Movimiento de abejorros entre cultivos de frutos rojos y hábitats naturales:

Influencia en la producción de fresa, explotación de recursos florales silvestres y prevalencia de parásitos

Tesis Doctoral

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Influencia en la producción de fresa, explotación de recursos florales silvestres y prevalencia de parásitos

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Influencia en la producción de fresa, explotación de recursos florales silvestres y prevalencia de parásitos

Memoria presentada por el Licenciado en Ciencias Ambientales Alejandro Trillo Iglesias para optar al título de Doctor por la Universidad de Jaén

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CERTIFICA

Que Alejandro Trillo Iglesias, Licenciado en Ciencias Ambientales por la Universidad de Jaén y Máster en Etología por la Universidad de Córdoba, ha realizado bajo su dirección la presente Memoria de Tesis Doctoral, titulada "Movimiento de abejorros entre cultivos de frutos rojos y hábitat naturales: Influencia en la producción de fresa, explotación de recursos florales silvestres y prevalencia de parásitos" y que a su juicio reúne los méritos suficientes para optar al grado de Doctor por la Universidad de Jaén.

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Resumen

Movimiento de abejorros entre cultivos de frutos rojos y hábitats naturales:

Influencia en la producción de fresa, explotación de recursos florales silvestres y prevalencia de parásitos

Los insectos polinizadores son uno de los grupos de animales más importantes para la agricultura al igual que para la mayoría de plantas silvestres por su contribución en la producción de frutos y semillas. En las últimas décadas, el área dedicada a cultivos que se benefician de polinizadores ha aumentado desproporcionadamente comparado con la de los cultivos que no dependen de polinizadores. Esto ha conllevado un aumento paralelo en el uso de polinizadores comerciales para cubrir los servicios de polinización.

Generalmente los cultivos proporcionan pulsos florales que alcanzan elevadas densidades florales monoespecíficas. Los espacios naturales, en cambio, proporcionan recursos florales diversos, aunque frecuentemente dispersos y que varían a lo largo del año. La variabilidad en la oferta de los recursos florales que proporcionan ambos tipos de hábitats en el tiempo o espacio a través del paisaje, promueve el movimiento entre hábitats, tanto de polinizadores comerciales como de silvestres, con el fin de satisfacer sus necesidades de alimentación. El movimiento de polinizadores en los agroecosistemas puede jugar un papel importante en multitud de procesos ecológicos tal como la reproducción de las plantas silvestres o de los cultivos, e incluso el de los polinizadores.

El objetivo general de esta Tesis Doctoral es investigar el movimiento de polinizadores comerciales y silvestres entre hábitats naturales y agrícolas promovido por la variabilidad de recursos florales que dependen tanto de la estación del año como de las características del paisaje. Concretamente, se estudia la influencia de los polinizadores comerciales y silvestres en la producción de un cultivo de fruta. A la vez, se cuantifica la presencia y densidad de polinizadores comerciales en comparación con taxones nativos en el hábitat natural adyacente a los cultivos. Además, a modo de posibles riesgos ecológicos, se estudia el solapamiento en la explotación de los recursos florales entre los polinizadores comerciales y nativos, y la prevalencia de parásitos en las colonias comerciales usadas en los cultivos. Para ello, se realizaron muestreos y experimentos de campo en cultivos y en hábitats naturales en el Valle del Guadalquivir, en la provincia de Huelva (SO España). El sistema de estudio consistió en extensas áreas dedicadas al cultivo de frutos rojos, mayoritariamente fresas (*Fragaria* × *ananassa*) en invernaderos semiabiertos, que florecen durante el invierno y la primavera. En este cultivo, los agricultores utilizan colmenas de abejorros *Bombus terrestris* mayoritariamente en invierno. Estos cultivos se encuentran ubicados en una matriz de espacios naturales con sotobosques mediterráneos arbustivos ricos en plantas polinizadas por insectos, los cuales florecen en su mayoría en primavera.

El uso de abejorros comerciales produjo un aumento en las visitas a las flores de fresa especialmente en invierno, pero no en el peso de la fruta. Los servicios de polinización están cubiertos por la comunidad de visitantes florales presentes tanto en invierno como en primavera. Estos fueron mayoritariamente abejas de la miel (*Apis mellifera*) pero también sírfidos y otros dípteros. Gran parte de los abejorros comerciales diversificaron su dieta en los hábitats naturales adyacentes a los cultivos, aunque su movimiento fue mayor en primavera cuando la riqueza y abundancia de especies de plantas silvestres con flores es alta. En el hábitat natural, la dieta de los abejorros comerciales solapó en gran medida con la de la subespecie nativa de abejorro (*B. t. lusitanicus*). Además, la presencia del abejorro nativo en el hábitat natural, al contrario que la del abejorro comercial, disminuyó con un aumento del área dedicada al cultivo de frutos rojos en el paisaje. Por último, gran parte de las colonias de abejorros comerciales que procedían directamente del productor presentaron prevalencias elevadas del parásito intracelular, *Nosema* spp. Sin embargo, dicha prevalencia no cambió a través del tiempo en estos paisajes agrícolas. *Nosema* spp. podría afectar negativamente a la salud de los abejorros y tiene el potencial de transmitirse al resto de la comunidad de polinizadores a través de las flores que visitan.

Los resultados de esta Tesis indican que es necesario profundizar en la contribución de los polinizadores comerciales en la producción de los cultivos con el fin de optimizar su uso y prevenir los riesgos para la entomofauna y la flora silvestre. Debido al declive de algunas especies de polinizadores y por tanto de los servicios de polinización que proporcionan, es importante que en los paisajes agrícolas se priorice la implementación de medidas agroambientales que fomenten la diversidad de los visitantes florales silvestres en los cultivos. Solo en el caso de que la contribución en la polinización por parte de los polinizadores comerciales sea importante, resulta esencial promover la cría de subespecies nativas libres de parásitos.

Abstract

Spillover of bumblebees between berry crops and natural habitats:

Impact on strawberry production, exploitation of wild floral resources and prevalence of parasites

Insect pollinators are among the most important animals for agriculture as well as for most wild plant species due to their contribution to fruit and seed production. During the last few decades, the area devoted to crops that benefits from pollinators has disproportionately increased compared to non-dependent crops. This has led to a parallel increase in the use of commercial pollinators for pollination services.

Crops usually provide pulsed floral resources that achieve high monospecific flower densities. Natural habitats, in contrast, provide diverse floral resources, but often scattered food plants, that vary throughout the year. Variability in the availability of floral resources provided by both habitat types over time or space across the landscape, promotes the spillover of either commercial or wild pollinators between habitats, in order to satisfy their feeding needs. The spillover of pollinators in agroecosystems can play an important role in multiple ecological processes such as reproduction of wild plants or crops, or even that of pollinators.

This Thesis aims to investigate the spillover of commercial and wild pollinators between natural and agriculture habitats promoted by the variability of floral resources, which depends on season and the surrounding landscape. Specifically, the ways in which commercial and wild pollinators contribute to fruit production are assessed. In parallel, the presence and density of commercial pollinators compared to native taxa in natural habitats adjacent to crops is quantified. Moreover, in terms of possible ecological risks, the overlap in floral resource exploitation between commercial and native pollinators, and the parasite prevalence in commercial bumblebee colonies used in crops, are studied. To do this, sampling and field experiments were carried out both in agricultural and in natural habitats in the Guadalquivir Valley, province of Huelva (SW Spain). The study system is characterized by large areas cultivated with berry crops, mostly strawberries (*Fragaria* × *ananassa*) under semi-open polytunnel greenhouses, that flower from winter to spring. In this crop, farmers use colonies of commercial bumblebees *Bombus terrestris* mostly in the winter. These crops are located within a matrix of natural habitats with a Mediterranean understorey rich in insect-pollinated plants, most of which flower in the spring.

The use of commercial bumblebees resulted in an increase in strawberry flower visitation, mostly in winter, but not in fruit weight. The pollination services are fulfilled by the community of flower visitors present in both the winter and spring. These were mostly honeybees (*Apis mellifera*) but also hoverflies and other Diptera. Many commercial bumblebees diversified their diet by foraging in natural habitats adjacent to crops, although their spillover was higher in spring than in winter, when the abundance and richness of wild plant species are high. In the natural habitat, commercial bumblebee diet overlapped with that of the native bumblebee subspecies (*B. t. lusitanicus*). Moreover, the presence of native bumblebees in natural habitats, in contrast to commercial bumblebees, decreased with an increase in the area devoted to berry crops in the landscape. Lastly, most commercial bumblebee colonies from the producer showed a high prevalence of an intracellular parasite, *Nosema* spp. However, this prevalence did not change over time in these agricultural landscapes. *Nosema* spp. may negatively affect bee health and may be transmitted to other pollinators through visited flowers.

The results found in this Thesis highlight the important of studying the contribution of commercial pollinators in crop production in order to optimize their use and prevent risks posed to wild entomofauna and flora. Due to global declines in pollinator species and the pollination services they provide, it is important to prioritize agri-environment schemes that promote diverse crop flower visitors. When the contribution of commercial pollinators is important, it is essential to promote breeding programs of parasite-free native subspecies.

Introducción general

Muchos animales se mueven entre hábitats a través del paisaje (*cross-habitat spillover*) con el objetivo de satisfacer sus necesidades vitales, y por tanto, optimizar su éxito reproductivo. Este movimiento puede darse por diferencias entre la oferta de recursos que proporcionan distintos hábitats en el tiempo o en el espacio (Hansson & Åkesson, 2014). Cuando los movimientos de animales entre hábitats resultan en el logro de una función se le denomina movimiento funcional (*functional spillover*, en adelante lo llamaremos “movimiento”) (Blitzer *et al.*, 2012). El movimiento de animales entre hábitats, no solo va a afectar al éxito reproductivo de los animales involucrados, sino que también puede influenciar multitud de procesos ecológicos que son esenciales para la dinámica de las comunidades. En especial son aquellos relacionados con la interacción entre especies como puede ser la dispersión de semillas, la depredación, el parasitismo o la polinización (Rand *et al.*, 2006; Blitzer *et al.*, 2012; Tscharntke *et al.*, 2012).

La actividad humana es causante de grandes cambios en la oferta de recursos a través del paisaje. La expansión de las tierras agrícolas es una de las causas principales que ha conllevado a la pérdida de hábitat natural y la fragmentación del paisaje (Defries *et al.*, 2004). De hecho, el área dedicada a cultivos ocupa alrededor de un tercio de la superficie terrestre no cubierta por hielo (Ellis *et al.*, 2010) y continua aumentando para abastecer la demanda de una población humana en crecimiento (revisado en Ramankutty *et al.* 2018). Por tanto, los paisajes agrícolas promueven el movimiento de animales entre hábitats naturales y agrícolas como mecanismo para prosperar en estos paisajes altamente cambiantes (Smith *et al.*, 2014).

Los insectos polinizadores son uno de los grupos de animales más importantes para la agricultura actual. La producción de alrededor del 70% de las principales especies de cultivo depende de insectos polinizadores (Klein *et al.*, 2007). Es decir, la producción de frutos y semillas en estos cultivos depende de forma obligatoria o facultativa de una polinización mediada por insectos polinizadores. En general, estos servicios de polinización han sido valorados en alrededor de 153 billones de euros anuales (Gallai *et al.*, 2009). Entre 1961 y 2006, la expansión agrícola fue del 23% e implicó principalmente cultivos dependientes de polinización mediada por insectos (Aizen *et al.*, 2008). Esto ha conllevado un aumento en el uso de polinizadores comerciales, principalmente la abeja de la miel (*Apis mellifera*) y los abejorros (*Bombus* spp.), para suplementar los servicios de polinización (Velthuis & van Doorn, 2006; Aizen & Harder, 2009; Geslin *et al.*, 2017b). Sin embargo, el papel que desempeñen estos polinizadores comerciales en la producción de los cultivos va a estar influenciado, no solo por la identidad de la especie de polinizador usado, sino también por la del tipo y variedad de cultivo (Rader *et al.* 2009 o trabajos incluidos en Garibaldi *et al.* 2013). Además, también va a ser clave el tipo de paisaje en el que se localizan estos cultivos, a través de la identidad y diversidad de la comunidad de visitantes florales (Kremen *et al.*, 2002; Lye *et al.*, 2011; Ellis *et al.*, 2017). A pesar del uso frecuente de polinizadores comerciales para los servicios de polinización, hoy en día se desconoce su contribución en la mayoría de cultivos teniendo en cuenta los factores citados anteriormente. Además, la mayoría de trabajos existentes hasta la fecha se basan en el uso y eficiencia de una única especie, la abeja de la miel (ver trabajos incluidos en Garibaldi *et al.* 2013).

Los insectos polinizadores requieren principalmente de néctar y polen, además de lugares de nidificación para completar su ciclo biológico. No obstante, no todos los grupos de polinizadores presentan igual grado de susceptibilidad frente a los cambios que se puedan dar en el paisaje y por tanto, en los recursos. Por ejemplo, gran parte de las especies de dípteros no suelen ser tan susceptibles a la pérdida de hábitat natural en el paisaje como las especies de himenópteros (Rader *et al.*, 2016). Esto se justifica en parte porque los dípteros no suelen nidificar en lugares fijos y presentan hábitos de alimentación muy diversos a lo largo de su ciclo de vida (Raymond *et al.*, 2014). En cambio, los himenópteros como las abejas y los abejorros suelen nidificar en lugares fijos (*central place foragers*) y por tanto, su rango de forrajeo va a estar limitado por la localización de su nido (Beutler & Loman, 1951; Stephens & Krebs, 1986). Es por ello que, la distribución temporal y espacial de los recursos florales en el paisaje, puede adquirir una mayor importancia en el éxito de la progenie de los himenópteros (Dukas & Edelstein-Keshet, 1998; Schmid-Hempel & Schmid-Hempel, 1998). Además, gran parte de estos polinizadores necesitan, no solo de recursos florales en abundancia, sino que sean diversos (Alaux *et al.*, 2010; Brodschneider & Crailsheim, 2010; Di Pasquale *et al.*, 2013).

El movimiento de polinizadores entre hábitats naturales y agrícolas está promovido principalmente por la variabilidad en la oferta floral entre hábitats (revisado en Blitzer *et al.* 2012). Por un lado, los hábitats naturales proporcionan recursos florales diversos, el periodo de floración de las plantas silvestres coincide con la fenofase de vuelo de los polinizadores, no obstante a menudo las especies de plantas que ofrecen recursos para los polinizadores se encuentran dispersas en el espacio. Por otro lado, los cultivos proporcionan recursos florales monoespecíficos que alcanzan altas densidades de floración y que en su mayoría florecen durante periodos cortos de tiempo, días o semanas (*mass-flowering crops*) (Westphal *et al.*, 2003). Estos cultivos son por ejemplo los de colza (*Brassica napus*), la patata (*Solanum tuberosum*) o el girasol (*Helianthus annuus*). Sin embargo, también existen cultivos que proporcionan largos periodos de floración (meses), que incluso se puede prologar en el tiempo si son cultivados en invernaderos. El movimiento de polinizadores en paisajes agrícolas con este tipo de cultivos de larga floración ha pasado en gran parte inexplorado. Los cultivos de larga floración son por ejemplo la fresa (*Fragaria × ananassa*), el tomate (*Solanum lycopersicum*) o el pimiento (*Capsicum annuum*).

El movimiento de polinizadores puede ocurrir en ambas direcciones, es decir, desde los hábitats naturales hacia los cultivos y viceversa. Originariamente, la comunidad científica se ha enfocado más en el estudio del movimiento de polinizadores silvestres desde los hábitats naturales hacia los cultivos. Sólo recientemente, el movimiento en la dirección opuesta ha recibido más atención (Holzschuh *et al.*, 2011; Blitzer *et al.*, 2012). Este movimiento se ha estudiado, no solo en su composición taxonómica, sino también en la función de polinización que los polinizadores realizan en los cultivos y en las plantas silvestres que se localizan en las zonas adyacentes a los cultivos. En la Figura 1 se ilustra el movimiento de polinizadores silvestres entre un hábitat natural (bosque) y un cultivo de floración masiva (colza) según Blitzer *et al.* (2012). En esta ilustración podemos observar como gran parte de los polinizadores presentes en el hábitat natural se espera que se desplacen hacia el cultivo en su pico de floración, aprovechando la abundancia de recursos florales, y como este movimiento podría repercutir positivamente en la producción de semillas del cultivo y negativamente en la reproducción de las plantas silvestres que se quedan sin visitar (Fig. 1a). Se espera el proceso contrario cuando cesa la floración del cultivo, causando un efecto positivo en la reproducción de las plantas silvestres (Fig. 1b). Sin embargo, estos son escenarios simplificados ya que solo se centran en la floración del cultivo y la atracción que ejerce sobre los polinizadores. Otros factores como la abundancia y la diversidad de los recursos florales en el hábitat natural, que son necesarios para los polinizadores, han sido poco explorados y se espera que jueguen un papel determinante en la dirección y magnitud del movimiento de polinizadores entre hábitats.

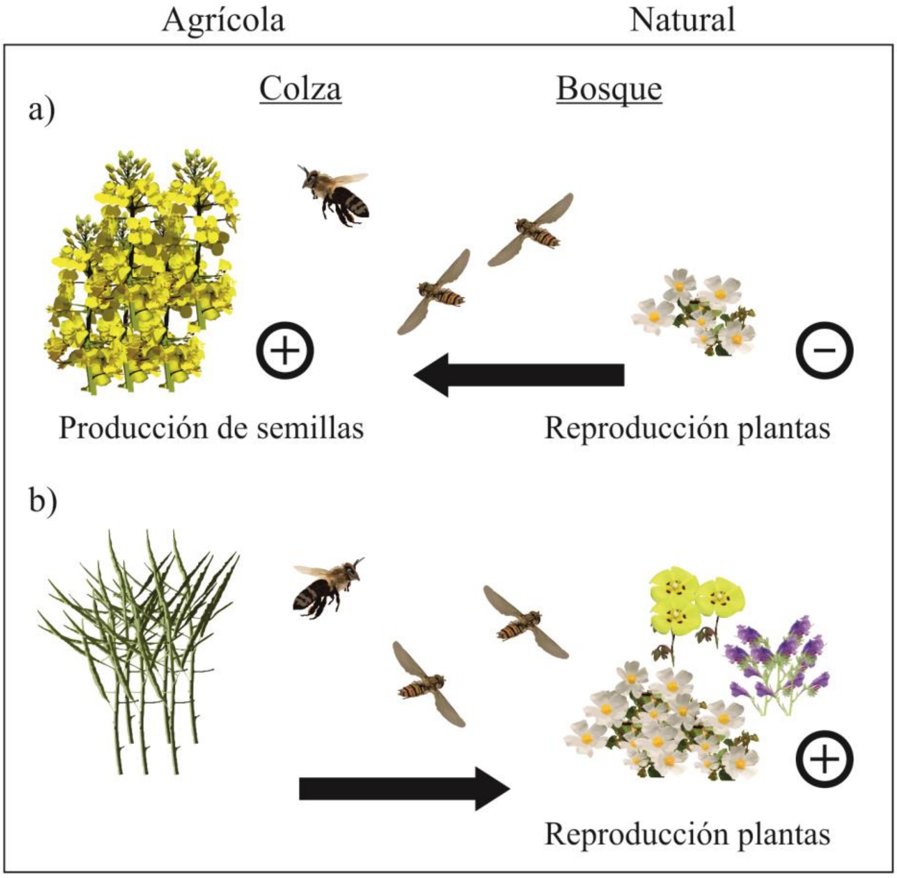


Figura 1. Ilustración del movimiento de polinizadores silvestres (a) desde un hábitat natural (bosque) hacia un cultivo en floración (colza, *Brassica napus*) y (b) el proceso contrario cuando cesa la floración del cultivo. Este movimiento se espera que afecte (a) positivamente a la producción de semillas del cultivo y negativamente a la reproducción de las plantas silvestres cuando los polinizadores se mueven principalmente hacia el cultivo y (b) positivamente a la reproducción de las plantas silvestres cuando los polinizadores se mueven principalmente hacia el hábitat natural porque ha cesado la floración en el cultivo (modificado de Blitzer *et al.* 2012).

Además, a pesar del uso, hoy en día, frecuente de polinizadores comerciales para los servicios de polinización en cultivos, su movimiento se ha explorado mucho menos que el de los polinizadores silvestres. Las especies de polinizadores comerciales más usados son generalistas (Goulson, 2010; Requier *et al.*, 2015) y suelen ampliar su dieta forrajeando en plantas silvestres localizadas en zonas adyacentes a los cultivos (Whittington *et al.*, 2004; Murray *et al.*, 2013; González-Varo & Vilà, 2017). Esto se ha asociado a una serie de posibles riesgos indeseados en la entomofauna y flora local (Goulson, 2003; Montero-Castaño *et al.*, 2018). A continuación se mencionan algunos ejemplos:

(1) Competencia por los recursos florales: Al aumentar la abundancia de polinizadores comerciales en el hábitat natural puede reducir la disponibilidad de néctar y polen de las flores para los polinizadores silvestres. Por ejemplo, el uso de abejorros comerciales en Japón para la polinización de cultivos de tomate y su movimiento al medio natural se ha relacionado con una partición de nicho entre esta especie comercial y varias especies de abejorros nativos. Esto indica que hubo una fuerte competencia por los recursos florales (Ishii *et al.*, 2008).

(2) Competencia por los lugares de nidificación: Los polinizadores comerciales pueden usar los mismos espacios para nidificar que usan los polinizadores silvestres. Por ejemplo, se ha observado en Argentina una gran similitud entre los lugares de nidificación que elige el abejorro nativo *B. dahlbomii* y los comerciales *B. ruderatus* y *B. terrestris* (Morales, 2007).

(3) Transmisión de parásitos: Los polinizadores comerciales albergan parásitos que pueden ser transmitidos a los polinizadores silvestres a través de las flores que visitan. Por ejemplo, se ha mostrado en Irlanda una mayor prevalencia de parásitos en zonas adyacentes a cultivos donde se usan abejorros comerciales en comparación con zonas alejadas (Murray *et al.*, 2013).

(4) Hibridación: El uso de polinizadores comerciales de taxones filogenéticamente próximos (es decir, congenéricos o intraespecíficos) a los nativos puede resultar en un apareamiento entre ellos. Por ejemplo, se ha demostrado en la Península Ibérica que la subespecie comercial de abejorro *B. t. terrestris* hibrida con la subespecie nativa *B. t. lusitanicus* (Cejas *et al.*, 2018).

(5) Cambios en la producción de semillas: Al aumentar la tasa de visitas de polinizadores comerciales en las plantas silvestres puede repercutir de manera positiva o negativa en la reproducción de las plantas silvestres. Por ejemplo, se ha demostrado en España que una tasa muy elevada de visitas de la abeja de la miel disminuye la producción de semillas de dos especies de cistáceas (Magrach *et al.*, 2017).

A pesar de las crecientes pruebas acerca del impacto que causan los polinizadores comerciales en el hábitat natural todavía se desconoce en gran medida, la influencia de factores claves como, la disponibilidad de recursos florales en el hábitat adyacente o el número de colonias usadas a nivel de paisaje, en la magnitud del movimiento de polinizadores comerciales hacia el medio natural.

Objetivos de la Tesis Doctoral

El objetivo general de esta Tesis Doctoral es investigar el movimiento de polinizadores comerciales y silvestres entre hábitats naturales y agrícolas promovido por la variabilidad de recursos florales en el tiempo y en el espacio. Se estudia cómo estos movimientos se ven influidos por la cantidad de cultivo en el paisaje y cómo varían estacionalmente.

Los objetivos específicos son:

1. Estudiar la contribución de un polinizador comercial en la producción de un cultivo de fruta teniendo en cuenta al resto de la comunidad de visitantes florales.

2. Describir la presencia, densidad y explotación de los recursos florales de un polinizador comercial y una subespecie nativa en los hábitats naturales que rodean a los cultivos.

3. Cuantificar de forma experimental el movimiento bidireccional de polinizadores entre hábitats naturales y agrícolas mediado por la variabilidad estacional en la oferta floral y la cantidad de área del paisaje dedicada al cultivo.

4. Evaluar la prevalencia de parásitos en colonias de un polinizador comercial en los paisajes agrícolas donde se usa.

Sistema de estudio

Todo el trabajo de campo se ha llevado a cabo en el valle del Guadalquivir dentro de la provincia de Huelva, al suroeste de España (Fig. 2). El clima es típicamente mediterráneo, con inviernos suaves y veranos calurosos. La precipitación media anual en la región es de 525 mm y las temperaturas medias de las mínimas y máximas en enero y en julio son de 5.9 ºC/16.2 ºC y 18.9 ºC/32.7 ºC, respectivamente (AEMET).

El área de estudio está caracterizada por presentar espacios naturales dominados por plantaciones de pino piñonero (*Pinus pinea*) con un sotobosque muy diverso de plantas que atraen a insectos polinizadores (Fig. 2 y Fig. 3a). Entre estas plantas destacan las que pertenecen a las familias Fabaceae, Cistaceae o Asteraceae. Concretamente, algunas de las especies y géneros más representativos son las herbáceas *Echium*, *Leontodon* y *Linaria*, y arbustos *Cistus ladanifer*, *C. salvifolius*, *Lavandula stoechas* (Fig. 3b), *Halimium calycinum* (Fig. 3c), *Rosmarinus officinalis* (Fig. 3d) y *Ulex australis*. La mayoría de estas plantas florecen durante la primavera, con excepción de las tres últimas, entre alguna otra no citada, que presentan picos de floración durante el invierno. Estos bosques albergan una gran diversidad de insectos polinizadores. Más de 240 especies se han cuantificado en los pinares del Valle del Guadalquivir. Entre ellas, cabe destacar a las especies de sírfidos *Eristalis tenax* o *Episyrphus balteatus*, y una gran diversidad de abejas solitarias del género *Eucera*, *Andrena* y *Lasioglossum*. Los abejorros son raros en general, representan menos del 1% de las visitas a flores silvestres (Magrach *et al.*, 2017) y están representados únicamente por la subespecie nativa *Bombus terrestris lusitanicus* (Rasmont *et al.*, 2008).

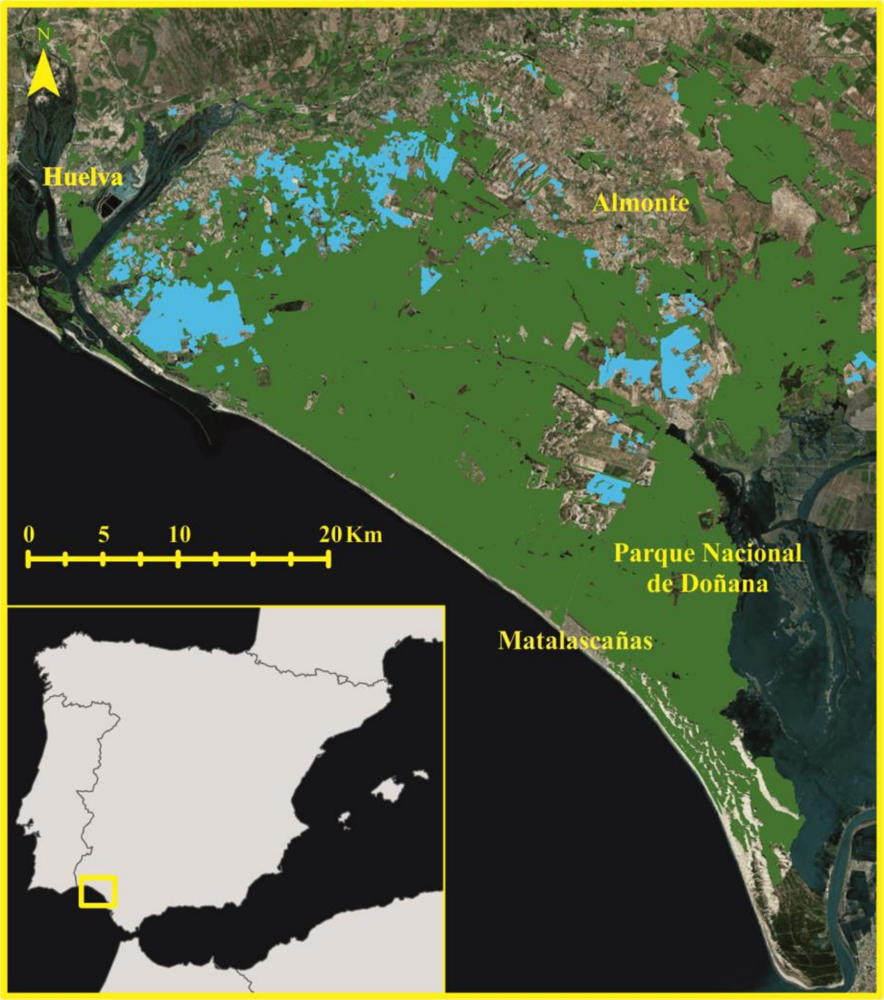


Figura 2. Fotografía aérea de la región de estudio localizada en la provincia de Huelva (SO España). Las áreas de color azul represente invernaderos en los que se cultivan frutos rojos, mientras que las áreas de color verde oscuro representan espacios naturales.

Figura 3. (a) Pinar (*Pinus pinea*) con sotobosque típico Mediterráneo. Al fondo se pueden observar invernaderos con frambuesas (*Rubus idaeus*), (b) lavanda (*Lavandula stoechas*) floreciendo durante la primavera, (c) jaguarzo amarillo (*Halimium calycinum*) y (d) romero (*Rosmarinus officinalis*) floreciendo en invierno.

En la actualidad, una de las actividades agrícolas más importantes de esta región de estudio es el cultivo de frutos rojos (Fig. 2 y Fig. 4b). La historia de este cultivo es relativamente reciente. Las primeras referencias datan de finales de los años 40 y principios de los 50. En 1956 el área dedicada al cultivo de fresas (*Fragaria* × *ananassa*) en secano era de tan solo 2 hectáreas (Medina Mínguez, 2003). Los años 60 fueron clave para el impulso de esta actividad agrícola en la zona, debido a varios factores que se dieron simultáneamente: la llegada de nueva tecnología a la región procedente de California; la presencia del Dr. Dieter Wimberg, científico pionero en el sector, y de D. Antonio Medina Lama, abogado sevillano que poseía una finca agrícola en el término municipal de Moguer (Fig. 4a). Las fresas se empezaron a cultivar bajo invernadero en caballones acolchados con plásticos, los suelos se desinfectaban y se regaban. A partir de los años 70/80, el cultivo de fresa bajo invernadero se fue extendiendo por las diferentes comarcas de la provincia. También aparecieron las primeras Sociedades Cooperativas que consolidaban dicha actividad agrícola en la región (López Aranda, 2010). En la actualidad, la superficie cultivada en la región es de aproximadamente 11,145 hectáreas (campaña 2017/2018). Además del cultivo de fresas (Fig. 4c), se cultivan otros frutos rojos, principalmente la frambuesa (*Rubus idaeus*) y el arándano (*Vaccinium corymbosum*; Fig. 4d), aunque en menor proporción (45%; Diariosur). No obstante, estas cifras varían ligeramente cada temporada.

