002; Knight et al. 2005), la densidad de individuos y su estructura (Sork et al. 2002; Knight 2003; Sanz and Pulido 2015) o la presencia de otras especies (Mugnaini et al. 2007; Aderkas et al. 2012). De hecho, a una escala geográfica amplia es el clima quien determina la variabilidad en la producción de frutos que existe entre diferentes poblaciones (García et al. 2000; Obeso 2002; Montesinos et al. 2010), mientras que, a una escala local, variaciones temporales del clima determinan la inversión reproductiva (Lee y Bazzaz 1982; Herrera 1991; Crone y Lesica 2006). Para especies con una fuerte variación interanual en su producción de frutos hay una clara correlación entre las condiciones climáticas y una alta producción (Kelly and Sork 2002).

El éxito reproductor no es, por tanto, ajeno a la configuración espacial de la vegetación, de modo que los cambios en las características del territorio pueden afectarla. La conversión de bosques en pastos y cultivos es uno de los principales motores del cambio global, especialmente en países en vías de desarrollo (Gibbs et al. 2010, Phelps et al. 2013; Fig. 2). Sin embargo, el patrón opuesto se puede observar en países desarrollados, donde el éxodo rural y la intensificación de las prácticas agrarias ha llevado a un abandono de los usos tradicionales y las zonas menos productivas (Rey Benayas et al. 2007, Valladares et al. 2014). Como resultado, se está produciendo la expansión de los bosques hacia antiguos campos de cultivo (Gimeno et al. 2012), la densificación de las masas forestales (Rey Benayas et al. 2007, Améztegui et al. 2010) o cambiando su composición específica (Hansen et al. 2001, Chauchard et al. 2007, Vayreda et al. 2016). Por un lado, estos cambios en la estructura de los bosques llevan a una mayor competencia por los recursos, a nivel intraespecífico por la densificación (Kenkel 1988, Getzin et al. 2006, Wang et al. 2016) e interespecífico por la entrada de nuevas especies (Costa et al. 1997, Montesinos and Fabado 2015). Por el otro lado, la regeneración del bosque y colonización de nuevas áreas reduce la fragmentación y aumenta la eficiencia en la polinización, tanto por el viento como por animales, así como

Fig. 2 Bosque de araar (*Tetraclinis articulata*) adehesado en Marruecos.

de la dispersión (Santos and Tellería 1994, González-Varo et al. 2009). Por el contrario, una densificación de los bosques puede resultar también en una mayor depredación predispersiva de frutos y semillas (Sholes 2008; Guyot et al. 2016) y en el caso de bosques mixtos, la presencia de otras especies puede reducir la eficiencia en la polinización, especialmente por el viento, debido a un efecto barrera o por interferencia polínica entre especies que solapen sus periodos reproductivos (Mugnaini et al. 2007, Aderkas et al. 2012, Millerón et al. 2012).

¿ESTOICISMO O RESIGNACIÓN? EL CASO DE LA SABINA ALBAR

La sabina albar (*Juniperus thurifera* L.; Fig. 3) es un endemismo mediterráneo occidental con sus principales poblaciones en la Península Ibérica y Marruecos, presentando relictos en Francia, Italia y Argelia (Costa et al. 1997). Se trata de una especie que aparece en zonas de clima mediterráneo de marcada continentalidad y sequía estival, generalmente en suelos pobres predominantemente calcáreos, aunque también en suelos silíceos (p.e. Sierra de Guadarrama en Segovia o Barrios de Luna en León) (Costa et al. 1997). Además, los sabinares albares están incluidos en el anexo IV de la directiva hábitats (código 9560) y si bien la especie está catalogada como preocupación menor (LR/lc) según la Unión

Internacional para la Conservación de la Naturaleza (UICN) sus poblaciones se encuentran severamente fragmentadas y en continuo decrecimiento (Farjon 2013). La estructura actual de los sabinares albares es consecuencia de un manejo tradicional ligado a la ganadería ovina y caprina (Olano et al. 2008; Fig. 3 A y B), uso que aún persiste en el Atlas marroquí si bien en España no ha desaparecido por completo todavía. Durante la segunda mitad del siglo XX los usos tradicionales de los sabinares han ido cesando su actividad lo que está generando cambios en la estructura de estos bosques (Fig. 3C). Así, están entrando nuevas especies arbóreas como encinas, robles o pinos y se está produciendo una densificación de los sabinares (Olano et al. 2012).

La dispersión de la sabina albar se basa en el consumo de sus pseudofrutos carnosos, gálbulos o conos por un amplio grupo de vertebrados, principalmente aves del género *Turdus* (Tellería et al. 2011) y carnívoros como el zorro (*Vulpes vulpes* (Linnaeus 1758)) o la garduña (*Martes foina* (Erxleben 1777)) que son muy eficientes para la dispersión en espacios abiertos (Escribano-Ávila et al. 2012). Sin embargo, la cantidad de gálbulos viables listos para la dispersión está afectada por múltiples factores y puede ser muy baja (Montesinos 2010). Parte de esta limitación reside en la fuerte variabilidad interanual, ligado a la estrategia de reducción de las elevadas tasas de depredación predispersiva por artrópodos (Mezquida y Olano 2013). Mientras que, por otra parte, la sabina albar presenta una reducida viabilidad de las semillas (Montesinos et al. 2010) lo que puede estar relacionado tanto con la eficiencia de la polinización y la disponibilidad de recursos. En general, estos efectos podrían agudizarse en un futuro cercano, como consecuencia de los cambios de uso y del cambio climático. En otras especies de *Juniperus* se ha predicho un aumento en la tasa de pérdida de gálbulos por factores bióticos y abióticos, reduciendo el número de semillas viables como se ha indicado para otras especies del género (Gruwez et al. 2013), además de una posible disminución de las tasas de dispersión de frutos y semillas. Aunque la sabina albar muestra una gran capacidad de adaptación a variaciones en las condiciones climáticas, haciéndola muy resiliente frente a un futuro escenario de clima más cálido y seco (Camarero et al. 2010), las tasas de depredación predispersiva por artrópodos y aborto de semillas son especialmente sensibles a las condiciones ambientales (Montesinos et al. 2010), pudiendo ser un cuello de botella para el futuro de la especie.

Los insectos que se alimentan de frutos y semillas normalmente muestran preferencias por determinados caracteres (Sallabanks y Courtney 1992). En el caso de la sabina albar, existe un diverso grupo de artrópodos que se alimentan de los gálbulos, reduciendo la cantidad

Fig. 3. A: Sabinas albares en Siguero (Segovia). B: Sabinar albar en Calatañazor (Soria). C: Sabinar albar mixto en Megina (Guadalajara)

de ellos que llegan a un estado óptimo para su dispersión (Roques 1984; ver pág. 69-70). En el caso de la península Ibérica este grupo de artrópodos está formado principalmente por: los ácaros *Trisetacus quadrisetus* (Thomas 1889) (Acari, Phytoptidae), las polillas *Pammene juniperana* (Millière 1858) (Lepidoptera, Tortricidae) y *Mesophleps oxycedrella* (Milière 1871) (Lepidoptera, Gelechiidae) y la avispa *Megastigmus thuriferana* Roques & El Alaoui 2006 (Hymenoptera, Torymidae). Estas especies atacan secuencialmente los gálbulos durante su desarrollo mostrando preferencias por determinadas características de los gálbulos. Esta depredación de gálbulos secuencial lleva a una aborción selectiva de los gálbulos infestados en las fases tempranas de su desarrollo. Además, cuando los gálbulos son atacados en fases de desarrollo similares a la madura, los artrópodos depredadores podrían seleccionar gálbulos con características similares a las preferidas por los dispersores, generando presiones selectivas conflictivas (Siepielski y Benkman 2007).

OBJETIVOS

El objetivo principal de esta tesis es comprender los diferentes factores que limitan la producción de gálbulos y semillas viables en la sabina albar, así como comprender el impacto y selección de frutos por parte de los depredadores predispersivos, determinando su respuesta a diferentes escalas temporales y ambientales. Los trabajos de investigación llevados a cabo para esta tesis se organizan en cuatro capítulos cuyos objetivos específicos son:

Capítulo 2. Realizar un seguimiento del proceso de maduración de los gálbulos de sabina albar, así como del proceso de entrada y biología de los depredadores predispersivos. De esta forma, podremos observar cómo se ajustan los ciclos vitales de los depredadores al desarrollo de los gálbulos y cuáles son los criterios de selección de frutos para cada predador. El seguimiento de los gálbulos y de su tasa de depredación en diferentes momentos permitirá detectar procesos de aborción selectiva de los gálbulos infestados, que pudiera implicar una subestimación de los niveles de depredación predispersiva ejercidos por cada especie. Con ello se contribuirá a mejorar nuestra comprensión de las presiones selectivas que ejercen los depredadores cuando basamos nuestras observaciones e interpretaciones sólo en fases maduras de los frutos.

Capítulo 3. Discernir el papel que juegan los niveles de recursos, el éxito en la polinización y la depredación predispersiva y cómo interactúan entre ellos para determinar el éxito reproductivo final en la sabina albar. Con ello se pretende comprender mejor cómo interactúan entre sí los diferentes filtros que determinan el número final de gálbulos que llegan a madurar. Este trabajo modificó experimentalmente los niveles de polen y de depredadores predispersivos.

Capítulo 4. Evaluar a una escala geográfica amplia el impacto combinado de las condiciones climáticas y de la depredación predispersiva sobre la producción de gálbulos y la calidad de las semillas. Con ello se trata de comprender cómo se comportan la sabina y sus depredadores bajo un gradiente de condiciones climáticas y cómo estas condiciones interactúan con los sucesos locales.

Capítulo 5. Evaluar cómo la configuración de los sabinares puede afectar a la producción de gálbulos y niveles de depredación predispersiva. Se realizó un diseño muestral comprendiendo bosques puros y mixtos de sabina en diferentes regiones geográficas. En este trabajo se trata de observar de qué modo la tendencia de los sabinares a convertirse en bosques mixtos pueden afectar el éxito reproductor de la especie.

METODOLOGÍA

Esta tesis aborda la cuestión con un enfoque observacional, combinado con un diseño experimental en el capítulo 3. Se recolectaron gálbulos en diferentes fases de desarrollo de diversos sabinares de la península Ibérica en los que se ha realizado trabajo de campo entre los años 2012 y 2016 durante la duración de la tesis. Además, en el capítulo 4 se dispuso de muestras recolectadas en años anteriores por el equipo de trabajo.

En el capítulo 2 se monitorizó el desarrollo de los frutos de sabina albar. Se seleccionaron cien ramas en veinte árboles, a razón de cinco ramas por árbol, en el sabinar de Villaciervos (Soria) y se muestreó regularmente entre febrero 2013 y octubre de 2016 durante el desarrollo de tres cohortes de frutos. En cada muestreo se contaron los frutos presentes en las ramas seleccionadas. Además, se recolectaron regularmente gálbulos de otras ramas para su disección en laboratorio con el fin de observar el desarrollo de las larvas de los depredadores, estimar la depredación predispersiva y evaluar la selección de frutos por los artrópodos en diferentes fases fenológicas. Estos datos se compararon con los resultantes de los gálbulos maduros que se recogían en octubre de su segundo año de desarrollo.

En el capítulo 3 se trató de discernir experimentalmente el papel que juegan los niveles de recursos, el éxito en la polinización y la depredación predispersiva en el desarrollo de gálbulos. Para ello se realizó un experimento con un diseño factorial completo que incluía diferentes tratamientos: A) control, B) aporte extra de polen, C) aporte de pesticidas y D) aporte extra de polen + pesticidas. Se realizó en el mismo sabinar que el estudio anterior, en Villaciervos (Soria). Para ello se seleccionaron al azar cuarenta árboles. En cada árbol se seleccionaron 8 ramas y se aplicó cada uno de los tratamientos a dos ramas por árbol. El experimento se realizó para la cohorte de gálbulos del año 2014. Se realizó además un seguimiento mensual de la pérdida de frutos. En octubre de 2015 se recolectaron todos los gálbulos maduros de las ramas tratadas para estimar la depredación por artrópodos y analizar la calidad de las semillas. Con el fin de observar el efecto del aporte extra de polen sobre la fertilización en junio de 2015 se recolectaron 100 gálbulos inmaduros (50 control y 50 con aporte extra de polen). Estas muestras fueron procesadas para la observación, en microscopio óptico, de la formación de tubos polínicos (ver metodología en Capítulo 3). La influencia del esfuerzo reproductivo inicial, el aporte extra de polen y la aplicación de pesticidas sobre el desarrollo de gálbulos, la calidad de las semillas y la depredación predispersiva se testó mediante modelos mixtos.

En el capítulo 4 se evalúo cómo influyen las condiciones ambientales en el desarrollo de gálbulos y su efecto combinado sobre la calidad de las semillas y la depredación predispersiva a una escala geográfica amplia. Para ello se analizaron muestras provenientes de catorce sabinares a lo largo de su distribución en la península Ibérica recolectadas durante los años 2007 y 2008. En cada sabinar fueron seleccionados al azar treinta sabinas en las que se estimó la producción de gálbulos y se recolectaron treinta gálbulos para la estima de la depredación predispersiva y diez para el análisis de sus características y de la calidad de sus semillas. La influencia combinada de las condiciones ambientales y la producción de gálbulos sobre la calidad de las semillas y la depredación predispersiva se evaluó mediante modelos mixtos.

En el capítulo 5 se evaluó cómo pueden afectar los cambios en la estructura y composición específica de los sabinares a la reproducción final. Se seleccionaron bosques puros con una clara dominancia de la sabina albar (> 90 % de representación en pies) y bosques con baja representación (< 50 %) obviando los estados intermedios (51 – 89 %). Con este esquema, durante otoño de 2014 se realizó un diseño pareado con sabinares puros y mixtos en diferentes localidades del centro de la Península Ibérica: Megina, Pinilla de Molina y Valhermoso (Guadalajara); Arcones, Prádena y Siguero (Segovia); Bayubas de Abajo, Calatañazor y Cabrejas del Pinar (Soria). En cada sabinar fueron seleccionadas al azar treinta sabinas en las que se estimó la producción de gálbulos y se recolectaron treinta gálbulos para la estima de la depredación predispersiva y diez para el análisis de sus características y calidad de sus semillas. El efecto de la composición del bosque sobre la producción de gálbulos y la calidad de sus semillas se evaluó mediante modelos mixtos.

Los procedimientos de análisis de los gálbulos recolectados fueron semejantes para todos los capítulos. Se tomaron medidas del tamaño con un calibre digital y se identificó la actividad de los artrópodos depredadores mediante las señales que estos dejan en los gálbulos maduros o bien por su presencia en el interior del fruto (ver una clave detallada en el capítulo 2). Además, en una porción de los gálbulos fueron extraídas las semillas y tras su secado en estufa a 60 ºC durante 48 horas se pesaron pulpa y semillas por separado. Para las semillas se observó su estado de desarrollo y su calidad. En función de dichos parámetros y siguiendo la clasificación propuesta por Gruwez et al. (2013) fueron asignadas a una de las siguientes tres categorías: Llena (Fig. 4A), si la semilla muestra un desarrollo completo con un embrión y megagametofito bien desarrollados; vacía (Fig. 4B y C) si la semilla no muestra un desarrollo completo del embión o megagametofito o estas estructuras están ausentes pero la semilla se muestra externamente como una semilla viable y abortada (Fig 4D) si la semilla fue abortada antes de la fertilización, siendo reconocible como una semilla deformada, no completamente desarrollada. Los datos obtenidos del análisis de las muestras se han analizado empleando modelos mixtos. Se denominan así porque combinan efectos fijos y aleatorios (Zuur et al. 2009). Este tipo de modelos estadísticos siguen la misma lógica que otros tipos de modelos por la que se trata de describir la relación entre una variable respuesta y una o varias variables (efectos) explicativas fijas. Estos modelos se emplean cuando dada la naturaleza de la toma de datos en los experimentos, las observaciones pueden no ser independientes entre sí. Así, por ejemplo, cuando se disponen de datos provenientes de “n” poblaciones, las observaciones realizadas en una determinada población se parecerán más entre sí que con las de otras poblaciones. El efecto que tienen las diferentes poblaciones sobre la variable respuesta puede no ser de interés para el

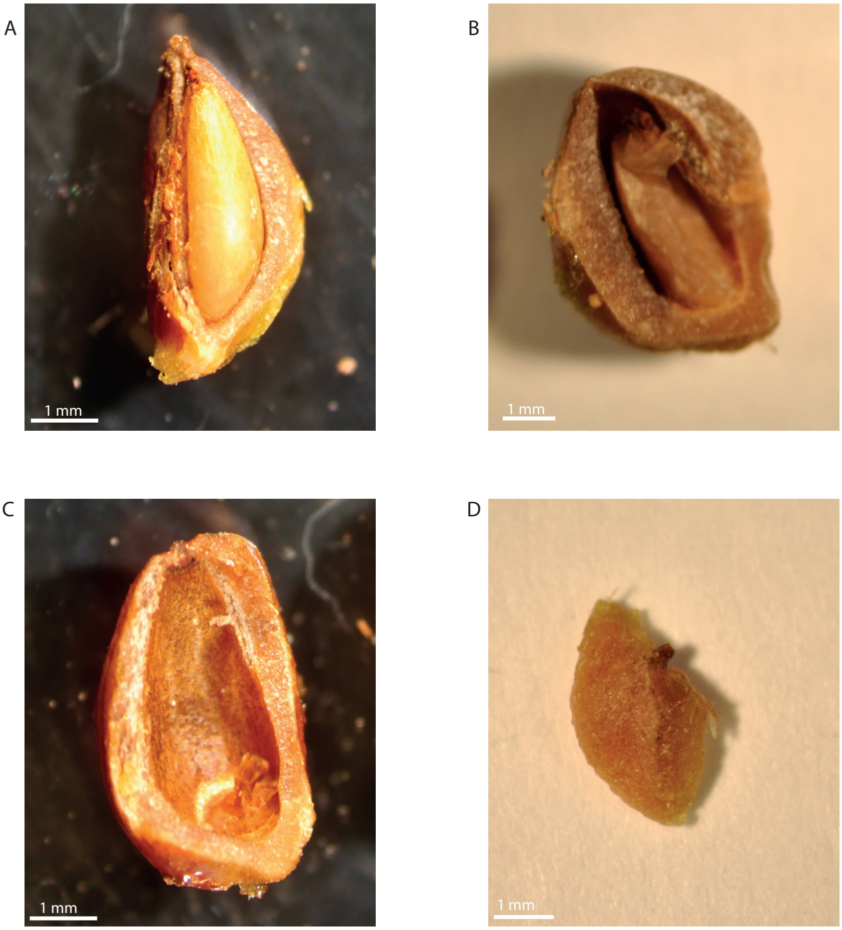

Fig 4. Diferentes estados identificados en las semillas de sabina albar. A: Semilla llena, mostrando un embrión y megagametofito bien desarrollados. B: Semilla vacía con embrión y megagametofito sin desarrollar por completo. C: Semilla vacía sin embrión ni megagametofito desarrollados. D: Semilla abortada sin desarrollo post-fertilización..

experimentador, sino que interesa conocer en qué medida la estimación de los valores de la variable respuesta pueden variar en función de la población (niveles del factor) que se seleccione de un modo aleatorio. De esta forma el factor "población" representaría el efecto aleatorio en el modelo mixto. Estos modelos pueden ser empleados tanto para variables que siguen una distribución normal (modelos mixtos lineales; capítulo 4 y 5) como para variables que siguen otro tipo de distribuciones, como la binomial o la Poisson (modelos mixtos lineales generalizados; capítulos 2 a 5).

En cada capítulo de la tesis, los efectos fijos se eligieron en base a los conocimientos biológicos previos. En el capítulo 2 con el fin de simplificar la comparación de modelos obtenidos en las diferentes fases fenológicas sólo se consideraron modelos saturados. En el capítulo 3 se realiza una selección de modelos eliminando secuencialmente las variables menos explicativas hasta obtener un modelo óptimo sencillo. En el capítulo 4 con el fin de obtener información general de los posibles efectos fijos a una escala geográfica amplia sólo se consideraron modelos saturados. Finalmente, en el capítulo 5 se realizó primero una selección de la estructura de efectos aleatorios sobre los modelos saturados y, segundo, una selección de estructura de efectos fijos tal como se indica en Zuur et al. (2009). Esta selección de estructuras se realizó en base al criterio de información bayesiana (BIC) recomendable cuando el objetivo es evaluar hipótesis (Aho et al. 2014).

PRINCIPALES RESULTADOS

En el capítulo 2 se describió el ciclo vital, asociado al desarrollo de los gálbulos, de los depredadores. Los diferentes artrópodos depredadores atacaron secuencialmente los conos y semillas de la sabina albar. Los ácaros colonizaron las flores a la espera, tras la polinización, que se inicie el desarrollo de semillas para su infestación. Salieron del interior de las semillas durante el invierno en busca de nuevas flores que colonizar. Se identificaron las dos especies de polillas, *M. oxycedrella* y *P. juniperana*. Estas realizaron la puesta de huevos sobre los gálbulos, de primer y segundo año, desde finales de mayo a mediados de junio, entrando sus larvas en los gálbulos desde mediados de junio a finales de julio. Las larvas se desarrollaron en el interior del gálbulo y salieron de su interior a partir de finales de octubre. Las cochinillas colonizan nuevos gálbulos en su fase de ninfa durante el mes de junio. Una vez seleccionado el lugar idóneo sufren la metamorfosis a la fase sedentaria y adulta completando su ciclo vital. Finalmente, las avispas depositaron huevos en el interior de las semillas desde finales de julio hasta mediados de septiembre. Las larvas se desarrollaron en el interior de las semillas a lo largo de doce meses, saliendo de su interior desde finales de julio hasta finales de agosto.

La identificación de los criterios de selección de gálbulos por parte de los adultos fue diferente si se basaba en gálbulos inmaduros o maduros, siendo en general más robusto cuando se escogían gálbulos inmaduros recogidos poco después de la entrada del depredador. Así durante el primer año de formación del cono: la polilla *M. oxycedrella* selecciona conos de mayor tamaño y con un mayor número de semillas; mientras la polilla *P. juniperana* selecciona los mismos árboles que *M. oxycedrella* pero selecciona gálbulos previamente ocupados por ácaros de los que previsiblemente se alimenta; la avispa *M. thuriferana* selecciona gálbulos de mayor tamaño, característica que se relaciona con un mayor contenido de semillas viables; las cochinillas no seleccionan gálbulos en base a su tamaño y/o contenido en semillas. Durante el segundo año de formación del cono: la polilla *M. oxycedrella* seleccionó los gálbulos de mayor tamaño, pero con un menor número de semillas en su interior. Por el contrario, cuando la evaluación de la selección se basó en gálbulos maduros no fue detectado ningún efecto para entrada de las dos especies de polillas, mientras que la avispa seleccionó gálbulos con un mayor contenido en semillas.

Los resultados correspondientes al capítulo 3 muestran que existe una asociación entre un mayor nivel de recursos estimado como número de flores por rama y éxito reproductor. Estimado tanto en términos de número final de gálbulos, como de tasa de éxito por flor y de número de semillas llenas por gálbulo. La polinización no resultó ser un facor limitante, además, la adición manual de polen no mejoró el éxito reproductivo e incluso supuso un menor número de gálbulos que completaron su desarrollo. Sin embargo, las flores que fueron tratadas con extra de polen mostraron una menor tasa de aborción en sus semillas. La exclusión de los artrópodos depredadores mediante biocidas resultó en un aumento en el cuajado de gálbulos, así como una mejor calidad de las semillas.

Los resultados correspondientes al capítulo 4 muestran que las condiciones ambientales favorables se asocian con una mayor eficiencia en la polinización, incrementando la producción de gálbulos y la calidad de sus semillas. Así, condiciones de humedad superiores a la media durante el primer año de formación del gálbulo incrementó su producción total. A su vez, los árboles con una mayor producción de gálbulos presentaron más semillas llenas y menos vacías y abortadas. Árboles con una alta producción de gálbulos generaron un efecto de saciado en los depredadores predispersivos menos móviles como los ácaros mientras que polillas y avispas, con una capacidad de dispersión mayor, buscan árboles con una mayor producción. Además, las polillas y avispas seleccionaron árboles con gálbulos más grandes y con un mayor contenido en semillas a la vez que evitaron árboles con una mayor incidencia de otros depredadores.

