UNIVERSIDAD POLITÉCNICA DE MADRID

ESCUELA TÉCNICA SUPERIOR DE INGENIERÍA AGRONÓMICA,
ALIMENTARIA Y DE BIOSISTEMAS

Evaluation of Flowering Plant Strips and the Risk of Pesticides on Pollinators in Melon Agro-ecosystems

TESIS DOCTORAL

Celeste Azpiazu Segovia

Ingeniera Técnica Agrícola
Máster en Tecnología Agroambiental para una Agricultura Sostenible

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DEPARTAMENTO DE PRODUCCIÓN AGRARIA

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Tribunal nombrado por el Magfco. Y Excmo. Sr. Rector de la Universidad
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Fdo.:

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LOS VOCALES

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A mis padres y a mi hermana

«Mucha gente pequeña, en lugares
pequeños, haciendo cosas pequeñas,
pueden cambiar el mundo».

Eduardo Galeano

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Abstract

There is a growing concern worldwide about pollinator decline, because it threatens both the maintenance of biodiversity conservation and the sustainable food production on a global scale. While it is universally agreed that the origin of pollinator loss is multifactorial, in intensive agricultural areas, the lack of flower resources and nesting opportunities, as well as the use of pesticides have been identified as one of the main causes. In this context, the goals of this thesis are to increase understanding of how changes in agricultural practices aiming at improving biodiversity could have an effect on pollinator-dependent crops, as well as to provide knowledge of the pesticide risk to pollinators in a realistic scenario.

Planting flower strips adjacent to crops is among the habitat management practices employed to offer alternative floral resources to pollinators beyond the flowering of the crop itself. This measure seeks to improve pollinator population in agro-ecosystems and consequently pollination services. One of the greatest challenges of the design of flower-rich areas is the selection of the plants for the mix, with attractive potential for pollinator groups in the region and with hardly interspecific competition between them. In addition more information is needed to understand whether flower margins may play the double role of contributing to enhance the presence of pollinators in the crop environment and their potential spillover of pollinators on nearby pollinated dependent crops such as melon (*Cucumis melo* L.). Over the course of two consecutive years, the suitability of a flower mixture of 10 herbaceous plants for pollinators was evaluated on a weekly basis in a randomized block design of 2 melon plots with or without 1m-wide flower strips. We measured the floral coverage and pollinator visits to the plant species, as well as visits, yield, and quality of the crop. We identified four herbaceous plant species suitable to provide resources to pollinators and to be included in a flower strip in Central

Spain based on their attractiveness, staggered blossom and coverage: *Coriandrum sativum* L., *Diplotaxis virgata* L., *Borago officinalis* L. and *Calendula officinalis* L. However, the plant composition must be carefully chosen, especially in the concurrence of blooming in the flower strip and melon crop. Flower strips can act either as pollinator competitor or facilitator to the crop depending on their floral coverage and/or the predominant species during the crop bloom period. We suggest that the concurrence of blooming of the rewarding plant *C. officinalis* with the melon crop should be avoided in our area, due to their attractive potential to the main pollinator taxa that visit the crop, thus preventing their displacement to the melon flowers. Moreover the selected mixture was tested during one year in a commercial melon field in order to know how far the flower strip could influence visitors in the crop. In the commercial plot, bee visitation rate in the melon flowers decreased with the distance to the flower strip. No influence due to the specific flower strip evaluated on crop productivity or quality was found either in the experimental plots or in the commercial farm.

Not only the agricultural intensification lower the availability of suitable habitats and food sources for pollinators, but agricultural habitats may also be further degraded due to the use of toxic pesticides to pollinators. Accordingly, for pesticides regulation, laboratory studies are required to assess the risk for pollinators, but nowadays the process presents some limitations. Traditionally, most laboratory studies on bee ecotoxicology test lethal and sublethal effects of single compounds following a short-term exposure. However, under field conditions, bees are often chronically exposed to a variety of chemicals, with potential synergistic effects. In addition, adult bees also can ingest important amounts of pollen. Therefore, to simulate pesticide oral exposure, the risk assessment should include exposure via pollen in combination with exposure via nectar. To overcome these limitations and to assess the impact of pesticides

under a more realistic field scenario, first of all it is essential to know to which field concentrations pollinators are exposed. Secondly, we must know which are the most frequent pesticide combinations. In our work we have identified the pesticide residues found in pollen and nectar of commercial melon fields in Central Spain, and we have chosen the most probable combinations to perform chronic oral tests via pollen and nectar. Foraging bees in selected melon fields, had been exposed to a high number of pesticides (nine insecticides, nine fungicides and one herbicide). The highest pesticide residue concentrations (above 25 ppb) in melon flowers corresponded to pesticides sprayed by farmers. However, residues of eleven agrochemicals, not used by farmers during the current crop cycle were also detected in the samples showing that melon agro-ecosystem can be a pesticide contaminated environment. Pesticide concentrations in melon flowers were one or two orders of magnitude higher in pollen than in nectar. Three insecticides (acetamiprid, imidacloprid, chlorpyrifos) and one fungicide (myclobutanil) were detected in nectar. The most probable pesticide co-occurrence (>60%) in the study area was between four insecticides (acetamiprid, oxamyl, imidacloprid and chlorpyrifos) and two fungicides (metalaxil-m and azoxystrobin). Based on these results, we selected one of the most frequent pesticide mixtures in our field-realistic scenario (acetamiprid, chlorpyrifos, oxamyl) to assess the impact on *Bombus terrestris* L. micro-colonies through a chronic oral toxicity test. We found neither an effect in the pollinator parameters measured (mortality, pollen and syrup collected and reproduction fitness), nor a synergy among compounds at concentration tested.

Moreover, we studied the effects of three of the detected pesticides in the commercial melon fields (acetamiprid, imidacloprid and myclobutanil) on the solitary bee *Osmia bicornis* L. We orally exposed females of this species throughout their life span via pollen and sugar syrup. We measured pollen and syrup consumption, longevity, ovary maturation and thermogenesis. At the

tested concentrations, no synergistic effects emerged, and we found no effects on longevity and ovary maturation. However, all treatments containing imidacloprid resulted in suppressed syrup consumption and drastic decreases in thoracic temperature and bee activity. Our results have important implications for pesticide regulation. If we had measured only lethal effects we would have wrongly concluded that the pesticide combinations containing imidacloprid were safe to *O. bicornis*. The incorporation of tests specifically intended to detect sublethal effects in bee risk assessment schemes should be an urgent priority. Moreover, pesticide intake was three orders of magnitude higher via syrup than pollen. It is reasonable to consider that the contamination via pollen may not have had a significantly influence on the results of the chronic oral toxicity tests with adults of this solitary bee at field realistic concentration.

In addition, we have used bumblebee (*B. terrestris*) micro-colonies in order to evaluate the influence of imidacloprid exposure at field-realistic concentrations through the double route of contamination (nectar and pollen), and to assess whether contamination via pollen shows the same results as those found with *O. bicornis* adults. The micro-colony studies allowed us to measure effects on adults, on brood development and on offspring (males). Pollen is the essential protein source to larvae. Therefore we hypothesized that the double route of contamination could have an influence on the development of the micro-colony, especially on the brood production and male progeny. We found no effects on workers survival. However micro-colonies exposed to imidacloprid collected less syrup and pollen, produced less brood, and consequently less males were emerged. In agreement with the *O. bicornis* study, these effects mainly come from contaminated nectar treatments, because no differences were found between micro-colonies exposed to imidacloprid via nectar and pollen (N+P),

and those exposed just via nectar (N), neither between the control and the micro-colonies exposed to imidicloprid via pollen (P).

Resumen

Existe una creciente preocupación en todo el mundo sobre el declive de los polinizadores, siendo una amenaza tanto para el mantenimiento y la conservación de la biodiversidad como la producción de alimentos a escala mundial. Si bien el origen de las pérdidas de polinizadores está universalmente aceptado que es multifactorial, en áreas de agricultura intensiva, la falta de recursos florales y oportunidades de anidación, así como el uso de pesticidas, se han identificado como una de las principales causas. En este contexto, los objetivos de esta tesis se centran en aumentar el conocimiento de cómo los cambios en las prácticas agrícolas destinadas a mejorar la biodiversidad podrían tener un efecto en los cultivos que dependen de los polinizadores, así como evaluar el riesgo de pesticidas sobre estos insectos beneficiosos en un escenario realista.

La implementación de márgenes florales adyacentes a los cultivos es una de las prácticas de manejo del hábitat que se está fomentando para ofrecer fuentes alternativas de polen y néctar a los polinizadores más allá de la floración del propio cultivo. Esta medida busca mejorar las poblaciones de polinizadores en agro-ecosistemas y, en consecuencia, los servicios de la polinización. Uno de los mayores desafíos en el diseño de estas áreas ricas en flores es la selección de las plantas que formarán parte de la mezcla floral. Estas especies deberán contar con un adecuado potencial atractivo para los grupos de polinizadores de la zona y con una baja competencia interespecífica entre ellas. Además, es necesaria más información para comprender si los márgenes florales pueden desempeñar el doble papel de contribuir a mejorar la presencia de polinizadores en el entorno agrario y su potencial para desplazar los polinizadores hacia los cultivos cercanos con polinización entomófila como el melón (*Cucumis melo* L.). Durante dos años consecutivos, se evaluó la idoneidad de una mezcla de flores de 10 plantas herbáceas para polinizadores en un

diseño de bloques al azar con 2 parcelas de melón con o sin márgenes florales de 1 m de ancho. Medimos la cobertura floral y las visitas de polinizadores en las diferentes especies de plantas, así como las visitas, el rendimiento y la calidad en el cultivo. Identificamos cuatro especies de plantas herbáceas adecuadas para proporcionar recursos a los polinizadores, y con potencial para ser incluidas en márgenes florales en el centro de España, en función de su poder atractivo, floración escalonada y cobertura floral: *Coriandrum sativum* L., *Diplotaxis virgata* L., *Borago officinalis* L. y *Calendula officinalis* L. Sin embargo, la composición de las plantas debe elegirse cuidadosamente, especialmente cuando la floración de los márgenes florales y el cultivo de melón coinciden. Los márgenes florales pueden actuar como competidores o como facilitadores de polinizadores al cultivo, dependiendo de su cobertura floral y/o de las especies predominantes durante el período de floración del melón. En base a nuestros resultados, se recomienda evitar la concurrencia de la floración de *C. officinalis*, planta que ofrece gran cantidad de polen y néctar, con el cultivo de melón en nuestra área. Su potencial atractivo para los principales taxones de polinizadores que visitan el cultivo podría evitar su desplazamiento hacia las flores de melón. Además, la mezcla seleccionada se probó durante un año en una finca comercial de melón con el objetivo de conocer el alcance de influencia del margen floral en las visitas en el cultivo. En la parcela comercial, la tasa de visitas de polinizadores en las flores de melón disminuyó con la distancia al margen floral. No se encontró ninguna influencia de los márgenes florales en la productividad o calidad del cultivo, ni en las parcelas experimentales ni en la finca comercial.

No sólo la intensificación agrícola reduce la disponibilidad de hábitats y fuentes de alimentos adecuados para los polinizadores, sino que los hábitats agrícolas también pueden degradarse aún más debido al uso de pesticidas tóxicos para los polinizadores. En consecuencia, para la regulación de pesticidas, se

requieren estudios de laboratorio que evalúen su riesgo en los polinizadores, pero hoy en día el proceso presenta algunas limitaciones. Tradicionalmente, la mayoría de los estudios de laboratorio sobre ecotoxicología de abejas prueban los efectos letales y subletales de compuestos individuales después de una exposición a corto plazo. Sin embargo, en condiciones de campo, las abejas a menudo están crónicamente expuestas a una variedad de productos químicos, con posibles efectos sinérgicos. Además, las abejas adultas, también pueden ingerir cantidades importantes de polen. Por lo tanto, para simular la exposición oral de pesticidas, en las evaluaciones del riesgo se debería incluir la exposición a través del polen en combinación con la exposición a través del néctar. Para superar estas limitaciones y evaluar el impacto de los pesticidas en un escenario de campo más realista, en primer lugar es esencial saber a qué concentraciones de campo están expuestos los polinizadores. En segundo lugar, debemos conocer cuáles son las combinaciones de pesticidas más frecuentes. En nuestro trabajo hemos identificado los residuos de pesticidas encontrados en el polen y el néctar de campos comerciales de melón en el centro de España. Hemos elegido las combinaciones más probables para realizar pruebas de exposición crónicas vía oral (polen y néctar). Las abejas que se alimentaron en los campos seleccionados de melón estuvieron expuestas a una gran cantidad de pesticidas (nueve insecticidas, nueve fungicidas y un herbicida). Las concentraciones más altas de residuos de pesticidas (superiores a 25 ppb) en las flores de melón eran de pesticidas aplicados por los agricultores. Sin embargo, en las muestras también se detectaron residuos de once agroquímicos no utilizados durante el ciclo de cultivo, lo que revela que el agro-ecosistema de melón puede ser un ambiente contaminado con pesticidas. Las concentraciones de pesticidas en las flores de melón fueron uno o dos órdenes de magnitud más altas en polen que en néctar. Se detectaron tres insecticidas (acetamiprid, imidacloprid, clorpirifos) y un fungicida (miclobutanil) en el néctar. La concurrencia más probable de pesticidas (>60%) en el área de estudio fue entre

cuatro insecticidas (acetamiprid, oxamil, imidacloprid y clorpirifos) y dos fungicidas (metalaxil-m y azoxistrobina). Basándonos en estos resultados, seleccionamos una de las mezclas de pesticidas más frecuentes en nuestro escenario realista (acetamiprid, clorpirifos, oxamil) para evaluar el impacto en las micro-colmenas de *Bombus terrestris* L. a través de una prueba de toxicidad oral crónica. No encontramos ningún efecto en los parámetros medidos (mortalidad, polen y néctar recolectado y capacidad reproductiva), ni tampoco efectos sinérgicos entre los compuestos.

Además, estudiamos los efectos de tres de los plaguicidas detectados en los campos comerciales de melón (acetamiprid, imidacloprid y miclobutanil) en la abeja solitaria *Osmia bicornis* L. Las hembras fueron expuestas oralmente a los agroquímicos a lo largo de su vida a través del polen y del néctar. Medimos el consumo de polen y néctar, la longevidad, la maduración de los ovarios y la termogénesis. A las concentraciones probadas, no surgieron efectos sinérgicos, y no encontramos efectos sobre la longevidad y la maduración de los ovarios. Sin embargo, en todos los tratamientos que contenían imidacloprid se redujo el consumo néctar y hubo una disminución drástica de la temperatura torácica y la actividad de las abejas. Nuestros resultados tienen implicaciones importantes para la regulación de pesticidas. Si hubiéramos medido sólo los efectos letales, habríamos concluido erróneamente que las combinaciones de pesticidas que contenían imidacloprid eran seguras para *O. bicornis*. La incorporación de pruebas específicamente destinadas a detectar efectos subletales en los esquemas de evaluación de riesgo de abejas debería ser una prioridad urgente. Además, la ingesta de pesticidas fue tres órdenes de magnitud mayor a través del sirope que del polen. Es razonable considerar que la contaminación vía polen puede no tener una gran influencia en los resultados de las pruebas de toxicidad oral crónica con adultos de esta abeja solitaria a concentraciones realistas.

Asimismo, hemos utilizado micro-colmenas de *B. terrestris* con el objetivo de evaluar la influencia de la exposición a concentraciones realistas de campo de imidacloprid a través de la doble ruta de contaminación (néctar y polen) y evaluar si la contaminación a través del polen revelaba los mismos resultados que aquellos encontrados con adultos de *O. bicornis*. Los ensayos con micro-colmenas permiten medir los tanto efectos en adultos como en el desarrollo de la cría y en la descendencia (machos). El polen es la fuente esencial de proteínas para las larvas. Por lo tanto, planteamos la hipótesis de que la doble ruta de contaminación podría influir mayoritariamente en el desarrollo de la micro-colmena, especialmente en la producción de cría y la progenie masculina. No encontramos efectos sobre la supervivencia de los adultos. Sin embargo, las micro-colmenas expuestas al imidacloprid recolectaron menos néctar y polen, produjeron menos cría y, en consecuencia, la emergencia de los machos fue menor. Tal y como se observó en los ensayos con *O. bicornis*, el efecto procedió principalmente de los tratamientos de néctar contaminado, porque no se encontraron diferencias entre las micro-colmenas expuestas al imidacloprid a través del néctar y el polen (N+P) y aquellas expuestas solo a través del néctar (N); tampoco entre el control y las micro-colmenas expuestas al imidacloprid vía polen (P).

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CHAPTER I: GENERAL INTRODUCTION

1.1 Pollination and pollinator importance in agro-ecosystems.

Pollination is the transfer of pollen from the male anther to the female stigma of flowers to enable fertilization and reproduction, making possible the production of seeds and fruits. The majority of plants have evolved by developing mechanisms that prevent self-fertilization allowing the maintenance of genetic variability, so the role of pollinators to transport pollen from one plant to another is essential. Pollen can be biotic-borne by birds, insects, bats, etc. or abiotic-borne by water or wind. Insects are the main pollination vectors (Garibaldi et al., 2013; Rader et al., 2016) and feed on floral resources (i.e. pollen and nectar) as exchange for their pollination services.

Pollination is necessary for the maintenance of natural areas and agro-ecosystems (Ashman et al., 2004; Garibaldi et al., 2011; Klein et al., 2007). Approximately 87% of flowering plants world-wide (Ollerton et al., 2011) and 75 % of the crops rely on pollinators (Klein et al., 2007). These pollinator-dependent crops include horticultural crops (e.g. melon, tomato, watermelon, cucumber, etc.), fruit crops (e.g. almond, apple, pear, blueberry, mango, etc.), forage legumes (alfalfa, clover), oil seed plants (rapeseed or sunflower) or textile fibres (linen or cotton). All of them need pollinators to increase crop yield as well as to improve fruit quality (Garratt et al., 2014; Klatt et al., 2014). In terms of global food production, pollinator-dependent crops account for 35%. Other crops important in our diet, are however self-pollinated, pollinated by wind (e.g. cereals) or are parthenocarpic (Klein et al., 2007). However, the value (per ton) of pollinator-dependent crops is five times higher than the value of non-dependent crops. Consequently, recent estimates suggest that crop pollination by insects supposes \$361 billion worldwide (Lautenbach et al., 2012). Nevertheless, there is a reasonable doubt about the precision, reliability,

and value of pollinators concerning their role in maintaining the structure and functioning of natural ecosystems, and everything indicates that the real value has never been calculated.

1.2 Pollinator biodiversity.

Many crops may receive some benefit from insect pollination and therefore, farmers worldwide traditionally encourage the introduction of *Apis mellifera* L. hives in their crops. Apart from the honey bee, other managed bees are commercially available around the world [e.g. *Bombus* spp., *Osmia* spp., *Megachile rotundata* F., *Nosmia melendri* Cockerell; (Sgolastra et al., 2019)]. Managed bumblebees are most commonly used in enclosed production systems (greenhouses and tunnels), but the other managed species are predominantly used for field and orchard crops (Figure 1).

Figure 1: From left to right: *Apis mellifera* hives near to a melon crop, *Osmia bicornis* nest in a pear orchard, and *Bombus terrestris* hives in a tomato greenhouse.

Wild pollinators can visit crops with a high frequency and their role in crop pollination can be even more efficient than that of managed bees (Garibaldi et al., 2013). Bees (Hymenoptera, Apoidea) are a large group of insects with more than 20,000 species worldwide (Michener, 2007), and are considered the most important pollinators in the majority of ecosystems, because both adults and larvae feed on flower resources. In Spain the number of species cited so far

surpasses 1,100 (Ortiz-Sánchez, 2011), embracing six families: Apidae, Megachilidae, Andrenidae, Halictidae, Melittidae and Colletidae.

Bee species can have very different life histories. Around 75% of the total are solitary species; 10% are social with various degrees of social complexity, including the most known and recognized species such as honey bees, bumblebees and stingless bees (Apidae); and the remaining bees are cleptoparasitic (Danforth et al., 2013). In solitary bee species, each female builds and supplies her nest but has no contact with the offspring. The social bees however, live in colonies with one reproductive female and a number of non-reproductive workers. Social species are included in two families, the Halictidae and the Apidae. The genera *Halictus* spp. and *Lasioglossum* spp. (Halictidae), include both solitary and social species (Danforth et al., 2013). The cleptoparasitic species lay their eggs in the nest of other bee species and feed on their hosts' provisions. In Europe, three families of bees include some genera with cleptoparasitic species: Halictidae, Megachilidae and Apidae (Ortiz-Sánchez et al., 2018). Regarding nesting behaviour, approximately half of the bee species dig their nests in the ground (Michener, 2007). Among the non-excavating species, there is a great diversity of nesting behaviours: some open their galleries in plants, while others can build their nests with mud or resins. Other species nest in a variety of pre-existing natural cavities in rock fissures, created by other organisms (e.g. abandoned nests). And there are also other species that nest in empty shells or in accumulations of dry plants on the soil surface (Ortiz-Sánchez et al., 2018).

However, even though entomophilic pollination is preferentially associated with bees, other insect groups considered as "secondary" [coleoptera, lepidoptera, diptera and hymenoptera (wasps and ants)], also can play a fundamental role in the pollination of some crops (Stefanescu et al., 2018).

Maintaining the diversity of pollinators in agro-ecosystems is essential, since the increase in abundance and richness of species can improve the pollination services (Winfree, 2013). In several crops where large numbers of managed bees are present, the pollination contribution of wild bees is additive to that of managed bees (Carvalho et al., 2010) [e.g. pumpkin (Hoehn et al., 2008); almond (Brittain et al., 2013); coffee (Klein et al., 2003); apple (Mallinger and Gratton, 2015)]. For example, in sunflowers, wild bees facilitate the movement of honey bees across rows of male and female plants (Winfree and Kremen, 2009). Besides, a diverse guild of bees can exhibit temporal complementarity, because both the emergence dates and foraging periods can vary even within the day (Albrecht et al., 2012). Furthermore, pollinator biodiversity can mitigate the vulnerability of crop pollination against climate change or human-induced disturbances (Bartomeus et al., 2013). For all these reasons, the conservation and creation of diverse assemblages of wild insects is considered a key measure in agro-ecosystems.

1.3 Current status and bee decline drivers.

There is a growing concern about the pressure that both managed and wild pollinators suffer in modern world and the evidence of the pollinator decline has been thoroughly reviewed (Potts et al., 2010). The number of managed honey bee colonies have decreased in Europe (25% between 1985-2005; Potts et al., 2010) and North America (59% between 1947-2005; VanEngelsdorp et al., 2008). Although globally stocks have actually increased by ~45% between 1961 and 2008, it is only due to a major increase in numbers of hives in countries such as China and Argentina (Aizen and Harder, 2009). But there are many reports of unusually high rates of honey bee colony losses from many parts of the world, sometimes attributed to the *colony collapse disorder syndrome* (CCD) (VanEngelsdorp et al., 2009). Severe declines have been also reported in bumblebees in Europe (Goulson et al., 2008), North America (Gixti et al., 2009),

South Africa (Schmid-Hempel et al., 2014) or China (Williams et al., 2009). Data for wild bees is scarce but historic records also suggest declines in the United Kingdom, The Netherlands and Belgium (Biesmeijer et al., 2006; Carvalheiro et al., 2013) or Illinois, U.S. (Burkle et al., 2013). And the problem of bee decline is even worse since in parallel, the area of crops that needs pollination is increasing (Aizen et al., 2008).

Pollinator populations are limited by three main direct factors: the abundance of floral resources, the availability of nesting environments, and the incidental risk due to pathogens, predators and pesticides. On the other hand, several indirect factors can also affect bee populations: land management, landscape context, disturbance and invasive species (Roulston and Goodell, 2011) (Figure 2). In addition, climate change is one of the largest threats to the future. Temperature changes can affect the distribution of pollinator species and also the phenology of plants (Willmer, 2012).

Thereby, habitat loss, excessive use of pesticides, incidence of parasites and their combined effects often explain the decline of pollinator communities (Goulson et al., 2015). In this thesis we have focused mainly on the two first drivers of decline, because in intensive farming areas, scarcity of floral resources and nesting habitats and a high presence of pesticides have often been identified as the main causes of bee decline (Botías and Sánchez-Bayo, 2018; Goulson et al., 2015).

Figure 2: Direct and indirect effects in bee populations. Bee populations are driven by direct effects and modulated by species traits. Indirect effects act by their influence on underlying direct factors. Source: Roulston and Goodell, 2011.

1.3.1 *Habitat loss.*

Through agricultural intensification we have transformed complex natural ecosystems into simplified agro-ecosystems and this has caused a loss of favourable habitats for pollinators. Several studies have shown that the availability of natural or semi-natural habitats significantly increases pollinator populations (Holzschuh et al., 2006; Kremen et al., 2004; Le Féon et al., 2010) because there is a positive relationship between plant diversity and insect diversity (Winfree et al., 2009). Accordingly, heterogeneous habitats are important for the conservation of pollinator insects since there is greater availability of floral resources (nectar and pollen).

Floral resources are essential for bees since they feed on them throughout their life cycle. Adult bees consume nectar and small amounts of pollen (Cane, 2016) during the flight season and females collect large amounts of both resources to feed the larvae. Nectar provides energy and the pollen is the protein source.

Intensive farming areas include few wildflowers and therefore floral resources are limited to those offered by pollinator-dependent crops. Many of these crops can offer mass flowering (e.g. oilseed rape), and pollinator density in the landscape context are positively related to the availability of highly rewarding mass flowering crops (Westphal et al., 2003). However every crop provides spatially and temporally isolated flower resources and this can be a problem for many pollinator species when their flight periods do not coincide with the crop blossom or when they have periods of activity beyond the crop blossom (e.g. some social and multivoltin species). Furthermore, pollinators require pollen from a set of plant species (Tasei and Aupinel, 2008a). The diet of bees that inhabit intensive farmland areas is very little diverse and some essential nutrients may not be present (Roulston and Cane, 2000). This inadequate nutrition has raised concern about the detrimental effect on pollinator immune system (Alaux et al., 2010). In addition many bees also collect other plant resources for build their nests: leaves, resin, floral oil, plant sap, pith or dead wood, and they are likely to correlate with local vegetation structure and thus with floral resources as well (Roulston and Goodell, 2011). Most bees are ground nesters, thus soil conditions (e.g. compactness) and/or human activities (e.g. tilling) also can limit their populations. As compared with floral resources, much less is known about how wild bees might be limited by nesting resources (Roulston and Goodell, 2011).

Therefore, in order to maintain pollinator diversity and abundance in crop surroundings, there is a need to ensure a sustainable restoration of pollinator habitats within agro-ecosystems.

1.3.2 *Pesticides.*

The intensification of agriculture has involved worldwide a reliance on insecticides, fungicides and herbicides for the control of crop pests, diseases and

weeds. In the European Union (EU), there are around 500 active substances approved for their use as pesticides (EC, 2019). But pesticides are considered one of the most involved factors in the reduction of pollinator diversity and in the phenomenon of bee decline (Goulson et al., 2015), due to the lethal and sublethal toxic effects that they may cause (Williams and Osborne, 2009; Goulson et al., 2015). Because of that nowadays there is a growing concern about impacts of pesticides on non-target organisms (natural enemies and bees) and pest control is done under Integrated Pest Management (IPM), an holistic approach, ecologically based, which can be applied to any ecosystem (Alston, 2011). IPM is mandatory in the EU since it came into force the directive 2009/128/CE on the *Sustainable use of pesticides* (Doue, 2009) transposed in Spain to the RD 1311/2012 *Framework for a sustainable use of phytosanitary products* (BOE, 2012).

In agro-ecosystems pollinators can be exposed to pesticides through various routes after their application. They can enter in direct contact with droplets when pesticides are sprayed on crops (Thompson, 2001) or with the dust particles released during the sowing of seeds dressing with systemic insecticides (Nuyttens et al., 2013). In addition pollinators can also be contaminated by contact with treated plant surfaces while moving around in the search of floral resources (Krupke et al., 2012). Furthermore, pesticides can affect them via oral through the consumption of nectar and/or pollen contaminated from crops (Bonmatin et al., 2005; Dively and Kamel, 2012; Stoner and Eitzer, 2012) and from wild plants growing near agricultural areas (Botías et al., 2015; David et al., 2016; Tsvetkov et al., 2017). Guttation, the loss of drops of xylem sap with water and dissolved materials on the tips or edges of injured leaves, a common phenomenon in higher plants, can also contaminate pollinators (Shawki et al., 2018). Besides, feeding on honeydew excretions by phloem-feeding hemipteran insects such as aphids, mealybugs, whiteflies, and

psyllids can also impair pollinators (Calvo-Agudo et al., 2019). In addition, pesticide residues in soil are another important route of exposure in ground nesting species and bees that use mud to build their nests (e.g. *Osmia* spp.) (Anderson and Harmon-Threatt, 2019; Sgolastra et al., 2019). Also pesticides residues in other nesting materials from plant matrices such as leaves, resins, floral oils etc, need to be considered as potential contaminants (Sgolastra et al., 2019).

Great attention has been devoted to the direct toxic effects of pesticides on bees. Particularly the impact of the most commonly used insecticides, many of them neurotoxics: organophosphates, carbamates, pyrethroids, fipronil and neonicotinoids, has been addressed extensively. Toxicity is assessed with an acute oral or topical test in laboratory following a short-term exposure period (24 or 48 hours), and expressed by the lethal dose 50 (LD₅₀), which represents the dose of the substance that causes the death in 50% of the individuals. Systemic insecticides, such as neonicotinoids and fipronil, are generally more toxic to honey bees than other groups of insecticides (Simon-Delso et al., 2015). They have nanograms oral LD₅₀'s (ng/bee) [clothianidin: 3.5; imidacloprid: 13; thiametoxan: 5; fipronil: 1 (Sanchez-Bayo and Goka, 2014)] and accordingly they have been classified as highly toxic to bees (USEPA, 2014). For this reason, in 2018 the EU decided to extend the previous ban (EC, 2013) of the three most widely used neonicotinoids (imidacloprid, thiamethoxam and clothianidin) to all outdoors applications (EC, 2018a, 2018b, 2018c). Neonicotinoids target the insect central nervous system and activate the nicotinic acetylcholine receptors (nAChR) causing over stimulation, paralysis and death. The risk of these pesticides does not only lie in their high toxicity but also in their long persistence in the environment (Goulson, 2013), and the bee exposure to sub-lethal doses for long periods of time may result in lethal effects due to cumulative toxicity (Rondeau et al., 2014). Sublethal effects of neonicotinoid

exposure have also been observed in bees, such as impair in foraging behaviour (Gill and Raine, 2014; Schneider et al., 2012; Yang et al., 2008); homing (Bortolotti et al., 2003; Henry et al., 2012), locomotion (Tosi and Nieh, 2017; Williamson et al., 2014), navigation (Fischer et al., 2014), colony health (Tsvetkov et al., 2017), pollinator capacity (Stanley et al., 2015), reproduction (Laycock et al., 2012) and consumption inhibition (Thompson et al., 2015; Zhu et al., 2017). All these behavioural alterations could have negative consequences on the long-term survival and conservation of pollinators in agro-ecosystems.

Bees are not only exposed to neonicotinoids, but also to a wide variety of pesticides, as shown by the large number of different compounds frequently found in bee matrices (Mullin et al., 2010; Porrini et al., 2016). Since two or more compounds are often present in honey bee hives and in agricultural environments, multi-pesticide exposure in bees is a frequent scenario (Botías et al., 2017; Tosi et al., 2018). The researches carried out so far have mainly focused on the study of the effects of single compounds, probably underestimating the full risk of pesticides on bees. In fact, exposure to multi compounds may result in additive, and what it is more important, in synergistic toxic effects (Gill et al., 2012; Iwasa et al., 2004; Sgolastra et al., 2018a, 2017; Zhu et al., 2017). Several studies have shown that some fungicides (e.g. triazole) can increase the toxicity of insecticides to social and solitary bees (Gill et al., 2012; Iwasa et al., 2004; Sgolastra et al., 2018a, 2018b, 2017; Zhu et al., 2017) by reducing their detoxification capacity. In addition to synergies with insecticides, fungicides may have an indirect impact, since they can alter the microflora present in the pollen and nectar of the treated plants (Bartlewicz et al., 2016; Yoder et al., 2013), which in turn could have important consequences for their nutrition and health (Engel et al., 2016; Mattila et al., 2012). Besides, some pesticides can also alter the insect microbiota, which has a key role on the insect tolerance to them, as shown in the aphelinid parasitoid *Eretmocerus mundus* Mercet (Fernández et

al., 2019). The use of herbicides can also indirectly affect pollinators because they eliminate numerous wild plants and reduce floral diversity in agricultural areas (Bohnenblust et al., 2016), and as mentioned before, they constitute a fundamental food resource in agro-ecosystems.

Finally, it is important to note that the impacts of pesticides are different among bee species for two principal reasons. First of all, solitary and social bees have different life history traits and accordingly they may have different routes and levels of exposure to soil residues and plant residues matrices, important resources in solitary bee nests (Anderson and Harmon-Threatt, 2019; Sgolastra et al., 2019). Moreover, in solitary bees, the death of a nesting female has as a result a complete cessation of its reproductive output, whereas in social bees, the deaths of non reproductive individuals can be buffered by the survival of other colony members and the production of new members (i.e., super organism resilience) (Straub et al., 2015). Secondly, the available evidence points out towards a different sensitivity to pesticides among bee species (Arena and Sgolastra, 2014). In general, solitary bees (e.g. *Osmia* spp.) have higher vulnerability than honey bees and bumblebees (Rundlöf et al., 2015; Sgolastra et al., 2018b; Woodcock et al., 2017).