Hoy en día, todos los frutos rojos en la región se cultivan en invernaderos abiertos por sus extremos, los cuales generan condiciones óptimas para que la producción se prolongue en el tiempo. El cultivo de fresa produce floración continua desde noviembre hasta mayo. En cambio, la frambuesa y el arándano producen una floración pulsada de pocas semanas que depende de la variedad. En general, tanto la calidad como la cantidad de frutos producidos en estos cultivos, se ve beneficiada por una polinización mediada por insectos (Isaacs & Kirk, 2010; Ellis *et al.*, 2017). Por ejemplo, la fresa está compuesta por múltiples aquenios, los cuales si son polinizados producen un alargamiento en las celdas de los receptáculos (Csukasi *et al.*, 2011) que se traduce en un incremento en el tamaño y en una menor deformación de la fruta (Fig. 5) (Free, 1993).

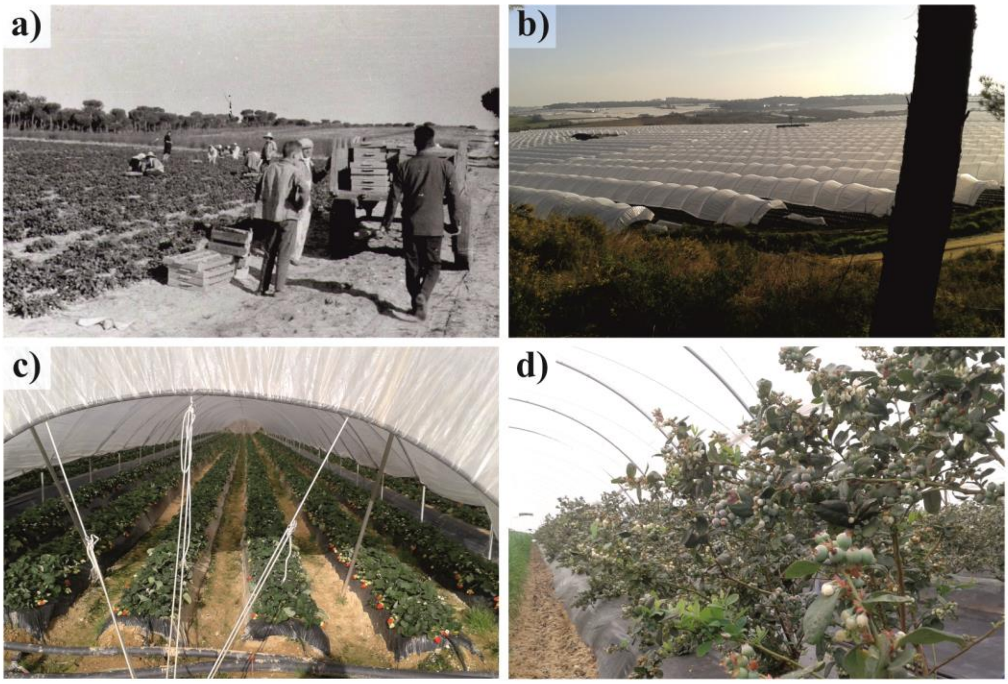


Figura 4. (a) Cultivo de fresas en los años 60 en una finca pionera llamada “Las Madres del Avitor”, en la comarca de Moguer. Imagen obtenida de un informe presentado por José Manuel López Aranda en unas jornadas de horticultura celebradas en Madrid en el año 2010; (b) cultivos de frutos rojos (mayoritariamente fresas, *Fragaria* × *ananassa*) en las proximidades de Moguer; (c) interior de un invernadero de fresa y (d) arándano (*Vaccinium corymbosum*).

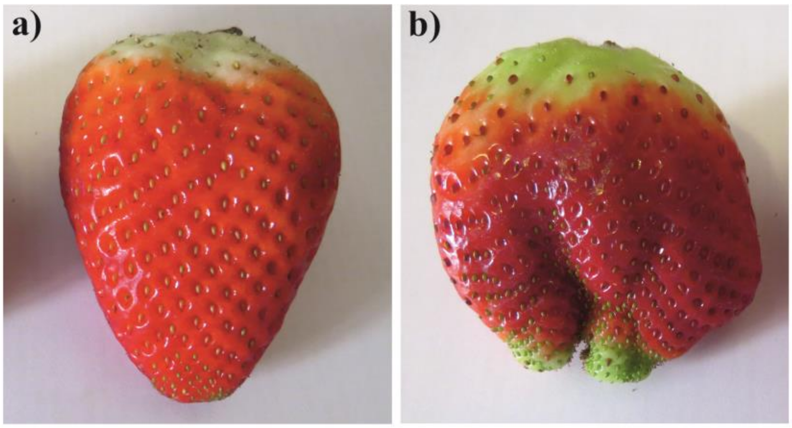


Figura 5. Fresas recolectadas en un invernadero de estudio. Se aprecian los aquenios que componen los frutos. La fresa de la izquierda (a) no presenta deformación, mientras que la de la derecha (b) presenta deformación porque no todos los aquenios fueron fecundados.

Tanto en esta región de estudio como en el resto del mundo, es frecuente el uso de polinizadores comerciales para suplementar la polinización de los cultivos de frutos rojos. Entre los polinizadores comerciales más utilizados cabe destacar a la abeja de la miel de la especie *Apis mellifera* y al abejorro de la especie *Bombus terrestris* (Fig. 6 a, b, c y d). La abeja de la miel se ha utilizado durante cientos de años como polinizador en la mayoría de cultivos dependientes de polinización mediada por animales (Aizen & Harder, 2009; Garibaldi *et al.*, 2013). Sin embargo, la comercialización de abejorros ha sido más reciente. Comenzó en Bélgica y en Holanda a finales de los años 80 promovido por la búsqueda de nuevas técnicas que reemplazasen la costosa polinización mecánica que se hacía en el cultivo de tomate (*Solanum lycopersicum*) bajo invernadero (Ravestijn & Nederpel, 1988; Ravestijn & Sande, 1991). Posteriormente, el logro de su producción industrial masiva, además de, su eficacia polinizando otros cultivos de hortalizas en invernaderos, impulsó su comercio y expansión mundial (Velthuis & van Doorn, 2006). En la actualidad, alrededor de dos millones de colmenas de abejorros son producidas anualmente para polinizar alrededor de 20 tipos de cultivo en todo el mundo (Velthuis & van Doorn, 2006; Graystock *et al.*, 2016a).

En nuestra región de estudio, el uso masivo de abeja de la miel y abejorros como polinizadores comerciales, fue impulsado alrededor del año 2000 (Medina-Mínguez, comunicación personal). En esta época, prácticamente casi la totalidad de fresa que se cultivaba en la región pertenecía a la variedad “Camarosa” procedente de California. Esta variedad, presentaba unas tasas de deformación muy elevadas (Ariza *et al.*, 2011). Es por ello que, se empezaron a usar colonias de abeja de la miel y/o de abejorros para intentar solventar este problema. Los abejorros se usaban principalmente cuando las condiciones climáticas eran adversas, sobre todo en invierno, debido a su gran capacidad de forrajeo a bajas temperaturas. Hasta el año 2009/10, “Camarosa” fue la variedad dominante en la región y ha estado presente casi hasta la actualidad (2016/17). No obstante, actualmente se plantan otras variedades (p. ej. Fortuna, Primoris y Rábida) que presentan bajas tasas de deformación pero se siguen usando estos polinizadores comerciales. Creemos pues que es interesante explorar la eficiencia de los abejorros en la polinización de variedades que se utilizan actualmente.

Figura 6. (a) Colmena de abejorros (*Bombus terrestris*) en cultivo de fresa (*Fragaria* × *ananassa*); (b) colmena de abejorros en cultivo de arándanos (*Vaccinium corymbosum*); (c) abejorros saliendo de su colmena y (d) abejorro visitando una flor de fresa.

Estructura de la Tesis Doctoral

Además de esta **Introducción general**, una Discusión general y unas Conclusiones generales, esta Tesis Doctoral consta de cuatro capítulos. Dos capítulos han sido recientemente publicados y los otros dos han sido enviados a revistas internacionales indexadas. A continuación, se detalla el contenido y finalidad de cada uno de los capítulos.

En el **Capítulo 1** (*Managed bumblebees increase flower visitation but not fruit weight in polytunnel strawberry crops*) se estudia la contribución de los abejorros comerciales y la de los polinizadores silvestres en la producción de fresas en invierno y en primavera en un gradiente de intensificación agrícola en el paisaje.

En el **Capítulo 2** (*Contrasting occurrence patterns of managed and native bumblebees in natural habitats across a greenhouse landscape gradient*) se describe la presencia, densidad y explotación de los recursos florales de abejorros comerciales y la subespecie nativa en parches de hábitat natural cercanos a los cultivos. El muestreo se realiza en invierno y en primavera en un gradiente de intensificación agrícola en el paisaje.

En el **Capítulo 3** (*Seasonality of bumblebee spillover between crops and natural habitats*) se cuantifica el movimiento de abejorros entre hábitats naturales y agrícolas teniendo en cuenta la variabilidad estacional en la oferta de recursos florales que ofrecen ambos tipos de hábitat y el contraste entre paisajes con alta y baja extensión de cultivo.

Finalmente, en el **Capítulo 4** (*Prevalence of Nosema microsporidians in commercial bumblebees Bombus terrestris is not related to the landscape intensity use of colonies*) se evalúa el estado inicial de infección de colonias de abejorros comerciales. Posteriormente se explora si hay cambios en la prevalencia de los parásitos con el tiempo en paisajes donde se usan colonias de abejorros de forma intensiva.

En la ilustración de la Figura 7 se puede observar la zona del paisaje abarcada en cada uno de los capítulos de esta tesis así como las especies de polinizadores más estudiados y las plantas más representativas.

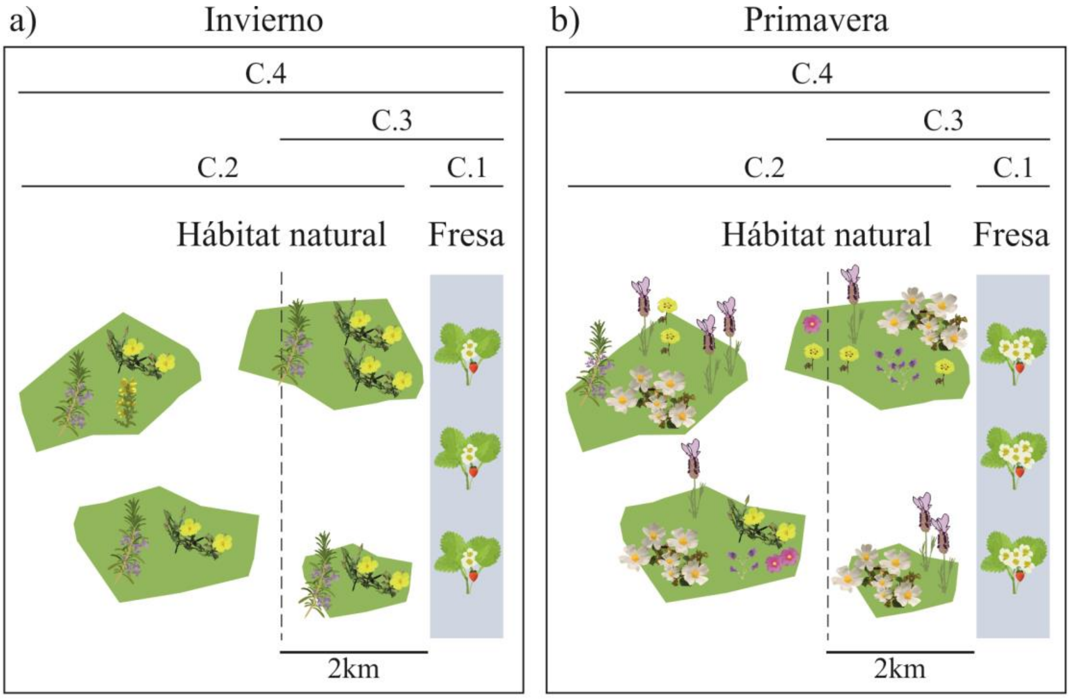


Figura 7. Ilustración del tipo de hábitat y de la escala del paisaje abarcada en cada capítulo de esta tesis así como de las especies de plantas más representativas que se espera que estén floreciendo (a) en invierno (*Halimium calycinum*, *Rosmarinus officinalis*, *Ulex australis* y *Fragaria* × *ananassa*) y (b) en primavera (*H. calycinum*, *R. officinalis*, *Lavandula stoechas*, *Tuberaria* sp., *Echium vulgare*, *Cistus salviifolius*, *C. crispus* y *F.* × *ananassa*). Los parches verdes representan el hábitat natural y los azules los invernaderos de fresa.

Capítulo 1

Managed bumblebees increase flower visitation but not fruit weight in polytunnel strawberry crops

Trillo, A., Herrera, J.M. & Vilà M. (2018) Managed bumble bees increase flower visitation but not fruit weight in polytunnel strawberry crops. *Basic and Applied Ecology*, **30**, 32–40.

Resumen

La polinización mediada por animales es esencial para la producción y calidad de frutos y semillas de muchos cultivos consumidos por el humano. Sin embargo, los servicios de polinización podrían estar comprometidos cuando los polinizadores silvestres son escasos. Los polinizadores comerciales son usados frecuentemente para suplementar estos servicios bajo la asunción de que mejoraran el rendimiento de los cultivos. Sin embargo, la información espaciotemporal de la dependencia a polinizadores de estos cultivos todavía es limitada. Nosotros evaluamos la contribución de colonias de abejorros comerciales comparado con el de la comunidad de polinizadores disponibles en la tasa de visitas a flores de fresa (variedad 'Fortuna') y en la calidad de sus frutos a lo largo de un gradiente de intensificación agrícola en el paisaje (es decir, cobertura de frutos rojos en invernaderos). Se usaron colonias de abejorros en invierno y en primavera, es decir, cuando probablemente los polinizadores silvestres en su fase vital de vuelo sean poco o muy abundantes, respectivamente. La colocación de colonias incrementó las visitas de abejorros a las flores de fresa, especialmente en invierno. El uso de colonias de abejorros no afectó a las visitas a flores de fresa de otros insectos, principalmente abejas de la miel, sírfidos y otros dípteros. La visita a las flores por parte de las abejas de la miel y de los insectos silvestres no varió entre estaciones ni estuvo relacionada con la cobertura de cultivos de frutos rojos. Las fresas fueron de la mayor calidad, es decir, de mayor peso, cuando se permitió polinización mediada por insectos, y estuvo positivamente relacionada con los visitantes florales silvestres en invierno pero no en primavera. Sin embargo, el incremento de visitas a las flores de fresa por parte de abejorros comerciales y de abejas de la miel no afectó al peso de la fresa. Nuestros resultados sugieren que los servicios de polinización que producen alta calidad en los frutos de fresa son proporcionados por la comunidad de visitantes florales presentes en la región de estudio sin la necesidad de usar abejorros comerciales.

Abstract

Animal-mediated pollination is essential for the production and quality of fruits and seeds of many crops consumed by humans. However, crop pollination services might be compromised when wild pollinators are scarce. Managed pollinators are commonly used in crops to supplement such services with the assumption that they will enhance crop yield. However, information on the spatiotemporal pollinator-dependence of crops is still limited. We assessed the contribution of commercial bumblebee colonies compared to the available pollinator community on strawberry (‘Fortuna’ variety) flower visitation and strawberry quality across a landscape gradient of agricultural intensification (i.e. polytunnel berry crop cover). We used colonies of bumblebees in winter and in spring, i.e. when few and most wild pollinators are in their flight period, respectively. The placement of colonies increased visits of bumblebees to strawberry flowers, especially in winter. The use of bumblebee colonies did not affect flower visitation by other insects, mainly honeybees, hoverflies and other Diptera. Flower visitation by both honeybees and wild insects did not vary between seasons and was unrelated to the landscape gradient of berry crop cover. Strawberries were of the highest quality (i.e. weight) when insect-mediated pollination was allowed, and their quality was positively related to wild flower visitors in winter but not in spring. However, increased visits to strawberry flowers by managed bumblebees and honeybees had no effect on strawberry weight. Our results suggest that the pollination services producing high quality strawberry fruits are provided by the flower visitor community present in the study region without the need to use managed bumblebees.

Introduction

Around 75% of world food crops require or benefit from animal-mediated pollination to increase the production and quality of fruits and seeds (Klein *et al.*, 2007). During the last half century, the area devoted to these crops has disproportionately increased compared with non-dependent crops (Aizen *et al.*, 2008). Several factors can affect the presence of pollinators in crops and compromise the service they provide. For instance, the reduction of natural habitats can decrease wild pollinator abundance and richness in agroecosystems (Williams *et al.*, 2010; Winfree *et al.*, 2011) because it decreases the availability of nesting sites and flower resources over time. Furthermore, the temporal variability of wild pollinators, which is due to their life-cycle and their activity depending on temperature, creates periods in which their abundance is low (Pisanty *et al.*, 2014; Ellis *et al.*, 2017). If crops bloom outside of the main pollinator flying phenophase period or for a long period, wild pollinators might not fulfill crop pollination services. For these reasons, many farmers do not solely rely on wild pollinators, but rather managed insect pollinators are used to supplement visitation rates on pollinator-dependent crops regardless of the occurrence of wild pollinators.

Honeybees (*Apis mellifera*) and bumblebees (*Bombus* spp.) are the most common managed pollinators used worldwide. Honeybees have been historically domesticated for honey production and crop pollination (Aizen & Harder, 2009; Garibaldi *et al.*, 2013), being present in many crop systems worldwide (Winfree *et al.*, 2007). In contrast, bumblebees have been domesticated more recently (i.e. last four decades) mainly to pollinate greenhouse tomato crops (Velthuis & van Doorn, 2006). Because bumblebees show higher activity when weather conditions are cool and cloudy and require less management effort compared with honeybees, their use has been extended to many other crops, such as berries and apples. Nowadays, over a million colonies are annually commercialized all over the world (Velthuis & van Doorn, 2006).

Contrasting results have been found in relation to the use of managed pollinators and crop yield. In fact, their contribution might depend on the spatiotemporal variability of wild pollinators in crops. For instance, when the services provided by managed pollinators are estimated in a scenario where wild pollinators are absent, an overall positive effect is found (Roldán Serrano & Guerra-Sanz, 2006; Albano *et al.*, 2009). However, in a scenario where wild pollinators are present, this relationship can vary. On the one hand, if wild pollinator populations are relatively small and do not complete the required pollination services, managed pollinators can make a significant contribution to crop yield as seen in blueberry

(Isaacs & Kirk, 2010), raspberry (Lye *et al.*, 2011) and sunflower crops (Pisanty *et al.*, 2014). On the other hand, if wild pollinator populations are large and diverse, managed pollinators may drop back to a secondary role and supplement the pollination services, which in many cases does not translate into an increased crop yield (Holzschuh *et al.*, 2012; Garibaldi *et al.*, 2013; Mallinger & Gratton, 2015). But even negative effects, for example in raspberry drupelet set, have been found when non-native bumblebees and also honeybees visit flowers quite frequently (Sáez *et al.*, 2014).

In addition, managed pollinators can also spillover into adjacent natural areas (Ishii *et al.*, 2008; González-Varo & Vilà, 2017). There, they can compete with native pollinators for floral and nesting resources (Inoue *et al.*, 2008; Ishii *et al.*, 2008), as well as drive parasite spread into native pollinator populations (Colla *et al.*, 2006; Fürst *et al.*, 2014). Furthermore, managed pollinators can disrupt plant-pollinator networks and impact the reproductive success of wild plants (Magrach *et al.*, 2017).

Huelva province in SW Spain is, currently, the second largest producer of strawberries (*Fragaria x ananassa*) in the world (~300,000 tons per year; Freshuelva). Farmers typically grow strawberries under semi-open polytunnels for a long period; the flowering period spans from November to May. Many farmers rely on managed pollinators (honeybees and/or bumblebees) to aid crop pollination, because studies have shown that strawberry fruit quality is enhanced when insect-pollinated (e.g. Klatt *et al.* 2014). In general, honeybee hives are used throughout the entire crop flowering period, while bumblebee colonies are mainly used in winter when it is cold. However, in this region wild pollinators are abundant in natural habitat patches, especially in spring (Magrach *et al.*, 2017).

Our goal in this study was to determine whether strawberry quality increases with the use of commercial bumblebee colonies, and the role of the native strawberry flower visitor community. For this purpose, we placed colonies of *Bombus terrestris* in 12 strawberry plots along a landscape gradient of polytunnel berry crop cover. We surveyed strawberry flower visitors when colonies were both absent and present, and evaluated the pollination services provided during winter (early-January to mid-February), a period when major revenues might be compromised by the scarcity of wild pollinators, and in spring (early-March to mid-April), a period when most wild plants bloom and pollinators are very active in adjacent natural habitats. We addressed the following questions: (a) Does the use of bumblebee colonies affect flower visitation rates in strawberries? (b) Are strawberry flower visitors affected by seasonal differences along a landscape gradient? (c) To what extent does strawberry quality rely on insect-mediated pollination? and (d) Does the use of bumblebee colonies and/or the increase of flower visitors enhance strawberry quality?

Materials and methods

Study system

The study was conducted in the Guadalquivir valley, province of Huelva (SW Spain), in 2016. The climate is typically Mediterranean with hot and dry summers and mild winters. Mean annual temperature and precipitation are 18.2 ºC and 525 mm, respectively (AEMET). In the study region, berry crops are quite widespread, especially strawberries (~75% of the area devoted to berry crops; Freshuelva), forming an intermingled mosaic with woodland patches composed of a rich entomophilous understorey that mostly blooms in spring.

Throughout the study area, we selected 12 strawberry semi-open polytunnel plots across a landscape gradient of agricultural intensification and natural habitat cover (Fig. 1). The distance between selected strawberry plots ranged from 2,532 to 17,406 m (5060 ± 1192 m, mean ± SE, hereafter). These distances are larger than the reported values of maximum foraging distances of most pollinators (Gathmann & Tscharntke, 2002; Osborne *et al.*, 2008), assuring independence of study plots. Agricultural intensification was measured as the percentage of berry crop cover in circular areas around each plot. Natural habitats, in contrast, consisted of the sum of grasslands, pinewoods and shrubland areas. We considered the cover of berry crops, rather than other agricultural lands such as olive trees and vineyards, a good proxy of agricultural intensification because these are the most extensive and most disturbed in the area in terms of labour and inputs. Multiple scales with radii of 500, 1000, 1500 and 2000 m were used to select the best scale predictor. For instance, at a scale with 2-km buffer radius, berry crop cover in the landscape ranged from 0.1 to 64% (24.0 ± 5.9%), while natural habitat cover ranged from 6 to 53% (33.4 ± 4.4%). We used the ArcGIS (ESRI, 2011) programme based on the land cover map of Andalucía from 2011 (Moreira *et al.*, 2011) to calculate the percentage of each land-use type.

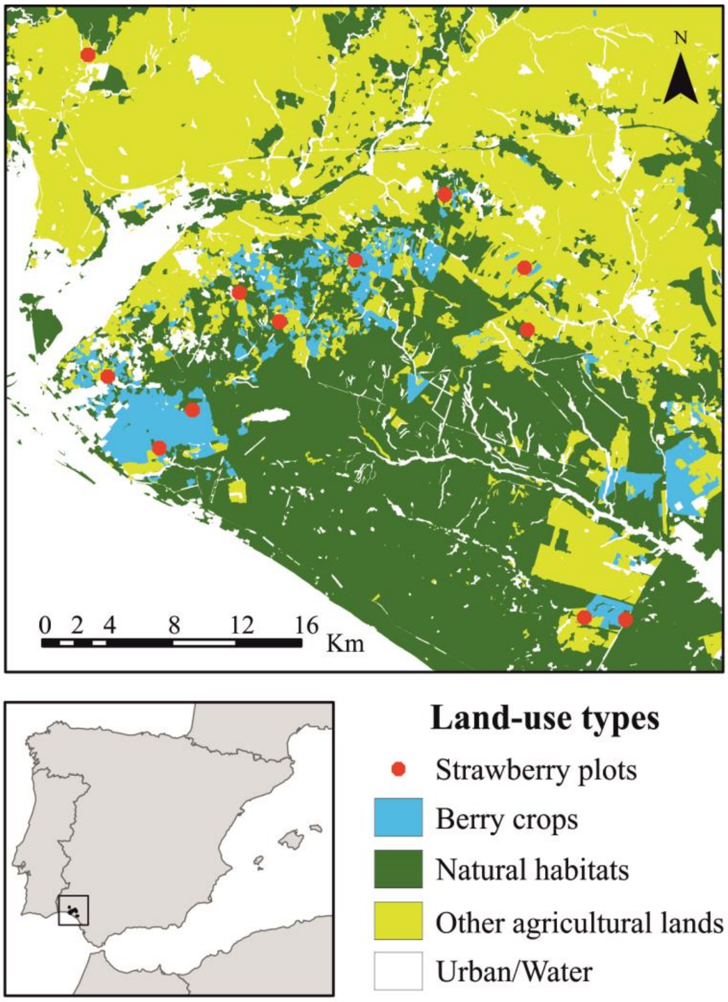


Figure 1. Geographical distribution of the 12 study strawberry plots in the province of Huelva (SW Spain). Latitude 37º23'24.19'' N to 37º19'14.65'' N and longitude 6º52'34.16'' W to 6º31'13.31'' W.

Within each strawberry plot we selected an area c. 25 m × 70 m (experimental plot), which consisted of five semi-open polytunnels. Each tunnel was 5 m wide, 70 m long and had five to six parallel 0.5 m wide ridges. Each ridge had two rows of strawberry plants and was elevated half a metre aboveground (see Appendix A: Fig. A1). Experimental plots met three criteria. First, the strawberry variety was 'Fortuna', because it is one of the most cultivated in this region and therefore important in the economic sector. Second, plots were adjacent to natural habitats. Third, there were no commercial bumblebee and honeybee colonies within 300 m of the experimental plots. This distance was the maximum for which we could ensure the absence of managed bee colonies without compromising farmers' demands.

Supplementation of Bombus terrestris colonies

We used 48 bumblebee colonies from Koppert Biological Systems, which were kept with a syrup solution ad-libitum in a plastic box covered by cardboard. Each colony included the founding queen and approximately 100 workers. From 7 to 26 January 2016 (i.e. 20 days of treatment; round one), we placed two colonies in six (i.e. half) of the selected strawberry plots (randomly assigned). One colony was positioned in the middle of the first tunnel (i.e. tunnel one) and the other in the middle of the last tunnel (i.e. tunnel five) in each plot. After this period, we removed the colonies and placed new colonies in the remaining six plots for the following 20 days (round two). We hereafter refer to round one and round two as the 'winter' season. Starting on 3 March 2016, we repeated the procedure to cover the period when wild pollinators thrive in natural habitats. We thus refer to rounds three and four, both also 20 days in length, as the 'spring' season. In sum, each plot had a consecutive absent/present colony treatment or vice-versa in winter and in spring.

Flower visitor censuses

Five days after the start of each round (i.e. after the placement of bumblebee colonies) we conducted two flower visitor censuses on two days per plot at different times: one day in the morning and another day in the afternoon. The same sampling procedure was also conducted in the six plots without colonies. Sampling was performed on sunny and calm days with minimum interior temperatures of 16 ºC in winter and 20 ºC in spring. Plots were selected at random on those days. For each census, we walked 200 m within tunnels two to four for 60 min. We recorded and identified managed bumblebees and honeybees visiting strawberry flowers along the four middle ridges of each tunnel, comprising a total area of 2 m × 200 m per census. For wild flower visitors, we reduced the observed area and sampled the two central ridges of each tunnel, comprising a total area of 1 m × 200 m per census. The difference in the survey area was justified given that managed flower visitors (i.e. bumblebees and honeybees) are highly active and larger than most wild flower visitors (i.e. Diptera), and therefore more easily detected.

As with honeybees, we assumed that all bumblebees observed were managed bumblebees. In fact, in this region, bumblebees are rare (Magrach *et al.*, 2017). Most wild flower visitors recorded were identified as hoverflies and other Diptera. Specimens are deposited at the EBD-CSIC. For analyses, flower visitors were assigned to one of the three following groups: bumblebees, honeybees and wild insects.

To quantify the availability of strawberry flowers, we surveyed two 50 m transects in tunnels two to four each census day. Along each transect we counted receptive flowers within 40 cm x 40 cm quadrates placed every five metres. Flower density was two times larger in spring than in winter but variability among plots was very low (16.97 ± 1.83 flowers/m^2 in winter and 32.18 ± 1.34 flowers/m^2 in spring). Therefore, we decided not to include flower density in the statistical analyses as a factor influencing spatial differences within a season.

Contribution of flower visitors to strawberry quality

To quantify the dependence of strawberry crops on insect-mediated pollination, and specifically to compare the contribution of managed bumblebees and other flower visitors to strawberry yield, we tagged 30 virgin primary flowers from different plants in each experimental plot. In each plot, virgin flowers were selected five to six days after the start of each round throughout tunnel three. Virgin flowers were identified as those not yet open and therefore not visited by insects before treatment. Each flower was randomly assigned to one of the following two treatments: net-bagged or open. For the net-bagged treatment, nylon tulle bags (size: 10 cm × 10 cm; openings: 1 mm × 1 mm) were used to cover virgin flower buds. Thus, the net-bagged treatment allowed for self- and wind-pollination, while the open treatment additionally permitted insect pollination. It should be noted that the treatments were only applied to primary flowers as they produce fruits with the largest commercial value. After fruit set, i.e. approximately 10 days after treatment, we bagged all fruits to avoid hand picking. Fruits were harvested and weighed in the field when ripe. We used weight and degree of deformation as measures of fruit quality (Klatt *et al.*, 2014). Degree of deformation was estimated based on European marketing criteria (European Commission 2011). All fruits showed extremely low deformation rates and overall 99.3% of them were classified into class I (good quality). Thus, differences in the degree of deformation were not compared.

Statistical analyses

For each season we calculated strawberry flower visitation by bumblebees, honeybees and wild insects as the average number of recorded interactions per census and round. To deal with differences in surveyed area for each flower visitor group we standardized the data for an area of 100 m^2.

First, we evaluated whether the use of commercial bumblebee colonies increased bumblebee visits to strawberry flowers. For this purpose, we built a generalized linear mixed model (GLMM; gamma error distribution and log link function) with bumblebee flower visitation as the response variable, and colony treatment (absent/present), season (winter/spring) and their interaction as fixed factors. A contrast matrix was built for post hoc comparison when significant interactions were found. Prior to this, we ascertained that there were no significant differences (Wilcoxon signed-rank test: p = 0.10 in winter and p = 0.06 in spring) in the placement of bumblebee colonies at different rounds in winter (round one *vs*. round two) and in spring (round three *vs*. round four).

