The potential impact of sulfoxaflor exposure on bumblebees (*Bombus terrestris*)

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I declare that the following work is my own, with the following exceptions. In Chapter 2 parts of the foraging data were collected by Emma Wrake, Miranda Burke and Sara Cobacho Jimenez. In Chapter 3 the radial-arm maze data was collected by Alfie Scott. In Chapter 4 I performed a Meta-analysis and the data was extracted from 23 published papers. In Chapter 5 Jacob Horner and I collected the microcolony data together. In Chapter 6, in experiments 3 & 4, I was assisted by Callum Martin & Ash Samuelson.

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Abstract

Sulfoxaflor is an increasingly important insecticide which has been licensed for use in 81 countries globally and is thought likely to replace neonicotinoids (the most commonly used insecticide in the world) over large geographical ranges. Despite this, the potential sub-lethal consequences of sulfoxaflor on important pollinators, such as bumblebees (*Bombus spp.*), has yet to be examined. Here, I begin by demonstrating, in Chapter 2, that bumblebee (*B. terrestris*) colonies exposed to sulfoxaflor (5ppb) over a two-week period produced fewer workers and reproductive offspring throughout their entire life cycle than colonies that had not been exposed. In Chapter 3 I found that acute sulfoxaflor exposure did not influence bumblebee olfactory learning or memory in a proboscis extension reflex experiment, and further found no effect on bumblebee performance in a radial-arm maze. In Chapter 4 however, using a meta-analysis technique, I did find that other insecticides do influence bee olfactory learning/memory. In chapter 5 I determined using a microcolony based design that chronic sulfoxaflor exposure (5ppb) can influence bumblebee egg laying and larvae production, which offers a potential underlying mechanism to the observed impacts of sulfoxaflor exposure on worker production (Chapter 2). In the final data chapter of this thesis I found in Chapter 6 that sulfoxaflor exposure also influenced bumblebee larval growth in an *in vitro* experimental design. I also investigated the potential interaction between the bumblebee fungal parasite *Nosema bombi* and sulfoxaflor but found no synergistic effects on larval growth or mortality. The results from this thesis demonstrate that sulfoxaflor exposure can have important sub-lethal impacts on bumblebees, and ultimately cautions against licencing insecticides for use without a true understanding of the potential impact they can have on important pollinators.

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Chapter 1: Introduction

1.1 Introduction to bumblebee natural history

There are an estimated 20,000 bee species worldwide, distributed over seven families (Ascher & Pickering 2012). Most bee species are solitary, but bees range remarkably in their sociality, with certain honeybee species (*Apis* spp.*)* colonies containing on average 50,000 workers in a colony. Other social bees include the charismatic bumblebees (*Bombus* spp.), with an estimated 250 bumblebee species described worldwide, and 25 thought to be present in the UK (Williams & Osborne 2009; Falk 2015).

Bees have been a particular focus of conservation efforts because they offer key ecosystem services for both agricultural crop production (Klein *et al.* 2007; Ricketts *et al.* 2008; Garibaldi *et al.* 2013; Rader *et al.* 2016) and wild flower pollination (Aguilar *et al.* 2006). The contribution of pollinators to global food production is increasing and in 2009 was valued at an estimated US$361 billon (Lautenbach *et al.* 2012) with an estimated 87.5% of angiosperms requiring animal pollination (Ollerton, Winfree & Tarrant 2011). While pollination services are provided by a wide variety of different species (Rader *et al.* 2016), the intensification of agriculture has resulted in a dependence on bees, in particular honeybees and bumblebees, to provide vital pollination services for agricultural crops (Klein *et al.* 2007; Potts *et al.* 2010b; Kleijn *et al.* 2015; Rader *et al.* 2016). Commercially managed bumblebees and honeybees can improve crop set, increasing both yield and marketability (Lye *et al.* 2011; Rader *et al.* 2016; Martin, Fountain & Brown 2019) but wild bees also provide important pollination services (Garibaldi *et al.* 2013; Mallinger & Gratton 2015; Rader *et al.* 2016; Landaverde-González *et al.* 2017; Horth & Campbell 2018), and further offer a pollination buffer in the case of declines in honeybees (Van Engelsdorp *et al.* 2007; Potts *et al.* 2016). Given the economic importance of bees, bee declines, both locally and globally, could therefore have important social, and economic ramifications.

Bumblebees, upon which this PhD thesis will largely focus, are vital for pollination of many agricultural crops as both wild bumblebees and commercially reared colonies can increase crop yields, and sometimes crop marketability (Lye *et al.* 2011; Garibaldi *et al.* 2013; Rader *et al.* 2016; Martin *et al.* 2019). Like many other bees, bumblebees have evolved adaptions for collecting pollen, such as pollen baskets (corbiculae), and are effective at pollinating certain angiosperms such at tomatoes, strawberries and raspberries (Bronstein, Alarcón & Geber 2006; Lye *et al.* 2011; Martin *et al.* 2019). Bumblebees are also 'buzz pollinators', and vibrate their flight muscles in order to access pollen hidden in poricidal anthers, pollinating the plant in the process, a type of pollination service that not all bees are capable of (De Luca & Vallejo-Marín 2013).

The vast majority of bumblebee species inhabit temperate regions, and colonies follow an annual cycle whereby the queens of most species will hibernate over the winter (Alford 1975). For most species, the annual colony cycle starts in early spring when mated queens will emerge from hibernation (Figure 1.1). Upon emergence queens will attempt to initiate a nest. If a suitable nest location is successfully found, the queen starts to collect nectar and pollen to feed her developing brood. She will incubate the brood by 'shivering' to generate heat, and approximately 4-5 days after laying, the first eggs hatch into larvae (Alford 1975). During this period the queen will continue to forage in order to provision the larvae, a particularly vulnerable phase of the colony life cycle, as a fall in the amount of locally available food could result in the death of either the larvae or queen (Goulson 2003). Bumblebee larvae have 4 instars and take approximately 10-14 days to develop a hard casing, in which pupation occurs. A further 14 days are required for pupation, meaning that the total development time is between 4-5 weeks, depending on the species (Alford 1975). At this point the newly emerged workers aid the queen in brood care and carry out all foraging, meaning that the founding queen does not leave the colony. The colony will grow and can reach up to approximately 350 workers per colony, again depending on the species (Goulson 2003). Once the nest contains a certain density of workers, depending on species, the queen will stop producing workers, and start producing gynes and males (Alford 1975; Bloch 1999). The switching point (time when the colony changes from producing workers to males and queens) can be artificially manipulated by increasing worker density (Bloch 1999) and is likely controlled by the queen pheromone production (Bourke & Ratnie 2001). Once developed, the gynes and males leave the nest, and the new queens will subsequently go on to hibernate and emerge in the next spring, hopefully to start their own nest. The founding queen and colony will eventually perish.