Finalmente, los resultados del capítulo 5 muestran que el cambio de la estructura del bosque afecta al éxito reproductor de la especie. Esto puede deberse a diferentes componentes. La eficiencia en la polinización resultaría mayor en sabinares puros, lo que se tradujo en una mayor cantidad de gálbulos que completan su desarrollo. Si bien la cantidad de semillas viables por gálbulo resultó similar en ambos tipos de sabinar, los puros presentaron una menor cantidad de semillas abortadas y mayor de vacías lo que indica que en sabinares puros hay una mayor cantidad de semillas que inician su desarrollo. Por otra parte, los artrópodos que consumen los frutos y semillas mostraron una respuesta más compleja. Ácaros y polillas mostraron mayores consumos de gálbulos y semillas en sabinares puros, mientras que la depredación por avispas fue mayor en bosques mixtos.

Principales conclusiones

La inclusión de la historia natural y fenología de los procesos de formación del gálbulo en sabina albar y su interacción los artrópodos depredadores de gálbulos proporciona una mejor comprensión de las relaciones planta-artrópodo. En primer lugar, nos permite observar cómo se ajustan entre sí los ciclos biológicos de la sabina albar y de los artrópodos depredadores. A la vez, nos permite discernir en qué momento se producen las respuestas defensivas por parte de la sabina albar, principalmente en forma de aborto selectivo. Si bien tras la polinización la cantidad de gálbulos que comienzan su desarrollo es elevada (ver Capítulo 2) hay una gran pérdida de frutos entre mayo y septiembre del primer año de formación. Esta aborción parece asociada a la entrada de ácaros y polillas (ver capítulo 2 y 3).

Por otra parte, en frutos cuyo proceso de desarrollo es largo, como es el caso de los gálbulos de sabina albar, el aborto selectivo en etapas tempranas de formación puede implicar que se subestime la capacidad depredadora de los artrópodos (ver capítulo 2). Es más, como la aborción selectiva no es igual para todas las especies, esto puede provocar cambios en la importancia relativa de los diferentes agentes implicados (ver capítulo 3). En tercer lugar, en esta tesis hemos visto cómo los modelos de selección de fruto basados en fases fenológicas cercanas a la ovoposición proporcionan una mejor comprensión de las presiones selectivas en aquellos depredadores que infestan los gálbulos en etapas tempranas (ver capítulo 2).

En la pérdida de gálbulos de la sabina albar intervienen además los niveles de recursos y el éxito en la polinización. Se observó cómo la inversión inicial en recursos favorece el éxito reproductor, en números absolutos y relativos. Por el contrario, el polen resultó no ser un factor limitante en el experimento del capítulo 3, pero es posible que este efecto sea contexto dependiente, ya que en dicho caso se trataba de un sabinar monoespecífico denso, con individuos vigorosos, mientras que en bosques mixtos (ver capítulo 5), hay diferentes indicios que sugieren una limitación polínica.

En este trabajo diferentes indicios sugieren la existencia de una interacción entre diferentes factores. Así, una mayor inversión en reproducción puede llevar a una saturación de los ácaros (ver capítulo 3), resultando en un mayor éxito reproductivo. Sin embargo, los depredadores con mayor capacidad dispersiva (polillas y avispas) son atraídos por los árboles que muestran una mayor producción de gálbulos (ver capítulos 3 y 4). Por otra parte, los diferentes depredadores interactúan entre sí (ver capítulos 3 y 4), de forma que la exclusión de ácaros y polillas conllevó una mayor tasa de gálbulos atacados por avispas (ver capítulo 3).

Cuando se abre el foco y se analiza a escalas espaciotemporales mayores, se observa cómo las condiciones ambientales en el año de floración e inicio de los frutos modulan la formación de gálbulos (ver capítulo 4). Así, hay una relación positiva entre la cantidad de gálbulos y de semillas viables con condiciones de mayor humedad con respecto de la media en las etapas tempranas de formación del gálbulo. Además, la depredación predispersiva está determinada por las condiciones ambientales de cada zona y por la producción y características de los gálbulos (ver capítulo 4). Así, por ejemplo, los ácaros depredan menor porcentaje de los gálbulos cuando los años son más húmedos.

Finalmente, se observó como el cambio en la composición específica de los sabinares puede promover cambios en el éxito reproductor de la sabina albar. Si bien la cantidad de semillas viables por gálbulo resultó similar en sabinares puros y mixtos, el determinante de un mayor éxito reproductivo en sabinares puros es la mayor producción de gálbulos. El hecho de una menor producción en sabinares mixtos se relacionó con la competencia interespecífica y con limitaciones en la polinización. Las relaciones planta-artrópodo también se ven afectadas al cambiar el contexto espacial para los depredadores. En términos generales existe una menor tasa de depredación en bosques mixtos, sin embargo, la respuesta a los cambios resultó diferente para cada especie (ver capítulo 5).

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CHAPTER 2

Ripe fruits may not be optimal: green fruits improve the detection of traits associated to predispersal predation risk

"Weeks in the field can save you minutes in the library"

Thomas Eisner

ABSTRACT

Predispersal seed predation by arthropods has been described as one of the main selective pressures on plants. Arthropods show preferences for certain fruit and seed traits to optimize their fitness. In response, plants have shaped their reproductive parts to reduce losses from seed predators along with enhancing their reproductive output. Fruit and seed preferences by arthropods are usually explored on ripe fruits, but fruit choices usually occur in early phases of fruit formation, and the implications of differences in the timing of analyses are not fully understood. Our aim was to explore whether the analysis of fruit preferences by predispersal seed predators differ when assessed using immature versus ripe fruits. To perform this task, we monitored fruit production and predispersal predation during three cohorts (four years) in a Spanish juniper (*Juniperus thurifera*) woodland in central Spain. We explored fruit preferences by arthropods using linear mixed models at different phenological stages: fruits collected shortly after the oviposition period of each species and ripe fruits. To perform this task, we assessed the timing of fruit development and predispersal predation. We monitored the fate of 11,480 flowers and analysed predation in 3,740 immature and 2,342 ripe fruits. A complex guild of arthropods predated on juniper fruits. Loss of fruit was very high during the first five months of fruit development, matching the entry of the main predispersal predators. Predation rates for most of the species declined from immature to ripe fruit indicating the selective abortion of infested fruits, although this response was species-specific leading to rank reversals in relative predation impact. Fruits collected just after arthropod entry provided better models for arthropod preferences for fruit traits for most of the species. Evaluation of preferences by different arthropods attacking fruits in the Spanish juniper was improved through multiple fruit sampling matching the timing of arthropods' entry. This result can probably be extrapolated to other species which have seeds with long maturation periods. and claims for the need of developing a deeper knowledge on natural history to improve our understanding of the biological interactions behind ecological and evolutionary processes.

INTRODUCTION

Arthropods and plants are among the largest taxa of living organisms in number of species and in terrestrial ecosystem biomass, respectively. Species in both groups interact in different ways with each other, and close interactions has been traditionally considered as the promoters of the current diversity of terrestrial life forms (Ehrlich and Raven 1964). In fact, 90 % of flowering plants are estimated to be pollinated by animals, mainly by insects (Ollerton et al. 2011) and 26 % of the insect species feed on vascular plants (Schoonhoven et al. 2005). Interactions between plants and insects had critical economic implications especially in agricultural systems (Rechcigl and Rechcigl 2000; Schoonhoven et al. 2005, Badii et al. 2015) and are basic for the functioning of natural systems from an ecological and evolutionary point of view (Janzen 1971; Kolb et al. 2007; Suchan and Alvarez 2015).

Predispersal seed predators may limit plant reproductive success (Crawley 2000). Indeed, predispersal seed predation has been described as one of the main selective pressures in plants (Kolb et al. 2007). Plants have developed multiple evolutionary strategies to minimize the detrimental effects of seed predators, including the accumulation of secondary metabolites, changes in fruit and seeds traits or inter-annual variation in fruit crop (Janzen 1969, 1971; Crawley 2000; Kolb et al. 2007; Schoonhoven et al. 2005). Natural history is critical to decipher the ecological and evolutionary consequences of plant-seed predators' interactions. This process becomes more complex when multiple species share the same host plant. Predators must develop life history traits to maximize predation and minimize interspecific competition (Espelta et al 2009; Bonal et al. 2011), whereas plants must deal with contrasting selective pressures (Gagic et al. 2016). The different agents must adjust the phenology of their vital events, including those involving predation pressures, to optimize their fitness (Gillot 2005; Bonal et al. 2010, 2011; Ehrlén 2015). Nevertheless, the inclusion of natural history in ecological research to explain plant-insect relationships and its consequences is scarce (see Östergård et al. 2007; Espelta et al. 2009, Xia et al. 2016). In fact, predispersal seed predation is routinely evaluated on ripe fruits and seed predator preferences assessed based on the characteristics of ripe fruits (Sperens 1997; García et al. 2000; Mezquida and Olano 2013; Mezquida et al. 2016; Moreira et al. 2016), assuming that this relationship reflects the processes occurring at earlier vital stages.

Our aim was to explore whether the common practice of exploring predator-plant selection using ripe fruits provides similar information to evaluating this relationship at the phenological stage corresponding to the timing of fruit use by each seed predator. To perform this task, we selected the conifer Spanish juniper (*Juniperus thurifera*), which produces pseudo-fruits (hereafter fruits) that mature over a 20 months period when they are attacked by a wide array of specialized arthropods, including mites, moths, chalcid wasps and scale insects (Roques et al. 1984). The ecological and evolutionary implications of seed predation in this species and other junipers has received an intense research effort which has been based on the evaluation of ripe fruits (Mezquida and Olano 2013; Roques et al. 2013; El Alaoui et al. 2013; Mezquida et al. 2016). We hypothesized that plants having long seed maturiation periods, levels of seed predation might be underestimated due to selective fruit losses of infested fruits with different abortion rates determining changes in their relative infestations ranks. Moreover, we predicted that the set of traits associated to predator preferences may differ from the traits evaluated on ripe fruits, and that these differences would be more intense for seed predators showing higher losses of damaged fruits.

MATERIAL AND METHODS

Natural history

Spanish juniper (*Juniperus thurifera* L.) is a dioecious juniper species, endemic to the Western Mediterranean where it inhabits continental summer-dry environments. Wind pollination occurs in late winter-early spring. Pollen germination and megagametophyte formation starts after pollination, but fertilization is delayed until late spring. Embryo development begins after fertilization and seeds are almost fully grown by mid-summer, when they start to harden their seed coat. Megagametophyte and embryo final maturation takes 12-13 additional months (Gruwez et al. 2013). Juniper fleshy cones (hereafter fruits) ripe 20 months after pollination in next year autumn (Montesinos et al. 2012). Thus, individuals display two fruit cohorts from March to November.

A variety of arthropods can damage Spanish juniper fruits during this long development period (Roques et al. 1984; El Alaoui et al. 2013). However, in the Iberian Peninsula, three arthropod taxa are the main predispersal seed predators: mites, moths and chalcid wasps.

Mites, *Trisetacus quadrisetus* (Thomas 1889) (Acari, Phytoptidae), uses seeds as a growth chambers where they feed and reproduce (Roques et al. 1984). The larvae of two moth species: *Mesophleps oxycedrella* (Millière 1871) (Lepidoptera, Gelechiidae) (yellow juniper moth hereafter) and *Pammene juniperana* (Millère 1858) (Lepidoptera, Tortricidae) (brown juniper moth hereafter) feed on juniper fruit pulp and seeds (Roques et al. 1984). The larva of a chalcid wasp, *Megastigmus thuriferana* Roques & El Alaoui 2006 (Hymenoptera, Torymidae) feeds on well-developed megagametophyte and embryo (Rouault et al. 2004). Other frequent arthropods that feed on Spanish juniper fruits include the pulp sucker scale insect *Carulaspis juniperi* (Bouché 1851) (Hemiptera, Diaspididae) and the pulp eater juniper fly *Rhagoletis zerny* Hendel 1927 (Diptera, Tephritidae).

Study area

Sampling site was located in Villaciervos, Soria province, Central Spain (41º 44′ N, 2º 40′ W; 1150 m.a.s.l.). Climate is Mediterranean continental, mean annual precipitation is 533 mm. Mean monthly temperatures range from 2.8 ºC in January to 20 ºC in July. The rock parent material is Cretaceous limestone covered by deep soil with small areas of exposed bedrock. Vegetation is dominated by a *J. thurifera* woodland with open areas covered by xeric grasslands with shrubs, including *Cistus laurifolius* L. *J. communis* L. and *Thymus zygis* Loefl.

Sampling design and fruit analysis

Two monitoring schema were preformed simultaneously. First monitoring evaluated fruit success on 20 randomly selected female junipers. We considered fruit success as the number of initial flowers that complete their development to ripe fruits. Five flowering branches from all around the canopy were selected and tagged for each tree. Monitoring was initiated in March 2013 and maintained until October 2016. Thus, monitoring comprised three fruit cohorts (2013, 2014 and 2015). Initial reproductive effort for each branch was estimated by counting the number of female flowers for each cohort in March from 2013 to 2015. Fruit development during the first year was monitored by counting the fruits within the branches monthly from May to September for the three cohorts. Fruit development during the second year was monitored monthly from March to October for the 2013 and 2014 cohorts and in March, April, September and October for the 2015 cohort. In October, when fruits are ripe, all second-year fruits remaining in the branches were collected to characterize fruits and quantify pre-dispersal fruit damage.

Second monitoring was aimed to assess the timing of attack by different seed predator, quantify their impact and to characterize fruits and seeds during seed development. We sampled fruits at different stages of seed development for the 2013 and 2014 cohorts. We collected 20 fruits from all around the canopy from 10 randomly selected female juniper trees. In 2013, sampled trees were different from the trees where we followed flower and fruit development. In 2014, fruits were collected from a subset of ten trees from those twenty used to monitor fruit success, taking care of gathering fruits from branches away from those monitored. During the first and second year of each cohort, fruits were collected monthly from June to September. Sampled fruits were measured to the nearest 0.01 mm with a digital caliper and opened in the laboratory under a dissecting microscope. We counted the total number of seeds and the number of 'full' seeds (i.e., those showing a completely developed embryo and megagametophyte). Fruits were also inspected to detect arthropod activity. Arthropods attacking Spanish juniper fruits can be identified through their aspect and/or feeding behavior (see Appendix 1 for details). Mites' colonies deform the seeds causing an elongation of their tip that usually stick out of the fruit surface. Yellow juniper moth larva is white with a brown cephalic capsule and a black post-cephalic mark. Brown juniper moth larva is white with a dark cephalic capsule and four black post-cephalic marks. Both moth species showed a different feeding pattern on seed and fruit pulp. Scale insects are found on the fruit surface: nymphs are yellow with red eyes and sedentary adults have circular and ellipsoidal shapes, and are white-colored. Chalcid wasp larva is white and develops inside the seeds, making a circular hole in the apical region of the fruit when emerging as adults. The larvae of juniper flies are apod, flat and yellow, and they feed only on fruit pulp.

Statistical analysis

We defined four critical stages during fruit development: 'Flowers' that were counted in March; 'initial fruits' that could be identified in May; 'unripe' that occurred when fruits reached the final size in September of the first year of development and 'ripe fruits' when fruits turned to dark blue in October of the second year.

We used linear mixed models with a binomial error structure to analyze the effect of different factors on transition success between the different stages. We consider as success the number of fruits per branch that persisted from between two consecutive stages. Firstly, we analyzed the full transition from 'flowers' to 'ripe fruits'. Besides, we analyzed the three transitions between the critical stages (flower to initial fruit, initial to unripe fruit and unripe to ripe fruit). Fixed term for all models included initial flower number per branch. All transitions from initial fruits onwards included also number of fruits per branch in the previous stage. Random component for all models included branches nested within trees and cohort.

We used fruit success monitoring dataset to test whether the initial reproductive effort affected the final number of full seeds per fruit. We used linear mixed models with Poisson error structure. Fixed terms in the model included fruit size and the number of flowers and ripe fruits per branch. Random components for the model included branches nested within trees and cohort.

We used linear mixed models with a binomial error structure to analyze in predispersal predation monitoring dataset which fruit traits were preferred by different arthopods during their oviposition based on collected fruits from 10 randomly selected trees. Arthropods use fruits traits, such as fruit size and seed number, as cues to oviposit eggs, so we included fruit size and number of seeds per fruit as fixed terms in all models. Since different arthropods may interact at tree and fruit level, we considered mutual effects in the models (Mezquida and Olano 2013). Effects were included according to the timing of fruit attack. Mites were the first to attack fruits, so model for mites did not include the incidence of other arthropods. Moth species can attack fruits at two distinct phases of fruit development, on initial fruits between May and June (see results; first entry hereafter) and on unripe fruits between May and June of second year of fruit development (second entry hereafter). Models for both moths first entry included the incidence of mites as well as moth mutual interaction. Chalcid wasps' model included the incidence of mites, both moths and scale insects. Model for yellow juniper moth second entry included the incidence of arthropods previously attacking fruits (mites, both moth species, scale insects and chalcid wasps). The model for scale insects did not include other arthropods because they only occur in the fruit surface and are expected not to be affected by them. No model was run for brown juniper moth second entry since its incidence was very low. In those cases of positive species interaction at tree level we run a post-hoc Chi-square analysis to evaluate the interaction at fruit level.

To compare results from models using fruits collected shortly after arthropod entry with those from models based on predation rates on ripe fruits as is the common practice, we performed linear mixed models for each predispersal predator using ripe fruits to quantify damage rates and fruit traits from the branches of the 20 trees of our fruit success monitoring. All models included the same fixed and random terms as analyzed previously: fruit size, seed number per fruit and competing predators' infestation rates at tree level according to entry order. Tree and year were included as random factors. Moths entering during the first and second year of fruit development were analyzed separately, albeit statistical analysis was only performed for yellow juniper moth second entry. Model calculations were performed using the nlme packages in R environment (R Development Core Team, 2015).

RESULTS

Phenological sequence of events

The small, scale-shaped, *J. thurifera* female flowers started to grow in September and finished their development in next late February to early March. Pollination took place in March. Fruit growth after pollination was fast and fruits reached their final size by September of the first year (Fig. 1). Seed fertilization took place during May and June. Seeds gained size relatively fast after fertilization and reached their final size in September of the first year. Seed coat hardening was initiated in September. Fruits and seeds matured during the second year and in late September, green fruits turned to dark blue.

Different arthropods attacked fruits during their development (Fig. 1; see Appendix 2 for details). Mites were the first species to colonize the fruits by entering the flowers during winter, waiting for seed development after pollination. As a result, mites were already inside the seeds in first year June. Mites used the seed as a growth chamber feeding on the nucellus. They had several generations and in first year September, mites ceased their reproduction (no eggs could be found within the colonies). Adults exited the seed in midwinter and searched new female flowers to colonize.

The yellow juniper moth is an annual species with only one generation per year. Adult females lay one egg over fruit surface in June. Females can use first and second year fruits.

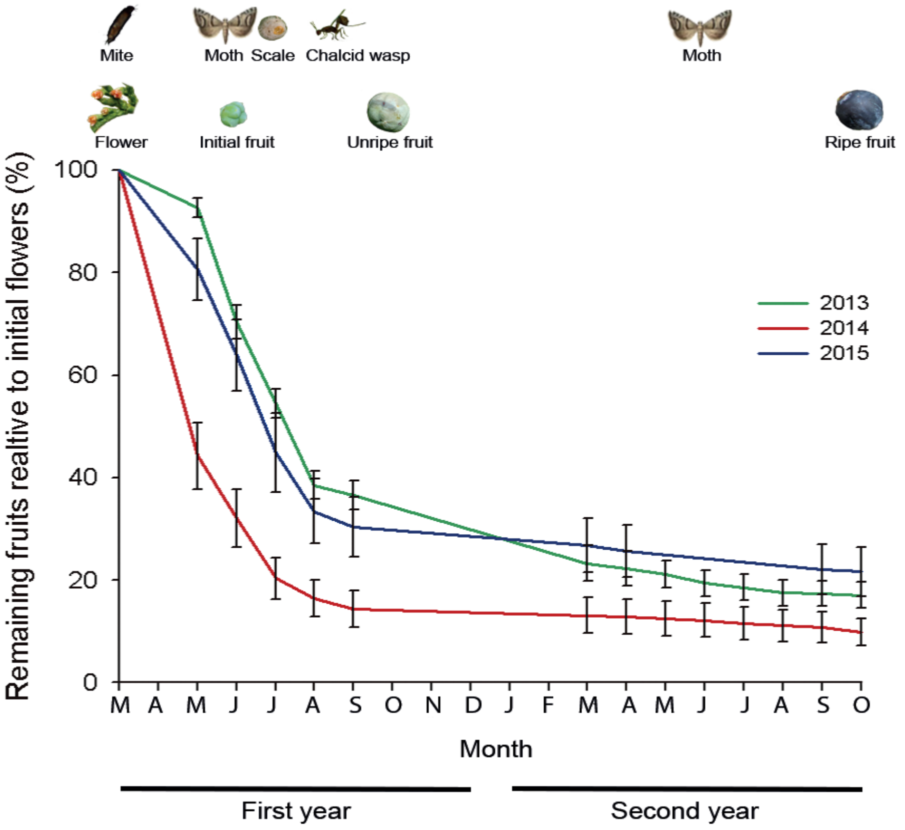

Fig. 1. Fruits remaining in the branch relative to initial flowers for 2013 (green line), 2014 (red line) and 2015 (blue line) cohorts. Initial flowers were measured in March and fruit number monitored until second year October for each cohort. Data presented as percentage relative to initial flowers. Bars represent ± 1 standard error. Above pictures marks the different phenological stages of fruit development and arthropods attacks.

Hatching occurred from mid-June to late July. Larvae entered the fruit leaving the egg corion glued to the fruit surface. When larva was infesting a first-year fruit, it made a gallery and entered into the seed, feeding first on seed corion and later on the megagametophyte. Larvae exited the seed in September and fed on pulp. Eggs laid in second year fruits showed a distinct behavior with larvae feeding only on pulp. In both cases, larvae left the fruit at mid-autumn of their respective year by making an irregular hole in fruit surface.

Brown juniper moth is also an annual species with only one generation per year. They had the same activity period as yellow juniper moth but they differed in feeding behavior. Females laid one egg on the fruit surface during June and hatching occurred from mid-June to late July. Larvae fed on egg corion after hatching. Larvae that hatched in a first years' fruit, usually ate one seed (in some cases two). Afterwards they built a nest with seed remains, silk and fecal deposits, resting during August. The larvae reactivated in September and fed on pulp. Larvae hatching in second year fruits only fed on pulp. In both cases, larvae exited the fruit from late October to early November of their respective year.

Scale insects are annual species with only one generation per year. Adult males flied during late-May and June in search of sedentary females for mating. Females produced one to forty eggs that were laid under the scale cover. Nymphs hatched in July, colonizing first and second year fruits. Once the nymph selected the feeding site they started to suck pulp and became sedentary. Individuals passed the winter as an adult.

Chalcid wasp is an annual species with only one generation per year. Females selected first year fruits and laid one egg per seed from late July to late September. Larvae fed on the seed and stayed within the seed until late June when they pupated during a month. Adults emerged from the fruit in late July.

Fruit and full seed production

A total of 11,480 flowers were monitored in the 100 branches from 20 female juniper trees of fruit success monitoring. All the tagged branches in 2013 presented flowers. However, the number of re-flowering branches was 93 for 2014 and 52 for 2015. Flower number differed between years: 2013 (3,215), 2014 (6,086) and 2015 (2,390). Largest number of flowers per branch (64.3 ± 9.8; mean ± standard error) occurred in 2014 with decreasing flowering effort in 2015 (46.0 ± 8.1) and 2013 (32.2 ± 3.3).