1.4 Opportunities and conservation measures.

There is universal agreement that we must ensure adequate pollinator conditions in agro-ecosystems in order to boost their populations, which in turn will result in a crop amelioration and increased yield in some of them. Within agricultural areas, the increase on the availability of floral resources and nesting sites, as well as a reduction of the impact of some agricultural practices (e.g. the pesticides), are essential measures for the conservation of pollinators.

1.4.1 *Increase on the availability of floral resources and nesting sites.*

Actions to improve and restore biodiversity and environmental quality are being introduced from the European Union (EU) (EC, 2017). The Common Agricultural Policy is being designed to build a more competitive and sustainable agriculture, where a high percentage of direct payments will go to those farmers who provide environmental services (EC, 2018d). The EU agri-environment schemes have encouraged farmers to dedicate 5% of arable land to ecologically beneficial elements with the creation of flower-rich habitats adjacent to the crops (Figure 3). Flower margins or strips interspersed with crops (Wratten et al., 2012), can boost the presence of pollinators in commercial farms. Specially in intensive agricultural areas (Morrison et al., 2017) flowering plants can provide continuous resources to pollinators beyond the crop bloom interval, covering the flight period of many pollinator species (Garibaldi et al., 2014) and satisfying the wild pollinator needs. Therefore, they can improve wild bee reproduction rates (Carvell et al., 2015), and contribute to enhancing their abundance (Jönsson et al., 2015), species richness (Scheper et al., 2015) and population persistence (M'Gonigle et al., 2015).

Figure 3: Flower strips along a field margin of melon crop to provide benefits for pollinator populations.

Many efforts have been made to select the plant species with attractive potential for beneficial insects (Ambrosino et al., 2006; Barbir et al., 2016, 2015; Colley and Luna, 2000; Hogg et al., 2011; Pontin et al., 2006) and even computational tools

are being applied to select an optimal mix (M'Gonigle et al., 2017). Depending on the flowering plants selected, the pollinator attraction capacity varies just like the richness and abundance of the different pollinator species that visit the area (Barbir et al., 2016, 2015; Carreck and Williams, 2002; Carrié et al., 2012; Hogg et al., 2011). Species belonging to families Boraginaceae, Brassicaceae, Apiaceae, Polygonaceae and Hydrophyllaceae have been described as attractive to beneficial insects (Barbir, 2016; Pontin et al., 2006). The candidate flowering plants should exhibit different phenology and morphology (Balzan et al., 2014) in order to attract many and diverse pollinators, because the access to nectar and pollen resources depends, among other factors, on the pollinator flight period, and tongue length and body size, respectively (Campbell et al., 2012; Fontaine et al., 2006). Accordingly, some plants can be widely attractive to cosmopolitan pollinators, while other plants have flowers that are more attractive to specific groups of pollinators (e.g. open flowers are attractive to hoverflies and plants from Boraginaceae family are very attractive to bumblebees). Therefore, it is crucial to have a deep basic knowledge of the abundance and diversity of pollinator groups in the region before choosing the plant species that will contribute to an efficient management of pollinator habitats within the landscapes. In addition, other ecological and physiological characteristics of plants should be considered before being introduced into agro-ecosystems. First of all, plant species must be preferably native or naturalized to ensure a good adaptation to edaphic and climatic conditions, to have a good interaction with the local pollinators and a lesser risk of becoming a weed and compete with the crop (Isaacs et al., 2009; Wratten et al., 2012). Secondly, they do not have to be a pest or pathogen reservoir. And thirdly, they should have an easy management and be commercially available. Moreover, competition for the space among species in a mixed flower strip should also be minimal because the floral coverage of a very attractive plant can be lesser compared to that reached in a mono specific strip and pollinator visits may

decrease consequently (Barbir et al., 2015). However, mixed flower strip can support a greater pollinator diversity (Amy et al., 2018).

Although it is well known that flower strips support higher insect abundance and diversity than crops (Haaland et al., 2011; Kohler et al., 2008) their potential spillover of pollinators on nearby insect-pollinated crops is less recognized. The effect of plant species in the flower strip, which bloom simultaneously with the crop, is controversial. Whilst some flowering plants can compete for pollinators, many others do not have negative effect on the neighboring plants and may even play a positive role in facilitation (e.g. when the presence of one plant in bloom increases the presence of pollinators in a nearby plant) (Carvalheiro et al., 2016). Therefore, the flower plant strips can have a dual role: they can act as exporters of pollinators to adjacent crops thus enhancing the abundance of crop flower visitors (Carvalheiro et al., 2012; Morandin and Kremen, 2013) or they can act as concentrators of pollinators when they offer enough resources, and thus they do not have a positive effect on the crop (Kohler et al., 2008). Some studies show that pollination services can be increased in nearby crops [e.g. mango (Carvalheiro et al., 2012); tomato (Balzan et al., 2016) or blueberry (Blaauw and Isaacs, 2014)] but some others show a lack of effect [e.g. cider apple orchards (Campbell et al., 2017), cucumber (Quinn et al., 2017); summer vegetable crops, (Winfrey et al., 2008)] or even contradictory results in the same crop (strawberries) depending on the scale [positive at small scale (Ganser et al., 2018); negative in commercial plantations (Hodgkiss et al., 2019)].

Therefore research is needed to examine the contribution of flower mixtures to pollinator visits and services in nearby pollinator-dependent crops, and their influence at a regional level (**Experiment 1**).

1.4.2 *Measures to mitigate the impact of pesticides: filling gaps in risk assessment schemes.*

The use of pesticides has been indicated as one of the main causes of bee decline in agro-ecosystems. Therefore, the reduction of their use seems to be a necessary measure to prevent greater negative consequences (Garibaldi et al. 2014), especially if a decrease in pesticide use hardly reduces the crop productivity (Lechenet et al., 2017). Nowadays, in the EU pest control is undertaken with IPM, an holistic approach ecologically based, that integrates multiple pest management practices (e.g. physical, mechanical, biological, cultural, chemical, etc.) to reduce the status of pests to tolerable levels while maintaining a quality environment, once the economic thresholds have been surpassed. Tactics are sequentially applied, firstly all the non-chemicals and secondly, the pesticides, choosing only those selective and environmentally sounded. Recently, pollinator's health has been proposed to be included in this paradigm (Biddinger and Rajotte, 2015). Therefore in this context, Integrated Pest and Pollinator Management (IPPM) praises the use of pesticides that are less toxic to beneficial insects (natural enemies and pollinators), if needed, and farmers are requested to only apply them in the late afternoon or with low temperatures, in order to avoid the coincidence with the bee activity periods. Also attention must be paid to reduce drift by doing the applications either localized or with calm wind, or to avoid coincide with the flowering pick of the crop.

1.4.2.1 *Limitations of risk assessment.*

Current risk assessment schemes in the U.S. and Europe, have an important limitation: they test only effects of single pesticides (EPPO, 2010; USEPA, 2014), even though bees in agricultural areas are likely to be exposed to combinations of them (Botías et al., 2017; Tosi et al., 2018).

To overcome this problem and to assess the impact of pesticides under a more realistic field scenario, the first essential thing is to know to which field concentration levels pollinators are exposed and what is the probability of pesticide occurrence in the environment (Sanchez-Bayo and Goka, 2014).

One of the primary routes of bee exposure to pesticides is via consumption of contaminated pollen and nectar from flower crops (Bonmatin et al., 2005; Dively and Kamel, 2012; Stoner and Eitzer, 2012) and from wild plants growing near treated crops (Botías et al., 2015; Botías and Sánchez-Bayo, 2018; David et al., 2016; Tsvetkov et al., 2017). Most studies attempting to test field realistic concentrations in laboratory have used the residues detected in honey bee-collected pollen (beebread or corbicular pollen) as proxy for the field pesticide exposure (Laycock et al., 2012; Zhu et al., 2017). However, this approach underestimates the actual risk that pesticide residues might pose to bees for three reasons. First of all, pollen collected by honey bees for transportation to the hive is mixed with nectar and glandular secretions, and therefore, the pesticide residues are diluted. Secondly, bees exposed to lethal or sublethal concentrations might not be able to come back to the hive (Fischer et al., 2014; Henry et al., 2012; Stanley et al., 2016). Thirdly, honey bee-collected pollen from different hives is usually pooled before chemical analysis and come from both collect pollen from treated and untreated plants (Bonmatin et al., 2015; Rolke et al., 2016). Therefore, the analysed samples may have lower contamination than the real. Dilution of chemical residues in beebread defined as the pollen stored in comb cells in the hive, is even stronger due to phenomenon of pesticide degradation. Overall, as a result, the best estimation of the pesticide risk for pollinators is to analyse the residues found on the pollen directly collected from plants, because they are usually higher than those found in the pollen collected by the bees (Kyriakopoulou et al., 2017).

Currently, the information of pesticide residues in the flowers is limited to a few crops: maize (Bonmatin et al., 2005), pumpkin (Dively and Kamel, 2012; Stoner and Eitzer, 2012) or rapeseed (Botías et al., 2015; David et al., 2015). Most of these studies focused on few active ingredients or chemical groups (e.g. neonicotinoids) overlooking the multi-pesticide exposure. Testing all possible pesticide combinations seems unaffordable, however knowing the most likely combinations in real field can help selecting which pesticide combinations must be assessed in chronic exposure laboratory studies. Accordingly, the information of pesticide residues in real scenarios is essential for being including in pesticide risk assessment schemes in order to ensure the conservation of these important organisms (**Experiment 2**).

Secondly, once the most frequent pesticide mixture has been identified in a real scenario it is important to incorporate in the risk assessment scheme the multi-pesticide exposure. Focusing on single compounds may underestimate the risk of pesticide use on bees because the exposure to multiple compounds may result not only in additive but also in synergistic adverse effects (Gill et al., 2012; Iwasa et al., 2004; Sgolastra et al., 2018a, 2018b, 2017; Zhu et al., 2017).

Thirdly, most bee ecotoxicological studies assess lethal and/or sublethal effects following short-term (acute) exposure (Decourtye et al., 2005; Laurino et al., 2011). However, due to pesticide persistence in the environment, bees in field conditions are often exposed for long periods of time (chronic exposure) (Botías et al., 2015). Exposure to very low doses for long periods of time may result in lethal effects due to cumulative toxicity (Rondeau et al., 2014), and for that reason, the inclusion of chronic exposure test in the risk assessment scheme is also necessary.

Finally, to simulate oral exposure to pesticides, most studies expose bees to contaminated “nectar” (sugar-water solution laced with the desired amounts of pesticide). However, adult bees also ingest considerable amounts of pollen (Cane, 2016; Cane et al., 2017). Because pollen from flowers growing in agricultural areas has been shown to contain pesticide residues (Bonmatin et al., 2005; Botías et al., 2015; Dively and Kamel, 2012), exposure via pollen should be tested in combination with exposure via nectar.

Therefore, improvements are needed to incorporate these limitations in the testing schemes in order to properly assess the impact of pesticides under realistic scenarios (**Experiments 2 and 3**).

Moreover the current risk assessment scheme focuses on *A. mellifera*, but both the differences in the level of exposure and the response to pesticides may vary significantly between different bee taxa (Arena and Sgolastra, 2014). This differences highlight the need to include more bee species in the standard ecotoxicological data set required for the authorization of pesticides in order to achieve the same level of protection for honey bees and wild bees alike. In addition, many life-history traits influencing the vulnerability to pesticides suggest that bumblebees and solitary bees could be more susceptible than honey bees, in particular, when the application of a pesticide coincides with the nesting period in solitary bees or with colony establishment in bumblebees (Kopit and Pitts-Singer, 2018; Sgolastra et al., 2019). As such, the European Food Safety Authority recommends to include bumblebees and solitary bees in the risk assessment schemes of agrochemicals (EFSA, 2013). For that reason in this thesis we have evaluated the risk of pesticides in a real scenario, in the social bumblebee *Bombus terrestris* L. (**Experiments 2 and 4**) and in the solitary bee *Osmia bicornis* L. (**Experiment 3**).

However, there is a lack of consensus on whether field-realistic levels of exposure might have a significant impact on pollinators (Goulson, 2013). A long list of sublethal effects of neonicotinoid in bees has been reported (Bortolotti et al., 2003; Fischer et al., 2014; Gill and Raine, 2014; Henry et al., 2012; Schneider et al., 2012; Stanley et al., 2016; Tosi and Nieh, 2017; Williamson et al., 2014; Yang et al., 2008; Zhu et al., 2017). Others studies however, have reported no significant sublethal effects at field-realistic concentrations (Laycock et al., 2014; Schmuck et al., 2001; Tasei et al., 2001). In particular the chronic exposure of bumblebee microcolonies to imidacloprid, has given contradictory results. Tasei et al., (2000) showed a significant reduction in the production of larvae and males of 43 and 41% respectively, but in contrast, Mommaerts et al. (2010) did not find any reduction. The lack of similarity could be partly explained by differences in the methodologies of both studies. The last study, only exposed bees to imidacloprid via syrup contaminated, instead of testing the double route of exposure via syrup and pollen, more representative of field conditions. Recently, a study with adults of a solitary bee (*O. bicornis*) chronically exposed to field-realistic concentrations of pesticides, showed that effect of the active ingredient of imidacloprid via pollen was very low, due to the overall low amounts of pollen ingested by the bees (Azpiazu et al., 2019). Therefore, it is reasonable to consider that the contamination via pollen may not have a significantly influence on the results of chronic oral toxicity tests with adults of this solitary bee. In the bioassays with micro-colonies compared to individual adult exposure tests, social organization can be considered, which is a more reliable indicator of trends in larger queenright colonies (Tasei and Aupinel, 2008b). Moreover, the use of micro-colonies in the risk assessment studies with bumblebees can be justified by the low cost, the ease of use, and the possibility to work with several replicates in standardised conditions (Mommaerts and Smagghe, 2011). In the micro-colonies one worker becomes dominant and starts to lay haploid eggs after one week, hence it allows to measure effects of an

active compound on workers, brood development and offspring (males). Pollen is the essential protein source to larvae. Therefore the double route of contamination (syrup and pollen) may influence the development of the microcolony, especially the brood production and male progeny (**Experiment 4**).

1.5 Melon crop.

Melon (*Cucumis melo* L.) is an important pollinator-dependent summer crop from an economic perspective. Spain is the main European producer and accounts for 35% of the total European production (FAO, 2018). Melon production (17,950 ha outdoors and 2,523 ha protected crops mainly located in Central and South Spain; 655 thousand tons production) was valued at 217 million € in 2017, being the main cultivated varieties the toad skin, the cantaloupe and the smooth skin types (MAPA, 2018). In general, melon plants are andromonoecious, with both male staminate and hermaphrodite flowers producing pollen and nectar (Maroto, 1995). Melon requires bee pollination to improve fruit quality and quantity (Bomfim et al., 2016; Tschoeke et al., 2015) and in their absence, production reductions up to 90% have been recorded (Klein et al., 2007). Farmers usually introduce 2-5 *A. mellifera* hives/ha because production can be increased [e.g. by 40% in rockmelon in Australia; (Beeaware, 2016)]. This crop is also visited around the world by a wide variety of pollinators, mainly wild bee species (Klein et al., 2007; Rodrigo Gómez et al., 2016; Tschoeke et al., 2015; Winfree et al., 2008), being outstanding the Halictidae genus *Lasioglossum* in Central Spain (Rodrigo Gómez et al., 2016). Melon crops are frequently sprayed with insecticides to control mainly aphids and whiteflies and with fungicides during bloom to control powdery mildew and other fungal diseases (Duncan and Ewing, 2015; Khetereli et al., 2016).

1.6 Biology of the species tested in laboratory.

1.6.1 *Bombus terrestris*

The bumblebee species are a well-known pollinator of wild flowers (Goulson, 2010) and they have also become economically important due to their use for decades in the commercial pollination (Velthuis and Van Doorn, 2006). These species are an effective pollinators of several agricultural crops such as tomato (Ahmad et al., 2015; Morandin et al., 2001), pepper (Shipp et al., 1994) or blueberry (Javorek et al., 2006). Specially in tomato crops, bumblebees are excellent pollinators, in contrast to honey bees, because of their ability to buzz pollinate (Buchmann, 1983): they are capable of vibrating their body at a frequency of 400 Hz (Harder and Barclay, 1994) close to the flower's anther, causing the flower to release pollen. In addition, commercial bumblebees also improve fruit yields when they are implemented alongside wild pollinators in the field (Lye et al., 2011). However, the importation of commercial bumblebee colonies and their release into the environment have been questioned (Ings et al., 2006) because they can compete with the native species (Ings et al., 2006; Matsumura et al., 2004) or transfer pathogens (Colla et al., 2006) and parasites (Goka et al., 2006) into native populations. To reduce the risks of the impact of the commercial bee trade, mitigations strategies should be consider, including controls on international movement of bee, improved hygiene and parasite screening of colonies before and after shipping (Goulson and Hughes, 2015), as well as native species and subspecies should be locally reared (Ings et al., 2006).

Bombus terrestris is an eusocial bee belonging to the family Apidae (orden Hymenoptera). The majority of bumblebee species worldwide such as *B. terrestris*, have an annual life-cycle (Goulson, 2010; Figure 4). New queens are the individuals that survive during the winter in a state of diapausa. In the autumn, at the end of the cycle, the old queen and all of her workers die, and the new queens after being fertilized by males, wintering in underground sites.

The queen emerge from diapause in the spring when temperature is favourable and seeks out a suitable nest place, often in pre-existing holes of rodents or other species. The queen starts foraging and filling the nests with pots full of nectar and a pollen clumps into which she lays approximately ten eggs that progress into larvae, pupae and female worker bees. The pollen lump is covered on the outside with a layer of wax (secreted from the ventral abdominal surface of the queen) mixed with pollen. In this early stage, she also incubates the eggs and keeps the nest warm by her body temperature (Goulson, 2010). The larvae have four instars, when they increase in size, they develop in independent cells. After approximately 10–14 days of development they spin a strong silk cocoon and pupate. The pupae take a further 14 days approx. for emergence. The total development time is about 4–5 weeks. The new-emerged workers have white hairs and they develop their characteristic coloration after approximately 24 h. Once the new workers emerge, the queen ceases to forage, and workers acquire the majority of nectar and pollen resources, feed the larva and maintain the nest. The queen, the biggest member of the colony, is the responsible for continuing laying the eggs. There is evidence that ovarian development in young workers is prevented in presences of the queen to not allow the existence of new queen that compromise their leadership. From this point onward the size of the colony grows rapidly, the nest can increase in weight by 10-fold within 3-4 days. Colonies of *B. terrestris* can grow to contain up to 350 workers. When the colony cycle comes to its end, the production of new queens and drones is assessed. The queen loses her leadership and the *switch point* occurs in the nest. From this moment, the old queen and the workers enter in competition, and both start to lay unfertilized eggs (haploid), from which males emerge. The queen also continues to lay diploid eggs to the production of the new queens. Developing queens require more food over a longer period than worker larvae, so they can only be produced if sufficient food is available, and if there are sufficient workers to feed the larvae. The

young queens leave the nest to forage, consume large quantities of pollen and nectar, and incorporate fat reserves. Males after a few days within the colony leave to feeding on flowers and with to searching for a mate. After mating, young queens continue feeding for a while and then they begin to search for suitable hibernation sites (Goulson, 2010; Figure 4).

Figure 4: Life cycle of *B. terrestris*; modified after www.bumblebee.org.

1.6.2 *Osmia* spp.

Osmia genus belongs to the family Megachilidae (orden Hymenoptera) and comprises more than 300 species (Michener, 2007). They are among the first bees to emerge after winter, therefore they are important pollinators of early-blooming plants. For this reason, management methods have been developed to use various *Osmia* species as commercial pollinators of orchard with spring-blooming around the world: *Osmia cornifrons* Radoszkowski (the hornfaced bee) in eastern Asia (Sekita and Yamada, 1993), *Osmia lignaria* Say (the blue orchard bee) in North America (Bosch and Kemp, 2002a, 2002b); and *Osmia cornuta*

Latreille (the horned mason bee) and *Osmia bicornis* (= *rufa*) L. (the red mason bee) in Europe (Ladurner et al., 2004; Vicens and Bosch, 2000).

In general, the solitary *Osmia* bees are univoltine, i.e. produce only one generation per year, as *O. lignaria*, *O. cornifrons*, and *O. cornuta*, the three species whose developmental biology is best known (Bosch et al., 2008). These *Osmia* spp. overwinter as cocooned adults and when they are exposed to warm temperature (spring) emerge from the cocoons, leave their natal nest and mate. Males emerge 2-4 days before females. Once the females leave the nest, mating take place. During a brief period (2-5 days), females mature their ovaries prior to nesting (Lee et al., 2015; Sgolastra et al., 2016; Wasielewski et al., 2011). After this period, females start to select their nesting sites in pre-established cavities and repeatedly enter in their prospective nest cavities. After the selection of a nesting cavity, females initiate their nesting activities. Females begin collecting nesting materials (mud) to build a series of separated cells and pollen and nectar for larval provision. Each cell contains a loaf of pollen mixed with nectar, on top of which an egg is laid. A nest is completed when the entrance is sealed with a thick mud plug. Females are active for 20-25 days (including the prenesting period) and build 0.5 to 1.5 cells per day under field conditions (Bosch et al., 2008). Each cavity contained around 8-12 cells, of which 2.5 to 5 contain female progeny. The first cells correspond to female progeny and they are generally larger than those of the males and contain more provision. Eggs take more than a week to hatch. Larval development proceeds through five larval instars and takes one month approx. The fifth instar spins a thick multilayered cocoon with secretions originating from the salivary glands. Cocooned larvae (prepupae) enter a dormant stage in early summer to the end of summer (1-3 months). Newly formed pupae take 1-1.5 month to acquire adult pigmentation and the pupal development. At the end of the pupal period the adults eclose inside their cocoons and remain exposed to warm

temperatures at the end of the summer and beginning of autumn (prewintering period), so the cocooned adults are still exposed to temperatures warm enough for development. This period allows slowly developing individuals within a population, to achieve the adulthood and be ready for wintering when temperatures start to decline. Wintering is the period where cocooned adults are exposed to cold temperatures. This is necessary for adult diapause development. *Osmia* adults not exposed to wintering temperatures are unable to survive and get out of the cocoons. In spring, when the temperatures increase, a incubation preemergence time is required to emerge from the cocoons (Bosch et al., 2008; Figure 5).

Figure 5: Life cycle of *Osmia* spp. Source: Sgolastra et al. (2019).

CHAPTER II: OBJECTIVES

The **general objective** of this thesis is to provide knowledge on pollinator conservation in melon agro-ecosystems in Central Spain. Two important measures within agricultural areas must be addressed to ensure adequate pollinator and pollination services:

1) Increase on the availability of floral resources and nesting sites. This thesis aims to increase understanding on how changes in agricultural practices, specifically the implementation of flower strips near the crop can have an effect on this pollinator-dependent crop and on the pollinators who visit it.

2) Reduction of the impact of agricultural practices such as the use of pesticides. The study aims at providing knowledge on the pesticide risk to pollinators under realistic scenarios.

To achieve the general objective, several **specific objectives** have been proposed:

To optimize a regionally adapted flower strip that supports pollinators in melon agro-ecosystems based on the attractiveness, flower coverage, phenology and competition among the different plant species (**Experiment 1**).

To evaluate the contribution of the flower strip to the pollination services of a nearby melon crop and to ascertain its role as pollinator competitor or facilitator to the crop (**Experiment 1**).

To study in a melon commercial field how far the flower strip could influence visitors in the crop (**Experiment 1**).

To evaluate the influence of the flower strip on the melon crop productivity and quality (**Experiment 1**).

- To identify the pesticide residues in pollen and nectar, and to identify the most likely pesticide combinations in commercial melon fields in Central Spain (**Experiment 2**).
- To assess the impact of one very likely pesticide mixture in the field, on the micro-colonies of the social bee *Bombus terrestris* L. through a chronic oral toxicity test (**Experiment 2**).
- To establish whether the exposure to combinations of two neonicotinoids and a triazole fungicide at field-realistic concentrations found in commercial melon crops, cause lethal and/or sublethal effects in the solitary bee *Osmia bicornis* L. (**Experiment 3**).
- To determine whether the double route of exposure (via nectar or/and pollen) to imidacloprid at field-realistic doses affects the development of *Bombus terrestris* L. micro-colonies (**Experiment 4**).

CHAPTER III: MATERIALS AND METHODS

3.1 EXPERIMENT 1: The role of annual flowering plant strips on a melon crop in Central Spain. Influence on pollinators and crop.

3.1.1 *Study site.*

The study was carried out in two different areas of Central Spain, both with continental Mediterranean climate [cold winters, hot summers and scant rainfall ($\approx$ 400-450 mm per year)]: in small plots at the experimental farm La Poveda [Arganda del Rey, Madrid; 40°19'N and 3°29'W, elevation 536 m above sea level (a.s.l.)] and in a commercial plot located at the typical productive Spanish area of Corral de Almaguer (Toledo; 39°45'N and 3°11'W, elevation 708 m a.s.l.). The meteorological data, precipitation and temperatures, (daily mean, maximum mean and minimum mean temperature), were obtained from the nearest weather stations (1 km from the plots) and are available online at Agro-climatic Information System for Irrigation (SIAR, 2015).

3.1.2 *Experimental design.*

A 2-year study (2013–2014) was set up in small experimental plots (10 x 10 m²) at La Poveda (Madrid). It consisted of a randomized block design of 3 blocks 10 m apart, west–east orientated, with 2 drip-irrigated melon plots 10 m apart each (control and flower plots). The location within the farm was changed every year, depending on the soil availability and irrigation facilities. The spontaneous vegetation in the margins of the control plots was weeded periodically. Flower strips 1 m wide were placed on the two north–south sides of the plots (Figure 6).

A one-year study (2014) was carried out in a commercial field of 24 ha at Corral de Almaguer, where a flower strip (2 m wide x 280 m long) was established in an area of approx. 2.8 ha (100 x 280 m²). The experimental design consisted of 4

blocks 70 m apart and with 7 different distances from the flower strip each—1.75, 10.5, 19.25, 28, 45.50, 71.75, and 99.75 m—based on the melon-planting pattern (1.75 m x 1.50 m) and the possible influence of the flower strip (a highest concentration of distances near to the flower strip) (Figure 6).

3.1.1 *Melon crop.*

Sancho, a hybrid melon cultivar (toad skin type) widely planted in Central Spain, was selected for the study and managed following typical crop cultivation techniques. Pesticide treatments were not applied, except localized, anti-powdery mildew treatments of Flint® (50% trifloxystrobin, water-dispersible granules, 0.25 l/ha, Bayer) up to 3 days before harvesting. Fruits were ready for harvesting at the end of July.

In the experimental plots, melon plants with 2–3 mature leaves were transplanted from mid-May (planted in rows 2 m apart and 1 m between plants). The commercial field was planted on 16 May (planting pattern 1.75 m x 1.50 m). Around the commercial melon field, honey bee hives were routinely managed in order to increase the pollination of this and other adjacent melon fields.

Figure 6: Experimental design and location of La Poveda experimental plots (Madrid; Central Spain)(a and c) and commercial melon field at Corral de Almaguer (Toledo; Central Spain) with the sampling area (2.8 ha, continuous line) (b and d).

3.1.2 *Flower strip composition and evolution.*

We used seed mixes of flowering annual herbaceous plants of different botanical families, based on previous works (Azpiazu et al., 2017; Viñuela et al., 2012) (Table 1 and Figure 7). All the species were commercially available (Semillas Silvestres SL, Cordoba Spain) and with a seed size suitable for

mechanical sowing after adaptation of the machinery, two of the bottle necks when selecting plants for flower mixes. The plant species were native or naturalized, well adapted to the climate in Central Spain, and had different phenology and floral morphology features (colour, size, corolla shape and depth) (Flora ibérica, 2013) in order to attract pollinator diversity. The plants also differed in the floral nectary position, which determines the nectar availability: ‘concealed’ (in deep corollas or spurs), ‘partly-concealed’ (in short corollas) or ‘opened’ (in short corollas or in extra-floral nectaries) (Campbell et al., 2017). For every individual species, we evaluated the attractiveness to visitors, flower coverage, blooming duration and phenology. Based on these parameters, in the experimental farm, the composition of the mix was slightly modified the second year. In the commercial field of Corral de Almaguer, a flower seed mix under commercial development and slightly different to that used in the experimental plots was sown.

Table 1: Characteristics of the specific taxa that make up the flower strips.

Species	Family	Floral nectaries	Height (cm) ¹	La Poveda	Corral de Almaguer
<i>Calendula officinalis</i> L.	Compositae	open	20-50	2013, 2014	2014
<i>Coriandrum sativum</i> L.	Umbelliferae	open	40-60	2013, 2014	2014
<i>Nigella gallica</i> L.	Ranunculaceae	open	10-40	2013	2014
<i>Lobularia maritima</i> L.	Cruciferae	open	2-20	2014	-
<i>Borago officinalis</i> L.	Boraginaceae	partly concealed	30-70	2013, 2014	2014
<i>Diplotaxis virgata</i> Cav.	Cruciferae	partly concealed	50-100	2013	-
<i>Diplotaxis catholica</i> L.	Cruciferae	partly concealed	<80	2014	2014
<i>Medicago sativa</i> L.	Leguminosae	concealed	30-70	2013, 2014	2014
<i>Salvia verbenaca</i> L.	Labiatae	concealed	5-60	2013	2014
<i>Silene vulgaris</i> Moench.	Caryophyllaceae	concealed	24-80	2013, 2014	2014
<i>Vicia sativa</i> L.	Leguminosae	concealed	<80	2013	2014

¹Data from Flora Ibérica website (Flora ibérica, 2013).

Figure 7: Plant species of the flower strip: *D. virgata*, *C. sativum*, *B. officinalis*, *C. officinalis*, *M. sativa*, *S. vulgaris*, *L. maritima*, *S. verbenaca*, *V. sativa* and *N. gallica*.

Prior to sowing, the soil was prepared by tilling and then the seed mix was broadcast-sown and the seeds were covered using a shallow stubble plough. In the experimental plots, the sowing was done before winter began (December 21st), aiming at reaching 13 plants per m² approx. (optimal density in order to allow every plant to have enough space, 25 x 30 cm). In the commercial field however, the sowing was done at the beginning of May (6th) because before winter, the farmer did not know the exact location of the crop within the farm yet.

In the flower strips, direct visual sampling was performed once a week during the bloom period to assess the floral coverage in every plant species, based on an adapted scale (Braun-Blanquet et al., 1979): 1 (>0-1%), 2 (1-10%), 3 (10-25%), 4 (25-50%), 5 (50-100%) and the number of flowers (*B. officinalis*, *N. gallica*, *S.vulgaris*, *V.sativa*,) or inflorescences (*C. officinalis*, *C. sativum*, *D. catholica*, *D.*

virgata, *L. maritima*, *S. verbenaca*) was also counted. In La Poveda, 3 marked areas (1 x 1 m²) were randomly distributed in each of the 2 flower strips of each flower plot (6 in total per plot). Because the flower coverage and season can have an influence on the plant attractiveness and on the activity of the pollinator species, the total sampling period was divided into 3, in order to allow for comparison of the pollinator groups visiting the plant species with simultaneous bloom: (1) early spring blooming flowers; (2) late spring-early summer blooming flowers; (3) summer co-blooming flowers with the crop. In the commercial field of Corral de Almaguer, 4 marked areas (1 x 1 m), ≈70 m apart, were distributed in the flower strip.

3.1.3 *Visitor sampling.*

Visual samplings of pollinator visits were performed weekly in the flower strips and crop, between 09:00 and 14:00 under suitable weather conditions for foraging visitors (temperature above 16 °C, clear skies and calm wind). Every week we started in a different block in both the experimental and the commercial plots. Depending on the peculiarities of experimental or commercial field and flower strip or crop, the samplings were made differently.

In the experimental plots (La Poveda), the flower visits in the flower strip or to melon crop were assessed. In the flower strips, observations were done in the marked areas previously described (6/plot, 3 minutes/marked area, 18 minutes/plot in total). Visits in the melon crop were assessed in transects 10 m long x 1 m wide (3/plot, 3 minutes/transect, 9 minutes/plot in total) which yielded more visits. In preliminary samplings with fixed marked areas, the number of melon flowers could be zero in some sampling dates, due to its staggered bloom.

In the commercial field of Corral de Almaguer, the flower emergence in the flower strip was not as homogeneous as in the experimental plots, and pollinators were recorded in transects 15 m long x 1 m wide over 3 minutes. Four transects 70 m apart, were located in the flower strip and in the melon crop in each of the 7 different distances from the flower strip (Figure 1). To minimize the influence of the number of flowers in the flower strip, the bee visitation rate (visits·flower⁻¹) was used to compare visits to the flower strip and crop. Therefore, the number of flowers was also counted in 3 marked areas (1 x 1 m²) per transect in the flower strip, and in each of the 28 transects of the melon crop, in order to estimate the number of flowers observed per transect.