Second, we evaluated whether the use of commercial bumblebee colonies affected flower visitation by honeybees and wild insects using paired Wilcoxon signed-rank tests. Because colony treatment (absent/present) had no effect on flower visitation by honeybees and wild insects (see results), data from colony supplementation treatments were pooled. Then, we tested whether flower visitation by honeybees and wild insects varied between seasons and/or was related to the landscape gradient. For this purpose, we built GLMMs (gamma error distribution and log link function) with flower visitation by honeybees and wild insects as the response variables, and season (winter/spring), land use-type (berry crop or natural habitat cover) and their interaction as fixed factors. Multiple scales for each land use-type were tested in separate models. Models were selected based on the minimum value given by the second order Akaike Information Criterion (AICc; Burnham *et al.*, 2011) and compared with null models (models without explanatory variables).

Third, we assessed the degree of strawberry dependence on insect-mediated pollination using linear mixed models (LMMs) with a Gaussian error distribution. In the models, the log transformed (log x+1) strawberry weight was used as a response variable, and colony treatment (absent/present), season (winter/spring), and pollination treatment (net bagged/open flowers) were included as fixed factors. In the model, we also included the interaction between colony treatment and season.

Finally, we evaluated the direct contribution of each flower visitor group (bumblebees, honeybees and wild insects) and also total flower visitors to strawberry fruit weight in winter and in spring, using separate LMMs for each season. The difference in the weight of fruits from open flowers and net-bagged flowers was used as the response variable. The indirect contribution of the landscape to strawberry weight was not compared as it had no effect on flower visitors (see results).

For GLMMs and LMMs, ‘plot’ was included as a random factor to account for re-sampled plots between rounds and/or seasons. The statistics yielding both F- and p-values were calculated using Satterthwaite’s approximations to determine denominator degrees of freedom. Moran’s I index was used to check for spatial autocorrelation of each flower visitor group and no signal was found (I < 0.4). All statistical analyses were computed in R (v.3.1.3, R Core Team, 2014) using packages *lmerTest* (Kuznetsova *et al.*, 2013), *lme4* (Bates *et al.*, 2014), *multcomp* (Hothorn *et al.*, 2013), *MuMIn* (Barton, 2009) and *Ncf* (Bjørnstad, 2013).

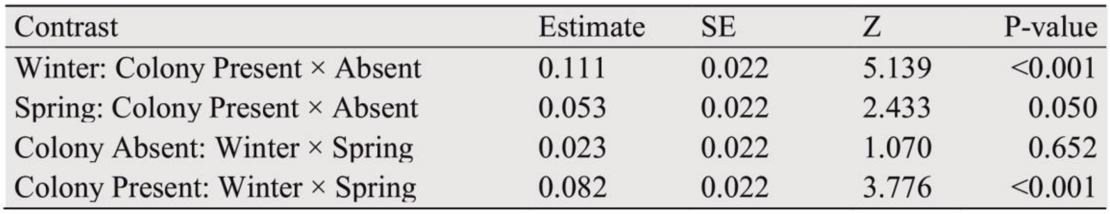
Results

Strawberry flower visitors

Overall, we recorded 790 and 1092 strawberry flower visitors belonging to 20 and 27 species (or morphospecies) in winter and in spring, respectively. Despite the apparent high richness of insect taxa visiting strawberry flowers, five taxa accounted for 94.5% of all records (see Appendix A: Table A1). These included managed bumblebees (*Bombus terrestris*) and honeybees (*Apis mellifera*), and wild insects such as *Eupeodes corollae* (47.4% of total recorded wild insects), Diptera sp1 (morphotype 1; 9.3%) and *Episyrphus balteatus* (7.9%). Overall, the total number of visits was not significantly different between winter and spring (mean ± se = 0.47 ± 0.08 ind/100 m^2 in winter and 0.70 ± 0.13 ind/100 m^2 in spring; paired Wilcoxon signed-rank tests: n = 12, p = 0.09).

When commercial bumblebee colonies were absent, strawberry flower visitation by bumblebees did not differ significantly between winter and spring (Table 1, Fig. 2). Most of the records (~78%) derive from two strawberry plots in winter with the highest area of berry crops in the landscape. However, the placement of colonies significantly increased strawberry flower visitation by bumblebees (Table 1, Fig. 2), being higher in winter than in spring (Table 1, Fig. 2).

Table 1. The effects of colony treatment (absent/present) and season (winter/spring) on strawberry flower visitation by bumblebees.



Contrast	Estimate	SE	Z	P-value
Winter: Colony Present × Absent	0.111	0.022	5.139	<0.001
Spring: Colony Present × Absent	0.053	0.022	2.433	0.050
Colony Absent: Winter × Spring	0.023	0.022	1.070	0.652
Colony Present: Winter × Spring	0.082	0.022	3.776	<0.001

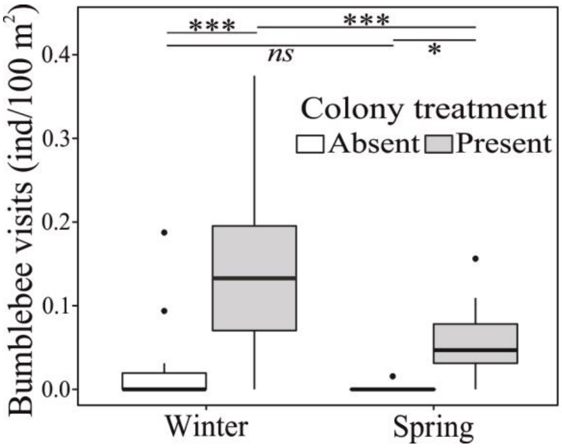


Figure 2. Box-plot showing strawberry flower visitation by bumblebees (visits per 100 m^2) when commercial bumblebee colonies were absent and present in winter and in spring. Significance levels: ***p = 0.001; **p = 0.01; *p = 0.05; *ns* = p > 0.05.

Flower visitation by both honeybees and wild insects was not affected by colony treatment in any season (paired Wilcoxon signed-rank tests: n = 12, p > 0.05). Honeybees and wild insects were recorded visiting strawberry flowers in all plots throughout the sampling dates. Overall, honeybees were the most abundant flower visitors. Flower visitation by honeybees was 0.36 ± 0.07 ind/100 m in winter and 0.56 ± 0.11 ind/100 m^2 in spring, while flower visitation by wild insects was 0.07 ± 0.01 ind/100 m^2 in winter and 0.12 ± 0.05 ind/100 m^2 in spring. There were no significant effects of season (winter/spring) and land-use type (berry crop or natural habitat cover) on either honeybee or wild insect flower visitation at any of the scales examined, as null models showed the lowest AICc values.

Strawberry weight

A total of 1296 fruits were weighed (144 fruits were excluded from analyses because damage and/or fungus was visible). Strawberries from bagged flowers were, overall, 15% smaller than fruits developed from open flowers ($F_{1,\ 1281.3} = 104.37$, $p < 0.001$) (Fig. 3). Strawberries were also smaller in spring than in winter ($F_{1,\ 1282.8} = 153.78$, $p < 0.001$) (Fig. 3) because flowers become smaller throughout the growing season (see Appendix A: Fig. A2). Colony treatment had no effect on strawberry weight in any season ($F_{1,\ 1281.7} = 2.28$, $p = 0.131$).

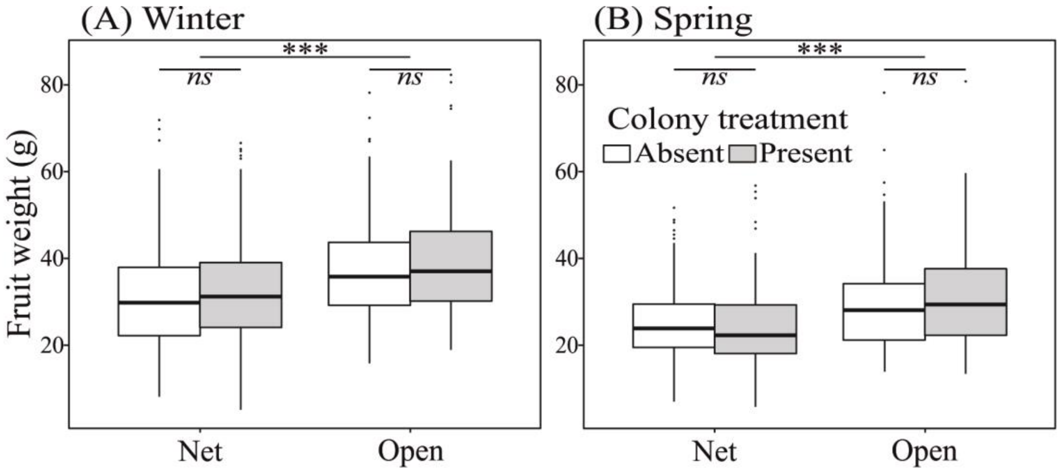


Figure 3. Box-plots showing strawberry weight of net-bagged and open-pollinated flowers when commercial bumblebee colonies were absent and present in winter (A) and in spring (B). Box-plots show medians and, 25th and 75th percentiles. The whiskers indicate the 5th and 95th percentiles, while black dots denote outliers. Significance levels: ***p = 0.001; **p = 0.01; *p = 0.05; *ns* = p > 0.05.

Table 2. The effects of flower visitation by bumblebees, honeybees and wild insects and also total insects on strawberry weight in winter and in spring. Satterthwaite's approximations were performed for statistic results.

Models	Flower visitor group	Df	F	P-value
Winter				
Model1				
	Bumblebee	1, 14.9	0.21	0.656
	Honeybee	1, 18.3	0.25	0.621
	Wild insects	1, 19.9	5.01	**0.037**
Model2				
	Total visitors	1, 19.4	0.02	0.899
Spring				
Model3				
	Bumblebee	1, 13.5	0.35	0.565
	Honeybee	1, 20.0	0.02	0.891
	Wild insects	1, 19.9	2.48	0.131
Model4				
	Total visitors	1, 20.7	1.66	0.212

In winter, the difference in strawberry weight between net-bagged and open flowers was not related to flower visitation by bumblebees (Fig. 4A) or honeybees (Fig. 4B), but rather by wild insects (Fig. 4C) (Table 2). However, in spring there were no significant relationships with total visits of pollinators in any season (Table 2, Fig. 4D).

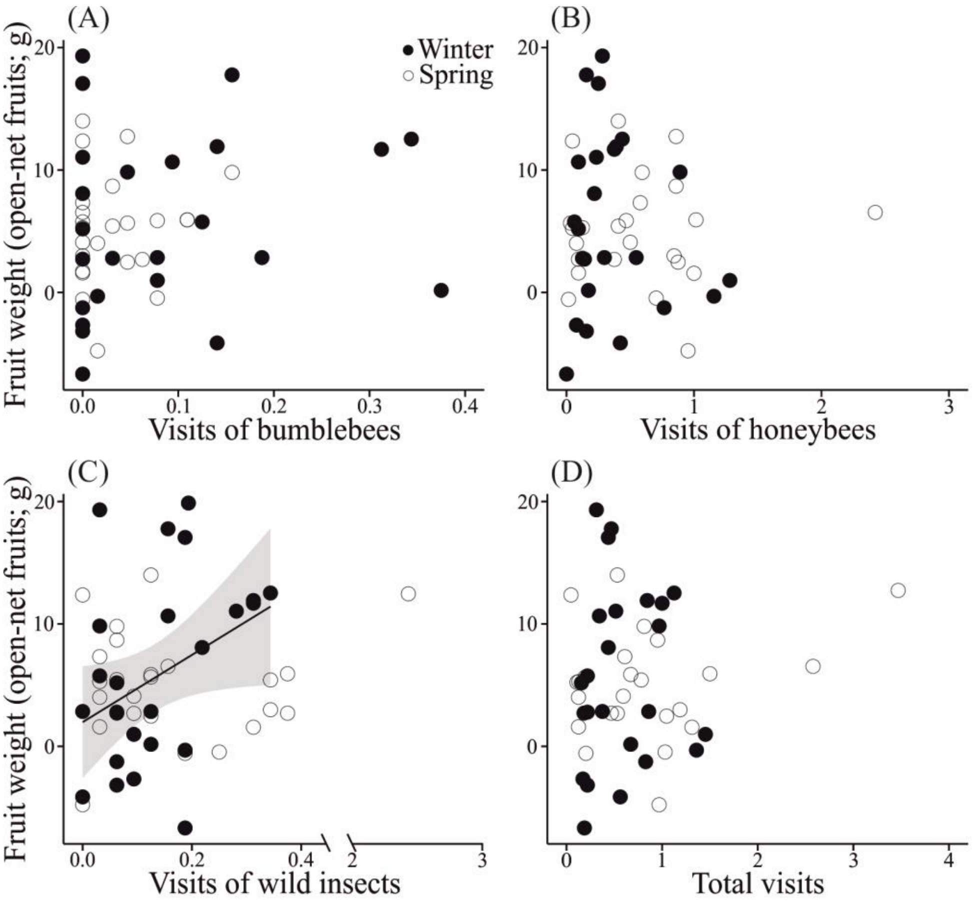


Figure 4. Relationships between difference of strawberry fruit weight (open - net fruits) and flower visitation (n/100 m^2) by bumblebees (A), honeybees (B), wild insects (C) and total insects (D) in winter (black dots) and in spring (open dots). The line indicates a significant relationship and the shaded area indicates the 95% confidence interval.

Discussion

The use of commercial bumblebee colonies in strawberry crops increased bumblebee strawberry flower visitation, especially in winter. This increase did not affect flower visitation by other insects, composed mainly of honeybees, hoverflies and other Diptera. Surprisingly, strawberry weight, which was higher when insect pollination was allowed, did not increase when bumblebee colonies were used. Moreover, strawberry weight was not related to increases in the number of flower visits by bumblebees and honeybees in any season. However, in winter, strawberry weight increased when wild flower insects proliferated, although such an effect did not occur in spring.

Strawberry flower visitors

Flower visitation by bumblebees was higher in winter than in spring. Seasonal differences are probably due to a spillover of bumblebees from crops to adjacent habitats in spring, a period when most wild plants bloom (Whittington et al., 2004). However, the flower visitor community present in strawberry crops was neither affected by the use of commercial bumblebee colonies in winter nor in spring. Lye and colleagues (2011) also reported no competition when bumblebee colonies were used in raspberry crops in Scotland. It is likely that the massive and continuous bloom offered by these crops provides sufficient floral resources for all flower visitors.

Even when we used commercial bumblebee colonies and did not observe honeybee hives in the area, honeybees were the most common flower visitors, as also reported by other authors (Chagnon *et al.*, 1993; Bartomeus *et al.*, 2014). Honeybee hives harbour a high number of individuals (~60 K individuals; Seeley & Morse, 1976), approximately 50 times that of bumblebee colonies. Honeybees fly long distances (~1.5 km; Steffan-Dewenter & Kuhn, 2003) and thus could have come from other areas. We noticed that flower visitation by honeybees was quite similar among strawberry plots. In our study region, most honeybee hives are maintained over the crop flowering period and it is likely that the number of honeybee hives is related to berry crop cover in the landscape (González-Varo & Vilà, 2017). Thus, although in spring honeybees are attracted to forage in natural habitats, because more attractive resources are available (Free, 1993), the growth of honeybee hive populations from winter to spring could maintain similar numbers of individuals foraging in strawberry crops between seasons.

Unexpectedly, flower visitation by wild insects did not vary between seasons and it was unrelated to natural habitat cover. Although there is a large amount of evidence reporting that land-use intensification negatively affects wild pollinators, mostly bees (Isaacs & Kirk, 2010; Klein *et al.*, 2012; Holzschuh *et al.*, 2016), in our study the community of observed flower visitors was composed of hoverflies and other Diptera. Pollinators show variable sensitivity to landscape composition depending on their life history. Non-bee insects can be less reliant on natural areas than bees (Rader *et al.*, 2016); for instance, hoverflies and other Diptera are able to exploit resources from habitat types highly altered (Winfree *et al.*, 2011; Raymond *et al.*, 2014). This, together with the mild winter weather conditions in the study region, could favour wild flower visitor populations to be sustained throughout the strawberry flowering period regardless of changes in landscape characteristics.

Strawberry weight

As expected, strawberry crops produced heavier fruits when insect-pollinated (Klatt *et al.*, 2014), but the measures that we recorded as proxies for fruit quality (shape and weight) did not increase with the use of bumblebee colonies nor with an increase in visitation frequency of managed bumblebees and honeybees. There are several studies which have found that managed pollinators promote crop yield, but they are less valuable than wild pollinators (Holzschuh *et al.*, 2012; Garibaldi *et al.*, 2013). This is widely related to flower damage when pollinators are at saturation levels or, as is likely in our system (although we lacked these measures), to the transfer of low-quality or incompatible pollen (Morris *et al.*, 2010; Aizen *et al.*, 2014; Sáez *et al.*, 2014). Honeybees were the most frequently recorded flower visitor. Thus, it is likely that honeybees provided the pollination services to produce larger fruits when insect-pollinated, but increases in flower visitation frequency did not produce an added effect (Garibaldi *et al.*, 2013; Mallinger & Gratton, 2015). A similar process could have occurred when colonies of bumblebees were present or even, as their recorded visits to strawberry flowers were low, bumblebee effects on strawberry weight may have been diluted. Importantly, when the number of wild non-bee flower visitors increased, they provided supplementation of the pollination services by increasing the weight of fruits. But this positive effect was only significant in winter. A possible explanation for such seasonal differences could be related to the time spent foraging by wild flower visitors. It is likely that in spring, when weather conditions are optimal, the visitation rates of each individual are greater, providing the maximum pollination service across the landscape.

Conclusions

Our study indicates that bumblebee colony supplementation in the most cultivated strawberry variety, Fortuna, in Huelva (SW Spain) does not yield an increase in fruit weight. We found that the community of flower visitors present, composed of honeybees, hoverflies and other Diptera, provides the necessary pollination service to this strawberry variety. It is important to take into account that most flower visitors were honeybees which probably play an important role in the pollination function, and that bumblebees are not common pollinators in this Mediterranean region. Moreover, the strawberry plots in which we conducted our experiments were located adjacent to natural habitats where the presence of wild flower visitors could be greater than at larger distances within the crop. In any case, our findings emphasize the need to consider the spatiotemporal variability of managed and wild pollinators in pollinator-dependent crops. The unnecessary use of managed pollinators reduces farmers' profits. It is very important, therefore, that crop type, fruit variety and region are considered before the widespread use of commercial bumblebee colonies.

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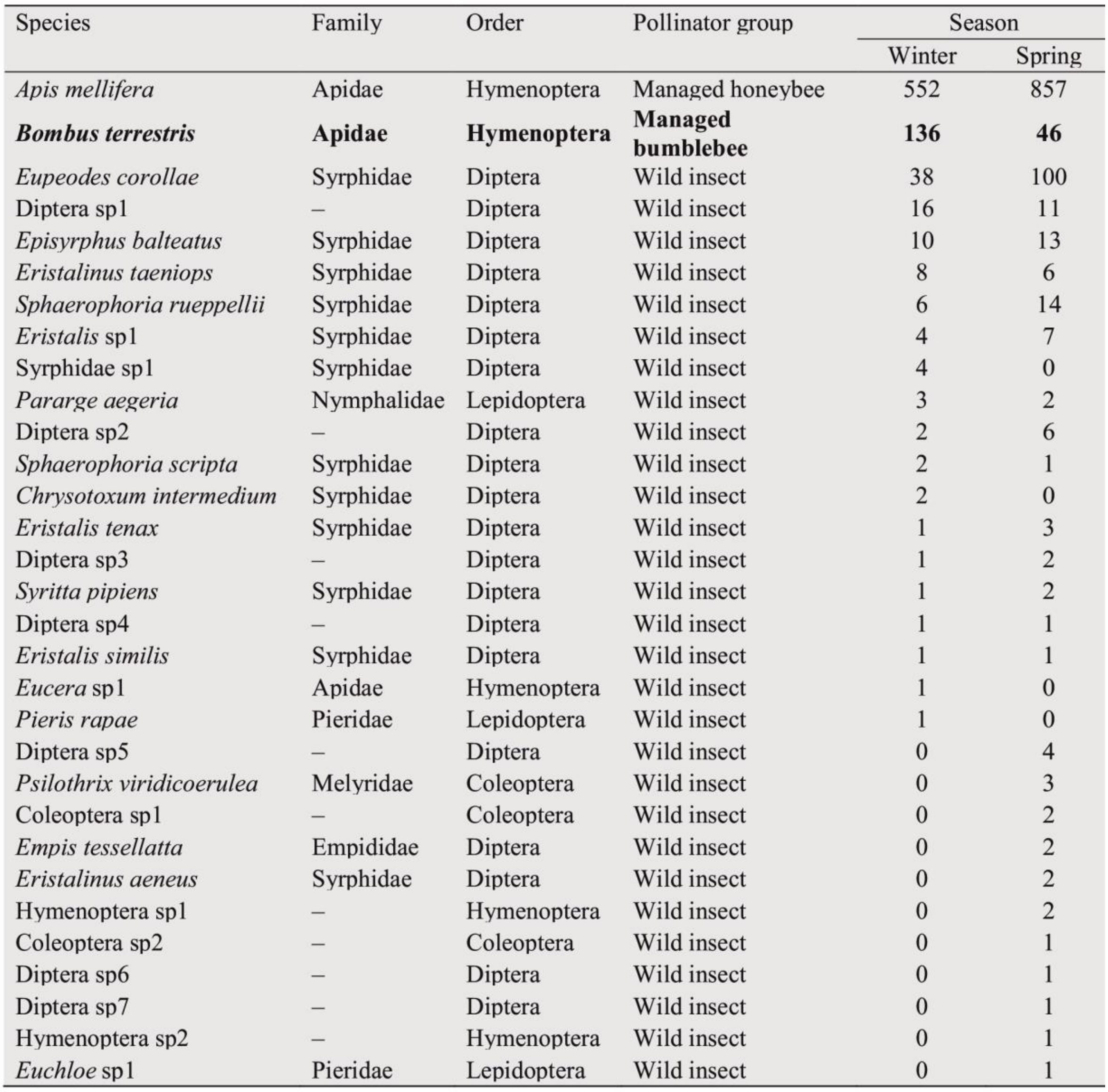
Appendix

Table A1. Total number of interactions registered between each pollinator species (or morphospecies) and strawberry flowers (*Fragaria x ananassa*) in winter and in spring throughout the study.



Species	Family	Order	Pollinator group	Season	
				Winter	Spring
Apis mellifera	Apidae	Hymenoptera	Managed honeybee	552	857
Bombus terrestris	**Apidae**	**Hymenoptera**	**Managed bumblebee**	**136**	**46**
Eupeodes corollae	Syrphidae	Diptera	Wild insect	38	100
Diptera sp1	–	Diptera	Wild insect	16	11
Episyrphus balteatus	Syrphidae	Diptera	Wild insect	10	13
Eristalinus taeniops	Syrphidae	Diptera	Wild insect	8	6
Sphaerophoria rueppellii	Syrphidae	Diptera	Wild insect	6	14
Eristalis sp1	Syrphidae	Diptera	Wild insect	4	7
Syrphidae sp1	Syrphidae	Diptera	Wild insect	4	0
Pararge aegeria	Nymphalidae	Lepidoptera	Wild insect	3	2
Diptera sp2	–	Diptera	Wild insect	2	6
Sphaerophoria scripta	Syrphidae	Diptera	Wild insect	2	1
Chrysotoxum intermedium	Syrphidae	Diptera	Wild insect	2	0
Eristalis tenax	Syrphidae	Diptera	Wild insect	1	3
Diptera sp3	–	Diptera	Wild insect	1	2
Syritta pipiens	Syrphidae	Diptera	Wild insect	1	2
Diptera sp4	–	Diptera	Wild insect	1	1
Eristalis similis	Syrphidae	Diptera	Wild insect	1	1
Eucera sp1	Apidae	Hymenoptera	Wild insect	1	0
Pieris rapae	Pieridae	Lepidoptera	Wild insect	1	0
Diptera sp5	–	Diptera	Wild insect	0	4
Psilothrix viridicoerulea	Melyridae	Coleoptera	Wild insect	0	3
Coleoptera sp1	–	Coleoptera	Wild insect	0	2
Empis tessellatta	Empididae	Diptera	Wild insect	0	2
Eristalinus aeneus	Syrphidae	Diptera	Wild insect	0	2
Hymenoptera sp1	–	Hymenoptera	Wild insect	0	2
Coleoptera sp2	–	Coleoptera	Wild insect	0	1
Diptera sp6	–	Diptera	Wild insect	0	1
Diptera sp7	–	Diptera	Wild insect	0	1
Hymenoptera sp2	–	Hymenoptera	Wild insect	0	1
Euchloe sp1	Pieridae	Lepidoptera	Wild insect	0	1

Figure A1. Left: Example of polytunnels and adjacent natural habitats. Right: View inside the polytunnels.

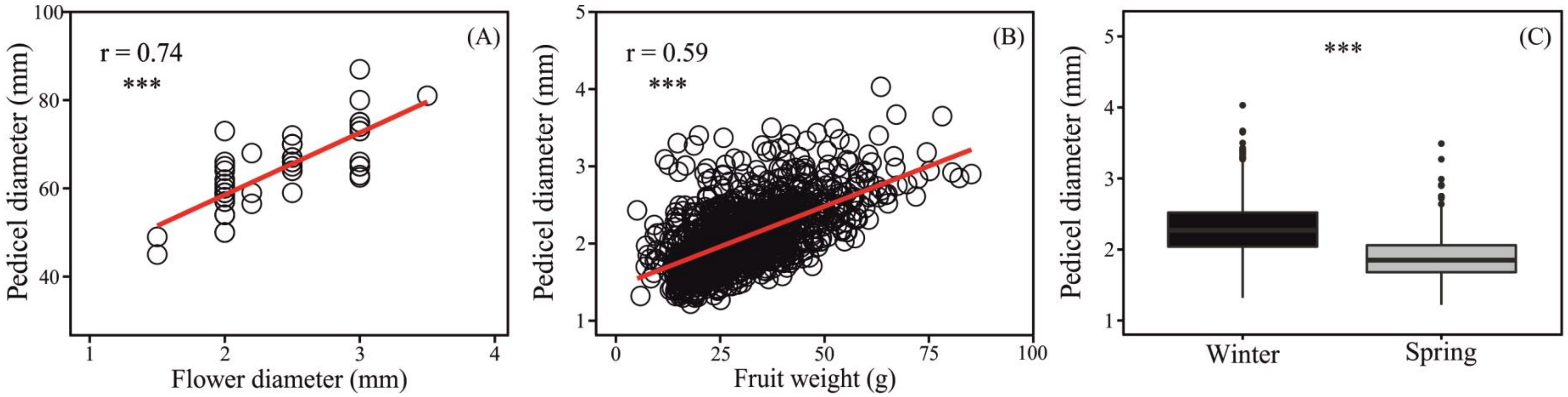


Figure A2. Spearman rank correlation between the diameter of flowers randomly selected in the strawberry plots and the diameter of their pedicel measured 10 mm from their receptacle (n = 48) (A). Spearman rank correlation between fruit weight (all study fruits; n = 1296) and the diameter of their pedicel (B). Box-plot from Wilcoxon signed-rank test comparing pedicel diameter of strawberry fruits (n = 1296) in winter and in spring (C) Significance level: ***p = 0.001.

Capítulo 2

Contrasting occurrence patterns of managed and native bumblebees in natural habitats across a greenhouse landscape gradient

Trillo, A., Montero-Castaño, A., González-Varo, J.P., González-Moreno, P., Ortiz-Sánchez, F.J. & Vilà, M. (2019) Contrasting occurrence patterns of managed and native bumblebees in natural habitats across a greenhouse landscape gradient. *Agriculture, Ecosystems & Environment*, **272**, 230–236.

Resumen

Durante las últimas décadas se ha producido una importante expansión de cultivos dependientes de polinizadores. El incremento en el uso de colonias comerciales de polinizadores asociado a estos cultivos podría promover el movimiento de polinizadores manejados hacia hábitats naturales cercanos. Allí, estos polinizadores manejados podrían explotar recursos florales similares a los de los polinizadores silvestres, y por tanto, incrementar la competencia por los recursos locales. Sin embargo, el movimiento de polinizadores manejados ha sido poco estudiado y la investigación se ha centrado mayoritariamente en solo una especie, la abeja de la miel occidental (*Apis mellifera*). En el suroeste de España, nosotros investigamos la presencia, densidad y explotación de los recursos de abejorros manejados (*Bombus terrestris*) y nativos (*B. t. lusitanicus*) en 19 pinares con matorral mediterráneo a lo largo de un gradiente de cobertura de invernadero de fresa. El muestreo se realizó en dos años consecutivos durante dos estaciones: invierno, cuando las fresas comienzan a florecer y los agricultores usan frecuentemente colonias, y en primavera, cuando hay una mayor disponibilidad de recursos florales y los polinizadores silvestres prosperan. En invierno, la densidad de los abejorros manejados en los pinares fue mayor que la de los nativos. La presencia de los abejorros manejados y la de los nativos en los pinares mostró patrones contrarios en relación con la cobertura de invernadero en el paisaje. La presencia de abejorros manejados se relacionó positivamente con la cobertura de invernadero, mientras que la de abejorros nativos se relacionó negativamente. En general, la presencia y densidad de los abejorros no difirió entre estaciones. Las dos subespecies de abejorro mostraron preferencias similares hacia plantas silvestres particularmente en invierno, cuando las especies de plantas en floración son escasas. Concluimos que aunque los abejorros manejados son colocados en los invernaderos, su papel se extiende más allá de estos cultivos. Son necesarios estudios futuros para evaluar la función de los polinizadores manejados en los cultivos con el objetivo de reducir su movimiento hacia los hábitats naturales, y por tanto, el riesgo que suponen para los polinizadores nativos.

Abstract

In recent decades, there has been a remarkable expansion of pollinator-dependent crops. An increase in the use of commercial pollinator colonies associated with these crops may promote the spillover of managed pollinators into nearby natural habitats. There, these managed pollinators can exploit floral resources similar to those of wild pollinators, and thus increase competition for local resources. Nonetheless, managed pollinator spillover has been poorly studied and research has focused on only one species, the western honeybee (*Apis mellifera*). In south-western Spain, we investigated the presence, density and exploitation of floral resources by managed (*Bombus terrestris*) and native bumblebees (*B. t. lusitanicus*) in 19 Mediterranean pinewood understories across a landscape gradient of strawberry polytunnel greenhouse cover. Sampling was performed over two consecutive years in two seasons: winter, when strawberries begin flowering and farmers frequently use colonies, and spring, when there is greater availability of floral resources and wild pollinators thrive. In winter, the density of managed bumblebees in pinewoods was higher than that of native bumblebees. The presence of managed and native bumblebees in pinewoods showed contrasting patterns in relation to greenhouse cover in the landscape. The presence of managed bumblebees was positively associated with greenhouse cover, whereas that of native bumblebees was negatively associated with greenhouse cover. Overall, the presence and density of bumblebees did not differ between seasons. The two bumblebee subspecies showed similar wild flowering plant preferences, particularly in winter, when flowering plant species are scarce. We conclude that, although managed bumblebees are placed in greenhouse crops, their pollination role extends beyond these crops. Further studies are needed to assess the pollination function of managed pollinators in crops in order to reduce their spillover into natural habitats and thus, the risks posed to native pollinators.