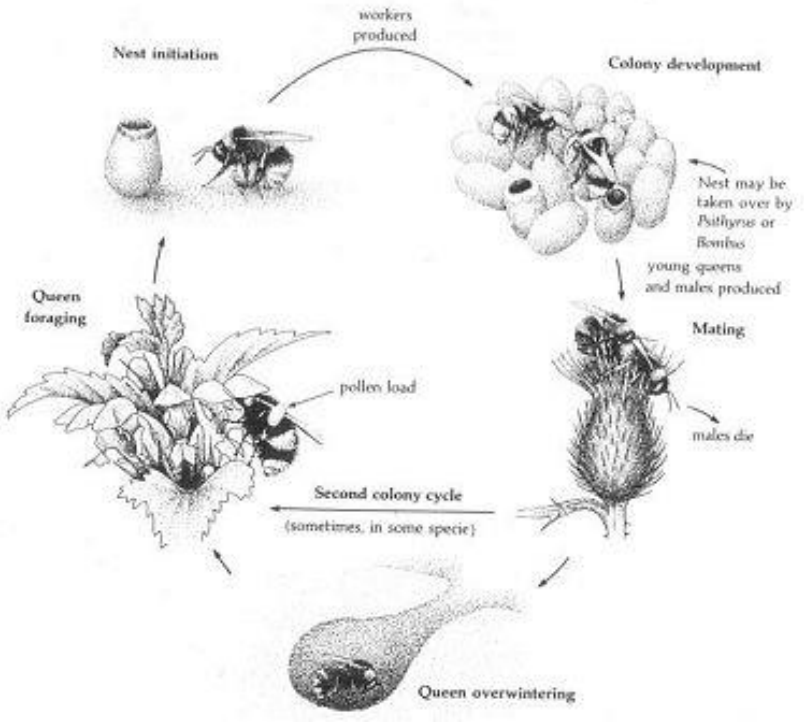

Figure 1. 1: The bumblebee life cycle. Taken from (Prys-Jones & Corbet 2011)

1.2 Bee population-level trends

Overall, there is evidence for general trends in insect declines globally. For example, data collected over 27 years in protected areas in Germany demonstrated that there was 82% decline in the number of flying insects found within national parks across all the land use categories considered (Hallmann *et al.* 2017). In a similar study, Lister & Garcia (2018) recorded arthropod biomass in a Puerto Rican National Park in 2011, 2012 and 2013, and compared it to data collected in 1976 and 1977. They found a significant drop in arthropod biomass, including within the Hymenoptera order, but also found similar declines in specific reptiles, birds and mammals (Lister & Garcia 2018).

While some bumblebee species are increasing their geographical range, either by deliberate/ accidental introduction (*B. terrestris*) (Schmid-Hempel *et al.* 2014) or by responding to changing environments (*B. hypnorum*) (Goulson & Williams 2001), the long-term data that we do have suggests that bumblebee declines are occurring globally and there are documented declines in the UK (Williams 1982; Biesmeijer *et al.* 2006; Powney *et al.* 2019), Ireland (Fitzpatrick *et al.* 2007), central and western Europe (Biesmeijer *et al.* 2006; Kosior *et al.* 2007), North America (Colla & Packer 2008; Grixti *et al.* 2009; Cameron *et al.* 2011), South America (Schmid-Hempel *et al.* 2014), China (Xie, Williams & Tang 2008; Williams & Osborne 2009) and Japan (Inoue, Yokoyama & Washitani 2008).

Furthermore, data collected in the UK from the Bees, Wasps and Ants Recording Society (BWARS) has documented a number of solitary bee extinctions in the UK since the 19th century (and two cases of bumblebee extinctions) (Ollerton *et al.* 2014), with similar observations reported in the Netherlands (Biesmeijer *et al.* 2006). A more recent analysis of this data has also demonstrated that solitary bees species (in contrast to eusocial species) have a reduced occupancy (proportion of 1km grid cells that the bees are present) in the UK (Powney *et al.* 2019). In the rest of Europe, the most recent assessment of the status of European Bees found that 9.1% of bee species within the European Union are threatened with extinction within Europe, with a further 5.2% classified as near threatened. A further 55.6 % of 1,101 species are also data deficient, meaning no formal evaluation could be conducted for these species, although the authors suggest that it is likely that many of these species are threatened with extinction (Nieto *et al.* 2014).

In contrast to the majority of wild bees, honeybee (*Apis mellifera*) numbers are increasing globally (Aizen & Harder 2009), despite localised declines, in North America (Van Engelsdorp *et al.* 2007; Oldroyd 2007; Potts *et al.* 2010b). Given the dependency of intensive agricultural on honeybees, localised honeybee declines are also of great concern (Potts *et al.* 2010a).

1.3 Drivers of wild bee declines

1.3.1 Habitat loss and the intensification of agriculture

While many of the potential drivers of bee declines remain controversial (Goulson *et al.* 2015), habitat loss is undoubtedly a major contributing factor (Ricketts *et al.* 2008; Brown & Paxton 2009; Winfree *et al.* 2009; Potts *et al.* 2010a; Goulson *et al.* 2015). In the UK it is thought that 97% of flower rich grasslands were lost in the 20th century as a result of an increase in intensive agriculture (Howard *et al.* 2003), and there is further evidence that between 1978 and 1998 the ranges of 71% of plant species used as bumblebee forage were reduced (Carvell *et al.* 2006). A recent study using molecular genetics and habitat assessments has been conducted on the survival of family lineages, of summer bumblebee workers and emerging queens in spring (*B. terrestris, B. lapidarius and B. pascuorum)* (Carvell *et al.* 2017). Carvell et al. (2017) collected bumblebee DNA non-lethally by clipping the tarsal tip of the mid-leg of bees and genotyping the bees at 13-14 microsatellite loci per species, which was used to estimate family lineages between bees. Carvell et al. (2017) found that bumblebee family lineage survival was higher in areas which had a great quantity of floral resources available (estimated floral and plant cover), and specifically found that agri-environment schemes and habitat restoration have a positive impact on bumblebee survival. Furthermore, a meta-analysis that analysed effects of various anthropogenic stressors on bee species abundance and diversity, which generated 130 effect sizes from 54 papers, showed that habitat loss, and fragmentation, had the largest negative influence when

compared to other anthropogenic factors (Winfree *et al.* 2009). Furthermore, while urbanisation could be cited as a reason for loss of habitat, evidence suggests that gardens offer more floral resources than certain agricultural environments (Goulson *et al.* 2002; Cane *et al.* 2006; Carré *et al.* 2009; Samuelson *et al.* 2018). Given this, it seems increasingly likely that both the intensification of farming, and habitat loss in agricultural environments (Howard *et al.* 2003; Carvell *et al.* 2006) are important contributing factors to wild bee declines.