The visitor groups considered in the visual samplings were beetles, hoverflies and bees. Lepidopterans and Vespoidea were very scarce and were not included in analysis. Bee species were only identified up to family or genus but for statistical purposes, they were classified following the widely accepted phylogenetic family classification based on the proboscis length (Michener, 2007), which helps in structuring bee communities and plant-pollinator networks as it is related to the ability of pollinators to access floral rewards. The specimens in the visual sampling were assigned to the group of long-tongue (L-T) bees (tongues normally longer than 6 mm; Apidae and Megachilidae) or short-tongue (S-T) bees (tongues normally shorter than 5 mm; Colletidae, Halictidae, Melittidae, Andrenidae) (Biesmeijer et al., 2006; Michener, 2007). The last group included bees with very different body sizes, so finally we considered 3 functional bee groups: small (< 1cm) short-tongued (S-T), large S-T and long-tongued (L-T) bees, because body size, alongside proboscis length, matters for the choice of the flower.

To get acquainted with the most common pollinators in our study areas, and to help us to understand the species richness in every visitor group of the visual

samplings, some destructive samplings were also done. In the first year of the study, bi-weekly captures with an entomological sweep net, or 3 square methacrylate wet pan traps (25 cm side) with the bottom painted in fluorescent yellow (F201 yellow®, Paintusa, Valencia, Spain) were performed. Additionally, throughout the years, individual species were captured with an entomological net and taken to the lab for identification. Those visitors captured in the melon flowers were noted down. Species present in Central Spain were identified according to the Atlas of Hymenoptera (2017) and Ortiz-Sánchez (2011), and some of them were already well-known (Azpiazu et al., 2017; Barbir et al., 2015; Rodrigo Gómez et al., 2016).

3.1.4 *Melon productivity and quality.*

Even though melon has staggered ripening, a single harvest when the first fruits were fully mature was performed. All fruit from every plot in the experimental farm (La Poveda) were collected (13 August 2013 and 5 August 2014). In the commercial field of Corral de Almaguer, 5 distances from the flower strip (1.75, 10.5, 28, 45.50 and 71.75 m) and 3 randomly located transects, 15 m long per distance, were selected. All mature melons were collected inside 3 frames (1 × 1 m²) randomly located per transect (31 July 2014). Collected fruits were weighed in the field to calculate the mean fruit weight and yield per ha (dynamometer ProScale® Versa 77, US).

A selection of 12 typically sized melons for the variety (2-4 kg) without external defects, randomly selected in every plot at La Poveda and 9 melons in every distance at Corral de Almaguer were taken to the lab for quality parameter measurements [following Cabello et al.(2009)]. In the lab, fruit diameter and length were recorded along with the following quality parameters (Figure 8): flesh thickness from the placenta to the beginning of the exocarp (caliber Krefting, Germany); flesh firmness (penetrometer fitted with an 8 mm diameter

probe Bertuzzi FT 327®, Facchini, Italy); percentage pulp juice or juiciness, measuring the pulp fresh (electronic balance FY-3000®, A&D, Japan) and pulp liquefied weight (blender model 753®, Moulinex, France); pH of the juice (pH meter Basic 20®, Crison, Spain) and total soluble solids (°BRIX) (refractometer Palette 100®, Atago, Japan). To evaluate the efficiency of insect pollination, in 2013 we weighted placenta and seeds, while in 2014 we calculated the total number of seeds per fruit as a more precise indicator (Walters and Taylor, 2006).

Figure 8: Quality parameters measured in the lab: a) flesh thickness b) flesh firmness c) and total soluble solids (°BRIX) d) pH of the juice e) placenta and seeds f) number of seeds.

3.1.5 *Statistical analysis.*

In La Poveda experimental farm, linear mixed-effect models (LMM) (Littell et al., 1998; Wang and Goonewardene, 2004) were used to analyze the pollinator visits. In the flower strips, in order to assess the attractiveness of the different plants species to each visitor group, we considered the mean number of visits in the 6 marked areas per plot (18 min in total) as the dependent variable and visitor group (beetles, hoverflies, L-T bees, small S-T bees and large S-T bees) and plant species as the fixed factors. Plants that did not have a high percentage of coverage (<1%) and/or attracted few pollinators were not considered for

statistical analysis (*N. gallica*, *S. verbenaca.*, *S. vulgaris*, *V. sativa* and *D. catholica*). The block was considered as random factor and the sampling dates as the repeated measures factor. We included as covariate the number of flowers or inflorescences of each plant species. Separate analyses were carried out in every bloom period—(1) early spring blooming flowers; (2) late spring-early summer blooming flowers; (3) summer co-blooming flowers with the crop—and year, because the plant composition, associated visitors and plot location within the farm changed yearly. In the melon crop, to compare the visits between melon plots with and without flower strips and to ascertain its role as pollinator competitor or facilitator, the mean number of visits in the 3 transects per plot (9 minutes in total) was the dependent variable; the treatments (control and flower strip plots) and visitor group (L-T bees, small S-T bees and large S-T bees) were the fixed factors; block was the random factor and the sampling dates were the repeated measures factor. Facilitation occurred when visits to melon plots with flower strips were significantly higher than to control plots; competition, if the number was significantly lower; and no effect if statistical differences were not observed between treatments. Data in all cases were transformed to $[\ln(x+1)]$ for normality prior analysis. The lowest value of Akaike's Information Criterion (AICc) was used to select the best covariance structure for the repeated measures factor (Littell et al., 1998; Wang and Goonewardene, 2004) and the linearly independent pairwise comparisons of estimated marginal means were performed using the Fisher least significant difference (LSD) test ($P < 0.05$).

In the Corral de Almaguer commercial field, a generalized linear mixed model (GzLMM) was performed in R version 3.0.2 using the glmmADMB package to evaluate whether the bee visitation rate in the melon crop declined with increasing distance from the flower strips. We accounted for overdispersion by fitting a negative binomial error distribution and using a log link function. We used the visits per transect (3 minutes) as a dependent variable, the number of

flowers as an offset and the distance (8 measured distances, range 0–100 m) as a continuous fixed effect. To account for non-independence of data collected we included the block and the sample dates as random factors. We tested the significance of the main effect using likelihood ratio two-sided test, and Tukey-test ($P < 0.05$) to contrast the distant levels.

In La Poveda experimental farm, the melon productivity and quality parameters in control and flower strip plots were compared every year independently using a Student's t-test ($\alpha < 0.05$). If any of the assumptions was violated after variable transformation [$\ln(x + 1)$], a non-parametric Mann–Whitney/Wilcoxon test was applied. In Corral de Almaguer commercial field, the melon productivity and quality parameters at different distances from the flower strip were analysed with one-way analysis of variance (ANOVA). Means were separated with the LSD multiple range test ($P < 0.05$). The non-parametric Kruskal–Wallis test ($P < 0.05$) was used to establish differences when data violated the premises of the ANOVA.

3.2 EXPERIMENT 2: Pesticide residues in nectar and pollen of commercial melon fields and their effects on *Bombus terrestris* micro-colonies.

3.2.1 *Determination of pesticide levels in pollen and nectar of melon flowers.*

3.2.1.1 *Melon flowers sampling: nectar and pollen collection.*

During July 2017 we collected melon flowers in five commercial fields on the basin of the Madrilenian Tajo River, a typical and recognized area for melon cultivation in the southeast of Madrid, Spain (Figure 9). Based on farmer's information, the fields have been treated with different pesticides during the crop cycle (Appendix 1).

Figure 9: Location of the five conventional commercial melon fields in the study area, southeast of Madrid, Spain.

The flower collection was made in 3 points separated by at least 100 m in each field. Both male and hermaphrodite flowers were collected. Nectar was extracted from 30-70 flowers, using microcapillaries (5 μ l, Blaubrand® intraMARK) and 50 μ l samples were stored at -80 °C (Figure 10). Hundreds of additional flowers were frozen at -80 °C for later pollen collection. In October, the stored flowers were dried in an incubator at 37 °C during 24 h to facilitate the removal of the pollen from the anthers (following Botías et al., 2015).

Anthers were then collected and the pollen was extracted using a sieve of 150 μm pore size [melon pollen grain Ø = 50-100 μm (PalDat, 2017)] (Figure 10). We used 140-250 flowers to obtain approximately 0.1g of pollen per sample.

Figure 10: Nectar and pollen extraction: a) nectar collection with microcapillaries b) 50 μl nectar sample c) pollen extracted from the anthers using a sieve.

3.2.1.2 Multi-residual analyses of pesticides.

The multi-residual analyses were carried out in the Laboratorio Químico Microbiológico de Sevilla an official accredited analytical testing company (www.lqmsa.com). High performance liquid chromatography with quantification and confirmation by triple-quadrupole mass spectrometer detector (HPLC-QQQ) and gas chromatography with triple-quadrupole mass spectrometer detector (GC-QQQ) were used to target more than 200 compounds (Appendix 2). The recoveries were over the detection limit (LOD) of 3 ng/g for each analyte.

3.2.1.2.1 Pollen and nectar preparation.

Pollen preparation was performed using a modified version of the QuEChERS methodology described by David et al. (2015). Pollen samples (100-200mg) were weighed and introduced in 15 ml Eppendorf tubes with a ceramic microbead. Two ml of water were added to each sample to form an emulsion and extracted by adding 2ml of acetonitrile (CH_3CN , Baker 9012 "HPLC Analyzed"), mixing in Agytax® SR2 for 2 minutes followed by sonicating for 10 minutes. Then, 1 g of magnesium sulphate/sodium acetate mix (4:1) (QuEChERS Salts, Agilent 5982-

0650) was added followed by immediate shaking (Agytax® SR2) for 2 min and sonication without heating for 10 min. The supernatant was transferred into a 4 ml polypropylene tube with a ceramic microbead and Agilent QuEChERS dispersive 5982-5056 was added to match the weight of the sample and shaken for 2 min (Agytax® SR2) and centrifugated (3000 rpm for 5 min). The supernatant was measured and transferred to a centrifuge tube and dried with high purity N₂ stream. The extract was reconstituted with 100 µL of CH₃CN.

Nectar preparation followed a similar procedure. To calculated nectar weight, nectar samples (ca. 50 µL) were inserted in pre-weighed Eppendorf tubes. The volume of water added was adjusted to 1 mL.

3.2.1.2.2 Instruments.

HPLC-QQQ was performed with an Agilent Technologies 1200 HPLC consisting of a binary pump G1312A, an autosampler, a vacuum degasser and a thermo column compartment (G1316A). The analytical column for the separation of the analytes was an Agilent Technologies Poroshell 120 C18 (10 cm × 2.1 mm × 2.7 µm) with adequate guard column maintained at 40 °C. The separation of the analytes, was performed by applying a gradient of components A (CH₃CN with 0.1% formic acid) and B (2 mM ammonium formate in water with 0.1% formic acid) with a flow rate of 0.6 mL·min⁻¹. The injection volume was 5 µL. The gradients started with 20% of component A and 80% of component B for 1 min and then the component A was increased to 100% within 10 min. After 4 min, the component A was decreased to 20% and the component B increased to 80% within 1 min, followed by equilibration for 5 min. Total run time was 22 min.

The Agilent Technologies 6410 mass spectrometer working in dynamic multi reaction monitoring (MRM) mode is equipped with an electrospray ionization

(ESI) interface for the introduction of solvated samples. Triple Quadrupole Mass Spectrometer was used for the detection and quantification of pesticides. Capillary positive voltage was 3000 V. Nitrogen was the nebulising gas with a flow rate of 9L·min⁻¹ and temperature 345 °C. Nebulizer gas pressure was set at 40 psi.

To perform the GC-QQQ, we used a GC Agilent Technologies GC7890A with an automatic liquid autosampler and a split-splitless injector. A DB-5 (5%Phenyl 95% Methylpolysiloxane) column Agilent 19091S-433 (30 m × 0.25 mm × 0.25 µm) was used. A sample volume of 1 µL was injected into the GC in splitless mode at an injector temperature of 250 °C. The oven temperature program was as follows: initial temperature 70 °C (held for 2 min) increased by 25 °C/min to 150 °C; increased by 3 °C /min to 200 °C (held for 1 min); increased by 8 °C/min to 280 °C (held for 10 min). High Purity Nitrogen gas was used as collision gas with a flow rate of 1.5mL/min and Helium at 1.35 mL/min for quenching effect into the collision cell (octopole). Calibration standards concentrations ranged from 2 to 100 µg/L.

3.2.2 *Chronic bioassay with *Bombus terrestris* micro-colonies.*

A laboratory bioassay were carried out to quantified the lethal and sublethal effects and reproduction fitness of bumblebee's micro-colonies under chronic oral exposure. Commercial bumblebee colonies were purchased from Agrobio S.L. (La Venta del Viso, Almería, Spain). Newly emerged workers (hairs silver in colour and wings crumpled and soft) were collected from the queenright colonies and in groups of five were placed in a circular plastic box (diameter 12 cm, height 9.5 cm) with a mesh in the cover to allow air ventilation (Figure 11). After 1 week, a hierarchy become established; the ovaries of the dominant worker (pseudo-queen) were developed and it started to lay haploid eggs that developed into males. A maximum of 24 micro-colonies per day were formed in

3 consecutive days and they were weekly rotated within the chamber to ensure there was no positional influence. Queenless micro-colonies were maintained in a walk-in chamber at 28-30 °C temperature, 50-60 % relative humidity (RH) and continuous darkness throughout the entire study period.

Figure 11: Micro-colonies of *Bombus terrestris*.

After 4 days to acclimatise to their new environment, the micro-colonies were assigned to one of the eight treatments (see Results Experiment 2 section 4.2.2.1, to pesticide selection justification,): control (CON), acetamiprid (A), chlorpyrifos (C), oxamyl (O) and the mixtures (A+C, A+O, C+O, A+C+O). Before the food contaminated was offered, dead bees were replaced with workers from the same original queenright colony [as Laycock et al. (2012)]. We used eight micro-colonies per treatment and excluded those with no egg laying the first week (without egg cells covered of wax) and as well two micro-colonies where all the individuals had died because the syrup had spilled out from the feeder.

Commercial syrup (Api 65®: 1.21 g/ml, fructose/glucose/saccharose solution; Agrobio S.L., Spain) and organic honey bee pollen (Bona Mel®, Alicante, Spain; mean mass: 6.32 g, SE = 0.07 g;) contaminated at field-realistic concentrations (Table 5 in Results chapter) were offered *ad libitum* during the study period. Stock pesticide solutions were prepared by mixing 500 mg of Epik® (acetamiprid, 20% w/w; Sipcam Inagra S.A.), 500 mg of Chas® (chlorpyrifos, 5 %

w/w, FMC Agricultural Solutions, S.A.U) and 100 µl of Afromyl® (oxamyl, 10 % w/v, Industrias Afrasa S.A.) with 50 ml of distilled water. These solutions were diluted in the syrup or in the distilled water used for the pollen preparation to reach the desired concentrations.

The pollen was renewed every 3-4 days, and the syrup was replaced once a week (Fauser-Misslin et al., 2014) throughout the 11 week study period. Forty millilitres of the commercial syrup were offered to the bumblebees in bird drinking troughs (capacity 70 ml, diameter 4 cm, height 8.5 cm). Following Dance et al. (2017), instead of pollen and syrup consumption we used as parameter pollen collection because some syrup was stored in the wax honey pots and some pollen was used as provision brood. Accordingly, every 3-4 days, pollen was weighed and the volume of syrup was measured weekly. Six identical plastic boxes to those used for micro-colonies were kept with full syrup feeders and pollen but without bumblebees in order to control the amount of evaporation. Twice per week, mortality was evaluated in order to estimate the bumblebee survival and male reproduction was also assessed. The male offspring was removed in each observation and kept in the freezer (-20 °C), to latter measure the thorax width (inter-tegulae span) using a stereomicroscope (S6E Leica®) at 20x magnification and an ocular micrometer (precision ±0.01 mm). The measurements were made in the 3 of the last drones emerged in each micro-colony, considering as the most unfavourable case. To assess brood production, at the end of the bioassay, the micro-colonies were dissected and the number of larvae and pupae were counted.

3.2.3 *Statistical analysis.*

We used one-way analysis of variance (ANOVA, $P < 0.05$) with statistical software package SPSS Statistics® (IBM Corp. Released, 2013) to analyse the effect of treatments on total pollen and syrup collected, on brood and male

production and on their thorax width. The linearly independent pairwise comparisons of estimated marginal means were separated using the LSD test. The nonparametric tests of Kruskal-Wallis ($P < 0.05$) were used to establish differences on mortality, because data violated the premises of the ANOVA after variable transformation $[\ln (x + 1)]$.

3.3 EXPERIMENT 3: Chronic oral exposure to field-realistic pesticide combinations via pollen and nectar: effects on feeding and thermal performance in the solitary bee *Osmia bicornis*.

3.3.1 *Bee population and test conditions.*

All tests were conducted with newly-emerged females of *O. bicornis*, a cavity-nesting solitary bee. This species has not been recorded visiting melon flowers but we decided to work with this species for various reasons. First, it can be easily reared under controlled conditions; second, it has been proposed by the European Food Safety Authority as a test species for risk assessment (EFSA, 2013); third, a fair amount of information is available on *Osmia* ecotoxicology (Abbott et al., 2008; Artz and Pitts-Singer, 2015; Biddinger et al., 2013; Ladurner et al., 2008, 2005; Scott-Dupree et al., 2009; Sgolastra et al., 2018a, 2017, 2015) and the available evidence indicates a higher vulnerability to pesticides than honey bees and bumblebees (Rundlöf et al., 2015; Sgolastra et al., 2017; Woodcock et al., 2017).

Osmia bicornis cocoons from a population reared in a pesticide-free area of the Kazimierz Landscape Park (Poland) were shipped to the laboratory of Agricultural Entomology at the University of Bologna (Italy) in January 2018 and kept at 3-4 °C until May 2018. At that time, large cocoons (presumed to contain females) were incubated at 22-23 °C until emergence. A previous study showed that emergence time influences sensitivity to pesticides in *O. bicornis* [females taking longer to emerge are more sensitive (Sgolastra et al., 2019)]. For this reason, we only used bees that emerged over two consecutive days during the peak of the emergence period (days 4-5). Upon emergence (<24 h), bees were transferred to a Plexiglas holding cage (50 × 50 × 50 cm³) for ca. 4 hours to allow them to deposit the meconium. Then, bees were individually caged in plastic ice cream cups (diameter: 5.5–8 cm; height: 7 cm) with transparent lids

perforated with a pin to allow air exchange. Each cup contained a syrup feeder and a pollen feeder. The syrup feeder was a 1-ml calibrated syringe (Tuberculin Beroject® III, Beromed; accuracy: 0.01 ml) inserted through the lid. A petal of *Euryops* (Asteraceae) was attached to the tip of the syringe to enhance location of the feeder by the bee (Figure 12). The pollen feeder was a 1.5-ml Eppendorf tube inserted through the side of the cage with the upper half of the bottom cut off (Figure 12). Bees were maintained at 23.6 ± 0.3 °C and 50-60% relative humidity. Cups were kept under natural light conditions throughout the experiment but direct sunlight exposure was avoided to reduce pesticide degradation (Maienfisch et al., 2001).

Figure 12: Individual cages and close-up of syrup and pollen feeders

3.3.2 *Treatments.*

Previous analysis of the pollen and nectar of melon flowers from 5 commercial fields from the southeast of Madrid, Spain, yielded 19 pesticides (acetamiprid, imidacloprid, oxamyl, metalaxil-m, chlorpyrifos, abamectin, azoxystrobin, myclobutanil, boscalid, flonicamid, atrazina, quinomethionato, cloranthraniliprol, difenoconazole, kresoxim-methyl, chlorothalonil, thiacloprid, *alfa*-cypermethrin, quinoxifen). Because it was not feasible to test so many compounds, we decided to work with three of them: the triazole fungicide,

myclobutanil, and two neonicotinoid insecticides, imidacloprid and acetamiprid (see Results Experiment 2, Table 6). These three compounds were selected because: 1) they are the pesticides most commonly applied to melon fields in the study area; 2) their occurrence in the pollen/nectar samples was very high; 3) the two neonicotinoids have different detoxification pathways and differ in their toxicity to bees (Manjon et al., 2018); and 4) several studies have found synergistic effects between mixtures of neonicotinoid insecticides and triazole fungicides (Biddinger et al., 2013; Iwasa et al., 2004; Sgolastra et al., 2018a, 2017; Thompson et al., 2014).

We exposed bees to the mean active ingredient concentrations found in the nectar and pollen of melon flowers in commercial fields (Table 2). Females emerging on any given day were evenly distributed among eight treatments: control (CON), acetamiprid (A), imidacloprid (I), myclobutanil (M) and the mixtures A+I, A+M, I+M, A+I+M. Each treatment group received the specific food for the entire test period, i.e. until the natural death of the bees.

The syrup was prepared by diluting sucrose in water (33% w/w). Honey bee pollen pellets were obtained from an organic beekeeper (Bona Mel®) and stored at 3-4 °C until use. Pellets were then ground with a coffee grinder and mixed with distilled water (pollen/water 3:1 w/w) to obtain a single uniform pollen source. Although honeybee-collected pollen could be a potential source of pathogens (Higes et al., 2008; Singh et al., 2010), we did not irradiate the pollen pellets (Graystock et al., 2016). Nonetheless, we are confident that this did not affect our results because mean longevity of control bees in our study (19 days) was similar to mean longevity recorded in previous *O. bicornis* laboratory studies (17 days) in which bees were only fed with syrup (Sgolastra et al., 2018a). This longevity is also similar to the mean life span of adult *Osmia*

females nesting in field and semi-flied conditions (17.5–24 days) (Bosch and Vicens, 2006; Sandrock et al., 2014; Sgolastra et al., 2016).

Stock solutions of each pesticide were prepared by diluting 500 mg of Epik® (acetamiprid, 20% w/w), 100 µl of Confidor® (imidacloprid, 20% w/v) and 100 µl of Systhane Forte® (myclobutanil, 24% w/v) in 50 ml of purified distilled water. These solutions were diluted in the syrup or in the distilled water used for the pollen preparation to reach the desired concentrations identified in the pollen and/or nectar of the melon flowers (Table 2).

3.3.1 *Syrup consumption, pollen consumption and longevity.*

Cups were inspected daily to monitor syrup consumption (assessed by checking the level of syrup in the calibrated syringe) and bee mortality. Pollen consumption was assessed once a week and whenever a bee died. For each cup, we weighed with an analytical scale (accuracy = 0.0001 g) the remaining pollen in the Eppendorf tube along with any pollen crumbs scattered over the bottom of the holding cage. Average daily pollen consumption was estimated by dividing pollen consumption by the number of days elapsed between measurements. Eight additional containers with syrup and pollen feeders but without bees were used as controls to measure and account for potential evaporation from the syrup and pollen sources. Five additional cages without bees were used to measure the evaporation of the pollen crumbs scattered over the bottom of the holding cage. Syrup was renewed every 3-4 days and pollen once a week.

Bees that had not begun feeding by the fourth day of exposure were discarded. Sample sizes in each treatment are shown in Table 7 (See Results Experiment 3). At the end of the experiment, we measured the head width of each bee under a stereomicroscope at 240x as a proxy of body size (Bosch and Vicens, 2002).

Table 2: Concentration and occurrence of Acetamiprid, Imidacloprid and Myclobutanil in the pollen and nectar of melon flowers from 5 commercial melon fields near Madrid (Spain). (See details of analytical techniques in M&M Experiment 2).

Pesticide	Class	N of fields sprayed	Mean ($\pm$ SE) concentration (ppb) in melon flowers		Frequency (% samples)	Days between field application and pollen/nectar surveys	Analytical technique
			pollen	nectar			
Acetamiprid (A)	Neonicotinoid insecticide	5	482.93 $\pm$ 215.85	6.41 $\pm$ 3.53	100.00%	2-11	HPLC-QQQ
Imidacloprid (I)	Neonicotinoid insecticide	4	369.36 $\pm$ 186.31	15.34 $\pm$ 7.62	66.70%	45-71 ^a	HPLC-QQQ
Myclobutanil (M)	Triazole fungicide	4	0	5.58 $\pm$ 0.70	26.70%	2-15	GC-QQQ

^aTiming of application unknown for one of the 5 fields.

HPLC-QQQ: high performance liquid chromatography with triple-quadrupole mass spectrometer detector; GC-QQQ: gas chromatography with triple-quadrupole mass spectrometer detector.

3.3.1 *Thoracic temperature.*

Some bees showed clear signs of apathy. For this reason, we decided to measure thoracic temperature as a proxy of muscular activity. Thermogenesis in bees is mainly achieved by shivering of the flight muscles (Heinrich, 1993). We used a compact thermal imaging camera FLIR e60bx (320x240 pixels; range: -20 °C to 120°C; sensitivity: <0.045°C at 30 °C) to take thermal photographs of the bees in their cages in a dark room at 24.6 °C (Figure 13). These measures were taken on the 17th day of exposure in 6 bees per treatment.

Figure 13: Thermal photograph of an *Osmia bicornis* female at room temperature (24.6 °C) reaching a thoracic temperature of 32.1 °C.

3.3.2 *Ovary maturation.*

Upon emergence from the cocoon, *Osmia* females take about 3 days to fully mature their ovaries (Lee et al., 2015; Sgolastra et al., 2016; Wasielewski et al., 2011). On day 3 of the exposure phase, we took other 14 bees per treatment and froze them at -24 °C. These bees were later dissected in Ringer's physiological solution (NaCl 9 g, KCl 0.2 g, NaHCO₃ 0.2 g, CaCl₂ 0.2 g in 1 litre of distilled water), and the length of the most mature oocyte in each of the 6 ovarioles was measured under a stereo microscope at 500x (precision, ± 0.01 mm) (Figure 14). We use the mean length of these 6 oocytes as a measure of ovary maturation. At the end of the experiment, the head width of each bee was measured as described above.

Figure 14: Ovaries of an *Osmia bicornis* female upon emergence. The length of the most mature oocyte in each of the 6 ovarioles was measured.

3.3.3 *Statistical analysis.*

We used Gehan-Breslow Kaplan-Meier (K-M) survival analysis with pairwise multi comparison procedures (Log-Rank Test, $P < 0.05$) to compare survival curves among treatments. We used general linear models (GLM) to analyze the effect of treatment on longevity (square-root transformed), mean daily syrup consumption and ovary maturation (log-transformed). Body size was included as covariate in all these models and pairwise comparisons were conducted with the Fisher's LSD test ($P < 0.05$). Pollen consumption followed a clear two-phase temporal pattern (see results). For this reason, to analyse the effect of treatment on mean daily pollen consumption (log-transformed), we used a general linear mixed model (GLMM) with treatment (fixed factor), period (fixed factor repeated within subjects), their interaction, and body size as a covariate. Means were separated using Fisher's LSD test ($P < 0.05$). Thoracic temperature data were not normally distributed and could not be appropriately transformed. For this reason, we used a non-parametric Kruskal-Wallis test followed by Dunn's multiple pairwise comparisons ($P < 0.05$) to establish differences in thoracic temperature among treatments.

3.4 EXPERIMENT 4: Can the development of *Bombus terrestris* micro-colonies be different when they are exposed to imidacloprid via syrup or/and via pollen?

3.4.1 *Treatments and food contamination.*

Based on 20 studies, Godfray et al. (2014) calculated the average neonicotinoid residual concentrations in nectar and pollen from different seed-treated crops: 1.9 ppb in nectar and 6.1 ppb in pollen, being variable depending on the crop and on the neonicotinoid used. Besides, a meta-analysis of imidacloprid nectar residues in several seed-treated crops, found that the range of residual concentrations in nectar was 0.7-10 ppb (Cresswell, 2011). This concentration can be even higher in crops where treatments are made after transplantation (foliar or drip irrigation water applications), as shown in two studies in *Cucurbita pepo* L. Besides, Dively and Kamel (2012) showed a range of imidacloprid concentrations of 0.1-12 ppb in nectar and 0.1-80 ppb in pollen depending on the treatment timing, the concentration and year; while Stoner and Eitzer, (2012) calculated an average of 10 ± 3 ppb in nectar and 14 ± 8 ppb in pollen, for different varieties and application times.

Micro-colonies were exposed to the mean field-realistic concentrations reported for imidacloprid in a cucurbit crop: 6 ppb in nectar and 30 ppb in pollen (Dively and Kamel, 2012). Stock solutions of imidacloprid were prepared by diluting 100 μ l of Confidor® (imidacloprid, 20% w/v) in 50 ml of purified distilled water. These solutions were later diluted in the syrup or in the distilled water (in the case of pollen preparation) until the desired concentrations in the nectar and/or pollen were reached. A commercial syrup (Api 65®: 1.21 g/ml, fructose/glucose/saccharose solution; Agrobio S.L., Spain) and organic pollen (Bona Mel®, mean mass: 6.32 g, SE = 0.07 g) were used. The four treatments considered were: 1) Control (untreated syrup and pollen); 2) Nectar (N), chronic

exposure to imidacloprid via treated syrup at 6 ppb; 3) Pollen (P), chronic exposure to imidacloprid via treated pollen at 30 ppb and 4) Nectar+Pollen (N+P), chronic exposure to imidacloprid via treated syrup and pollen.

3.4.1 *Chronic bioassay with *Bombus terrestris* micro-colonies.*

Commercial bumblebee colonies were purchased from Agrobio S.L. (La Venta del Viso, Almería, Spain). Newly emerged workers (hairs silver in colour and wings crumpled and soft) were collected from the queenright colonies and in groups of five were placed in a circular plastic box (diameter 12 cm, height 9.5 cm) with a mesh in the cover to allow air ventilation. After 1 week, a hierarchy become established, the ovaries of the dominant worker (pseudo-queen) were developed and it started to lay haploid eggs that developed into males. The micro-colonies were formed in 2 consecutive days and 10 micro-colonies per treatment were used and excluded those with no egg laying the first week (without wax covered egg cells) and as well three micro-colonies where all the individuals had died because the syrup has spilled from the feeder. They were rotated within the chamber weekly to ensure there was no positional influence. Queenless micro-colonies were maintained in a walk-in chamber at 28-30 °C temperature, 50-60 % relative humidity (RH) and continuous darkness throughout the entire study period.

After 4 days to acclimatise to their new environment, the micro-colonies were assigned to one of the four treatments: control, N, P or N+P. The small number of dead bees prior the string of the tests, were replaced with workers from the same original queenright colony as indicated by Laycock et al. (2012). Contaminated pollen and nectar were offered *ad libitum* during the 11 week study period.

The pollen was renewed every 3 days, and the syrup was replaced once a week (Fauser-Misslin et al., 2014) throughout the study period. Forty millilitres of the commercial syrup were offered to the bumblebees in bird drinking troughs (capacity 70 ml, diameter 4 cm, height 8.5 cm). Following Dance et al. (2017), instead of pollen and syrup consumption we used pollen collection as an indicative of bumblebee activity because some syrup was stored in the wax honey pots and some pollen was used as provision brood. Accordingly, every 3 days, pollen was weighed and the volume of syrup was measured weekly. Six identical plastic boxes to those used for micro-colonies were kept with full syrup feeders and pollen but without bumblebees in order to control the amount of evaporation. Mortality and male reproduction were assessed every 3 days in order to estimate the survival and the effect on reproduction respectively. The offspring males were removed in each observation. To assess brood production, at the end of the bioassay the micro-colonies were dissected and the number of larvae and pupae were counted.

3.4.2 *Statistical analysis.*

We used one-way analysis of variance (ANOVA, $P < 0.05$) with statistical software package SPSS Statistics® (IBM Corp. Released, 2013) to analyse the effect of treatments on total pollen and syrup collected and male production. The linearly independent pairwise comparisons of estimated marginal means were separated using the LSD test. The nonparametric tests of Kruskal-Wallis ($P < 0.05$) were used to establish differences on mortality and brood production, because data violated the premises of the ANOVA after variable transformation $[\ln(x + 1)]$.

CHAPTER IV: RESULTS

4.1 EXPERIMENT 1: The role of annual flowering plant strips on a melon crop in Central Spain. Influence on pollinators and crop.

4.1.1 Bloom period and flower coverage in the flower strips.

The floral coverage together with the precipitation and the daily mean, maximum-mean and minimum-mean temperatures during the sampling period in both farms, are presented in Figure 13.

4.1.1.1 La Poveda experimental plots.

The year 2013 was rainier (145.9 mm) than 2014 (65.4 mm) and milder (mean $20.2 \pm 3.0^\circ\text{C}$; maximum mean $34.0 \pm 2.4^\circ\text{C}$) than 2014 (mean $21.4 \pm 1.9^\circ\text{C}$; maximum mean $29.1 \pm 2.0^\circ\text{C}$), which also had a more delayed spring (minimum mean temperature up to mid May was $5.9 \pm 2.6^\circ\text{C}$).

The bloom sampling period of the flower strip lasted 13 weeks both years (Figure 13). In the first bloom period, in terms of floral coverage, *D. virgata* and *C. sativum* were predominant in 2013 and *C. sativum* and *L. maritima* in 2014. Due to an error by the seed supply company, in 2014 a different species of *Diplotaxis* emerged: *D. catholica* L., with a longer bloom, yet a height and coverage considerably lower than that of *D. virgata*. Over the two years, the most flower rich species in the second and third bloom periods were *C. officinalis* and *B. officinalis*. In the second period of 2013, there was competition for space among some plant species: *D. virgata* produced a huge amount of dried matter, and the floral coverage of *C. officinalis* and *B. officinalis* was much lower than in 2014. *Medicago sativa* reached the maximum bloom in the third period, coinciding with the melon bloom. The plant species with the highest floral coverage in the flower strip had open nectaries, except *B. officinalis* and *M. sativa*, which had partly concealed or concealed nectaries, respectively. The rest of species contributed less to the floral coverage (Figure 15).