Introduction

Human demand for fruits and seeds has increased over the past few decades(Aizen & Harder, 2009; Gallai *et al.*, 2009). Consequently, the area devoted to flowering crops, many of which require or benefit from animal-mediated pollination (Klein *et al.*, 2007), has disproportionately increased compared to that of non-pollinator dependent crops (Aizen *et al.*, 2008). These expanding agricultural systems offer floral resources that can attract pollinators from nearby natural habitats (Blitzer *et al.*, 2012).

From an economic point of view, the spillover of wild pollinators from natural habitats to crops may not be sufficient to meet the pollination services required. In other words, there may be insufficient pollinators visiting crop flowers to achieve maximum seed and fruit set, thereby affecting the quality and quantity of crop yield (Klein *et al.*, 2007; Ricketts *et al.*, 2008). For instance, the remaining small patches of natural habitat may not support enough pollinators to pollinate large areas of flowering crops (Garibaldi *et al.*, 2011; Holzschuh *et al.*, 2016; Eeraerts *et al.*, 2017). In addition, the bloom period of crops often does not overlap with the flying period of wild pollinators because crops are cultivated to complete their life cycle in a different period than pollinators. Further, greenhouse crops may not be accessible to pollinators. For these reasons, managed pollinators are frequently used in crops to supplement wild pollinator visitation to flowers (Morse, 1991; Velthuis & van Doorn, 2006; Rucker *et al.*, 2012).

Commercial production of bumblebees began in the late 1980s to replace hand- and mechanical-pollination of tomato crops (Ravestijn & Sande, 1991). This quickly led to the mass production and worldwide transport of these bees to supplement the pollination of about 20 different crops (Velthuis & van Doorn, 2006). Bumblebees are generalist pollinators (Memmott, 1999) and require a diverse pollen diet to thrive (Brunner *et al.*, 2014; Roger *et al.*, 2017). Despite their use in crops, they frequently escape and spillover into adjacent natural habitats to forage (Whittington *et al.*, 2004; Inari *et al.*, 2005; Murray *et al.*, 2013), as these areas typically provide more consistent and diverse floral resources. Managed bumblebees exploit a wide range of wild flowering plant species, increasing the likelihood of competitive interactions with local pollinator species (Whittington *et al.*, 2004; Ishii *et al.*, 2008), hybridisation with congeners (Ings *et al.*, 2005; Kraus *et al.*, 2011), as well as the spread of parasites (Goka *et al.*, 2006).

In addition to the evidence of bumblebees moving from crops to natural habitats, spillover has been shown to vary spatially and temporally. Spillover from crops to natural habitats may depend on crop cover in the surrounding landscape (e.g. Klein *et al.*, 2012; Gaines-Day & Gratton, 2016; Magrach *et al.*, 2017). Large crop areas will require a larger number of commercial colonies to meet pollination demands, eventually spilling over into natural habitats (González-Varo & Vilà, 2017). Furthermore, other factors such as the temporary management of colonies and floral resource availability throughout the year may mediate the magnitude of the spillover, with potential detrimental impacts on local pollinator fauna (González-Varo & Vilà, 2017).

Huelva (SW Spain) is the second largest strawberry producer in the world after California. In Spain, the strawberry flowering period spans from November to May, and commercial bumblebee colonies are used for pollination services. We investigated the presence, density and exploitation of floral resources by managed (*Bombus terrestris*) and native bumblebees (*B. t. lusitanicus*) in Mediterranean pinewood understories across a gradient of strawberry greenhouse cover, over two consecutive years. We surveyed plant-bumblebee interactions in understorey pinewoods during the two seasons in which managed and native bumblebees overlap: in winter, when farmers use more bumblebee colonies to overcome a shortage in wild pollinators, and in spring, when most wild plants bloom and wild pollinators thrive. Specifically, we addressed the following questions: (1) Are the presence and density of managed bumblebees in pinewoods across the landscape similar to those of native bumblebees? (2) Are the presence and density of managed and native bumblebees associated with greenhouse cover in the landscape? (3) Does their presence and density differ between seasons? (4) Do managed and native bumblebees exploit similar flowering plant taxa?

We expect the presence and density of managed bumblebees in pinewoods to be greater than those of native bumblebees in winter and the reverse in spring. We expect the presence and density of managed bumblebees to be positively associated with greenhouse cover and to be greater in winter than in spring. We expect the reverse to be true for native bumblebees. Finally, we expect both managed and native bumblebees to forage on similar wild flowering plants, as well as on strawberry flowers.

Materials and methods

Study area and bumblebee species

The study was conducted in a 40 km × 20 km area in the Guadalquivir Valley in the province of Huelva (SW Spain) (Fig. 1) in 2014 and 2015. Since 1965, in the province of Huelva there has been an increase in the production of berries, especially strawberries (*Fragaria* × *ananassa*). This area has become one of the major producers worldwide (i.e. around 7330 ha producing 308,500 tons of strawberries in 2014-2015; see FYH). The study area is characterised by a mosaic of intensive strawberry crops under semi-open polytunnel greenhouses and remaining patches of natural habitat. These patches are either shrublands or pinewoods (*Pinus pinea*), which are composed of a rich understorey vegetation (González-Varo *et al.*, 2016). Amongst the most representative entomophilous species are the shrubs *Cistus* spp., *Erica* spp., *Halimium* spp., *Lavandula stoechas*, *Rosmarinus officinalis* and *Ulex australis* and the herbs *Echium* spp., *Leontodon* spp. and *Linaria* spp. The climate is Mediterranean with warm and dry summers and mild winters (mean annual temperature and precipitation are 18.2 ºC and 525 mm, respectively; AEMET).

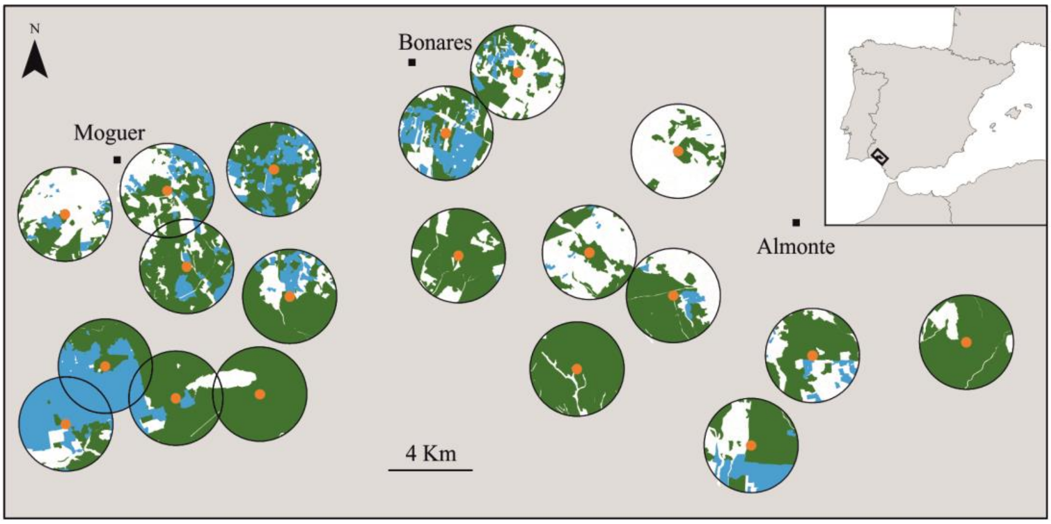


Figure 1. Study area in the province of Huelva (SW Spain). Circles indicate the 2 km landscape radius surrounding each pinewood plot. Greenhouse (black colour), natural habitat (grey colour) and other land-use types such as agricultural fields, wetlands and urban areas (white colour) represent the main land covers in the study area. Names denote towns.

Strawberry fruit quality benefits from insect-mediated pollination (Zebrowska, 1998; Klatt *et al.*, 2014; Chapter 1). To secure maximum revenue in south-western Spain and worldwide (Velthuis & van Doorn, 2006), farmers frequently use commercial *B. terrestris* colonies. Here, the strawberry flowering period spans from November to May. Notably, the use of bumblebee colonies is typically more intensive in winter, because wild pollinators are scarce and the weather conditions do not favour their activity (although see Chapter 1).

Bombus terrestris is native to Europe, North Africa and West and Central Asia, and is represented by 9 subspecies (Estoup *et al.*, 1996; Rasmont *et al.*, 2008; Lecocq *et al.*, 2016). Two subspecies represent the bulk of the bumblebees traded around the world for pollination services. Before the 2000s, the subspecies *terrestris* was the most widely traded, while the subspecies *dalmatinus* has dominated in the last decade (Ings *et al.*, 2006, 2009; Owen *et al.*, 2016). Until recently, it has not been common for countries to use managed bumblebees from their native subspecies (e.g. *B. t. audax* in UK; see Graystock *et al.*, 2016a).

In the study region, the managed bumblebees used are not the native bumblebee *B. t.* subsp. *lusitanicus*. Here, bumblebees occur at very low frequency (Magrach *et al.*, 2017), as they are at the limit of their distributional range (Goulson, 2010).

Sampling design

We selected 19 pinewood plots (50 m × 50 m) within the study area. The 2 km landscape radius surrounding each plot represented a gradient of greenhouse cover ranging from 0% to 64% (mean ± SD = 20.3 ± 19.4%), and a gradient of natural habitat ranging from 10% to 99% (mean ± SD = 47.9 ± 26.6%). These two main land-use types are negatively correlated (Spearman rank correlation: R = -0.50, n = 19, p = 0.03) and thus, we considered greenhouse cover the key factor influencing bumblebee patterns in natural habitats. On average, the remaining land-use types (e.g. denuded areas, urban areas, wetlands and other agricultural fields) ranged from 0.7% to 89% (mean ± SD = 31.6 ± 23.6%).

Plots were located at different distances from the closest greenhouse, ranging from 25 m to 4389 m. The average (± SD) distance between study plots was 4246 ± 946 m (range = 2977–6577 m) and most plots (~70%) were separated by >4 km, with no overlap in their 2 km radius buffers. However, the overlap for the closest plots was negligible, affecting only 11.5% (range = 4.1–23.5%) of their buffer area (see Fig. 1). Moreover, 2 km is considered to be the maximum foraging range of workers from most bumblebee species (see Osborne *et al.*, 2008), as bumblebees often limit their foraging range at the local scale if floral resources are abundant (Osborne *et al.*, 2008; Bommarco *et al.*, 2010). Thus, we are confident that our study plots meet independence criteria in terms of the individual bumblebees recorded during our surveys. The percentages of greenhouse cover were calculated using ArcGIS (ESRI, 2011) based on the land-cover map of Andalucía from 2011

(Moreira *et al.*, 2011), and updated with Google maps (2013) and field surveys (Table A1).

To ensure similar flowering plant species identity across the landscape gradient, all sampling plots shared at least five species. These plant species were *Halimium calycinum*, *R. officinalis* and *U. australis*, which bloom mainly in winter but also in spring, and *Cistus salvifolius* and *L. stoechas*, which bloom only in spring.

We conducted bumblebee censuses in 2014 and 2015 during two seasons: winter (early-January to early-February) and spring (late-March to late-April). We sampled each plot on four random days per season and year, two in the morning (09:30–13:00) and two in the afternoon (13:00–17:00), by walking 5-8 parallel transects (on average 7.91 transects per plot, 50 m × 5 m each) for 15 min, selecting a different transect each sampling time. Along each transect, we recorded the number of bumblebees of each subspecies foraging on flowers, and noted the plant taxa. Bumblebees were visually identified by colouration patterns according to Rasmont et al. (2008) and Ornosa & Ortiz-Sánchez (2004). Native bumblebees are characterised by reddish hair, especially on the scutellum and legs, while managed subspecies (hereafter, 'managed bumblebees') have black hair. In total, we conducted 2348 transect walks on 78 days accounting for a total of 587 sampling hours. Sampling was conducted on sunny and non-windy days with temperatures above 11 ºC.

Prior to this study, we conducted pilot sampling from November 2013 to May 2014 to test the proposed protocol and explore the distribution of native and managed bumblebee populations across the landscape (Appendix: Pilot sampling, Table A1 and Fig. A1).

To quantify floral resource availability for pollinators, in each plot and on each census day, we walked five randomly placed 50 m parallel transects. We counted and identified receptive flowers within 40 cm × 40 cm quadrants placed every two meters. Therefore, the number of flowers was counted in 130 quadrants per plot and day. Overall, nine flowering plant taxa were recorded in winter (2.49 ± 0.13 plants per plot, mean ± SE, hereafter), and 29 flowering plant taxa in spring (6.39 ± 0.34 plants per plot). The average floral density (flowers m^2) was 10.18 ± 3.18 in winter, and 30.87 ± 6.14 in spring (see Table A2 for more details).

Pollen loads

In addition to recorded plant-pollinator interactions and to amplify the resolution of floral resource exploitation, we captured both managed and native bumblebees during the censuses to identify their pollen loads. Bumblebees were captured while foraging using aerial nets and were frozen in individual clean vials for later identification of pollen loads. Sample sizes were small, particularly for native bumblebees, due to their low abundance and our intention to avoid disrupting their populations (see results).

The body of each bumblebee was rubbed with a cube (0.3 cm × 0.3 cm) of fuchsin jelly (Beattie, 1971). The cube was then mounted on a slide to identify pollen grains under a microscope. For each slide, we counted and identified all pollen grains in 20 fields at ×200 magnification. For the identification of pollen grains, we used a reference pollen collection prepared during the sampling period. Because some plant taxa cannot be identified to the species level via the microscope, some pollen was assigned to the genus level (Table A3).

For the plant-bumblebee interactions recorded, we identified pollen loads in a total of 33 (0 in 2014 and 33 in 2015) managed bumblebees in winter and 23 (10 in 2014 and 13 in 2015) in spring. We also collected a total of 4 (0 in 2014 and 4 in 2015) native bumblebees in winter and 12 (8 in 2014 and 4 in 2015) in spring. Overall, we counted 28,039 and 37,840 pollen grains from the bodies of bumblebees. They belonged to 10 different plant taxa in winter and 20 plant taxa in spring, respectively. We identified 76.2% of the pollen to the species level (Table A3).

Statistical analyses

Bumblebee presence and density data were pooled over the two sampling years because there were no significant differences between years for any season (Wilcoxon signed-rank paired tests, all $p > 0.05$).

Differences in presence and plot density (bumblebees per 100 m^2) between managed and native bumblebees in each season were analysed by Wilcoxon signed-rank paired tests. Plot density differences between seasons were also compared by Wilcoxon signed-rank paired tests. Because of small sample sizes (see results), we could not perform more complex analyses on bumblebee density.

The relationship between presence/absence of managed and native bumblebees with greenhouse cover, season (winter/spring) and their interaction were analysed using generalised linear mixed models (GLMM). Models were fitted with binomial error structures. The site was included as a random factor to account for re-sampling plots in winter and in spring. Models were evaluated based on the minimum value given by the second order Akaike Information Criterion (AICc; Burnham *et al.*, 2011) and the model with the lowest AICc score was considered ‘the best model’ (see Table A4). Autocorrelation was checked using the variograms of the residuals.

To quantify similarity in the exploitation of floral resources between managed and native bumblebees, we separately analysed the observed plant-bumblebee interactions and body pollen loads. We pooled the data for each season during the two sampling years due to the small sample size per plot. Data were analysed with the proportional similarity index (PS; Hurlbert, 1978). PS was calculated as: $PS = \sum_i \min (p_{i,m}, p_{i,n})$, where $p_{i,m}$ is either the proportion of interactions on plant taxa *i* or the proportion of pollen grains of plant taxa *i* for managed bumblebees and $p_{i,n}$ is either the proportion of interactions on plant taxa *i* or the proportion of pollen grains of plant taxa *i* for native bumblebees. PS values can range from 0 (no niche overlap between bumblebees) to 1 (complete niche overlap).

Furthermore, the number of bumblebees carrying strawberry pollen and the percentage of strawberry pollen grains were compared between managed and native bumblebees, and between seasons using Chi square tests.

All statistical analyses were run with R (v. 3.1.2; R Core Team, 2014). We used the R packages *bipartite* (Dormann *et al.*, 2009), *lme4* (Bates *et al.*, 2014) and *MuMIn* (Barton, 2009).

Results

Bumblebee presence and density in pinewoods

We found managed bumblebees in 12 (8 in 2014 and 10 in 2015) pinewood plots in winter and in 9 (6 in 2014 and 7 in 2015) pinewood plots in spring. Native bumblebees were present in 7 (7 in 2014 and 3 in 2015) pinewood plots in winter and in 7 (4 in 2014 and 4 in 2015) pinewood plots in spring. There were no significant differences between the presence of managed and native bumblebees in pinewoods in either winter ($W = 10$, $P = 0.11$) or spring ($W = 13.5$, $P = 0.53$).

The presence of managed bumblebees in pinewood plots was positively related to greenhouse cover in the landscape, whereas that of native bumblebees was negatively related, although both patterns were significant at the 0.10 significance level (managed bumblebees: $z = 1.823$, $P = 0.068$ and native bumblebees: $z = -1.728$, $P = 0.084$; Fig. 2a and 2b). Season had no significant effect on the presence of bumblebees, as this variable was not selected in the models with the lowest AICc (i.e. only the variable greenhouse cover remained in the models, see Table A4).

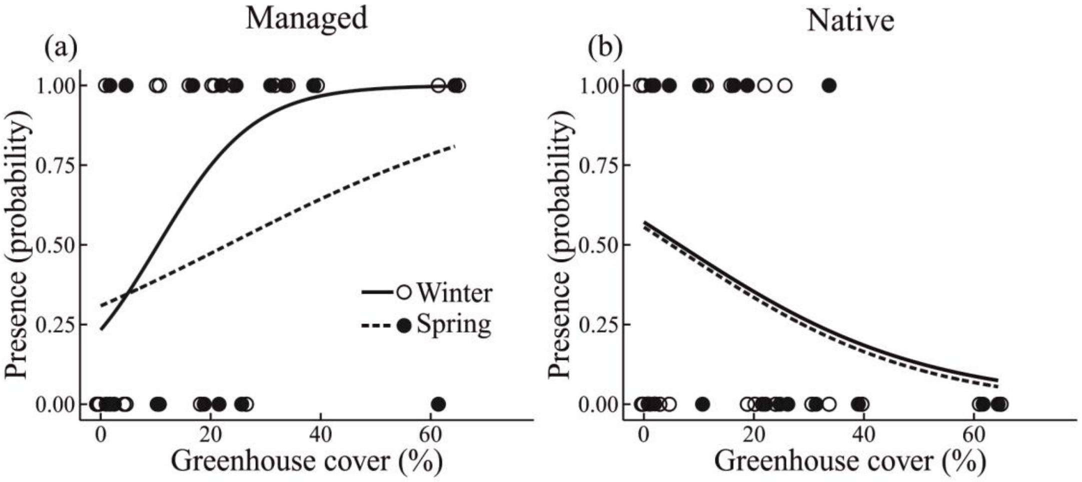


Figure 2. Patterns of the occurrence of (a) managed (*Bombus terrestris*) and (b) native bumblebees (*B. t. lusitanicus*) in pinewood plots across a gradient of greenhouse cover in a 2 km landscape radius in winter and in spring.

Over the two sampling years, we recorded a total of 73 (17 in 2014 and 56 in 2015) managed bumblebees in winter and 37 (12 in 2014 and 25 in 2015) in spring. Native bumblebees were even less abundant with a total of 15 (10 in 2014 and 5 in 2015) bumblebees in winter and only 13 (7 in 2014 and 6 in 2015) in spring. In winter, the density of managed bumblebees in the plots was significantly higher than that of native bumblebees (0.48 ± 0.17 bumblebees per 100 m^2, mean ± SE, hereafter, and 0.10 ± 0.04 bumblebees per 100 m^2, respectively; W = 16, $P = 0.04$), but similar in spring (0.26 ± 0.11 bumblebees per 100 m^2 and 0.09 ± 0.03 bumblebees per 100 m^2, respectively; W = 15, $P = 0.22$). Further, managed and native bumblebee density did not differ significantly between seasons (W = 58, $P = 0.14$ and W = 34, $P = 0.96$, respectively).

Floral resource use

In winter, *R. officinalis* (79.8%) and *U. australis* (17.8%) were the most common flowering plants of the 9 plant taxa recorded in the pinewoods (Table A2). These plant species were the most visited by both managed (~88%) and native bumblebees (100%), and were represented by the most abundant pollen types recorded on bumblebee bodies (overall, ~56% of managed bumblebee pollen loads and ~97% of native bumblebee pollen loads). Overall, managed bumblebees were observed on a total of 5 plant taxa while their pollen loads included pollen from a total of 10 plant taxa (half of them were not present in our study plots). Native bumblebees were observed on a total of 2 plant taxa and their pollen loads contained pollen from 3 plant taxa (one of them was not present in our plots) (Fig. 3 and Table A3).

In spring, *L. stoechas* (46.1%) and *Erica* spp. (20.8%) were the most common flowering plants of the 29 plant taxa recorded in the pinewoods (Table A2). However, there was great variability in plant-bumblebee interactions and bumblebee pollen loads. Most managed bumblebees were observed visiting *L. stoechas* (43.2%) and *H. calycinum* (24.3%), whereas native bumblebees continued to visit *R. officinalis* (61.5%) and *L. stoechas* (38.5%) as well. Managed bumblebees mainly carried pollen from *Cytisus grandiflorus* (25.1%) and *Linaria* spp. (33.0), while native bumblebees carried pollen from *Cerinthe gymnandra* (21.7%), *C. grandiflorus* (35.8%) and *U. australis* (24.9%) (Fig. 3 and Table A3). Managed bumblebees were observed visiting a total of 9 plant taxa, while their pollen loads showed a total of 16 plant taxa, most of which were absent or in low abundance in our plots. Native bumblebees were observed visiting a total of 2 plant taxa and their pollen loads contained a total of 9 plant taxa, most of them absent or in low abundance in our plots (Table A2 and Table A3).

The similarity index in exploited floral resources between managed and native bumblebees was, overall, larger for plant-bumblebee interactions than for pollen loads, and also in winter compared to spring. In fact, in winter, the overlap was almost complete for observed plant-pollinator interactions with PS = 0.83, while for pollen loads, it was PS = 0.56. In spring, the overlap for interactions was PS = 0.41 and for pollen loads it was PS = 0.28.

Finally, we found strawberry pollen only on the bodies of managed bumblebees. In winter, both the number of bumblebees carrying strawberry pollen and the percentage of strawberry pollen grains were higher than in spring (49% of individuals in winter and 13% in spring, $\chi^2 = 6.10$, $P = 0.01$; 3.4% of strawberry pollen in winter and 0.3% in spring, $\chi^2 = 592.8$, $P < 0.001$) (Table A3).

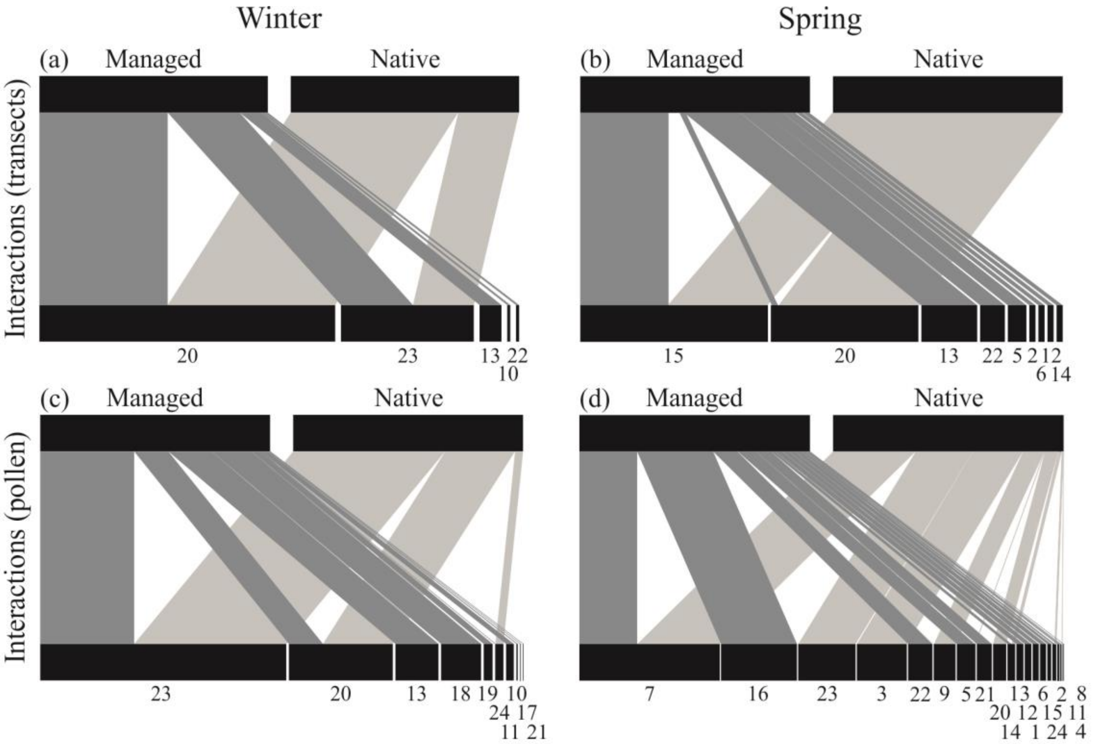


Figure 3. Illustration of plant-bumblebee (a, b) and body pollen loads (c, d) interaction networks in winter and in spring. As sample sizes varied between managed (*Bombus terrestris*) and native bumblebees (*B. t. lusitanicus*) and between seasons, interaction networks were proportionally homogenized (i.e. interaction bar widths sum the unity for each bumblebee group). The width of the links is proportional to the number of interactions observed or pollen grains counted for managed and native bumblebees. The numbers represent the following plant taxa: (1) *Acacia* spp., (2) *Centaurea* spp., (3) *Cerinthe gymnandra*, (4) *Cistus cryspus*, (5) *Cistus ladanifer*, (6) *Cistus salviifolius*, (7) *Citisus grandiflorus*, (8) *Citrus* spp., (9) *Equium* spp., (10) *Erica* spp., (11) *Fragaria* × *ananassa*, (12) *Genista triacanthos*, (13) *Halimium calycinum*, (14) *Halimium halimifolium*, (15) *Lavandula stoechas*, (16) *Linaria* spp., (17) Brassicaceae, (18) *Eucalyptus* spp., (19) *Prunus* spp., (20) *Rosmarinus officinalis*, (21) *Rubus idaeus*, (22) *Stauracanthus genistoides*, (23) *Ulex australis* and (24) *Vaccinium corymbosum.* See Table A3 for more details.

Discussion

Managed bumblebees used in polytunnel greenhouse crops also foraged in nearby pinewoods. The density of managed bumblebees in pinewoods was greater than that of native bumblebees in winter but similar in spring. The presence of managed bumblebees in pinewoods was positively related to crop cover in the landscape. In contrast, the presence of native bumblebees in pinewoods was negatively related to crop cover in the landscape. Remarkably, managed bumblebees foraged on a wide range of flowering plant species, many of which were shared with native bumblebees, especially in winter, when flowering plant species were scarce.

The occurrence of managed and native bumblebees in pinewoods showed opposite patterns in relation to greenhouse cover in the landscape. As expected, managed bumblebees were positively associated with greenhouse cover. An increase in polytunnel greenhouse cover at the landscape scale may be linked to greater use of commercial bumblebee colonies in the landscape (e.g. Klein *et al.*, 2012; Gaines-Day & Gratton, 2016). A greater abundance of colonies may subsequently result in a higher probability of occurrence of managed bumblebees in nearby small pinewoods, which become isolated within a large matrix of greenhouses. However, the relationship we found was not very strong. One explanation for this is that the presence of greenhouses in the landscape associated with colony use could drive the presence of managed bumblebees in pinewoods, regardless of greenhouse cover. Another possible and complementary explanation for this lack of a strong pattern might be that bumblebees have the potential to fly long distances when foraging (Cresswell *et al.*, 2000) and thus, they were present in many of our selected agricultural landscapes.

In contrast to managed bumblebees, native bumblebees were negatively related to greenhouse cover in the landscape. This pattern was also likely the result of a decrease in natural habitat cover in the landscape, as both habitat types were negatively correlated, as mentioned previously. Several causes may explain this pattern. Overall, bumblebees prefer to forage in natural habitats rather than in crops (Collado *et al.*, 2018). In this study, we did not find any strawberry pollen on native bumblebee bodies, although this result does not directly indicate that native bumblebees do not forage in strawberry crops, as they were collected in pinewoods surrounded by greenhouses. However, a parallel study that recorded strawberry flower visitors has shown the absence of native bumblebees foraging in greenhouses (Chapter 1). Therefore, an increase in greenhouse cover linked to a decrease in natural habitats in the landscape could reduce the availability of floral resources for bumblebees. In parallel, greenhouse crops are unsuitable nesting sites due to frequent disturbances (e.g. Holzschuh *et al.* 2007). Other causes, such as the intensive use of pesticides in strawberry crops and their probable spread into adjacent natural habitats (Botías *et al.*, 2016), could be responsible for the contrasting pattern found between the presence of native bumblebees in pinewoods and crop cover in the landscape. All of these possibilities should be tested, and our results should be interpreted with caution since the relationship that was found was not very strong.

Despite the fact that we performed intensive and extensive sampling over two years (i.e. 2348 transect walks on 78 days for a total of 587 h), the density of bumblebees was too low to assess variation across the greenhouse gradient. Bumblebees are rare in this region; for instance, Magrach *et al.*, (2017) showed that bumblebees accounted for only 0.3% of the total visits recorded in an exhaustive plant-pollination network analysis. Surprisingly, in the pilot sampling performed the year before this study, we found similar numbers of bumblebees but with much less sampling effort than in the present study (i.e. 74 h compared to 587 h). Despite methodological differences between the two samplings (i.e. an active search for bumblebees in the pilot sampling), we believe that the low densities observed here probably arose as a result of the strong attraction of bumblebees to plant taxa such as *Acacia* spp. and *C. gymnandra* (Trillo, personal observation). Remarkably, these taxa were absent or in low abundance in the study plots but well represented in the pollen loads (e.g. *C. gymnandra*, *C. grandiflorus* and *Eucalyptus* spp.). Plants from these taxa were isolated and in most cases located in human-modified areas, such as roads and their surroundings, close to our plots.

Unexpectedly, the presence and density of both managed and native bumblebees in pinewoods did not differ between winter and spring. However, this shared pattern is due to the result of different processes operating in the two bumblebee groups. Because commercial bumblebee colonies placed in winter are active for a short period of time, there are farmers that also use new colonies in spring, regardless of crop flower visitation by wild insects (Trillo, personal observation). The use of bumblebees throughout the strawberry flowering period leads to a constant spillover of managed bumblebees from crops into natural habitats in both winter and spring. Even if the use of colonies is higher in winter than in spring, a greater number of bumblebees may spillover into natural areas in spring, prompted by the attraction of a high diversity and abundance of wild flowering plant species (Whittington *et al.*, 2004; Chapter 1). Indeed, bumblebees collected less strawberry pollen in spring than in winter. Native bumblebees are adapted to begin their life-cycle when temperatures are mild, extending from spring to summer in temperate regions (Goulson, 2010). In contrast, in our lowland Mediterranean study region, temperatures are extreme (up to 44 ºC; AEMET) from late-spring to late-summer. Mild most of the winter coinciding with the bloom of several abundant flowering plant species (e.g. *R. officinalis* and *U. australis*) that are highly attractive to bumblebees.