1.3.2 Pathogens & parasites

Human management of both honeybees and bumblebees has increased the prevalence of bee pathogens within the environment (Colla *et al.* 2006; van Engelsdorp & Meixner 2010). The dependency on commercial honeybees for their pollination services in some intensively farmed agricultural environments, such as almond pollination in California (Sumner & Boriss 2006), means that colonies are routinely transported across large geographical areas. The high densities of honeybees within these localised environments can increase parasite prevalence/abundance but can also result in the spread of non-native pathogens. For example, the varroa mite (*Varroa destructor)*, which was originally associated with the Asian honeybee (*Apis cerana),* is now also found in Europe (*A. mellifera*) and is thought to be a major contributor to honeybee declines in Europe and North America (Rosenkranz, Aumeier & Ziegelmann 2010; Nazzi *et al.* 2012). The mite is an external parasite and attaches itself to the body of a bee to feed on the fat reserves (Ramsey *et al.* 2019) and reproduces by laying its eggs on developing larvae. In doing so the mite acts as a vector for other pathogens, such as deformed wing virus (DWV), making colonies vulnerable to collapsing (Rosenkranz *et al.* 2010; Nazzi *et al.* 2012). Furthermore, evidence is now also emerging demonstrating disease transmission between honeybees, bumblebees and solitary bees (Klee *et al.* 2007; Chen *et al.* 2008; Plischuk *et al.* 2009; Li *et al.* 2012; Fürst *et al.* 2014; Ravoet *et al.* 2014; McMahon *et al.* 2015).

Commercially reared bumblebee colonies can often contain parasites and diseases, including *Nosema bombi, N.ceranae, Apicystis bombi* and deformed wing virus, which can then be passed into wild populations (Goka *et al.* 2001; Colla & Packer 2008; Otterstatter & Thomson 2008; Graystock *et al.* 2013). One hypothesis is that 'exotic' strains of certain pathogens, such as *N. bombi*, when moved to a new environment are more detrimental to native bumblebees, as they have not evolved adequate defence mechanisms to the new pathogens (Meeus *et al.* 2011; Arbetman *et al.* 2013). A study on museum specimens in North America however failed to detect an increase in prevalence of alien strains of *N. bombi,* suggesting that the commercial bumblebee trade merely increased the prevalence and opportunity of infection transmission (Cameron *et al.* 2016). Parasites such as *N. bombi* & *C. bombi* are known to have negative effects on colony growth, reproductive output and navigational ability

(Brown, Loosli & Schmid-Hempel 2000; Brown, Schmid-Hempel & Schmid-Hempel 2003; Wolf *et al.* 2014), which suggests an increase of pathogens into the environment is likely to have a negative effect on bee populations (Cameron *et al.* 2011).

1.3.3 Invasive species

In recent times bumblebees such as *B. terrestris* and *B. impatiens* have also been introduced into non-native habitats to improve crop pollination and seed set of non-native and enclosed crops (Inoue & Yokoyama 2010; Schmid-Hempel *et al.* 2014). As mentioned above, non-native commercial bee colonies not only risk increasing the prevalence of pathogens within an environment, but may also potentially outcompete native pollinators, and in certain cases have devasting impacts on wild bee populations (Schmid-Hempel *et al.* 2014). For example, *B. terrestris* was first introduced to Japan in 1991 for agricultural greenhouse pollination services (Velthuis & van Doorn 2006) and is now found across the country, where they outcompete native bees for floral resources and nest sites (Inoue & Yokoyama 2010). Likewise, *B. terrestris* was first introduced to Chile in 1998 (Velthuis & van Doorn 2006) and was sighted on the Atlantic coast of Argentina in 2011 (Schmid-Hempel *et al.* 2014) where it appears to be driving declines in South American native such as *B. dahlbomii,* which are seldom found when the invasive *B. terrestris* is present.

1.3.4 Climate change

Climate change is likely to influence biodiversity on a global scale, but data directly linking bee declines and climate change are few. Extreme weather patterns such as increased flooding, forest fires and predicted increases in heatwaves and droughts, as a consequence of climate change, will undoubtedly negatively influence bumblebees and other pollinators (Rasmont *et al.* 2015). One way in which climate change can influence bees is by changing the phenology of either bee colonies, or the flowers they visit (Bartomeus *et al.* 2011; Willmer 2012; Kudo & Cooper 2019). There is some evidence to suggest that climatic changes may favour more generalist feeders (short-tonged bumblebees), which can feed on a greater variety of flowers than specialists feeders (long-tonged bumblebees) which are dependent on fewer plants (Miller-Struttmann *et al.* 2015). Climate change could also result in bumblebees specifically becoming more range restricted, and Kerr *et al.* (2015) demonstrated with long-term data taken over 110 years from North America and Europe, that bumblebee species in southerly regions are experiencing range loses and are shifting to higher elevations, potentially making species more vulnerable to extinction.

1.4 Insecticides and introduction of neonicotinoids

1.4.1 Historical pesticide use

During the second world war the organochlorine pesticides, such as the infamous DDT, were developed to control mosquito numbers in the battle against malaria in South East Asia and Africa. The effectiveness of DDT as an insecticide was first discovered by the swiss chemist Paul Hermann Muller in 1939, who went on to win the noble prize in 1948 for his discovery. After the second world war these systemic pesticides were used globally in agriculture as plant protection products. However, evidence of unwanted side effects on both vertebrate and invertebrate wildlife, as synthesised by Rachel Carson in 1962 in her book *Silent Spring*, resulted in bans in agricultural organochlorine pesticides use in many countries around the world (Carson 1962).

Despite this, intensive agriculture had already become reliant on insecticides for crop protection, so there was a demand for the production on novel insecticides to replace organochlorines. During the 1970's and 80's a range of insecticides including the organophosphates, pyrethroids and methylcarbamates were developed and widely used until their effectiveness diminished over time as a result of rising pest resistance (Michigan State University 2018). Instead, neonicotinoid-based insecticides are now the most commonly used insecticide group in the world (Simon-Delso *et al.* 2015). Neonicotinoids are highly soluble and are thus systemic insecticides that act as agonists of nicotinic acetylcholine receptors (NAChRs) in the insect central nervous system, altering synaptic functioning (Palmer *et al.* 2013; Moffat *et al.* 2016). High doses of neonicotinoids overstimulate and block acetylcholine receptors leading to paralysis and death (Matsuda *et al.* 1998). Differences in the binding sites of NAChRs of invertebrates and vertebrates (Tomizawa & Casida 2009) mean that the neonicotinoids that are in common use as insecticides are toxic at low dosage for insects but not vertebrates, and so their use at field realistic applications is thought to be less hazardous to vertebrate wildlife and humans (Cimino *et al.* 2017) (but see (Gibbons, Morrissey & Mineau 2015). The first neonicotinoid insecticide that was licensed for use was Imidacloprid in 1991 but numerous others have now been manufactured such as acetamiprid, clothianidin, thiacloprid, thiamethoxam (Bass *et al.* 2015).

Although neonicotinoids are effective at targeting many pest species, the repeated use of these agrochemicals has resulted in certain targets pests beginning to show evidence for the evolution of resistance (Bass *et al.* 2015). The first recorded case of pest resistance to neonicotinoids was reported in 1996, when Cahill *et al* (1996) reported that imidacloprid was not effectively controlling populations of cotton whiteflies (*Bemisia tabaci)* in Spanish greenhouses. A review conducted by Bass *et al.* (2015) has confirmed that more than 500 peer reviewed papers have been published on the topic of pest

resistance to neonicotinoids. The Arthropod Pesticide Resistance Database (APRD), which describes cases of pest species resistance, at the time of writing has 501 cases of pest resistance to imidacloprid, spanning 23 different species, with other neonicotinoids such as acetamiprid and thiamethoxam having 119 & 212 documented cases of pest resistance across 16 and 15 different pest species respectably (Michigan State University 2018).