4.1.1.2 *Commercial field at Corral de Almaguer.*

The year 2014 was drier in this area compared to La Poveda (25.87 mm of rain). The mean temperature was (20.09 ± 0.36 °C); the maximum mean temperature 28.49 ± 0.43 °C, and the minimum mean temperature 11.09 ± 0.32 °C.

The bloom period of the flower strip was recorded over seven weeks. Only *C. sativum*, *C. officinalis* and *B. officinalis* reached coverage percentages >1%. The flowering of *C. sativum* (mid-May to mid-June) was not coincident with melon blossom but those of *C. officinalis* (three in floral coverage; Figure 15) and *B. officinalis* (1–2 in flower coverage; Figure 15) were.

Figure 15: Floral coverage in the flower strips of La Poveda experimental plots (Madrid) during the three bloom periods and of the commercial melon field at Corral de Almaguer (Toledo) and meteorological conditions (temperature and precipitation, SIAR, 2018). The plants in bold were used in the statistical analyses of comparison between visitor groups and plant species each year.

4.1.1 *Visitors.*

During the bloom period of the flower strips and crop, the destructive sampling with sweep nets and pan traps illustrated the great richness of insects visiting the flowers: bees (69 species from 20 genera), beetles (19 species) and hoverflies (nine species) (Table 3). In the bee group, the families Apidae, Megachilidae, Andrenidae and Halictidae were well represented. The identified visitors in the melon flowers from the destructive sampling with the sweep net are shown in bold in Table 3.

Table 3: Visitor species identified after destructive sampling (sweep net and pan traps) in experimental and commercial melon fields of Central Spain.

Beetles			
Bruchidae	<i>Spermophagus</i> sp.	Curculionidae	<i>Apion</i> sp. ¹
	<i>Anthaxia anatolica</i> Chevrolat 1837 ¹	Dasytidae	<i>Enicopus calcaratus</i> Kiesenwetter 1859 ¹
Cerambycidae	<i>Agapanthia cardui</i> (Linnaeus 1767) ¹		<i>Psilothrix viridicoerulea</i> (Geoffroy 1785) ¹
	<i>Agapanthia annularis</i> (Olivier 1795) ¹	Dermestidae	<i>Attagenus fasciatus</i> (Thunberg 1795) ¹
	<i>Certallum ebulinum</i> (Linnaeus 1767) ¹	Meloidae	<i>Cerocoma schaefferi</i> (Linnaeus 1758) ¹
Cetoniidae	<i>Oxythyrea funesta</i> (Poda 1761) ¹	Nitidulidae	<i>Meligetes</i> sp. ¹
	<i>Tropinota hirta</i> (Poda 1761) ¹	Oedemeridae	<i>Oedemera podagrariae</i> (Linnaeus 1767) ¹
Chrysomelidae	<i>Altica</i> sp. ¹		<i>Oedemera simplex</i> (Linnaeus 1767) ¹
	<i>Clytra</i> sp. ¹	Tenebrionidae	<i>Heliotaurus ruficollis</i> (Fabricius 1781) ^{1,2}
Coccinellidae	<i>Coccinella septempunctata</i> Linnaeus 1758 ^{1,2}		
Hoverflies			
Syrphidae	<i>Ceriana vespiformis</i> (Latreille 1804) ¹		<i>Scaeva</i> sp. ^{1,2}
	<i>Episyrphus balteatus</i> (De Geer, 1776) ^{1,2}		<i>Sphaerophoria scripta</i> (Linnaeus, 1758) ^{1,2}
	<i>Eristalis tenax</i> (Linnaeus, 1758) ^{1,2}		<i>Sphaerophoria rueppellii</i> (Weidemann, 1820) ¹
	<i>Eupeodes corollae</i> (Fabricius, 1794) ²		
Long-Tongue (L-T) bees			
Apidae	<i>Amegilla quadrifasciata</i> (de Villers, 1789) ^{1,2}		<i>Eucera notata</i> Lepeletier, 1841 ¹
	<i>Anthophora agama</i> Radoszkowski, 1869 ¹		<i>Habropoda zonatula</i> Smith, 1854 ¹
	<i>Anthophora atroalba</i> Lepeletier, 1841 ¹		<i>Xylocopa violacea</i> (Linnaeus, 1758) ^{1,2}
	<i>Anthophora fulvotarsis</i> Brullé, 1832 ¹	Megachilidae	<i>Anthidium florentinum</i> (Fabricius, 1775) ¹
	<i>Apis mellifera</i> Linnaeus, 1758 ^{1,2}		<i>Coelioxys echinata</i> Förster, 1853 ^{*,1}
	<i>Bombus terrestris</i> (Linnaeus, 1758) ^{1,2}		<i>Hoplitis antigae</i> (Pérez, 1895) ^{*,1}
	<i>Ceratina chalcites</i> Germar, 1839^{*,1}		<i>Hoplitis</i> sp. ^{*,1}
	<i>Ceratina cucurbitina</i> (Rossi, 1792)^{*,1,2}		<i>Megachile pilidens</i> Alfken, 1924 ^{1,2}
	<i>Ceratina nigrolabiata</i> Friese, 1896 ¹		<i>Megachile rotundata</i> (Fabricius 1787) ¹
	<i>Eucera elongatula</i> Vachal, 1907 ^{1,2}		<i>Megachile versicolor</i> Smith, 1844 ¹

(Continued)

Table 3. Continued

Small Short Tongue (S-T) bees			
Andrenidae	<i>Andrena bicolor</i> Fabricius, 1775 ²		<i>Halictus maculatus</i> Smith, 1848 ¹
	<i>Andrena djelfensis</i> Pérez, 1895 ^{1,2}		<i>Halictus seladonius</i> (Fabricius, 1794) ¹
	<i>Andrena tenuistriata</i> Pérez, 1895 ¹		<i>Halictus tetrazonius</i> Klug in Germar, 1817 ¹
	<i>Andrena</i> sp.1 ¹		<i>Halictus</i> sp. 1 ¹
	<i>Andrena</i> sp.2 ¹		<i>Halictus</i> sp. 2 ¹
	<i>Panurgus calcaratus</i> (Scopoli, 1763) ¹		<i>Lasioglossum discum</i> (Smith 1853) ¹
	<i>Panurgus canescens</i> Latreille, 1811 ¹		<i>Lasioglossum leucozonium</i> (Schrank, 1781) ^{1,2}
	<i>Panurgus</i> sp. ¹		<i>Lasioglossum clypeare</i> (Schenck 1853) ¹
Halictidae	<i>Ceylalictus variegatus</i> (Fabricius, 1798) ¹		<i>Lasioglossum malachurum</i> (Kirby, 1802)^{1,2}
	<i>Halictus crenicornis</i> Blüthgen 1923 ¹		<i>Lasioglossum minutulum</i> (Schenck 1853)^{1,2}
	<i>Halictus gemmeus</i> Dours, 1872 ¹		<i>Sphecodes croaticus</i> Meyer 1922 ¹
Large Short Tongue (S-T) bees			
Andrenidae	<i>Andrena albopunctata</i> ssp. melona Warncke,		<i>Halictus quadricinctus</i> (Fabricius, 1776) ^{1,2}
	<i>Andrena bicolorata</i> (Rossi, 1790) ¹		<i>Halictus rubicundus</i> (Christ, 1791) ¹
	<i>Andrena bimaculata</i> (Kirby, 1802) ¹		<i>Halictus scabiosae</i> (Rossi, 1790) ^{1,2}
	<i>Andrena carbonaria</i> (Linnaeus, 1767) ^{1,2}		<i>Halictus tridivisus</i> Blüthgen, 1924 ¹
	<i>Andrena flavipes</i> Panzer, 1799¹		<i>Lasioglossum aegyptiellum</i> (Strand, 1909) ¹
	<i>Andrena florea</i> Fabricius, 1793 ¹		<i>Lasioglossum albocinctum</i> (Lucas 1846)^{1,2}
	<i>Andrena nigroaenea</i> (Kirby, 1802) ¹		<i>Lasioglossum pygmaeum</i> (Schenck, 1853)^{1,2}
	<i>Andrena ovatula</i> (Kirby, 1802) ¹		<i>Pseudapis bispinosa</i> (Brullé, 1832) ^{1,2}
	<i>Andrena thoracica</i> (Fabricius, 1775) ^{1,2}		<i>Pseudapis diversipes</i> (Latreille 1806) ¹
	<i>Panurgus banksianus</i> (Kirby, 1802) ¹		<i>Sphecodes albilabris</i> (Fabricius, 1793) ¹
	<i>Halictus asperulus</i> Pérez, 1895 ¹		<i>Sphecodes gibbus</i> (Linnaeus, 1758) ¹
Halictidae	<i>Halictus consobrinus</i> (Perez, 1895) ¹		<i>Sphecodes gibbus</i> (Linnaeus, 1758) ¹
	<i>Halictus crenicornis</i> Blüthgen, 1923 ^{1,2}	Melittidae	<i>Dasypoda visnaga</i> (Rossi, 1790) ²
	<i>Halictus fulvipes</i> (Klug, 1817) ¹		

Visitors were classified in five groups [beetles, hoverflies, L-T bees (Apidae and Megachilidae species), small S-T bees (Andrenidae and Halictidae species ≤ 1 cm) and large S-T bees (Andrenidae, Halictidae and Melittidae species > 1 cm)]. Bees were categorized according to the size and length of the proboscides (Michener, 2007): S-T= short tongue and L-T= long tongue bees. *Species considered for statistical analysis within S-T bees prior to identification, due to their small size. ¹Species present in La Poveda, Madrid; ²Species present in Corral de Almaguer, Toledo. Melon visitors captured with the sweep net are in bold. The taxonomic species name follows Atlas Hymenoptera (2017) and Ortiz-Sánchez (2011).

4.1.1.1 *La Poveda experimental plots.*

In the flower strips, significant differences were found between visitor groups, plant species and their interaction in all blooming periods and years (Table 4). The most visited plants for each pollinator group are shown in Figure 16. In every bloom period and year, the different plant species were visited significantly more by certain visitor groups. In the first bloom period of 2013, *D.*

virgata was highly attractive to small S-T bees and hoverflies, and *B. officinalis* to small S-T bees. In 2014, however, *D. virgata* was not present in the flowering plant mix and the small S-T bees preferentially visited *C. sativum*. In the second bloom period, *B. officinalis* and *C. officinalis* were the most attractive plant species to small S-T bees in 2013 but in 2014, *B. officinalis* was highly visited by L-T bees, and *C. officinalis* by both small and large S-T bees. In the third bloom period, *B. officinalis* was the most visited plant by small S-T bees in 2013, and in 2014, the highest number of visits of small and large S-T bees was recorded in *C. officinalis* (Figure 16). Number of flowers in the plant species also affected the pollinator visits and those with the highest number of flowers received more visits (Table 4).

Table 4: Influence of visitor groups and plant species on the number of visits to the flower strips of La Poveda experimental plots in the different years and bloom periods.

	1 st Bloom period			2 nd Bloom period			3 rd Bloom period		
2013	df	F	P	df	F	P	df	F	P
Visitor groups (V)	4,71.2	32.15	<0.001	4,39.1	79.88	<0.001	2,22.9	68.46	<0.001
Plant species (S)	3,101.5	5.70	0.001	3,39.9	35.89	<0.001	3,26.2	141.33	<0.001
V x S	12,71.9	19.55	<0.001	12,39.0	7.26	<0.001	6,22.9	20.94	<0.001
N flowers	1,141.8	37.01	<0.001	1,41.5	10.18	0.002	1,90.5	70.38	<0.001
2014									
Visitor groups (V)	4,48.4	3.95	0.007	4,37.4	18.17	<0.001	2,59.8	39.21	<0.001
Plant species (S)	3,72.8	4.03	0.010	3,46.8	57.99	<0.001	3,64.3	24.46	<0.001
V x S	12,48.3	3.06	0.003	12,37.3	18.48	<0.001	6,59.8	10.47	<0.001
N flowers	1,135.9	5.24	0.024	1,110.0	24.02	<0.001	1,96.8	17.99	<0.001

Visitor groups: beetles, hoverflies, small (< 1cm) short-tongued (S-T); large S-T and long-tongued (L-T) bees. Means are observations of 3 blocks (6 marked areas/block during 3 minutes). Linear mixed-effects model; $P < 0.05$. Number of flowers of each plant species included as covariate.

In general, beetles appeared early in the season in our area, were abundant in the first bloom period of the flower strip in La Poveda in 2014 (25% of visits), and their populations lowered in the second period (6%). They practically disappeared in the third period, which coincided with the melon bloom. Their lowest abundance was recorded in 2013 (8%), the year with the longest and coolest winter and the latest flower strip bloom. Hoverflies followed a similar pattern to that of beetles, but their abundance was generally much lower. The L-T bees and the large S-T bees were especially abundant in the second and third bloom periods. In general, the small S-T bee group had the highest number of visits in flowering plant strips in all bloom period and years (32%–79%) (Figure 16).

Figure 16: Visits (mean $\pm$ SE) of beetles, hoverflies, small (< 1cm) short-tongued (S-T), large S-T and long-tongued (L-T) bees to different plants of a flower strip in La Poveda experimental plots, in different years and bloom periods. Means are observations of 3 blocks (6 marked areas (1 x 1 m²)/plot, 3 minutes/marked area, 18 min in total) and those followed by the same letter are not significantly different within bloom periods and years. Linear mixed-effects model; Fisher's LSD post hoc; $P < 0.05$. Number of flowers of each plant species was included as covariate. The pie-charts show the percentage of the different visitor groups or plant species within bloom periods and years.

In the melon crop, bee species (L-T, small S-T, large S-T bees) were the only visitors of melon flowers (Figure 17) and significant differences were detected among visitor groups both years (2013: $F_{2,87.6} = 232.8$, $P < 0,001$; 2014: $F_{2,12.1} = 17.4$, $P < 0,001$; Figure 17). Only in 2013 did the flower strip acted as facilitator and the total number of visits was significantly higher in the flower melon plots with flower strips (2013: $F_{1,87.6} = 5.70$, $P = 0.019$; 2014: $F_{1,12.1} = 0.05$, $P = 0.823$; Figure 17). The interaction visitor group- treatment was not significant in either year (2013: $F_{2,87.6} = 0.44$, $P = 0.664$; 2014: $F_{2,12.1} = 0.17$, $P = 0.849$). The visits of small S-T bees were the most abundant in both melon control plots and melon with flower strip plots.

Figure 17: Bee visits (mean $\pm$ SE) to melon flowers in control plots or plots with flower strip of La Poveda experimental farm. *Indicates significant differences between treatments. NS= non-significant difference. Bee groups: small (< 1cm) short-tongued (S-T); large S-T and long-tongued (L-T) bees. Means are observations of 3 blocks (3 transects/plot, 3 min/transect, 9 min in total) and those followed by the same letter are not significantly different within years. Linear mixed-effects model, Fisher's LSD post hoc, $P < 0.05$.

4.1.1.2 Commercial field at Corral de Almaguer.

Bee visitation rate in the flower strip was significantly higher than in the melon flowers where they were significantly affected by the distance to the flower strip ($\chi^2_{(df)} = 1117.5_{(7)}$, $P < 0,001$, Figure 18). Visitation rate to the melon flowers decreased with the increase in distance to the flower strip and were

significantly higher at the first distance (1.75 m) compared to other distances (10.5 to 100 from the flower strip) except for the third (19.25 m).

Figure 18: Bee visitation rate (mean visits·flower⁻¹ ± SE) in the flower strip and at different distances of the melon crop in the commercial field of Corral de Almaguer (Central Spain). Means are observations per flower and transect 15 m in length during 3 min, and those followed by the same letter within each distance are not significantly different. Generalized linear mixed model; Tukey-tests post hoc; $P < 0.05$.

4.1.2 Melon quality and production.

In general, both in the small experimental plots at La Poveda and in the commercial field at Corral de Almaguer, we did not find any statistically significant difference in the production (fruit yield in tons per ha) or in the quality parameters of the melon fruits (fruit weight in Kg; fruit diameter and fruit length in cm; flesh thickness in cm; flesh firmness in Newtons; % juiciness; °BRIX; pH; placenta plus seed weight in g and/or number of seeds) in the years of the study between control melon plots and plots with flower strips or between or distance to the flower strip. In 2014, in the experimental plots at La

Poveda, control melon plots yield more fruit tons ha⁻¹ (31.0 ± 0.2) than flower plots (24.4 ± 0.1) ($t = -2.43$, $P = 0.024$) with a fruit weight (Kg) significantly higher (Control plots: 2.3 ± 0.1 ; Flower plots 2.1 ± 0.1) ($W = 1482.5$, $P = 0.015$). In 2013 in La Poveda and in 2014 in the commercial field of Corral de Almaguer, no statistically significant difference was detected.

4.2 EXPERIMENT 2: Pesticide residues in nectar and pollen of commercial melon fields and their effects on *Bombus terrestris* micro-colonies.

4.2.1 *Pesticides found in the melon flowers.*

The results of the multi-residual analyses of pesticides in melon flowers are shown in Table 5. The frequency of appearance of each compound in the samples is shown in Table 5 and Figure 19.

A total of nine insecticides, nine fungicides and one herbicide were detected in the pollen and nectar collected of the five commercial melon fields. The mean concentration found was variable among active ingredients, in a range of 3 to 480 ppb. Pesticides residues were mainly found in pollen and at a higher concentration than in nectar. Only three insecticides were detected in both pollen and nectar (acetamiprid, imidacloprid, chlorpyrifos) and only one fungicide (myclobutanil) was exclusively found in nectar. Farmers had only applied during the current crop cycle, eight out of the 19 detected pesticides. The highest concentrations detected in flowers corresponded to foliar spraying applications (acetamiprid, imidacloprid, boscalid, abamectin, kresoxil-methyl and flonicamid). The most frequent pesticides in the flowers were: acetamiprid, imidacloprid, abamectin, oxamyl, metalaxil-m, and chlorpyrifos, and only the first three had been applied by the farmers in the current crop cycle (Appendix 1). The most probable pesticide combinations in the study area were: acetamiprid, metalaxil-m, oxamyl (co-occurrence in the 100% of the fields) then between these three pesticides and imidacloprid (co-occurrence in the 80% of the fields) and in the next place these four pesticides plus chlorpyrifos and azoxystrobin (co-occurrence in the 60% of the fields) (Figure 19).

Table 5: Concentration, frequency and application information on the pesticides detected in the pollen and nectar of the melon crop (see details on application treatments in the Appendix 1). In black pesticides selected for the chronic bioassay with *Bombus terrestris* micro-colonies.

Compound	Pesticide type	N of fields sprayed	Days between field application and pollen/nectar surveys	N of fields where the pesticide was detected	Concentration (ppb) in melon flowers (mean±S.E.)		Frequency (% samples)
					Pollen	nectar	
acetamiprid (A)	insecticide	5	2 - 11	5	482.93 ± 215.85	6.41 ± 3.53	100.00%
imidacloprid	insecticide	4	45 - 71 ^b	4	369.36 ± 186.31	15.34 ± 7.62	66.70%
oxamyl (O)	insecticide	-	-	5	< 3^c	0	46.70%
metalaxil-m	fungicide	-	-	5	< 3 ^c	0	46.70%
chlorpyrifos (C)	insecticide	-	-	3	3.97 ± 0.93	1.45 ± 1.45	40.00%
abamectin	insecticide-acaricide	2	2 - 5	2	32.67 ± 12.83	0	40.00%
azoxystrobin	fungicide	-	-	3	5.92 ± 2.92	0	36.70%
myclobutanil	fungicide	4	2 - 15	2	0	5.58 ± 0.70	26.70%
boscalid	fungicide	2	5 ^b	2	266.38 ± 152.77	0	26.70%
flonicamid	insecticide	1	18	1	27.10 ± 6.08	0	20.00%
atrazina ^a	herbicide	-	-	1	5.10 ± 0.74	0	20.00%
quinomethionato ^a	fungicide	-	-	1	52.50 ± 19.62	0	16.70%
cloranthraniliprol	insecticide	-	-	2	5.65 ± 2.65	0	13.30%
difenoconazole	fungicide	-	-	2	3.80 ± 0.70	0	13.30%
kresoxim-methyl	fungicide	2	5 ^b	1	29.60 ± 15.20	0	13.30%
chlorothalonil	fungicide	-	-	1	25.85 ± 5.75	0	13.30%
thiacloprid	insecticide	-	-	1	< 3 ^c	0	13.30%
alfa-cypermethrin	insecticide	-	-	1	153	0	6.70%
quinoxifen	fungicide	3	2 - 11	1	< 3 ^c	0	6.70%
triadimenol	fungicide	1	5	0	0	0	0%

^a Unauthorized pesticides in Spain according to the phytosanitary products registry (MAPA, 2017).

^b Timing of application unknown for one of the 5 fields.

^c < LOQ: Limit of quantification.

Figure 19: Co-occurrence network of pesticide residues detected in pollen and nectar samples of 5 conventional commercial melon fields (n=15). Node sizes indicate the pesticide frequency. Links between pesticides represent the probability of co-occurrence. We performed a co-occurrence analysis between the active ingredients found in the melon flowers using a probabilistic model for pair-wise patterns and the co-occur package in R.

Details of dissipation time in soil, octanol-water partition coefficient and solubility in water of pesticides found in melon flowers are reported in Table 6. These parameters are important for the evaluation of the pesticide persistence, bioaccumulation and solubility.

Table 6: Soil degradation, octanol-water partition coefficient and solubility in water for the pesticides found in the melon flowers. Data from Bio-Pesticide database website (PPDB, 2019).

Active ingredient	Pesticide class	Systemic properties	Soil degradation (days) DT ₅₀ (lab 20 °C) ^b	Octanol-water partition coefficient: Log K _{ow} (pH 7, 20°C) ^c	Solubility in water at 20 °C (mg l ⁻¹) ^d
acetamiprid	neonicotinoid insecticide	yes	1.60	0.80	2,950
imidacloprid	neonicotinoid insecticide	yes	187	0.57	610
oxamyl	carbomate insecticide	yes	5.30	-0.44	148,100
metalaxil-m	acylalanines fungicide	yes	6.50	1.71	26,000
chlorpyrifos	organophosphate insecticide	no	386	4.70	1.05
abamectin	avermectins acaricide	no	25.30	4.40	0.020
azoxystrobin	methoxyacrylates fungicide	yes	84.50	2.50	6.7
myclobutanil	triazole fungicide	yes	365	2.89	132
boscalid	pyridine-carboxamide fungicide	no	484.40	2.96	4.6
flonicamid	flonicamid insecticide	yes	1.10	-0.24	5,200
atrazina ^a	triazine herbicide	yes	66	2.70	35
quinomethionato ^a	quinoxaline fungicide	no	3*	3.78	1
cloranthraniliprol	diamides insecticide	no	597	2.87	0.88
difenoconazole	triazole fungicide	yes	130	4.36	15.0
kresoxim-methyl	oximino-acetates fungicide	no	0.87	3.40	2.0
chlorothalonil	chloronitriles fungicide	no	3.53	2.94	0.81
thiacloprid	neonicotinoid insecticide	yes	300	1.26	184
alfa-cypermethrin	pyrethroid insecticide	no	23.40	5.80	0.004
quinoxifen	aryloxyquinoline fungicide	yes	308	5.10	0.047
triadimenol	triazole fungicide	yes	136.7	3.18	72

^a Unauthorized pesticides at present in Spain, according to the phytosanitary product registration (MAPA, 2017).^b < 30 = Non-persistent; 30 - 100 = Moderately persistent; 100 - 365 = Persistent; > 365 = Very persistent.^c < 2.7 = Low bioaccumulation; 2.7 - 3 = Moderate; > 3.0 = High.^d < 50 = Low solubility; 50 - 500 = Moderate; > 500 = High.* DT₅₀ residue degradation under aerobic field conditions was not available and typical DT₅₀ was reported.

4.2.2 *Effects on Bombus terrestris micro-colonies.*

4.2.2.1 *Pesticide mixture selected and chemicals.*

Two of the most frequent pesticide residues found in the pollen and nectar samples analysed, coming from products with different mode of action, were selected for the assays (acetamiprid a neonicotinoid and nicotinic acetylcholine receptor (nAChR) competitive modulator; and chlorpyrifos a organophosphate and acetylcholinesterase (AChE) inhibitor). Additionally we also selected the pesticide oxamyl (a carbamate and acetylcholinesterase (AChE) inhibitor) because it was detected in all sampling fields. Commercial formulations of these pesticides registered for pest control in melon in Spain (MAPA, 2017) were used and tested at the concentration levels found in the nectar and pollen (Table 5).

4.2.2.2 *Worker mortality.*

Over the 11 weeks of the experiment, a total of 13 worker bees died after chronic exposure to 3 pesticides and their mixtures. Accordingly, the mortality was not significantly different among treatments ($\chi^2 = 6.96$, $df = 7$, $P = 0.43$) and the average was 95.4%.

4.2.2.3 *Pollen and syrup collected.*

No significant differences were found in the total amounts of pollen and syrup collected during the bioassay period (pollen: $F_{7, 49} = 0.84$, $P = 0.56$; syrup: $F_{7, 49} = 0.54$, $P = 0.1$; Figure 20).

4.2.2.4 *Brood and male production.*

The male number and brood production after 11 weeks of exposure to the cocktail of pesticides was no significant different among treatments (male: $F_{7,49} = 0.67$, $p = 0.70$; brood production: $F_{7,49} = 0.34$, $P = 0.70$, Figure 20). The thorax width (inter-tegulae span) in the males emerged last week was not different among treatments either ($F_{7,163} = 2.04$, $P = 0.06$, Figure 20).

Figure 20: Total pollen collected (g) (a); total syrup collected (ml) (b) and total number of males produced (c) by the micro-colonies throughout the 11-week period study. Thorax male width (mm) (d); number of egg cells during the first week (e) and brood production (f) in every treatment (A= acetamiprid, n:6; C= clorphyrifos, n:8; O= oxamyl, n:8; A+C= n:7; A+O= n:7; C+O= n:7; A+C+O= n:8; CONTROL= n:6). Boxplots indicate the lower quartile, median and upper quartile, with whiskers extending to the most extreme data point that is no more than 1.5 times the interquartile range from the edge of the box. Not significantly differences (Fisher's LSD post hoc; $p < 0.05$) were found in any of the evaluated parameters.

4.3 EXPERIMENT 3: Chronic oral exposure to field-realistic pesticide combinations via pollen and nectar: effects on feeding and thermal performance in the solitary bee *Osmia bicornis*.

4.3.1 *Survival and longevity.*

Chronic exposure to the three pesticides and their mixtures at field-realistic concentrations had no effect on survival and longevity of *Osmia* females. Cumulative survival curves did not differ significantly among treatments (Log Rank test: $F = 6.53$, $df = 7$, $P = 0.42$, Figure 21). Longevity (overall mean = 16.32 ± 0.86 days) did not significantly differ among treatments (GLM: $F = 1.22$, $df = 7$, $P = 0.30$), and was not influenced by body size (GLM: $F = 0.03$, $df = 1$, $P = 0.89$). There were no differences among treatments in body size (ANOVA: $F = 0.746$; $df = 7$; $P = 0.63$, Table 7).

Figure 21: Cumulative survival probability in *O. bicornis* females chronically exposed to eight pesticide oral treatments at field-realistic concentrations. A: acetamiprid, I: imidacloprid, M: myclobutanil. Log Rank test: $\chi^2 = 6.53$, $df = 7$, $P = 0.48$.

4.3.2 Syrup and pollen consumption.

In all treatments containing imidacloprid (I, A+I, I+M, A+I+M), bees consumed approximately 80% less syrup per day than in the rest of the treatments including the control (GLM: $F = 38.16$, $df = 7$, $P < 0.001$; Figure 22a). The effect of imidacloprid on syrup consumption began on day 2 (Figure 22b); differences among treatments were not significant on day 1; (GLM: $F = 0.52$, $df = 7$, $P = 0.82$). Body size affected syrup consumption (GLM: $F = 4.22$, $df = 1$, $P = 0.04$), with larger bees tending to consume more syrup in all the treatments except M and A+I.

Figure 22: Mean ($\pm$ SE) daily syrup consumption ($\mu\text{l} \cdot \text{day}^{-1}$) (a) and syrup consumption ($\mu\text{l} \cdot \text{bee}^{-1}$) over time until 50% mortality (b) in *O. bicornis* females chronically exposed to eight pesticide oral treatments at field-realistic concentrations. A: acetamiprid, I: imidacloprid, M: myclobutanil. Means with the same letter are not significantly different (Fisher's LSD post hoc; $P < 0.05$).

Daily pollen consumption ranged between 1 and 4 mg per bee during the first week of exposure, and then abruptly decreased in all treatments (Figure 23b). We found significant differences between these two periods (GLMM: $F = 137.97$, $df = 1$, $P < 0.001$) and among treatments (GLMM: $F = 3.62$, $df = 7$, $P = 0.002$), as well as a significant interaction between period and treatment (GLMM: $F = 3.41$, $df = 7$, $P = 0.002$) (Figure 23a). During period 1, only bees of treatment M consumed significantly less pollen than control bees whereas, in period 2, pollen consumption was significantly low in all treatments compared to the control (Figure 23a). Body size had no effect on pollen consumption (GLMM: $F = 0.30$, $df = 1$, $P = 0.59$).

The total amounts of pesticide ingested via syrup and pollen by bees of each treatment throughout the entire exposure are reported in Table 7. In all cases, exposure via syrup was three orders of magnitude higher than exposure via pollen.

Figure 23: Mean ($\pm$ SE) daily pollen consumption (mg day^{-1}) (a) and pollen consumption (mg bee^{-1}) over time until 50% mortality (b) in *O. bicornis* females chronically exposed to eight pesticide oral treatments at field-realistic concentrations. A: acetamiprid, I: imidacloprid, M: myclobutanil. Period 1: first week; Period 2: remainder of the bioassay. Means with the same letter are not significantly different (Fisher's LSD post hoc; $P < 0.05$).

Table 7: Body weight and amount of active ingredient ingested via syrup and pollen in *O. bicornis* females exposed to various pesticide combinations (treatments) throughout their adult life span (chronic exposure). A: acetamiprid, I: imidacloprid, M: myclobutanil. Period 1: first week; Period 2: remainder of the bioassay.

Treatment	n bees	Body weight (mean $\pm$ SE mg)	Acetamiprid (mean ng bee ⁻¹)				Imidacloprid (mean ng bee ⁻¹)				Myclobutanil (mean ng bee ⁻¹)	
			Syrup ¹	Pollen ²		Total	Syrup ¹	Pollen ²		Total	Syrup ¹	
				Period 1	Period 2			Period 1	Period 2			
A	20	70.67 $\pm$ 1.87	2.88	0.007	0.0003	2.8902						
I	16	68.93 $\pm$ 1.58					1.63	0.008	0.0006	1.6375		
M	17	71.09 $\pm$ 2.03									3.42	
A+I	13	69.08 $\pm$ 2.85	0.58	0.005	0.00004	0.5883	1.40	0.004	0.00003	1.4002		
A+M	16	72.31 $\pm$ 1.72	3.34	0.01	0.0003	3.3520					2.91	
I+M	12	71.50 $\pm$ 2.37					1.59	0.008	0.0003	1.5999	0.58	
A+I+M	14	66.72 $\pm$ 2.16	0.53	0.007	0.0002	0.5356	1.27	0.005	0.0001	1.2707	0.46	
CONTROL	13	68.19 $\pm$ 3.01										

¹Total active ingredient consume in the syrup (ng bee⁻¹) = daily consumption (μ l bee⁻¹day⁻¹) $\times$ longevity (days) $\times$ concentration (μ g l⁻¹) / 10³

²Total active ingredient consume in the pollen (ng bee⁻¹) = daily consumption (mg bee⁻¹day⁻¹) $\times$ longevity (days) $\times$ concentration (ng g⁻¹) / 10⁶

4.3.3 Thoracic temperature.

Thoracic temperature significantly differed among treatments (Kruskal-Wallis: $\chi^2 = 38.83$, $df = 7$, $P < 0.001$; Figure 24). The lowest temperatures were registered in bees of the four treatments containing imidacloprid (I) although only in treatments I and A+I+M differed significantly from the control (Figure 24). Low temperatures were accompanied by clear signs of apathy in bees of these four treatments. These signs were not observed in any of the other treatments.

Figure 24: Mean ($\pm$ SE) thoracic temperature ($^{\circ}\text{C}$) in *O. bicornis* females after 17 days of chronic exposure to eight pesticide oral treatments at field-realistic concentrations. A: acetamiprid, I: imidacloprid, M: myclobutanil. Means with the same letter are not significantly different (Kruskal-Wallis test followed by Dunn's post hoc; $P < 0.05$).

4.3.4 Ovary maturation.

No significant differences were found in mean basal oocyte length among treatments (GLM: $F = 1.45$, $df = 7$, $P = 0.20$). Oocyte length was positively related to body size in all treatments (GLM: $F = 24.7$, $df = 1$, $P < 0.00$).

4.4 EXPERIMENT 4: Can the development of *Bombus terrestris* micro-colonies be different when they are exposed to imidacloprid via syrup or/and via pollen?