The transition from 'flowers' to 'initial fruits' showed high inter-annual variation (Fig. 1). The lowest initial fruit success occurred in 2014 when 59.0 ± 0.6 % of the flowers became initial fruits, whereas success levels were higher in 2013 (91.9 ± 0.5 %) and 2015 (84.3 ± 0.7 %). In contrast, fruit loss during first summer from initial to unripe fruit (May to September) was higher in 2013 (62.3 ± 0.9 %) and 2014 (60.2 ± 0.8 %) than in 2015 (50.8 ± 1.2 %). Fruit loss per branch from unripe to ripe fruits (1st year September to 2nd year October) was high in 2013 (45.8 ± 1.2 %), intermediate in 2015 (31.5 ± 1.5 %) and lower in 2014 (25.8 ± 0.8 %). When flower to ripe fruits success was globally evaluated, had a maximum in 2015 (28.4 ± 0.9 %), being lower for 2013 (18.8 ± 0.7 %) and 2014 (17.4 ± 0.5 %) cohorts.

Table 1. Generalized linear mixed models testing the effect of flowers per branch on the transition probabilities between flower and ripe fruits in *Juniperus thurifera*. First model evaluates the transition probability between flowers and ripe fruits, whereas second to fourth models indicate the transition probabilities between intermediate fruiting stages. Models for transitions from initial to final size fruits and from final size to ripe fruits also include fruit number per branch as a covariate. Significant P-values in bold. SE: Standard error, SD Standard deviation of random factor estimates.

Fruiting %	Fixed terms	Estimate	SE	*P*	Random	SD
Flower to ripe fruit	Intercept	-2.389	0.359	**<0.001**	Tree:Branch	1.267
March 1st year to	Flowers per branch	0.007	0.001	**<0.001**	Branch	< 0.001
October 2nd year					Year	0.562
Flower to initial fruit	Intercept	1.331	0.807	0.099	Tree:Branch	1.262
March 1st year to	Flowers per branch	0.005	0.001	**<0.001**	Branch	$2.9·10^{-5}$
June 1st year					Year	1.375
Initial to final size fruit	Intercept	-0.627	0.241	**0.009**	Tree:Branch	0.881
June 1st year to	Flowers per branch	$3.8·10^{-5}$	0.002	0.982	Branch	$1.3·10^{-5}$
September 1st year	Fruits per branch	0.003	0.002	0.156	Year	0.375
Final size to ripe fruit	Intercept	0.220	0.242	0.364	Tree:Branch	1.282
September 1st year	Flowers per branch	0.003	0.002	0.074	Branch	$3.0·10^{-5}$
to October 2nd year	Fruits per branch	0.001	0.004	0.851	Year	0.298

Probability of a flower becoming a ripe fruit increased with branch flower number (Table 1). This effect depended on the transition between flowers and initial fruits, since transition probabilities between initial to unripe fruits and from unripe to ripe fruits showed no relation with either the number of flowers or fruits per branch. Fruit success monitoring revealed a higher number of full seeds within the fruits in 2014 cohort (0.51 ± 0.02 seeds per fruit) than in 2015 (0.27 ± 0.02) and 2013 (0.16 ± 0.02). Number of full seeds per fruit in ripe fruits was positively associated to fruit size and flower number per branch (Table 2).

Fruit damage

A total of 3,740 immature fruits collected from 2013 to 2015 were used to evaluate monthly damage during fruit development and 2,342 ripe fruits were used to evaluate the final fruit damage by arthropods. As much as 60.6 ± 3.5 % of the 2013 cohort fruits were damaged by any of the arthropods in September 2013. Damage rates decreased during winter, but recovered at slightly lower levels due to arthropods second entry to 54.5 ± 4.0 % in August 2014. Damage rates were lower for the 2014 cohort (39.2 ± 3.6 % in August 2014 and 37.2 ±

Table 2. Linear mixed models assessing the effect of flowers and fruit per branch and fruit size on the number of full seeds in ripe fruits in *Juniperus thurifera*. Significant P-values in bold. SE: Standard error, SD Standard deviation of random factor estimates

	Fixed terms	Estimate	SE	*P*	Random	SD
# Full seeds in ripe fruits	Intercept	-2.775	$8.5\cdot10^{-4}$	**<0.001**	Tree:Branch	0.990
	Flowers per branch	0.004	$5.8\cdot10^{-4}$	**<0.001**	Branch	$3.2\cdot10^{-4}$
	Fruits per branch	$-4.2\cdot10^{-4}$	$8.3\cdot10^{-4}$	0.615		
	Fruit size	0.068	$8.5\cdot10^{-4}$	**<0.001**		

3.6 % in August 2015). Estimation of damage rates using ripe fruits was considerably lower for the 2013 cohort (25.4 ± 1.8 %) but of similar magnitude for the 2014 cohort (32.4 ± 1.4 %).

Scale insects showed the highest incidence, reaching a maximum of 40.4 ± 3.5 % in September 2013 (Table 3). Yellow juniper moth was the next in importance, reaching 19.2 ± 2.8 % in July 2013. Mites (15.4 ± 2.3 % in June 2013) and chalcid wasps (16.0 ± 2.7 % in September 2014) showed damage rates in the same range. Brown juniper moths had lower, but still significant damage rates with a peak in 2013 August with 7.7 ± 1.7 %. Juniper flies had a moderate impact in 2013 (9.3 ± 2.1 % in September 2013), but was negligible for the other cohorts. Comparison of these values with predispersal damage rates based just on ripe fruits from fruit success monitoring revealed large underestimations of predispersal damage rates for all species but chalcid wasps.

Fruit damage monitoring revealed variation in damage rates at seasonal and inter-annual scales. At seasonal scale percentage of fruit damage peaked one or two months after arthropods entry and then declined for all species except for chalcid wasp that maintained similar damage levels along fruit maturation cycle. In species with two entries, a secondary damage peak occurred during second year of fruit development. Differences between maximum damage rates in first year of fruit development and second year September at the end of fruit damage monitoring were rather high especially for both moths first year entry (Yellow juniper moth: from 19.2 ± 2.8 % to 0.6 ± 0.6 %; Brown juniper moth: from 7.7 ± 1.7 % to 0.6 ± 0.6 %) scale insects (from 40.4 ± 3.5 % to 5.6 ± 1.7 %) and for mites (from 15.4 ± 2.3 % to 7.5 ± 2.4 %) and much lower for second entry of arthropods (Yellow juniper moth: from 11.7 ± 2.6 % to 2.8 ± 1.2 %; Brown juniper moth: from 2.6 ± 1.3 % to 3.3 ± 1.3

Table 3. Predation rates (percentage ± standard error) for the main predispersal predators founded within the fruits of *Juniperus thurifera*. Data represent the maximum observed during the first and second year of fruit development and on ripe fruits for the 2013, 2014 and 2015 fruit cohorts. Data for second year of fruit development for 2015 cohort was not available.

	2013 fruit cohort			**2014 fruit cohort**			**2015 fruit cohort**	
Species	**1st year**	**2nd year**	**Ripe**	**1st year**	**2nd year**	**Ripe**	**1st year**	**Ripe**
Mites	15.4 ± 2.3	11.4 ± 2.5	2.5 ± 0.6	14.0 ± 2.6	7.9 ± 2.1	2.2 ± 0.4	16.0 ± 4.1	4.0 ± 0.7
Yellow Moth 1st	19.2 ± 2.8	7.8 ± 2.5	6.1 ±1.0	5.4 ±1.7	2.4 ±1.2	1.1 ± 0.3	4.8 ± 2.1	4.4 ± 0.8
Yellow moth 2nd		11.7 ± 2.6	6.6 ±1.0		4.4 ±1.5	5.9 ± 0.7		6.9 ± 1.0
Brown moth 1st	7.7 ± 1.7	3.0 ± 1.3	0.7 ±0.3	4.5 ±1.6	1.1 ±0.7	0	3.8 ± 1.7	0.7 ± 0.3
Brown moth 2nd		2.6 ± 1.3	3.5 ±1.7		3.3 ±1.3	1.7 ± 0.4		2.2 ± 0.6
Scale insect	40.4 ±3.5	23.4 ±3.4	22.2 ±1.7	25.3 ±3.2	13.3 ± 2.5	24.3 ± 1.3	23.7 ± 4.0	34.9 ± 1.8
Chalcid wasp	2.1 ± 1.0	14.9 ±2.9	8.3 ±1.1	9.5 ±2.5	16.0 ± 2.7	23.3 ± 1.3	21.5 ± 3.6	14.7 ± 1.4
Juniper fly	9.3 ± 2.1	2.6 ± 1.3	0.3 ±0.2	2.8 ±1.2	0.6 ± 0.6	0.7 ± 0.2	1.5 ± 1.1	0.7 ± 0.3
Total predation	60.6 ± 3.5	54.4 ±4.0	25.4 ±1.8	39.2 ±3.6	37.2 ± 3.6	32.4 ± 1.4	58.5 ± 4.3	56.6 ± 1.9

%; scale insects: 3.9 ± 1.4 % to 0.8 ± 0.8 %) and chalcid wasps (from 16.0 ± 2.7 % to 12.5 ± 3.0 %). Predispersal damage rate also showed some degree of variability between both monitored years with higher level of infestation for first year moths' and scale insect entry in 2013 cohort (Table 3).

Linear mixed models for fruit preferences for the different taxa differed when the analyses were based on fruit characteristics measured shortly after oviposition compared to models based on ripe fruits. In its first entry, yellow juniper moth preferred larger fruits with more seeds (Table 4). However, the model yellow juniper moth preferences based on ripe fruits did not find any effect of either fruit size or number of seeds per fruit, even with a much larger sample size. In its second entry, yellow juniper moth showed a positive effect of fruit size, but a negative effect of number of seeds per fruit. Interestingly, a positive effect of the incidence of chalcid wasps was also found. In contrast, analysis of yellow juniper moth second entry preferences based on ripe fruits detected no effect of fruit size, seed number or the incidence of chalcid wasps (Table 4). When coexistence at fruit level was evaluated presence of moth yellow juniper moth was marginally positively associated to chalcid wasp presence ($X^2 = 7.81$; $P = 0.0501$). The linear mixed model for brown juniper moth first entry revealed that their incidence increased in trees with higher incidence of mites and yellow juniper moths. When this analysis was performed on ripe fruits the effects of the incidence of mites or yellow juniper moths were not detected (Table 5). Interestingly, whereas the presence of mites was strongly positively associated to brown juniper moth infestation at fruit level ($X^2 = 29.13$; $P < 0.001$), both moths showed a random pattern ($X^2 = 0.20$; $P = 0.978$). No significant effects were detected for scale insects' preferences during their first entry (Table S2). However, when this analysis was performed on ripe fruits a positive effect of number of seeds per fruit was found (Table S2). Finally, predation monitoring revealed the preference of chalcid wasps for larger fruits. The same analysis performed on ripe fruits found a positive signal of seed number per fruit and of yellow juniper moth fruit damage rate (Table 6).

DISCUSSION

Monitoring of Spanish juniper fruit development revealed that fruit set was largely determined by initial reproductive effort. Initial investment in flowers enhanced ripe fruit set levels and number of full seeds per fruit. Several arthropods damaged fruits and depredated the seeds during the developing period, greatly diminishing ripe crop size. The sequential attack of mites, moths and scale insects exerted a strong pressure during the first five months of fruit development. Selective abortion of damaged fruits caused that an important proportion of damaged fruits were not detected in ripe fruits. Moreover, fruit preferences by arthropods differed when analyses were based on developing compared to ripe fruits.

Higher resource availability enhances the investment in reproductive structures and supports the demands of fruits and seeds during their development (Obeso 2002; Knight et al. 2005; Pickup and Barret 2012, see chapter 3). Initial flower production, a proxy of reproductive effort, was related to final crop size in absolute terms (Spearman $\rho = 0.654$; $P = < 0.001$): branches with more flowers bore more fruits. But also in relative terms: the probability of a flower becoming a ripe fruit increased with number of flowers per branch (Table 2) and in terms of fruit quality, branches with more flowers produced more full seeds per fruit.

Table 4. Linear mixed models evaluating fruit preferences in the yellow juniper moth *Mesophleps oxycedrella*. Separate models are performed for the first and second year of oviposition as well as for fruit traits measured for fruits gathered during the oviposition period of moths and for ripe fruits. Significant P-values in bold. SE: Standard error, SD Standard deviation of random factor estimates.

Fruits damaged by Yellow juinper moth	**Fixed terms**	**Estimate**	**SE**	***P***	**Random**	**SD**
First entry	Intercept	-7.389	1.035	**<0.001**	Tree	<0.001
1st year July fruits	Fruit size	0.441	0.133	**<0.001**	Year	0.125
	Number of seeds	0.353	0.144	**0..014**		
	Mite incidence	-1.389	1.054	0.188		
	Brown moth incidence	5.256	3.875	0.175		
First entry	Intercept	-3.549	0.661	**<0.001**	Tree	0.878
Ripe fruits	Fruit size	$2.9\cdot10^{-5}$	$3.3\cdot10^{-5}$	0.379	Year	0.653
	Number of seeds	-0.026	0.133	0.842		
	Mite incidence	0.866	2.132	0.685		
	Brown moth incidence	0.022	3.668	0.995		
Secon entry	Intercept	-7.429	3.166	**0.019**	Tree	1.031
2nd year July fruits	Fruit size	0.646	0.322	**0.045**	Year	0.006
	Number of seeds	-0.741	0.323	**0.022**		
	Mite incidence	-1.370	3.016	0.650		
	Chalcid wasp incidence	6.474	3.201	**0.043**		
	Yellow moth 1st year incidence	-0.381	10.649	0.972		
	Brown moth 1st year incidence	12.127	6.670	0.069		
	Scale incidence	-2.980	3.157	0.345		
Second entry	Intercept	-3.101	0.688	**<0.001**	Tree	1.258
Ripe fruits	Fruit size	$2.4\cdot10^{-5}$	$2.2\cdot10^{-5}$	0.277	Year	0.547
	Number of seeds	0.067	0.101	0.511		
	Mite incidence	-5.536	4.743	0.243		
	Chalcid wasp incidence	1.470	1.503	0.328		
	Yellow moth 1st year incidence	-1.359	2.843	0.633		
	Brown moth 1st year incidence	2.098	3.005	0.485		
	Scale incidence	-0.520	1.226	0.672		

Table 5. Linear mixed models testing for *Juniperus thurifera* fruit selection by brown juniper moth *Pammeme juniperana* during oviposition phase based on fruits in first year July and ripe fruits. Significant P-values in bold. SE: Standard error, SD Standard deviation of random factor estimates

Fruits dadmaged by Brown juniper moth	Fixed terms	Estimate	SE	*P*	Random	SD
First entry	Intercept	-2.636	1.200	**0.028**	Tree	<0.001
1st year July fruits	Fruit size	-0.248	0.180	0.167	Year	<0.001
	Number of seeds	-0.001	0.200	0.995		
	Mites incidence	2.650	0.587	**<0.001**		
	Yellow moth incidence	6.732	2.895	**0.020**		
First entry	Intercept	-7.824	1.768	**<0.001**	Tree	1.530
Ripe fruits	Fruit size	$1.3\cdot10^{-5}$	$1.0\cdot10^{-4}$	0.895	Year	0.938
	Number of seeds	0.241	0.375	0.521		
	Mite incidence	3.832	4.527	0.397		
	Yellow moth incidence	6.286	5.081	0.216		

Fruit loss concentrated on the first five months of fruit development matching the main period of entry of predispersal fruit predators. The presence of higher predispersal damage rates in developing than in ripe fruits indicates a selective abortion of damaged fruits, with higher investment of resources in high-quality fruits (Stephenson 1981; Ayre and Whelan 1989; Meyer et al. 2014; Riba-Hernández et al. 2016). However, this effect was contingent on the identity of predispersal predators. Mites, moths and scale insects' damage rates were much higher at early phases of fruit development than in ripe fruits supporting the selective abortion of fruits damaged by these arthropods. In contrast, chalcid wasps and moths second entry fruit damages did not differ from damage rates in ripe fruits. These differences may be related to the stages in which both predators group occur. Chalcid wasps oviposit when seeds are well-developed (Rouault et al. 2004), and probably the cost of fruit abortion at that stage is high compared to previous development phases (Stephenson 1981, Guitian et al. 1992). In addition, during moths' second year entry, seeds are close to maturity and protected by a thick seed coat, so pulp consumption by moth larvae do not directly damage the seeds, although may negatively affect fruit dispersal (García et al. 1999). Thus, selective abortion did not only lead to a large underestimation of the selective pressure exerted by predispersal predators, but also to changes in their relative importance.

Table 6. Linear mixed models testing for *Juniperus thurifera* fruit selection by chalcid wasp *Megastigmus thuriferana* during oviposition phase based on growing fruits and ripe fruits. Significant P-values in bold. SE: Standard error, SD Standard deviation of random factor estimates.

Fruits damaged by Chalcid wasps	Fixed terms	Estimate	SE	*P*	Random	SD
Entry 1st year September	Intercept	-9.006	2.466	**<0.001**	Tree	1.969
fruits	Fruit size	0.652	0.273	**0.017**	Year	1.047
	Number of seeds	0.199	0.197	0.313		
	Mite incidence	-5.465	3.828	0.153		
	Yellow moth incidence	3.706	3.481	0.287		
	Brown moth incidence	5.779	6.063	0.341		
	Scale incidence	-1.650	1.979	0.404		
Entry	Intercept	-3.116	0.565	**<0.001**	Tree	1.314
Ripe fruits	Fruit size	$7.6 \cdot 10^{-5}$	$1.7 \cdot 10^{-5}$	0.654	Year	0.434
	Number of seeds	0.211	0.077	**0.006**		
	Mite incidence	-6.332	4.387	0.149		
	Yellow moth incidence	-10.140	3.667	**0.006**		
	Brown incidence	7.404	3.804	0.052		
	Scale incidence	0.977	1.019	0.338		

The analyses based on ripe fruits showed no preferences by the yellow juniper moth, whereas developing fruits revealed preferences in fruit selection. Yellow juniper moths preferred large fruits and with more seeds in the first year of fruit development. Bigger fruits provide to yellow moths more pulp resources. Indeed, they star breeding directly on seeds that were soft and available for breeding at this phenological stage providing. Thus, pulp and seeds provide the necessary resources, increasing larvae survival chances (Sallabanks and Courtney 1992; Desouhant et al. 2000). Preferences were somewhat different in second year entry. Large fruits were still preferred, but seed number exerted a negative effect. Yellow juniper moths are unable to feed on second-year hardened seeds, so fruits with less seeds have more pulp providing more resources to the larvae. The analyses of fruit preferences by brown juniper moth based on developing fruits revealed an association to yellow moth and mites' presence. Tree level association with yellow juniper moth would indicate that they use signals emitted either by yellow juniper moth as a cue for favourable oviposition places, a common phenomenon in Lepidoptera (Raitanen et al. 2014). In contrast, tree and fruit level association to mites would indicate an omnivorous behaviour that has been described for other Tortricidae species (see Pierce 1995; Wang and Daane 2014).

Scale insects reached the largest fruit damage rate (40%), however their detection in ripe fruits was lower (22 %). The underestimation of the importance of scale insects cannot be attributed to low detectability in ripe fruits (E. Rodríguez pers. obs.), so it could indicate a selective abortion and therefore an impact on final crop size. The inability to detect preferences for the fruit traits we measured may be related to the feeding behaviour these insects as pulp suckers, that probably choose fruits by their water content, as occur in J. communis (García 1998), and not by size or seed related traits.

Chalcid wasp was the only taxa where ripe fruit provided more information in traits selection than immature fruits. This discrepancy may be related to the low fruit abortion associated to this species, and the larger statistical power provided from nearly four times larger sample size of ripe fruits. Chalcid wasps selected fruits with more seeds while avoiding trees and fruits already occupied by other arthropods, especially from seed eaters as yellow juniper moth (Mezquida and Olano 2013; Mezquida et al. 2016).

Fruit preferences by predispersal seed predators is commonly based on the analysis of ripe fruits (Sperens 1997; García et al. 2000; Mezquida and Olano 2013; Mezquida et al. 2016), however several mechanisms can bias this approach. 1) Selective abortion of damaged fruits produced a high underestimation of damage (from 60.6 ± 3.5 % to 25.4 ± 1.8 %) with species-specific abortion rates altering the relative ranking of predispersal predators. 2) Changes in fruit characteristics from the moment of arthropod entry to ripe fruit that may complicate the identification of preferences. Despite the lower sample size (600 unripe fruits vs. 2342 ripe fruits), fruits collected just after arthropod entry provided better models of arthropod preferences for most species. 3) Finally, species with that can oviposit at different stages of fruit development might differ in their preferences depending on the phenological fruit phase selected, exerting contrasted fruit preferences. Evaluation of preferences by different arthropods attacking fruits in the Spanish juniper was improved through multiple fruit sampling matching the timing of arthropods' entry. This result can probably be extrapolated to other species which have seeds whit long maturation periods. Overall our results claim for the need of developing a deeper knowledge on natural history to improve our understanding of the biological interactions behind ecological and evolutionary processes.

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SUPPLEMENTARY MATERIAL

Appendix 1. Identification key for the main arthropods observed developing inside the fruit and seeds of *Juniperus thurifera*:

1a Arthropod developing inside the fruit. 2.

1b White scale insect or yellowish nymphs with red eyes developing on the fruit surface: *Carulaspis juniperi* (Fig. A).

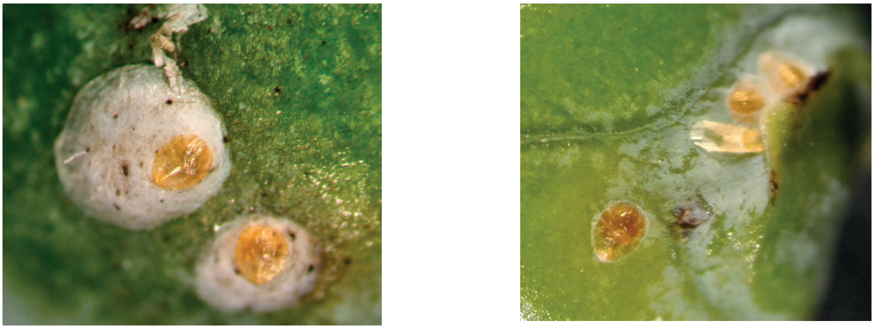


Fig A. Adults and sedentary nymphs of *Carulaspis juniperi* developing on fruit surface.

2a Arthropod developing inside the seeds. 3

2b Arthropod developing in the pulp. 6

3a Deformed seeds with elongated tips having red and/or white mites and spherical white eggs in variable number in the inside: *Trisetacus quadrisetus* (Fig. B).

3b Normal seed, without elongated tip. 4

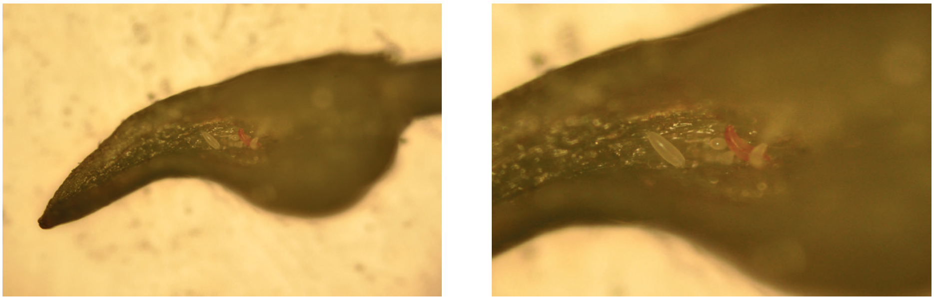


Fig B. Left: Early-seed attacked by *Trisetacus quadrisetus*, showing elongated tip. Right: Red and white mites plus eggs within a seeed

4a White legless larva, white to brownish (male) or yellowish (female) pupa developing inside of a well-developed seed: *Megastigmus thuriferana* (Fig C.a).

4b Legged larva, with pairs of pseudopods in mid body and in anal region, developing inside of seed corion or feeding on megagametophyte. 5 (Fig C.b)

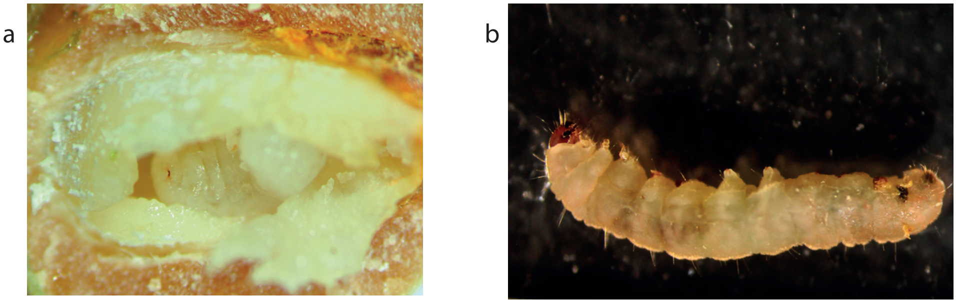


Fig C. a: *Megastigmus thuriferana* larva developing within seed. b: Legged larva of *Mesophleps oxycedrella.*

5a White larva showing a dark post cephalic mark – sometimes divided in two –, the seed shows galleries in the corion but does not have big holes: *Mesophleps oxycedrella* (Fig D.a).