1.4.2 Exposure routes

Bees are exposed to insecticides in various ways, with the most obvious route of exposure occurring when bees feed on the nectar and pollen of treated crops (oral exposure) (Bonmatin *et al.* 2015; Kyriakopoulou *et al.* 2017). Bees can also be directly sprayed with insecticides, or come into contact with insecticides on plant surfaces (contact exposure), (Greig-Smith *et al.* 1994), although spraying prior or post flower bloom can reduce the risk to bees (Centner, Brewer & Leal 2018), as can spraying at night-time, when bees are not foraging (although impacts on non-bee pollinators are likely to occur and residues may remain in the morning). Spray drift can also contaminate crop margins, and non-target flowers, such as weeds and wildflowers that are present in orchards in-between trees rows so spraying pre or post-bloom can still contaminate floral resources (David *et al.* 2016). Bees can also be directly exposed to insecticides from the dust generated during the sowing of seed treatments (Greig-Smith *et al.* 1994; Krupke *et al.* 2012), with documented cases of dust generated from seed treatments resulting in honeybee mortality (Greig-Smith *et al.* 1994; Pistorius, Bischoff & Heimbach 2009). Some bee species, such as honeybees, also collect water in addition to nectar and pollen, meaning bees can potentially be exposed through contaminated water sources, through guttation and puddles in fields (Girolami *et al.* 2009; Samson-Robert *et al.* 2014). Furthermore, contaminated soil in crop margins could influence ground nesting bee species, for which the surrounding soil could be contaminated, although data do not exist on this potential exposure route to my knowledge.

When referring to oral insecticide exposure we consider two types of exposure time course, (i) acute and (ii) chronic. Acute pesticide exposure occurs when a foraging bee forages on a flower that is contaminated with an insecticide, and in doing so, consumes a dose of the insecticide. Chronic exposure occurs when bees are routinely exposed over a longer period of time (e.g. an oilseed rape bloom). For example, foraging adults that routinely feed on a treated mass flowering unit, and bees/larvae in the colony that are exposed through contaminated nectar and pollen stores over a long period of time, could be subject to chronic insecticide exposure.

Acute pesticide exposure is often suggested to be more field realistic, as bees can forage on a wide variety of different flowers, reducing the likelihood of chronic exposure (Garbuzov *et al.* 2015). However, certain bee species (such as long-tongued bumblebees) are floral specialists and only feed

on a few plant species, which, if the plants are exposed to insecticides, could result in bees routinely feeding on nectar that contains residues (Johnson & Steiner 2000). Some bees, such as bumblebees, also show high flower constancy, repeatedly feeding in the same foraging patch (Woodgate *et al.* 2016). If systemic insecticides persist within that foraging patch (Bonmatin *et al.* 2015; Kyriakopoulou *et al.* 2017), then the likelihood of chronic exposure increases. Indeed, systemic insecticides present in the soil of treated fields will degrade over time, but the time it takes for an insecticide to degrade varies according to environmental conditions, such as temperature, moisture, soil pH and soil type (Bonmatin *et al.* 2015). Neonicotinoids break down much more quickly than organochlorine pesticides, such as DDT, which can still be found in the residue of crops in Europe, despite having being banned in the 1970's (Silva *et al.* 2019). The half-life is still high for certain neonicotinoids (Bonmatin *et al.* 2015). For example, the lowest reported half-life for imidacloprid is 107 days, in a subtropical, humid environment (Cox 2001), but these values are extremely variable, and, in contrast, a US EPA study (1993) found that the half-life on imidacloprid ranged from 3-4 months to 1 year. The half-life for other neonicotinoids, such as clothianidin, is just as variable and can range between 148 days and 7,000 days (Decant & Barrett 2010). Furthermore, laboratory experiments demonstrating that bumblebees *(B. terrestris*) and honeybees (*A. mellifera*) prefer neonicotinoid treated sucrose over untreated sucrose suggest that bees might preferentially feed on crops treated with neonicotinoids (Kessler *et al.* 2015; Arce *et al.* 2018), increasing the possibility of chronic exposure.

Insecticide exposure risk for bees is not limited to agricultural crops. Botías *et al.* (2016) analysed residue levels in non-target wildflower crops, in the field margins of oil seed rape fields that had been treated with various neonicotinoids, and found that they were also contaminated with neonicotinoids (range: ≤ 0.02–106 ng/g). The residues found in the crop margins were high enough to have potentially lethal consequences for certain predatory insects that could aid in pest control (Botías *et al.* 2016). Outside of the agricultural environment, research in urban areas has found that both bumblebee (*B. terrestris)* nests and honeybee colonies (*A. mellifera*) within urban environments contain neonicotinoid residues, with half of the samples collected containing at least one neonicotinoid (Nicholls *et al.* 2018), at concentrations that are known that have sub-lethal impacts on bumblebees (Gill, Ramos-Rodriguez & Raine 2012; Samuelson *et al.* 2016; see below). Garden centres and supermarkets sell insecticides for horticultural use, and while it is difficult to determine how often bees forage on flowers which have been treated with garden insecticides, the results from Nicholls *et al.* (2018) suggest that bees can be exposed. Another possible route of exposure is from ornamental plants sold in garden centres which regularly contain a wide variety of different agrochemicals within their nectar (Lentola *et al.* 2017). Gardens and allotments are an increasingly important source of food

for bees (Samuelson *et al.* 2018; Baldock *et al.* 2019) suggesting this is a considerable exposure route. Finally, a study by Mitchell *et al.* (2017) which examined the residue levels of 198 honey samples taken from around the world found that 75% of samples contained at least one of five tested neonicotinoid insecticides with, 45% of samples containing more than one, suggesting that bees are being exposed to neonicotinoids on a global scale.

1.4.3: Individual and colony-level impacts of neonicotinoids

The regulatory process in North America and Europe has a tiered-based system for establishing whether an agrochemical should be licenced for use or not (Sanchez-Bayo & Tennekes 2017). At the lowest tier (tier 1), short-term mortality experiments (in which some behavioural measures are recorded) in the form of LD50 or LC50 experiments are conducted to determine the hazard quotient (HQ) of an agrochemical. These experiments use honeybees (*A. mellifera*) as a model species and typically run for a maximum on 96 hours, although there are calls to extend this time to 10 days (Hesketh et al. 2016; OECD 2017). From these experiments, mortality endpoints are established, and if the HQ exceeds 0.1 the agrochemical will be further tested at Tier 2. Tier 2 based experiments include conducting more laboratory experiments on other bee species (such as *Bombus*) and also semi-field experiments in polytunnels. If the results from Tier 2 are inconclusive then further, Tier 3 field experiments can also be conducted (eg Campbell et al. 2016).

Therefore, in its current form the regulatory process does not consider the potential sub-lethal consequences of pesticide exposure at Tier 1. If a novel insecticide has a HQ lower than 0.1 the sub-lethal consequences of an insecticide will not be considered further, meaning that neonicotinoids and other agrochemicals can potentially be licensed for use despite having unknown sub-lethal impacts on pollinators at the colony and landscape scale (Sanchez-Bayo & Tennekes 2017). Furthermore, the potential impact of chronic exposure on honeybees and of insecticide exposure (both lethal and sub-lethal) on non-*Apis* bees such as bumblebees and solitary bees can potential go undetected.