4.4.1 *Worker mortality.*

Over the 11 weeks of the experiment a total of 12 worker bees died after chronic exposure to field realistic concentrations of imidacloprid via nectar or/and pollen. The mortality was significantly different across treatments ($\chi^2 = 8.79$, $df = 3$, $P = 0.03$) and the averages per treatment were: 13.3 % in the control, 0% in N, 4.4% in P and 5% in N+P treatments. The mortality in the control was significantly different to that of N treatment ($P = 0.028$). Validation criteria of the Organisation for Economic Co-operation and Development (OECD) guideline to chronic oral toxicity test (OECD, 2017) were followed (control mortality <15%).

4.4.2 *Pollen and syrup collected.*

In treatments with bee exposure to imidacloprid via syrup (N and N+P), adult bees collected approximately 20% less syrup and 50% less pollen (total syrup collected: $F_{3,30} = 3.70$, $P = 0.022$, Figure 25a; total pollen collected $F_{3,30} = 22.03$, $P < 0.001$, Figure 25b). In treatments with bee exposure to imidacloprid via pollen, no significant differences were found in the total pollen and syrup amounts collected compared with control micro-colonies. Differences were neither detected between micro-colonies exposed via nectar and those exposed by the double via of nectar and pollen (Figure 25).

Figure 25: Mean ($\pm$ SE) of total syrup (a) and pollen (b) collected in bumblebee micro-colonies throughout the 11 week study period: Control (untreated syrup and pollen; $n=10$), Nectar (chronic exposure to imidacloprid via treated syrup at 6 ppb; $n=7$), Pollen (chronic exposure to imidacloprid via treated pollen at 30 ppb; $n=9$) and Nectar+Pollen (chronic exposure to imidacloprid via treated syrup and pollen; $n=8$). Means with the same letter are not significantly different (Fisher's LSD post hoc; $P < 0.05$).

4.4.3 Brood and male production.

Brood production was significantly different among treatments ($F_{3,30} = 7.37$, $P = 0.001$, Figure 26). It was lower in micro-colonies exposed to imidacloprid via nectar (N and N + P) compared to those micro-colonies where had been exposed to imidacloprid via pollen and to control ones. Between these last two treatments no significant differences were found (Figure 26).

Figure 26: Brood production in bumblebee micro-colonies throughout the 11 weeks study period from each treatment: Control (untreated syrup and pollen; n=10), Nectar (chronic exposure to imidacloprid via treated syrup at 6 ppb; n=7), Pollen (chronic exposure to imidacloprid via treated pollen at 30 ppb; n=9) and Nectar+Pollen (chronic exposure to imidacloprid via treated syrup and pollen; n=8). Data followed by the same letter are not significantly different (Fisher's LSD post hoc; $p < 0.05$).

The number males after micro-colonies exposure for 11 weeks to imidacloprid was also significant different depending on the via of contamination ($F_{3,30} = 5.78$, $P = 0.003$, Figure 27). Imidacloprid exposure via nectar (N and N+P) decreased the number of males in the micro-colonies of *B. terrestris*. No additive effect was found in micro-colonies exposed via nectar and pollen compared to those exposed just via nectar (Figure 27).

Figure 27: Cumulative male production in bumblebee micro-colonies throughout the 11 weeks study period from each treatment: Control (untreated syrup and pollen), Nectar (chronic exposure to imidacloprid via treated syrup at 6 ppb), Pollen (chronic exposure to imidacloprid via treated pollen at 30 ppb) and Nectar+Pollen (chronic exposure to imidacloprid via treated syrup and pollen). Means with the same letter are not significantly different (Fisher's LSD post hoc; $P < 0.05$).

CHAPTER V: DISCUSSION

5.1 EXPERIMENT 1: The role of annual flowering plant strips on a melon crop in Central Spain. Influence on pollinators and crop.

5.1.1 *Selection of suitable flowering plants.*

Flowering plant strips contribute to supply foraging habitats to many pollinators by offering food, shelter and nesting resources. The increased plant diversity and the availability of flowers throughout the season (e.g., plants with staggered and/or precocious bloom) contributes to the enhancement of bee populations over time (Balzan et al., 2014; Blüthgen and Klein, 2011; Haaland et al., 2011; Nicholls and Altieri, 2013; Rosa García and Miñarro, 2014). In our experimental plots, the sequential bloom lasted for 13-14 weeks and allowed the bee, hoverfly and beetle species to visit the flower strips and to use their nectar and pollen resources during two seasons (spring and/or summer). The beginning of the bloom in the flower strips was earlier than usual in the second year (the mean and the maximum mean temperatures increased during the years of the study) and this affected the pollinator groups associated with the different bloom periods.

The most suitable species in terms of coverage and attractiveness were *C. sativum* and *D. virgata* in spring and *B. officinalis* and *C. officinalis* in summer. All these species are well known for their attractiveness to pollinators when sown as mono-specific plots (Barbir et al., 2015; Bugg et al., 2008; Carreck and Williams, 2002; Hogg et al., 2011) and they behaved also very well in our mix. All these plants had the highest floral coverage, and they were the tallest in the mixture (between 50-100 cm in our case), which probably facilitated the ability of pollinators to find them. They have open or partly concealed nectaries and were visited mostly by S-T bees, also predominant in the melon crop, because the presence of specialised feeding structures in the pollinators (eg. long

proboscis) are not required to obtain a flower reward. However, *B. officinalis* was also visited by a wide range of L-T bees but only before the melon bloom. Some L-T bees are considered more efficient pollinators of *B. officinalis* than the S-T Halictidae species, which did not touch the flower stigma while drinking nectar (Gorenflo et al., 2017).

On the contrary, at both locations (La Poveda and Corral de Almaguer) other species were not considered good candidates for the mixture and were not sown the following years due to several reasons. Pollinators were not to these plants most likely because they did not exhibit some of the required features for attraction (e.g. high floral coverage and/or good plant height) or because the blooming period was short. The species *N. gallica*, *S. verbenaca*, *V. sativa*, *S. vulgaris*, *D. catholica* and *M. sativa* had little contribution to the total coverage due to their low bloom and received fewer visits than the most attractive plants. All these plants, except *N. gallica*, have concealed nectaries, which means extra work to get the reward compared to the most attractive plants in our study (*C. sativum*, *D. virgata*, *B. officinalis*, *C. officinalis*), which have open or partially concealed nectaries. Moreover, the short bloom duration and low height of *N. gallica* and *V. sativa*, also most likely contributed to the low number of visits recorded. In La Poveda, the species *L. maritima*, was sown for the first time in 2014 because it is highly attractive to hoverflies (Colley and Luna, 2000; Pineda and Marcos-García, 2008). This species emerged well and exhibited a high percentage of floral coverage but in agreement with Barbir et al (2015) received almost no visits within the mixture. Its low height (<20 cm) seems to have accounted for this because taller plants hide the visual flower signals of color, which are particularly important for pollinator recognition (Gumbert, 2000).

Other factors to take into account for the success of a given species within a mixture are the speed of senescence, and the final dried mass reached. The

species *D. virgata* ended its development and dried in early summer (end of June - mid July), reaching a large mass that occupied the space needed for the regular growing of the nearby species, therefore making them invisible to the pollinator's eye. In 2014, when the *Diplotaxis* species of lower height was present by error in La Poveda (*D. catholica*), the coverage percentage of *B. officinalis* and *C. officinalis* was higher probably because they had more space for growing and were not shaded by the taller *D. virgata*. On the contrary, the dried mass of other plant species on the contrary, reached a much lower volume.

5.1.2 *Visits to the melon crop and the role of the flower strip as competitor or facilitator.*

In the experimental plots of La Poveda, visits to melon flowers were significantly higher in the melon plots with flower strip only in the year 2013, suggesting that the flower strip was acting as an exporter. In 2014, no statistically significant differences were found between the two types of plots, suggesting that its role was neutral for pollinators. The total flower coverage and attractiveness of the blooming species in the flower strip may have accounted for this. Floral coverage in the flower strips was higher in 2014 during the third bloom period compared to 2013, the year in which significant differences were found, probably because the resources in the flower strips were not enough to satisfy the pollinator needs. On the contrary if resources are high in the flower strips, pollinators would probably not be interested in searching for food in the melon crop due to its less attractive flowers (Bomfim et al., 2016) and therefore, the flower strip could not act as a facilitator. The number of pollinator visits to the flower strip was similar in 2013 and 2014; however, differences in the visits to melon flowers between flower and control plots were only observed in 2013. The attractiveness of *C. officinalis* and *B. officinalis* to the small S-T bee group might have accounted for this. We have focused on this group because it was the most abundant in both the flower strip and the melon

flowers, and because some small bees belonging to the family Halictidae (genus *Lassioglossum* spp.) have been previously identified as a key pollinators of the melon crop in Central Spain, such as the eusocial *L. malachurum* (Kirby, 1802) (Rodrigo Gómez et al., 2016), which is also seen in our study. In 2013 the most visited plant for these bees was *B. officinalis*, which does not have high pollen content and mainly supplies nectar (Carreck and Williams, 1997) and therefore, this could have probably generated an increased in pollinator foraging activity trying to seek pollen in the nearby resources such as the melon crop. The eusocial *Lassioglossum malachurum* (Kirby, 1802) is a major pollen-forager species (Pisanty et al., 2014; Rodrigo Gómez et al., 2016). Contrastingly, according to Hicks et al. (2016), *C. officinalis*, the most visited plant in 2014, produces a lot of both pollen and nectar compared to the other 65 plant species, thus preventing the displacement of bees to the melon crop. In Mediterranean landscapes, it is known that Compositae was the most exploited family for the species *L. malachurum* (Polidori et al., 2010). Besides, melon has a low number of open flowers each day, the flowers are relatively hidden and unattractive to pollinators compared to wildflowers, and only offer a small amount of nectar and pollen (Bomfim et al., 2016). The results highlight the fact that both resource quantity and quality matter to flower visitors (Fowler et al., 2016), because pollinators are able to distinguish between plant species and learn which ones provide the greatest reward (Goulson, 1999).

In the commercial field of Corral de Almaguer, the flower strip was as expected, much more attractive to bees than the nearby melon flowers and this agrees with the results of other studies (Haaland et al., 2011; Hodgkiss et al., 2019; Jönsson et al., 2015; Scheper et al., 2013). The landscape context seems to be important in determining the density of some pollinators in the flower strips, e.g. bumblebees (Heard et al., 2007), which were not very common in our commercial farm. In agreement with Kohler et al (2008) we also found that the

effect of our flower-rich strip was spatially limited. Visits in the first distances in the melon crop (<2m) were higher compared to the farthest distances. The decline of visits to the crop with the increase in distance to natural or semi-natural areas has also been reported (Campbell et al., 2017; Carvalheiro et al., 2010; Kohler et al., 2008). Our finding supports the results of La Poveda in 2014, because the plant with the highest floral coverage in the flower strip of the commercial field was *C. officinalis*, which seemed not to act as exporter of pollinators beyond 2 m.

5.1.3 *Melon productivity and quality.*

The significant differences recorded on the bee visits between flower and control plots in 2013 could have had an influence on the productivity and quality of the melon fruits, as has been shown for other crops (Balzan et al., 2016; Blaauw and Isaacs, 2014; Carvalheiro et al., 2012; Ganser et al., 2018). Nevertheless, the presence of flower strips in our small and commercial plots was not associated with an improvement in melon production or quality. In agreement with our findings, the yield of tomato and pepper (Winfrey et al., 2008), cider orchards (Campbell et al., 2017), cucumber (Quinn et al., 2017) or commercial strawberry (Hodgkiss et al., 2019) was not affected either. Only in the experimental farm in 2014, the yield and weight of melons from the control plots was higher, in spite of the fact that the number of visits were equal between the two kinds of plots. Therefore, this difference seems to be unrelated to flower visitors but with other factors, such as crop management practices, soil quality, etc. The results in the commercial farm could have also been affected by the presence of *A. mellifera* hives, which could have been enough for an optimal melon pollination. Hence, the flower strip would not have offered an extra advantage. However, wild bees can improve pollination services, in spite

of the presence of *A. mellifera* hives (Garibaldi et al., 2013), and this could have happened in our farm.

In some studies effects have been detected in the years following to the strip establishment (Blaauw and Isaacs, 2014). Probably because wild bee populations need time to colonize new habitats (Williams et al., 2001), permanent flowering strips in crop fields when possible would also enhance the presence of pollinators in the area over time. However, this initiative seems to be a challenge in annual crops (e.g. melon) in intensive agro-ecosystems with a rotation period between years (Quinn et al., 2017) because the distance between the flower strip and crop within the farm can exceed its possible area of influence, especially in small bees species, which usually forage within an area of few hundred meters from the nest (Zurbuchen et al., 2010). Further, pollination services in melon fields could be enhanced by including soil patches, alone or in combination with flowers, with adequate features for *Lasioglossum* females to build nests (e.g. compact soil almost avoid of vegetation (Polidori et al., 2010), because species of this genus are key pollinators of the crop.

In conclusion, our study provides a list of S-T and L-T pollinators that visit melon fields in Central Spain and identifies some good plants species of high floral coverage and staggered bloom, to be included in flower strips: *C. sativum*, *D. virgata*, *C. officinalis* and *B. officinalis*. Based on our results, the plant composition in the mixture must be carefully chosen. Even though the present study was not designed to evaluate interspecific competition between flower strip plant species, the shorter plant *L. maritima* remained hidden under the highest plants and received a low number of visits, even though its floral coverage was high. Additionally, *D. virgata* produced a high amount of dry matter, which could have diminished the floral coverage of the nearby species.

Moreover, in choosing the optimal mix, it is also essential to take into account which species support the key crop pollinator taxa, and to facilitate their movement from the flower strip to the crop. In our area, we suggest that the concurrence of blooming in the rewarding *C. officinalis* with the melon crop should be avoided; otherwise the flower strip may not act as a pollinator exporter of the main pollinator taxa to the melon crop. However, further long-term studies with mono-specific flower strips are needed to confirm our hypothesis.

In our area, the presence of the specific flower strips evaluated in experimental and commercial melon farms did not have an influence on melon productivity and quality. However, offering nesting structures and flowering plants on a regional scale might increase bee pollinator populations and so help to provide adequate pollinator services over the years.

5.2 EXPERIMENT 2: Pesticide residues in nectar and pollen of commercial melon fields and their effects on *Bombus terrestris* micro-colonies.

Current pesticide risk assessment schemes for pollinators could not represent a realistic pesticide exposure due to several reasons. Firstly, in acute oral toxicity tests the exposure time is short and only mortality is evaluated, therefore obviating the wide range of sublethal effects that can be detected when bees are chronically exposed to low pesticide concentrations (Azpiazu et al., 2019). Secondly, tests are based on effects of single products while bees in agro-ecosystems can be exposed to multiple pesticides at the same time or along their life cycles (Mullin et al., 2010; Porrini et al., 2016; Sanchez-Bayo and Goka, 2014; Tosi et al., 2018), which may have synergistic effects (Gill et al., 2012; Iwasa et al., 2004; Sgolastra et al., 2017, 2018a, 2018b; Zhu et al., 2017). To our knowledge, this is the first time that a multi-residual analysis of pesticides in pollen and nectar in crop flowers have been tested, while other studies have focused on a few active ingredients or chemical groups (e.g. neonicotinoids) overlooking the multi-pesticide exposure. Our results have revealed that the bees foraging in melon fields of the study area were exposed to an high number of pesticides (nine insecticides, nine fungicides and one herbicide), and we have evaluated their impact of one of the most likely pesticide mixture on *B. terrestris* micro-colonies through a chronic oral toxicity test.

In general, the highest pesticide residue concentrations (above 25 ppb) in melon flowers corresponded to pesticides sprayed by farmers 2-18 days before the flower sample collection (i.e. acetamiprid, imidacloprid, abamectin, boscalid, flonicanid and kresoxim-methyl) suggesting that pesticide applications close to crop bloom period or in blooming have a significant impact on the residue levels. Other studies also found that long time periods between treatment and bloom led to a higher degradation of the pesticide active ingredient (Dively and

Kamel, 2012; Pohorecka et al., 2012). Some fungicides applied by farmers, were not detected in the melon flowers of some of the fields (myclobutanil, kresoxim-methyl, quinoxyfen, triadimenol) because they may have degraded prior to the monitoring of pollen and nectar.

However, residues of eleven active ingredients, not used by farmers during the current crop cycle, were detected in the samples. Pesticides applied to agricultural fields are subject to a number of fate processes including degradation, volatilization followed by off-site vapor drift, or accumulation in soil, and transport to surface or groundwater (Goulson, 2013; Krupke et al., 2012; Sarmah et al., 2004). These final fates make agro-ecosystems as pesticide contaminated environments and from the different reservoirs, where pesticides can last for extended periods of time, pesticides can be moved to the pollen and nectar of crops and therefore, bees can be exposed to them during prolonged periods of time (Figure 28).

Two insecticides (oxamyl and chlorpyrifos) and two fungicides (metalaxil, and azoxystrobin) were present in more than 25% of the samples and had not been applied by farmers. Based on the pesticide parameters shown in Table 6 we discuss their possible origin sources. First, irrigation water seems to have been the contamination route for the highly soluble and non-persistent compounds oxamyl (carbamate insecticide) and metalaxil (acylalanine fungicide). In agreement, oxamyl had been detected in ground water in many parts of the U.S. (EPA, 2000) and metalaxil and oxamyl are two of the most frequent pesticides in surface waters in Ontario, Canada (Struger et al., 2016) due to spray drift and runoff, respectively. On the other hand, accumulation in soil could have been the contamination route for chlorpyrifos, an insecticide with high Log K_{ow} values (i.e. Octanol-water partition coefficient used to measured bioaccumulation chemical properties) and persistence in this medium. Other

studies have been also reported chlorpyrifos loss rates from soil following application (Das et al., 2019; Ngan et al., 2005) or chlorpyrifos volatilization rates from a potato field (Leistra et al. 2006).

Source: designed by Francesca Alessandro.

Figure 28: Possible origin sources of pesticide residues in pollen and nectar of the melon crop. 1) Spray application, 2) Drip irrigation, 3) Soil; 4) drift. Source: designed by Francesca Alessandro.

Even though chlorpyrifos is non-systemic suggesting that there is no translocation to the plant, several surveys in North America (Mullin et al., 2010), Italy (Chiesa et al., 2016; Porrini et al., 2016; Tosi et al., 2018), Spain (Bernal et al., 2011), Uruguay (Pareja et al., 2011), Brasil (Pacífico da Silva et al., 2015) and Egypt (Al Naggar et al., 2015) have also frequently detected chlorpyrifos in bee hive matrices. Moreover, imidacloprid and thiacloprid, two neonicotinoid pesticides particularly persistent in soil (Table 2, Botías et al., 2015; Goulson, 2013), were found in our samples, which suggest that soil

reservoirs could have contributed to the flower contamination. Pesticide exposure in soils is a major concern for bees nesting underground or for those using soil substrates in the nest building as mason bees (Chan et al., 2019; Sgolastra et al., 2019).

Moreover, it is notorious that high concentrations of some pesticides were detected in pollen and nectar of the melon crop, although they had not been applied during the current crop cycle. This is the case of imidacloprid, which use outdoors has been recently banned by the EU (EC, 2018b). The residues found probably come from the application of this pesticide to melon seedlings in the nursery previous to the transplant to the field. There is evidence that the imidacloprid plant uptake is not fast and consequently its residues can accumulate in the pollen (Cowles and Eitzer, 2017) or nectar (Byrne et al., 2014), which could explain our results. In addition, some farmers could have also applied this product in the field without including the application in their pesticide record book. High concentrations levels of *alfa*-cypermethrin and chlorothalonil were also found, and both compounds had not been applied during the current crop cycle. The presence of these pesticides residues in pollen and nectar of the melon crop could also be due to a pesticide misuse. Our results highlight the need that the incorporate measures to control malpractices of pesticide application, farmer's training and awareness (Misganaw et al., 2017) in order to reduce the risk of pesticides.

Pesticide concentrations were one or two orders of magnitude higher in pollen than in nectar of the melon flowers. Our results agree with those of other authors that used similar methodology (Botías et al., 2015; Dively and Kamel, 2012; Kyriakopoulou et al., 2017; Mullin et al., 2010; Stoner and Eitzer, 2012) or even with those who analyzed bee-collected matrices (i.e, pollen and honey) (Sanchez-Bayo and Goka, 2014). The reason of this difference is not clear. In the

melon crop, the higher exposure of the anthers to a foliar pesticide application, could have contributed to a higher residue accumulation in pollen compared to nectaries that are less exposed than anthers (Willmer 2011). Cowles and Eitzer (2017) have also suggested that residues in pollen and nectar are related to the kind of tissue that provision for them: xylem for pollen but phloem, both phloem and xylem or neither for nectaries, depending on the crop. Especially when systemic pesticides are applied, we can expect an influence on the accumulation residues in nectar. We found four pesticide residues in nectar (acetamiprid, imidacloprid, myclobutanil and chlorpyrifos). The first three compounds have systemic properties and were applied by farmers. The remaining compounds have been found only in pollen. However, consumption of pollen is considered to be about 3% that of consumption of nectar in adult bees (USEPA, 2014), so the probability of pollinator contamination by feeding pollen is much lower than by feeding nectar. Among nectar and pollen consumed by larvae, the differences are less remarkable, and therefore, the wide range of pesticides detected in pollen melon flower could have led to the development of important lethal or sublethal effects in larvae bees exposure to them.

The most probable pesticide co-occurrence (>60%) in the study area were between the 4 insecticides (acetamiprid, oxamyl, imidacloprid and chlorpyrifos) and two fungicides (metalaxil-m and azoxystrobin). These studies contrast with those who have analyzed the pollen from bee hives, where acaricides used to control varroa have been the most frequent pesticides (coumaphos and tau-fluvalinate) and chlorpyrifos has been found in 14% of the samples (Sanchez-Bayo and Goka, 2014). So this study is important because shows the most frequent combinations to which other non-*Apis* species can be exposed.

The key question to be answered is whether the detected pesticide residues in pollen and nectar and their combinations are likely to have a significant impact on bees. In our work, the chronic exposure of *B. terrestris* micro-colonies via pollen and nectar contaminated with the concentration levels of acetamiprid, chlorpyrifos, oxamyl and their combinations detected in the commercial melon fields of Central Spain, showed no effect in the pollinator parameters measured (mortality, pollen and syrup collected and reproduction fitness), and no synergy among compounds. In contrast, another most likely combination of pesticides in this study area caused important sublethal effects in the solitary bee *Osmia bicornis* (Azpiazu et al., 2019).

The results of our work provide essential information that should be taken into account when establishing pesticide regulations aiming at guaranteeing the conservation of pollinators. Further research are needed to evaluated the effects of pesticide in the melon agro-ecosystems in order to ascertain the possible implications and synergistic effects with other pesticide combinations found.

5.3 EXPERIMENT 3: Chronic oral exposure to field-realistic pesticide combinations via pollen and nectar: effects on feeding and thermal performance in the solitary bee *Osmia bicornis*.

Bees in agro-ecosystems are chronically exposed to combinations of pesticides (Botías et al., 2017; Tosi et al., 2018). However, the effects of this exposure scenario are not well understood because most laboratory studies test acute exposure to single products at concentrations that often are not field-realistic (Carreck and Ratnieks, 2014). In addition, most studies addressing oral exposure only consider the nectar route, overlooking pesticide ingestion via pollen consumption. We tested chronic exposure to pollen and syrup contaminated with field-measured concentrations of pesticide combinations found in pollen and/or nectar in commercial melon plots. To our knowledge, this is the first time pesticide exposure via pollen is tested in adult solitary bees. Two previous studies have exposed *Osmia* larvae to neonicotinoids via pollen (Abbott et al., 2008; Nicholls et al., 2017). At field-realistic doses, these studies did not find any effects on larval survival or adult performance.

With the exception of myclobutanil, which was not detected in pollen, pesticide concentrations (ppb) were one or two orders of magnitude higher in pollen than in nectar of melon flowers. Other studies measuring pesticide levels from pollen and nectar have found similar results (Botías et al., 2015; Dively and Kamel, 2012; Mullin et al., 2010; Stoner and Eitzer, 2012). However, because solitary bee adults consume much greater amounts of nectar than pollen (ca. 93% of total food weight consumed by bees in our study was via syrup), the amount of active ingredient ingested per bee in our study was about three orders of magnitude higher via syrup than pollen. This is important because some laboratory studies expose bees via syrup to pesticide concentrations found in pollen (Laycock et al., 2012; Zhu et al., 2017), thus exposing bees to doses presumably higher than those encountered by bees under field conditions.

Contrary to other studies testing mixtures of neonicotinoid insecticides and triazole fungicides on *O. bicornis* (Sgolastra et al., 2018a, 2017) and other bee species (Biddinger et al., 2013; Iwasa et al., 2004; Sgolastra et al., 2017; Thompson et al., 2014) we did not find synergistic effects between these two classes of pesticides. This discrepancy may be due to the identity of the compounds involved. In general, cyano-substituted neonicotinoids (including acetamiprid and thiacloprid) show higher synergism than nitro-substituted neonicotinoids (including imidacloprid, clothianidin and thiamethoxam) (Biddinger et al., 2013; Iwasa et al., 2004). However, even within these two subgroups of neonicotinoids differences among compounds have been found. In agreement with our results, Thompson et al. (2014) did not observe synergism between triazole fungicides and imidacloprid but they found synergism between these fungicides and two other nitro-substituted neonicotinoids (clothianidin and thiamethoxam) in honey bees. Differences between our results and those of other studies can also be explained by differences in the route of exposure. Iwasa et al. (2004) and Biddinger et al. (2013) found synergism between triazole fungicides and acetamiprid applied topically, as opposed to orally in our study. Finally, differences between our results and those of other studies may also be explained by differences in the concentrations to which bees were exposed. Synergism between triazole fungicides and neonicotinoids has been shown to be concentration-dependent (Thompson et al., 2014). In our study, the dose of myclobutanil consumed by *O. bicornis* throughout their lifespan in treatment A+M was 2.91 ng bee⁻¹. This dose is 8-153 times lower than the triazole fungicide doses tested in Thompson et al. (2014) (propiconazole: 22.4 ng bee⁻¹; tebuconazole: 447 ng bee⁻¹). In treatments containing imidacloprid (I+M and A+I+M), due to the inhibitory effect of this compound on syrup feeding, the levels of myclobutanil ingested by bees were even lower. Overall, the doses of myclobutanil ingested by *O. bicornis* in our

study are ca. 1000 times lower than the lethal doses estimated by Han et al., (Han et al., 2018) in *Apis cerana* F. (acute oral toxicity: $LD_{50}=2,154$ ng bee⁻¹ and $LD_5=1,085$ ng bee⁻¹).

Following emergence, *Osmia* females undergo a short period (2-5 days) prior to initiating nesting activities (Bosch and Vicens, 2006; Sgolastra et al., 2016). During this period, females consume pollen (Cane, 2016) and complete ovary maturation (Lee et al., 2015; Sgolastra et al., 2016; Wasielewski et al., 2011). The high levels of pollen consumption recorded during the first seven days of exposure in our study are congruent with the results of Cane (2016). During this phase (period 1), treatment M showed significantly lower pollen consumption than control bees. On first sight, the M result may seem surprising because pollen in this treatment was not contaminated (no myclobutanil residues were found in the pollen of melon flowers) (Table 2). However, this treatment resulted in the highest ingestion of myclobutanil via syrup (Table 7). We also found differences in pollen consumption during the second week following exposure. In this case, all treatments yielded significantly lower feeding levels than the control. Nevertheless, the differences found in pollen consumption among treatments did not result in differences in ovary maturation, which did not vary across treatments. This in contrast to a previous study that found a lower ovary maturation in *Osmia* females co-exposed to clothianidin and propiconazole (Sgolastra et al., 2018a). Again, this discrepancy may be explained by the different compounds, as well as by the concentrations tested. Because they were interested in exposure right after fungicide application to a flowering crop, Sgolastra et al. (2018a) tested propiconazole at the field application rate (62.5 mg L⁻¹). By contrast, we tested myclobutanil at the concentration found in the nectar of melon flowers 2-15 days after application (5.58 µg L⁻¹). Under field conditions, pesticides degrade over time and this

process has not been considered in our laboratory study. At any rate, toxic effects are expected to be greater right after application and therefore the concentrations used in our study do not represent the worst case scenario for bees. Studies evaluating pesticide degradation under field conditions are needed to better understand the extent of chronic exposure of bees to pesticides in agricultural landscapes.

Imidacloprid had a clear inhibitory effect on syrup consumption. On the other hand, we did not detect any changes in pollen consumption, possibly due to overall low amounts of pollen ingested in all treatments. *Osmia bicornis* females ingested approximately 80% less syrup in all treatments containing imidacloprid compared to the other treatments, including the control. As a result, the dose of imidacloprid (alone and in mixtures) ingested by *O. bicornis* females throughout their life-span was ca. 1.5 ng. This amount is 10-20 times lower than the acute oral LD₅₀ reported in honey bees [13 ng bee⁻¹ (Sanchez-Bayo and Goka, 2014)] and bumblebees [27 ng bee⁻¹ (Sanchez-Bayo and Goka, 2014)]. For the same reason, the amounts of acetamiprid and/or myclobutanil ingested by bees in A+I, I+M and A+I+M were also reduced by 80% when compared to treatments containing acetamiprid and myclobutanil but not imidacloprid (Table 2). Feeding suppression following exposure to this neonicotinoid has also been reported in *A. mellifera* (Zhu et al., 2017) and *Bombus terrestris* L. (Cresswell et al., 2012; Laycock et al., 2012; Thompson et al., 2015). Because bees cannot taste neonicotinoids (Kessler et al., 2015), feeding suppression is likely to be due to the toxicity of the neonicotinoid rather than repellence. Kessler et al., (2015) found that honey bees and bumblebees preferred syrup containing imidacloprid to control solutions, even though ingestion of this compound caused them to eat less syrup overall. We found feeding suppression in *O. bicornis* exposed to imidacloprid at doses as low as

1.27-1.64 ng bee⁻¹. In agreement with our results, the anti-feeding response caused by imidacloprid ingestion has been shown to be greater under chronic exposure (Cresswell et al., 2012; Thompson et al., 2015).

Feeding suppression in imidacloprid-exposed *O. bicornis* was accompanied by decreased thoracic temperature and apathy. These symptoms could be caused by a general lack of energy due to low feeding levels. However, there is accumulating evidence that imidacloprid directly affects muscular activity. A transcriptome study showed significant down-regulation of twenty-two genes related to muscle function in imidacloprid (10 ppb) treated bees (Wu et al., 2017). Thoracic muscles (the largest in a bee body) are involved in thermoregulation and flight. Other studies document disrupted thermogenic capacity in honey bees (Tosi et al., 2016) and bumblebees (Potts et al., 2018) following exposure to imidacloprid and thiamethoxam. These studies show that ingestion of small doses of neonicotinoids results in an initial short-term stimulation followed by decreased thoracic temperature the day after exposure (Tosi et al., 2016). Other studies have shown that acute exposure to field-realistic doses of neonicotinoids causes excitation (hyperactivity), whereas chronic exposure causes depression (hypoactivity) and impairs flight ability (Suchail et al., 2001; Tosi et al., 2017; Tosi and Nieh, 2017; Williamson et al., 2014). In agreement with our results, Crall et al., (2018) showed that workers orally exposed to 6 ppb of imidacloprid were less active compared to control workers. Studies in bumblebees at colony level, have demonstrated that exposure to imidacloprid impairs colony thermoregulation, alters nursing behaviour, social and spatial dynamics (Crall et al., 2018) and decreases pollen intake (Feltham et al., 2014; Gill et al., 2012).

Our results show clear differences between the two neonicotinoids tested. Acetamiprid yielded no negative effects, even though the amounts of this

compound ingested in treatments A and A+M were twice as high as amounts of imidacloprid ingested in any of the treatments containing imidacloprid. Other studies have found acetamiprid to be less toxic to bees than imidacloprid (Iwasa et al., 2004). These findings are particularly relevant in the context of the IPPM (Biddinger and Rajotte, 2015), which aims to include pollinator health into the IPM paradigm. Whenever effective non-chemical alternatives are not available, IPPM advocates for the use of pesticides that are less toxic to bees and other beneficial insects. IPPM relies on information on lethal and sublethal toxicity of commonly applied pesticides to wild and managed bees.

Our results also have important consequences for bee risk assessment. Current bee risk assessment schemes rely on estimates of LD₅₀ (dose at which half of the population dies) at 48 h following exposure. None of the compounds or mixtures tested in our study resulted in increased mortality. Therefore, if we had considered only lethal effects, we would have wrongly concluded that, at field-realistic doses, all compounds and mixtures tested were safe to bees. Yet, some of our treatments profoundly impaired thermoregulation and bee activity. It is important to note that this effect was not restricted to the immediate post-exposure period, since thoracic temperature was measured on the 17th day of exposure. Although, the ecological consequences of this effect should be confirmed in field conditions, we conclude that incorporating tests specifically intended to detect sublethal effects into risk assessment schemes is essential to evaluate the impact of pesticide exposure on the dynamics of bee populations in agro-ecosystems.

5.4 EXPERIMENT 4: Can the development of *Bombus terrestris* micro-colonies be different when they are exposed to imidacloprid via syrup or/and via pollen?