5b White larva showing four dark post cephalic marks, seed almost complete eaten or showing a big hole, usually covered with fecal deposits and silk: *Pammene juniperana* (Fig D.b).

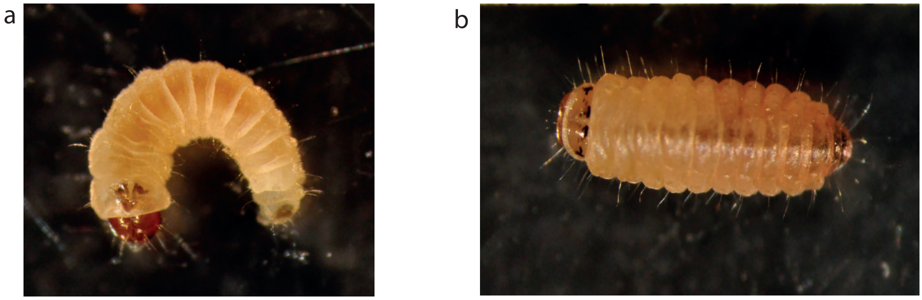


Fig D. a: *Mesophleps oxycedrella* larva showing the dark postcephalic mark divided. b: *Pammene juniperana* larva showing the four postcephalic marks.

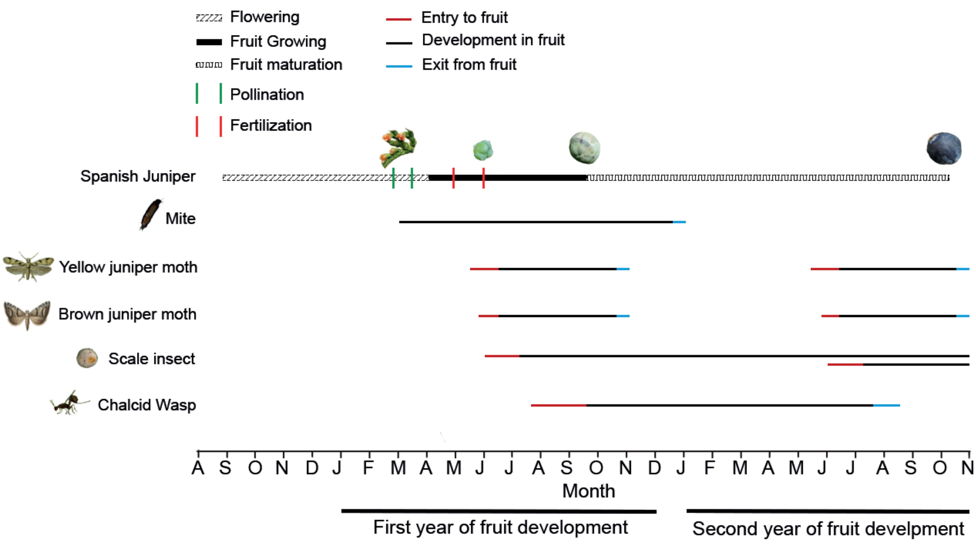
6a Legged Larva 7

6b Legless yellow larva: Tephritidae (Diptera) *Rhagoletis* cf. *zernyi.*

7a White larva showing a dark post cephalic mark – sometimes divided in two – showing pink and green iridescence in late summer and autumn: *Mesophleps oxycedrella* (Fig. D.a).

7b White larva showing four dark post cephalic marks: *Pammene juniperana* (Fig. D.b).

Appendix 2. Phenological sequence of events observed during *J. thurifera* fruit development and its predispersal predators' activity.

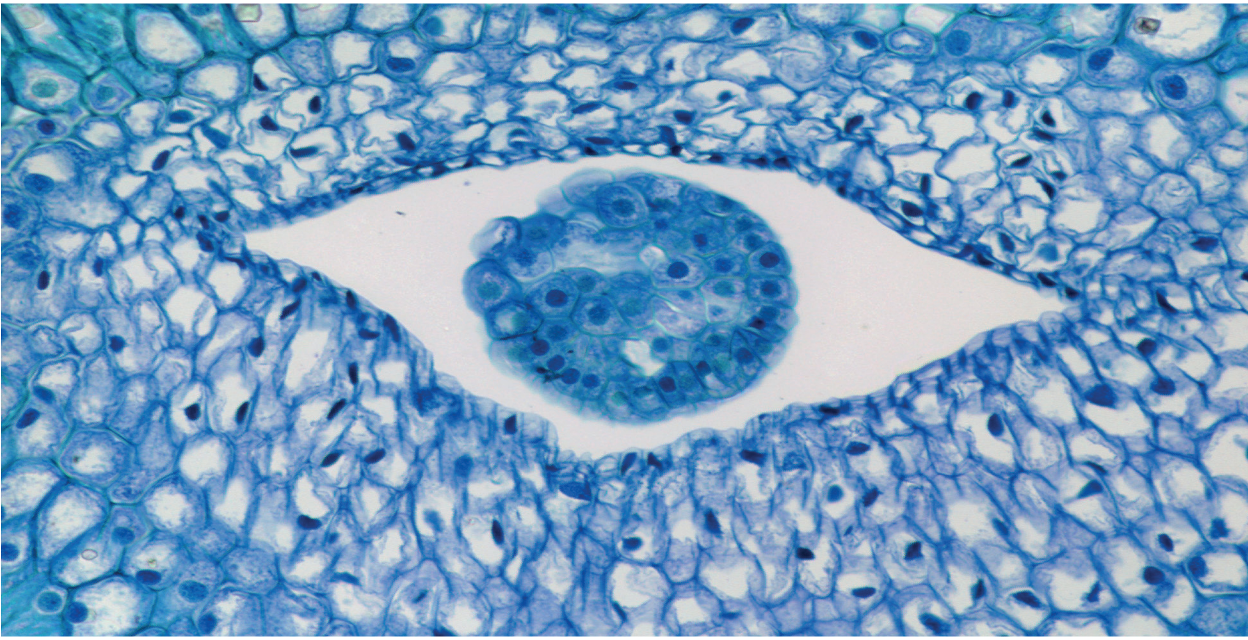


Appendix 3. Differences in fruit development transitions between years. Table also showed the differences in number of full seeds per fruit for each year. Significant *P*-values in bold. SE: Standard error.

Comparing items	Year pairs	Estimate	SE	*P*
Flower to ripe fruit	2013 - 2014	-0.090	0.056	0.109
	2013 - 2015	0.542	0.064	< **0.001**
	2014 - 2015	0.632	0.057	< **0.001**
Flower to initial fruit	2013 - 2014	-2.057	0.070	< **0.001**
	2013 - 2015	-0.744	0.086	< **0.001**
	2014 - 2015	1.313	0.062	< **0.001**
Initial to unripe fruits	2013 - 2014	0.088	0.051	0.083
	2013 - 2015	0.472	0.059	< **0.001**
	2014 - 2015	0.384	0.056	< **0.001**
Unripe to ripe fruits	2013 - 2014	0.888	0.085	< **0.001**
	2013 - 2015	0.608	0.091	< **0.001**
	2014 - 2015	-0.280	0.091	**0.002**
# Full seeds in ripe fruits	2013 - 2014	1.131	0.109	< **0.001**
	2013 - 2015	0.512	0.124	< **0.001**
	2014 - 2015	-0.619	0.085	< **0.001**

CHAPTER 3

Deciphering the role of reproductive investment, pollination success and predispersal seed predation on reproductive output in *Juniperus thurifera*



"All creative people want
to do unexpected"

Hedy Lamarr

ABSTRACT

Reproductive output is determined by a complex set of factors including resources available to invest in reproduction, successful pollination and fertilization, and fruit and seed loss from predators during fruit development and ripening. Disentangling the relative contribution of each limiting factor is critical to underpin the factors determining plant fitness. We performed an experimental approach to assess the relative contribution of initial reproductive effort, pollination success and predispersal seed predation on plant reproductive success in the wind-pollinated dioecious conifer *Juniperus thurifera*. We set a full factorial design with two treatments: pollen addition and pesticide application on 40 female juniper trees, and monitored the full process of fruit development during 20 months. The influence of initial reproductive effort, hand-pollination and pesticide application on fruit set at different stages, seed viability and fruit damage by several specialized arthropods was tested by mixed models. We also set up an additional experiment to test the effect of hand-pollination on pollen tube formation. Most reproductive structures were lost at the early stages of reproduction, particularly during the initial growing and development of the fruits. Resources invested in reproduction had important consequences for fruit and seed set. Production of more flowers reduced the incidence of low-mobile seed predators through a satiation effect, increased the proportion of fruits set and the quality of ripe fruits (having more viable seeds). Pollination was not limiting in the population and year of study and manual addition of pollen diminished levels of fruit set, although fruits that ripen had more viable seeds. Predispersal seed predation by different arthropods was an important limiting factor to fruit set in this juniper species. The initial investment in surplus flowers allowed plants to selectively abort damaged and depredated fruits during the early phase of development and allocate resources to the remaining fruits. Overall, our experiment indicated that levels of fruit set in Spanish junipers were largely determined by initial reproductive effort, predispersal seed predation exerted by arthropods and the interaction between both factors.

INTRODUCTION

The number of viable seeds produced by plants is an important determinant of plant recruitment and individual fitness (Herrera 1991; Louda and Potvin 1995). Multiple factors influence seed production and limit reproduction, thus potentially playing important roles in plant population dynamics and evolution (Hainsworth 1984;, Price et al. 2008; Boieiro et al. 2012). Plants invest resources in reproduction by producing flowers that must be pollinated to form fruits, ovules must be fertilized and sustained with resources to develop into viable seeds, and seeds and fruits must escape predation during maturation to produce sound seeds ready for dispersal (Kaye 1999; Pías et al. 2007). From the initial reproductive investment, losses occur sequentially due to factors such as pollination failure, lack of fertilization and seed predation, although their relative influence on reproduction is difficult to disentangle because several components may act in combination (Hainsworth 1984; Herrera 1991; Gruwez et al. 2013; Walsh et al. 2014).

Resources allocated to reproduction compete with demands from other plant functions as survival or growth (Obeso; 2002, Hirayama et al. 2008; Teitel et al. 2016), leading to trade-offs between different requirements, with resource allocation priorities determined by life strategy (Pickup and Barret 2012; Martín et al. 2015). Under high availability of resources, plants usually invest more energy in reproductive structures by, for example, producing more ovules (Obeso 2002) or higher pollen loads (Pearse et al. 2015), overall enhancing their reproductive success (Barringer et al. 2013). Nevertheless, the cost of reproduction may extend well beyond the flowering period due to the high energetic demand of fruit development (Obeso 2002; Hirayama et al. 2008; Martín et al. 2015; Teitel et al. 2016). Thus, the investment in energy required for large fruit crops may exhaust current and stored resources compromising resource availability for the next reproductive season (Karlsson et al 1990; Lyles et al. 2015; Pessendorfer et al. 2016). Under these premises, inter-annual changes in fruit production (masting) may respond to inter-annual variation in resource levels as well as inter-cohort resource competition (Knight et al. 2005; Crone et al. 2009).

A high initial investment in reproduction may be curtailed if pollination fails. Pollination success depends on the concert of multiple factors and high inter-annual variation is the norm (Koenig and Knops 2014; Koenig et al. 2015). Success at this reproductive stage is affected by individual plant traits, such as total flower production (Kudo and Harder 2005), flowering synchrony (Albert et al. 2001; Obeso 2002; Koenig et al. 2015; Lyles et al. 2015) or pollen quality and quantity (Knight 2003; Labouche et al. 2016). Floral traits and environmental factors are important determinants of pollination efficiency. For example, abundant precipitations during the pollination period diminishes pollination success in wind-pollinated plants, whereas warm conditions favor the mobility of invertebrate pollinators potentially enhancing pollination in animal-pollinated plants (Knops et al. 2007; Rech et al. 2016). Pollination success is also modulated by factors at higher spatial scales like plant density and structure (Sork et al. 2002; Knight 2003; Sanz and Pulido 2015; see chapter 5), habitat fragmentation (Sork et al. 2002; Knight et al. 2005), interspecific competition for pollinators (Mitchell et al. 2009) or interference from heterospecific pollen (Mugnaini et al. 2007; Aderkas et al. 2012). Thus, fruit set and number of fruits produced may be low even in the presence of high resource levels if conditions for pollination and early fruit development are not satisfactory.

Plants accumulate nutritious resources in the embryo and its surrounding tissues during fruit and seed development. The high and concentrated nutritional value of fruits and seeds are targeted by specialized predispersal predators, mainly arthropods. Moreover, arthropods developing inside fruits and seeds are provided with a protective place against desiccation and natural enemies during its development (Sallabanks and Courtney 1992). Thus, predispersal predation may compromise successful plant reproduction and become a strong evolutionary force. In response to predispersal predation, plants develop different strategies to defend their fruit and seeds from attacks. Short-term strategies involve physical and chemical defenses (Janzen 1971; Schoonhoven et al. 2005; Boivin and Auger-Rozenberg 2016), fruit and seed abortion after infestation (Bonal et al. 2010; Meyer et al. 2014; Boivin and Auger-Rozenberg 2016) or delay in fertilization to avoid embryo loss by predator oviposition (Rouault et al. 2004; Aderkas et al. 2005). Long-term responses include higher inter-annual variations in fruit production to constrain the ability of specialist predators to match its demographic cycles with fruit production (Janzen 1971), boosting plant reproductive success during high production years (Turgeon 1994).

In the present work, we designed an experimental approach to determine the relative contribution of initial reproductive effort, pollination success and predispersal seed predation on plant reproductive success in the wind-pollinated dioecious conifer *Juniperus thurifera*.

We estimated fruit set levels at different stages of fruit development (fruits will be used for the fleshy cones produced by this juniper), and quantified seed predation and seed characteristics in ripe fruits. We address the hypothesis that resources, as represented by the initial individual investment in reproduction, improve reproductive success by counteracting losses at different stages and enabling the selective abscission of damaged or low quality fruits. We also test the hypothesis that, if pollination is limiting, the addition of supplementary pollen would increase fruit or seed set levels. Finally, we assess the relative role of predispersal seed predators in depressing the reproductive potential in this juniper by experimentally reducing their impact and estimating the resulting fruit and seed set levels.

MATERIAL AND METHODS

Natural history

Spanish juniper (*J. thurifera*) is a dioecious juniper species, endemic to the Western Mediterranean where it inhabits continental summer-dry environments. Junipers, as is common in other gymnosperms, exhibit a long interval between pollination and seed ripening. Wind pollination occurs in late winter-early spring. Pollen germinates and megagametophyte formation starts after pollination, but fertilization is delayed until late spring. Embryo development begins after fertilization and seeds are almost fully grown by mid-summer, when seed coat starts to harden. Maturation of the megagametophyte and embryo takes 12-13 additional months (Gruwez et al. 2013). Therefore, the fruits (fleshy cones) produced by this juniper species ripen about 20 months after pollination in next year autumn (Montesinos et al. 2012).

A variety of arthropods can damage Spanish juniper fruits during this long development period (Roques et al. 1984; El Alaoui et al. 2013). In the Iberian Peninsula, three arthropod taxa are the main predispersal predators: mites, moths and chalcid wasps. Mites, *Trisetacus quadrisetus* (Thomas 1889) (Acari, Phytoptidae), colonize the seeds at the very beginning of their development and use them as growth chambers where they feed and reproduce (Roques 1984; see chapter 2). Two moth species: *Mesophleps oxycedrella* (Millière 1871) (Lepidoptera, Gelechiidae) and *Pammene juniperana* (Millère 1858) (Lepidoptera, Tortricidae) feed on juniper fruits. Females oviposit usually one egg on the fruit surface from late-May to early July during the first and second year of fruit development and, by late July, larvae have entered the fruit and seeds. Chalcid wasps *Megastigmus thuriferana* Roques & El Alaoui 2006 (Hymenoptera, Torymidae) feed on well-developed megagametophyte and embryo (Rouault et al. 2004). Females oviposit one egg inside the seeds of developing fruits from late July-early August to late September.

Study area

The study took place in Villaciervos, Soria province, Central Spain (41º 44′ N, 2º 40′ W; 1150 m.a.s.l.). Climate is Mediterranean continental with mean annual precipitation of 595 mm, only 110 mm occurring during summer. Mean monthly temperatures ranged from 1.7 ºC in January to 19.7 ºC in July. The rock parent material is Cretaceous limestone covered by deep soil with small areas of exposed bedrock. Vegetation is dominated by the Spanish juniper with open areas among trees covered by xeric grasslands with shrubs, including *Cistus laurifolius* L., *J. communis* L. and *Thymus zygis* Loefl.

Sampling design and sample analyses

We randomly chose 40 female juniper trees within the study area. For each tree, eight flowering branches from all around the canopy were selected and tagged. We set a full factorial design with two treatments: pollen addition and pesticide application. Two branches per tree were subjected to each of the treatment combinations: pollen, pesticide, pollen plus pesticide and control. Pollen addition consisted on manual addition of pollen collected from 10 different male trees and applied onto female cones with a soft paintbrush shortly after pollen collection. Pollen addition was performed twice during March 2014, coinciding with the pollination period. The pesticide treatment consisted in applying a mixture of acaricide and insecticide that was prepared diluting the acaricide (Fenpyroximate 5 % w/v) and the insecticide (Chlorpyrifos 48 % w/v) in water at 0.2 % v/v. Pesticide was applied twice per month between May and September in 2014 and 2015. Junipers are sensitive to the deposition of particles, other than juniper pollen, during pollination (Mugnaini et al. 2007), so timing of pesticide spraying was chosen to avoid interfering with pollen reception on female flowers during pollination while encompassing the oviposition period of arthropods. Branches subjected to the pollination plus pesticide treatment were supplemented with pollen and later sprayed with pesticide as explained above. Control branches were manipulated as the experimental branches, but no pollen or pesticide was applied.

We counted the number of female flowers produced in each tagged branch in March 2014 as a measure of the initial reproductive effort at branch level. The number of developing fruits in each branch were counted monthly from June to September during the first year of development (2014), when most of the fruits are lost (see chapter 2), and in May, July and October during the second year of development (2015). All fruits remaining in the tagged branches were collected when ripen in late October 2015. We selected five sound fruits (i.e., without signs of damage by arthropods) from each branch (when available) to count and characterize seeds. Fruits were opened to separate and count the number of seeds, and each seed was dissected under a microscope and assigned to one of the following categories: 'full', for seeds showing a completely developed embryo and megagametophyte; 'empty', for seeds that interrupted the development of the megagametophyte and/or embryo after fertilization, and that are externally similar to fully developed seeds although they do not contain (or only some remnants) megagametophyte or embryo; and 'aborted', for seeds that interrupted their development between pollination and fertilization, and that are visually recognizable as small, not completely developed seeds. The rest of the fruits were opened and examined in the laboratory under a dissecting microscope to detect signs of arthropod attack. Damage by arthropods can be easily assigned to each of the three arthropod taxa (Roques et al. 1984; Mezquida and Olano 2013). Mites deform the seeds causing the elongation of their tips that usually stick out of the fruit surface, moths make an irregular hole in different parts of the fruit surface and chalcid wasps make a circular hole in the apical region of the fruit.

To further explore the effect of pollen addition on seed development, we evaluated whether the manual addition of pollen increased the likelihood of pollen germination and pollen tube growing. In March 2015, we conducted another pollen addition experiment to a subset of 10 experimental trees. Two branches from each tree were selected and tagged, one was subjected to the pollen addition treatment and the other used as control. In June 2015, we collected five sound developing fruits from each branch (two fruits were damaged or had no viable seeds, so final sample size was 98 developing fruits, 50 and 48 fruits for natural and manual pollination, respectively). The presence of pollen tube was evaluated under light microscopy. Sampled fruits were stored in 96% v/v ethanol until processing and then embedded in Technovit 7100 resin (Heraeus Kulzer, Wehrheim, Germany) following Leroux et al. (2007). First, we excised outer tissues of the juniper fruits to ensure good resin infiltration and stored them in 99 % v/v ethanol for 2 days to full dehydration. After dehydration was completed, samples were infiltrated with Technovit 7100 infiltration liquid (2-hydroxyethylmethacrylate) diluted to 30%, 50%, and 70% with 99% v/v ethanol. Once the samples were infiltrated with these solutions (1 day each), they were transferred to 100% Technovit 7100 infiltration liquid for 2 days and embedded in polypropylene flat bottom capsules (TAAB laboratories, Berkshire, UK). Transverse sections of 4 µm were cut with a Microm HM360 microtome (Microm International GmbH, Walldorf, Germany), collected on glass slides, stained with a 0.05% w/v solution of toluidine blue O (Merck, Darmstadt, Germany, C.I. No. 52040) in 0.1% w/v Na2B4O7, and mounted in DePeX (Gurr, BDH Laboratory, UK). Sections were observed with a Nikon Eclipse E600 microscope and images were recorded using a Nikon digital camera DXM1200.

Statistical analysis

We defined four critical stages during fruit development: 'flowers', as the number of flowers produced and counted in March; 'initial fruits', as the number of initiated fruits that could be identified in May; 'unripe fruits', as the number of unripe green fruits that have reached their final size in September of the first year of development, and 'ripe fruits', as the number of mature fruits that turn to dark blue in October of the second year. We used linear mixed models with a binomial error structure that accounts for differences in sample size to analyze the influence of experimental treatments on transition success between flowers and ripe fruits. We consider as success the number of fruits that persisted between two stages for each treatment. Firstly, we analyzed the full transition from flowers to ripe fruits using the number of ripe fruits relative to the number of flowers produced. Fixed terms in this model included pollen, pesticide and the interaction between both treatments, and number of flowers per branch was included as a continuous predictor quantifying the initial reproductive effort. Besides, we analyzed the three transitions between the intermediate stages: flower to initial fruits, initial to unripe fruits and unripe to ripe fruits. Model structure was similar to the above model, although number of fruits per branch (initial or unripe fruits; instead of number of flowers) was included as a fixed continuous variable in the models for unripe fruit set and ripe fruit set. Tree was included as a random factor in the four models.

Linear mixed models with Poisson error structure were used to test whether experimental treatments affected the number of total, full, empty and aborted seeds in ripe fruits. Fixed terms in the models included pollen and pesticide treatments, their interaction and number of flowers per branch. Tree was included as a random factor. Finally, to test whether pollen addition influenced the proportion of fruits showing a grown pollen tube, we fitted a linear mixed model with binomial error structure. Pollen treatment, number of flowers per branch and number of viable seeds per fruit were included in the fixed part of the model, and tree was included as a random factor.

To test for the influence of experimental treatments on levels of fruit damage by each arthropod, linear mixed models with a binomial error structure were used. Because fruit abundance may affect damage rates (Mezquida and Olano 2013), we included the number of developing fruits in the branch when each arthropod oviposits or enters the fruit or seed as a covariate. Therefore, we included number of flowers in the mixed model for fruit damage by mites, number of initial fruits (counted in May 2014) in the model for fruit damage by moths and number of developing fruits (July 2014) in the model for fruit damage by chalcid wasps. Because moths and chalcid wasps tend to avoid ovipositing in fruits already infested by other arthropods (Mezquida and Olano 2013), we included the proportion of fruits depredated by other arthropods as explanatory variables in the models for moths and chalcid wasps. Tree was included as a random factor in the models. Count variables used as covariates, such as number of flowers and fruits at different development stages, were standardized to zero mean and unit variance to rescale them and reduce their variance. Mixed models for seed characteristics, pollen tube development and fruit damage by arthropods were simplified by progressively removing the least significant terms. Model calculations were performed using the lme4 package in R environment (R Development Core Team, 2014).