Bumblebees are generalist pollinators (Memmott, 1999) that require diverse pollen sources to thrive (Brunner *et al.*, 2014; Roger *et al.*, 2017). In pinewoods, managed bumblebees exploited a wide range of flowering plant species, as did native bumblebees. Both bumblebee groups showed similar floral resource exploitation, especially in winter, when flowering plant species tend to be scarce. These comparable patterns were expected as they are morphologically similar subspecies (Rasmont *et al.*, 2008). Their capacity to exploit most flower types, such as short- and long-tubed flowers (Inouye, 1980), probably led to a decrease in floral resource overlap in spring, when most flowering plants bloom.

Conclusions

This study provides correlational evidence that managed bumblebees spillover from pollinator-dependent crops into natural habitats. We found that increasing coverage of these crops at the landscape scale increased the probability of presence of managed bumblebees, which forage on a wide range of plant taxa, and in contrast, decreased that of native bumblebees in natural habitats.

The use of managed pollinators for pollination services has become a common practice worldwide (Velthuis & van Doorn, 2006; Aizen & Harder, 2009). However, it is important to highlight that, on the one hand, their pollination function in crops varies greatly for each particular system (Lye *et al.*, 2011; Petersen *et al.*, 2013; Chapter 1) and depends on the community of crop flower visitors (e.g. Garibaldi *et al.*, 2011). On the other hand, a range of managed pollinators forage in natural habitats where they can interfere with wild pollinators in multiple ecological processes, such as resource competition, hybridisation and parasitism (Geslin *et al.*, 2017b). Research on the impacts of agricultural intensification and the introduction of managed pollinators on the native pollinator community is of great importance as the area of pollinator-dependent crops is increasing worldwide (Aizen *et al.*, 2008; González-Varo *et al.*, 2013; Essl *et al.*, 2018). Further studies are needed to optimize the use of managed pollinators in crops in order to reduce or prevent their presence in agricultural landscapes.

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Appendix

Pilot sampling

<u>Objective:</u> Pilot sampling to detect if managed (*Bombus terrestris*) and native bumblebees (*B. t. lusitanicus*) temporally overlap in pinewoods throughout the strawberry flowering period.

<u>Methods:</u> We undertook a pilot sampling in four pinewood plots. Plots differed in the percentage of greenhouse cover within a 2 km radius; there were two pinewood plots with high greenhouse cover and two without greenhouse cover in their surrounding landscape (Table A1). These four plots were selected to be representative of the most and least altered landscapes in our study area, respectively.

We conducted weekly bumblebee surveys from November 2013 to May 2014—when strawberries bloom and farmers start to use commercial bumblebee colonies until the end of crop production, which coincided with the end of blooming of most wild plant species in the study region. In fact, there were two consecutive weeks in which we did not observe bumblebees (see Fig. A1 a and b). Censuses were performed once per week: one week in the morning (10:00-13:00 h) and the following week in the afternoon (13:00-16:00 h). Each census consisted of searching for bumblebees in the centre (100 m × 100 m) of each selected pinewood plot foraging on flowers for one hour, capturing them for *in-situ* identification and releasing them afterwards. Overall, our surveys accounted for 74 sampling hours.

<u>Results & Discussion:</u> We observed a total of 164 bumblebees, and the vast majority of them (94.5%) were managed bumblebees. All managed bumblebees except three were observed in plots with high greenhouse cover (Fig. A1 a and b). They likely escaped from greenhouses to forage in adjacent attractive wild floral resources. We started to observe managed bumblebees at the time that strawberry plants bloomed (November) and they disappeared when strawberries and wild flowers dried out (late May). In plots with high greenhouse cover, a higher number of managed bumblebees was observed during the first half of the sampling period (until mid-February; Fig. A1 a). In contrast, the nine native bumblebees were only observed in plots without greenhouse cover: one in winter (January) and the rest in spring (March) (Fig. A1 b). Taking into account that the selected pinewood plots were representative of the most and the least altered landscapes in our study region, our results suggest that managed and native bumblebees have the potential to overlap in time and space in the pinewoods surrounded by greenhouse crops. In winter, the

abundance of managed bumblebees will likely be greater than in spring, whereas the opposite pattern exists for the native bumblebee.

Table A1. Study plot locations, main land-use types and mean (± SE) flower density in each study plot.

Site	Experimental design	Latitude	Longitude	% Land-use types		Flower density (flowers m^2)	
				Greenhouse	Natural habitat	Winter	Spring
Almonte	Censuses	37º13'46.28'' N	6º33'45.15'' W	4.6	56.4	2.2	6.0
Bonares	Censuses	37º18'44.42'' N	6º38'26.45'' W	10.7	26.2	45.2	44.1
El Rocío	Censuses	37º10'22.02'' N	6º31'24.52'' W	31.3	31.6	1.2	22.9
Ermita	Censuses	37º15'46.12'' N	6º48'23.04'' W	25.6	34.3	2.8	65.5
Mazagón 2	Censuses	37º11'38.27'' N	6º50'00.44'' W	61.4	35.8	3.1	28.6
Mazagón 3	Censuses	37º10'56.52'' N	6º47'53.19'' W	18.8	73.4	6.4	26.6
Mazagón Control	Censuses	37º11'08.20'' N	6º45'33.48'' W	0.0	99.1	2.9	110.9
Merendero	Censuses	37º12'01.88'' N	6º36'18.64'' W	0.0	93.2	1.5	7.2
Monturrio	Censuses	37º16'19.63'' N	6º45'23.62'' W	33.7	49.8	5.0	53.0
Motocross	Censuses	37º12'31.48'' N	6º29'39.35'' W	10.7	48.5	10.6	1.7
Niebla	Censuses	37º14'34.45'' N	6º39'55.60'' W	1.3	61.3	9.5	32.1
Palos Moguer	Censuses	37º15'05.55'' N	6º51'20.40'' W	22.0	17.2	6.9	37.2
Peral	Censuses	37º14'00.10'' N	6º47'46.32'' W	16.3	62.1	6.3	29.7
Playeros	Censuses	37º13'23.96'' N	6º44'43.19'' W	24.2	61.5	7.5	12.9
Rociana	Censuses	37º17'02.89'' N	6º33'51.99'' W	0.7	9.9	18.5	12.8
Rociana Sur	Censuses	37º14'41.38'' N	6º36'13.84'' W	21.5	21.1	7.6	13.2
Arrayán	Censuses + Pilot sampling	37º13'28.19'' N	6º23'35.92'' W	0.0	82.7	3.2	9.4
Mazagón 1	Censuses + Pilot sampling	37º10'17.82'' N	6º51'05.19'' W	64.4	14.1	2.8	58.5
Urbanización	Censuses + Pilot sampling	37º17'18.23'' N	6º40'29.21'' W	39.0	32.5	50.1	14.2
Pinares Palacio	Pilot sampling	36º59'21.33'' N	6º27'09.12'' W	0.0	87.0	-	-

Table A2. List of the flowering plant taxa recorded across the pinewood plots over the two sampled years. Mean flower density for all the pinewood plots pooled in winter and in spring is shown.

Family	Species	% Flower density	
		Winter	Spring
Amaryllidaceae	*Leucojum trichophyllum*	0.000	0.220
Asteraceae	*Centaurea sphaerocephala*	0.000	0.007
Asteraceae	*Leontodon* spp.	0.000	0.015
Asteraceae		0.000	1.650
Boraginaceae	*Echium* spp.	0.000	0.045
Brassicaceae	*Malcolmia* spp.	0.210	5.145
Caryophyllaceae	*Silene* spp.	0.000	0.029
Cistaceae	*Cistus crispus*	0.000	0.035
Cistaceae	*Cistus ladanifer*	0.000	0.115
Cistaceae	*Cistus libanotis*	0.000	0.166
Cistaceae	*Cistus salviifolius*	0.000	0.777
Cistaceae	*Halimium calycinum*	1.946	3.760
Cistaceae	*Halimium halimifolium*	0.000	0.688
Cistaceae	*Tuberaria* spp.	0.086	0.653
Ericaceae	*Erica* spp.	0.026	20.843
Fabaceae	*Cytisus grandiflorus*	0.015	0.668
Fabaceae	*Genista triacanthos*	0.000	8.171
Fabaceae	*Lotus* spp.	0.000	0.152
Fabaceae	*Lupinus* spp.	0.000	0.001
Fabaceae	*Stauracanthus genistoides*	0.038	0.194
Fabaceae	*Ulex australis*	17.841	0.141
Geraniaceae	*Erodium* spp.	0.000	0.240
Lamiaceae	*Lavandula stoechas*	0.008	46.111
Lamiaceae	*Rosmarinus officinalis*	79.830	8.876
Liliaceae	*Asphodelus* spp.	0.000	0.009
Liliaceae	*Leopoldia comosa*	0.000	0.001
Primulaceae	*Anagallis arvensis*	0.000	0.002
Ranunculaceae	*Ranunculus* spp.	0.000	0.017
Scrophulariaceae	*Linaria* spp.	0.000	0.646

Table A3. Percentage of plant-bumblebee interactions and pollen loads in managed (*Bombus terrestris*) and native bumblebees (*B. t. lusitanicus*) in winter and in spring, pooled for the two sampling years. Dashes indicate absence of pollen taxa for both managed and native bumblebees in that season, and zero values indicate absence of pollen taxa for one of the bumblebee groups in that season.

Family	Species	Interactions (%) / Pollen loads (%)			
		Winter		Spring	
		Managed (n = 73 / 33)	Native (n = 15 / 4)	Managed (n = 37 / 23)	Native (n = 13 / 12)
Asteraceae	*Centaurea* spp.	- / -	- / -	2.7 / 1.7	0.0 / 0.0
Boraginaceae	*Cerinthe gymnandra*	- / -	- / -	- / 0.0	- / 21.7
Boraginaceae	*Echium* spp.	- / -	- / -	- / 0.1	- / 9.4
Brassicaceae		- / 0.1	- / 0.0	- / -	- / -
Cistaceae	*Cistus crispus*	- / -	- / -	- / 0.0	- / 0.1
Cistaceae	*Cistus ladanifer*	- / -	- / -	8.1 / 7.9	0.0 / 0.2
Cistaceae	*Cistus salviifolius*	- / -	- / -	2.7 / 2.6	0.0 / 0.0
Cistaceae	*Halimium calycinum*	9.6 / 18.8	0.0 / 0.0	24.3 / 3.1	0.0 / 0.0
Cistaceae	*Halimium halimifolium*	- / -	- / -	2.7 / 1.8	0.0 / 1.5
Ericaceae	*Erica* spp.	1.4 / 0.6	0.0 / 0.0	- / -	0.0 / -
Ericaceae	*Vaccinium corymbosum*	- / 0.1	- / 3.5	- / 0.6	- / 0.0
Fabaceae	*Acacia* spp.	- / -	- / -	- / 2.9	- / 0.0
Fabaceae	*Cytisus grandiflorus*	- / -	- / -	- / 25.1	- / 35.8
Fabaceae	*Genista triacanthos*	- / -	- / -	2.7 / 2.7	- / 0.0
Fabaceae	*Stauracanthus genistoides*	1.4 / -	0.0 / -	- / 10.6	- / 0.0
Fabaceae	*Ulex australis*	31.5 / 40.9	26.7 / 66.1	10.8 / 0.0	0.0 / 24.9
Lamiaceae	*Lavandula stoechas*	- / -	- / -	43.2 / 1.0	38.5 / 0.6
Lamiaceae	*Rosmarinus officinalis*	56.2 / 14.9	73.3 / 30.4	2.7 / 0.0	61.5 / 5.7
Myrtaceae	*Eucalyptus* spp.	- / 17.4	- / 0.0	- / -	- / -
Rosaceae	***Fragaria* × *ananassa***	**- / 3.4**	**- / 0.0**	**- / 0.3**	**- / 0.0**
Rosaceae	*Prunus* spp.	- / 3.9	- / 0.0	- / -	- / -
Rosaceae	*Rubus idaeus*	- / -	- / -	- / 6.5	- / 0.0
Rutaceae	*Citrus* spp.	- / -	- / -	- / 0.1	- / 0.0
Scrophulariaceae	*Linaria* spp.	- / -	- / -	- / 33.0	- / 0.0

Table A4. Summary of model (presence/absence) selection by AICc. All possible combinations are shown, as well as the null model (without independent variables).

Response variable	Model	AICc	ΔAICc
Managed			
	Greenhouse	50.4	-
	Greenhouse + Season	51.2	0.8
	Greenhouse × Season	51.7	1.3
	Season	54.2	3.8
	Null Model	53.6	3.2
Native			
	Greenhouse	52.2	-
	Greenhouse + Season	54.7	2.5
	Season	56.6	4.4
	Greenhouse × Season	57.4	5.2
	Null Model	54.3	2.1

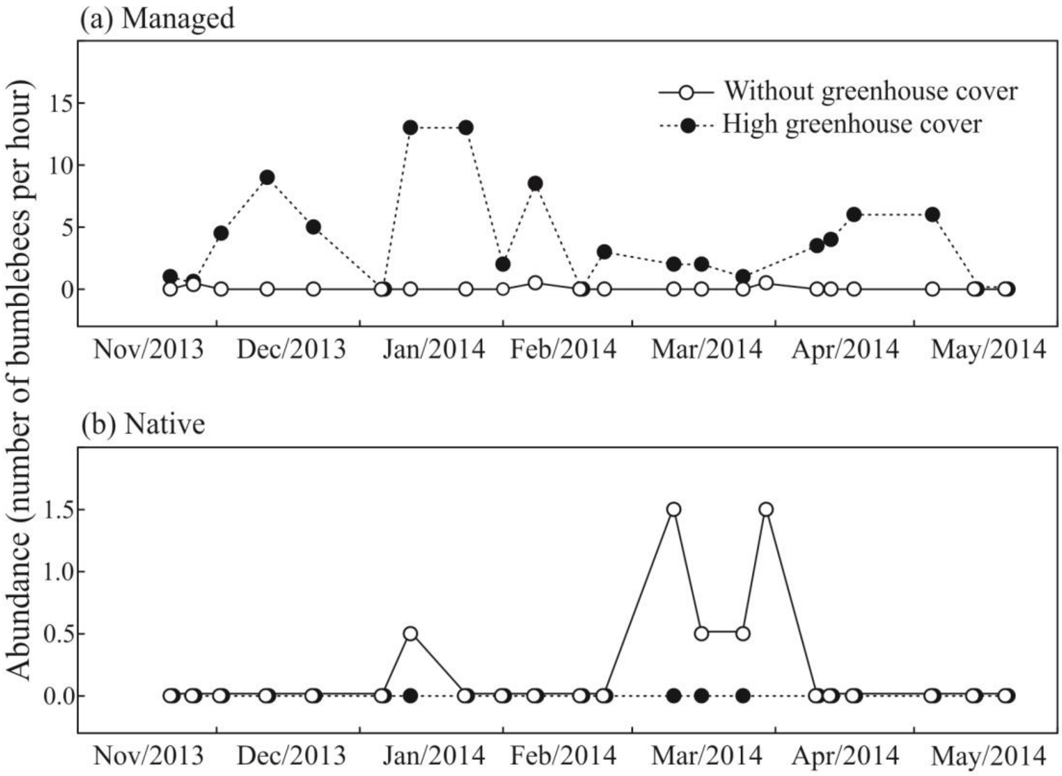


Figure A1. Abundance (number of bumblebees per hour) of (a) managed (*Bombus terrestris*) and (b) native bumblebees (*B. t. lusitanicus*) recorded in four pinewood plots (two with high and two without greenhouse cover in the surrounding 2 km radius) during the pilot sampling (covering one year of the strawberry flowering period).

Capítulo 3

Seasonality of bumblebee spillover between crops and natural habitats

Trillo, A., Montero-Castaño, A. & Vilà, M. Seasonality of bumblebee spillover between crops and natural habitats. Enviado a *Ecological Applications*.

Resumen

En los paisajes agrícolas, diferencias en los recursos entre cultivos y hábitats naturales promueve el movimiento de organismos funcionalmente importantes, como por ejemplo los insectos polinizadores, para satisfacer sus necesidades de alimentación. Sin embargo, poco se sabe acerca de las diferencias en los recursos florales (p. ej. densidad floral y riqueza) entre hábitats que determina este movimiento y su efecto en la dieta de los polinizadores. El movimiento de polinizadores entre hábitats complementarios se estudia normalmente en paisajes con hábitats con periodos de floración consecutivos, tal es el caso de los cultivos de floración masiva. Esto impide conocer cuáles son las características de los recursos florales preferidos por los polinizadores. Aquí, nosotros exploramos el movimiento de polinizadores entre dos hábitats con periodos largos y sincrónicos de floración, pero con diferente densidad y riqueza de recursos florales. Colocamos colonias de abejorros en invernaderos abiertos con cultivos de fresa y en bosques adyacentes a los cultivos en paisajes con baja y alta cobertura de cultivos de frutos rojos. Repetimos el experimento en invierno y en primavera. La densidad floral en los bosques es similar y ligeramente más rica que la de los cultivos en invierno, y mayor y mucho más rica en primavera. El movimiento se estimó como el porcentaje de granos de polen en los abejorros procedente del otro tipo de hábitat. En general, los abejorros tanto de las colonias en los cultivos como en los bosques forrajearon a través de ambos tipos de hábitats mostrando dietas similares. Las dietas fueron más ricas en primavera que en invierno. En primavera, la mayoría de abejorros forrajearon en los hábitats naturales independientemente del tipo de hábitat donde se localizó la colonia. En cambio, en invierno, la mayoría de abejorros forrajeó en los cultivos, pero los abejorros localizados en los bosques en paisajes con baja cobertura de cultivo forrajearon principalmente en los hábitats naturales. Mediante el chequeo de los recursos florales en esos paisajes fragmentados y dinámicos, los abejorros parecen diversificar su dieta de polen de acuerdo con la disponibilidad de recursos. El comportamiento de los abejorros varía de acuerdo con el tipo de hábitat donde la colonia se localiza y la cobertura de cultivo en el paisaje cuando las diferencias en la densidad y riqueza floral son bajas entre hábitats. Se necesitan más estudios que evalúen las implicaciones ecológicas de estos movimientos entre hábitats.

Abstract

In agricultural landscapes, resource differences between crops and natural habitats promote spillover of functionally relevant mobile organisms, like insect pollinators, to satisfy their feeding needs. However, little is known about the differences in floral resources (e.g. floral density and richness) between habitats driving this spillover and their effect on pollinator diet. Pollinator spillover across complementary habitats is typically studied in landscapes of habitats with consecutive flowering periods, such as mass-flowering crops, preventing the disentanglement of characteristics of the floral resources preferred by pollinators. Here, we explore pollinator spillover between two habitats with long and synchronous flowering periods, but with different floral resource density and richness. We placed bumblebee colonies in open polytunnel strawberry crops and in woodlands adjacent to crops in landscapes with low and high berry crop cover. We repeated the experiment in winter and spring. Floral density in woodlands is similar to and slightly richer than that of crops in winter, and higher and much richer in spring. Spillover was estimated as the percentage of pollen grains from the other habitat type found in bumblebee pollen loads. Overall, bumblebees from both crop and woodland colonies foraged across both habitat types showing similar diets that were richer in spring than in winter. In spring, most bumblebees foraged in natural habitats independent of habitat colony location. In contrast, in winter, most bumblebees foraged in crops, but bumblebees located in woodlands in landscapes with low crop cover mainly foraged in natural habitats. By tracking floral resources in these dynamic fragmented landscapes, bumblebees seem to diversify their pollen diet according to available resources. Bumblebee foraging behaviour varies according to habitat colony location and crop cover in the landscape only when the differences in floral density and richness are low between habitats. Further studies are needed to assess the ecological implications of this spillover between habitats.

Introduction

Differences in the availability of resources among habitat types can promote the cross-habitat movement of organisms to satisfy their needs (Dunning *et al.*, 1992). When these movements result in the achievement of their vital functions, it is called functional spillover (hereafter, spillover) (Blitzer *et al.*, 2012). The spillover of organisms not only contributes to the maintenance of their populations, but also plays an important role in multiple ecological processes, such as biological control, seed dispersal and pollination (Rand *et al.*, 2006; Tscharntke *et al.*, 2012; Driscoll *et al.*, 2013).

The spillover of mobile organisms is very important in agricultural landscapes, which represent about one-third of emerging lands worldwide (Ellis *et al.*, 2010). In these landscapes, many mobile organisms exploit the resources offered by crops and isolated natural habitats, probably allowing them to thrive in these highly dynamic, and typically fragmented, landscapes (Smith *et al.*, 2014). Remarkably, insect pollinators are one of the most influential organisms. Globally, they provide pollination services to 70% of the major crop species (Klein *et al.*, 2007). Over the past few decades, the area devoted to crops that are attractive to and benefit from pollinators has disproportionately increased compared to non-insect dependent crops (Aizen *et al.*, 2008). Further, insect pollinators are essential for the reproduction of many wild plant species (Ollerton *et al.*, 2011).

Bees are the primary pollinators in most ecosystems (Neff & Simpson, 1993; Winfree, 2010). In general, bees require feeding resources (i.e. pollen and nectar) from diverse plant species (Westrich, 1996; Cane, 2001). In addition, unlike other taxa such as flies or butterflies, bees are central place foragers; i.e., they have fixed nest sites that constrain the distances to foraging locations (Beutler & Loman, 1951; Stephens & Krebs, 1986). Therefore, the spatial distribution and temporal availability of floral resources within their flying ranges can play an important role in their foraging dynamics (Dukas & Edelstein-Keshet, 1998; Schmid-Hempel & Schmid-Hempel, 1998; Steffan-Dewenter & Kuhn, 2003).

Natural habitats provide diverse, though often scattered, plant species which in most cases flower during the flying period of pollinators. Crops, in contrast, provide monospecific, but highly abundant, floral resources, which mostly flower over short periods of time (Westphal *et al.*, 2003). These habitat differences in floral resources can explain why commercial bees, which are frequently used for pollination services, and wild bees spillover between habitats to optimise their food intake (Whittington *et al.*, 2004; Montero-Castaño *et al.*, 2016; González-Varo & Vilà, 2017).

Although bee spillover between natural and crop habitats is a common process, little is known about the influence of the temporal changes in floral resources between habitats on spillover and ultimately on bee pollen diet. Bee spillover has largely been studied between habitats with sudden changes in their floral abundance, such as mass-flowering crops with consecutive bloom periods. However, the spillover in landscapes with habitats providing synchronous floral resources for a long period of time, remains largely unexplored (see Blitzer *et al.*, 2012; Geslin *et al.*, 2017b).

By placing bumblebee colonies in habitats with contrasting floral resources, crops (monospecific) and woodlands (diverse), we experimentally quantified bidirectional pollinator spillover and thus complementarity in habitat use through the analysis of bumblebee pollen diet at the individual and colony levels. By conducting the experiment in landscapes with contrasting crop cover and in two seasons, we also tested how pollen diet and thus spillover depend on differences in floral resources that change in terms of density and richness over space and time. We addressed the following questions: (a) Do bumblebees complement their diet by foraging across both habitat types? If so, (b) do bumblebees from crop and woodland colonies have similar pollen diets? (c) Do pollen diet and spillover differ with increasing crop cover in the landscape and between seasons?

We expect that (i) bumblebees from crop and woodland colonies will forage across both habitat types and exploit similar resources to maximise their pollen diet; (ii) the use of crop resources will be larger when their cover in the landscape is high; and (iii) crops will be a more important food resource in winter than in spring to compensate for low and scattered wild floral resources. Therefore, the spillover from woodlands to crops will be higher and from crops to woodlands lower in winter than in spring.

Materials and methods

Study system

The study was conducted in a 30 km × 20 km area in the Guadalquivir Valley, province of Huelva (SW Spain, Table A1 for specific locations), in January and April 2015. The climate is typically Mediterranean with very mild winters. In January, the coldest month, the mean of the maximum temperatures is 16.2 ºC (AEMET), allowing the foraging activity of commercial bumblebees (Chapter 1).

The area is characterised by large areas of berry crops, mostly strawberries (*Fragaria* × *ananassa*) (~70% of the cultivated area), but also raspberries (*Rubus idaeus*) and blueberries (*Vaccinium corymbosum*) in open polytunnel greenhouses (Freshuelva). The flowering period for strawberries is long, spanning November to May, with the flowering peak in spring, when the floral density is almost twice that in winter (early-January to mid-February: 9.26 ± 1.00 flowers m^2, mean ± SE hereafter; early-March to mid-April: 17.55 ± 0.73 flowers m^2; see Chapter 1). The main natural areas in the study region are pinewoods with a rich understorey of insect-pollinated plants (González-Varo & Vilà, 2017). In these natural areas, the flowering peak is also in spring when the wild floral richness and density are almost triple than in winter (mid-January to mid-February: up to 9 plant taxa and 10.18 ± 3.18 flowers m^2; mid-March to late-April: up to 29 plant taxa and 30.87 ± 6.14 flowers m^2; Chapter 2).

In this region, farmers frequently use commercial pollinators, such as honeybees (*Apis mellifera*) and/or bumblebees (*Bombus terrestris*), to supplement berry crop pollination. In this study, we focussed on bumblebees for several reasons: (i) they are generalist pollinators known to visit both crops and wild species worldwide (Corbet *et al.*, 1991; Osborne & Williams, 1996); (ii) they have large foraging ranges (Osborne *et al.*, 2008) making them suitable for studies at the landscape scale; (iii) they have an annual life-cycle and thus colonies can be easily compared; and (iv) their commercial production allows handling colonies at similar phenological stages (e.g. similar feeding needs).

Experimental design

To explore the bidirectional spillover between crops and natural habitats, we selected 14 2-km-radius landscapes. This buffer area represents the maximum flying distance of most bumblebee foragers (Osborne *et al.*, 2008). Our study plots were located in strawberry crops adjacent (~50 m) to natural habitats and at the centre of six of these landscapes (crops, hereafter); the centre of the remaining eight plots was located in pinewoods adjacent (~50 m) to strawberry crops (woodlands, hereafter). The average distance between the centres of the study crops was 5461 ± 1350 m (range = 2426–8751 m) and between the centres of the study woodlands was 4895 ± 554 m (range = 3121–7470 m).

To explore whether the pollen diet and spillover between crops and woodlands was influenced by landscape crop cover, we selected study crops and woodlands with contrasting berry crop cover (i.e. low *versus* high) and similar woodland cover (Fig. 1). For low crop cover landscapes (2 crop and 4 woodland), the average berry crop cover was 5.6 ± 1.5% (range = 1.8–10.4%); for high crop cover landscapes (4 crop and 4 woodland), it was 43.8 ± 5.7% (range = 27.9–63.9%) (Table A1). Land use types were characterised using ArcGIS (ESRI, 2011) based on the land use map of Andalusia from 2011 (Moreira *et al.*, 2011).

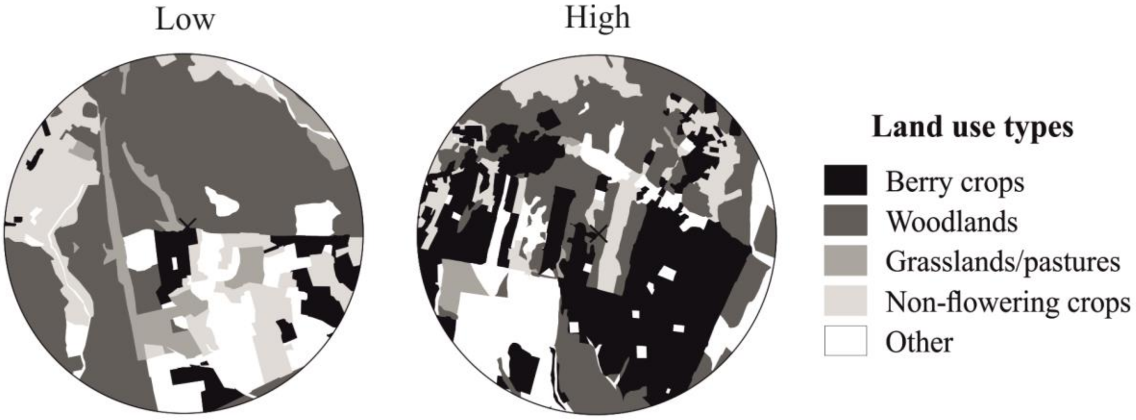


Figure 1. Two examples of study woodlands with low (left) and high (right) berry crop cover within a 2 km buffer area.

In the centre of each study landscape, we placed two commercially produced *Bombus terrestris* colonies (Koppert Biological Systems) for one month. We used two colonies per plot to minimise any contingencies in colony loss or malfunction. Moreover, we expected individuals from different colonies but in the same landscapes to exploit similar floral resources (Saifuddin & Jha, 2014). All colonies were at a similar phenological stage and included a queen and 50-100 workers each. They were supplied with a syrup solution ad-libitum in a plastic box covered with cardboard. In crops, colonies were hung (~20 m from each other) inside the polytunnel strawberry greenhouses, and in the woodlands, they were placed on the ground hidden in wooden boxes to avoid predation.

To explore the seasonality of the spillover of bumblebees between crops and woodlands and its importance in pollen diet, the study was repeated in January and April (hereafter referred to as winter and spring, respectively). As mentioned in the *Study system* section above, in winter, the floral density is similar between strawberry crops and woodlands, and in spring, there is higher floral density in woodlands than in crops. In woodlands, floral richness is always greater than in crops.

Bumblebee survey and pollen loads

We analysed pollen loads on bumblebees to explore pollen diet (i.e. richness) and spillover (i.e. pollen from the other habitat type) between crops and woodlands and thus complementary habitat use.

In each study plot and in each season, we collected bumblebee workers returning to their colony on two different days: one during the second week and another during the third week after the colonies were placed in the field. Individuals were collected on two different days, within a one-week interval, to obtain a broad representation of the visited plant taxa, while controlling for colony phenology.

In total, we collected 526 bumblebees (265 in winter and 261 in spring). On average, 18.8 ± 0.6 (range = 10–25) bumblebee individuals per plot (i.e. two colonies pooled) and season were collected. Bumblebees were captured using aerial nets and frozen (-20 ºC) in individual clean vials for later preparation of pollen load samples. Captures were conducted on sunny, warm (minimum temperature of 14 ºC) days with minimal wind conditions.

A cube (0.3 cm × 0.3 cm) of fuchsin jelly was rubbed on the body of each bumblebee (Beattie, 1971). We avoided scopas due to their high concentration of pollen, which can hinder identification. We assumed that body and scopas pollen are representative of visited plant species such that including pollen from scopas would not change the results in relative terms. Each cube was mounted on a slide and all pollen grains within 20 random fields at 200x magnification were counted and identified. For pollen identification, we used a reference pollen collection from the study area. However, when identification could not be performed at the species level, pollen grains were assigned to a higher taxonomic level, such as genus or family (Table A2). We considered as proof of a visited plant taxa more than ten pollen grains of that taxa in the 20 fields per individual (for a similar approach see Bosch *et al.*, 2009).