Bees in agro-ecosystems are exposed at low concentrations of pesticides such as neonicotinoids, in the nectar and/or pollen of crops (Bonmatin et al., 2005; Dively and Kamel, 2012; Stoner and Eitzer, 2012) and wildflowers growing in agricultural field margins (Botías et al., 2015; David et al., 2016; Tsvetkov et al., 2017). Nevertheless, most toxicity studies using bumblebee micro-colonies only have considered nectar contamination (sugar-water solution laced with the desired amounts of pesticide) (Laycock et al., 2014; Mommaerts et al., 2010), and sublethal effects differed from results of studies where exposure via nectar in combination with via pollen have been studied (Elston et al., 2013; Tasei et al., 2000). Because of that, in our work we have investigated the possible combined effect of the double food route contamination in bumblebees micro-colonies by exposing them chronically to field realistic concentrations of imidacloprid (nectar 6 ppb and/or pollen 30 ppb) in order to clarify the different results in these studies.

Adult bees consume nectar and small amounts of pollen (Cane, 2016) and females also collect large amounts of both resources to feed the larvae. However, we did not find effects in the evaluated parameters between microcolonies exposed to imidacloprid via nectar and pollen or just exposed via nectar. And in general no differences were found between pollen and control treatments. These results suggest that the deleterious effects of imidacloprid in bees mainly come from the consumption of contaminated syrup.

According to other studies on honey bees (Cresswell, 2011), bumblebees (Mommaerts et al., 2010) and solitary bees (Azpiazu et al., 2019), no lethal effects have been observed after imidacloprid exposure at low concentrations.

However, important sublethal effects have been detected in our study on the feeding behaviour (i.e. nectar and pollen collection) as well as on the progeny production (i.e. the number of males).

Micro-colonies exposed to imidacloprid via syrup (N and N+P) collected approximately 20% less syrup compared to the pollen and control treatments. Our results are in agreement with other studies that have also reported inhibitory effect of this neonicotinoid in *A. mellifera* (Zhu et al., 2017), *B. terrestris* (Cresswell et al., 2012; Laycock et al., 2012; Thompson et al., 2015) and *O. bicornis* (Azpiazu et al., 2019). Moreover consistent results have been reported from queenless micro-colonies where reduction values of 10-30% in syrup consumption (Laycock et al., 2012), were similar to those obtained in our study. However, the syrup ingested by *Osmia* bees exposed to imidacloprid (15.34 ppb) was reduced by 80% compared to the control (Azpiazu et al., 2019). The different concentration, species tested and methodologies used might have accounted for the results. The consumption reduction has been reported to be dose-dependent (Laycock et al., 2012) and the concentration used in Azpiazu et al (2019) study with *O. bicornis* was more than double compared to that used in our bumblebee micro-colonies. Moreover, the differences among studies may be also explained for the different bee species, because the genus *Osmia* sp is more vulnerable to pesticides than honey bees and bumblebees (Rundlöf et al., 2015; Sgolastra et al., 2017; Woodcock et al., 2017). Finally, Azpiazu et al, (2019) only exposed individual females, while in the present study we have used micro-colonies formed initially by 5 workers but 4 weeks later, males began to emerge. Male progeny may also have consumed considerable amounts of syrup, and as the anti-feeding response caused by imidacloprid ingestion has been shown to be greater under chronic exposure (Cresswell et al., 2012; Thompson et al., 2015), the fact that males were present in the micro-colonies

just for a maximum of three days, could have contributed to buffering the effect.

Bees exposed to imidacloprid via nectar, also collected 50% lesser pollen than the controls, being reductions greater than those registered in exposure via syrup. The difference can also be explained by the presence of male progeny. Workers consume between 60-95% more nectar than pollen (Gradish et al., 2019), therefore in this case we suggest that the pollen consumption of males is not buffering the effect, because pollen collected is mainly to feed the larvae and to provide substrate for nest building. Other studies with queenright colonies exposed to sublethal concentrations of imidacloprid, have also shown impairment in their pollen foraging efficiency (Feltham et al., 2014; Gill et al., 2012). Besides, a reduction in the pollen amount consumed has been reported in micro-colonies studies as well (Laycock et al., 2012).

Such pollen and nectar constraints in treatments N and N+P seem to affect brood development and as result the male production is reduced. The lack of the needed amounts of protein and carbohydrates appears to be the main reason, because is well known that imidacloprid at low concentrations does not affect ovary development in bumblebees (Laycock et al., 2012) and in solitary bees (Azpiazu et al., 2019).

Because food collection is a task exclusively performed by workers, we could expect a lack of increase on this effect in treatments via the double route of exposure (nectar and pollen). As explained above, the amount of pollen consumed is lesser than the amount of nectar, and as a result the amount of active ingredient ingested is also lower. Thus, the double route of exposure as shown in adults of *Osmia* bees (Azpiazu et al., 2019), may not be significant.

The difference between nectar and pollen consumed by larvae is not as remarkable as in the case of workers [nectar: 24-60 mg/larva/day; pollen 10-40 mg/larva/day; (Gradish et al., 2019)]. Therefore, in treatments with imidacloprid contaminated pollen it was expected that the progeny production would be reduced by the direct effect of the neonicotinoid combined with the lack of food. We have found a trend to reduce the progeny production in the micro-colonies exposed to imidacloprid via pollen, but not significant differences could be detected compared control and nectar contaminated treatments. Previous studies did not detect any effect on larval survival after exposure at field-realistic concentrations of pesticides (Abbott et al., 2008; Nicholls et al., 2017), suggesting that effects of neonicotinoid exposure may be less severe for larvae than for adults. Certainly in honey bees it has been suggested that larvae may be more tolerant to neonicotinoids than adult bees (Yang et al., 2012), based on the different nACh-receptor expression across developmental stages (Thany et al., 2003; Thany and Gauthier, 2005).

In conclusion, based on our results the exposure of *B. terrestris* micro-colonies to field-realistic concentrations of imidacloprid via pollen have not shown any significant effect on micro-colonies development, suggesting that the effects mainly come from the contaminated syrup. However, these results should be corroborated by evaluating pesticide mixtures. Fungicides may have an indirect impact on bees by altering the microflora present in the pollen the treated plants (Bartlewicz et al., 2016; Yoder et al., 2013). This could have important consequences for the bee nutrition and health (Engel et al., 2016; Mattila et al., 2012) even during larval development (Dharampal et al., 2019).

CHAPTER VI: CONCLUSIONS

Based on the research objectives described in Chapter II, the following conclusions can be drawn:

- The best species for a flower strip in central Spain based on their attractiveness, staggered blossom and coverage were *Coriandrum sativum* L., *Diplotaxis virgata* L., *Borago officinalis* L. and *Calendula officinalis* L. The plant composition in the mixture must be carefully chosen. Interspecific competition can prevent the shorter plants from attracting pollinators (e.g. *Lobularia maritima*) and plants with high dry matter can diminish the floral coverage of the nearby species (**Experiment 1**).
- The flower strip can act as facilitator to the crop depending on the predominant species in the flower strip and their floral coverage during the crop bloom period. In our area, the concurrence of blooming in the rewarding *C. officinalis* with the melon crop should be avoided; otherwise the margin may not act as a pollinator exporter of the main pollinator taxa to the melon crop (**Experiment 1**).
- The effect of the flower strip in the commercial farm was spatially limited. Pollinator visits in the first distances in the melon crop (< 2m) were higher respect to the farthest distances. The predominant plant species was *C. officinalis*, which seemed to not act as exporter of pollinators beyond 2 m (**Experiment 1**).
- The presence of flower strips in experimental o commercial melon plots did not have an influence on melon productivity and quality (**Experiment 1**).

Foraging bees in melon fields of the study area were exposed to a high number of pesticides (nine insecticides, nine fungicides and one herbicide). The highest pesticide residue concentrations (above 25 ppb) in melon flowers corresponded to pesticides sprayed by farmers. However, residues of eleven active ingredients, not used by farmers during the current crop cycle, were detected in the samples, and therefore, melon agro-ecosystem can be a very pesticide contaminated environment. Pesticide concentrations were one or two orders of magnitude higher in pollen than in nectar of the melon flowers. The most probable pesticide co-occurrence (>60%) in the study area were between the 4 insecticides (acetamiprid, oxamyl, imidacloprid and chlorpyrifos) and two fungicides (metalaxil-m and azoxystrobin) (**Experiment 2**).

The chronic exposure of *B. terrestris* micro-colonies via pollen and nectar contaminated with the concentration levels of acetamiprid, chlorpyrifos, oxamyl and their combinations detected in the commercial melon fields of Central Spain, showed no effect in the pollinator parameters measured (mortality, pollen and syrup collected and reproduction fitness), and no synergy among compounds (**Experiment 2**).

The exposure to combinations of two neonicotinoids and a triazole fungicide mixture at field-realistic concentration found in melon flowers in adults of the solitary bee *O. bicornis*, showed neither synergistic effects nor and nor effects on longevity and ovary maturation. However, all treatments containing imidacloprid resulted in suppressed syrup consumption and drastic decreases in thoracic temperature and bee activity. Our results have important implications for pesticide regulation. If we had measured only lethal effects we would have wrongly concluded that the pesticide combinations containing imidacloprid were safe to *O. bicornis*. The

incorporation of tests specifically intended to detect sublethal effects in bee risk assessment schemes should be an urgent priority. In this way, the effects of pesticide exposure on the dynamics of bee populations in agro-ecosystems will be better assessed. **(Experiment 3).**

Bumblebees micro-colonies exposed to imidacloprid at field-realistic concentration collected less syrup and pollen, produced less brood, and consequently less males were emerged. These effects mainly come from contaminated nectar treatments, because no differences were found between micro-colonies exposed to imidacloprid via nectar and pollen (N+P), and those exposed just via nectar (N), neither between the control and the micro-colonies exposed to imidicloprid via pollen (P) **(Experiment 4).**

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Azpiazu, C., Medina, P., Adán, Á., Sánchez-Ramos, I., del Estal, P., Fereres, A., & Viñuela, E. (2020). The Role of Annual Flowering Plant Strips on a Melon Crop in Central Spain. Influence on Pollinators and Crop. *Insects*, 11(1), 66.

Azpiazu, C., Bosch, J., Viñuela, E., Medrzycki, P., Teper, D., Sgolastra, F., 2019. Chronic oral exposure to field-realistic pesticide combinations via pollen and nectar: effects on feeding and thermal performance in a solitary bee. *Sci. Rep.* 9, 13770.

REFERENCES

- Abbott, V.A., Nadeau, J.L., Higo, H.A., Winston, M.L., 2008. Lethal and sublethal effects of imidacloprid on *Osmia lignaria* and clothianidin on *Megachile rotundata* (Hymenoptera: Megachilidae). *J. Econ. Entomol.* 101, 784–796.
- Ahmad, M., Bodlah, I., Mehmood, K., Aslam Sheikh, U.A., Aziz, M.A., 2015. Pollination and foraging potential of European Bumblebee, *Bombus terrestris* (Hymenoptera: Apidae) on tomato crop under greenhouse system. *Pak. J. Zool.* 47, 1279–1285.
- Aizen, M.A., Garibaldi, L.A., Cunningham, S.A., Klein, A.M., 2008. Long-Term Global Trends in Crop Yield and Production Reveal No Current Pollination Shortage but Increasing Pollinator Dependency. *Curr. Biol.* 18, 1572–1575.
- Aizen, M.A., Harder, L.D., 2009. The Global Stock of Domesticated Honey Bees Is Growing Slower Than Agricultural Demand for Pollination. *Curr. Biol.* 19, 915–918.
- Al Nagggar, Y., Codling, G., Vogt, A., Naiem, E., Mona, M., Seif, A., Giesy, J.P., 2015. Organophosphorus insecticides in honey, pollen and bees (*Apis mellifera* L.) and their potential hazard to bee colonies in Egypt. *Ecotoxicol. Environ. Saf.* 114, 1–8.
- Alaux, C., Ducloz, F., Crauser, D., Le Conte, Y., 2010. Diet effects on honeybee immunocompetence. *Biol. Lett.* 6, 562–565.
- Albrecht, M., Schmid, B., Hautier, Y., Müller, C.B., 2012. Diverse pollinator communities enhance plant reproductive success. *Proc. R. Soc. B Biol. Sci.* 279, 4845–4852.
- Alston, D.G., 2011. The Integrated Pest Management (IPM) Concept. Utah State Univ. Ext. Utah Plant Pest Diagnostic Lab. 1–2.
- Ambrosino, M.D., Luna, J.M., Jepson, P.C., 2006. Relative frequencies of visits to selected insectary plants by predatory hoverflies (Diptera: Syrphidae), other beneficial insects, and herbivores. *Environ. Entomol.* 35, 394–400.
- Amy, C., Noël, G., Hatt, S., Uyttenbroeck, R., Van De Meutter, F., Genoud, D., Francis, F., 2018. Flower strips in wheat intercropping system: Effect on pollinator abundance and diversity in Belgium. *Insects* 9, 114.
- Anderson, N.L., Harmon-Threatt, A.N., 2019. Chronic contact with realistic soil concentrations of imidacloprid affects the mass, immature development speed, and adult longevity of solitary bees. *Sci. Rep.* 9, 1–9.
- Arena, M., Sgolastra, F., 2014. A meta-analysis comparing the sensitivity of bees to pesticides. *Ecotoxicology* 23, 324–334.
- Artz, D.R., Pitts-Singer, T.L., 2015. Effects of fungicide and adjuvant sprays on nesting behavior in two managed solitary bees, *Osmia lignaria* and *Megachile rotundata*. *PLoS One* 10, e0135688.
- Ashman, T.-L., Knight, T.M., Steets, J.A., Amarasekare, P., Burd, M., Campbell, D.R., Dudash, M.R., Johnston, M.O., Mazer, S.J., Mitchell, R.J., Morgan, M.T., Wilson, W.G., 2004. Pollen limitation of plant reproduction: ecological and evolutionary causes and consequences. *Ecology* 85, 2408–2421.
- Atlas Hymenoptera, 2017. Atlas Hymenoptera [WWW Document]. URL <http://www.atlashymenoptera.net> (accessed 5.3.17).
- Azpiazu, C., Bosch, J., Viñuela, E., Medrzycki, P., Teper, D., Sgolastra, F., 2019. Chronic oral

- exposure to field-realistic pesticide combinations via pollen and nectar: effects on feeding and thermal performance in a solitary bee. *Sci. Rep.* 9, 13770.
- Azpiazu, C., Morales, I., Adán, Á., Medina, P., Fereres, A., 2017. Identifying a suitable annual floral mixture and its relative attractiveness to pollinators in Central Spain. *IOBC-WPRS Bull.* 122, 79–82.
- Balzan, M. V., Bocci, G., Moonen, A.-C., 2014. Augmenting flower trait diversity in wildflower strips to optimise the conservation of arthropod functional groups for multiple agroecosystem services. *J. Insect Conserv.* 18, 713–728.
- Balzan, M. V., Bocci, G., Moonen, A.C., 2016. Utilisation of plant functional diversity in wildflower strips for the delivery of multiple agroecosystem services. *Entomol. Exp. Appl.* 158, 304–319.
- Barbir, J., 2016. Managing Semi-natural Habitats of Pollinators within Agro-ecosystems. *Entomol. Ornithol. Herpetol. Curr. Res.* 5, 1–3.
- Barbir, J., Azpiazu, C., Badenes-Pérez, F.R., Fernández-Quintanilla, C., Dorado, J., 2016. Functionality of Selected Aromatic Lamiaceae in Attracting Pollinators in Central Spain. *J. Econ. Entomol.* 4, 1–8.
- Barbir, J., Badenes-Pérez, F.R., Fernández-Quintanilla, C., Dorado, J., 2015. The attractiveness of flowering herbaceous plants to bees (Hymenoptera: Apoidea) and hoverflies (Diptera: Syrphidae) in agro-ecosystems of Central Spain. *Agric. For. Entomol.* 17, 20–28.
- Bartlewicz, J., Pozo, M.I., Honnay, O., Lievens, B., Jacquemyn, H., 2016. Effects of agricultural fungicides on microorganisms associated with floral nectar: susceptibility assays and field experiments. *Environ. Sci. Pollut. Res.* 23, 19776–19786.
- Bartomeus, I., Park, M.G., Gibbs, J., Danforth, B.N., Lakso, A.N., Winfree, R., 2013. Biodiversity ensures plant-pollinator phenological synchrony against climate change. *Ecol. Lett.* 16, 1331–1338.
- Beeaware, 2016. BeeAware, Melons [WWW Document]. URL <http://beeaware.org.au/pollination/pollinator-reliant-crops/melons/> (accessed 12.3.16).
- Bernal, J., Garrido-Bailón, E., Del Nozal, M.J., González-Porto, A. V., Martín-Hernández, R., Diego, J.C., Jiménez, J.J., Bernal, J.L., Higes, M., 2011. Overview of Pesticide Residues in Stored Pollen and Their Potential Effect on Bee Colony (*Apis mellifera*) Losses in Spain. *J. Econ. Entomol.* 103, 1964–1971.
- Biddinger, D.J., Rajotte, E.G., 2015. Integrated pest and pollinator management-adding a new dimension to an accepted paradigm. *Curr. Opin. Insect Sci.* 10, 204–209.
- Biddinger, D.J., Robertson, J.L., Mullin, C., Frazier, J., Ashcraft, S.A., Rajotte, E.G., Joshi, N.K., Vaughn, M., 2013. Comparative toxicities and synergism of apple orchard pesticides to *Apis mellifera* (L.) and *Osmia cornifrons* (Radoszkowski). *PLoS One* 8, e72587.
- Biesmeijer, J.C., Roberts, S.P.M., Reemer, M., Ohlemüller, R., Edwards, M., Schaffers, A.P., Peeters, T., Potts, S.G., Kleukers, R., Thomas, C.D., Settele, J., Kunin, W.E., 2006. Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. *Science* 313, 351–354.
- Blaauw, B.R., Isaacs, R., 2014. Flower plantings increase wild bee abundance and the pollination services provided to a pollination-dependent crop. *J. Appl. Ecol.* 51, 890–898.
- Blüthgen, N., Klein, A.-M., 2011. Functional complementarity and specialisation: The role of biodiversity in plant–pollinator interactions. *Basic Appl. Ecol.* 12, 282–291.

- BOE, 2012. Agencia Estatal Boletín Oficial del Estado. Real Decreto 1311/2012, de 14 de septiembre, por el que se establece el marco de actuación para conseguir un uso sostenible de los productos fitosanitarios. Minist. la Pres. BOE-A-2012-11605 11–13.
- Bohnenblust, E.W., Vaudo, A.D., Egan, J.F., Mortensen, D.A., Tooker, J.F., 2016. Effects of the herbicide dicamba on nontarget plants and pollinator visitation. *Environ. Toxicol. Chem.* 35, 144–151.
- Bomfim, I., Freitas, B., de Aragão, F., Walters, S., 2016. Pollination in cucurbit crops, in: Pessarakli, M. (Ed.), *Handbook of Cucurbits: Growth, Cultural Practices, and Physiology*. CRC Press, Boca Raton, pp. 181–200.
- Bonmatin, J.M., Giorio, C., Girolami, V., Goulson, D., Kreutzweiser, D.P., Krupke, C., Liess, M., Long, E., Marzaro, M., Mitchell, E.A., Noome, D.A., Simon-Delso, N., Tapparo, A., 2015. Environmental fate and exposure; neonicotinoids and fipronil. *Environ. Sci. Pollut. Res.* 22, 35–67.
- Bonmatin, J.M.B., Archand, P.A.M., Harvet, R.C., Oineau, I.M., 2005. Quantification of imidacloprid uptake in maize crops. *J. Agric. Food Chem.* 53, 5336–5341.
- Bortolotti, L., Montanari, R., Marcelino, J., Medrzycki, P., Maini, S., Porrini, C., 2003. Effects of sub-lethal imidacloprid doses on the homing rate and foraging activity of honey bees. *Bull. Insectology* 56, 63–67.
- Bosch, J., Kemp, W., 2002a. How to Manage the Blue Orchard Bee As an Orchard Pollinator, Sustainable Agricultural Network. Sustainable Agricultural Network, Beltsville, Maryland.
- Bosch, J., Kemp, W.P., 2002b. Developing and establishing bee, species as crop pollinators: The example of *Osmia* spp. (Hymenoptera: Megachilidae) and fruit trees. *Bull. Entomol. Res.* 92, 3–16.
- Bosch, J., Sgolastra, F., Kemp, W.P., 2008. Life cycle ecophysiology of *Osmia* mason bees used as crop pollinators, in: *Bee Pollination in Agricultural Ecosystems*. Oxford University Press, Oxford, UK, pp. 83–104.
- Bosch, J., Vicens, N., 2006. Relationship between body size, provisioning rate, longevity and reproductive success in females of the solitary bee *Osmia cornuta*. *Behav. Ecol. Sociobiol.* 60, 26–33.
- Bosch, J., Vicens, N., 2002. Body size as an estimator of production costs in a solitary bee. *Ecol. Entomol.* 27, 129–137.
- Botías, C., David, A., Hill, E.M., Goulson, D., 2017. Quantifying exposure of wild bumblebees to mixtures of agrochemicals in agricultural and urban landscapes. *Environ. Pollut.* 222, 73–82.
- Botías, C., David, A., Horwood, J., Abdul-Sada, A., Nicholls, E., Hill, E., Goulson, D., 2015. Neonicotinoid residues in wildflowers, a potential route of chronic exposure for bees. *Environ. Sci. Technol.* 49, 12731–12740.
- Botías, C., Sánchez-Bayo, F., 2018. Papel de los plaguicidas en la pérdida de polinizadores. *Ecosistemas* 27, 34–41.
- Braun-Blanquet, J., Lalucat Jo, J., Bolòs, O. de, 1979. *Fitosociología : bases para el estudio de las comunidades vegetales*. Spanish edition of *Pflanzensoziologie: Grundzüge der Vegetationskunde*. Blume, Madrid.
- Brittain, C., Williams, N., Kremen, C., Klein, A.M., 2013. Synergistic effects of non-*Apis* bees and

- honey bees for pollination services. *Proc. R. Soc. B Biol. Sci.* 280, 1–7.
- Buchmann, S.L., 1983. Buzz pollination in angiosperms., *Handbook of experimental pollination biology*. Van Nostrand Reinhold Company, New York, NY.
- Bugg, R.L., Colfer, R.G., Chaney, W.E., Smith, H. a., Cannon, J., 2008. Flower Flies (Syrphidae) and Other Biological Control Agents for Aphids in Vegetable Crops. UC ANR 8285, 1–25.
- Burkle, L.A., Marlin, J.C., Knight, T.M., 2013. Plant-pollinator interactions over 120 years: Loss of species, co-occurrence, and function. *Science*. 340, 1611–1615.
- Byrne, F.J., Visscher, P.K., Leimkuehler, B., Fischer, D., Grafton-Cardwell, E.E., Morse, J.G., 2014. Determination of exposure levels of honey bees foraging on flowers of mature citrus trees previously treated with imidacloprid. *Pest Manag. Sci.* 70, 470–482.
- Cabello, M.J., Castellanos, M.T., Romojaro, F., Martínez-Madrid, C., Ribas, F., 2009. Yield and quality of melon grown under different irrigation and nitrogen rates. *Agric. Water Manag.* 96, 866–874.
- Calvo-Agudo, M., González-Cabrera, J., Picó, Y., Calatayud-Vernich, P., Urbaneja, A., Dicke, M., Tena, A., 2019. Neonicotinoids in excretion product of phloem-feeding insects kill beneficial insects. *Proc. Natl. Acad. Sci.* 116, 16817–16822.
- Campbell, A.J., Biesmeijer, J.C., Varma, V., Wäckers, F.L., 2012. Realising multiple ecosystem services based on the response of three beneficial insect groups to floral traits and trait diversity. *Basic Appl. Ecol.* 13, 363–370.
- Campbell, A.J., Wilby, A., Sutton, P., Wäckers, F.L., 2017. Getting more power from your flowers: Multi-functional flower strips enhance pollinators and pest control agents in apple orchards. *Insects* 8, 1–18.
- Campbell, A.J., Wilby, A., Sutton, P., Wäckers, F.L., 2017. Do sown flower strips boost wild pollinator abundance and pollination services in a spring-flowering crop? A case study from UK cider apple orchards. *Agric. Ecosyst. Environ.* 239, 20–29.
- Cane, J.H., 2016. Adult pollen diet essential for egg maturation by a solitary *Osmia* bee. *J. Insect Physiol.* 95, 105–109.
- Cane, J.H., Dobson, H.E.M., Boyer, B., 2017. Timing and size of daily pollen meals eaten by adult females of a solitary bee (*Nomia melanderi*) (Apiformes: Halictidae). *Apidologie* 48, 17–30.
- Carreck, N.L., Ratnieks, F.L.W., 2014. The dose makes the poison: have “field realistic” rates of exposure of bees to neonicotinoid insecticides been overestimated in laboratory studies? *J. Apic. Res.* 53, 607–614.
- Carreck, N.L., Williams, I.H., 2002. Food for insect pollinators on farmland: insect visits to flowers of annual seed mixtures. *J. Insect Conserv.* 6, 13–23.
- Carreck, N.L., Williams, I.H., 1997. Observations on two commercial flower mixtures as food sources for beneficial insects in the UK. *J. Agric. Sci.* 128, 397–403.
- Carrié, R.J.G., George, D.R., Wäckers, F.L., 2012. Selection of floral resources to optimise conservation of agriculturally-functional insect groups. *J. Insect Conserv.* 16, 635–640.
- Carvalho, L.G., Kunin, W.E., Keil, P., Aguirre-Gutierrez, J., Ellis, W.N., Fox, R., Groom, Q., Hennekens, S., Van Landuyt, W., Maes, D., Van de Meutter, F., Michez, D., Rasmont, P., Ode, B., Potts, S.G., Reemer, M., Roberts, S.P.M., Schaminée, J., Wallisdevries, M.F., Biesmeijer, J.C., 2013. Species richness declines and biotic homogenisation have slowed

- down for NW-European pollinators and plants. *Ecol. Lett.* 16, 870–878.
- Carvalho, L.G., Saraiva, A.M., Giannini, T.C., 2016. Establishing Knowledge Management System for Ecological Interactions. The case of crop pollinators, in: Gemmill-Herren, B. (Ed.), *Pollination Services to Agriculture: Sustaining and Enhancing a Key Ecosystem Service*. Routledge, New York, pp. 74–94.
- Carvalho, L.G., Seymour, C.L., Nicolson, S.W., Veldtman, R., 2012. Creating patches of native flowers facilitates crop pollination in large agricultural fields: Mango as a case study. *J. Appl. Ecol.* 49, 1373–1383.
- Carvalho, L.G., Seymour, C.L., Veldtman, R., Nicolson, S.W., 2010. Pollination services decline with distance from natural habitat even in biodiversity-rich areas. *J. Appl. Ecol.* 47, 810–820.
- Carvell, C., Bourke, A.F.G., Osborne, J.L., Heard, M.S., 2015. Effects of an agri-environment scheme on bumblebee reproduction at local and landscape scales. *Basic Appl. Ecol.* 16, 519–530.
- Chan, D.S.W., Prosser, R.S., Rodríguez-Gil, J.L., Raine, N.E., 2019. Assessment of risk to hoary squash bees (*Peponapis pruinosa*) and other ground-nesting bees from systemic insecticides in agricultural soil. *Sci. Rep.* 9, 1–13.
- Chiesa, L.M., Labella, G.F., Giorgi, A., Panseri, S., Pavlovic, R., Bonacci, S., Arioli, F., 2016. The occurrence of pesticides and persistent organic pollutants in Italian organic honeys from different productive areas in relation to potential environmental pollution. *Chemosphere* 154, 482–490.
- Colla, S.R., Otterstatter, M.C., Gegear, R.J., Thomson, J.D., 2006. Plight of the bumble bee: Pathogen spillover from commercial to wild populations. *Biol. Conserv.* 129, 461–467.
- Colley, M.R., Luna, J.M., 2000. Relative Attractiveness of Potential Beneficial Insectary Plants to Aphidophagous Hoverflies (Diptera: Syrphidae). *Environ. Entomol.* 29, 1054–1059.
- Cowles, R.S., Eitzer, B.D., 2017. Residues of neonicotinoid insecticides in pollen and nectar from model plants. *J. Environ. Hort.* 35, 24–34.
- Crall, J.D., Switzer, C.M., Oppenheimer, R.L., Ford Versypt, A.N., Dey, B., Brown, A., Eyster, M., Guérin, C., Pierce, N.E., Combes, S.A., de Bivort, B.L., 2018. Neonicotinoid exposure disrupts bumblebee nest behavior, social networks, and thermoregulation. *Science*. 362, 683–686.
- Cresswell, J.E., 2011. A meta-analysis of experiments testing the effects of a neonicotinoid insecticide (imidacloprid) on honey bees. *Ecotoxicology* 20, 149–157.
- Cresswell, J.E., Page, C.J., Uygun, M.B., Holmbergh, M., Li, Y., Wheeler, J.G., Laycock, I., Pook, C.J., de Ibarra, N.H., Smirnoff, N., Tyler, C.R., 2012. Differential sensitivity of honey bees and bumble bees to a dietary insecticide (imidacloprid). *Zoology* 115, 365–371.
- Dance, C., Botías, C., Goulson, D., 2017. The combined effects of a monotonous diet and exposure to thiamethoxam on the performance of bumblebee micro-colonies. *Ecotoxicol. Environ. Saf.* 139, 194–201.
- Danforth, B.N., Cardinal, S., Praz, C., Almeida, E.A.B., Michez, D., 2013. The Impact of Molecular Data on Our Understanding of Bee Phylogeny and Evolution. *Annu. Rev. Entomol.* 58, 57–78.
- Das, S., Hageman, K.J., Taylor, M., Michelsen-heath, S., 2019. Fate of the organophosphate insecticide, chlorpyrifos, in leaves, soil, and air following application. *Chemosphere*. 243,

125194

- David, A., Botías, C., Abdul-Sada, A., Goulson, D., Hill, E.M., 2015. Sensitive determination of mixtures of neonicotinoid and fungicide residues in pollen and single bumblebees using a scaled down QuEChERS method for exposure assessment. *Anal. Bioanal. Chem.* 407, 8151–8162.
- David, A., Botías, C., Abdul-Sada, A., Nicholls, E., Rotheray, E.L., Hill, E.M., Goulson, D., 2016. Widespread contamination of wildflower and bee-collected pollen with complex mixtures of neonicotinoids and fungicides commonly applied to crops. *Environ. Int.* 88, 169–178.
- Decourtye, a., Devillers, J., Genecque, E., Le Menach, K., Budzinski, H., Cluzeau, S., Pham-Delègue, M.H., 2005. Comparative sublethal toxicity of nine pesticides on olfactory learning performances of the honeybee *Apis mellifera*. *Arch. Environ. Contam. Toxicol.* 48, 242–250.
- Dharampal, P.S., Carlson, C., Currie, C.R., Steffan, S.A., 2019. Pollen-borne microbes shape bee fitness. *Proc. R. Soc. B Biol. Sci.* 286.
- Dively, G.P., Kamel, A., 2012. Insecticide residues in pollen and nectar of a cucurbit crop and their potential exposure to pollinators. *J. Agric. Food Chem.* 60, 4449–4456.
- Doue, 2009. Directive 2009/128/ec of the European parliament and the council of 21 Octobre 2009 establishing a framework for Community action to achieve the sustainable use of pesticides. *D. Of. la Unión Eur.* 24-Xi-2009 309, 71–86.
- Duncan, J., Ewing, J., 2015. Specialty melon production for small and direct-market growers. *ATTRA Sustain. Agric.* 1–16.
- EC, 2019. EU Pesticides database - European Commission [WWW Document]. URL <https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=homepage&language=EN> (accessed 10.7.19).
- EC, 2018a. European Commission implementing regulation (EU) 2018/ 785 of 29 May 2018 amending Implementing Regulation (EU) No 540 / 2011 as regards the conditions of approval of the active substance thiamethoxam 2018, 40–44.
- EC, 2018b. European Commission implementing regulation (EU) 2018/783 of 29 May 2018 amending Implementing Regulation (EU) No 540/2011 as regards the conditions of approval of the active substance imidacloprid. *Off. J. Eur. Union* 132, 31–34.
- EC, 2018c. European Commission implementing regulation (EU) 2018/784 of 29 May 2018 amending Implementing Regulation (EU) No 540/2011 as regards the conditions of approval of the active substance clothianidin 132, 35–39.
- EC, 2018d. European Commission, Agriculture and Rural Development. Greening [WWW Document]. URL https://ec.europa.eu/agriculture/direct-support/greening_en (accessed 12.1.16).
- EC, 2017. Biodiversity Strategy - Environment - European Commission [WWW Document]. URL https://ec.europa.eu/environment/nature/biodiversity/strategy/index_en.htm (accessed 8.14.17).
- EC, 2013. European Commission implementing regulation (EU) No 485/2013 of 24 May 2013 amending Implementing Regulation (EU) No 540/2011, as regards the conditions of approval of the active substances clothianidin, thiamethoxam and imidacloprid, and prohibiting the . *Off. J. Eur. Union L* 139, 12–26.
- EFSA, 2013. European Food Safety Authority. Guidance on the risk assessment of plant