RESULTS

We monitored 40,532 flowers in 320 branches from 40 female juniper trees in 2014. The number of fruits that started to develop in May were 23,473 (57.9 % of the initial flowers). Major fruit losses occurred during the next two months of development (Fig. 1), with only

10,977 fruits remaining in July (27.1 % of the initial flowers; 46.8 % of the initial fruits). Losses from July to September 2014 were lower with 9,218 fruits remaining (22.7 % of the initial flowers; 39.3 % of initial fruits). Fruit losses continued during the next year until fruit ripening with 5,614 ripe fruits counted in October 2015 (13.9% of the initial flowers; 23.9% of the initial fruits).

Trees and branches producing more flowers set a higher proportion of ripe fruits (Table 1). Hand-pollination of flowers decreased, whereas spraying of pesticide increased, the proportion of fruits that ripen, and there was a positive interaction between pollen and pesticide treatment (Table 1; Fig. 2). The analysis of transitions between different developmental stages showed similar results, although branches with more initial fruits set a higher proportion of unripe fruits whereas branches with more unripe fruits set a lower proportion of ripe fruits (Table 1). In addition, the interaction between pollen addition and pesticide treatment was not significant for the transition between initial to unripe fruits, and fruit losses between unripe to ripe fruits did not differ between branches treated with pesticide and control ones (Table 1; Fig. 2). Overall, the proportion of ripe fruits relative to the number of flowers produced were higher in branches treated with pesticide followed by those hand-pollinated plus sprayed with pesticide, control branches and hand-pollinated ones (Fig. 1; Fig. 2).

Ripe fruits developed in branches that produced more flowers had more seeds, including more filled and less aborted seeds (Table 2; Fig. 3). Manual addition of pollen and pesticide treatment did not influence the number of seeds per fruit, but both treatments increased the number of full seeds and reduced the number of aborted seeds in ripe fruits (Table 2; Fig. 3). The number of empty seeds was not affected by either the initial number of flowers produced or any treatment (Table 2; Fig. 3). The mixed model to assess the effect of pollen addition on pollen tube development showed that the proportion of seeds with grown pollen tube was higher for fruits in hand-pollinated branches (Mean ± SE: 0.78 ± 0.08) than fruits in control branches (0.61 ± 0.06, $Z = 3.1$, $P = 0.002$, $n = 98$ fruits, 229 seeds). The presence of pollen tube was not influenced by the number of flowers produced by each branch or the number of seeds per fruit ($P > 0.15$, in both cases).

Damage by arthropods affected to 25.4 % of the overall ripe fruits. Chalcid wasps were by far the most abundant seed predator affecting to 17.3 % of the fruits, whereas mites

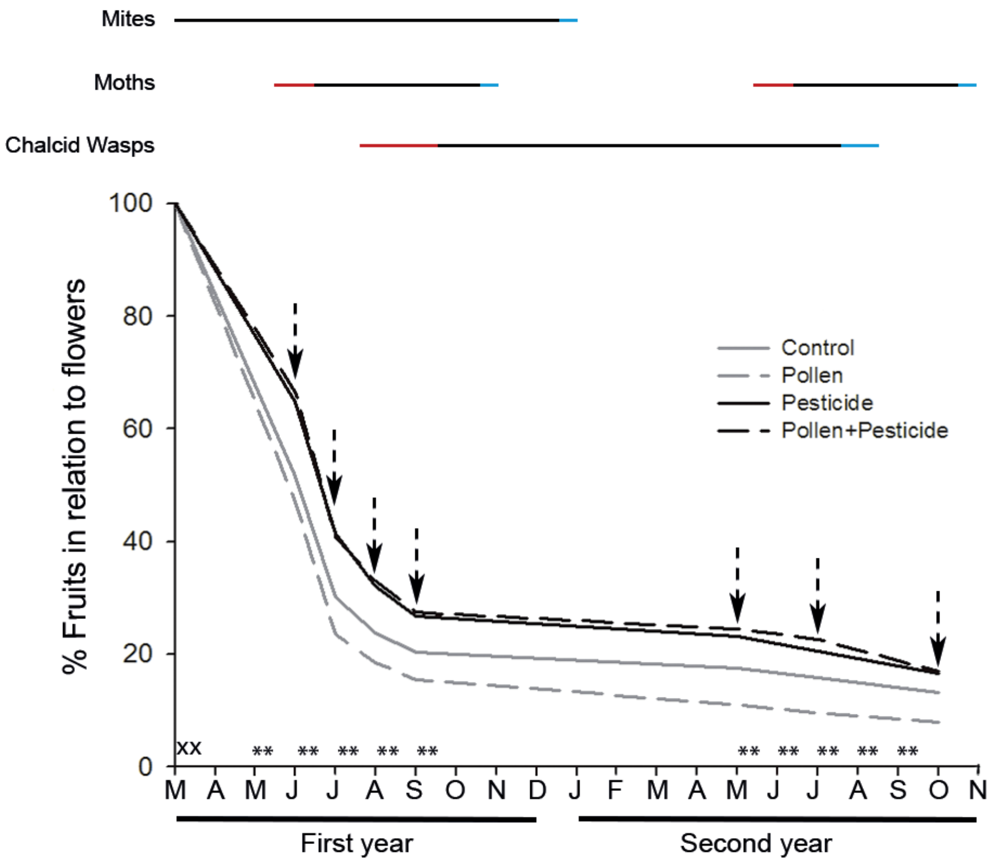


Fig. 1. Percentage of fruits in relation to initial flowers for the 2014-2015 fruit cohort. Lines represent the combination of pollen and pesticide treatments (see inlet legend). Vertical arrows indicate sampling dates. xx: timing of hand-pollination; **: timing of pesticide spraying. The upper panel shows the timing when three arthropod taxa enter (red line), stay (black line) and leave (blue line) the damaged fruits.

(3.9 %) and moths (4.9 %) had low predation rates. The mixed model for the incidence of mites in ripe fruits showed that branches producing more flowers and those treated with pesticide yielded fruits with lower levels of damage by this arthropod (Table 3; Fig. 4). Fruit and seed predation by moths increased with the number of fruits available during the period of oviposition and was reduced in branches treated with pesticide. The incidence of chalcid wasps did also show a negative effect on predation rates by moths (Table 3; Fig. 4). The model for seed predation by chalcid wasps showed that branches with more fruits available during oviposition and those treated with pesticide had higher levels of predation by chalcid wasps. In addition, the incidence of the other two arthropods (mites and moths) negatively influenced damage rates by chalcid wasps (Table 3; Fig. 4).

Table 1. Linear mixed models testing the effect of treatment (pollen addition, pesticide and the interaction between both treatments) and number of flowers or fruits on the transition probabilities between flower and ripe fruits. The first model evaluates the transition probability between flowers and ripe fruits, whereas second, third and fourth models assess the intermediate transition between different critical phenological stages. Significant P-values in bold. S.E.: Standard error, S.D. Standard deviation of random factor estimates.

	Fixed effects	**Estimate**	**SE**	***P***	**Random**	**SD**
Flower to ripe fruits	Intercept	-2.533	0.192	**<0.001**	Tree	1.183
	N. flowers	0.112	0.025	**<0.001**		
	Pollen	-0.575	0.052	**<0.001**		
	Pesticide	0.422	0.045	**<0.001**		
	Pollen x Pesticide	0.488	0.066	**<0.001**		
Flowers to initial fruits	Intercept	-0.178	0.179	0.318	Tree	1.119
	N. flowers	0.039	0.018	**0.032**		
	Pollen	-0.158	0.033	**<0.001**		
	Pesticide	0.596	0.035	**<0.001**		
	Pollen x Pesticide	0.291	0.047	**<0.001**		
Initial to unripe fruits	Intercept	-0.996	0.133	**<0.001**	Tree	0.797
	N. initial fruits	0.329	0.021	**<0.001**		
	Pollen	-0.216	0.048	**<0.001**		
	Pesticide	0.231	0.044	**<0.001**		
	Pollen x Pesticide	0.021	0.064	0.742		
Unripe to ripe fruits	Intercept	1.060	0.186	**<0.001**	Tree	1.047
	N. unripe fruits	-0.366	0.029	**<0.001**		
	Pollen	-0.662	0.092	**<0.001**		
	Pesticide	0.052	0.081	0.527		
	Pollen x Pesticide	0.557	0.112	**<0.001**		

DISCUSSION

Results from our experiment indicated that levels of fruit set in Spanish junipers were largely determined by initial reproductive effort and predispersal predation exerted by arthropods. The initial investment in flowers enhanced ripe fruit set levels and was also positively correlated with the absolute number of ripe fruits produced. Manual pollination unexpectedly caused higher fruit losses, although pollen addition improved seed set and reduced seed abortion. The spraying of pesticide successfully reduced the incidence of mites and moths, but not chalcid wasps, increased fruit set levels and ripe fruits set more viable seeds.

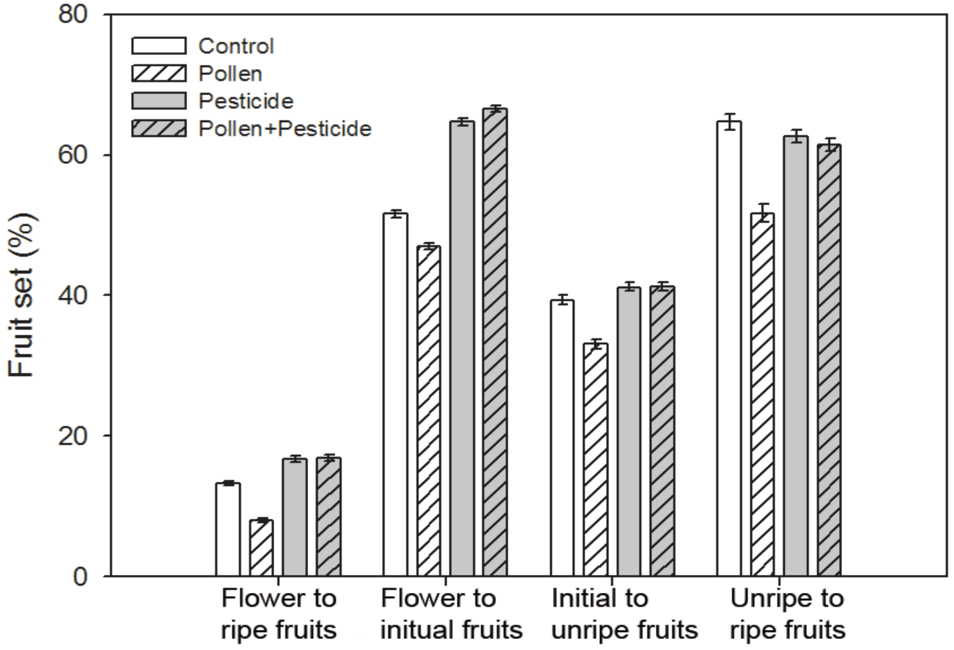


Figure 2. Percentage of fruits remaining in branches at different phenological stages for the experimental treatments (control, pollen addition, pesticide, pollen+pesticide). Bars represent the percentage of fruits relative to the number of flowers or fruits at the beginning of each transition. Total sample size for each developmental stage: flowers = 40,532; initial fruits = 23,473; unripe fruits = 9,128; ripe fruits = 5,614.

Our results supported our first hypothesis relating reproductive success to resource levels. Greater availability of resources allows higher investment in the production of reproductive structures as well as on the later maintenance of fruits and seeds during their development (Obeso 2002; Knight et al. 2005; Pickup and Barret 2012). Fruit set levels were positively correlated with flower production, indicating that the initial investment in reproduction led to greater fruit production in relative rates. Large flower displays may increase visits by pollinators in animal-pollinated plants, thus improving pollination and leading to higher reproductive output (Knight 2003; Kudo and Harder 2005). However, in dioecious wind-pollinated species, such as the Spanish juniper, pollination efficiency is associated with the quantity and quality of male pollen transported by wind. Thus, in wind-pollinated species a higher initial investment in reproductive structures may overcome later uncertainties during pollination, such us adverse weather conditions, and predispersal fruit and seed predation (Stephenson 1981; Ehrlén et al. 2015). The positive effect of the initial investment in reproductive structures was found during the first two transitions, from flower to initial

Table 2. Linear mixed models testing the effect of treatment (pollen addition, pesticide and the interaction between both treatments) and number of flowers per branch on the number of total, full, empty and aborted seeds in ripe fruits. Models include significant fixed effects after simplification of the initial model.SE: Standard error, SD Standard deviation of random factor estimates

	Fixed effectd	**Estimate**	**SE**	***P***	**Random**	**SD**
No. Seeds	Intercept	1.286	0.025	<0.001	Tree	0.078
	N. Flowers	0.056	0.022	0.010		
No. full seeds	Intercept	-1.519	0.179	<0.001	Tree	0.648
	N. flowers	0.247	0.061	<0.001		
	Pollen	0.281	0.122	0.022		
	Pesticide	0.387	0.136	0.004		
No. empty seeds	Intercept	0.972	0.024	<0.001	Tree	0.020
No. aborted seeds	Intercept	-0.450	0.133	<0.001	Tree	0.440
	N. flowers	-0.207	0.077	0.007		
	Pollen	-0.354	0.113	0.002		
	Pesticide	-0.249	0.118	0.035		

fruits and from these to unripe fruits, when most losses occur. The initial production of female flowers did not only increase the proportion and number of ripe fruits but also seed set. The production of fruits with more full and less aborted seeds in trees and branches that produced more flowers suggests the selective abortion of fruits during pollination and fruit predation, and the investment of resources to mature the high-quality fruits (Stephenson 1981; Ayre and Whelan 1989; Meyer et al. 2014; Riba-Hernández et al. 2016). In addition, greater initial investment in flowers reduced the incidence of mites. Mites are specialized seed predators with low dispersal capacity and are thus sensitive to crop variations at both population and individual tree level (Mezquida and Olano 2013). This satiating effect would favor individual variations in crop size, with high investment in flower and fruit production in some years and low in others (Linhart et al. 2014).

Our second hypothesis was not supported; manual pollination of flowers did not increase fruit set and thus pollen was not apparently limited during our experiment. On the contrary, hand-pollination had a negative effect on fruit set levels, and this effect was observed for the three transitions we tested during fruit development from flowers to ripe fruits. Hand-pollination experiments usually have positive or neutral outcomes, leading to either an increase

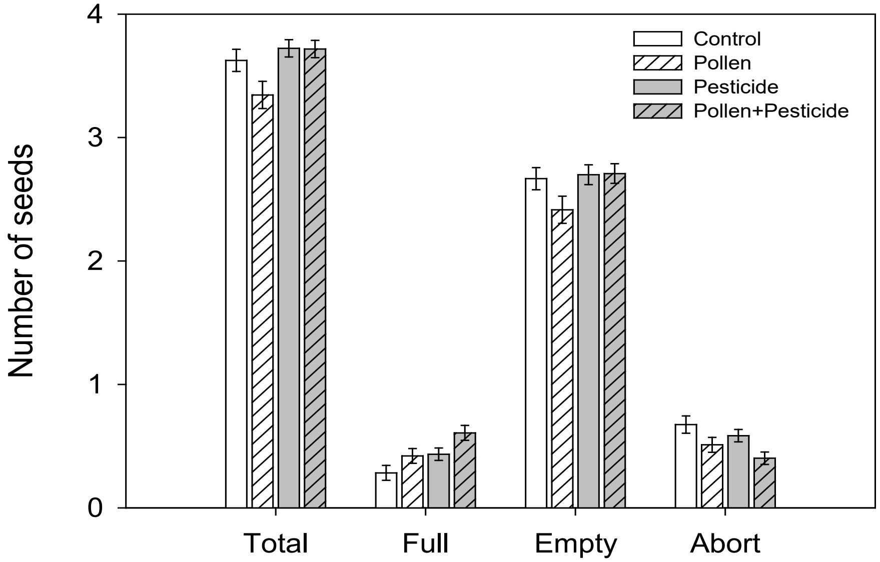


Figure 3. Number of total, full, empty and aborted seeds per fruit for different treatments: control (n = 120 fruits), pollen addition (n = 119 fruits), pesticide (n = 191 fruits), pollen+pesticide (n = 237 fruits). Error bars represent ±1 standard error.

or no change in fruit set or other component of reproductive success (Knight et al. 2005). However, several pollination experiments have found a reduction in reproductive success and several mechanisms have been proposed to explain that decrease (Young and Young 1992). For example, large pollen loads on flowers may have negative effects on pollen tube growth (Cruzan et al. 1986; Acar and Eti 2008). Our second pollination experiment showed that this was not the cause of lower fruit set because fruits developing from hand-pollinated flowers had proportionally more grown pollen tubes than fruits from natural-pollinated flowers. Moreover, hand-pollination had a positive effect on seed set, so that ripe fruits had more viable and less aborted seeds. Other explanations for our results are that manipulation during hand-pollination experiments may harm some flowers and increase abortion rates, and that low diversity of pollen, compared to natural pollination, or excess of pollen and pollen tubes may reduce fertilization (Young and Young 1992). Therefore, pollen did not seem to be limiting during our experiment in this population with a relatively high density of conspecifics, although may be limiting in some years in more open or mixed forests (see chapter 5). Manipulation or excess pollen during hand-pollination caused higher abortion rates or selective abscission of flowers and developing fruits, resulting in lower fruit set levels, although seed viability increased in ripe fruits.

Table 3. Linear mixed models testing the effect of treatment (pollen addition, pesticide and interaction) and flowers per branch on the incidence of mites, moths and chalcid wasps. Models include significant fixed effects after simplification of the initial model. SE: Standard error, SD Standard deviation of random factor estimates

Fruits damaged by	Fxed effects	Estimate	SE	*P*	Random	SD
Mites	Intercept	-3.209	0.321	<0.001	Tree	1.481
	N. flowers	-1.042	0.136	<0.001		
	Pesticide	-0.550	0.179	0.002		
Moths	Intercept	-2.661	0.227	<0.001	Tree	1.040
	N. initial fruits	0.230	0.101	0.023		
	Pesticide	-0.727	0.148	<0.001		
	Chalcid Wasp	-1.643	0.727	0.024		
Chalcid wasps	Intercept	-3.394	0.372	<0.001	Tree	1.690
	N. developing fruits	0.171	0.060	0.005		
	Pesticide	0.523	0.103	<0.001		
	Moths	-3.200	1.512	0.034		
	Mites	-3.355	1.441	0.020		

Our third hypothesis was supported; the spraying of pesticide enhanced fruit set levels and ripe fruits had more viable and less aborted seeds. The mixture of acaricide and insecticide was successful in reducing the incidence of mites and moths, but not chalcid wasps. The pesticide effect was observed soon after its application increasing the proportion of fruits that started to develop, likely affecting mites and perhaps early oviposition by moths. Pesticide treatment was also effective in reducing fruit loss during the oviposition period by moths, when fruits grow and most losses occur. During the last period of fruit development, from unripe to ripe fruits, losses were similar for branches treated with pesticide and untreated ones even though moths also depredate fruits during this phase, although the incidence tends to be lower (E. Rodríguez-García pers. obs., see chapter 2). Seed predation by chalcid wasps was not reduced by the treatment with pesticide, likely because females insert the ovipositor into the fruit to reach the seeds and lay eggs, so that eggs and larvae are not exposed to the pesticide. On the contrary, the incidence of chalcid wasps increased with pesticide treatment. This increase likely resulted from the lower incidence of mites and moths after pesticide application, reducing the interference and competition effect by these earlier predators and increasing the availability of sound fruits (Espelta et al. 2009; Bonal et al. 2010). Overall, pesticide treatment resulted in a greater proportion of fruits ripening

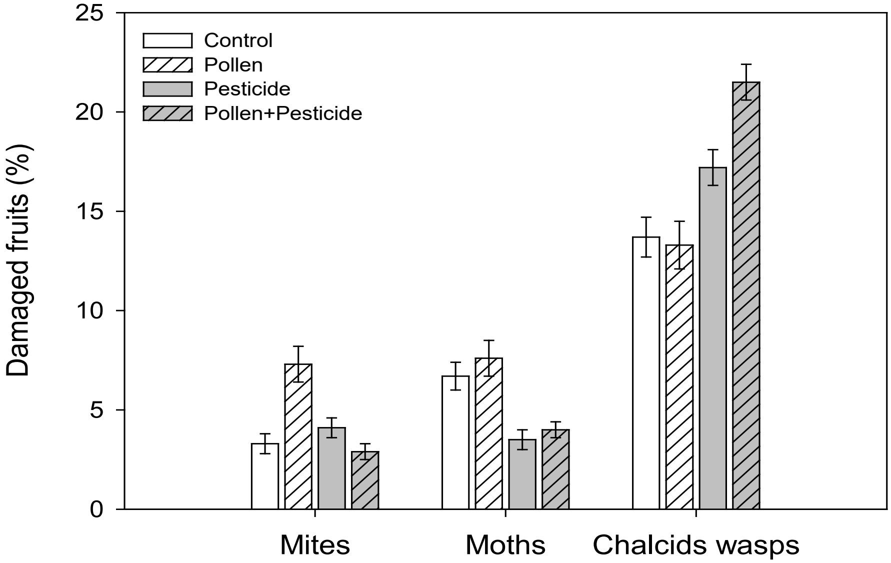


Figure 4. Percentage of fruits damaged by mites, moths and chalcid wasps for different treatments: control (n = 1258 fruits), pollen addition (n = 781 fruits), pesticide (n = 1642 fruits), pollen+pesticide (n = 1933). Error bars represent ±1 standard error.

and more viable seeds per fruit. Lower levels of fruit damage by mites and moths during earlier stages of fruit development, when most abortions occur (Stephenson 1981), under pesticide treatment may have led to selective abscission of damaged fruits or those with low seed numbers and the allocation of resources to high quality fruits (Meyer et al. 2014). Chalcid wasps oviposit when fruits are almost fully grown, but before the seed coat hardens, and fruit abortion is unlikely (Stephenson 1981; Meyer et al. 2014; Boivin et al. 2016), thus the incidence of chalcid wasps increased under pesticide treatment and probably did not decline by selective abortion of attacked fruits (see chapter 2).

In conclusion, we found that in the dioecious wind-pollinated Spanish juniper most reproductive structures are lost at the early stages of reproduction, particularly during the initial growing and development of fruits until they reach their final size by the end of the first summer. Pollination was not limiting in the population and year of study and manual addition of pollen diminished levels of fruits set, although fruits that ripen had more viable seeds suggesting a positive effect of pollination or more resources directed to seed set and development (i.e., a quality component). Predispersal seed predation by different arthropods seems to be an important limiting factor to fruit set in this juniper species. The initial investment in surplus flowers allowed plants to selectively abort damaged and depredated fruits during the early phase of development and allocate resources to the remaining fruits. Resources invested in reproduction had important consequences for fruit and seed set (Montesinos et al. 2012, Mezquida et al. 2016). Production of more flowers reduced the incidence of the low-mobile mites through a satiation effect, increased levels of fruit set and the quality of ripe fruits (having more viable seeds). Seed predators with greater dispersal capacity, however, were attracted to trees producing more fruits, which would favor individual as well as population variations in seed production between reproductive episodes to satiate seed predators, as found in this mast-seeding species (Herrera et al. 1998; Montesinos et al. 2012; Mezquida and Olano 2013).

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CHAPTER 4

Efficiency of pollination and satiation of predators determine reproductive output in Iberian *Juniperus thurifera* woodlands.

"I've watched them dance
To the music that the feeling brings
Then dig a hole
For the music when the feeling's gone"

Maika Makovski

ABSTRACT

Fruit production in animal-dispersed plants has a strong influence on fitness because large crops increase the number of seeds dispersed by frugivores. Large crops are costly, and environmental control of plant resources is likely play a role in shaping temporal and spatial variations in seed production, particularly in fluctuating environments such as the Mediterranean. The number of fruits that start to develop and the proportion of viable seeds produced are also linked to the number of flowers formed and the efficiency of pollination in wind-pollinated plants. Finally, large fruit displays also attract seed predators, having a negative effect on seed output. We assessed the relative impact of environmental conditions on fruit production, and their combined effect on seed production, abortion and seed loss through three predispersal predators in *Juniperus thurifera* L., sampling 14 populations across the Iberian Peninsula. Wetter than average conditions during flowering and early fruit development led to larger crop sizes; this effect was amplified at tree level, with the most productive trees during more favourable years yielding fruits with more viable seeds and less empty and aborted seeds. In addition, large crops satiated the less mobile seed predator. The other two predispersal predators responded to plant traits, the presence of other seed predators and environmental conditions, but did not show a satiation response to the current-year crop. Our large-scale study on a dioecious, wind-pollinated Mediterranean juniper indicates that pollination efficiency and satiation of seed predators, mediated by environmental conditions, are important determinants of reproductive output in this juniper species.