Data analyses

Pollen richness was calculated as the number of pollen taxa at the colony level and at the individual bumblebee level.

Pollen richness at the colony level was estimated using the first-order jackknife species-richness estimator. We used this estimator because it shows high precision when measures are small and sampling effort varies (Hortal *et al.*, 2006). For each habitat (crop and woodland), we compared differences in colony pollen richness between landscapes with high and low crop cover, and between seasons using Mann-Whitney U tests.

Differences in pollen richness at the individual level were analysed using a generalised linear mixed model (GLMM; Poisson error distribution) for each habitat separately. Crop cover in the landscape (low/high) and season (winter/spring) were included in the model as fixed factors, as well as their interaction. Study landscape was included as a random factor to account for the re-sampled landscapes in winter and spring and within seasons. Post hoc comparisons among treatments were conducted by building contrast matrices.

The spillover was calculated at the individual bumblebee level as the percentage of pollen grains from the habitat type not occupied by the colony. That is, the spillover of bumblebees from colonies placed in woodlands was estimated as the percentage of berry pollen of the total on their bodies, while spillover of bumblebees from colonies placed in crops was estimated as the percentage of wild pollen of the total. Berry pollen included *Fragaria* × *ananassa*, *Rubus idaeus* and *Vaccinium corymbosum*.

Differences in spillover of bumblebees were also analysed using a generalised linear mixed model (GLMM; Binomial error distribution) for each habitat separately. The response variable ‘bumblebee spillover’ was coded as a two-column matrix (crop pollen/wild pollen or vice versa). Crop cover in the landscape (low/high) and season (winter/spring) were included in the model as fixed factors, as well as their interaction. Study landscape was included as a random factor to account for the re-sampled landscapes in winter and spring and within seasons. Post hoc comparisons among treatments were conducted by building contrast matrices.

For each season, we estimated the similarity in the use of floral resources between habitats and between landscapes with low and high berry crop cover within each habitat. For this purpose, we calculated the proportional similarity index (PS; Hurlbert, 1978). $PS = \sum_i \min(p_{i,a}, p_{i,b})$ where $p_{i,a}$ is the proportion of pollen grains of plant taxa i in bumblebees from pool a and $p_{i,b}$ is the proportion of pollen grains of plant taxa i in bumblebees from pool b. PS values range from 0 (no overlap) to 1 (complete overlap of pollen taxa identity and proportion). For each season, three PS indexes were calculated: PS_{habitat} for pollen taxa similarity between bumblebees in crops and in woodlands, irrespective of the crop cover in the landscape; PS_{crops} for pollen similarity between crop bumblebees from low and high crop cover landscapes; and PS_{woods} for pollen similarity between woodland bumblebees from low and high crop cover landscapes.

Statistical analyses were computed in R (v.3.1.3, R Core Team, 2014). For GLMMs, we used the *lme4* package (Bates *et al.*, 2014), for contrast matrices the *multcomp* package (Hothorn *et al.*, 2013) and for illustrative networks the *bipartite* package (Dormann *et al.*, 2009).

Results

Overall, we collected 526 bumblebees from 54 colonies. Due to extremely low colony activity, we missed data from two colonies placed in crops, one each season. In total, we counted 868,945 pollen grains belonging to 41 plant taxa. Bumblebees from colonies placed either in crops or in woodlands shared pollen plant taxa from both habitat types. In winter, bumblebees exploited more similar floral resources ($PS_{habitat}$ = 0.66) than in spring ($PS_{habitat}$= 0.48) (Table A2 and Fig. 2).

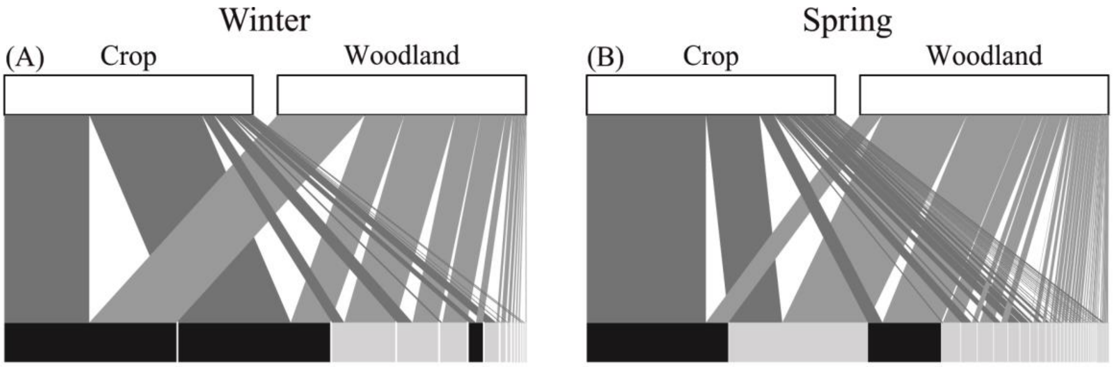


Figure 2. Illustration of bumblebee body pollen load interaction networks in colonies placed in crops and woodlands in winter (A) and spring (B). As sample sizes varied in both habitats, the networks were proportionally homogenised (i.e. for each habitat, the top bar widths provide the sum of the whole). The width of the links is proportional to the number of pollen grains counted. Numbers in the lower bar indicate pollen plant taxa according to Table A2. Black rectangles in the lower bar represent pollen from berry crop species (*Fragaria* × *ananassa*, *Rubus idaeus* and *Vaccinium corymbosum*) and light rectangles represent pollen from wild plant taxa. See Table A2 for more detailed pollen plant taxa.

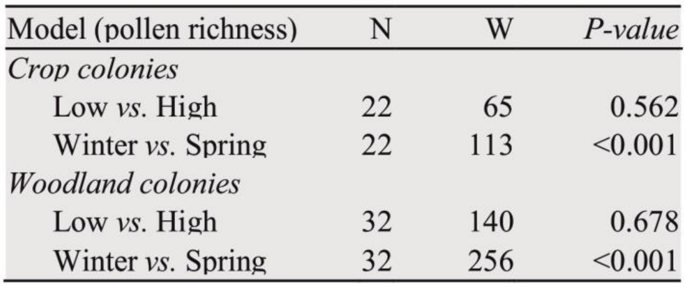
Pollen richness and spillover in crops

A total of 11 and 30 plant taxa were found in the pollen loads of bumblebees in crops, in winter and spring, respectively. Overall, berry crop species were the most represented pollen in bumblebees in winter (77.5 ± 4.0%, mean ± SE hereafter), with 35.3 ± 4.4% of pollen loads represented by berry crops in spring. Wild plant pollen taxa were mostly represented by *Ulex australis* (10.6 ± 2.8%), Myrtaceae (4.6 ± 2.0%) and Brassicaceae (2.8 ± 1.6%) in winter, and by *Echium* spp. (19.4 ± 3.5%), *Vicia* spp. (6.9 ± 2.1%) and *Cistus ladanifer* (6.6 ± 1.9%) in spring. Bumblebees exploited similar pollen resources in low and high landscapes in both winter (PS_{crops} = 0.65) and spring (PS_{crops} = 0.72) (Table A2 and Fig. A1).

Pollen richness at the colony level (i.e. first-order jackknife) was significantly higher in spring (15.00 ± 1.42 plant taxa) than in winter (7.23 ± 0.85 plant taxa). However, there was no significant difference between landscapes with low (9.84 ± 1.41) and high (11.85 ± 1.66) crop cover (Table 1). Pollen richness at the individual level was also significantly higher in spring than in winter but only in landscapes with high crop cover (Table 2 and Fig. 3A).

Post-hoc analysis revealed that the spillover of bumblebees from crops to woodlands was overall significantly higher in spring than in winter. However, the significance was marginal in landscapes with low crop cover (Table 2 and Fig. 3B).

Table 1. Mann-Whitney U tests for differences in bumblebee colony pollen richness in study crops and woodlands between landscapes with low and high berry crop cover and between winter and spring.



Model (pollen richness)	N	W	*P-value*
Crop colonies			
Low *vs*. High	22	65	0.562
Winter *vs*. Spring	22	113	<0.001
Woodland colonies			
Low *vs*. High	32	140	0.678
Winter *vs*. Spring	32	256	<0.001

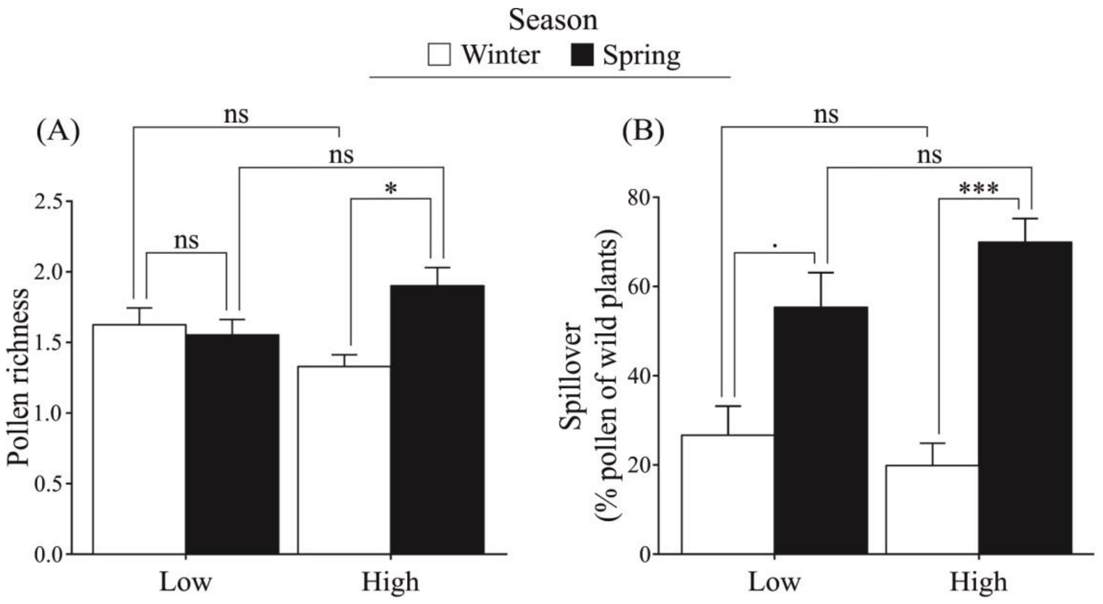


Figure 3. Mean + SE (A) pollen richness and (B) spillover of bumblebees from crops (i.e. % pollen of wild plants) in winter (white bars) and spring (black bars) in landscapes with low and high crop cover. $^{***}p = 0.001$, $^{*}p = 0.05$, $^{\cdot}p = 0.10$, ns: $p > 0.10$.

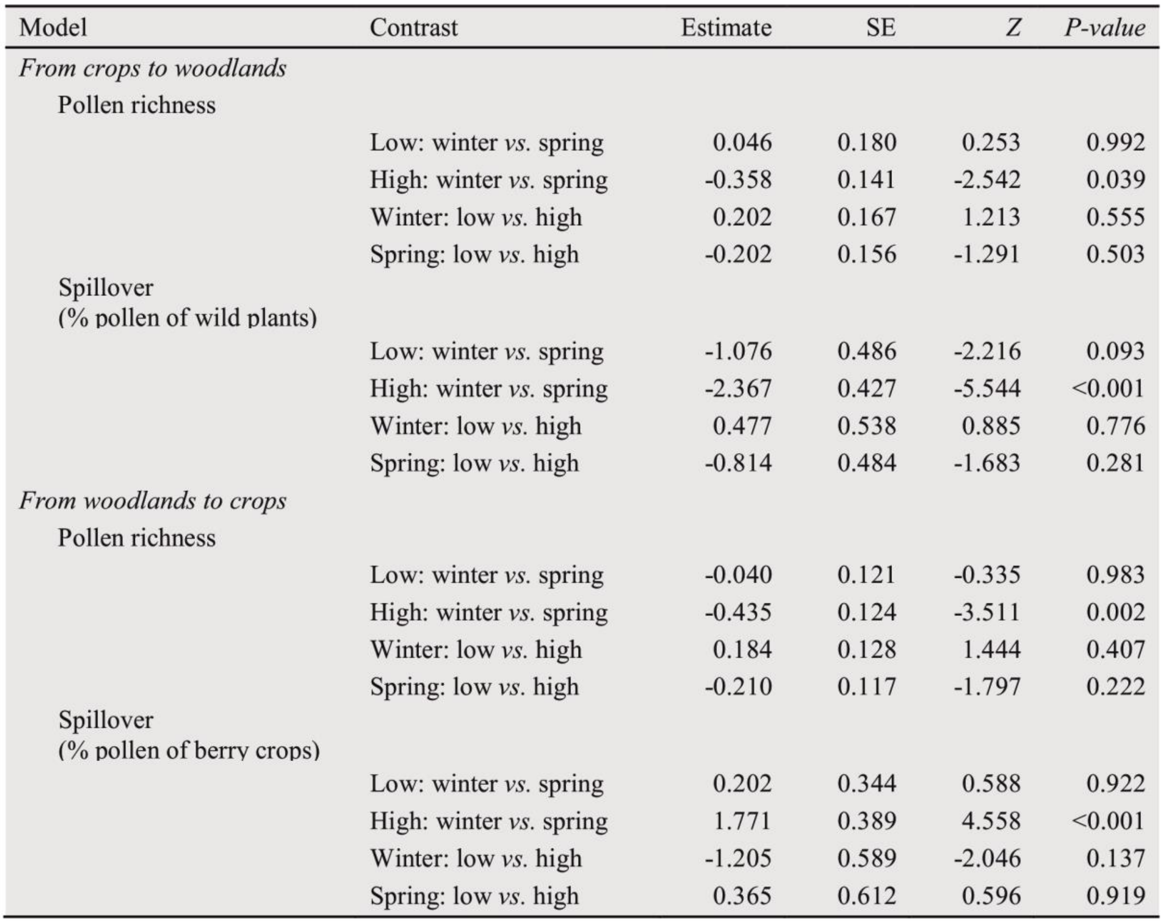
Pollen richness and spillover in woodlands

A total of 13 and 37 plant taxa were found in the pollen loads of bumblebees, in winter and spring, respectively. Berry crop species represented 48.1 ± 3.8% and 27.6 ± 3.5% of pollen loads in winter and spring, respectively. In winter, the most represented wild plant taxa were *Ulex australis* (21.6 ± 3.0%), *Rosmarinus officinalis* (9.7 ± 2.0%) and Brassicaceae (7.5 ± 2.0%). In spring, they were *Echium* spp. (18.3 ± 2.9%), *Cistus ladanifer* (8.6 ± 2.0%) and *Vicia* spp. (5.7 ± 1.8%). Bumblebees exploited similar pollen resources in low and high crop cover landscapes in both seasons (winter: $PS_{woods} = 0.60$; spring: $PS_{woods} = 0.59$) (Table A2 and Fig. A1).

Pollen richness at the colony and individual level mirrored the patterns found in crops. That is, pollen richness at the colony level was significantly higher in spring (16.20 ± 0.91 plant taxa) than in winter (7.95 ± 0.33 plant taxa), and did not differ between landscapes with low (11.29 ± 1.14) and high (12.86 ± 1.36) crop cover (Table 1). Pollen richness at the individual level was also significantly higher in spring than in winter, but only in landscapes with high crop cover (Table 2 and Fig. 4A).

The spillover of bumblebees from woodlands to crops was higher in winter than in spring only in landscapes with high crop cover (Table 2 and Fig. 4B).

Table 2. Generalised linear mixed model (GLMM) results for differences in pollen richness and spillover (% pollen from the other habitat type) of bumblebees in crops and woodlands in landscapes with low and high berry crop cover within a 2 km buffer area in winter and spring.



Model	Contrast	Estimate	SE	*Z*	*P-value*
From crops to woodlands					
Pollen richness					
	Low: winter *vs.* spring	0.046	0.180	0.253	0.992
	High: winter *vs.* spring	-0.358	0.141	-2.542	0.039
	Winter: low *vs.* high	0.202	0.167	1.213	0.555
	Spring: low *vs.* high	-0.202	0.156	-1.291	0.503
Spillover (% pollen of wild plants)					
	Low: winter *vs.* spring	-1.076	0.486	-2.216	0.093
	High: winter *vs.* spring	-2.367	0.427	-5.544	<0.001
	Winter: low *vs.* high	0.477	0.538	0.885	0.776
	Spring: low *vs.* high	-0.814	0.484	-1.683	0.281
From woodlands to crops					
Pollen richness					
	Low: winter *vs.* spring	-0.040	0.121	-0.335	0.983
	High: winter *vs.* spring	-0.435	0.124	-3.511	0.002
	Winter: low *vs.* high	0.184	0.128	1.444	0.407
	Spring: low *vs.* high	-0.210	0.117	-1.797	0.222
Spillover (% pollen of berry crops)					
	Low: winter *vs.* spring	0.202	0.344	0.588	0.922
	High: winter *vs.* spring	1.771	0.389	4.558	<0.001
	Winter: low *vs.* high	-1.205	0.589	-2.046	0.137
	Spring: low *vs.* high	0.365	0.612	0.596	0.919

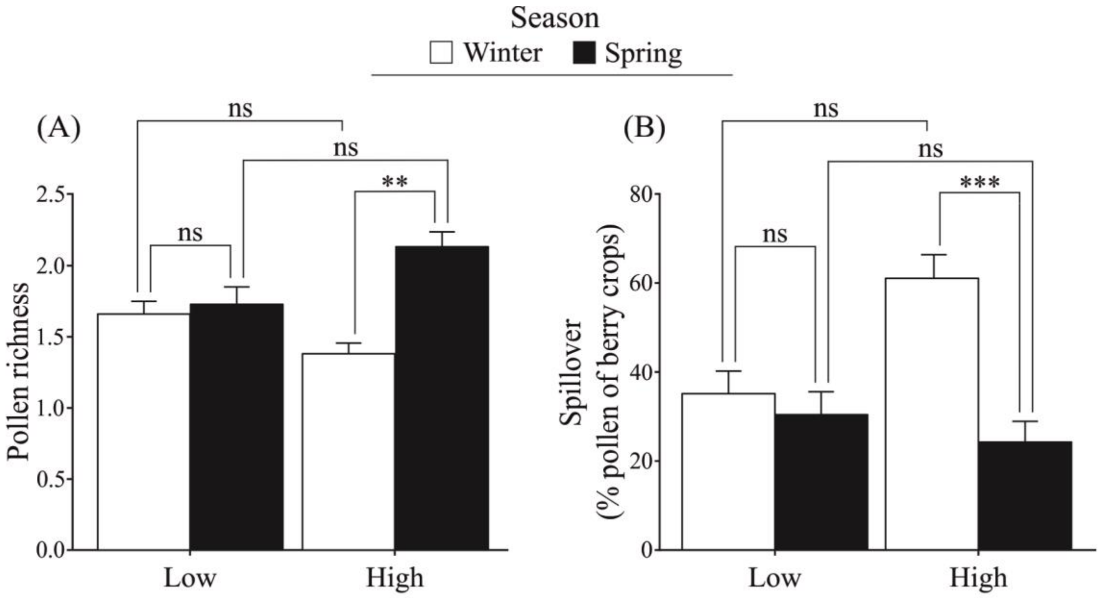


Figure 4. Mean + SE (A) pollen richness and (B) spillover of bumblebees in woodlands (i.e. % pollen of berry crops) in winter (white bars) and spring (black bars) in landscapes with low and high crop cover. $^{***}p = 0.001$, $^{*}p = 0.05$, ns: $p > 0.10$.

Discussion

We experimentally quantified the bidirectional spillover of bumblebees and thus the complementary habitat use through their pollen loads in landscapes with contrasting crop cover. Our results show that irrespective of habitat colony location, i.e. crops or woodlands, and season, bumblebees complemented their diet by foraging across both habitat types. By exploiting floral resources in crop and natural habitats, bumblebees seem to diversify their pollen diet. This flexible behaviour, tracking resources at the landscape scale, may enable bumblebees to thrive in these dynamic and fragmented landscapes (Westphal *et al.*, 2003, 2006, 2009).

At the colony level, bumblebee pollen diet was rich and quite similar between habitats. Bumblebees are generalist pollinators (Memmott, 1999). A diverse pollen diet may ensure larval bee growth (Génissel *et al.*, 2002; Tasei & Aupinel, 2008) and colony health (Brunner *et al.*, 2014; Roger *et al.*, 2017). Diet richness varied with the seasonal availability of floral resources in the landscape, rather than with differences in landscape crop cover (Jha *et al.*, 2013). This is consistent with other studies that have shown ample pollen diets in bumblebees even in agricultural landscapes with large areas of monospecific floral resources (Foulis & Goulson, 2014; Bobiwash *et al.*, 2017).

Ample diets at the colony level result from the sum of individual bumblebees' diets, rather than from average differences among individuals. It is well known that individuals are fairly flower species constant (Free, 1970). Flower constancy can maximise foraging by reducing the time spent handling new flower types (e.g. Woodward & Laverty 1992; Chittka & Thomson 1997). Surprisingly, at the individual level, bumblebees carried higher pollen richness in spring than in winter only in landscapes with high crop cover. One explanation for this pattern may be an intense diversified foraging strategy in isolated and patchily distributed natural habitats in landscapes with high crop cover. Bumblebees may frequently switch to forage on another plant species when conspecific flowers are not available nearby (e.g. Chittka *et al.*, 1997). This could maximise their rate of resource acquisition by minimising travel time between patches (Pyke, 1980). Notably, time is more constraining than energy (Beutler & Loman, 1951; Heinrich, 1979). However, according to this explanation, one may also expect this pattern to occur in winter. However, in winter there were no differences in pollen richness between landscapes possibly because the low species richness of wild flowering plants (only 1-3 abundant plant species) masked a pattern.

As expected, in spring, most bumblebees foraged in natural habitats independent of habitat colony location. That is, bumblebees from crops did spillover to woodlands, while most bumblebees in woodlands remained in the woodlands. These patterns matched the high floral density and species richness in natural habitats compared to that of crops (Westphal *et al.*, 2006; Chapter 1). Unfortunately, we could not discern between the relative importance of floral density versus species richness for bumblebees because both parameters were higher in natural habitats than in crops. In addition, bumblebee spillover did not differ between landscapes with contrasting crop cover. As the selected study landscapes had similar natural habitat cover and this habitat seemed to be the key factor influencing bumblebee spillover in this season, it is likely that bumblebees fulfilled their pollen needs regardless of landscape crop cover.

In contrast, in winter, most bumblebees foraged in crops, except bumblebees located in woodlands in landscapes with low crop cover, which mainly foraged in natural habitats. In winter, the density of floral resources between habitats is similar, while floral richness is higher in natural habitats than in crops, as in spring. Yet, only 1-3 wild plant species provide abundant floral resources in this season with more scattered plant species distribution compared to that of crops. Thus, as expected, crops were a more important food resource in winter than in spring. Most bumblebees in winter located in woodlands in landscapes with low crop cover forage in natural habitats. One explanation for this may be that crop cover was too low compared to natural habitats. Therefore, bumblebees preferred to forage in the most extensive habitats that matched with the natural habitat (Collado *et al.*, 2018). This result suggests different foraging behaviour for bumblebees whose colony is located in distinct habitats through trade-offs between resource density and richness when their differences are low at the landscape scale. However, further studies are needed to disentangle the resource cues for spillover.

Our results are congruent with observations of bee complementary habitat use in other agricultural landscapes (Mandelik *et al.* 2012; Martins *et al.* 2018). However, most previous studies have been carried out in mass-flowering crops with contrasting temporal floral resources compared to the surrounding natural habitats. Our study focussed on agricultural landscapes in which natural and crop habitats provide floral resources for a long period of time. Moreover, our experimental approach and the analysis of pollen diet allowed us to infer diet complementarity within a colony rather than temporal habitat use of individuals from the same species (e.g. Mandelik *et al.*, 2012; Martins *et al.*, 2018; but also see Danner *et al.*, 2014). We were also able to infer the relative influence of floral density and species richness on diet and spillover, as they change in space and time at the landscape scale.

Determining how pollinators utilise different habitats in the landscape to fulfil their needs is vitally important to understanding their diet (Westphal *et al.*, 2009; Jauker *et al.*, 2012). In turn, this has consequences for other interacting plant and pollinator species. The bidirectional spillover of pollinators can play an important role in crop production (Garibaldi *et al.*, 2013; Petersen *et al.*, 2013). Based on our results and other studies worldwide (e.g. Whittington *et al.* 2004; Riedinger *et al.* 2014), when wild floral resources are scarce within the agricultural matrix, it is likely that crops benefit from an increase in flower visitation by both commercial and wild pollinators. In addition, the bidirectional spillover of pollinators can play an important role in multiple ecological processes in natural habitats. For example, native pollinators can be subject to an increasing competition for floral resources with commercial pollinators when the bulk of them spillover from crops to natural habitats (Goulson, 2003; Geslin *et al.*, 2017b; González-Varo & Vilà, 2017). Changes in the pollinator community can also impact wild plant visitation rates and thus, seed set (Blitzer *et al.*, 2012; Geslin *et al.*, 2017b). More studies are needed on the influence of changes in pollen diet due to spillover on wild pollinators, as well as the influence of seasonal spillover on crop production and wild plant seed set.

Acknowledgements

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Appendix

Table A1. Location and characteristics of the 14 study habitats (crops or woodlands) where bumblebee colonies were placed, according to the landscape berry crop cover (low or high) and main land use types (others refers to integrated wetlands, urban areas and denuded soil) in a 2 km radius buffer area.

Habitat	Latitude	Longitude	Landscape	Land use types (%)				
				Berry crops	Woodlands	Grasslands	Non-flowering crops	Others
Crop	37º10'19.52'' N	6º51'1.00'' W	High	63.6	14.6	2.2	2.0	17.6
Crop	37º17'19.02'' N	6º40'26.56'' W	High	36.5	30.7	1.9	12.1	18.8
Crop	37º16'31.58'' N	6º46'1.14'' W	High	32.1	46.4	3.7	5.9	11.9
Crop	37º15'29.93'' N	6º47'8.43'' W	High	27.9	37.4	2.5	6.7	25.5
Crop	37º14'30.33'' N	6º48'5.55'' W	Low	8	58.0	1.5	4.6	27.9
Crop	37º14'37.62'' N	6º35'43.73'' W	Low	1.8	22.7	7.1	58.4	10.0
Woodland	37º10'18.97'' N	6º51'5.96'' W	High	63.9	13.9	2.1	2.0	18.1
Woodland	37º11'31.78'' N	6º50'0.36'' W	High	61.1	35.8	1.1	0.2	1.8
Woodland	37º17'18.15'' N	6º40'29.83'' W	High	37.1	30.1	1.9	12.5	18.4
Woodland	37º16'19.07'' N	6º45'21.52'' W	High	28.3	51.0	4.1	7.3	9.3
Woodland	37º14'1.33'' N	6º47'48.59'' W	Low	10.4	63.1	1.6	4.1	20.8
Woodland	37º12'28.43'' N	6º29'33.72'' W	Low	7.4	49.0	8.7	18.2	16.7
Woodland	37º13'40.82'' N	6º33'40.31'' W	Low	3.9	60.4	0.6	29.0	6.1
Woodland	37º14'34.40'' N	6º36'13.82'' W	Low	1.9	22.3	7.4	58.6	9.8

Table A2. Plant origin and total number of pollen grains counted on bumblebees (*Bombus terrestris*) collected in winter and spring from commercial colonies placed in strawberry crops (*Fragaria × ananassa*) and woodlands whose surrounding landscapes have low or high berry crop cover within a 2 km buffer area. The number of bumblebees collected in each case is given in parentheses. Dashes indicate absence of pollen taxa in colonies placed in strawberry crops or woodlands. Pollen loads of berries (strawberry, raspberry or blueberry crops) are highlighted in bold.

Plant taxa	Family	Strawberry crops		Woodlands	
		Winter Low/High (n = 40 / 61)	Spring Low/High (n = 38 / 70)	Winter Low/High (n = 85 / 79)	Spring Low/High (n = 77 / 76)
(1) *Acacia* spp.	Fabaceae	- / -	0 / 1,749	- / -	1,038 / 1,805
(2) Apiaceae spp.	Apiaceae	- / -	0 / 6,353	- / -	- / -
(3) *Arctotheca calendula*	Asteraceae	- / -	459 / 0	- / -	0 / 196
(4) Asteraceae	Asteraceae	- / -	- / -	- / -	0 / 25
(5) Asteraceae (Liguliflorae)	Asteraceae	- / -	- / -	- / -	11 / 0
(6) Asteraceae (Liguliflorae)	Asteraceae	- / -	- / -	- / -	66 / 0
(7) Asteraceae (Tubuliflorae)	Asteraceae	- / -	0 / 268	- / -	610 / 12
(8) *Borago officinalis*	Boraginaceae	- / -	- / -	- / -	760 / 0
(9) Brassicaceae Type I	Brassicaceae	- / -	- / -	11 / 0	- / -
(10) Brassicaceae Type II	Brassicaceae	110 / 10,912	500 / 120	54,837 / 16,376	- / -
(11) *Carpobrotus edulis*	Aizoaceae	- / -	0 / 5,048	- / -	136 / 128
(12) *Centaurea* spp.	Asteraceae	- / -	0 / 485	- / -	0 / 693
(13) *Cerinthe gymnandra*	Boraginaceae	- / -	0 / 57	0 / 1,561	0 / 691
(14) *Cistus albidus*	Cistaceae	- / -	0 / 181	- / -	179 / 1,418
(15) *Cistus cryspus*	Cistaceae	- / -	139 / 588	- / -	246 / 64
(16) *Cistus ladanifer*	Cistaceae	- / -	2,153 / 1,792	- / -	1,937 / 2,213
(17) *Citrus* spp.	Rutaceae	- / -	0 / 244	- / -	1,408 / 248
(18) *Cytisus grandiflorus*	Fabaceae	- / -	0 / 1,088	1,677 / 0	5,323 / 3,482
(19) *Equium* spp.	Boraginaceae	0 / 497	18,323 / 11,354	- / -	39,744 / 14,983
(20) *Erica* spp.	Ericaceae	- / -	- / -	- / -	65 / 0
(21) Fabaceae spp.	Fabaceae	- / -	- / -	- / -	279 / 1,636

(Cont.) **Table A2.** Plant origin and total number of pollen grains counted on bumblebees (*Bombus terrestris*) collected in winter and spring from commercial colonies placed in strawberry crops (*Fragaria* × *ananassa*) and woodlands whose surrounding landscapes have low or high berry crop cover within a 2 km buffer area. The number of bumblebees collected in each case is given in parentheses. Dashes indicate absence of pollen taxa in colonies placed in strawberry crops or woodlands. Pollen loads of berries (strawberry, raspberry or blueberry crops) are highlighted in bold.