1.4.3.1: Sub-lethal effects on behaviour

In one of the first experiments to examine the potential sub-lethal effects of pesticides on bee behaviour, Henry *et al.* (2012), using individual RFID tags, tested the homing ability of 653 individual honeybees that were exposed to 1.34 ng of thiamethoxam. Exposed honeybees were less likely to return to the nest successfully and those that did were slower than control bees. Experiments on bumblebee homing success have received mixed results, with Stanley *et al.* (2016) demonstrating that bumblebees exposed to thiamethoxam, and displaced 1km from the nest were actually more successful at homing than bees that had not be exposed. Stanley *et al.* (2016) suggest that these results can be attributed to the increased knowledge of the surrounding environment, as bumblebees

exposed to the thiamethoxam performed longer, less efficient, foraging bouts than bumblebees that were not exposed. In contrast to the 1Km data, Stanley *et al.* (2016) found no effect of thiamethoxam exposure on bumblebee homing when released from 2km away from the nest.

Bee foraging efficiency is likely to be important for colony fitness, as more food within the nest results in a greater reproductive output (Génissel *et al.* 2002). Gill, Ramos-Rodriguez & Raine (2012) demonstrated that chronic exposure to imidacloprid reduced the pollen foraging score (size of the pollen load scored by the experimenter) and increased worker mortality. Gill *et al.* (2012), using a colony-based design, compared a control group that were fed untreated sucrose solution with 3 other treatment groups that were either fed for four weeks on either (i) sucrose contaminated with imidacloprid (10ppb), (ii) untreated sucrose placed on a filter paper that had been sprayed with a pyrethroid insecticide (λ-cyhalothrin solution), or (iii) a combination of both the pyrethroid and imidacloprid. Gill *et al.* (2012) monitored the colonies daily and found that worker mortality was significantly higher in the treatment groups that had been exposed to imidacloprid. Furthermore, using a combination of RFID tags and foraging observations, Gill *et al.* (2012) found that bumblebee colonies exposed to imidacloprid were more likely to return with less pollen than control bees, and that they had, perhaps as a result of the reduced pollen intake per individual worker, more foragers than control bees. Follow-up experiments with other chemicals have yielded similar results. Feltham, Park & Goulson (2014) demonstrated that bumblebee colonies chronically exposed to imidacloprid returned with less pollen than control bees, and Stanley *et al.* (2016) (as mentioned above) demonstrated that chronic exposure to thiamethoxam at a lower dosage, (2.4ppb) resulted in workers returning with pollen less often than control bees. Interestingly, a follow up study to Gill *et al.* (2012), which used the same data, showed that when analysing individual foraging performance over time, bumblebees that had been chronically exposed to imidacloprid did not improve their foraging performance over time, while control bees did (Gill & Raine 2014).

Neurotoxic insecticides that act as NAChR agonists, such as neonicotinoids, are neurotoxins, can alter synaptic function in the insect central nervous system (see section 1.5.1]) and strong evidence exists demonstrating that insecticide exposure can inactivate, or impair the development of neural cells (Palmer *et al.* 2013; Moffat *et al.* 2016; Peng & Yang 2016). It is therefore perhaps not surprising that a plethora of research has now demonstrated that pesticide exposure can influence bee learning and memory (Decourtye *et al.* 2004b; Stanley, Smith & Raine 2015b; Goñalons & Farina 2015; Piiroinen & Goulson 2016; Samuelson *et al.* 2016). The majority of experiments investigating this topic have used a proboscis extension reflex protocol, whereby bees (usually honeybees (*A. mellifera, A. cerana*) are either acutely or chronically exposed to an insecticide, after which forward-paired olfactory

conditioning is conducted to allow bees to learn to associate a sucrose reward with an olfactory stimulus. More recent research has used a radial-arm maze to understand the impact of insecticide exposure on bee working memory (also known as short-term memory). Samuelson *et al.* (2016) showed that bumblebees (*B. terrestris*) that received an acute dosage of thiamethoxam were more likely to re-visit already visited flowers than bees that were fed untreated sucrose solution, reducing their foraging efficiency. Furthermore, insecticide exposure, can also influence non-cognitive traits such as bumblebee foraging motivation (Lämsä *et al.* 2018; Muth & Leonard 2019).

While much attention has focused on the impact of neonicotinoid exposure on bee behaviour outside the nest, Crall *et al.* (2018) demonstrated that chronic exposure to imidacloprid can also influence worker behaviour inside the nest as well. Crall *et al.* (2018) used an automated robotic platform to monitor the behaviour of individual bumblebees (*B. impatiens)* within colonies that had, or had not, been provided with sucrose solution containing imidacloprid (9.6ppb). Exposed colonies had reduced activity (time spent moving) and workers spent less time in proximity to the brood, suggesting that brood care was reduced (although this is based purely on spatial position within the nest, and direct observations of behaviour were not conducted). Crall *et al.* (2018) also found that time of day had a significant impact on their results, with differences in activity between control and treatment colonies significantly greater at night when compared to day-time activity. The same effect was observed in reference to brood care. Social network density (number of interactions) was also reduced in treated colonies, similar to results observed in honeybees (Forfert & Moritz 2017), and follow up experiments demonstrated that acute imidacloprid exposure also had similar effects on brood care and colony activity. Furthermore, in other follow up experiments, whereby bumblebee colonies were allowed to forage outside, Crall *et al.* (2018) found that exposed colonies were less able to thermoregulate than unexposed colonies, with the nest air temperature, and the brood temperature significantly lower in exposed colonies.

1.4.3.2: Sub-lethal effects on physiology

Insecticide exposure, can also influence bee physiology. Laycock *et al.* (2012), for example, used a microcolony-based design to demonstrate that chronic exposure to imidacloprid at field realistic dosages can reduce bumblebee (*B. terrestris*) brood production by one third. Similar experiments using similar methodologies have found contrasting results (Laycock & Cresswell 2013; Laycock *et al.* 2014) but studies using wild bumblebee queens have demonstrated that neonicotinoid exposure can influence bumblebee egg laying and ovary development (*B. lucorum, B. pascuorum, B. pratorum, B. terrestris*) (Baron, Raine & Brown 2017b; Baron *et al.* 2017a). Similar effects are also observed with honeybee queens (*A. mellifera*) exposed to neonicotinoids (Williams *et al.* 2015). Neonicotinoid

exposure can also influence drone quality, with honeybee drones exposed to either thiamethoxam or clothianidin having a lower sperm viability (percentage of sperm that is alive) and a reduced life spam, than unexposed drones (Straub *et al.* 2016).

As mentioned above, bumblebee and honeybee nests routinely contain insecticide residues in nectar, pollen and honey stores within colonies (Mitchell *et al.* 2017; Nicholls *et al.* 2018) and these will be fed to developing larvae. In an experiment to examine the impact of pesticide exposure on honeybee larvae (*A. mellifera*), Wu, Anelli & Sheppard (2011) demonstrated that larvae exposed to high levels of different insecticides had delayed development, resulting in later adult emergence and in some cases reduced adult longevity. Follow up experiments have shown that synergistic pesticide use can sometimes increase the likelihood of honeybee (*A. mellifera*) larval mortality (Zhu *et al.* 2014).