INTRODUCTION

The number of fruits produced by individual fleshy-fruited plants frequently has important consequences for plant fitness (Jordano 1995; Martíınez et al. 2007; Ortiz-Pulido et al. 2007; Sobral et al. 2010). Individuals producing larger crops attract more frugivorous animals and, as a result, more seeds can be dispersed from the parent plant (Laska & Stiles 1994; Ortiz-Pulido et al. 2007; Blendinger et al. 2008). However, plants that produce more fruits, also normally attract more predispersal seed predators (Herrera 1986; Sallabanks & Courtney 1992), which may lower plant reproductive success by reducing the number of healthy fruits available for dispersal (Jordano 1987). Fruit production is also dependent on the number of flowers formed, which sets an upper limit to the number of fruits starting to develop. Moreover, flower production largely determines pollination success and embryo development after fertilisation in wind-pollinated species (Nilsson & Wastljung 1987; Kelly & Sork 2002).

Reproductive investment during years of large fruit crops is costly in terms of energy devoted to reproduction (Obeso 2002), particularly for female plants in dioecious species that have to allocate resources to both flower and fruit production (Obeso 1997; Montesinos et al. 2012). Environmental conditions and resource levels influence reproductive investment at different spatial and temporal scales (Agren et al. 2008). At a geographic scale, climate largely determines inter-population variability in the resources available to plants for growth and reproduction (García et al. 2000; Obeso 2002; Montesinos et al. 2010; DeSoto et al. 2012). At a local scale, temporal variations in environmental (weather) conditions modulate annual reproductive investment by woody perennials (Lee & Bazzaz 1982; Herrera 1991; Crone & Lesica 2006). For species with high fluctuations in reproductive output, environmental conditions are clearly linked to large crops (Kelly & Sork 2002). However, this connection is not straightforward (Kelly & Sork 2002), and temporal variations in reproduction do not entirely match the availability of resources and may also depend on the allocation of reserves to the current seed crop (Koenig & Knops 2000; Crone et al. 2009; Sala et al. 2012; Hoch et al. 2013; Ida et al. 2013). Therefore, the number of viable seeds produced by individual plants is determined by different factors acting during the reproductive cycle, such as environmental conditions, flower and fruit production, the efficiency of pollination and the amount of seed lost to predators, which typically vary among populations and years in many woody perennials.

In this study, we assess the relative impact of environmental conditions on fruit production, and their combined effect on seed production, abortion and seed loss by predispersal predators in the Spanish juniper (*Juniperus thurifera* L.), a long-lived dioecious Mediterranean tree producing berry-like cones (fruits, henceforth). We sampled 14 Spanish juniper populations throughout the Iberian Peninsula, which comprises the largest part of its distribution range (Gauquelin et al. 1999), covering a wide gradient of climate conditions (DeSoto et al. 2012; Tellería et al. 2014). Seed production in the Spanish juniper exhibits large temporal fluctuations (Montesinos et al. 2012; Mezquida & Olano 2013); therefore the geographic scale of our sampling allows us to examine the influence of contrasting climate conditions and crop sizes, as well as to assess the effect of current-year local environmental (weather) conditions for each population.

Seed viability in junipers is usually low due to pollination failure and predation by specific seed predators (Fuentes & Schupp 1998; Chambers et al. 1999; García et al. 2000; Mezquida & Olano 2013). First, in order to address the hypothesis that large crop sizes and/or wetter environmental conditions would positively affect the production of viable seeds as a result of more resources being available for the developing seeds and/or improved pollination efficiency, we quantified individual seed production per fruit and seed viability for each population. Second, we quantified the intensity of fruit damage by three arthropods with different dispersal capacities, and characterised individual fruit traits in all populations, to test whether crop size, environmental conditions, interaction between predator species and fruit traits influence the levels of damage caused by each arthropod. We predicted that less mobile arthropods would be negatively affected by tree crop size (Kelly & Sork 2002; Mezquida & Olano 2013), and that the incidence of each arthropod, particularly those that colonise the fruit early in its development, would negatively influence the incidence of the others (Mezquida & Olano 2013). We also expected that the fruit traits of each tree would affect arthropod incidence, depending on the fruit parts their larvae feed upon during development. To test this, we examined arthropod preferences by considering contrasting crop sizes, rather than just a good crop year, as was the case in a previous study (Mezquida & Olano 2013). Because environmental conditions may directly or indirectly impact these small arthropod species (Ward 1973; Montesinos et al. 2010), we also included average and current- year climate variables for each population as predictors in our models. Finally, we estimated the average number of viable seeds surviving the predispersal period for each population, in order to assess the relative impacts of crop size, environmental conditions during that crop year and seed loss through arthropods on population-level seed output.

MATERIAL AND METHODS

Natural history of the system

The Spanish juniper is a long-lived dioecious tree endemic to the western Mediterranean Basin, with its main distribution range located in the Iberian Peninsula and Morocco. Fruit production in this species is highly variable from year to year, with masting episodes followed by several years of very low to medium levels of fruit production (Montesinos et al. 2012; Mezquida and Olano 2013). Female flowers are wind-pollinated during late winter. After fertilisation, fruits grow until reaching their full size in late summer and then mature for over a year, ripening during the autumn of the year after pollination. During this period, fruits are vulnerable to damage at different stages of their development, largely through the actions of three arthropod species (Roques et al. 1984; Mezquida & Olano 2013). Mites (*Trisetacus quadrisetus*, Acari, Phytoptidae) enter the fruit early after pollination and use the seeds as growth chambers. Seeds used by mites show a characteristic fluted seed wall and a deformed, elongated tip (Roques et al. 1984; Montesinos et al. 2010). Chalcid wasps (*Megastigmus thuriferana*, Hymenoptera, Torymidae) (Auger-Rozenberg et al. 2006) are seed predators that oviposit their eggs into seeds during mid- to late summer when fruits are almost fully grown, but before the seed coat hardens (Roualt et al. 2004). Larvae develop inside seeds for a year, with a diapause period during winter, and adults emerge from fruits leaving a characteristic exit hole (Roques et al. 1984). Moths (*Mesophleps oxycedrella*, Lepidoptera, Gelechiidae and *Pammene juniperana*, Lepidoptera, Tortricidae) oviposit on the fruit surface during the first summer, and their oviposition period overlaps with that for chalcid wasps. Larvae hatch, enter the fruit and develop for about a year while feeding on the pulp and seeds, before leaving the fruit during the ripening phase to pupate in the soil (Roques et al. 1984). Damaged fruits present galleries with faecal pellets from the larva and an exit hole on the fruit surface, which is easily distinguishable from chalcid wasps' emergence holes.

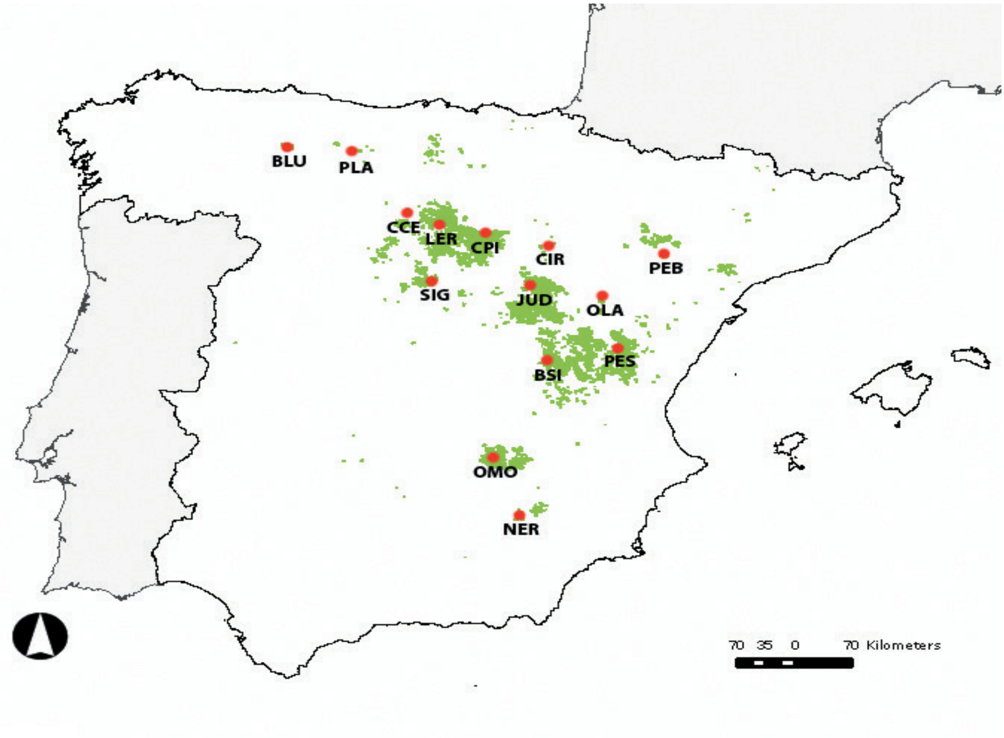

Fig 1. Distribution of Spanish juniper in the Iberian Peninsula and location of the sampled populations. Abbreviations for each population are indicated in Table 1.

Population variation in fruit production and seed set

We estimated fruit production and categorised seeds in each of 14 populations across the whole distribution of Spanish juniper in the Iberian Peninsula (Fig. 1) during early autumn in 2007 or 2008. In each population, we chose 30 female trees at random while walking through the forest, and estimated fruit production using an index of relative fruit abundance. Each tree was scored from 0 to 5 according to its crop size after visually inspecting the whole canopy (Koenig et al. 1994). Fruit crop estimated using this index is reliable and highly related to crop size calculated from fruit counts (Mezquida & Olano 2013).

To characterise fruit traits in each population, we collected ten sound fruits without signs of arthropod damage, again from 30 female trees at each site. Because fruit or seed traits cannot be characterised for trees with no or very few fruits, sampled trees include those with an index of relative fruit abundance of 1 or higher (i.e. excluding the 0 category). In

Table 1. Characteristics of the 14 Spanish juniper populations sampled in the Iberian Peninsula: population (abbreviation), geographic location, elevation, average annual precipitation, drought index (estimated for a 12-month period starting in October before the first year of fruit development using a standardised index, as explained in Material and Methods), sampling year and number of trees sampled

Population	Geogrpahic location	Elevation (m.a.s.l.)	Precipitation (mm)	Drought index	Sampling year	no. of trees
Barios de Luna (BLU)	42°52′ N 5°51′ W	1170	848	-0.550	2008	30
Peña Lampa (PLA)	42°50′ N 4°51′ W	1200	1050	-0.483	2008	28
Cobos de Cerrato (CCE)	42°3′ N 4°0′ W	820	495	0.391	2008	29
Lerma (LER)	41°55′ N 3°31′ W	1090	591	0.285	2008	30
Pina de Ebro (PEB)	41°29′ N 0°15′ W	360	361	-0140	2008	27
Puerto de Escandón (PES)	40°16′ N 0°59′ W	1240	570	1.634	2008	30
Olalla (OLA)	40°57′ N 1°10′ W	1120	511	0.929	2008	28
Ciria (CIR)	41°37′ N 1° 56′ W	1118	515	-0.810	2007	27
Cabrejas del Pinar (CPI)	41°47′ N 2°50′ W	1250	680	-0.285	2008	30
Judes (JUD)	41°7′ N 2°11′ W	1250	566	-0804	2007	29
Sigueruelo (SIG)	41°10′ N 3°39′ W	1140	653	-0.709	2007	30
Buenache de la Sierra (BSI)	40°8′ N 1°57′ W	1360	774	-0.660	2007	30
Ossa de Montiel (OMO)	38°53′ N 2°46′ W	910	448	-0.347	2008	30
Nerpio (NER)	38°7′ N 2°24′ W	1310	466	-0.551	2008	30

some populations, fruit production was very low and we could not obtain 30 trees with enough sound fruits to characterise fruit traits, so the final sample of trees ranged from 27 to 30 (Table 1). For each fruit, maximum length and width were measured in the laboratory to the nearest 0.01 mm with digital calipers and averaged to calculate fruit diameter. Pulp mass and total seed mass were measured to the nearest 0.01 mg with a digital scale after fruits had been oven-dried for >36 h at 60 °C. Seeds were examined individually under a dissecting microscope. For each fruit, we counted the total number of seeds and the number of seeds in each of three categories: 'full', referring to seeds with an undamaged, well-developed embryo; 'empty', fully developed seeds but with an undeveloped or no embryo; and 'aborted', referring to small, not fully developed seeds. For each tree, we calculated the number of seeds per fruit as the average for all fruits, and the total number of full, empty and aborted seeds relative to the total number of seeds in the sample of fruits.

To assess whether the number of seeds per fruit in each population was affected by individual fruit production and/or environmental conditions, we used linear mixed models in which individual trees were nested within population, which was a random factor, and parameters were estimated using restricted maximum likelihood. Fruit production calculated using the average for trees in which we collected fruits (mean ± SE for the 14 populations: 2.5 ± 0.1) was larger than that estimated for each population (1.7 ± 0.1), due to the latter including trees that did not produce fruit. Because both estimates were highly correlated ($r = 0.77$, $P = 0.001$), and the first estimate represents the actual fruit crop for trees in which we gathered fruits, we excluded the estimate of crop size including trees that did not produce fruit from further analyses. We also included in the model two environmental variables representing average climate conditions and weather conditions during the 12 months (November to October) encompassing the period of flowering, pollination and fruit growth of the sampled crop for each population. Annual precipitation for each site was extracted from the climatic atlas of the Iberian Peninsula (Ninyerola et al. 2000), which is based on data from 2285 meteorological stations for a period ranging from 15 to 50 years, depending on the locality. Annual precipitation was square-root transformed to improve normality and used as a predictor in the linear mixed model. Drought stress may be an important factor influencing juniper fruit and seed development (Verheyen et al. 2009), and in order to have an estimate of drought stress for each population during the first year of fruit formation and growth, we used the Standardised Precipitation Evapotranspiration Index. This is a standardised index that estimates the intensity of drought at different temporal scales in relation to normal conditions in each site (see Vicente-Serrano et al. 2010), and thus it is related to local variations in water availability. Positive values of the index indicate wetter than average conditions for a given site independent of average annual precipitation for that site. Data were obtained from SPEIbase version 2.2:1 (http://sac.csic.es/spei/ database.html#p2). This data set provides a 0.5º spatial resolution and a monthly time resolution. We used the value calculated from a 12-month period starting in October before the first year of fruit development. In this way, we can consider the drought tendency of an annual cycle that includes pollination, fruit growth and arthropod oviposition and early larvae development.

We used generalised mixed models to test whether the proportions of full, empty and aborted seeds were influenced by individual fruit production and environmental conditions. Models were similar to those described above, although assumed a binomial error. The dependent variable was the number of seeds in each of the three categories, and the binomial denominator was the total number of seeds (Zuur et al. 2009).

Population variation in fruit damage by arthropods

To quantify fruit damage by arthropods, we collected 30 additional fruits from around the canopy of the same trees for which we had estimated fruit production and characterised fruits. Fruits were inspected and opened in the laboratory under a dissecting microscope to detect signs of damage by arthropods. For each tree, we calculated the number of fruits damaged by mites, chalcid wasps and moths relative to the total number of fruits examined.

To test whether variation in fruit damage by each arthropod is determined by individual fruit production, interaction between arthropods, fruit characteristics of each tee, and/or environmental conditions, we used generalised mixed models with a binomial error structure. Initial parameters in the models were selected according to previous knowledge of the biology of each arthropod. Mites colonise the fruits shortly after pollination, when the cones are starting to grow; hence predictor variables in the mixed model for fruit damage by mites included fruit production per tree and the two environmental variables (annual precipitation and drought index; using the 12-month standardised index explained above), but not interaction with other arthropods or ripe fruit traits (Mezquida & Olano 2013). Population was included as a random factor. Chalcid wasps and moths avoid ovipositing on fruits already damaged by other arthropods, particularly when when there are enough undamaged fruits available (Mezquida and Olano 2013). Consequently, we included the proportion of fruits damaged by mites as explanatory variables in the mixed models for seed predation by chalcid wasps and moths, as well as their mutual interaction. Fruit traits included in the models were: fruit diameter, number of full, empty and aborted seeds, and total seed mass. To avoid multicollinearity, we excluded pulp mass due to its high correlation with fruit diameter ($r = 0.83$, $P < 0.001$). Finally, annual precipitation and drought index were included in both models.

Variation in seed output across populations

We calculated seed output for each tree as crop size x proportion of fruits not damaged by arthropods x number seeds per fruit x proportion of full seeds, and then used these to estimate an average reproductive output for each population. To assess the relationships between drought index, fruit production, fruit damage by arthropods and seed output at the population level, we used correlations between averaged values of these variables for the 14 populations.

RESULTS

Variation in seed number

Overall, seed numbers per fruit ranged from 1.5 to 6.0 seeds (n = 409 trees), while the average number of seeds per fruit in each of the 14 populations ranged from 3.0 to 4.1. The mixed model indicated that individual fruit production was the main predictor of the number of seeds per fruit (Table 2), so that trees that produced more fruits also had more seeds per fruit. The number of seeds per fruit increased linearly from trees with low to those with large crops (Fig. 2), except for the few trees we sampled with the largest crops of all (n = 4 trees). Drought index and annual precipitation had no effect on seed number (Table 2).

Variation in the proportion of full, empty and aborted seeds

The proportion of viable to non-viable seeds was very variable among populations. The lowest proportion of full seeds per fruit was recorded in Ciria (0.07) and the highest in the northern populations of Barrios de Luna and Peña Lampa (0.52 and 0.51, respectively). Barrios de Luna and Peña Lampa also had a low proportion of empty seeds (0.38, in both populations), while Ciria had the highest proportion (0.78). The proportion of aborted seeds per fruit ranged from 0.06 in Puerto de Escandón to 0.21 in Nerpio.

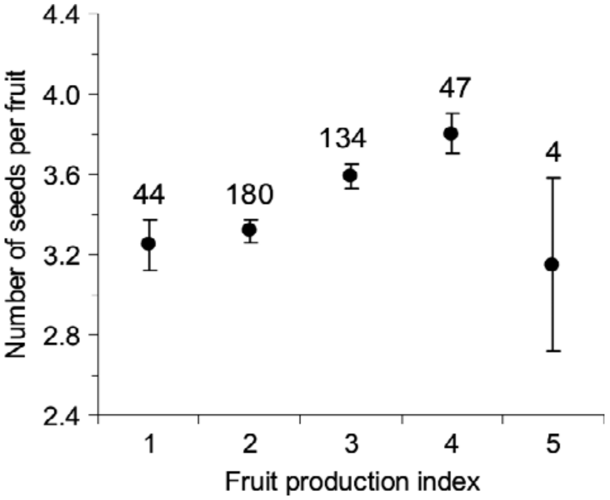

Fig. 2. Number of seeds per fruit for Spanish juniper trees (n = 409) producing diverse crop sizes (as estimated by an index of relative fruit abundance) in 14 populations in the Iberian Peninsula. Numbers above bars indicate the sample of trees for each index of fruit production.

Table 2. Estimates and SE from mixed models for number of seeds per fruit and the proportion of full, empty or aborted seeds. Significant P-values are shown in bold. The SD of the population effect on the estimates of the intercept as random factor is also shown

	Fixed effects	Estimate	SE	*P*	Random	SD
Number of seeds per fruit	Intercept	3.583	0.608	**<0.001**	Population	0.273
	Fruit production per tree	0.128	0.043	**0.003**		
	Drought index	0.202	0.121	0.122		
	Annual precipitation	-0.017	0.024	0.502		
Proportion of full seeds	Intercept	-2.917	1.253	**0.020**	Population	0.625
	Fruit production per tree	0.126	0.025	**<0.001**		
	Drought index	0.499	0.250	**0.046**		
	Annual precipitation	0.074	0.051	0.149		
Proportion of empty seeds	Intercept	1.564	0.913	**0.087**	Population	0.453
	Fruit production per tree	-0.065	0.023	**0.005**		
	Drought index	-0.157	0.182	0.387		
	Annual precipitation	-0.051	0.037	0.173		
Proportion of aborted seeds	Intercept	-1.120	0.596	**0.060**	Population	0.278
	Fruit production per tree	-0.099	0.034	**0.003**		
	Drought index	-0.484	0.120	**<0.001**		
	Annual precipitation	-0.024	0.024	0.315		

Mixed models indicated that fruit production per tree was a significant predictor of the proportion of full, empty and aborted seeds (Table 2). Trees that produced more fruits had a higher proportion of full seeds and lower proportion of empty and aborted seeds (Table 2, Fig. 3). In addition, drought index had a significant effect on the proportion of full and aborted seeds. Drier than average conditions during the first year of fruit formation and growth (i.e. low values of the drought index) significantly increased the proportion of aborted seeds and decreased the proportion of full seeds per fruit (Table 2).

Variation in fruit damage by arthropods

Fruit damage by arthropods was varied highly among populations (see Appendix S1). Levels of fruit damage by mites ranged from 6.4% in Puerto de Escandón to 22.5% in Ciria (Appendix S1). Seed predation by chalcid wasps was lowest in Cabrejas del Pinar (3.3%) and highest in Pina de Ebro (29.7%; Appendix S1). Moths were the main predispersal predators in most populations, with predation rates ranging from 9.8% in Judes to 52.% in Peña Lampa (Appendix S1).

Mixed models showed that fruit damage from mites was negatively influenced by individual fruit production and by wetter than average conditions during the first year of fruit formation, and marginally so with higher annual precipitation (Table 3). Seed predation by chalcid wasps was negatively affected by the incidence of the other two arthropods at the same tree, and increased with fruit production per tree (Table 3). Chalcid wasps preferred trees that produced smaller fruits with more aborted and also heavier seeds, but they predated less in sites with higher average annual precipitation (Table 3). Moths preferred trees with larger fruits and heavier seeds, and avoided trees with higher levels of fruit damaged by mites and chalcid wasps (Table 3).

Seed output across populations

Population-level crop sizes for the surveyed populations were generally medium to low, with large crops occurring in just two populations (e.g. Cobos del Cerrato and Lerma, with an average index of relative fruit abundance >3), as expected for a species with important

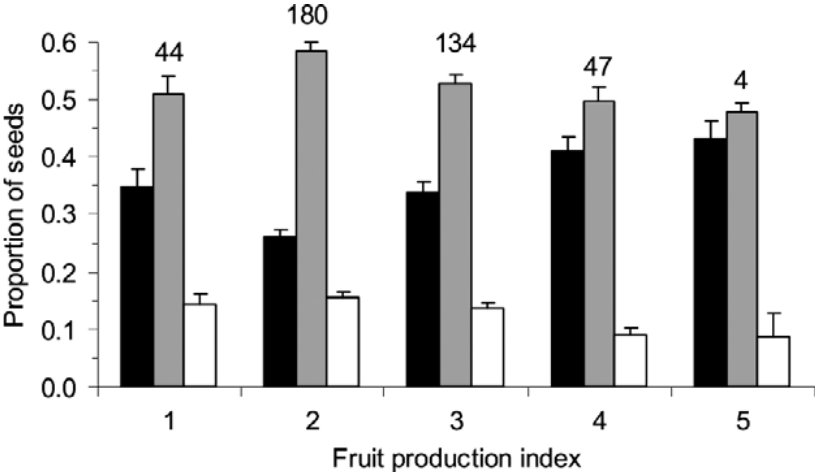

Fig. 3. Proportion of full (black), empty (grey) and aborted (white) seeds per fruit for Spanish juniper trees (n = 409) producing diverse crop sizes (as estimated by an index of relative fruit abundance) in 14 populations in the Iberian Peninsula. Error bars represent 1 SE. Numbers above columns indicate the sample of trees for each index of fruit production.

inter-annual fluctuations in seed production. Fruit crop size at the population level was positively correlated with drought index (r = 0.70, P = 0.005, n = 14 populations), with wetter than average conditions for each site leading to larger crops. Larger crops, in turn, led to increased population-level seed output (r = 0.84, P < 0.001; Fig. 4). Seed output for the 14 populations showed a significant negative relationship with damage by mites (r = -0.76, P = 0.001), but no relationship with seed predation by chalcid wasps (r = -0.39, P = 0.17) or fruit damage by moths (r = 0.05, P = 0.86).