		Strawberry crops		Woodlands	
Plant taxa	Family	Winter Low/High (n = 40 / 61)	Spring Low/High (n = 38 / 70)	Winter Low/High (n = 85 / 79)	Spring Low/High (n = 77 / 76)
(22) ***Fragaria x ananassa***	**Rosaceae**	**23,889 / 77,230**	**25,365 / 40,912**	**4,617 / 50,683**	**38 / 14,378**
(23) *Genista triacanthos*	Fabaceae	- / -	0 / 24	- / -	822 / 788
(24) *Halimium calycinum*	Cistaceae	169 / 30	845 / 1,115	3,762 / 225	636 / 479
(25) *Halimium halimifolium*	Cistaceae	- / -	205 / 175	- / -	1,172 / 257
(26) *Lathyrus* spp.	Fabaceae	- / -	0 / 31	- / -	0 / 133
(27) *Lavandula* spp.	Lamiaceae	- / -	0 / 137	- / -	1,844 / 663
(28) *Linaria* spp.	Scrophulariaceae	831 / 1,281	324 / 1,858	34,222 / 0	1,627 / 331
(29) *Myoporum* spp.	Malvaceae	- / -	- / -	- / -	0 / 591
(30) *Myrtaceae* spp.	Scrophulariaceae	2,550 / 8,052	28 / 571	93 / 3,192	11,059 / 276
(31) *Prunus* spp.	Rosaceae	0 / 501	- / -	- / -	- / -
(32) *Psoralea bituminosa*	Fabaceae	- / -	- / -	- / -	35 / 0
(33) *Quercus perennifolio*	Fagaceae	- / -	573 / 0	- / -	659 / 0
(34) *Rosmarinus officinalis*	Lamiaceae	451 / 57	0 / 95	2,682 / 3,492	40 / 52
(35) ***Rubus idaeus***	**Rosaceae**	**39,943 / 36,499**	**2,168 / 5,970**	**64,995 / 56,913**	**25,463 / 12,337**
(36) *Stauracanthus genistoides*	Fabaceae	- / -	0 / 229	- / -	1,594 / 209
(37) *Tamarix* spp.	Tamaricaceae	- / -	0 / 15	- / -	0 / 1,437
(38) *Teucrium* spp.	Lamiaceae	- / -	- / -	- / -	0 / 120
(39) *Ulex australis*	Fabaceae	9,301 / 4,630	0 / 217	18,381 / 17,557	83 / 0
(40) ***Vaccinium corymbosum***	**Ericaceae**	**1,063 / 5,591**	**347 / 84**	**1,610 / 7,952**	**299 / 50**
(41) *Vicia* spp.	Fabaceae	- / -	1,263 / 4,715	268 / 1,880	882 / 2,455

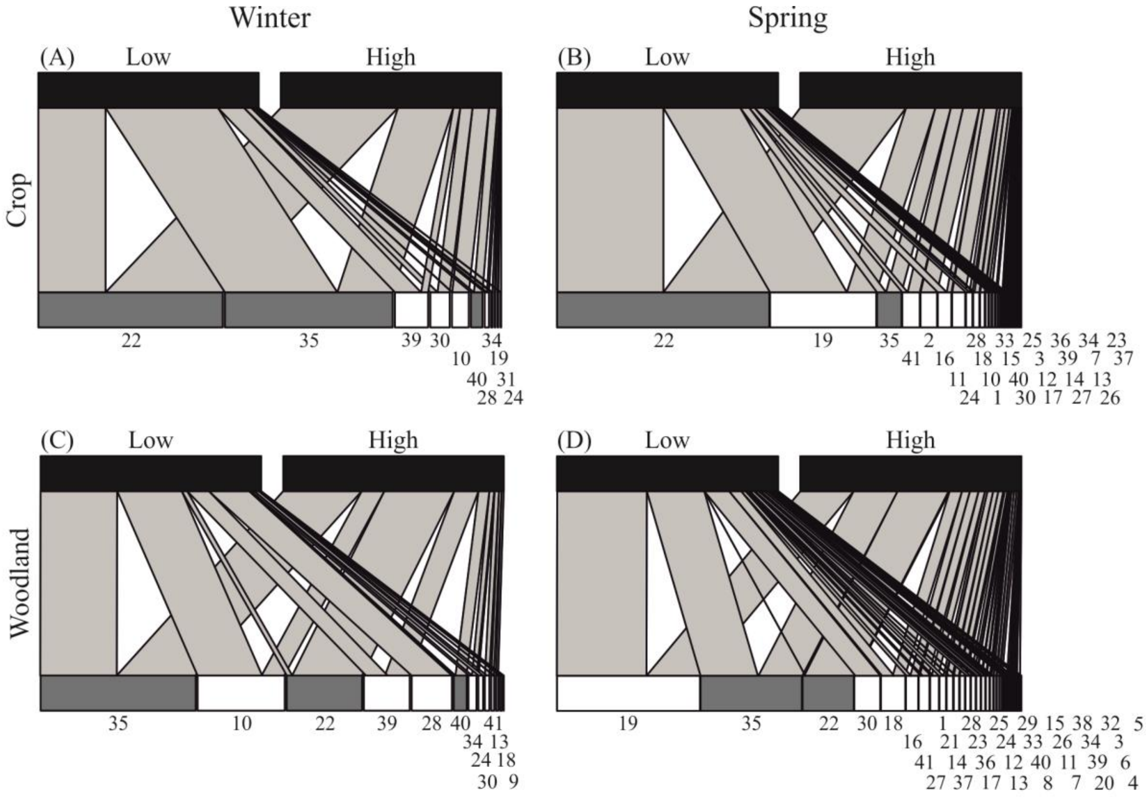


Figure A1. Illustration of bumble bee body pollen load interaction networks in landscapes with low and high berry crop cover in colonies placed in crops (A and B) and woodlands (C and D) in winter and spring. As sample sizes varied in the two habitats and in landscapes with low and high berry crop cover, the networks were proportionally homogenised (i.e. for each habitat, the top bar widths provide the sum of the whole). The width of the links is proportional to the number of pollen grains counted. Numbers in the lower bar indicate pollen plant taxa according to Table A2. Dark grey rectangles represent pollen from berry crop species.

Capítulo 4

Prevalence of *Nosema* microsporidians in commercial bumblebees (*Bombus terrestris*) is not related to the landscape intensity use of colonies

Trillo, A., Brown, M.J.F. & Vilà, M. Prevalence of *Nosema* microsporidians in commercial bumblebees (*Bombus terrestris*) is not related to the landscape intensity use of colonies. *Apidologie* (en revisión).

Resumen

El uso de abejorros comerciales para ayudar a la polinización de los cultivos podría resultar en una sobrepoblación tanto en los campos de cultivo como en los hábitats adyacentes. Consecuentemente, la transmisión de parásitos entre los polinizadores a través de las flores podría ser substancial. Aquí, nosotros evaluamos el estado inicial de infección de colonias de *Bombus terrestris* producidas comercialmente. Luego exploramos la influencia espacial y estacional en los cambios de prevalencia de parásitos a través de un paisaje donde las colonias de abejorros son usadas intensivamente para polinizar los cultivos de frutos rojos en el suroeste de España. Las colonias fueron colocadas dentro de los invernaderos de fresa y en matorrales adyacentes y distantes a los cultivos. El experimento se repitió en enero (invierno) y en abril (primavera) como periodos representativos de un alto y bajo uso de colonias de abejorros comerciales, respectivamente. Colectamos obreras de abejorros de las colonias a su llegada procedente del productor y 30 días después de estar colocadas en el campo. El abdomen de cada abejorro fue inspeccionado para identificar larvas de moscas parásitas (Conopidae, Diptera) y avispas del género *Syntretus* (Braconidae, Hymenoptera). Los sacos de aire fueron inspeccionados para encontrar el ácaro traqueal *Locustacarus buchneri* (Podapolipidae), mientras que el cuerpo graso fue inspeccionado para esporas del parásito *Apicystis bombi* (Lipotrophidae) y del microsporidio del género *Nosema* (Nosematidae). A su llegada el 71% de las colonias de abejorros estaban infectadas por esporas de *Nosema*. Tres abejorros procedentes de dos colonias situadas en matorrales adyacentes a los cultivos portaban esporas de *A. bombi*. La prevalencia de *Nosema* en las colonias no cambió significativamente entre sitios ni entre estaciones indicando que la intensidad del uso de colonias en el paisaje no influyó en la prevalencia de *Nosema*. Nuestros resultados destacan el riesgo potencial de la transmisión de parásitos de los abejorros comerciales hacia polinizadores nativos.

Abstract

The use of commercial bumblebees to aid crop pollination may result in overcrowding of crop fields and adjacent habitats by pollinators. Consequently, transmission of parasites between pollinators via shared flowers may be substantial. Here we assessed the initial infection status of commercially produced *Bombus terrestris* colonies, and then explored spatial and seasonal influences on changes in parasite prevalence across a landscape where bumblebee colonies are intensively used to pollinate berry crops in SW Spain. Colonies were placed inside strawberry greenhouse crops and in woodlands adjacent and distant to crops. We repeated the experiment in January (winter) and in April (spring) as representative periods of high and low use of commercial bumblebee colonies, respectively. Worker bumblebees were collected from colonies upon arrival from a producer and 30 days after being placed in the field. The abdomen of each bumblebee was morphologically inspected for larvae of parasitic conopid flies (Conopidae, Diptera) and braconid wasps of the genus *Syntretus* (Braconidae, Hymenoptera). The air sacs were inspected for the tracheal mite *Locustacarus buchneri* (Podapolipidae), while the fat body was inspected for spores of the neogregarine *Apicystis bombi* (Lipotrophidae) and microsporidians of the genus *Nosema* (Nosematidae). Upon arrival 71% of the bumblebee colonies were infected by spores of *Nosema*. Three bumblebees from two colonies harbored *A. bombi* spores at the end of their placement in woodlands adjacent to crops. *Nosema* colony prevalence did not change significantly either among sites or between seasons indicating that the intensity of landscape colony use did not influence *Nosema* prevalence. Our results highlight the potential risk for parasites to be transmitted from commercial bumblebees to native pollinators.

Introduction

In the last half century there has been an increase in the expansion of pollinator-dependent crops (Aizen *et al.*, 2008) that has required a parallel demand for commercially produced bees (e.g. Klein *et al.*, 2012; Gaines-Day & Gratton, 2016). Bumblebees (*Bombus* sp.) started to be commercially produced in Europe in the late 1980s, to replace the costly mechanical-pollination of tomatoes (*Solanum lycopersicum*) (Ravestijn & Nederpel, 1988; Ravestijn & Sande, 1991). Quickly, bumblebee breeding techniques advanced and colonies were mass-produced and transported worldwide, where they currently pollinate over 20 different pollinator-dependent crops. Over two million bumblebee colonies are produced annually (Graystock *et al.*, 2016a).

The use of commercial pollinators such as bumblebees to aid crop pollination is not free of environmental risks. For instance, queens of commercial bumblebees have become established in many parts of the world (Matsumura *et al.*, 2004; Morales *et al.*, 2013), and there is empirical evidence showing competition for nest sites with other native bumblebee queens in the lab (Ono, 1997) and in the field (Inoue *et al.*, 2008). In addition, commercial bumblebees may compete for food with other native pollinators (Matsumura *et al.*, 2004; Morales *et al.*, 2013), as well as promote the spread of parasites via shared flowers(Colla *et al.*, 2006; Meeus *et al.*, 2011; Schmid-Hempel *et al.*, 2014).

Several bee parasite species have been found in commercial bumblebee colonies. In 1999, Goka and colleagues found for the first time the presence of a parasite, *Locustacarus buchneri*, in commercially produced *Bombus terrestris* colonies upon arrival from a supplier in Japan (Goka *et al.*, 2000). The presence of this parasite has been linked to shorter lifespan (Otterstatter & Whidden, 2004) and changes in behavior of bumblebees (Otterstatter *et al.*, 2005). Further studies have reported that commercial bumblebee colonies frequently have a range of bumblebee parasites (Otterstatter & Thomson, 2006; Graystock *et al.*, 2013a; Murray *et al.*, 2013) and even honeybee parasites (Graystock *et al.*, 2013a), with the latter probably via the consumption of honeybee pollen in reared bumblebees (Goulson & Hughes, 2015). Importantly, the use of commercial pollinators in crops produces high densities of pollinators not only in the agricultural fields or greenhouses, but in adjacent natural areas as well (Ishii *et al.*, 2008; González-Varo & Vilà, 2017; Chapter 1). Presumably, in those areas, the rate of parasite transmission among pollinators will rise, because high densities of hosts provide ideal conditions for the spread of parasites (Arneberg *et al.*, 1998). In fact, several studies have shown, through the collection of free-flying bumblebees, high prevalence of parasites in sites adjacent to greenhouses where commercial bumblebees are used compared with sites distant to those greenhouses (Colla *et al.*, 2006; Murray *et al.*, 2013) or in greenhouses absent of such commercial bumblebees (Graystock *et al.*, 2014), although there is also evidence against this (Whitehorn *et al.*, 2013).

To partially reduce the impact of commercial bumblebees on native pollinator populations and because healthy bumblebees may perform better, as is seen with honeybees (Geslin *et al.*, 2017a), producers are under pressure to produce parasite-free bumblebee colonies. In this study, we first examined whether commercially produced *B. terrestris* colonies, used to pollinate berry crops in Huelva (SW Spain), carried parasites upon arrival from a producer. We morphologically searched for five common internal bee parasites: larvae of the family Conopidae and Braconidae, *L. buchneri*, *Apicystis bombi* and parasites of the genus *Nosema*, which all potentially affect bumblebee health. For instance, larvae of parasitic flies lead to bee death (Schmid-Hempel & Schmid-Hempel, 1996), *L. buchneri* likely reduces lifespan of individual infected host (Otterstatter & Whidden, 2004), *A. bombi* is linked to deterioration of the fat body (Schmid-Hempel, 2001) and *Nosema* reduces worker survival and colony size (Otti & Schmid-Hempel, 2007; Rutrecht & Brown, 2009; Graystock *et al.*, 2013a). We then experimentally tested spatial and seasonal influences on changes in the prevalence of these parasites across a landscape where bumblebee colonies are intensively used. Importantly, *L. buchneri*, *A. bombi* and *Nosema* are likely to be transmitted among pollinators via shared flowers (Durrer & Schmid-Hempel, 1994; Goka *et al.*, 2006; Graystock *et al.*, 2015). Colonies were placed inside strawberry crops and in woodlands adjacent and distant to those crops in January (winter) and repeated in April (spring), as representative periods of high and low use of bumblebee colonies in berry crops, respectively. We expect parasite prevalence to be highest with high densities of commercial bumblebees in the landscape, that is, 1) higher levels of prevalence at sites inside and adjacent to greenhouse crops than distant, and 2) higher levels in winter than in spring because of the greater use of colonies in winter.

Materials and methods

Study system

The study was conducted in the Guadalquivir Valley in the province of Huelva (SW Spain). In this region there are large intensively cultivated areas of berries, especially strawberries (~70% of the area devoted to berry crops; Freshuelva). Strawberries are cultivated in semi-open polytunnel greenhouses with open sides from November to May. In order to aid crop pollination, farmers use commercial bumblebees (*Bombus terrestris*). Notably, bumblebee colonies are especially used at the beginning of the flowering period (i.e. in winter; personal observations) due to major revenues and worse weather conditions than in spring. The most common remaining natural habitat patches across berry crops are woodlands composed of a rich flora of entomophilous Mediterranean shrubs and herbs, which provide flowers throughout the strawberry cultivation period (Herrera, 1988).

Experimental design

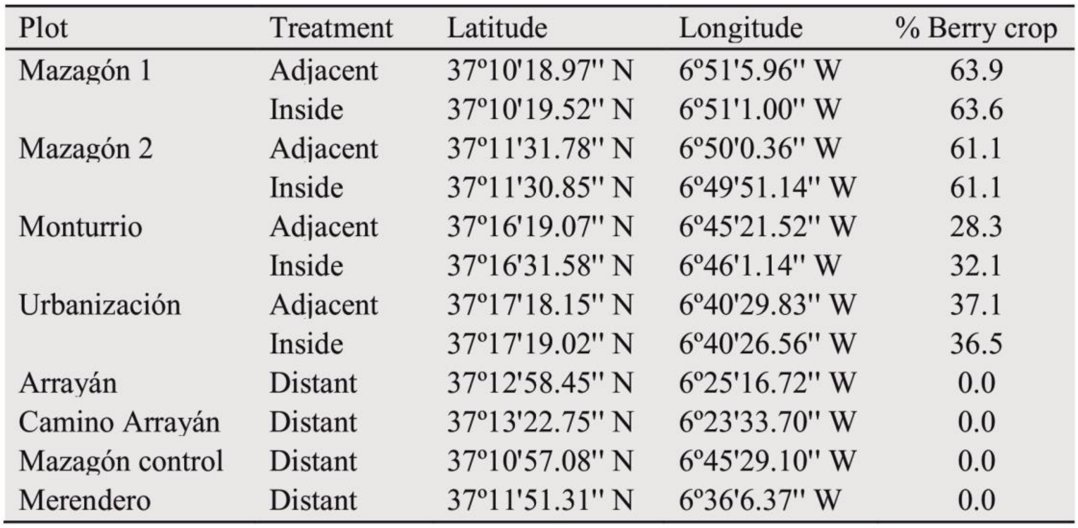
In 2015, we purchased 48 *B. terrestris* colonies from Koppert Biological Systems, one of the main producers in Europe and specifically in this region. Each colony consisted of a plastic box within a cardboard container, with syrup solution provided *ad libitum*. Each colony included a queen and ~100 workers.

First, to quantify colony parasite prevalence, at the arrival of the colonies (period 'before'), we collected 10 workers from each colony. Each worker was frozen in an individual clean vial at -20 ºC for later analyses.

Second, to investigate changes in colony parasite prevalence across the landscape, we placed colonies in four strawberry crops ('inside') and in eight woodlands, four adjacent to the selected strawberry crops (~50 m; 'adjacent') and four without berry crops in the surrounding 2 km radius landscape ('distant'). We chose a 2 km buffer radius because most bumblebee foraging flights do not exceed this distance (Osborne *et al.*, 2008). The surrounding landscape for inside and adjacent plots had a high berry crop cover (overall mean ± SE = 48 ± 5.6%; see Table 1). The average (± SE) distance between adjacent and distant woodland plots was 5903 ± 1038 m (range = 3.1–11.4 km). This distance meets independence criteria to avoid spatial pseudoreplication between non-paired plots.

Third, to investigate seasonal change effects, the experiment was conducted in January ('winter') and repeated in April ('spring'), as representative periods of high and low use of bumblebee colonies in strawberry crops, respectively. These two seasons also differ in climatic conditions (AEMET), availability of wild floral resources, and diversity of native pollinator species (Herrera, 1988).

Table 1. Location and berry crop cover in the surrounding landscape (2 km buffer radius) of plots inside, adjacent (~50 m) and distant (>2 km) to berry greenhouse crops.



Plot	Treatment	Latitude	Longitude	% Berry crop
Mazagón 1	Adjacent	37º10'18.97'' N	6º51'5.96'' W	63.9
	Inside	37º10'19.52'' N	6º51'1.00'' W	63.6
Mazagón 2	Adjacent	37º11'31.78'' N	6º50'0.36'' W	61.1
	Inside	37º11'30.85'' N	6º49'51.14'' W	61.1
Monturrio	Adjacent	37º16'19.07'' N	6º45'21.52'' W	28.3
	Inside	37º16'31.58'' N	6º46'1.14'' W	32.1
Urbanización	Adjacent	37º17'18.15'' N	6º40'29.83'' W	37.1
	Inside	37º17'19.02'' N	6º40'26.56'' W	36.5
Arrayán	Distant	37º12'58.45'' N	6º25'16.72'' W	0.0
Camino Arrayán	Distant	37º13'22.75'' N	6º23'33.70'' W	0.0
Mazagón control	Distant	37º10'57.08'' N	6º45'29.10'' W	0.0
Merendero	Distant	37º11'51.31'' N	6º36'6.37'' W	0.0

In each season, we placed two colonies of bumblebees in the center of each plot. Bumblebees were allowed to forage for 30 days. In strawberry crops the two colonies were hung between four separate greenhouses. The distance between the two colonies within a plot was ~20 m. In woodland plots the two colonies were hidden in wooden boxes to avoid predation. At the end of the experiment, we collected 10 bumblebee workers per colony returning to it (period ‘after’) over two days using aerial nets. Bumblebees were kept in individual clean vials with ice until arrival at the lab where they were stored at -20 ºC.

Parasite screening

The abdomen of each bumblebee was dissected and inspected under a magnifying lens for larvae of parasitic conopid flies (Conopidae, Diptera) and braconid wasps of the genus *Syntretus* (Braconidae, Hymenoptera), and the air sacs were specifically inspected for the tracheal mite *Locustacarus buchneri* (Podapolipidae) (Yoneda *et al.*, 2008). Then, a piece (0.2 cm × 0.2 cm, approx.) of the fat body was dissected out from each of the bumblebee and mounted on a slide. We completely screened each slide at ×400 magnification for the presence of spores of the neogregarine *Apicystis bombi* (Lipotrophidae) and microsporidians of the genus *Nosema* (Nosematidae). We estimated parasite prevalence (presence or absence) instead of individual infection levels (abundance) because the latter is influenced by many confounding factors that drive infection intensity (Rutrecht & Brown, 2009).

Statistical analyses

Only *Nosema* infections were statistically analyzed, because the remaining parasites showed no or very low prevalence in the colonies (see results). *Nosema* prevalence was calculated estimating the percentage of bumblebees infected per colony. A linear mixed model (LMM; Gaussian error distribution based on homogeneity in the residuals) was used to analyze whether changes in *Nosema* prevalence were related to our experimental setting. The difference in *Nosema* prevalence in the colonies before and after being placed in the field was used as the response variable. Season (winter/spring), plot type (inside, adjacent and distant), and their interaction were included as fixed factors in the model, while study plot was included as a random factor to account for the paired design between inside and adjacent plots and the re-sampled plots in winter and in spring. All statistical analyses were conducted in R (v.3.1.3, R Core Team, 2014). We used the package *lmerTest* (Kuznetsova *et al.*, 2013) for the LMM and Satterthwaite's approximations for F- and p- values.

Results

In total, over the two seasons we screened 919 bumblebee workers. We missed one colony and several individuals from other colonies due to low colony activity. On average (± SE) we collected 19.55 ± 0.13 (range = 16–20) bumblebees per colony. None of the bumblebees were infected by larvae of parasitic conopid flies (Conopidae, Diptera), braconid wasps of the genus *Syntretus* (Braconidae, Hymenoptera), or the tracheal mite, *Locustacarus buchneri*. The prevalence of *Apicystis bombi* was extremely low; only three bumblebees harbored spores in their fat body, and these were collected from two colonies at the end of their placement in adjacent woodlands.

In contrast, spores of *Nosema* were found in 58.3% (14 out of 24 colonies) of colonies in winter and in 83.3% (20 out of 24) in spring at the start of each experimental block, that is, upon arrival from the producer prior to their placement in the field. The average *Nosema* prevalence in the before period was 14.0 ± 3.4% (mean ± SE, hereafter) in winter, and 19.7 ± 3.2% in spring. The average *Nosema* prevalence in the after period was 10.2 ± 2.3% in winter and 26.4 ± 6.6% in spring. Neither the season ($F_{1, 35} = 2.88$, $p < 0.10$) nor the distance ($F_{2, 19} = 0.25$, $p < 0.79$) or their interaction ($F_{2, 35} = 0.50$, $p < 0.61$) had a significant effect on changes in *Nosema* colony prevalence between periods (Fig. 1A and 1B).

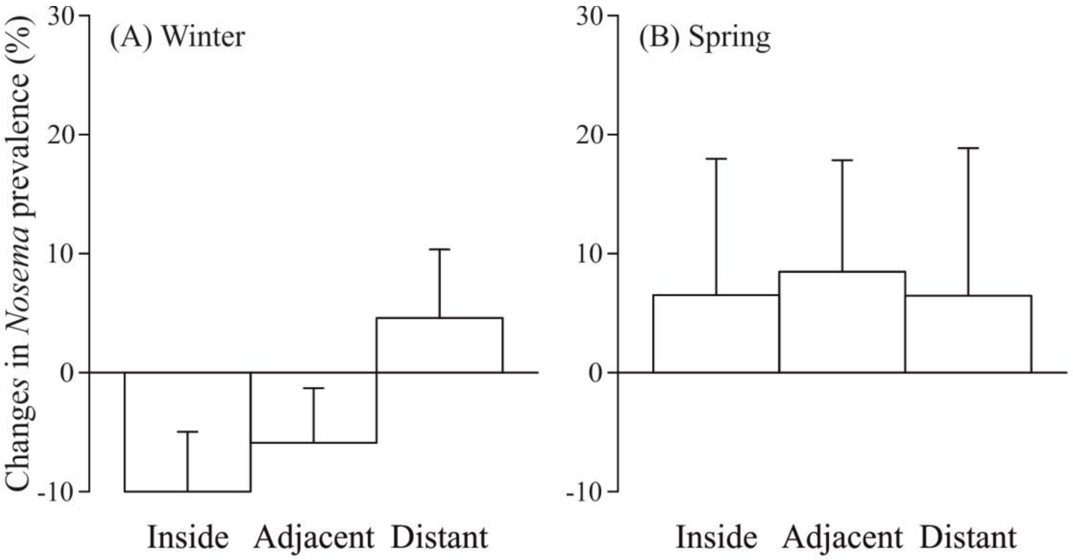


Figure 1. Mean (+SE) change in *Nosema* prevalence in commercially produced bumblebee colonies before and after being placed in plots inside, adjacent (~50 m) and distant (>2 km) to berry crops in winter (A) and in spring (B). Differences were not significant.

Discussion

The use of commercial bumblebees has been linked to the decline of several native pollinator species (Cameron *et al.*, 2011; Morales *et al.*, 2013; Schmid-Hempel *et al.*, 2014). Among the mechanisms behind this decline, parasite spillover from commercial to native pollinator populations may play a substantial role (Meeus *et al.*, 2011). Mass breeding programs may facilitate the probability of parasite transmission among hosts, as companies usually handle high densities of bumblebees in their facilities. In parallel, the provision of *ad libitum* food may facilitate the reproduction of infected hosts (Brown *et al.*, 2000). Furthermore, even in the case that commercial bumblebees are parasite-free, they may act as reservoirs for parasites in the field, through a spill-back mechanism, leading to an increase in parasite prevalence (Stout & Morales, 2009; Meeus *et al.*, 2011).

Upon arrival, we found no evidence for the presence of larvae of parasitic conopid flies (Conopidae, Diptera) and braconid wasps of the genus *Syntretus* (Braconidae, Hymenoptera), or the tracheal mite, *Locustacarus buchneri*, in the screened *Bombus terrestris* colonies. Although the presence of larvae of parasitic insects has never been reported in commercial bumblebees, the tracheal mite, *L. buchneri*, was highly prevalent at the end of the 20th century (Goka *et al.*, 2000) spilling over to native bumblebees (Goka *et al.*, 2006). However, it seems that producers have largely eliminated this parasite from commercial bumblebee colonies

(Goka *et al.*, 2006; Murray *et al.*, 2013; although see Sachman-Ruiz *et al.*, 2015). In addition, neither these parasitoids nor the tracheal mite, *L. buchneri*, were observed in bumblebees from the colonies after being placed in the field for a month. One explanation for this is that parasitoids of bumblebees might be at low abundance in our study sites, because native bumblebees (*B. terrestris lusitanicus*) are rare (Magrach *et al.*, 2017; Chapter 1), as they are at the limit of their distributional range (Goulson, 2010). In fact, in this region, the density of commercial bumblebees is around four times greater than that of native bumblebees (Chapter 2). Another possible and complementary explanation for this low prevalence might be that when bumblebees are parasitized, they desert their colony (Schmid-Hempel & Müller, 1991). In addition, even though *L. buchneri* may be present in native bumblebees (although we note that there is no information in Spain; Jabal-Uriel *et al.* 2017) it might be very difficult to detect parasite spillover from native to managed bumblebees due to their differential abundance.

Similarly, there was no evidence for the presence of the neogregarine *Apicystis bombi* (Lipotrophidae) in the screened colonies upon arrival. However, three bumblebees were found to be infected after having been placed in the field. In other regions, the parasite *A. bombi* has been detected infecting commercial bumblebee colonies from producers, although in a low number of colonies (Graystock *et al.*, 2013b; Murray *et al.*, 2013; although again see Sachman-Ruiz *et al.*, 2015). Native bumblebees can host *A. bombi* (Jabal-Uriel *et al.*, 2017), but, as noted above, they are rare in our study region (Magrach *et al.*, 2017; Chapter 1). In contrast, thousands of commercial colonies, from at least three producers (Koppert, Biobest and Agrobio, personal observation), are used on an annual basis. Therefore, it is more likely that other commercial bumblebees infected by *A. bombi* transmitted the parasite to the bumblebee colonies we screened, rather than native bumblebees, or, more parsimoniously, our initial screen failed to detect it in arriving colonies.

In contrast, we found commercially produced bumblebee colonies to be heavily infected with parasites of the genus *Nosema* upon arrival from the producer. Other studies have also reported similar levels of prevalence with around three quarters of commercial colonies infected (Graystock *et al.*, 2013a; Murray *et al.*, 2013). Unfortunately, our methodology did not allow us to distinguish between the bumblebee parasite *N. bombi* and the honeybee parasite *N. ceranae*. Both can infect bumblebees (Graystock *et al.*, 2013a; Fürst *et al.*, 2014).

Unexpectedly, our results showed no significant variation in *Nosema* infection rate at a colony level over time, as in a previous study that monitored wild bumblebees (Goulson *et al.*, 2018), even in landscapes where commercial bumblebees were intensively used to pollinate crops. Even in parasite-free landscapes, one would expect that if commercial colonies are infected by a parasite, it spreads within the colonies across time due to the high density of host and low genetic variability (Schmid-Hempel, 1998). We propose two potential explanations. On the one hand, bumblebees, in line with other social insects, have evolved social immune systems that combine prophylactic and activated responses to avoid, control or eliminate parasite infections (reviewed by Cremer *et al.*, 2007). Both, colony and individual (i.e. immunocompetence, reviewed by Schmid-Hempel, 2005) defense mechanisms might be involved in maintaining roughly constant *Nosema* prevalence over time. On the other hand, it has been experimentally demonstrated that *Nosema*, specifically *N. bombi*, relies more on transmission through the larval stage than through transmission among adults (Rutrecht *et al.*, 2007). If we consider that colonies were placed in the field for a month period and that the total development of a bumblebee from larvae to adult is about 4-5 weeks (Alford, 1975), this could explain why we failed to detect an increase in prevalence. Imhoof & Schmid-Hempel (1999) showed an average delay to *Nosema* infection in commercial colonies placed in the field of ~30 days.

Our study showed, for the first time in Spain, that commercially produced bumblebee colonies can be infected by *Nosema* parasites prior to their deployment in the field. These parasites can reduce lifespan and have detrimental effects on bumblebee behavior (Otti & Schmid-Hempel, 2007; Rutrecht & Brown, 2009; Graystock *et al.*, 2013a). Because commercial bumblebees placed in semi-open greenhouses frequently forage in natural areas (Whittington *et al.*, 2004; Foulis & Goulson, 2014), they have the potential to spread the pathogen into native pollinator populations (Colla *et al.*, 2006; Murray *et al.*, 2013). Despite the fact that producers must screen their production for pathogens (Graystock *et al.*, 2013a; Murray *et al.*, 2013), we still found a high number of infected colonies. Therefore, there is a need for the enforcement of more stringent protocols to preserve the health of commercial and native pollinators.