1.4.3.3: Impacts of sub-lethal effects on colony fitness

In an experiment investigating the impact of chronic neonicotinoid exposure on bumblebee reproductive output, Whitehorn *et al.* (2012) exposed bumblebee colonies (*B. terrestris*) to either a low dosage of 6 μg kg^{-1} and 0.7 μg kg^{-1} of imidacloprid in the nectar and pollen or a high dosage which was double that of the low treatment. Colonies were exposed in a laboratory for two-weeks, before being moved outside and monitored for a six-week period. Colonies exposed to both the high and low dosage of imidacloprid gained less weight than control colonies, and had an overall 85% reduction in the number of gynes that were produced. Arce *et al.* (2017) obtained similar results in a colony-level experiment that chronically exposed bumblebees (*B. terrestris)* to clothianidin (5ppb), with bumblebee colonies exposed to the insecticide producing less gynes and males.

In one of the first large scale field experiments to examine the potential impact of neonicotinoid exposure on bees, Rundlöf et al. (2015) showed that bumblebee colonies (*B. terrestris*) located in proximity to oilseed rape fields treated with clothianidin and the pyrethroid b-cyfluthrin showed reduced colony growth and reproductive output compared to bumblebee colonies located next to untreated fields. Rundlöf *et al.* (2015) also placed solitary bee nesting tubes (*O. bicornis*) at each location and found that none of the nests in the treated fields contained brood cells and that fewer wild bees (bumblebees and solitary bees) were counted within the treated fields when conducting transects.

In a follow up experiment, bumblebee colonies (*B. terrestris)* were left to forage in an environment, for two weeks, with raspberries that had, or had not, been treated with thiacloprid, another neonicotinoid insecticide (Ellis *et al.* 2017). The bees were then transported to another environment that was either flower-rich or flower-poor. The results showed that in the poor environment, neither

treatment group did well, but in the flower rich environment treated colonies survived for less time than control colonies and produced 46% fewer sexuals (Ellis *et al.* 2017). Woodcock *et al.* (2017), in the largest field experiment to date, investigated the impact of neonicotinoid treated oilseed rape across 3 countries (UK, Germany & Hungry) on the reproductive output of bumblebee colonies (*B. terrestris)* and solitary bees (*O. bicornis*). No differences in solitary bee reproductive output were found between control and neonicotinoid treated fields (clothianidin & thiamethoxam) in any of the 3 countries tested, although there contrasting effects of neonicotinoid exposure on male production in Germany and the UK. However, while comparing control and treated fields produced contrasting results, there was a negative correlation between nest residue levels and both bumblebee and solitary bee reproductive output

The colony-level consequences of neonicotinoids exposure on honeybees is unclear, with varying results between field experiments (Cutler *et al.* 2014; Budge *et al.* 2015; Rundlöf *et al.* 2015; Tsvetkov *et al.* 2017; Woodcock *et al.* 2017; Osterman *et al.* 2019). Rundlöf et al. (2015) (described above) found no effect of treatment on honeybee colony strength (number of workers within the colony) and Woodcock *et al.* (2017) demonstrated both positive and negative impacts of neonicotinoid on honeybee health. In a semi-field experiment, Tsvetkov *et al.* (2017) recorded the residue levels found in the nests of honeybee colonies foraging near a treated corn crop and, in a subsequent experiment, mimicked these residue levels by feeding an artificial pollen supplement laced with clothianidin, and finding that exposed honeybee colonies had increased worker mortality and queenlessness and reduced social immunity. Despite this, and in contrast to evidence from wild bees (Rundlöf *et al.* 2015; Woodcock *et al.* 2016, 2017; Wintermantel *et al.* 2018), the landscape consequences of neonicotinoid exposure on honeybee colony health is still largely debated (Cutler *et al.* 2014; Osterman *et al.* 2019).

1.5 European legislation

Given the above-mentioned mounting evidence of detrimental effects of exposure on bees, the European Union (in 2013) voted in favour of a moratorium on the use of 3 commonly used neonicotinoids (clothianidin, imidacloprid and thiamethoxam) on bee-attractive crops. This was extended to a complete ban on their agricultural use, outside of permanent green-house structures, in December 2018. The neonicotinoids acetamiprid and thiacloprid can still be used for agriculture, and all neonicotinoids can still be used in gardens.

1.6 Sulfoximine-based insecticides

The restrictions placed on neonicotinoid use within Europe has largely been met with a positive response from scientists and conservationists alike (Goulson 2018). Despite this, there are growing concerns about the novel insecticides that could replace neonicotinoids (Brown *et al.* 2016).

Sulfoximine-based insecticides are an increasingly important agrochemical group for controlling unwanted pests (Sparks *et al.* 2013; Longhurst *et al.* 2013) and, despite first being described in the literature in the 1940's, have only recently been considered for agricultural use (Zhu *et al.* 2011). Sulfoxaflor is the first manufactured sulfoximine-based insecticide and has now been licenced for use in 81 countries around the world, including within the European Union (sulfoxaflor has been registered for use in the UK but only within commercial greenhouses). Sulfoxaflor shares the same mode of action as neonicotinoids, acting as an agonist of Nicotinic Acetyl Choline Receptors (NAChRs) but has been classed as its own unique group (group 4C) by the Insecticide Resistance Action Committee (IRAC), distinct from neonicotinoids (Group 4A) due to its unique structural activity relationships (Zhu *et al.* 2011; Sparks *et al.* 2013; Houchat *et al.* 2019). Specifically, the classification of an insecticide as a neonicotinoid is dependent on the presence of amine nitrogen (sp^3) (Tomizawa & Casida 2009, 2011), which is not present in sulfoxaflor (see Figure 1.2; Sparks *et al.* 2013). Furthermore, despite the similar modes of action between sulfoxaflor and neonicotinoids, sulfoxaflor has been shown to be effective at targeting neonicotinoid resistant pests such as silverleaf whiteflies (*Bemisia tabaci*), glasshouse whiteflies, (*Trialeurodes vaporariorum),* sweet potato whiteflies *(B. tabaci)*, cotton aphids (*Aphis gossypii)*, green peach aphids (*Myzus persicae*) and brown planthoppers, (*Nilaparvata lugens)* (Babcock *et al.* 2011a; Zhu *et al.* 2011; Longhurst *et al.* 2013). Cytochrome P450 monooxygenases are enzymes that have been shown to play a role in neonicotinoid pest resistance in a range of pests (*Musca persicae*, *M. domestica* & *B. tabaci*) (Karunker *et al.* 2008; Philippou, Field & Moores 2009; Wen *et al.* 2009). Zhu *et al.* (2011) found no cross-resistance between imidacloprid resistant strains of *B. tabaci* and sulfoxaflor, and suggested that the enzymes (monooxygenases) that are responsible for developing pest resistance to neonicotinoids are not capable of metabolising sulfoxaflor. This suggests that sulfoxaflor could be a useful insecticide for controlling unwanted neonicotinoid-resistant pests (Zhu *et al.* 2011; Babcock *et al.* 2011b; Sparks *et al.* 2013).