- protection products on bees (*Apis mellifera*, *Bombus* spp. and solitary bees). EFSA J. 11, 3295.
- Elston, C., Thompson, H.M., Walters, K.F.A., 2013. Sub-lethal effects of thiamethoxam, a neonicotinoid pesticide, and propiconazole, a DMI fungicide, on colony initiation in bumblebee (*Bombus terrestris*) micro-colonies. *Apidologie* 44, 563–574. d
- Engel, P., Kwong, W.K., McFrederick, Q., Anderson, K.E., Barribeau, Seth Michael Chandler, J.A., Cornman, R.S., Dainat, J., Miranda, J.R. de, Doublet, V., Emery, O., Evans, J.D., Farinelli, L., Flenniken, M.L., Granberg, F., Grasis, J.A., Gauthier, L., Hayer, J., Koch, H., Kocher, S., Martinson, V.G., Moran, N., Munoz-Torres, M., Newton, I., Paxton, R.J., Powell, E., Sadd, B.M., Schmid-Hempel, P., Schmid-Hempel, R., Song, S.J., Schwarz, R.S., VanEngelsdorp, D., Dainat, B., 2016. The bee microbiome: impact on bee health and model for evolution and ecology of host-microbe interactions. *MBio* 7, 1–9.
- EPA, 2000. Environmental Protection Agency. Interim Reregistration Eligibility Decision (IRED), Oxamyl, Prevention, Pesticides and Toxic Substances (7508C).
- EPPO, 2010. European and Mediterranean Plant Protection Organization. Environmental risks assesment scheme for plant protection products. Chapter 10: honeybees, EPPO Bulletin.
- FAO, 2018. Food and Agriculture Organization of the United Nations. FAOSTAT [WWW Document]. URL <http://www.fao.org/faostat/en/#data/QC> (accessed 11.2.18).
- Fausser-Misslin, A., Sadd, B.M., Neumann, P., Sandrock, C., 2014. Influence of combined pesticide and parasite exposure on bumblebee colony traits in the laboratory. *J. Appl. Ecol.* 51, 450–459.
- Feltham, H., Park, K., Goulson, D., 2014. Field realistic doses of pesticide imidacloprid reduce bumblebee pollen foraging efficiency. *Ecotoxicology* 23, 317–323.
- Fernández, M. del M., Meeus, I., Billiet, A., Van Nieuwerburgh, F., Deforce, D., Vandamme, P., Viñuela, E., Smagghe, G., 2019. Influence of microbiota in the susceptibility of parasitic wasps to abamectin insecticide: deep sequencing, esterase and toxicity tests. *Pest Manag. Sci.* 75, 79–86.
- Fischer, J., Müller, T., Spatz, A.K., Greggers, U., Grünewald, B., Menzel, R., 2014. Neonicotinoids interfere with specific components of navigation in honeybees. *PLoS One* 9, 1–10.
- Flora ibérica, 2013. Flora ibérica. Plantas vasculares de la Península Ibérica e Islas Baleares [WWW Document]. Real Jardín Botánico, CSIC. URL <http://www.floraiberica.es/> (accessed 1.25.13).
- Fontaine, C., Dajoz, I., Meriguet, J., Loreau, M., 2006. Functional diversity of plant–pollinator interaction webs enhances the persistence of plant communities. *PLoS Biol.* 4, e1.
- Fowler, R.E., Rotheray, E.L., Goulson, D., 2016. Floral abundance and resource quality influence pollinator choice. *Insect Conserv. Divers.* 9, 481–494.
- Ganser, D., Mayr, B., Albrecht, M., Knop, E., 2018. Wildflower strips enhance pollination in adjacent strawberry crops at the small scale. *Ecol. Evol.* 8, 11775–11784.
- Garibaldi, L. a, Steffan-Dewenter, I., Winfree, R., Aizen, M. a, Bommarco, R., Cunningham, S. a, Kremen, C., Carvalheiro, L.G., Harder, L.D., Afik, O., Bartomeus, I., Benjamin, F., Boreux, V., Cariveau, D., Chacoff, N.P., Dudenhöffer, J.H., Freitas, B.M., Ghazoul, J., Greenleaf, S., Hipólito, J., Holzschuh, A., Howlett, B., Isaacs, R., Javorek, S.K., Kennedy, C.M., Krewenka, K.M., Krishnan, S., Mandelik, Y., Mayfield, M.M., Motzke, I., Munyuli, T.,

- Nault, B. a, Otieno, M., Petersen, J., Pisanty, G., Potts, S.G., Rader, R., Ricketts, T.H., Rundlöf, M., Seymour, C.L., Schüepp, C., Szentgyörgyi, H., Taki, H., Tscharntke, T., Vergara, C.H., Viana, B.F., Wanger, T.C., Westphal, C., Williams, N., Klein, A.M., 2013. Wild pollinators enhance fruit set of crops regardless of honey bee abundance. *Science*. 339, 1608–1611.
- Garibaldi, L.A., Aizen, M.A., Klein, A.M., Cunningham, S.A., Harder, L.D., 2011. Global growth and stability of agricultural yield decrease with pollinator dependence. *Proc. Natl. Acad. Sci.* 108, 5909–5914.
- Garibaldi, L.A., Carvalheiro, L.G., Leonhardt, S.D., Aizen, M.A., Blaauw, B.R., Isaacs, R., Kuhlmann, M., Kleijn, D., Klein, A.M., Kremen, C., Morandin, L., Scheper, J., Winfree, R., 2014. From research to action: Enhancing crop yield through wild pollinators. *Front. Ecol. Environ.* 12, 439–447.
- Garratt, M.P.D., Breeze, T.D., Jenner, N., Polce, C., Biesmeijer, J.C., Potts, S.G., 2014. Avoiding a bad apple: Insect pollination enhances fruit quality and economic value. *Agric. Ecosyst. Environ.* 184, 34–40.
- Gill, R.J., Raine, N.E., 2014. Chronic impairment of bumblebee natural foraging behaviour induced by sublethal pesticide exposure. *Funct. Ecol.* 28, 1459–1471.
- Gill, R.J., Ramos-Rodriguez, O., Raine, N.E., 2012. Combined pesticide exposure severely affects individual- and colony-level traits in bees. *Nature* 491, 105–108.
- Godfray, H.C.J., Blacquie, T., Field, L.M., Hails, R.S., Petrokofsky, G., Potts, S.G., Raine, N.E., Vanbergen, A.J., Mclean, A.R., 2014. A restatement of the natural science evidence base concerning neonicotinoid insecticides and insect pollinators. *Proc. Biol. Sci. B: Biol. Sci.* 281, 20140558.
- Goka, K., Okabe, K., Yoneda, M., 2006. Worldwide migration of parasitic mites as a result of bumblebee commercialization: Special feature: Population ecology of biological invasion. *Popul. Ecol.* 48, 285–291.
- Gorenflo, A., Diekötter, T., Van Kleunen, M., Wolters, V., Jauker, F., 2017. Contrasting pollination efficiency and effectiveness among flower visitors of *Malva sylvestris*, *Borago officinalis* and *Onobrychis viciifolia*. *J. Pollinat. Ecol.* 21, 62–70.
- Goulson, D., 2013. An overview of the environmental risks posed by neonicotinoid insecticides. *J. Appl. Ecol.* 50, 977–987.
- Goulson, D., 2010. *Bumblebees: behaviour, ecology, and conservation*, Second Edi. ed. Oxford University Press on Demand.
- Goulson, D., 1999. Foraging Strategies of Insects for Gathering Nectar an Pollen and Implications for Plant Ecology and Evolution. *Perspect. plant Ecol. Evol. Syst.* 2, 185–209.
- Goulson, D., Hughes, W.O.H., 2015. Mitigating the anthropogenic spread of bee parasites to protect wild pollinators. *Biol. Conserv.* 191, 10–19.
- Goulson, D., Lye, G.C., Darvill, B., 2008. Decline and Conservation of Bumble Bees. *Annu. Rev. Entomol.* 53, 191–208.
- Goulson, D., Nicholls, E., Botías, C., Rotheray, E.L., 2015. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science*. 347, 1255957.
- Gradish, A.E., Van Der Steen, J., Scott-Dupree, C.D., Cabrera, A.R., Cutler, G.C., Goulson, D., Klein, O., Lehmann, D.M., Lückmann, J., O'Neill, B., Raine, N.E., Sharma, B., Thompson, H., 2019. Comparison of Pesticide Exposure in Honey Bees (Hymenoptera: Apidae) and

- Bumble Bees (Hymenoptera: Apidae): Implications for Risk Assessments. *Environ. Entomol.* 48, 12–21.
- Graystock, P., Jones, J.C., Pamminer, T., Parkinson, J.F., Norman, V., Blane, E.J., Rothstein, L., Wäckers, F., Goulson, D., Hughes, W.O.H., 2016. Hygienic food to reduce pathogen risk to bumblebees. *J. Invertebr. Pathol.* 136, 68–73.
- Gixti, J.C., Wong, L.T., Cameron, S.A., Favret, C., 2009. Decline of bumble bees (*Bombus*) in the North American Midwest. *Biol. Conserv.* 142, 75–84.
- Gumbert, A., 2000. Color choices by bumble bees (*Bombus terrestris*): Innate preferences and generalization after learning. *Behav. Ecol. Sociobiol.* 48, 36–43.
- Haaland, C., Naisbit, R.E., Bersier, L.-F., 2011. Sown wildflower strips for insect conservation: a review. *Insect Conserv. Divers.* 4, 60–80.
- Han, W., Wang, Y., Gao, J., Wang, S., Zhao, S., Liu, J., Zhong, Y., Zhao, D., 2018. Acute toxicity and sublethal effects of myclobutanil on respiration, flight and detoxification enzymes in *Apis cerana cerana*. *Pestic. Biochem. Physiol.* 147, 133–138.
- Harder, L. D., Barclay, R. M. R., 1994. The Functional Significance of Poricidal Anthers and Buzz Pollination: Controlled Pollen Removal. *Funct. Ecol.* 8, 509–517.
- Heard, M.S., Carvell, C., Carreck, N.L., Rothery, P., Osborne, J.L., Bourke, A.F.G., 2007. Landscape context not patch size determines bumble-bee density on flower mixtures sown for agri-environment schemes. *Biol. Lett.* 3, 638–641.
- Heinrich, B., 1993. *The Hot-Blooded Insects*. Springer Berlin Heidelberg, Berlin, Heidelberg.
- Henry, M., Béguin, M., Requier, F., Rollin, O., Odoux, J.F., Aupinel, P., Aptel, J., Tchamitchian, S., Decourtye, A., 2012. A common pesticide decreases foraging success and survival in honey bees. *Science*. 336, 348–350.
- Hicks, D.M., Ouvrard, P., Baldock, K.C.R., Baude, M., Goddard, M.A., Kunin, W.E., Mitschunas, N., Memmott, J., Morse, H., Nikolitsi, M., Osgathorpe, L.M., Potts, S.G., Robertson, K.M., Scott, A. V., Sinclair, F., Westbury, D.B., Stone, G.N., 2016. Food for pollinators: Quantifying the nectar and pollen resources of urban flower meadows. *PLoS One* 11, e0158117.
- Higes, M., Martín-Hernández, R., Garrido-Bailón, E., García-Palencia, P., Meana, A., 2008. Detection of infective *Nosema ceranae* (Microsporidia) spores in corbicular pollen of forager honeybees. *J. Invertebr. Pathol.* 97, 76–78.
- Hodgkiss, D., Brown, M.J.F., Fountain, M.T., 2019. The effect of within-crop floral resources on pollination, aphid control and fruit quality in commercial strawberry. *Agric. Ecosyst. Environ.* 275, 112–122.
- Hoehn, P., Tschardt, T., Tylianakis, J.M., Steffan-Dewenter, I., 2008. Functional group diversity of bee pollinators increases crop yield. *Proc. R. Soc. B Biol. Sci.* 275, 2283–2291.
- Hogg, B.N., Bugg, R.L., Daane, K.M., 2011. Attractiveness of common insectary and harvestable floral resources to beneficial insects. *Biol. Control* 56, 76–84.
- Holtschuh, A., Steffan-Dewenter, I., Kleijn, D., Tschardt, T., 2006. Diversity of flower-visiting bees in cereal fields: effects of farming system, landscape composition and regional context. *J. Appl. Ecol.* 44, 41–49.
- IBM Corp. Released, 2013. IBM SPSS Statistics for Windows, version 22.0.
- Ings, T.C., Ward, N.L., Chittka, L., 2006. Can commercially imported bumble bees out-compete

- their native conspecifics? J. Appl. Ecol. 43, 940–948.
- Isaacs, R., Tuell, J., Fiedler, A., Gardiner, M., Landis, D., 2009. Maximizing arthropod-mediated ecosystem services in agricultural landscapes: the role of native plants. Front. Ecol. Environ. 7, 196–203.
- Iwasa, T., Motoyama, N., Ambrose, J.T., Roe, R.M., 2004. Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*. Crop Prot. 23, 371–378.
- Javorek, S.K., Mackenzie, K.E., Vander Kloet, S.P., 2006. Comparative Pollination Effectiveness Among Bees (Hymenoptera: Apoidea) on Lowbush Blueberry (Ericaceae: *Vaccinium angustifolium*). Ann. Entomol. Soc. Am. 95, 345–351.
- Jönsson, A.M., Ekroos, J., Dänhardt, J., Andersson, G.K.S., Olsson, O., Smith, H.G., 2015. Sown flower strips in southern Sweden increase abundances of wild bees and hoverflies in the wider landscape. Biol. Conserv. 184, 51–58.
- Kessler, S.C., Tiedeken, E.J., Simcock, K.L., Derveau, S., Mitchell, J., Softley, S., Stout, J.C., Wright, G.A., 2015. Bees prefer foods containing neonicotinoid pesticides. Nature 521, 74–76.
- Khetereli, A., Baramidze, V., Kushad, M., 2016. Chemical management guidelines for control of pests and diseases of vegetables and melons in Georgia, Strengthening Extension and Advisory Services. Georgia, USA.
- Klatt, B.K., Holzschuh, A., Westphal, C., Clough, Y., Smit, I., Pawelzik, E., 2014. Bee pollination improves crop quality, shelf life and commercial value. Proc. R. Soc. B: Biol. Sci. 281, 20132440.
- Klein, A.M., Steffan-Dewenter, I., Tscharnkte, T., 2003. Fruit set of highland coffee increases with the diversity of pollinating bees. Proc. R. Soc. B Biol. Sci. 270, 955–961. d
- Klein, A.M., Vaissiere, B.E., Cane, J.H., Steffan-Dewenter, I., Cunningham, S.A., Kremen, C., Tscharnkte, T., 2007. Importance of pollinators in changing landscapes for world crops. Proc. R. Soc. B Biol. Sci. 274, 303–313.
- Kohler, F., Verhulst, J., van Klink, R., Kleijn, D., 2008. At what spatial scale do high-quality habitats enhance the diversity of forbs and pollinators in intensively farmed landscapes? J. Appl. Ecol. 45, 753–762.
- Kopit, A.M., Pitts-Singer, T.L., 2018. Routes of Pesticide Exposure in Solitary, Cavity-Nesting Bees. Environ. Entomol. 47, 499–510.
- Kremen, C., Williams, N.M., Bugg, R.L., Fay, J.P., Thorp, R.W., 2004. The area requirements of an ecosystem service: crop pollination by native bee communities in California. Ecol. Lett. 7, 1109–1119.
- Krupke, C.H., Hunt, G.J., Eitzer, B.D., Andino, G., Given, K., 2012. Multiple Routes of Pesticide Exposure for Honey Bees Living Near Agricultural Fields. PLoS One 7, e29268.
- Kyriakopoulou, K., Kandris, I., Pachiti, I., Kasiotis, K.M., Spyropoulou, A., Santourian, A., Kitromilidou, S., Pappa, G., Glossioti, M., 2017. Collection and analysis of pesticide residue data for pollen and nectar – Final Report. EFSA Support. Publ. 14.
- Ladurner, E., Bosch, J., Kemp, W.P., Maini, S., 2008. Foraging and nesting behavior of *Osmia lignaria* (Hymenoptera: Megachilidae) in the presence of fungicides: cage studies. J. Econ. Entomol. 101, 647–653.
- Ladurner, E., Bosch, J., Kemp, W.P., Maini, S., 2005. Assessing delayed and acute toxicity of five

- formulated fungicides to *Osmia lignaria* Say and *Apis mellifera*. *Apidologie* 36, 449–460.
- Ladurner, E., Recla, L., Wolf, M., Zelger, R., Burgio, G., 2004. *Osmia cornuta* (Hymenoptera Megachilidae) densities required for apple pollination: A cage study. *J. Apic. Res.* 43, 118–122.
- Laurino, D., Porporato, M., Patetta, A., Manino, A., 2011. Toxicity of neonicotinoid insecticides to honey bees: laboratory tests. *Bull. Insectology* 64, 107–113.
- Lautenbach, S., Seppelt, R., Liebscher, J., Dormann, C.F., 2012. Spatial and temporal trends of global pollination benefit. *PLoS One* 7, e35954.
- Laycock, I., Cotterell, K.C., O'Shea-Wheller, T.A., Cresswell, J.E., 2014. Effects of the neonicotinoid pesticide thiamethoxam at field-realistic levels on microcolonies of *Bombus terrestris* worker bumble bees. *Ecotoxicol. Environ. Saf.* 100, 153–158.
- Laycock, I., Lenthall, K.M., Barratt, A.T., Cresswell, J.E., 2012. Effects of imidacloprid, a neonicotinoid pesticide, on reproduction in worker bumble bees (*Bombus terrestris*). *Ecotoxicology* 21, 1937–1945.
- Le Féon, V., Schermann-Legionnet, A., Delettre, Y., Aviron, S., Billeter, R., Bugter, R., Hendrickx, F., Burel, F., 2010. Intensification of agriculture, landscape composition and wild bee communities: A large scale study in four European countries. *Agric. Ecosyst. Environ.* 137, 143–150.
- Lechenet, M., Dessaint, F., Py, G., Makowski, D., Munier-Jolain, N., 2017. Reducing pesticide use while preserving crop productivity and profitability on arable farms. *Nat. Plants* 3, 1–6.
- Lee, K.Y., Lee, K.S., Yoon, H.J., Jin, B.R., 2015. Ovarian development and secretion of vitellogenin protein during the wintering period and after emergence in the hornfaced bee, *Osmia cornifrons*. *J. Asia. Pac. Entomol.* 18, 515–523.
- Littell, R.C., Henry, P.R., Ammerman, C.B., Littell, R.C., Henry, P.R., Ammerman, C.B., 1998. Statistical analysis of repeated measures data using SAS procedures. *J. Anim. Sci.* 74, 1216–1231.
- Lye, G.C., Jennings, S.N., Osborne, J.L., Goulson, D., 2011. Impacts of the Use of Nonnative Commercial Bumble Bees for Pollinator Supplementation in Raspberry. *J. Econ. Entomol.* 104, 107–114.
- M'Gonigle, L.K., Ponisio, L.C., Cutler, K., Kremen, C., 2015. Habitat restoration promotes pollinator persistence and colonization in intensively managed agriculture. *Ecol. Appl.* 25, 1557–1565.
- M'Gonigle, L.K., Williams, N.M., Lonsdorf, E., Kremen, C., 2017. A Tool for Selecting Plants When Restoring Habitat for Pollinators. *Conserv. Lett.* 10, 105–111.
- Maienfisch, P., Angst, M., Brandl, F., Fischer, W., Hofer, D., Kayser, H., Kobel, W., Rindlisbacher, A., Senn, R., Steinemann, A., Widmer, H., 2001. Chemistry and biology of thiamethoxam: A second generation neonicotinoid. *Pest Manag. Sci.* 57, 906–913.
- Mallinger, R.E., Gratton, C., 2015. Species richness of wild bees, but not the use of managed honeybees, increases fruit set of a pollinator-dependent crop. *J. Appl. Ecol.* 52, 323–330.
- Manjon, C., Troczka, B.J., Zaworra, M., Beadle, K., Randall, E., Hertlein, G., Singh, K.S., Zimmer, C.T., Homem, R.A., Lueke, B., Reid, R., Kor, L., Kohler, M., Benting, J., Williamson, M.S., Davies, T.G.E., Field, L.M., Bass, C., Nauen, R., 2018. Unravelling the molecular determinants of bee sensitivity to neonicotinoid insecticides. *Curr. Biol.* 28, 1137–1143.e5.

- MAPA, 2018. Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente. Anuario de estadística 2018 [WWW Document]. URL <https://www.mapa.gob.es/es/estadistica/temas/publicaciones/anuario-de-estadistica/2018/default.aspx?parte=3&capitulo=07&grupo=6&seccion=21> (accessed 11.25.19).
- MAPA, 2017. Ministerio de Agricultura, Pesca y Alimentación. Registro de Productos Fitosanitarios. [WWW Document]. URL <https://www.mapa.gob.es/es/agricultura/temas/sanidad-vegetal/productos-fitosanitarios/registro/menu.asp> (accessed 5.11.17).
- Maroto, J.V., 1995. Horticultura Herbacea Especial. Mundi-Prensa, Madrid.
- Matsumura, C., Yokoyama, J., Washitani, I., 2004. Invasion status and potential ecological impacts of an invasive alien bumblebee, *Bombus terrestris* L. (Hymenoptera: Apidae) naturalized in Southern Hokkaido, Japan. Glob. Environ. Res. 8, 51–66.
- Mattila, H.R., Rios, D., Walker-Sperling, V.E., Roeselers, G., Newton, I.L.G., 2012. Characterization of the active microbiotas associated with honey bees reveals healthier and broader communities when colonies are genetically diverse. PLoS One 7. doi:10.1371/journal.pone.0032962
- Michener, C.D., 2007. The Bees of the World, second. ed. The Johns Hopkins University Press, Baltimore.
- Misganaw, M., Mengesha, G., Awas, T., 2017. Perception of Farmers on Importance of Insect Pollinators in Gozamin District of Amhara Region, Ethiopia. Biodivers. Int. J. 1. doi:10.15406/bij.2017.01.00029
- Mommaerts, V., Reynders, S., Boulet, J., Besard, L., Sterk, G., Smagghe, G., 2010. Risk assessment for side-effects of neonicotinoids against bumblebees with and without impairing foraging behavior. Ecotoxicology 19, 207–215. doi:10.1007/s10646-009-0406-2
- Mommaerts, V., Smagghe, G., 2011. Side-Effects of Pesticides on the Pollinator *Bombus*: An Overview. In: Stoytcheva M, editor. Pesticides in the Modern World – Pests Control and Pesticides Exposure and Toxicity Assessment. Rijeka, Croatia: InTech. 508–552.
- Morandin, L.A., Kremen, C., 2013. Hedgerow restoration promotes pollinator populations and exports native bees to adjacent fields. Ecol. Appl. 23, 829–839.
- Morandin, L.A., Lavery, T.M., Kevan, P.G., Khosla, S., Shipp, L., 2001. Bumble bee (Hymenoptera: Apidae) activity and loss in commercial tomato greenhouses. Can. Entomol. 133, 883–893.
- Morrison, J., Izquierdo, J., Hernández, E., González-andújar, J.L., 2017. Agriculture , Ecosystems and Environment The role of field margins in supporting wild bees in Mediterranean cereal agroecosystems : Which biotic and abiotic factors are important ? Agric. Ecosyst. Environ. 247, 216–224.
- Mullin, C.A., Frazier, M., Frazier, J.L., Ashcraft, S., Simonds, R., VanEngelsdorp, D., Pettis, J.S., 2010. High levels of miticides and agrochemicals in north American apiaries: implications for honey bee health. PLoS One 5, e9754.
- Ngan, C.K., Cheah, U.B., Abdullah, W.Y.W.A.N., Lim, K.P., Ismail, B.S., 2005. Fate of Chlorothalonil, Chlorpyrifos and Profenofos in a Vegetable Farm in cameron Highlands, Malaysia. Water, Air, Soil Pollut. Focus 5, 125–136.
- Nicholls, C.I., Altieri, M.A., 2013. Plant biodiversity enhances bees and other insect pollinators

- in agroecosystems. A review. *Agron. Sustain. Dev.* 33, 257–274.
- Nicholls, E., Fowler, R., Niven, J.E., Gilbert, J.D., Goulson, D., 2017. Larval exposure to field-realistic concentrations of clothianidin has no effect on development rate, over-winter survival or adult metabolic rate in a solitary bee, *Osmia bicornis*. *PeerJ* 5, e3417.
- Nuyttens, D., Devarrewaere, W., Verboven, P., Foqué, D., 2013. Pesticide-laden dust emission and drift from treated seeds during seed drilling: A review. *Pest Manag. Sci.* 69, 564–575.
- OECD, 2017. Guideline for the Testing of Chemicals, No. 245: Honeybees (*Apis mellifera* L.), chronic oral toxicity test (10-day feeding). *Guidel. Test. Chem.* 1–7.
- Ollerton, J., Winfree, R., Tarrant, S., 2011. How many flowering plants are pollinated by animals? *Oikos* 120, 321–326.
- Ortiz-Sánchez, F.J., 2011. Lista actualizada de las especies de abejas en España (Hymenoptera: Apoidea: Apiformes). *Boletín la Soc. Entomológica Aragon.* 49, 265–281.
- Ortiz-Sánchez, F.J., Aguado, L.Ó., Ornos, C., 2018. Diversidad de abejas en España, tendencia de las poblaciones y medidas para su conservación (Hymenoptera, Apoidea, Anthophila). *Ecosistemas* 27, 3–8.
- Pacífico da Silva, I., Oliveira, F.A.S., Pedroza, H.P., Gadelha, I.C.N., Melo, M.M., Soto-Blanco, B., 2015. Pesticide exposure of honeybees (*Apis mellifera*) pollinating melon crops. *Apidologie* 46, 703–715.
- PalDat, 2017. PalDat- Palynological Database [WWW Document]. URL <https://www.palдат.org/> (accessed 6.11.17).
- Pareja, L., Colazzo, M., Pérez-Parada, A., Niell, S., Carrasco-Letelier, L., Besil, N., Cesio, M.V., Heinzen, H., 2011. Detection of pesticides in active and depopulated beehives in Uruguay. *Int. J. Environ. Res. Public Health* 8, 3844–3858.
- Pineda, A., Marcos-García, M.Á., 2008. Use of selected flowering plants in greenhouses to enhance aphidophagous hoverfly populations (Diptera: Syrphidae). *Ann. la Soc. Entomol. Fr.* 44, 487–492.
- Pisanty, G., Klein, A.M., Mandelik, Y., 2014. Do wild bees complement honeybee pollination of confection sunflowers in Israel? *Apidologie* 45, 235–247.
- Pohorecka, K., Skubida, P., Miszczak, A., Semkiw, P., Sikorski, P., Zagibajło, K., Teper, D., Kołowski, Z., Skubida, M., Zdańska, D., Bober, A., 2012. Residues of neonicotinoid insecticides in bee collected plant materials from oilseed rape crops and their effect on bee colonies. *J. Apic. Sci.* 56, 115–134.
- Polidori, C., Rubichi, A., Barbieri, V., Trombino, L., Donegana, M., 2010. Floral resources and nesting requirements of the ground-nesting social bee, *Lasioglossum malachurum* (Hymenoptera: Halictidae), in a Mediterranean semiagricultural landscape. *Psyche*. 2010, 1–11.
- Pontin, D.R., Wade, M.R., Kehrli, P., Wratten, S.D., 2006. Attractiveness of single and multiple species flower patches to beneficial insects in agroecosystems. *Ann. Appl. Biol.* 148, 39–47.
- Porrini, C., Mutinelli, F., Bortolotti, L., Granato, A., Laurenson, L., Roberts, K., Gallina, A., Silvester, N., Medrzycki, P., Renzi, T., Sgolastra, F., Lodesani, M., 2016. The status of honey bee health in Italy: results from the nationwide bee monitoring network. *PLoS One* 11, e0155411.
- Potts, R., Clarke, R.M., Oldfield, S.E., Wood, L.K., Hempel de Ibarra, N., Cresswell, J.E., 2018.

- The effect of dietary neonicotinoid pesticides on non-flight thermogenesis in worker bumble bees (*Bombus terrestris*). *J. Insect Physiol.* 104, 33–39.
- Potts, S.G., Biesmeijer, J.C., Kremen, C., Neumann, P., Schweiger, O., Kunin, W.E., 2010. Global pollinator declines: trends, impacts and drivers. *Trends Ecol. Evol.* 25, 345–353.
- PPDB, 2019. Pesticides Properties DataBase. University of Hertfordshire [WWW Document]. URL <https://sitem.herts.ac.uk/aeru/ppdb/en/index.htm> (accessed 10.3.19).
- Quinn, N.F., Brainard, D.C., Szendrei, Z., 2017. Floral Strips Attract Beneficial Insects but Do Not Enhance Yield in Cucumber Fields. *J. Econ. Entomol.* 110, 517–524.
- Rader, R., Batomeus, I., Garibaldi, L., Garratt, M.P.D., Howlett, B., Cunningham, S.A., Mayfield, M.M., Arthur, A.D., Andersson, G.K.S., Blanche, R., Bommarco, R., Brittain, C., Carneiro, L.G., Chacoff, N., Entling, M.H., Foully, B., Freitas, B.M., Gemmill-Herren, B., Ghazoul, J., Griffin, S., Gross, C.L., Herbertsson, L., Herzog, F., Hipólito, J., Jaggar, S., Jauker, F., Klein, A.M., Kleijn, D., Krishnan, S., Queiroz Lemos, C., Lindström, S.A.M., Mandelik, Y., Magalhães Monteiro, V., Nelson, W., Nilsson, L., Pattemore, D., de Oliveira, N., Pisanty, G., Potts, S.G., Reemer, M., Rundlöf, M., Sheffield, C.S., Scheper, J., Schüepp, C., Taki, H., Vergara, C.H., Viana, B.F., Woyciechowski, M., Winfree, R., 2016. Non-bee insects are important contributors to global crop pollination. *Proc. Natl. Acad. Sci.* 113, 146–151.
- Rodrigo Gómez, S., Ornos, C., Selfa, J., Guara, M., Polidori, C., 2016. Small sweat bees (Hymenoptera : Halictidae) as potential major pollinators of melon (*Cucumis melo*) in the Mediterranean. *Entomol. Sci.* 19, 55–66.
- Rolke, D., Persigehl, M., Peters, B., Sterk, G., Blenau, W., 2016. Large-scale monitoring of effects of clothianidin-dressed oilseed rape seeds on pollinating insects in northern Germany: residues of clothianidin in pollen, nectar and honey. *Ecotoxicology* 25, 1691–1701.
- Rondeau, G., Sánchez-Bayo, F., Tennekes, H.A., Decourtye, A., Ramírez-Romero, R., Desneux, N., 2014. Delayed and time-cumulative toxicity of imidacloprid in bees, ants and termites. *Sci. Rep.* 4, 1–8.
- Rosa García, R., Miñarro, M., 2014. Role of floral resources in the conservation of pollinator communities in cider-apple orchards. *Agric. Ecosyst. Environ.* 183, 118–126.
- Roulston, T.H., Goodell, K., 2011. The Role of Resources and Risks in Regulating Wild Bee Populations. *Annu. Rev. Entomol.* 56, 293–312.
- Roulston, T.H.I., Cane, J.H., 2000. Plant Systematics Pollen nutritional content and digestibility for animals. *Plant Syst. Evol.* 222, 187–209.
- Rundlöf, M., Andersson, G.K.S., Bommarco, R., Fries, I., Hederström, V., Herbertsson, L., Jonsson, O., Klatt, B.K., Pedersen, T.R., Yourstone, J., Smith, H.G., 2015. Seed coating with a neonicotinoid insecticide negatively affects wild bees. *Nature* 521, 77–80.
- Sanchez-Bayo, F., Goka, K., 2014. Pesticide residues and bees-a risk assessment. *PLoS One* 9, e94482.
- Sandrock, C., Tanadini, L.G., Pettis, J.S., Biesmeijer, J.C., Potts, S.G., Neumann, P., 2014. Sublethal neonicotinoid insecticide exposure reduces solitary bee reproductive success. *Agric. For. Entomol.* 16, 119–128.
- Sarmah, A.K., Müller, K., Ahmad, R., 2004. Fate and behaviour of pesticides in the agroecosystem - A review with a New Zealand perspective. *Aust. J. Soil Res.* 42, 125–154.
- Scheper, J., Bommarco, R., Holzschuh, A., Potts, S.G., Riedinger, V., Roberts, S.P.M., Rundlöf,

- M., Smith, H.G., Steffan-Dewenter, I., Wickens, J.B., Wickens, V.J., Kleijn, D., 2015. Local and landscape-level floral resources explain effects of wildflower strips on wild bees across four European countries. *J. Appl. Ecol.* 52, 1165–1175.
- Scheper, J., Holzschuh, A., Kuussaari, M., Potts, S.G., Rundlöf, M., Smith, H.G., Kleijn, D., 2013. Environmental factors driving the effectiveness of European agri-environmental measures in mitigating pollinator loss – a meta-analysis. *Ecol. Lett.* 16, 912–920.
- Schmid-Hempel, R., Eckhardt, M., Goulson, D., Heinzmann, D., Lange, C., Plischuk, S., Escudero, L.R., Salathé, R., Scriven, J.J., Schmid-Hempel, P., 2014. The invasion of southern South America by imported bumblebees and associated parasites. *J. Anim. Ecol.* 83, 823–837.
- Schmuck, R., Schning, R., Stork, A., Schramel, O., 2001. Risk posed to honeybees (*Apis mellifera* L., Hymenoptera) by an imidacloprid seed dressing of sunflowers. *Pest Manag. Sci.* 57, 225–238.
- Schneider, C.W., Tautz, J., Grünewald, B., Fuchs, S., 2012. RFID tracking of sublethal effects of two neonicotinoid insecticides on the foraging behavior of *Apis mellifera*. *PLoS One* 7, 1–9.
- Scott-Dupree, C.D., Conroy, L., Harris, C.R., 2009. Impact of currently used or potentially useful insecticides for canola agroecosystems on *Bombus impatiens* (Hymenoptera: Apidae), *Megachile rotundata* (Hymenoptera: Megachilidae), and *Osmia lignaria* (Hymenoptera: Megachilidae). *J. Econ. Entomol.* 102, 177–182.
- Sekita, N., Yamada, M., 1993. Use of *Osmia cornifrons* for pollination of apples in Aomori prefecture, Japan. *Japan Agric. Res. Q.* 26, 264–270.
- Sgolastra, F., Arnan, X., Cabbri, R., Isani, G., Medrzycki, P., Teper, D., Bosch, J., Mediche, S., 2018a. Combined exposure to sublethal concentrations of an insecticide and a fungicide affect feeding, ovary development and longevity in a solitary bee. *Proc. R. Soc. B Biol. Sci.* 285, 20180887.
- Sgolastra, F., Arnan, X., Pitts-Singer, T.L., Maini, S., Kemp, W.P., Bosch, J., 2016. Pre-wintering conditions and post-winter performance in a solitary bee: does diapause impose an energetic cost on reproductive success? *Ecol. Entomol.* 41, 201–210.
- Sgolastra, F., Blasioli, S., Renzi, T., Tosi, S., Medrzycki, P., Molowny-Horas, R., Porrini, C., Braschi, I., 2018b. Lethal effects of Cr(III) alone and in combination with propiconazole and clothianidin in honey bees. *Chemosphere* 191, 365–372.
- Sgolastra, F., Hinarejos, S., Pitts-Singer, T.L., Boyle, N.K., Joseph, T., Luckmann, J., Raine, N.E., Singh, R., Williams, N.M., Bosch, J., 2019. Pesticide Exposure Assessment Paradigm for Solitary Bees. *Environ. Entomol.* 48, 22–35.
- Sgolastra, F., Medrzycki, P., Bortolotti, L., Renzi, M.T., Tosi, S., Bogo, G., Teper, D., Porrini, C., Molowny-Horas, R., Bosch, J., 2017. Synergistic mortality between a neonicotinoid insecticide and an ergosterol-biosynthesis-inhibiting fungicide in three bee species. *Pest Manag. Sci.* 73, 1236–1243.
- Sgolastra, F., Tosi, S., Medrzycki, P., Porrini, C., Burgio, G., 2015. Toxicity of spirotetramat on solitary bee larvae, *Osmia cornuta* (Hymenoptera: Megachilidae), in laboratory conditions. *J. Apic. Sci.* 59, 73–83.
- Shawki, M.A.-A., Titěra, D., Kazda, J., Kohoutková, J., Táborský, V., 2018. Toxicity to honeybees of water guttation and dew collected from winter rape treated with Nurelle D®. *Plant Prot. Sci.* 42, 9–14.