DISCUSSION

Production of viable seed in Spanish juniper in the Iberian Peninsula was highly influenced by fruit crop size and environmental conditions during the year of flowering and initial fruit formation. Fruit damage levels by arthropods responded to multiple factors, including crop size, fruit traits, environmental conditions for each population and interactions between arthropods. Overall, wetter than average conditions during early fruit development led to larger crops, which in turn led to increased population-level seed output and lower levels of fruit damage by mites, the least mobile of the three arthropods. These results suggest that female trees that invest more in reproduction during years with less water stress increase both their seed set and seed viability.

Table 3. Estimates and SE from generalised mixed models for the incidence of mites, chalcid wasps and moths. Significant P-values are shown in bold. The SD of the population effect on estimates of the intercept as random factor is also shown

Fruits damaged by	Fixed effects	Estimate	S.E.	*P*	Random	SD
Mites	Intercept	-0.657	0.598	0.272	Population	0.277
	Fruit production per tree	-0.090	0.036	**0.012**		
	Drought index	-0.413	0.120	**<0.001**		
	Annual precipitation	-0.047	0.024	0.051		
Chalcid wasps	Intercept	2.809	1.219	**0.021**	Population	0.543
	Mites	-1.392	0.245	**<0.001**		
	Moths	-0.734	0.195	**<0.001**		
	Fruit production per tree	0.074	0.036	**0.040**		
	Fruit diameter	-0.125	0.055	**0.022**		
	no. full seeds	-0.033	0.058	0.570		
	no. empty seeds	-0.011	0.050	0.825		
	no. aborted seeds	0.178	0.068	**0.009**		
	Seed mass	4.006	1.355	**0.003**		
	Drought index	-0.166	0.221	0.453		
	Annual precipitation	-0.158	0.046	**<0.001**		
Moths	Intercept	-2.298	1.312	**0.080**	Population	0.625
	Mites	-1.021	0.172	**<0.001**		
	Chalcid wasps	-0.753	0.199	**<0.001**		
	Fruit production per tree	-0.036	0.026	0.172		
	Fruit diameter	0.100	0.040	**0.012**		
	no. full seeds	-0.075	0.043	0.078		
	no. empty seeds	0.029	0.037	0.432		
	no. aborted seeds	0.032	0.054	0.553		
	Seed mass	2.106	1.001	**0.035**		
	Drought index	0.165	0.251	0.511		
	Annual precipitation	0.023	0.051	0.647		

Crop size for each population was positively correlated with more favourable local environmental conditions (i.e. wetter than average conditions during the period that included flowering, pollination and initial fruit formation), but not to mean local conditions. This result is not unexpected because good environmental conditions are commonly related to higher reproductive effort in woody perennials (Kelly & Sork 2002), and temporal variations within populations are likely to be dictated by available reserves (Koenig & Knops 2000; Crone et al. 2009). For instance, in the Spanish juniper, experimenta monthly addition of water

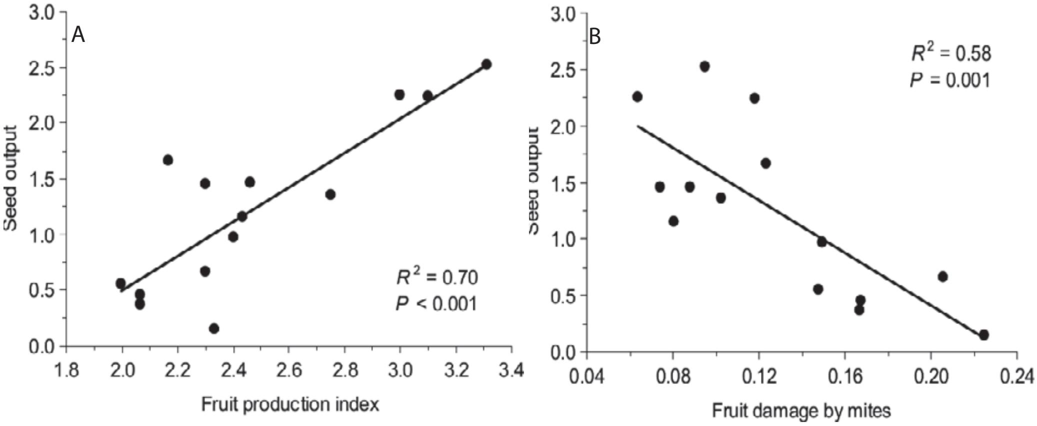

Fig. 4. Seed output estimated for 14 Spanish juniper populations in the Iberian Peninsula in relation to (A) crop size and (B) rate of fruits damaged by mites.

and nutrients to female trees from pollination to fruit ripening resulted in larger tree crops (Montesinos et al. 2012). Larger crops during favourable years were also associated with a higher number of seeds being set (a higher proportion of full seeds and lower abortion rates). This pattern was also observed in individual trees; trees that produced more fruit had fruits with more viable seeds and less empty and aborted seeds. Thus, the combined positive effect of large crops at the population and individual level on seed viability suggests that more efficient pollination combined with the availability of resources and reserves played a role in improving tree fitness.

Pollination failure is a significant cause of seed loss in conifers, particularly in dioecious wind-pollinated species, such as Spanish juniper (García et al. 2002; Gruwez et al. 2013), and many junipers and other Cupressaceae regularly produce high proportions of non-viable seeds (Fuentes & Schupp 1998; García et al. 2000; Wesche et al. 2005; Rumeu et al. 2009). Empty seeds are externally similar to full seeds and correspond to seeds aborted during or immediately after fertilisation (Gruwez et al. 2013). Causes for the production of empty seeds include lack of pollination (quantity) or problems with fertilization (quality), so that less empty seeds per fruit in trees with larger crop sizes would suggest a more efficient pollination. Our results suggest that larger crops were also associated with an increase in the number of viable seeds and a reduction in the number of aborted seeds, which is in agreement with the pollination efficiency hypothesis (Nilsson & Wastljung 1987).

Alternatively, seed abortion after fertilisation may be the result of selective allocation of resources to the developing seeds (Obeso 2004). Seed set may be limited by resources and reserves available to seed development in woody perennials (Koenig & Knops 1998; Crone et al. 2009; Ida et al. 2013). Indeed, overproduction of flowers and subsequent abortion of fruits and seeds is a frequent phenomenon (Obeso 2004). For example, 75% of Spanish juniper fruits that began to develop and grow were lost after the first summer (E. Rodríguez, personal observation) and < 6 % of the initial crop finished the ripening period (Tellería et al. 2011). Our results indicate that good local environmental conditions drove larger crops and that individuals producing more fruits showed increased pollination success and were likely to allocate more resources and reserves to fruits and seeds (leading to more viable seeds per fruit). In short, environmental conditions (resources) modulated crop size that, in turn, determined seed viability (pollen limitation), coupled with maternal resources for fruit development (resource limitation).

Damage by arthropods differed by a factor of 2.5 across the 14 populations, with even larger variations when damage by individual arthropod species was assessed. Variability was higher among trees within populations (see also Roques et al. 1984), and there was a strong relationship between individual tree characteristics and levels of fruit and seed damage by arthropods. Mites are important agents of seed damage in Spanish juniper (Roques et al. 1984). We found that populations producing larger crops during wetter than average years showed a lower proportion of fruits damaged by mites, and that the incidence of mites was negatively correlated with the number of viable seeds available for dispersers. Thus, satiation of mites at the population level is presumably a significant determinant of female reproductive output, as predicted by the predation satiation hypothesis (Janzen 1971; Nilsson and Wastljung 1987). The satiation effect is enhanced in large crops that follow years of low fruit production (Turgeon et al. 1994; Poncet et al. 2009; Mezquida & Olano 2013), so predator satiation would be better explored by examining the temporal functional response of the different predators (Linhart et al. 2014). The satiation effect may also occur at the scale of individual trees for specialised seed predators with low dispersal capacity, such as mites (Nilsson & Wastljung 1987; Linhart et al. 2014). In fact, we found that individual trees were able to satiate mites by producing more fruit, which would favour individual variability in seed production (Linhart et al. 2014). In support of satiation at this small scale, we previously demonstrated the influence of crop size on fruit damage by mites at the scale of individual trees, and consistent temporal variations in relative rates of fruit damage within individuals (Mezquida & Olano 2013).

Chalcid wasps did not show a functional response to population-level crops, but responded to crop size, fruit characteristics and the incidence of other arthropods on individual trees. Trees with abundant fruits attracted ovipositing female chalcid wasps, as is common in insect frugivores (Jordano 1987; Sallabanks & Courtney 1992). These small wasps are seed predators whose larvae develop inside juniper seeds so, after finding a tree, females avoid ovipositing in fruits already attacked by mites or moths. The avoidance of fruits occupied by other arthropods is stronger within large crops, when there are more undamaged fruits available to choose from (Mezquida and Olano 2013). Female chalcid wasps insert the ovipositor into the fruit to reach the seeds and lay eggs when fruits are almost fully grown (Roques et al. 1984; see chapter 2), and it follows that seeds inside smaller fruits (particularly those with less pulp) would be easier to reach by ovipositing females. Seed mass per fruit is largely determined by the number and size of full seeds, so the preference of chalcid wasps is likely to be for trees that have fruits with large, viable seeds, as they would logically improve larval development and survival rates (Napela & Grissell 1993; Fidgen et al. 1998). Finally, at a geographic scale the incidence of chalcid wasps was lower for populations with higher average annual precipitation, suggesting a direct impact of climate conditions on these small seed predators (Poncet et al. 2009; Montesinos et al. 2010).

As with chalcid wasps, the incidence of moths was not related to local crops nor to population reproductive output, yet showed preferences for certain fruit traits and the absence of other arthropods in individual trees. Fruit damage by moths increased in trees having larger fruits with heavier seeds, which is consistent with our earlier findings in a juniper woodland producing a large crop (Mezquida & Olano 2013). Moth larvae develop by feeding on the pulp and seeds before pupating in the soil, so larger fruits would be preferred in order to provide more resources for the larvae, increasing their chances of survival (Sallabanks & Courtney 1992; Desouhant et al. 2000). Ovipositing female moths seemed to avoid juniper trees with fruits already damaged by mites and chalcid wasps, as previously observed (Mezquida & Olano 2013).

The Spanish juniper is a masting species that produces significant seed crops once or twice every 10 years (Montesinos et al. 2012). Therefore, crop size figures recorded for the 14 populations we surveyed were only a 1-year snapshot of the inter-annual variation in fruit production (Montesinos et al. 2012; Mezquida & Olano 2013; Tellería et al. 2014). However, they provide a clear picture of the main factors determining reproductive output in this juniper. The increase in reproductive output in large crops seemed to be caused by a combination of pollination efficiency (quantity and quality components; Ida et al. 2013) and more resources being devoted to fruit development, resulting in more viable and less empty and aborted seeds. Environmental conditions modulated fruit production in female trees, although reserves accumulated during previous years are probably also important (Kelly & Sork 2002; Montesinos et al. 2012). There was a positive relationship between fruit production, at both tree and population level, and seed set, and large crops produced a satiation effect of the low-mobile predispersal predators (mites), thus leading to a positive feedback on their seed output before dispersal. The other two predispersal seed predator arthropods did not show this satiation effect as a consequence of their ability to cope with variable fruit crops through prolonged diapause (Turgeon et al. 1994), although their incidence can decrease after several years of no or low fruit production (Mezquida & Olano 2013). Our study supports the assertion that large crops during favourable local environmental conditions are associated with increased efficiency of pollination and the satiation of seed predators, which are important determinants of reproductive output in this dioecious, wind-pollinated juniper, inhabiting fluctuating environments (Kelly & Sork 2002; Crone et al. 2009; Montesinos et al. 2012).

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SUPPLEMENTARY MATERIAL

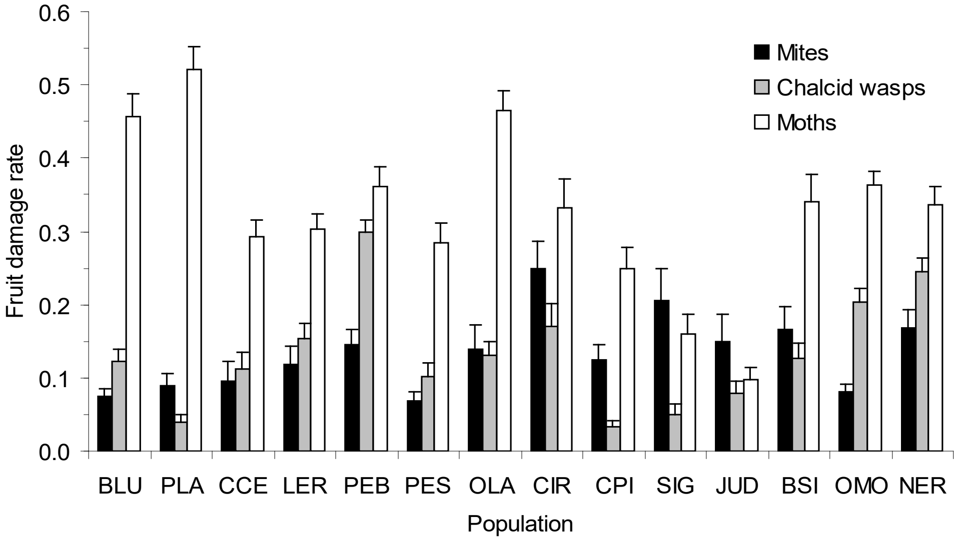

Fig. S1. Fruit damage rates by three predispersal pulp and seed predators for 14 Spanish juniper populations in the Iberian Peninsula. Error bars represent one standard error. BLU: Barrios de Luna; PLA: Peña Lampa; CCE: Cobos de Cerrato; LER: Lerma; PEB: Pina de Ebro; PES: Puerto de Escandón; OLA: Olalla; CIR: Ciria; CPI: Cabrejas del Pinar; JUD: Judes; SIG: Sigueruelo; BSI: Buenache de la Sierra; OMO: Ossa de Montiel; NER: Nerpio.

CHAPTER 5

You'd better walk alone: Changes in forest composition affect pollination efficiency and predispersal fruit damage in Iberian *Juniperus thurifera* forests.

"Déjame a solas
con mi sombra
que no tengo hueco
para nada
ni nadie más."

Javier Gallego Crudo

ABSTRACT

Changes in land use patterns are a major driver of global environmental change. In developed countries, cessation of traditional management practices led to forest expansion and shifts in forest composition: traditionally managed monospecific forests moved towards mixed forests. However, there is a scarce knowledge on how the presence of other tree species will affect reproduction of formerly dominant species. We explored this question in the wind-pollinated tree *Juniperus thurifera*. We hypothesized that the presence of heterospecific trees would have a negative effect on pollination due to pollen interference, however they would lead to a reduction in specialized fruit predators, and consequently also have a positive effect on reproduction. We assessed the relative importance of forest composition on fruit production, seed development and predispersal fruit damage on nine paired pure and mixed *J. thurifera* forests in three regions across the Iberian Peninsula. The effects of forest composition on crop size, fruit and seed characteristics, and damage by predispersal arthropods were tested by mixed models. Fruit production was lower and seed abortion higher in mixed forests, suggesting higher pollination failure. In contrast, fruit damage by arthropods was higher in pure forests, supporting the hypothesis that the presence of non-host plants reduces damage rates. Arthropods response to forest composition was species-specific, and relative damage rates varied depending on individual tree crops. Overall, enhanced crop size in pure forests compensated for increased damage rates, leading to greater net production of sound seeds. This study indicates that ongoing changes in forest composition after land abandonment may impact tree reproduction.

INTRODUCTION

Land use change is impacting on major ecosystem services such as climate regulation (Hansen et al. 2001, Foley et al. 2005) and the carbon cycle (Rey Benayas et al. 2007). Forest conversion into croplands and pasture is one of the most significant change in land use across the globe, particularly in non-developed countries (Gibbs et al. 2010, Phelps et al. 2013). However, the opposite pattern is occurring in developed countries, where urbanization and agricultural intensification are leading to the abandonment of low productivity lands and cessation of traditional management practices (Rey Benayas et al. 2007, Valladares et al. 2014). As a result, secondary succession is altering large tracts of land (Rey Benayas et al. 2007, Gimeno et al. 2012a), open areas encroachment (DeSoto et al. 2010, Ewers et al. 2013), the expansion of woodlands into abandoned lands (Gimeno et al. 2012b), forest densification (Rey Benayas et al. 2007, Améztegui et al. 2010), modification of perturbation patterns (Rey Benayas et al. 2007, Valladares et al. 2014) and changes in forest composition (Hansen et al. 2001, Chauchard et al. 2007, Olano et al. 2012, Vayreda et al. 2016). These changes are driving shifts in the abundance of different community components and ecosystem processes (Laiolo et al. 2004, Sirami et al. 2008, Herrando et al 2016).

Changes in forest structure and composition after abandonment modify competition levels among adult trees (Gimeno et al. 2012b, Vayreda et al. 2016). On the one hand, forest densification increases competition among conspecifics (Kenkel 1988, Getzin et al. 2006, Wang et al. 2016), while at the same time the colonization of forests by other woody species can intensify interspecific competition (Costa et al. 1997, Montesinos and Fabado 2015). Secondly, forest regeneration and the colonization of abandoned lands can enlarge the area covered by forest, thus reducing fragmentation and improving reproductive success by more effectively attracting animal pollinators and seed dispersers (Santos and Tellería 1994, González-Varo et al. 2009). Furthermore, increased tree density in formerly open forests or woodlands could also enhance reproductive success in wind-pollinated species due to a higher density of conspecifics that both increases pollen load and reduces the necessary pollen dispersal distances (Broadhurst 2015). However, a higher density of trees and fruits may also attract predispersal seed predators that can severely reduce the number of sound seeds before dispersal (Sholes 2008; Guyot et al. 2016). Nevertheless, these potential effects of forest densification could be different if tree density increase is driven by a higher frequency of heterospecifics, leading to mixed forests. The canopy of other tree species could hinder pollen dispersal in wind-pollinated species, whereas the presence of pollen from other species with overlapping flowering phenology could lead to reduced pollination efficiency and seed set (Mugnaini et al. 2007, Aderkas et al. 2012, Millerón et al. 2012). Conversely, fruit damage by predispersal predators in mixed forests might be lower than in pure, monospecific forests if the presence of heterospecifics prevents specialized fruit predators from finding their host plants due to physical or chemical interference from non-host plants (Sholes 2008, Barbosa et al. 2009).

In this study, we assess the consequences of changes in forest structure and composition on the reproduction of *Juniperus thurifera* L., an evergreen conifer tree endemic to the western Mediterranean. Rural exodus and changes in traditional management practices are driving the colonization of abandoned croplands and livestock pastures by *J. thurifera* within its distribution range (Rozas et al. 2008, Pías et al. 2014). Concurrently, the reduction in livestock density and grazing pressure is favoring colonization by oak and pine species that were historically scarce due to their lower tolerance of browsing (DeSoto et al. 2010). As a result, *J. thurifera* forests are rapidly shifting from pure, monospecific open forests to mixed, denser forests, over large geographical scales (Olano et al. 2012).

Our aim was to explore the impact of the transition from pure to mixed forests on *J. thurifera* reproductive success. We hypothesized that lower pollen load and greater interference from heterospecifics in mixed forests would lead to lower production of berry-like cones (fruits hereafter) and higher seed abortion rates. Contrastingly, we expected that mixed forests would experience lower levels of fruit damage, due to the increasing difficulty of predators to find host plants. However, this outcome may depend on the biology and dispersal capacity of the predator species (Barbosa et al. 2009). Finally, we assessed the combined effects of pollination success and predispersal fruit damage in order to evaluate the overall reproductive output of J. *thurifera* in pure and mixed forests.

MATERIAL AND METHODS

Study area and sampling design

We sampled *J. thurifera* forests in three regions in the Iberian Peninsula in Guadalajara, Segovia and Soria provinces (Fig. 1). The climate in these regions is classified as continental Mediterranean with mean annual temperatures of 10-11 ºC and mean annual precipitation ranging from 530 to 740 mm, with a two-month long drought in summer (Table 1). Lithology is calcareous in Guadalajara and Soria and granitic in Segovia. Within each region, we selected three populations and in each population, we sampled two forests differing in composition: monospecific (hereafter pure) and mixed.

A forest was considered pure if *J. thurifera* comprised more than 90 % of the tree stems and mixed if the proportion of juniper stems was below 50 %. To set cleared differences in the effect of composition, we deliberately avoided forests in which the proportion of juniper stems is between 51 and 89%. Three oak (*Quercus*) and two pine (*Pinus*) species were the other main tree species present in mixed juniper forests (Table 1). In each case, paired forests were located in the same mountain range and separated by between 500 and 1000 meters. Therefore, sampling design followed a double nested design with forest nested in population and population nested in region (Fig. 1, Table 1).

Study species

Juniperus thurifera is a long-lived dioecious tree, growing under a continental Mediterranean climate in Spain and Morocco, with smaller populations in France, Italy and Algeria. Female flowers are wind-pollinated; they flower during February-March, and fertilization takes place in March. Fruits grow until reaching their full size in September and then mature for over a year, ripening in October of the second year after pollination. Although a wide array of arthropods may feed on maturing *J. thurifera* fruits (Roques et al. 1984), predispersal fruit damage in the Iberian Peninsula is mainly driven by three arthropod taxa (Mezquida et al. 2016). A mite species (*Trisetacus quadrisetus*; Acari, Phytoptidae) uses *Juniperus* spp. seeds as growth chambers where the colony feeds and reproduces (Roques 1984, El Alaoui et al. 2013). Chalcid wasps (*Megastigmus thuriferana*; Hymenoptera, Torymidae) are seed

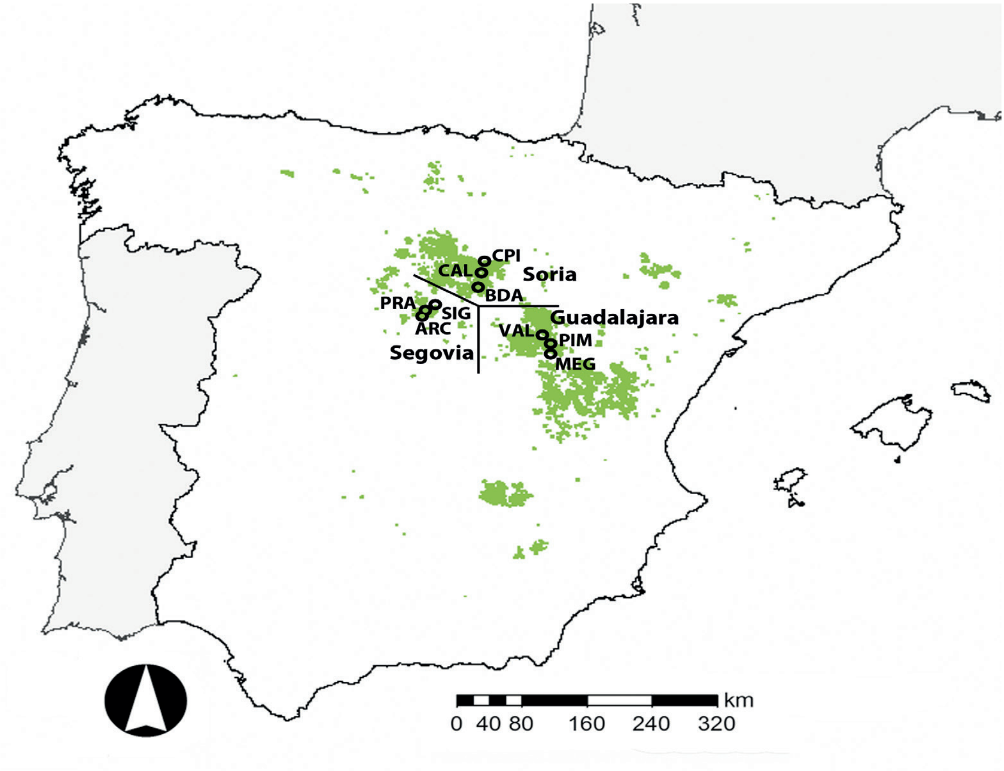

Fig. 1 Distribution of *Juniperus thurifera* sampled populations and regions. *J. thurifera* distribution is represented in green. Abbreviations for each population appear in Table 1

predators that only oviposit eggs inside developing seeds of *J. thurifera* before the seed coat hardens (Rouault et al. 2004, Auger-Rozenberg et al. 2006). Finally, two moth species (*Mesophleps oxycedrella*; Lepidoptera, Gelechiidae; and *Pammene juniperana*; Lepidoptera, Tortricidae) are pulp and seed eaters of *Juniperus* spp. fruits, and are common in *J. thurifera* (Roques et al. 1984).