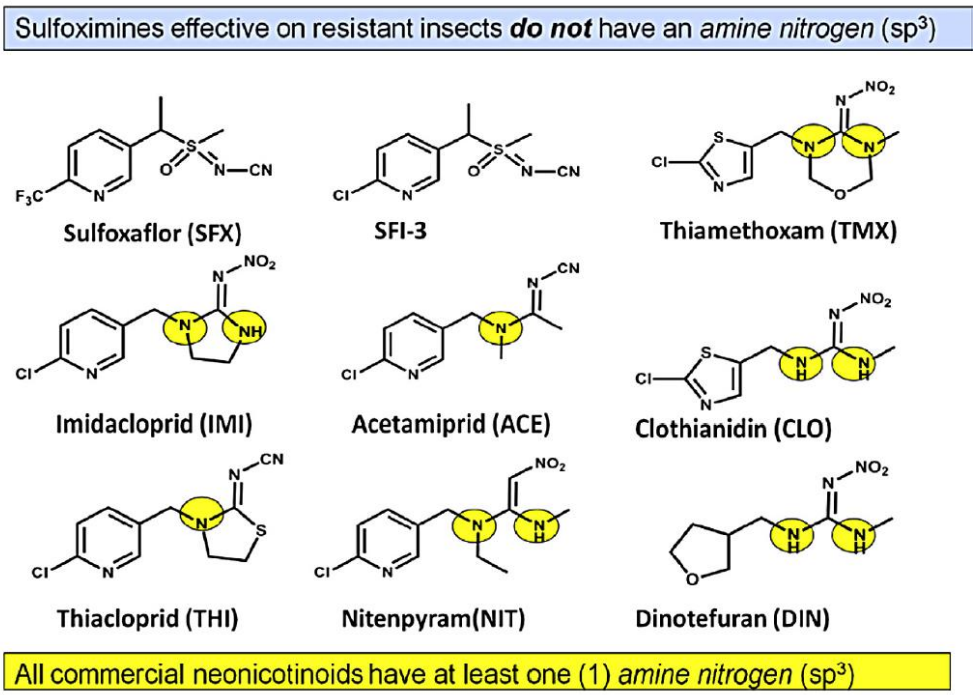

Figure 1. 2: Amine nitrogen (sp^3) is presnet in all neonicotinoids, but not sulfoxaflor. Taken from (Sparks *et al.* 2013)

Sulfoxaflor can be used as a spray or seed treatment, but its most common application is as a spray treatment and it has been registered for use on a range of bee-attractive crops, such as strawberries, raspberries, canola and cotton (Dow AgroSciences Ireland; Dow AgroSciences Australia Limited 2018; Syngenta Canada 2018). Given the rising pest resistance to neonicotinoids (Bass *et al.* 2015), and bans and restrictions on neonicotinoids use, alternative insecticides such as sulfoxaflor are thought likely to replace neonicotinoids over large geographical ranges (Brown *et al.* 2016).

Despite the growing global importance of sulfoxaflor there is still a limited amount of information on the residue levels that we would expect to find in the nectar and pollen of crops treated with sulfoxaflor at field realistic exposure rates. Cheng *et al.* (2018) conducted a semi-field experiment on a cucumber crop that was sprayed with sulfoxaflor twice over an 11-day period (100 grams of active ingredient/ha or 0.09 pound per acre) and found that the residue in flowers ranged between 0.155 – 0.304mg/kg (or 155 & 304ppb) on day 11 (the last day in which data is available). The pollen and nectar residue levels were not specified. In a similar experiment for the USA environmental protection agency (EPA) a cotton crop was twice sprayed with sulfoxaflor (2 X 0.45 pounds of active ingredient per acre over an 11-day period) with the residue levels not falling below 5ppb in the nectar of the treated crop (5.41-46.97ppb). The residue levels in the pollen were considerably higher and did not fall below 50ppb (57 -510ppb) (United States Environmental Protection Agency 2016).

Both of the above-mentioned studies obtained the residue data for crops that are treated with sulfoxaflor during flower bloom (United States Environmental Protection Agency 2016; Cheng *et al.* 2018). Spray during bloom is now prohibited in Europe (Dow AgroSciences Ireland; Dow AgroSciences USA 2018), potentially reducing the risk of pollinator exposure (Centner *et al.* 2018). Yet in many countries where sulfoxaflor is licensed for use, this is not the case (e.g New Zealand, South Africa, Australia, USA), with spray recommendations for large geographical ranges unclear (in particular for South America and Asia) (Dow AgroSciences Australia Limited 2018; Dow AgroSciences New Zealand 2018; Dow AgroSciences South Africa 2018; EPA 2019). The USA had previously restricted sulfoxaflor use but now sulfoxaflor is used across America, and can also be sprayed on certain bee attractive plants (strawberries, pumpkin & ornamental plants) whist crops are flowering (EPA 2019). In Europe, more recent data published by EFSA has also provided residue data for apples, pumpkin, strawberries and oilseed rape sprayed with sulfoxaflor (Abdourahime *et al.* 2019). The residue data for all crops varied (Apples, nectar, 0.181 to <0.003mg/kg; pollen = 5.19 – 0.0162mg/kg: pumpkin, nectar, 1.36 to 0.02mg/kg; pollen = 0.162 to 0.009mg/kg: strawberries, nectar, 0.707 to 0.009mg/kg; pollen = 12.7 to 0.011mg/kg: oilseed rape, nectar, 0.268 to 0.018mg/kg; pollen = 4.05 to 0.014mg/kg) but, as helpful as this data is, sulfoxaflor was still sprayed during flowering, so the field-realism of this data for Europe and North America is currently unclear. What is clear from the available residue data, is that if non-target flowers or weeds, in crop margins or between crop rows are present during spray application then bees are likely to be orally exposed to contaminated nectar. For example, sulfoxaflor is recommended for many orchard-based fruit crops, for which strips of non-crop plants are usually allowed to grow at ground level. If these flowers bloom during spray and are not subsequently mulched, foraging bees may be exposed.

In the only study to my knowledge to examine the residue levels of sulfoxaflor used as a seed treatment, a study from the Pest Management Regulatory Agency (Canada) (2016) found that the reside levels of a canola crop treated with a seed drench of 200 grams of active ingredient per 100 Kg of seed did not go above the limit of detection (0.56ppb). This could suggest that the residue levels found in crops laced with a sulfoxaflor seed treatment might have a lower risk to bees, than those observed with neonicotinoids (Bonmatin *et al.* 2015; Kyriakopoulou *et al.* 2017), potentially due to the lower half-life of sulfoxaflor when compared with certain neonicotinoids (EFSA 2014). Ultimately, as we only have one available study on one crop species (Pest Management Regulatory Agency (Canada) 2016), it is not clear what the residue levels that bees are likely to encounter in seed treated crops will be.