- Shipp, J.L., Whitfield, G.H., Papadopoulos, A.P., 1994. Effectiveness of the bumble bee, *Bombus impatiens* Cr. (Hymenoptera: Apidae), as a pollinator of greenhouse sweet pepper. *Sci. Hortic. (Amsterdam)*. 57, 29–39.
- SIAR, 2015. Sistema de información agroclimática para el regadío. Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente [WWW Document]. URL <http://eportal.magrama.gob.es/websiar/Inicio.aspx> (accessed 11.15.15).
- Simon-Delso, N., Amaral-Rogers, V., Belzunces, L.P., Bonmatin, J.M., Chagnon, M., Downs, C., Furlan, L., Gibbons, D.W., Giorio, C., Girolami, V., Goulson, D., Kreutzweiser, D.P., Krupke, C.H., Liess, M., Long, E., Mcfield, M., Mineau, P., Mitchell, E.A., Morrissey, C.A., Noome, D.A., Pisa, L., Settele, J., Stark, J.D., Tapparo, A., Van Dyck, H., Van Praagh, J., Van Der Sluijs, J.P., Whitehorn, P.R., Wiemers, M., 2015. Systemic insecticides (Neonicotinoids and fipronil): Trends, uses, mode of action and metabolites. *Environ. Sci. Pollut. Res.* 22, 5–34.
- Singh, R., Levitt, A.L., Rajotte, E.G., Holmes, E.C., Ostiguy, N., Vanengelsdorp, D., Lipkin, W.I., Depamphilis, C.W., Toth, A.L., Cox-Foster, D.L., 2010. RNA viruses in hymenopteran pollinators: Evidence of inter-taxa virus transmission via pollen and potential impact on non-*Apis* hymenopteran species. *PLoS One* 5.
- Stanley, D.A., Garratt, M.P.D., Wickens, J.B., Wickens, V.J., Potts, S.G., Raine, N.E., 2015. Neonicotinoid pesticide exposure impairs crop pollination services provided by bumblebees. *Nature* 528, 548–550.
- Stanley, D.A., Russell, A.L., Morrison, S.J., Rogers, C., Raine, N.E., 2016. Investigating the impacts of field-realistic exposure to a neonicotinoid pesticide on bumblebee foraging, homing ability and colony growth. *J. Appl. Ecol.* 53, 1440–1449.
- Stefanescu, C., Asís, J.D., Baños-Picón, L., Cerdà, X., García, M.A.M., Micó, E., Ricarte, A., Tormos, J., 2018. Diversidad de insectos polinizadores en la península ibérica. *Ecosistemas* 27, 9–22.
- Stoner, K.A., Eitzer, B.D., 2012. Movement of soil-applied imidacloprid and thiamethoxam into nectar and pollen of squash (*Cucurbita pepo*). *PLoS One* 7, e39114.
- Straub, L., Williams, G.R., Pettis, J., Fries, I., Neumann, P., 2015. Superorganism resilience: Eusociality and susceptibility of ecosystem service providing insects to stressors. *Curr. Opin. Insect Sci.* 12, 109–112.
- Struger, J., Grabuski, J., Cagampan, S., Sverko, E., Marvin, C., 2016. Occurrence and Distribution of Carbamate Pesticides and Metalaxyl in Southern Ontario Surface Waters 2007-2010. *Bull. Environ. Contam. Toxicol.* 96, 423–431.
- Suchail, S., Guez, D., Belzunces, L.P., 2001. Discrepancy between acute and chronic toxicity induced by imidacloprid and its metabolites in *Apis mellifera*. *Environ. Toxicol. Chem.* 20, 1–5.
- Tasei, J.-N., Aupinel, P., 2008a. Original article Nutritive value of 15 single pollens and pollen mixes tested on larvae produced by bumblebee workers (*Bombus terrestris*, Hymenoptera : Apidae). *Apidologie* 39, 397–409.
- Tasei, J.-N., Aupinel, P., 2008b. Validation of a Method Using Queenless *Bombus terrestris* Micro-Colonies for Testing the Nutritive Value of Commercial Pollen Mixes by Comparison with Queenright Colonies. *J. Econ. Entomol.* 101, 1737–1742.
- Tasei, J.-N., Ripault, G., Rivault, E., 2001. Hazards of Imidacloprid Seed Coating to *Bombus terrestris* (Hymenoptera: Apidae) When Applied to Sunflower. *J. Econ. Entomol.* 94, 623–

- 627.
- Tasei, J.N., Lerin, J., Ripault, G., 2000. Sub-lethal effects of imidacloprid on bumblebees, *Bombus terrestris* (Hymenoptera: Apidae), during a laboratory feeding test. *Pest Manag. Sci.* 56, 784–788.
- Thany, S.H., Gauthier, M., 2005. Nicotine injected into the antennal lobes induces a rapid modulation of sucrose threshold and improves short-term memory in the honeybee *Apis mellifera*. *Brain Res.* 1039, 216–219.
- Thany, S.H., Lenaers, G., Crozatier, M., Armengaud, C., Gauthier, M., 2003. Identification and localization of the nicotinic acetylcholine receptor alpha3 mRNA in the brain of the honeybee, *Apis mellifera*. *Insect Mol. Biol.* 12, 255–262.
- Thompson, H.M., 2001. Assessing the exposure and toxicity of pesticides to bumblebees (*Bombus* sp.). *Apidologie* 32, 305–321.
- Thompson, H.M., Fryday, S.L., Harkin, S., Milner, S., 2014. Potential impacts of synergism in honeybees (*Apis mellifera*) of exposure to neonicotinoids and sprayed fungicides in crops. *Apidologie* 45, 545–553.
- Thompson, H.M., Wilkins, S., Harkin, S., Milner, S., Walters, K.F.A., 2015. Neonicotinoids and bumblebees (*Bombus terrestris*): effects on nectar consumption in individual workers. *Pest Manag. Sci.* 71, 946–950.
- Tosi, S., Burgio, G., Nieh, J.C., 2017. A common neonicotinoid pesticide, thiamethoxam, impairs honey bee flight ability. *Sci. Rep.* 7, 1–8.
- Tosi, S., Costa, C., Vesco, U., Quaglia, G., Guido, G., 2018. A 3-year survey of Italian honey bee-collected pollen reveals widespread contamination by agricultural pesticides. *Sci. Total Environ.* 615, 208–218.
- Tosi, S., Démares, F.J., Nicolson, S.W., Medrzycki, P., Pirk, C.W.W., Human, H., 2016. Effects of a neonicotinoid pesticide on thermoregulation of African honey bees (*Apis mellifera scutellata*). *J. Insect Physiol.* 93–94, 56–63.
- Tosi, S., Nieh, J.C., 2017. A common neonicotinoid pesticide, thiamethoxam, alters honey bee activity, motor functions, and movement to light. *Sci. Rep.* 7, 1–13.
- Tschoeke, P.H., Oliveira, E.E., Dalcin, M.S., Silveira-Tschoeke, M.C.A.C., Santos, G.R., 2015. Diversity and flower-visiting rates of bee species as potential pollinators of melon (*Cucumis melo* L.) in the Brazilian Cerrado. *Sci. Hortic. (Amsterdam)*. 186, 207–216.
- Tsvetkov, N., Samson-Robert, O., Sood, K., Patel, H.S., Malena, D.A., Gajiwala, P.H., Maciukiewicz, P., Fournier, V., Zayed, A., 2017. Chronic exposure to neonicotinoids reduces honey bee health near corn crops. *Science*. 356, 1395–1397.
- USEPA, 2014. Guidance for Assessing Pesticide Risks to BeesU.S. Environmental Protection Agency (USEPA) Health Canada Pest Management Regulatory Agency (PMRA) and California Department of Pesticide Regulation, Office of Pesticide Programs USEPA, Health Canada PMRA, CDPR.
- VanEngelsdorp, D., Hayes, J., Underwood, R.M., Pettis, J., 2008. A survey of honey bee colony losses in the U.S., Fall 2007 to Spring 2008. *PLoS One* 3, 8–13.
- VanEngelsdorp, D., Evans, J.D., Saegerman, C., Mullin, C., Haubruge, E., Nguyen, B.K., Frazier, M., Frazier, J., Cox-Foster, D., Chen, Y., Underwood, R., Tarpy, D.R., Pettis, J.S., 2009. Colony collapse disorder: A descriptive study. *PLoS One* 4.

- Velthuis, H.H.W., Van Doorn, A., 2006. A century of advances in bumblebee domestication and the economic and environmental aspects of its commercialization for pollination. *Apidologie* 37, 421–451.
- Vicens, N., Bosch, J., 2000. Pollinating efficacy of *Osmia cornuta* and *Apis mellifera* on “Red Delicious” Apple. *Environ. Entomol.* 29, 235–240.
- Viñuela, E., Adan, A., Rodríguez, J., Hernando, S., Dorado, J., Fernández-Quintanilla, C., Canomanuel, G., Fereres, A., 2012. Provision of ecological infrastructures to increase pollinators and other beneficial organisms in rainfed crops in Central Spain. *IOBC/wrps Bull.* 79, 229–233.
- Walters, S.A., Taylor, B.H., 2006. Effects of honey bee pollination on pumpkin fruit and seed yield. *HortScience* 41, 370–373.
- Wang, Z., Goonewardene, L.A., 2004. The use of MIXED models in the analysis of animal experiments with repeated measures data. *Can. J. Anim. Sci.* 84, 1–11.
- Wasielewski, O., Giejdasz, K., Wojciechowicz, T., Skrzypski, M., 2011. Ovary growth and protein levels in ovary and fat body during adult-wintering period in the red mason bee, *Osmia rufa*. *Apidologie* 42, 749–758.
- Westphal, C., Steffan-Dewenter, I., Tscharntke, T., 2003. Mass flowering crops enhance pollinator densities at a landscape scale. *Ecol. Lett.* 6, 961–965.
- Williams, N.M., Minckley, R.L., Silveira, F.A., 2001. Variation in native bees used for detecting community changes. *Conserv. Ecol.* 5, 7.
- Williams, P., Tang, Y., Yao, J., Cameron, S., 2009. The bumblebees of Sichuan (Hymenoptera: Apidae, Bombini). *Syst. Biodivers.* 7, 491.
- Williamson, S.M., Willis, S.J., Wright, G.A., 2014. Exposure to neonicotinoids influences the motor function of adult worker honeybees. *Ecotoxicology* 23, 1409–1418.
- Willmer, P., 2012. Ecology: Pollinator-plant synchrony tested by climate change. *Curr. Biol.* 22, R131–R132.
- Winfree, R., 2013. Global change, biodiversity, and ecosystem services: What can we learn from studies of pollination? *Basic Appl. Ecol.* 14, 453–460.
- Winfree, R., Aguilar, R., Vázquez, D.P., LeBuhn, G., Aizen, M.A., 2009. A meta-analysis of bees’ responses to anthropogenic disturbance. *Ecology* 90, 2068–2076.
- Winfree, R., Kremen, C., 2009. Are ecosystem services stabilized by differences among species? A test using crop pollination. *Proc. R. Soc. B Biol. Sci.* 276, 229–237.
- Winfree, R., Williams, N.M., Gaines, H., Ascher, J.S., Kremen, C., 2008. Wild bee pollinators provide the majority of crop visitation across land-use gradients in New Jersey and Pennsylvania, USA. *J. Appl. Ecol.* 45, 793–802.
- Woodcock, B.A., Bullock, J.M., Shore, F.R., Heard, M.S., Pereira, M.G., Redhead, J., Ridding, L., Dean, H., Sleep, D., Henrys, P., Peyton, J., Hulmes, S., Hulmes, L., Sárospataki, M., Saure, C., Edwards, M., Genersch, E., Knäbe, S., Pywell, R.F., 2017. Country-specific effects of neonicotinoid pesticides on honeybees and wild bees Authors: Science. 365, 1393–1395.
- Wratten, S.D., Gillespie, M., Decourtye, A., Mader, E., Desneux, N., 2012. Pollinator habitat enhancement: Benefits to other ecosystem services. *Agric. Ecosyst. Environ.* 159, 112–122.
- Wu, Y.Y., Luo, Q.H., Hou, C.S., Wang, Q., Dai, P.L., Gao, J., Liu, Y.J., Diao, Q.Y., 2017. Sublethal effects of imidacloprid on targeting muscle and ribosomal protein related genes in the

- honey bee *Apis mellifera* L. Sci. Rep. 7, 1–8. doi:10.1038/s41598-017-16245-0
- Yang, E.C., Chuang, Y.C., Chen, Y.L., Chang, L.H., 2008. Abnormal Foraging Behavior Induced by Sublethal Dosage of Imidacloprid in the Honey Bee (Hymenoptera: Apidae). J. Econ. Entomol. 101, 1743–1748.
- Yoder, J.A., Jajack, A.J., Rosselot, A.E., Smith, T.J., Yerge, M.C., Sammataro, D., 2013. Fungicide contamination reduces beneficial fungi in bee bread based on an area-wide field study in honey bee, *Apis mellifera*, colonies. J. Toxicol. Environ. Heal. Part A 76, 587–600.
- Zhu, Y.C., Yao, J., Adamczyk, J., Luttrell, R., 2017. Feeding toxicity and impact of imidacloprid formulation and mixtures with six representative pesticides at residue concentrations on honey bee physiology (*Apis mellifera*). PLoS One 12, e0178421.
- Zurbuchen, A., Landert, L., Klaiber, J., Müller, A., Hein, S., Dorn, S., 2010. Maximum foraging ranges in solitary bees: only few individuals have the capability to cover long foraging distances. Biol. Conserv. 143, 669–676.

APPENDIX 1

List of products used in the year of sampling in the 5 commercial melon fields.

Pesticide	Comercial product	Active ingredient	Application day	Application method
FIELD 1 trasplant day: 09/05/2017; samping day: 05/07/2017				
ins	Teppeki®	Flonicamid	17/06/2017	Spray
fung	Collis®	Boscalid and Kresoxim-methyl	30/06/2017	
ins	Mospilan certis®	Acetamiprid	30/06/2017	
fung	Bayfidan®	Triadimenol	30/06/2017	
ins-ac	Vargas®	Abamectin	30/06/2017	
fung	Vivando®	Metrafenone	13/07/2017	
insc	Vargas®	Abamectin	26/07/2017	
fung	Bayfidan®	Triadimenol	27/07/2017	
insc	Mospilan certis®	Acetamiprid	27/07/2017	
fung	Quimaazufre®	Sulphur	12/08/2017	
ins	Confidor®	Imidacloprid		
FIELD 2 trasplant day:26/04/2017; samping day: 06/07/2017				
ins	Epik®	Acetamiprid	04/07/2017	Spray
fung	Arius®	Quinoxyfen	04/07/2017	
fung	Syshtane Forte®	Myclobutanil	04/07/2017	
ins	Confidor®	Imidacloprid		seedling spray
FIELD 3 trasplant day:20/05/2017; samping day: 12/07/2017				
ins	Epik®	Acetamiprid	01/07/2017	Spray
fung	Arius®	Quinoxyfen	01/07/2017	
fung	Syshtane Forte®	Myclobutanil	01/07/2017	
ins	Epik®	Acetamiprid	21/07/2017	
fung	Arius®	Quinoxyfen	21/07/2017	
fung	Syshtane Forte®	Myclobutanil	21/07/2017	
FIELD 4 trasplant day:12/06/2017; samping day: 27/07/2017				
fung	Arius®	Quinoxyfen		Spray
fung	Syshtane Forte®	Myclobutanil		
fung	Collis®	Boscalid and Kresoxim-methyl	data no available	
ins	Movento®	Spirotetramat		
ins	Epik®	Acetamiprid		
ins	Confidor®	Imidacloprid		

FIELD 5 trasplant day:19/06/2017; samping day: 06/08/2017				
fung	Arius®	Quinoxifen	23/07/2017	
fung	Syshtane Forte®	Myclobutanil	23/07/2017	
ins	Vargas®	Abamectin	23/07/2017	
fung	Arius®	Quinoxifen	04/08/2017	Spray
fung	Syshtane Forte®	Myclobutanil	04/08/2017	
ins	Vargas®	Abamectin	04/08/2017	
ins	Epik®	Acetamiprid	04/08/2017	
ins	Confidor®	Imidacloprid		seedling spray

APPENDIX 2

Pesticide type and family class of the compounds target in multi-residual analyses carried out in the Laboratorio Químico Microbiológico de Sevilla by high performance liquid chromatography with quantification and confirmation by triple-quadrupole mass spectrometer detector (HPLC-QQQ) and gas chromatography with triple-quadrupole mass spectrometer detector (GC-QQQ).

Compound	Pesticide type	Family/Class	Technique
ABAMECTIN	ins	avermectins	LC-QQQ
EMAMECTINA	ins	avermectins	LC-QQQ
DIFLUBENZURON	ins	benzoylureas	LC-QQQ
FLUFENOXURON	ins	benzoylureas	LC-QQQ
LUFENURON	ins	benzoylureas	LC-QQQ
TRIFLUMURON	ins	benzoylureas	LC-QQQ
ALDICARB	ins	carbamates	LC-QQQ
BENDIOCARB	ins	carbamates	LC-QQQ
BUTOXYCARBOXIM	ins	carbamates	LC-QQQ
CARBARYL	ins	carbamates	LC-QQQ
CARBOFURAN	ins	carbamates	LC-QQQ
ETHIOFENCARB	ins	carbamates	LC-QQQ
ETHIOFENCARB	ins	carbamates	LC-QQQ
FURATHIOCARB	ins	carbamates	LC-QQQ
METHIOCARB	ins	carbamates	LC-QQQ
METHOMYL	ins	carbamates	LC-QQQ
OXAMYL	ins	carbamates	LC-QQQ
PIRIMICARB	ins	carbamates	LC-QQQ
PROPOXUR	ins	carbamates	LC-QQQ
THIODICARB	ins	carbamates	LC-QQQ
CLOFENTEZINE	ins	clofentezine, diflovidazin, hexythiazox	LC-QQQ
METHOXYFENOZIDE	ins	diacylhydrazines	LC-QQQ
TEBUFENOZIDE	ins	diacylhydrazines	LC-QQQ
CLORANTRANILIPROL	ins	diamides	LC-QQQ
ETOXAZOLE	ins	etoxazole	LC-QQQ
FENOXYCARB	ins	fenoxycarb	LC-QQQ
FLONICAMID	ins	flonicamid	LC-QQQ
HEXYTHIAZOX	ins	hexythiarox	LC-QQQ
TEBUFENPYRAD	ins	meti acaricides and insecticides	LC-QQQ
ACETAMIPRID	ins	neonicotinoids	LC-QQQ
CLOTHIANIDIN	ins	neonicotinoids	LC-QQQ
IMIDACLOPRID	ins	neonicotinoids	LC-QQQ
NITENPYRAM	ins	neonicotinoids	LC-QQQ

THIACLOPRID	ins	neonicotinoids	LC-QQQ
THIAMETOXAM	ins	neonicotinoids	LC-QQQ
ACEPHATE	ins	organophosphates	LC-QQQ
FENAMIPHOS	ins	organophosphates	LC-QQQ
METHAMIDOPHOS	ins	organophosphates	LC-QQQ
OXYDEMETON-METHYL	ins	organophosphates	LC-QQQ
INDOXACARB	ins	oxadiazines	LC-QQQ
PROPARGITE	ins	propargite	LC-QQQ
PYMETROZINE	ins	pyridine azomethine derivatives	LC-QQQ
ROTENONE	ins	rotenone	LC-QQQ
SPINOSAD	ins	spinosyns	LC-QQQ
SPIROMESIFEN	ins	tetronic and tetramic acid derivatives	LC-QQQ
SPIROTETRAMAT	ins	tetronic and tetramic acid derivatives	LC-QQQ
METALAXIL-M	fung	acylalanines	LC-QQQ
MEPANIPYRIM	fung	anilino-pyrimidines	LC-QQQ
CARBENDAZIM	fung	benzimidazoles	LC-QQQ
THIABENDAZOLE	fung	benzimidazoles	LC-QQQ
METRAFENONE	fung	benzophenone	LC-QQQ
PROPAMOCARB	fung	carbamates	LC-QQQ
DIMETHOMORPH	fung	cinnamic acid amides	LC-QQQ
CYMOXANILO	fung	cianoacetamideoxime	LC-QQQ
CYAZOFAMID	fung	cyano-imidazole	LC-QQQ
DODINE	fung	guanidines	LC-QQQ
FENHEXAMID	fung	hydroxyanilides	LC-QQQ
IMAZALIL	fung	imidazoles	LC-QQQ
AZOXYSTROBIN	fung	methoxyacrylates	LC-QQQ
PYRACLOSTROBIN	fung	methoxy-carbamates	LC-QQQ
FENPROIMORPH	fung	morpholines	LC-QQQ
TRIDEMORPH	fung	morpholines	LC-QQQ
DIETHOFENCARB	fung	n-phenyl carbamates	LC-QQQ
FAMOXADONE	fung	oxazolidine-diones	LC-QQQ
OXADIXY	fung	oxazolidinones	LC-QQQ
MEPRONIL	fung	phenyl-benzamides	LC-QQQ
TRIFORINE	fung	piperazines	LC-QQQ
BOSCALID	fung	pyridine-carboxamide	LC-QQQ
FLUOPYRAM	fung	pyridinyl-ethyl-benzamides	LC-QQQ
ZOXAMIDE	fung	s toluamides	LC-QQQ
SPIROXAMINE	fung	spiroketal-amines	LC-QQQ
THIOPHANATE-METHYL	fung	thiophanates	LC-QQQ
DINICONAZOLE	fung	triazoles	LC-QQQ
EPOXICONAZOLE	fung	triazoles	LC-QQQ
BROMUCONAZOLE	fung	triazoles	LC-QQQ
FENBUCONAZOLE	fung	triazoles	LC-QQQ
IPROVALICARB	fung	valinamide carbamates	LC-QQQ
DIFLUFENICAN	herb	amides	LC-QQQ
OXADIAZON	herb	oxadiazolones	LC-QQQ
PROSULFOCARB	herb	thiocarbamates	LC-QQQ
ATRAZINA	herb	triazines	LC-QQQ

PROMETRYN	herb	triazines	LC-QQQ
SIMAZINE	herb	triazines	LC-QQQ
METAMITRON	herb	triazinones	LC-QQQ
BUTURON	herb	ureas	LC-QQQ
CHLORBROMURON	herb	ureas	LC-QQQ
CHLOROTOLURON	herb	ureas	LC-QQQ
CHLOROXURON	herb	ureas	LC-QQQ
DIFENOXURON	herb	ureas	LC-QQQ
DIURON	herb	ureas	LC-QQQ
FLUOMETURON	herb	ureas	LC-QQQ
ISOPROTURON	herb	ureas	LC-QQQ
LINURON	herb	ureas	LC-QQQ
METOBROMURON	herb	ureas	LC-QQQ
METOXURON	herb	ureas	LC-QQQ
MONOLINURON	herb	ureas	LC-QQQ
MONURON	herb	ureas	LC-QQQ
NEBURON	herb	ureas	LC-QQQ
BROMOPROPYLATE	ins	bromopropylate	GC-QQQ
BUPROFEZIN	ins	buprofezin	GC-QQQ
FORMETANATE	ins	carbamates	GC-QQQ
FORMOTHION	ins	carbamates	GC-QQQ
PIRIMICARB	ins	carbamates	GC-QQQ
PROPOXUR	ins	carbamates	GC-QQQ
CLOFENTEZINE	ins	clofentecine	GC-QQQ
ALDRIN	ins	cyclodiene organochlorines	GC-QQQ
DIELDRIN	ins	cyclodiene organochlorines	GC-QQQ
ENDOSULFAN	ins	cyclodiene organochlorines	GC-QQQ
ENDRIN	ins	cyclodiene organochlorines	GC-QQQ
HEPTACHLOR	ins	cyclodiene organochlorines	GC-QQQ
CYROMAZINE	ins	cyromazine	GC-QQQ
DICOFOL	ins	dicofol	GC-QQQ
ETOXAZOLE	ins	etoxazole	GC-QQQ
AZINPHOS-METHYL	ins	organophosphates	GC-QQQ
BROMOPHOS- METIL	ins	organophosphates	GC-QQQ
BROMOPHOS-ETIL	ins	organophosphates	GC-QQQ
CADUSAFOS	ins	organophosphates	GC-QQQ
CARBOPHENOTHION	ins	organophosphates	GC-QQQ
CHLORFENVINPHOS	ins	organophosphates	GC-QQQ
CHLORPYRIFOS	ins	organophosphates	GC-QQQ
DIAZINON	ins	organophosphates	GC-QQQ
DICHLOFENTHION	ins	organophosphates	GC-QQQ
DICHLORVOS	ins	organophosphates	GC-QQQ
DIMETHOATE	ins	organophosphates	GC-QQQ
DISULFOTON	ins	organophosphates	GC-QQQ
ETHION	ins	organophosphates	GC-QQQ
ETHOPROPHOS	ins	organophosphates	GC-QQQ
ETRIMFOS	ins	organophosphates	GC-QQQ
FENITROTHION	ins	organophosphates	GC-QQQ

FENPROIMORPH	ins	organophosphates	GC-QQQ
FENTHION	ins	organophosphates	GC-QQQ
FONOFOS	ins	organophosphates	GC-QQQ
HEPTENOPHOS	ins	organophosphates	GC-QQQ
MALATHION	ins	organophosphates	GC-QQQ
MECARBAM	ins	organophosphates	GC-QQQ
METHAMIDOPHOS	ins	organophosphates	GC-QQQ
METHIDATHION	ins	organophosphates	GC-QQQ
METHYL-PARTHION	ins	organophosphates	GC-QQQ
MEVINPHOS	ins	organophosphates	GC-QQQ
MONOCROTOPHOS	ins	organophosphates	GC-QQQ
OMETHOATE	ins	organophosphates	GC-QQQ
PARATHIO	ins	organophosphates	GC-QQQ
PARATHION-METHYL	ins	organophosphates	GC-QQQ
PHENTHOATE	ins	organophosphates	GC-QQQ
PHOSALONE	ins	organophosphates	GC-QQQ
PHOSMET	ins	organophosphates	GC-QQQ
PIRIMIPHOS-METHY	ins	organophosphates	GC-QQQ
PROFENOFOS	ins	organophosphates	GC-QQQ
PYRIDAPHENTHION	ins	organophosphates	GC-QQQ
QUINALPHOS	ins	organophosphates	GC-QQQ
TERBUFOS	ins	organophosphates	GC-QQQ
ACRINATHRIN	ins	pyrethroids, pyrethrins	GC-QQQ
ALPHA-CYPERMETHRIN	ins	pyrethroids, pyrethrins	GC-QQQ
BIFENTHRIN	ins	pyrethroids, pyrethrins	GC-QQQ
CYPERMETHRIN	ins	pyrethroids, pyrethrins	GC-QQQ
DELTAMETHRIN	ins	pyrethroids, pyrethrins	GC-QQQ
LAMBDA-CYHALOTHRIN	ins	pyrethroids, pyrethrins	GC-QQQ
PERMETHRIN	ins	pyrethroids, pyrethrins	GC-QQQ
TEFLUTHRIN	ins	pyrethroids, pyrethrins	GC-QQQ
ZETA-CYPERMETHRIN	ins	pyrethroids, pyrethrins	GC-QQQ
TETRADIFON	ins	tetradifon	GC-QQQ
ETRIDIAZOLE	fung	1,2,4-thiadiazoles	GC-QQQ
BENALAXYL	fung	acylalanines	GC-QQQ
CYPRODINIL	fung	anilino-pyrimidines	GC-QQQ
QUINOXYFEN	fung	aryloxyquinoline	GC-QQQ
OFURACE	fung	butyrolactones	GC-QQQ
CHINOMETHIONAT	fung	chinomethionat	GC-QQQ
CHLOROTHALONIL	fung	chloronitriles	GC-QQQ
CIAZOFAMIDA	fung	cianoimidazol	GC-QQQ
CIMOXANIL	fung	cyanoacetamide-oxime	GC-QQQ
PROCYMIDONE	fung	dicarboximides	GC-QQQ
VINCLOZOLIN	fung	dicarboximides	GC-QQQ
BUPIRIMATE	fung	hydroxy-(2-amino-) pyrimidines	GC-QQQ
PROCHLORAZ	fung	imidazoles	GC-QQQ
OXADIXY	fung	oxazolidinones	GC-QQQ
KRESOXIM-METHYL	fung	oximino-acetates	GC-QQQ

TRIFLOXYSTROBIN	fung	oximino-acetates	GC-QQQ
FLUDIOXONIL	fung	phenylpyrroles	GC-QQQ
PYRAZOPHOS	fung	phosphoro-thiolates	GC-QQQ
PYRIFENOX	fung	pyridines	GC-QQQ
FENARIMOL	fung	pyrimidines	GC-QQQ
NUARIMOL	fung	pyrimidines	GC-QQQ
QUINOMETHIONATO	fung	quinoxalines	GC-QQQ
DICHOLOFLUANID	fung	sulfamides	GC-QQQ
CYPROCONAZOLE	fung	triazoles	GC-QQQ
DIFENOCONAZOLE	fung	triazoles	GC-QQQ
ETACONAZOL	fung	triazoles	GC-QQQ
MYCLOBUTANIL	fung	triazoles	GC-QQQ
PENCONAZOLE	fung	triazoles	GC-QQQ
PROPICONAZOLE	fung	triazoles	GC-QQQ
TEBUCONAZOLE	fung	triazoles	GC-QQQ
TETRACONAZOLE	fung	triazoles	GC-QQQ
TRIADIMEFON	fung	triazoles	GC-QQQ
TRIADIMENOL	fung	triazoles	GC-QQQ
PROPANIL	herb	amide	GC-QQQ
CHLORTHAL-DIMETHYL	herb	benzoic acid	GC-QQQ
PROPHAM	herb	carbamates	GC-QQQ
ALACHLOR	herb	chloroacetamide	GC-QQQ
METOLACLOR	herb	chloroacetamide	GC-QQQ
BENFLURALIN	herb	dinitroaniline	GC-QQQ
PENDIMETHALIN	herb	dinitroaniline	GC-QQQ
PROFLURALIN	herb	dinitroaniline	GC-QQQ
NITROFEN	herb	diphenyl ether	GC-QQQ
NORFLURAZON	herb	pyridazinone	GC-QQQ
ATRAZINE	herb	triazine	GC-QQQ
SIMAZINA	herb	triazine	GC-QQQ
TERBUTHYLAZINE	herb	triazine	GC-QQQ
TERBUTRYN	herb	triazine	GC-QQQ
METRIBUZIN	herb	triazinone	GC-QQQ

ac= acaricide; ins= insecticide; fung= fungicide.

IRAC= Insecticide resistance action committee; FRAC= fungicide resistance action committee.

LOD of 3 ng/g for each analyte.