Fruit production and seed set

We estimated fruit production for each forest by randomly selecting 30 female trees selecting the females present along a linear transect in 2014 early autumn just prior to full ripening. Fruit production per tree was estimated after visually inspecting the whole canopy (Koenig et al. 1994) using an index of relative fruit abundance that ranged from 0 (no fruits) to 5 (very high fruiting). This qualitative test has been tested against fruit abundance measurements based on fruit counts showing high correlation (Koenig et al. 1994; Mezquida and Olano

Table 1. Characteristics of the nine *Juniperus. thurifera* populations sampled in the Iberian Peninsula: region, population (population abbreviation), geographic location, elevation, annual mean temperature, average annual precipitation and accompanying species in mixed forests.

Region	Population	Position	Elevation (m)	Annual mean T (ºC)	Precipitation (mm)	Accompanying species
Guadalajara	Megina (MEG)	40° 38′N 1° 54′W	1250	10	740	*Q. ilex, Q. faginea, P. nigra*
	Pinilla de Molina (PIM)	40° 40′N 1° 52′W	1370	10	648	*Q. ilex*
	Vahermoso (VAL)	40° 47′N 1° 56′W	1150	10	606	*P. pinaster*
Segovia	Arcones (ARC)	41° 6′N 3° 42′W	1200	11	653	*Q. pyrenaica, F. angustifolia*
	Prádena (PRA)	41° 7′N 3° 40′W	1250	10	739	*Q. pyrenaica, I. aquifolium*
	Siguero (SIG)	41° 10′N 3° 37′W	1100	11	694	*Q. pyrenaica, F. angustifolia*
Soria	Calatañazor (CAL)	41° 41′N 2° 48′W	1050	10	637	*Q. ilex*
	Cabrejas del Pinar (CDP)	41° 46′N 2° 50′W	1100	10	691	*Q. ilex, P. pinaster, P. sylvestris*
	Bayubas de Abajo (BDA)	41° 30′N 2° 54′	950	11	530	*P. pinaster*

2013). An advantage of this index allows to compare is its potential to compare tree fruiting effort irrespective of tree size. When available, we collected 40 ripe fruits from each tree by sampling all around the canopy. Fruit production was very low in Bayubas de Abajo (Soria province; Table 1), so this population was excluded from further analyses.

Seed and fruit traits were characterized at forest level for each of the 16 remaining forests by analyzing 10 fruits per tree in a subsample of eight randomly selected trees. For each fruit, maximum length and width were measured in the laboratory to the nearest 0.01 mm with a digital caliper and averaged to calculate fruit diameter. Fruits were opened to separate the seeds and pulp mass was measured to the nearest 0.01 mg with a digital scale after it had been oven-dried for 48 hours at 60ºC. Seeds were characterized according to the development stage of the megagametophyte. Every seed was examined under a dissecting microscope and assigned to one of the following categories according to Gruwez et al. (2013): 'full', for seeds showing a completely developed embryo and megagametophyte; 'empty', for seeds that interrupted the development of the megagametophyte and/or embryo after fertilization, and that are externally similar to fully developed seeds although they do not contain (or only some remnants of) the megagametophyte or embryo; and 'aborted', for seeds that interrupted their development between pollination and fertilization, and that are visually recognizable as small, not completely developed seeds. Parthenocarpic seeds have been described for junipers (Fuentes & Schupp 1998, García et al. 2000), however they can abort megagametophyte an embryo development at different stages along seed maturation process (Gruwez et al. 2013) making difficult to differentiate from those truly parthenocarpic at mature stage.

Predispersal fruit damage

In order to estimate rates of fruit damage by arthropods, for each tree we used 30 fruit samples (when available). When less than 30 ripe fruits were available, we estimated fruit damage rates whenever there was a minimum of 20 fruits. Forests within Cabrejas del Pinar (Soria) population were excluded from these calculations due to the low fruit availability in the mixed forest; analyses are therefore based on seven paired forests. Fruits were opened and examined in the laboratory under a dissecting microscope to detect signs of damage by arthropods. We counted the number of fruits damaged by mites, chalcid wasps and moths relative to the total number of fruits per tree. Signs of fruit damage can be easily assigned to each of the three arthropod groups (Roques et al. 1984, Mezquida and Olano 2013): mites deform the seeds causing the elongation of their tips that usually stick out of the fruit surface, chalcid wasps make a circular hole in the apical region of the fruit, and moths make an irregular hole in different parts of the fruit surface.

Seed output

We calculated total seed output for each tree as the result of multiplying fruit production x proportion of fruits not damaged by arthropods x number of seeds per fruit x proportion of full seeds. This calculation included seed traits, and thus seed output could only be estimated for the eight randomly selected trees for each forest (i.e., 128 trees from 16 forests). This index of seed output provides an estimate of reproductive output per individual (Mezquida et al. 2016).

Statistical analysis

We used linear mixed models to assess whether the type of forest (pure or mixed) influenced juniper fruit production and fruit traits. The random components of these and subsequent models consisted in population and population nested within region. We tested for differences in two fruit traits (diameter and pulp mass) that could covary with seed traits and influence fruit damage by predispersal predators (Mezquida and Olano 2013, Mezquida et al. 2016). Moreover, fruit production may affect seed production and seed set by influencing pollination success and resource investment (Mezquida et al. 2016), so we included the index of fruit production as a covariate in the mixed models for total number of seeds and number of full, empty and aborted seeds.

We used generalized mixed models with a binomial error structure to test for the effect of forest type on arthropod fruit damage. Fruit production for each tree was included as a covariate (Mezquida et al. 2016) as well as the interaction between fruit production and forest type. Since chalcid wasps and moths tend to avoid ovipositing on fruits already damaged by other arthropods (Mezquida and Olano 2013), we included the proportion of fruits damaged by each of the other arthropods as explanatory variables in the models for chalcid wasps and moths. Difference in total seed output between pure and mixed juniper forests were evaluated with a generalized mixed model with a Poisson error structure. Values were previously rounded to the nearest integer.

A similar procedure was followed for all mixed models. We included population nested in region as random factor in all models. The fixed part of the mixed model was then selected by sequentially removing non-significant terms (Zuur et al. 2009). Because our study was focused on hypothesis testing, we used the Bayesian information criterion instead of the Akaike information criterion (Aho et al. 2014). Model calculations were performed using the nlme package in R environment (R Development Core Team, 2014).

RESULTS

Fruit production was medium-low in all forests, with only three forests in the Guadalajara region having an average index of fruit production greater than 2 (Table S1). The mixed model indicated that fruit production was greater in pure than in mixed juniper forests (mean fruit production index ± SE, pure: 1.41 ± 0.07; mixed: 0.95 ± 0.05, n = 283 trees; Table 2, Table S1). There was no effect of forest type on fruit size or the amount of pulp (fruit diameter, pure: 9.57 ± 0.09 mm; mixed: 9.75 ± 0.10 mm, n = 128; pulp mass, pure: 0.24 ± 0.01 g; mixed: 0.25 ± 0.01 g, n = 128; Table 2). The total number of seeds per fruit did not differ between pure and mixed forests (pure: 3.46 ± 0.09 seeds; mixed: 3.27± 0.09 seeds, n = 128; Table 2). No differences between pure and mixed forests were found for the number of full seeds (Table 2, Figure 2). However, the number of empty seeds was higher in pure forests, whereas mixed forests had more aborted seeds per fruit (Table 2, Figure 2).

Predispersal damage affected to 24.5 ± 1.3 % of the fruits (n = 283 trees from 14 forests), and was higher in pure (26.4 ± 1.9 %, n = 156) than in mixed forests (22.2 ± 2.0 %, n = 127). Rates of mite (4.0 ± 0.5 %) and chalcid wasp damage (2.3 ± 0.4 %) were relatively low, and moths were the main predispersal predators (18.5 ± 1.2 %). The best mixed model for fruit damaged by mites included fruit production per tree and the interaction between fruit production and forest type, although it did not differ between pure and mixed forests (Table 3, Figure 3). Damage by mites increased in mixed forests with fruit production per tree but did not vary with fruit production per tree in pure forests (Figure 4A). The best model for fruit damage by chalcid included fruit production per tree and the interaction between fruit production and forest type, although it did not differ between pure and mixed forests (Table 3). Fruit damage by chalcid wasps increased with fruit production per tree in both, pure and mixed forests (Table 3, Figure 3 and 4B). Finally, the best model for fruit damage by moths included forest type and the interaction between fruit production per tree and forest type (Table 3). Moth damage was higher in pure than in mixed forests, although in the pure forests fruit damage decreased in trees that produced more fruits, a trend not observed in mixed forests (Figure 3 and 4C). The best models for chalcid wasps and moths did not include the damage of the other arthropods (Table 3).

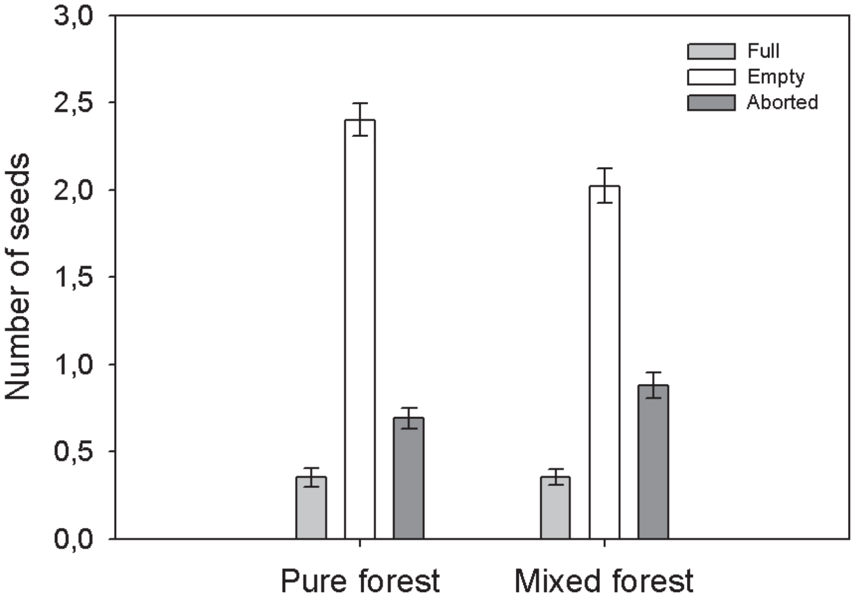

Fig. 2 Number of full, empty and aborted seeds per fruit for *Juniperus thurifera* trees from pure and mixed forests in 16 populations (n = 128 trees) in the Iberian Peninsula. Error bars represent ±1 standard error

Table 2. Generalized mixed models for *Juniperus thurifera* fruit traits: fruit production index, fruit size, pulp mass and number of seeds per fruit (total, full, empty or aborted seeds). SE: standard error, SD: Standard deviation of the random factor.

	Fixed effects	**Estimate**	**SE**	***P***	**Random**	**SD**
Fruit production per tree	Intercept	1.228	0.177	0.009	Population	0.340
	Pure forest	0.344	0.111	0.002	Region	0.180
Fruit diameter	Intercept	9.662	0.107	<0.001	Population	0.241
					Region	<0.001
Pulp mass	Intercept	0.248	0.008	<0.001	Population	0.011
					Region	<0.001
No. seeds per fruit	Intercept	3.264	0.099	<0.001	Population	0.074
					Region	0.074
No. full seeds	Intercept	0.247	0.101	0.024	Population	0.193
					Region	<0.001
No. empty seeds	Intercept	2.023	0.147	<0.001	Population	0.333
	Pure forest	0.379	0.125	0.003	Region	<0.001
No. aborted seeds	Intercept	0.872	0.109	0.005	Population	<0.001
	Pure forest	-0.189	0.088	0.034	Region	0.155

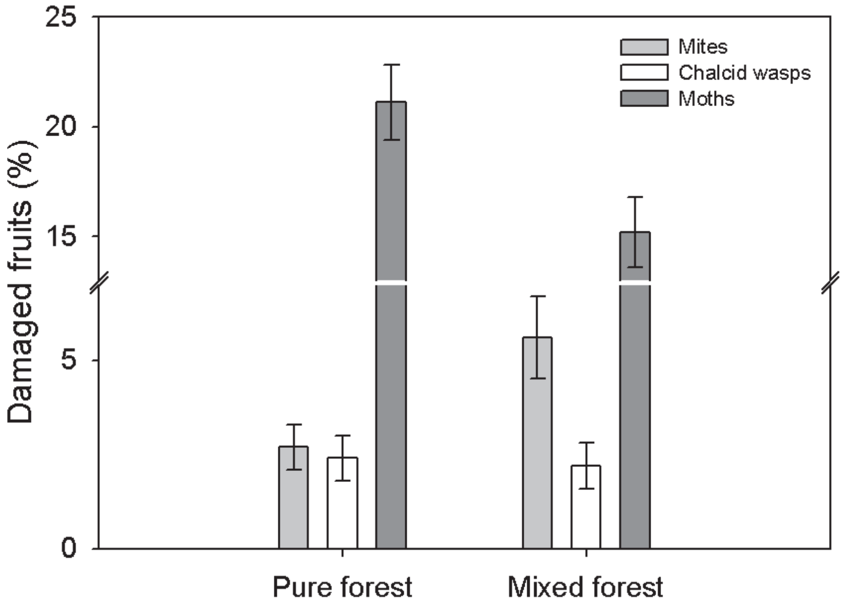

Fig. 3 Rates of *Juniperus thurifera* fruit predispersal predation in pure and mixed forests: mites, chalcid wasps and moths based on 14 populations in the Iberian Peninsula (n = 283 trees). Error bars represent ±1 standard error

Table 3. Generalized mixed models for the incidence of mites, chalcid wasps and moths in *Juniperus thurifera* fruits. Significant P-values are shown in bold. SE: standard error, SD: Standard deviation of the random factor.

Fruits damaged by	Fixed effects	Estimate	SE	*P*	Random	SD
Mites	Intercept	-3.942	0.680	<0.001	Population	0.667
	Fruit production per tree	0.477	0.100	<0.001	Region	1.019
	Pure forest	0.021	0.274	0.939		
	Fruit production per tree x Pure forest	-0.605	0.151	<0.001		
Chalcid Wasp	Intercept	-4.677	0.751	<0.001	Population	0.227
	Fruit production per tree	0.636	0.140	<0.001	Region	1.204
	Pure forest	0.084	0.335	0.802		
	Fruit production per tree x Pure forest	-0.350	0.172	0.042		
Moths	Intercept	-2.012	0.209	<0.001	Population	0.250
	Fruit production per tree	0.099	0.063	0.116	Region	0.255
	Pure forest	1.335	0.131	<0.001		
	Fruit production per tree x Pure forest	-0.545	0.076	<0.001		

Overall, the estimated seed output was higher for pure than mixed forests (pure: 0.50 ± 0.14; mixed. 0.28 ± 0.08, Z = 2.0, n=128, P = 0.045, including population as a random effect). The difference between forest types was mainly due to the greater fruit production in pure forests.

DISCUSSION

Our results indicate that variations in structure and composition in *J. thurifera* forests might influence the reproduction of this juniper species at distinct phases of seed and fruit development. Female *J. thurifera* trees produced less ripe fruits and had more aborted seeds in mixed than in pure forests suggesting detrimental effects of heterospecifics during pollination and early seed development. On the contrary, pure forests had higher rates of damaged fruits although the three arthropod groups showed contrasting responses to forest composition and fruit abundance. Overall, female trees in pure forests showed 63 % higher levels of seed output than mixed forests.

Fruit production was relatively low in all studied populations, a common pattern in this species, since it usually produces low to medium crops, with masting episodes every 7-10 years (Montesinos et al. 2012a). Higher fruit production in pure forests might reflect the sensitivity of *J. thurifera* to interspecific competition (Rozas and Olano 2013; Montesinos and Fabado 2015) with increased competition negatively influencing reproduction (García et al. 2000, Wesche et al. 2005). To better understand this, the effect of competition should be explored during years of high reproductive investment.

Dioecious juniper species usually present high abortion rates (Arista et al. 2001, García et al. 2000, Gruwez et al. 2013), with a lack of pollination being the main cause of early abortion of seeds in conifers (Owens 1995), and in particular in *J. thurifera* (see chapter 3). Pollen load is expected be lower in mixed forests, as junipers tend to have a lower conspecific density. Moreover, the presence of other tree species could physically interfere with the dispersal of juniper pollen (Millerón et al. 2012), with this barrier effect reducing pollen dispersal distance and the efficiency of pollen flow between trees. This effect may potentially diminish pollen load and the quality of pollen if it originates from nearby, closely related, individuals (Friedman and Barret 2009, Gruwez et al. 2013). In addition to the barrier effect, junipers are sensitive to aerial particles and heterospecific pollen during the period

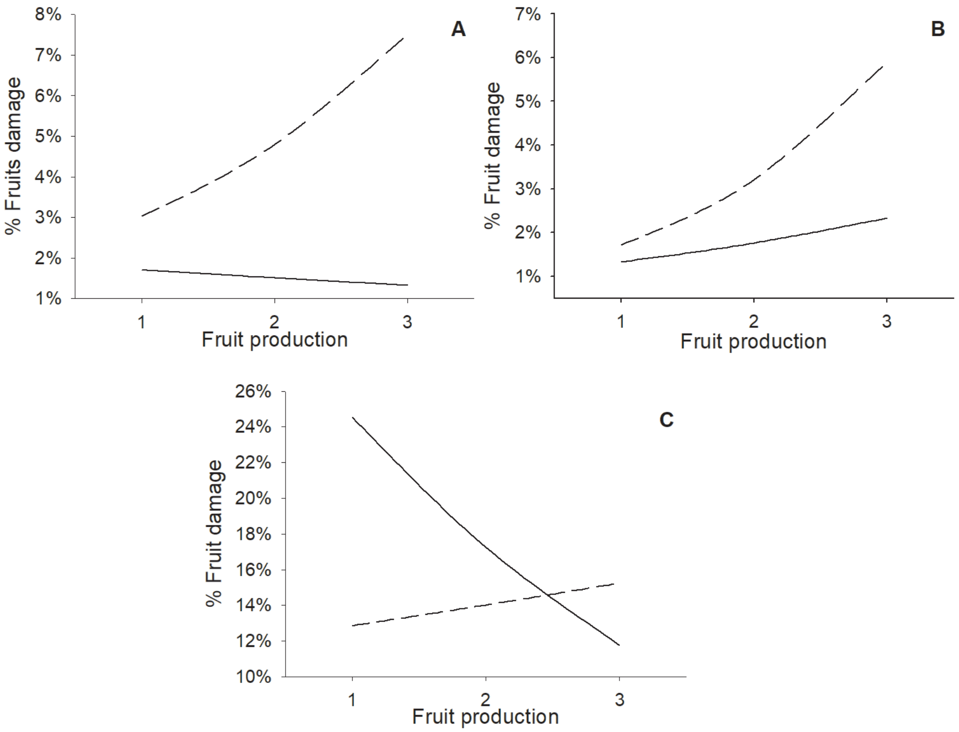

Fig. 4 Models predicting percentage of *Juniperus thurifera* fruits damaged by predispersal predators: (a) mites, (b) chalcid wasps and (c) moths in relation to fruit production per tree level in pure (solid line) and mixed forests (dashed line) in central Spain (n = 283 trees). Damage rate for trees with a fruit production index of 4 and 5 have not been modeled due to the small number of trees with such indices

of pollen reception in female flowers (Mugnaini et al. 2007). Therefore, the presence nearby of other wind-pollinated tree species with similar timing of pollen dispersal may reduce the probability of successful pollination (Mugnaini et al. 2007, Gruwez et al. 2013). Thus, the presence of oak species with pollen dispersal that partially overlaps with that of juniper could also have increased seed abortion rates in mixed forests. In contrast, pure forests showed higher numbers of empty seeds that may be associated with their being aborted during or immediately after fertilization (Gruwez et al. 2013), probably as a response to the selective allocation of resources during later developmental stages. In conclusion, although viable seed number per fruit did not differ between pure and mixed forests, factors driving seed losses were driven by different mechanisms.

Arthropod fruit damage rate was higher in pure forests, suggesting that the presence of other tree species (i.e., non-host plants) reduced damage rates. However, we found that responses varied depending on the arthropod group (Plath et al. 2012) and fruit abundance. Forest type did not affect the levels of damage caused by mites, indicating no apparent effect of the presence of non-host plants. Mites complete their entire life cycle in individual juniper trees, using seeds for reproduction, and passively colonizing other *J. thurifera* trees. Once they colonize a tree, mites develop their life cycle without the need to disperse to other trees; rates of fruit damage by these arthropods are therefore mostly determined by variations in fruit production rates in individual trees (Mezquida and Olano 2013). However, the interaction between forest composition and fruit production suggests that as tree fruit production augments, mite damage rate declines in pure forests, but increases in mixed forests. Chalcid wasps are specialized seed predators that oviposit eggs inside juniper seeds where the larvae develop. Female chalcid wasps use visual and olfactory cues to find their host plants (Turgeon et al. 1994). Chalcid wasps selected trees with higher fruit production, thus concentrating their oviposition on the available host-plants rather than moving and finding another juniper tree, especially in mixed forests where the presence of other tree species may difficult chalcid wasps from finding host-trees. Greater availability of juniper trees and higher fruit production in pure forests may have resulted in a resource dilution effect (Sholes 2008, Plath 2012), leading to lower effect of fruit production compared to mixed forests. However, moths were by far the most important pre-dispersal predator in the study site, and they determined the overall impact of forest type on arthropod damage. Interestingly, trees with low fruit production, as prevailed in our study sites, suffered higher fruit damage by moths in pure forests. Moths have a greater dispersal capacity than the other two arthropods and may avoid areas of mixed forests with a greater proportion of non-host trees, instead concentrating in pure forests where resource levels are higher. However, relative fruit damage levels reversed in forests with higher individual fruit production levels, with higher damage in mixed forests, indicating a lower satiation potential of low density forests and a greater concentration in high productivity trees.

Considering the different effects of the presence of heterospecific trees on fruit production and seed loss in *J. thurifera*, the reproductive output of female trees was higher in pure than in mixed forests. Since the number of viable seeds per fruit was similar for both forest types and fruit damage was lower in mixed forests, the main determinant of greater reproductive output in pure forests was fruit production. Therefore, during years when fruit production was generally low-medium, any further reduction in fruit production because of heterospecific competition and pollen limitation resulted in even lower reproductive output in mixed forests. This is consistent with findings from experimental alleviations of environmental stress to female trees during reproduction that resulted in larger crop sizes (Montesinos et al. 2012b). Furthermore, differences in reproductive success between pure and mixed forests may also become more pronounced during mast years when trees invest more in reproduction (Mezquida et al. 2016), and predispersal fruit damage rates may become higher in mixed than in pure forests.

Our study indicates that rapid changes in the structure of *J. thurifera* forests, and associated increases in interspecific competition (DeSoto et al. 2010, Gimeno et al. 2012a, Olano et al. 2012) might potentially affect juniper tree reproduction in the long term. This effect may act synergistically with increasing drought intensity (IPCC 2014), amplifying the negative effects of interspecific competition on *J.thurifera* under more xeric conditions (Gómez-Aparicio et al. 2011). Overall, our work highlights the need to explore the biological impact of current changes in landscape configuration in order to forecast species responses to global change.

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"Keep truckin' like the doodah man
Together, more or less in line
Just keep truckin' on"

Grateful Dead

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