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Discusión general

El estudio del movimiento de animales entre hábitats en los paisajes agrícolas es esencial para conocer como aprovechan los recursos que les ofrecen estos paisajes cambiantes y cómo esto afecta a su éxito reproductivo. A la vez, resulta fundamental conocer como este movimiento puede influenciar en los procesos ecológicos que se dan tanto en los cultivos como en los espacios naturales. Esto es de vital importancia para la economía del sector agrícola y para la conservación de la biota de los hábitats naturales, respectivamente (Rand *et al.*, 2006; Tscharntke *et al.*, 2012; Smith *et al.*, 2014).

El objetivo general de esta Tesis Doctoral ha sido investigar el movimiento de polinizadores comerciales y silvestres entre hábitats naturales y agrícolas promovido por la variabilidad de recursos florales que dependen tanto de la estación del año como de las características del paisaje. En el hábitat agrícola, se ha estudiado la influencia de los polinizadores comerciales y silvestres en la producción de fruta. En el hábitat natural, se ha cuantificado la presencia y densidad de polinizadores comerciales en comparación con taxones nativos. A modo de posibles riesgos ecológicos, se ha estudiado el solapamiento en la explotación de los recursos florales entre los polinizadores comerciales y nativos, y la prevalencia de patógenos en las colonias comerciales usadas en los cultivos. Para abordar los objetivos planteados en esta tesis se han realizado tanto observaciones muy intensivas y extensivas como experimentos de campo en paisajes agrícolas de cultivos de frutos rojos y parches de hábitat natural con gran diversidad de plantas entomófilas que se encuentran en el Valle del Guadalquivir (SO España).

El movimiento de polinizadores entre hábitats puede ocurrir en ambas direcciones. Inicialmente, su estudio se centró en el movimiento de polinizadores silvestres desde hábitats naturales hacia los cultivos, atraídos por la repentina abundancia floral. Más recientemente, se ha estudiado el proceso contrario cuando cesa la floración del cultivo (Blitzer *et al.*, 2012). Sin embargo, el estudio del movimiento de polinizadores comerciales entre hábitats ha pasado más desapercibido. En este sentido, esta Tesis avanza, especialmente, en el conocimiento del movimiento y la función de polinizadores comerciales, es decir, su influencia en la producción de los cultivos y su papel más allá del cultivo. Ya que el abejorro comercial de la especie *Bombus terrestris* ha sido el protagonista principal a lo largo de toda la tesis, nos vamos a centrar en él como hilo conductor de la discusión.

El uso de polinizadores comerciales en los cultivos no es siempre necesario

El uso de insectos polinizadores comerciales para los servicios de polinización, se ha convertido en una práctica cada vez más común a escala global que ha acompañado al incremento del área dedicada a cultivos dependientes de polinizadores para la producción de frutos y semillas (Velthuis & van Doorn, 2006; Aizen & Harder, 2009; Geslin *et al.*, 2017b). A pesar de ello, es fácil encontrar en la literatura trabajos que muestran tanto efectos positivos como neutros sobre su contribución en la producción de los cultivos. Casi todos estos estudios tratan del uso de la abeja de la miel (p. ej. Rader *et al.* 2009; Holzschuh, Dudenhöffer & Tscharntke 2012; Mallinger & Gratton 2015). El papel que desempeñen los polinizadores comerciales en los cultivos, no solo va a depender de las características intrínsecas del polinizador (Willmer *et al.*, 1994; Zhang *et al.*, 2015), sino también del tipo y variedad de cultivo, de factores ambientales y factores bióticos que van a mediar la presencia e interacción con otros polinizadores (Klein *et al.*, 2003; Garibaldi *et al.*, 2011; Garratt *et al.*, 2014). Generalmente, cultivos con una comunidad de visitantes florales abundante y rica producen frutos y semillas de la mayor calidad debido a procesos de complementariedad en el movimiento a través de la flor, entre otros (Garibaldi *et al.*, 2013).

En general, el uso de abejorros comerciales ha sido fomentado por los intereses económicos de las empresas que se dedican a su cría y por organismos públicos del sector agrícola, esto último al menos en España (Ariza *et al.*, 2016). Estas entidades promueven su uso en los cultivos en base a estudios realizados en condiciones controladas de invernaderos inaccesibles para la comunidad de polinizadores presentes en zonas adyacentes. En esas condiciones, se ha observado que el uso de abejorros mejora la calidad e incrementa la cantidad de frutos y semillas (ver Dag & Kammer 2001; Roldán Serrano & Guerra-Sanz 2006; Dimou *et al.* 2008). Ya que el coste de suplementar los cultivos con polinizadores comerciales no es excesivo en comparación con las ganancias potenciales, los agricultores que creen en el beneficio de su uso gestionan sus cultivos de forma similar. Es decir, el número de colonias usadas o incluso la necesidad de su uso es independiente a la limitación polínica que pueda haber en el cultivo, ya que no se mide (Cunningham *et al.*, 2016).

Tal y como se ha corroborado en el **Capítulo 1** en cultivos de fresa variedad Fortuna, el uso de colonias de abejorros comerciales puede ser poco eficiente en la producción cuando otros visitantes florales están presentes. Este mismo resultado se ha encontrado en cultivos de calabaza y arándano (Petersen *et al.*, 2013; Hicks & Sircom, 2016). Sin embargo, otros estudios sí han encontrado una importante contribución de los abejorros comerciales en la producción de cultivos de otras variedades de fresa y arándano, además de cultivos de frambuesa, pera y manzana (Desjardins & De Oliveira, 2006; Lye *et al.*, 2011; Zisovich *et al.*, 2012; Ellis *et al.*, 2017; Sapir *et al.*, 2017). Hay que tener en cuenta que variedades distintas de un mismo tipo de cultivo, incluso en la misma región de estudio, pueden ofrecer resultados contradictorios. Esto se debe a que cada variedad puede presentar una dependencia distinta a la polinización mediada por insectos para la producción de frutos y semillas (Klatt *et al.*, 2014). En este sentido, habría que priorizar los estudios en las variedades de cultivos más extendidos en cada región, ya que son los que van a demandar un mayor volumen de colonias.

Uno de los principales factores que se relaciona positivamente con la abundancia y riqueza de visitantes florales en los cultivos es la cantidad de espacios naturales en las zonas adyacentes a estos (Kremen *et al.*, 2004; Ricketts *et al.*, 2008; Winfree *et al.*, 2009). Esta relación suele estar ligada a la presencia de especies de abejas silvestres que nidifican en lugares fijos y que por tanto, necesitan de espacios naturales para nidificar (Winfree, 2010). No obstante, tal y como se ha observado en el **Capítulo 1**, y en otros trabajos en distintos continentes (Raymond *et al.* 2014; Rader *et al.* 2016), la distribución de otros insectos polinizadores, como puede ser la omnipresente abeja de la miel (*A. mellifera*) o insectos polinizadores que no son abejas, por ejemplo los dípteros, no suele ser tan dependiente de las características del paisaje en el que se encuentran. Esto se debe a que para el caso de la abeja de la miel, sus colonias se introducen en la mayoría de tipos de hábitats con el fin de producir miel y productos derivados, además de para polinizar cultivos (Garibaldi *et al.*, 2013; Hung *et al.*, 2018). En el caso de los dípteros, muchas de las especies explotan recursos de lugares muy diversos, incluso los alterados por el humano (Raymond *et al.*, 2014; Orford *et al.*, 2015). Cuando estos polinizadores están presentes en los paisajes agrícolas y además forrajean frecuentemente en los cultivos, su contribución en la función de polinización puede ser importante (**Capítulo 1**).

Según lo observado en el **Capítulo 1**, ya que la abeja de la miel lidera las visitas a los cultivos de fresa, es probable que su contribución en la producción sea destacada. Sin embargo, se necesitan experimentos de exclusión adicionales en los que se pudiese valorar su eficacia, por ejemplo a través de la deposición de polen por visita. La abeja de la miel es una especie cosmopolita. Su protagonismo no es exclusivo de nuestro sistema de estudio. Su papel en la polinización de cultivos ha sido destacada en muchísimos estudios, especialmente en escenarios en los que el resto de visitantes florales son escasos (Pisanty *et al.*, 2014; Lindström *et al.*, 2016; Geslin *et al.*, 2017a).

La presencia de la abeja de la miel y de dípteros en los cultivos de fresa, parece no estar influenciada por factores estacionales o de disposición de recursos florales, ya que visitaron los cultivos de manera similar tanto en invierno como en primavera. Estos resultados podrían ser similares en otras regiones con clima mediterráneo, ya que los inviernos suelen ser suaves y algunas plantas florecen. En cambio, si los polinizadores principales en los cultivos fuesen abejas silvestres, probablemente su presencia sería más susceptible incluso a los cambios a más corto plazo (Ellis *et al.*, 2017). Por lo tanto, en trabajos futuros sería interesante monitorizar con mayor intensidad todo el periodo de floración del cultivo, con el fin de obtener información precisa sobre los posibles cambios en la comunidad de polinizadores en el tiempo. A la vez, estos cambios podrían influir en el papel que desempeñen los polinizadores comerciales en los cultivos.

También habría que tener en cuenta que tanto en nuestro estudio como en gran parte de los estudios realizados hasta la fecha (p. ej. Connelly *et al.*, 2015; Eeraerts *et al.*, 2017), la mayoría de información proviene de zonas cultivadas cercanas a espacios naturales. Ahí es donde se espera una mayor abundancia y riqueza de polinizadores (revisado en Garibaldi *et al.* 2011). Sin embargo, a medida que nos alejamos de los espacios naturales, se espera que la comunidad de visitantes florales en los cultivos disminuya, y por tanto, la necesidad de polinizadores comerciales sea más importante.

Movimiento de polinizadores comerciales desde los cultivos hacia los espacios naturales adyacentes

Los polinizadores comerciales suelen ser generalistas (Coffey & Breen 1997; **Capítulo 2** y **3**). Varios estudios han demostrado que una dieta de polen diversa mejora su éxito reproductivo en comparación con una dieta pobre, por ejemplo a través de un aumento del tamaño de las larvas (Génissel *et al.*, 2002; Tasei & Aupinel, 2008) o un sistema inmune mejor (Brunner *et al.*, 2014; Roger *et al.*, 2017). Además, los polinizadores comerciales presentan amplios rangos de forrajeo. Es decir, pueden volar distancias de hasta 2 km en cada viaje (Osborne *et al.* 2008; Danner *et al.* 2016). Ambos rasgos, especies generalistas y amplios rangos de forrajeo, conllevan a que el papel que ejercen los polinizadores comerciales utilizados para la polinización de las flores en los cultivos se extienda más allá de los cultivos (González-Varo & Vilà 2017; **Capítulo 2** y **3**).

La magnitud del movimiento de estos polinizadores hacia el medio natural, y por tanto, su influencia en los procesos ecológicos, puede depender de las características del paisaje que les rodea. Un factor que podría determinar la presencia de estos polinizadores en el medio natural podría ser el área dedicada a cultivo en el paisaje, ya que teóricamente, el número de colonias usadas va a ser mayor. Recientemente y por primera vez, González-Varo & Vilà (2017) han demostrado que cuando cesa la floración del cultivo de naranjo, en paisajes donde el área dedicada a estos cultivos es mayor, también lo es la densidad de abejas de la miel en los parches de hábitat natural adyacentes. En esta tesis ofrecemos un caso de otro polinizador comercial.

En el **Capítulo 2** se muestra una correlación positiva entre la presencia de abejorros comerciales en los parches de hábitat natural adyacentes a los cultivos de frutos rojos y el área dedicada a cultivo en el paisaje. Sin embargo, la ausencia total o parcial de abejorros en gran parte de los parches muestreados impidió explorar si la densidad de abejorros también depende de la superficie de cultivos. Esta pregunta se podría abordar en el mismo sistema de estudio pero con una aproximación distinta. A través de muestreos focales en plantas que se ha observado que son muy atractivas para los abejorros, como es el caso de *Acacia* spp., *Echium vulgare* o *Cerinthe gymnandra*. También sería interesante contrastar nuestros resultados con otros lugares donde también se empleen abejorros en grandes cantidades. Podrían ser paisajes agrícolas con árboles frutales, otros cultivos de frutos rojos además de fresa y cultivos de invernadero con tomates y pimientos, y otras hortalizas (ver Velthuis & van Doorn 2006).

Otro factor clave que puede influenciar en la magnitud del movimiento de polinizadores comerciales hacia el medio natural está relacionado con la variación en el tiempo de la disponibilidad de recursos florales en el cultivo y en los hábitats naturales. Se espera que, aunque los cultivos estén en flor, los recursos florales silvestres sean más atractivos para los polinizadores, ya que son más diversos, pero siempre y cuando sean abundantes en el espacio (Whittington *et al.*, 2004).

Según el trabajo expuesto en el **Capítulo 2** en los parches de hábitat natural no se ha encontrado ningún patrón al respecto, probablemente debido a que un mayor uso de colonias de abejorros en invierno fue enmascarado por una mayor atracción de los abejorros hacia los recursos silvestres en primavera. En cambio según los datos del **Capítulo 3**, sí se ha encontrado un patrón. Quizás las diferencias se deben a que en el Cap. 2 el estudio fue observacional, en cambio en el Cap. 3 fue experimental, permitiendo establecer una relación causal. En el **Capítulo 3**, se observó en invierno que la gran abundancia de flores en los cultivos comparada con abundancias similares en el hábitat natural pero más dispersas actuó como un imán para los abejorros situados en colonias dentro de los cultivos. Sin embargo, en primavera, este efecto imán se diluyó y la mayoría de abejorros se movían hacia los parches de hábitat natural para forrajear, debido a que los recursos ya eran mucho más abundantes y ricos que en los cultivos.

A pesar de que en el **Capítulo 1** no se ha encontrado un efecto significativo de la contribución de los abejorros comerciales en el peso de la fresa, resulta fundamental tener en cuenta en trabajos futuros que factores como la disponibilidad de recursos florales en el paisaje pueden influir en la magnitud del movimiento de estos polinizadores y por tanto, en su tasa de visitas al cultivo (**Capítulo 1**). Cuando los recursos florales silvestres sean abundantes y ricos en las zonas adyacentes a los cultivos, el uso de polinizadores comerciales podría ser poco productivo para el agricultor, ya que la mayoría de individuos no van a forrajean en los cultivos (Whittington *et al.* 2004; **Capítulo 1**).

Riesgos ecológicos del uso de polinizadores comerciales: explotación de los recursos florales

Como se ha mencionado en la sección anterior, los polinizadores comerciales son generalistas y van a forrajear a los espacios naturales adyacentes a los cultivos. En este sentido, es de esperar que estos polinizadores forrajeen en las mismas plantas que lo hacen otros polinizadores silvestres generalistas taxonómicamente similares. Por ejemplo, en el Norte de Japón, se ha observado que alrededor de la mitad de las especies de plantas que visitan los abejorros nativos (*B. ardens sakagamii*, *B. hypocrita sapporoensis*, *B. diversus tersatus* y *B. pseudobaicalensis*) también son visitadas por el abejorro introducido (*B. terrestris*) (Matsumura *et al.*, 2004). Además, también en la misma región, se han observado procesos de exclusión de hábitat de especies nativas de abejorros donde el abejorro introducido es abundante (Ishii *et al.*, 2008).

En nuestra región de estudio, debido a la similitud morfológica de la subespecie de abejorro comercial (tanto *B. t. terrestris* como *B. t. dalmatinus*) y la subespecie nativa (*B. t. lusitanicus*) (ver Rasmont *et al.* 2008), uno de los riesgos asociados con el movimiento desde los cultivos hacia los hábitats adyacentes podría ser la competencia por los recursos florales entre estas subespecies. En el **Capítulo 2**, se ha corroborado que hay un solapamiento en el uso de los recursos florales, siendo mayor en invierno que en primavera, debido a que la riqueza de especies de plantas en flor es baja. Pero además de explotar recursos similares, se ha encontrado una relación negativa entre la probabilidad de presencia del abejorro nativo en los parches de hábitat natural y el área dedicada a cultivo en el paisaje. Esta relación, sin embargo, pudo estar asociada a otros múltiples factores, como por ejemplo a la pérdida de hábitat natural (ej. la cobertura de cultivo y de hábitat natural en el paisaje estaban ligeramente correlacionadas) que a su vez está ligada a una disminución de lugares para nidificar y de recursos florales silvestres (Potts *et al.*, 2010) o a una mayor exposición de pesticidas (Botías *et al.*, 2016). Se necesitan más estudios para dilucidar la importancia relativa de cada uno de los factores implicados afectando la presencia del abejorro nativo en el hábitat natural adyacente a los cultivos. Esta cuestión se podría abordar con diseños experimentales multifactoriales, aunque su ejecución en campo podría ser una tarea difícil de abordar.

De cualquier modo, con respecto al uso de los recursos florales en estos paisajes agrícolas, hay que destacar que no se han observado abejorros nativos en los cultivos de fresa (**Capítulo 1**) ni se ha registrado ningún grano de polen en los cuerpos de los abejorros nativos capturados en el hábitat natural (**Capítulo 2**). Sin embargo, es probable que el esfuerzo de muestreo no fuese suficiente para poder afirmar que los abejorros nativos no forrajean en cultivos de fresa, aunque tampoco fue este un objetivo principal de la tesis. Hay que destacar además que los abejorros son poco abundantes en nuestra región de estudio. De hecho, Magrach *et al.* (2017) observaron que representaban solo el 0.3% de las interacciones planta-polinizador.

En el **Capítulo 3**, usando colonias de abejorros comerciales, sí que se ha observado un porcentaje elevado de granos de polen de frutos rojos en los cuerpos de los abejorros que procedían de colonias ubicadas en los parches de hábitat natural. Esto fue incluso mayor en invierno en paisajes con alta cobertura de cultivo. Estos resultados sugieren la posibilidad de que el abejorro nativo también forrajee en los cultivos de una forma similar. Quedaría por estudiar si la ingesta de estos recursos repercute de alguna manera en el éxito reproductivo de los abejorros en particular, pero también de otros visitantes florales en general. Esta cuestión se podría abordar de dos maneras. Por un lado y similar al diseño experimental del **Capítulo 3**, mediante experimentos de campo en paisajes con alta y baja cobertura de cultivo, se evaluaría el éxito reproductivo de los polinizadores a través del peso y abundancia de larvas (Westphal *et al.*, 2009). Por otro lado, esta cuestión se podría abordar a través de experimentos en el laboratorio en los que se le suministrase a los polinizadores dietas ricas y pobres en polen procedente de cultivos de frutos rojos y se evaluase el impacto en su descendencia de una forma similar a la mencionada (Génissel *et al.* 2002).

Riesgos ecológicos del uso de polinizadores comerciales: prevalencia de parásitos

Otra de las amenazas que surge del uso de especies comerciales para la entomofauna nativa, sería a través de la transmisión de parásitos (Meeus *et al.*, 2011; Fürst *et al.*, 2014). En el caso concreto de los abejorros comerciales, a pesar de que se empezaron a utilizar a finales de los 80, no fue hasta comienzos del 2000 en Japón cuando se reportó por primera vez la presencia de ácaros (*Locustacarus buchneri*) en las colonias (Goka *et al.*, 2000). Ya en 2006, se reportó también su transmisión a especies de abejorros silvestres (Goka *et al.*, 2006). Ese fue el comienzo de una serie de trabajos realizados en todo el mundo en el que se han descrito un amplio abanico de organismos parásitos (hongos, bacterias, protistas, ácaros y virus) que pueden albergar las colonias de abejorros comerciales (Otterstatter & Thomson, 2006; Graystock *et al.*, 2013a; Murray *et al.*, 2013). Lo preocupante es que gran parte de estos parásitos pueden transmitirse a los insectos polinizadores silvestres (Durrer & Schmid-Hempel, 1994; Goka *et al.*, 2006; Graystock *et al.*, 2015). Se han observado correlaciones positivas entre la presencia de polinizadores comerciales y la prevalencia de parásitos en la comunidad de polinizadores silvestres (Colla *et al.*, 2006; Murray *et al.*, 2013; Graystock *et al.*, 2014). Algunos parásitos pueden tener efectos letales o subletales en los polinizadores que infectan (Brown *et al.*, 2000; Graystock *et al.*, 2016b; Brown, 2017).

Es por ello que, en la actualidad, se han impuesto una serie de medidas sanitarias para controlar la salud de las colonias de abejorros que se producen comercialmente (p. ej. para Europa ver European Commission 1992). Sin embargo, a pesar de todos los esfuerzos, tal y como se ha corroborado en el **Capítulo 4,** al igual que otros estudios recientes (Graystock *et al.*, 2013b; Sachman-Ruiz *et al.*, 2015), las medidas de control de las empresas son insuficientes para la producción de colonias libres de parásitos. Todavía siguen albergando elevadas cargas de algunos parásitos. En nuestro estudio se encontró que alrededor de un 71% de las colonias estaban infectadas por el microsporidio *Nosema* spp. Estos resultados destacan la necesidad de reforzar los protocolos sanitarios que se llevan a cabo en las empresas con el fin de preservar la salud de los polinizadores tanto comerciales como nativos, para preservar tanto la calidad de la producción y función como la conservación de la entomofauna, respectivamente.

En cualquier caso, sería interesante explorar en nuestra región de estudio si los polinizadores silvestres están afectados por la transmisión de parásitos procedentes de los abejorros comerciales, ya que para los sistemas mediterráneos aún no existe información. Esta pregunta se podría abordar con un diseño experimental similar al de Murray *et al.* (2013), donde recolectaron polinizadores silvestres en Irlanda en zonas cercanas y lejanas a cultivos de fresa donde se usaban abejorros comerciales. Murray *et al.* (2013) encontraron una mayor prevalencia de parásitos en la comunidad de polinizadores cerca de los cultivos sugiriendo procesos de transmisión de parásitos procedentes de abejorros comerciales.

Riesgos ecológicos del uso de polinizadores comerciales: retos futuros

Además de los trabajos propuestos en las secciones anteriores, tanto el movimiento de polinizadores comerciales hacia el hábitat natural como los cambios en la magnitud de dicho movimiento, pueden influenciar en otros procesos ecológicos que no han sido el foco de esta tesis.

Una de las amenazas posibles del uso de especies o subespecies no nativas es la posible naturalización de poblaciones o incluso la invasión en el entorno. Esto último ha ocurrido con *B. terrestris* en Japón (Matsumura *et al.*, 2004) y en Argentina (Morales *et al.*, 2013). Creemos que los abejorros comerciales consiguen naturalizarse en nuestra región de estudio, ya que hemos observado alguna colonia en el hábitat natural, apareamientos y reinas buscando nido. Sin embargo, su éxito debe ser muy bajo ya que su presencia en lugares alejados a los cultivos es bastante escasa, a pesar de los ya casi 20 años de su uso comercial en la región. No obstante, se necesitan de estudios futuros con el fin de obtener información más fina al respecto.

Sería interesante explorar como los polinizadores comerciales pueden influenciar la reproducción de las plantas silvestres localizadas en las zonas adyacentes a los cultivos. Un aumento en la abundancia de polinizadores generalistas podría resultar en un beneficio en el éxito reproductivo de algunas especies de plantas. En cambio, si las tasas de visitas a las plantas son muy elevadas esto podría incluso resultar en una reducción en la producción de semillas, por procesos de saturación polínica que previenen el desarrollo de los tubos polínicos (Sáez *et al.*, 2014; Magrach *et al.*, 2017).

Otro aspecto interesante surge de la posibilidad de que la subespecie de abejorro comercial (*B. t. terrestris* o *B. t. dalmatinus*) se aparee con la subespecie nativa (*B. t. lusitanicus*). Recientemente, estudios realizados por Cejas *et al.* (2018) sugieren que hay hibridación e introgresión genética entre ambas subespecies a lo largo de la Península Ibérica, aunque los resultados requieren de un mayor grado de resolución. Sería interesante llevar a cabo una aproximación similar a la propuesta en la sección anterior para la transmisión de parásitos (ver Murray *et al.* 2013) para poder elucidar esta cuestión a una escala espacial más fina y aportando información más robusta.

Finalmente, frente a la incertidumbre que genera el uso de polinizadores comerciales, no solo en la conservación de los polinizadores nativos, sino incluso en los servicios de polinización de los cultivos, sería preferible desarrollar vías alternativas donde fuesen posibles (Garibaldi *et al.*, 2014). Por ejemplo, una de las medidas agro-ambientales para reducir o incluso evitar el uso de polinizadores comerciales podría ser la creación de espacios entre los cultivos en los que se planten plantas nativas con flores que atraigan a polinizadores de las zonas adyacentes. A la vez, estos espacios los podrían usar los polinizadores como lugares de nidificación. Este tipo de medidas se ha visto en algunos estudios que se relaciona con una mayor tasa de visitas de los polinizadores a los cultivos (Carvalheiro *et al.*, 2011; Blaauw & Isaacs, 2014; Feltham *et al.*, 2015). En el caso de que los polinizadores comerciales fuesen necesarios, otras de las medidas para reducir su impacto sería que las empresas criasen subespecies nativas y que las colonias se vendiesen libres de parásitos.

Conclusiones generales

1. El uso de abejorros comerciales para suplementar los servicios de polinización es una práctica habitual en cultivos de frutos rojos localizados en el valle del Guadalquivir en la provincia de Huelva, al igual que en el resto del mundo en más de 20 tipos de cultivo. Sin embargo, existen numerosas lagunas en la literatura acerca de la contribución de estos y otros polinizadores comerciales en la producción de las distintas variedades de cultivos.

2. El uso de abejorros comerciales de la especie *Bombus terrestris* en los cultivos de fresa en invernaderos semiabiertos, concretamente en la variedad 'Fortuna', una de las variedades más cultivadas en esta región, supone un aumento de abejorros visitando las flores. La tasa de visitas es mayor en invierno que en primavera. Sin embargo, las visitas no se traducen en un incremento en el peso de la fresa en ninguna de las dos estaciones.

3. Los servicios de polinización en estos cultivos adyacentes a hábitats naturales están cubiertos por las visitas de otros polinizadores. Entre ellos destacan la abeja de la miel y los dípteros de las especies *Eupeodes corollae* y *Episyrphus balteatus* cuyas tasas de visita no dependen ni de la estación ni de la intensificación agrícola (cobertura de invernadero) en el paisaje.

4. En invierno, la mayor parte de abejorros comerciales forrajean en los cultivos, en cambio, en primavera, lo hacen en los hábitats naturales, debido a la mayor disponibilidad de recursos florales. Estos abejorros forrajean en una gran diversidad de flores silvestres al igual que lo hacen los abejorros nativos de la subespecie *B. t. lusitanicus*. El solapamiento en la explotación de los recursos florales entre abejorros comerciales y nativos es mayor en invierno que en primavera.

5. En los parches de pinares adyacentes a los cultivos, los abejorros comerciales pueden llegar a ser más abundantes que los abejorros nativos en invierno pero no en primavera. La presencia de abejorros comerciales en los pinares está positivamente relacionada con la cobertura de invernaderos en el paisaje, mientras que la presencia de abejorros nativos se relaciona negativamente.

6. El 71% de las colonias de abejorros comerciales albergan parásitos de microsporidios del género *Nosema*. La prevalencia de este parásito en las colonias no varía entre invierno y primavera, ni entre paisajes con alta cobertura de invernaderos y alejados a invernaderos. *Nosema* podría transmitirse a otros polinizadores silvestres a través de las flores que visitan. Es por ello que, se necesitan de protocolos sanitarios más exigentes con el fin de reducir la carga de parásitos en las colonias comerciales.

7. Es necesario valorar el uso de polinizadores comerciales para los servicios de polinización frente a otras medidas que fomenten la diversidad de polinizadores silvestres en los cultivos. En cualquier caso, con el objetivo de minimizar el riesgo que puede suponer el uso de polinizadores comerciales para la entomofauna nativa, las empresas deberían criar subespecies de abejorros nativos libres de parásitos.

General conclusions

1. The use of commercial bumblebees to supplement pollination services is common in berry crops located in the Guadalquivir Valley, province of Huelva, as well as in more than 20 types of crops worldwide. However, there are several knowledge gaps in the literature regarding the contribution of these and other commercial pollinators to crop production of different varieties.

2. The use of commercial bumblebees, *Bombus terrestris*, in semi-open strawberry greenhouse crops, specifically the 'Fortuna' variety, one of the most cultivated strawberry varieties in this region, increases bumblebee crop flower visitation. The rate of visits is higher in winter than in spring. However, crop flower visitation is not related to an increase in strawberry fruit weight in either of the two seasons.

3. The pollination services for these crops adjacent to natural habitats are fulfilled by the visitation of other pollinators. The honeybee and Diptera, such as *Eupeodes corollae* y *Episyrphus balteatus*, dominate crop flower visits whose rate depends on neither the season nor on agricultural intensification (greenhouse cover) in the landscape.

4. In winter, most commercial bumblebees forage in crops, whereas in spring most forage in natural habitats, due to the higher availability of floral resources. These bumblebees forage in a wide diversity of wild flowers as does the native bumblebee subspecies *B. t. lusitanicus*. The overlap in floral resource exploitation is higher in winter than in spring.

5. In the patches of pinewoods adjacent to crops, commercial bumblebees can be more abundant than native bumblebees in winter than in spring. The presence of commercial bumblebees in the pinewoods is positively related to greenhouse cover in the landscape, while the presence of native bumblebees is negatively related to greenhouse cover in the landscape.

6. Upon arrival from the producer, 71% of the bumblebee colonies are infected by microsporidian parasites of the genus *Nosema*. The prevalence of this parasite in the colonies does not change between winter and spring, nor between landscapes with high crop cover and those far from crops. *Nosema* may be transmitted to other wild pollinators through visited flowers. Thus, the enforcement of more stringent protocols is required in order to reduce parasite prevalence in commercial colonies.

7. There is a need to evaluate the use of commercial pollinators for pollination services against other measures that promote native pollinator diversity in crops. In any case, in order to minimize the risk associated with the use of commercial pollinators to native entomofauna, companies should breed native pollinator subspecies free of disease.

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“El problema de la agricultura actual, es que no es un sistema orientado a la producción de comida, sino a la producción de dinero” *Bill Mollison*

El objetivo general de esta Tesis Doctoral es investigar el movimiento de polinizadores comerciales y silvestres entre hábitats naturales y agrícolas promovido por la variabilidad de recursos florales que dependen tanto de la estación del año como de las características del paisaje. Concretamente, se estudia la influencia de los polinizadores comerciales y silvestres en la producción de un cultivo de fruta. A la vez, se cuantifica la presencia y densidad de polinizadores comerciales en comparación con taxones nativos en el hábitat natural adyacente a los cultivos. Además, a modo de posibles riesgos ecológicos, se estudia el solapamiento en la explotación de los recursos florales entre los polinizadores comerciales y nativos, y la prevalencia de parásitos en las colonias comerciales usadas en los cultivos.