A horizon-scanning exercise by 74 pollinator and pollination experts identified sulfoxaflor as the second greatest emerging threat to pollination services (Brown *et al.* 2016). The authors highlighted (i) the potential for sulfoxaflor to replace neonicotinoids over large geographical ranges, and (ii) the dearth of information on the potential sub-lethal impacts of sulfoxaflor on pollinators. As demonstrated in the literature review above, there is a plethora of evidence demonstrating that insecticides, although not always lethal at field application level, can have severe sub-lethal effects on bees. Prior to the data gathered as part of this thesis no studies to date had examined the potential sub-lethal consequences of sulfoxaflor on bees. Given the rising global importance of sulfoxaflor there was and is an urgent need to understand the potential consequences of this agrochemical on bees and other pollinators.

1.7 Aims of thesis

Based on the literature reviewed above, I decided to make the potential impacts of sulfoxaflor on bumblebees (*B. terrestris audax)*, the focus of my PhD thesis. Outlined below are the aims that I have set out to achieve in each chapter.

In **Chapter 2** I determined whether chronic exposure to sulfoxaflor (5ppb) influences the reproductive output of bumblebee colonies. Wild queens (*B. terrestris audax)* were collected and reared into colonies after which they were fed either a control or sulfoxaflor sucrose solution. After a two-week exposure period, colonies were moved into the field, where I conducted weekly colony census data until the end of the colony's life cycle. The results demonstrated that chronic sulfoxaflor exposure at 5ppb in nectar can reduce bumblebee colony reproductive output.

In **Chapter 3**, in an attempt to understand why sulfoxaflor exposure reduces colony-level reproductive output, I, based on studies conducted with neonicotinoids (Decourtye *et al.* 2004b; Stanley *et al.* 2015b; Samuelson *et al.* 2016), assessed whether acute sulfoxaflor exposure had an impact on (i) bumblebee/ honeybee learning and memory, using a proboscis extension reflex paradigm and (ii) bumblebee spatial working memory, using a radial-arm maze. I found no significant effect of sulfoxaflor exposure in either case.

Given that I found no effects of sulfoxaflor on learning and memory in Chapter 3, in **Chapter 4** I set out to examine the robustness of similar effects on bee cognition that have been described for neonicotinoids. I conducted a meta-analysis to determine whether insecticide exposure in general has an impact on bee learning and memory and found that other insecticides, in contrast to sulfoxaflor (Chapter 3), do consistently influence bee learning and memory.

In **Chapter 5,** I explored another potential explanation for the colony-level impacts of sulfoxaflor that I had described in Chapter 2. Using a microcolony-based design, I investigated whether sulfoxaflor exposure influenced the ovary development, egg laying, sucrose consumption and fecundity of bumblebee workers. I found that sulfoxaflor exposure can reduce bumblebee egg laying, suggesting a possible mechanism that caused the observed negative impacts of sulfoxaflor on bumblebee reproductive output (Chapter 2).

In **Chapter 6** I focused on understanding whether sulfoxaflor exposure has a direct impact on bumblebee larval mortality and growth when reared in artificial well-plates. I also considered the potential interaction between sulfoxaflor exposure and fungal *Nosema bombi*. I found that sulfoxaflor exposure reduced bumblebee larval growth, which could be a potential mechanism driving the results obtained in Chapter 2.

In **Chapter 7** I summarise and discuss the results obtained in this thesis, highlight points of interest and discuss areas of future research.

Chapter 2: Sulfoxaflor exposure reduces bumblebee reproductive success

Harry Siviter, Mark J F Brown, Ellouise Leadbeater

Siviter, H., Brown, M.J.F. & Leadbeater, E. (2018) Sulfoxaflor exposure reduces bumblebee reproductive success. *Nature*, **561**, 109–112.

Abstract

Intensive agriculture currently relies on pesticides to maximise crop yield (Tilman *et al.* 2002; Foley *et al.* 2005). Neonicotinoids are the most widely used insecticides worldwide (Simon-Delso *et al.* 2015), but increasing evidence of negative impacts on important pollinators (Whitehorn *et al.* 2012; Feltham *et al.* 2014; Rundlöf *et al.* 2015; Woodcock *et al.* 2016, 2017; Tsvetkov *et al.* 2017) and other non-target organisms (Pisa *et al.* 2017) has led to legislative re-assessment and created demand for the development of alternative products. Sulfoximine-based insecticides are the most likely successor (Brown *et al.* 2016), and are either licensed for use or under consideration for licensing in several worldwide markets (Simon-Delso *et al.* 2015), including within the European Union (Official Journal of the European Union 2015) where certain neonicotinoids (imidacloprid, clothianidin and thiamethoxam) are now banned for agricultural usage outside of permanent greenhouse structures. There is an urgent need to pre-emptively evaluate the potential sub-lethal effects of sulfoximine-based pesticides on pollinators (Brown *et al.* 2016), because such effects are rarely detected by standard ecotoxicological assessments, but can have major impacts at larger ecological scales (Bryden *et al.* 2013; Milner & Boyd 2017; Baron *et al.* 2017a). Here, we show that chronic exposure to sulfoxaflor (a sulfoximine-based insecticide), at dosages consistent with potential post-spray field exposure, has severe sub-lethal impacts on bumblebee *(Bombus terrestris*) colonies. Field-based colonies that were exposed to sulfoxaflor during the early growth phase produced significantly fewer workers than unexposed controls, and ultimately produced fewer reproductive offspring. Differences between the life-history trajectories of treated and control colonies first became manifest when individuals exposed as larvae began to emerge, suggesting that direct or indirect effects on a small cohort may have cumulative long-term consequences for colony fitness. Our results caution against the use of sulfoximines as a direct replacement for neonicotinoids. To avoid continuing cycles of novel pesticide release and removal, with concomitant impacts on the environment, a broad evidence base needs to be assessed prior to the development of policy and regulation.

Main text

The widespread global use of highly effective neonicotinoid-based pesticides has led to the evolution of resistance amongst several insect crop pests (Bass *et al.* 2015), and generated worldwide interest in emerging sulfoximine-based alternatives that have been shown to be effective in targeting some neonicotinoid-resistant species (Zhu *et al.* 2011; Perry *et al.* 2012; Longhurst *et al.* 2013). This potential lack of cross-resistance may reflect differences in three-dimensional molecular structure that preclude the breakdown of sulfoximines by enzymes involved in neonicotinoid metabolism (Sparks *et al.* 2012), supporting the claim that sulfoximines and neonicotinoids are chemically distinct (Zhu *et al.* 2011). However, as selective agonists of insect nicotinic acetylcholine receptors (NAChRs) (Zhu *et al.* 2011), the two pesticide groups share a common biological mode of action. This raises major concerns about potential effects on non-target species, and particularly bees. Neonicotinoids, while not lethal to bees at field realistic levels, have severe sub-lethal effects on both social and solitary bees, influencing cognition, foraging ability, homing ability, reproductive output, colony initiation (Gill *et al.* 2012; Henry *et al.* 2012; Whitehorn *et al.* 2012; Samuelson *et al.* 2016; Baron *et al.* 2017b; a; Tsvetkov *et al.* 2017; Woodcock *et al.* 2017; Siviter *et al.* 2018b), and, potentially, pollination services (Stanley *et al.* 2015a). Mathematical modelling has shown that these sub-lethal stressors can have drastic negative consequences for colony fitness downstream in the colony cycle (Bryden *et al.* 2013; Baron *et al.* 2017a).

To assess whether sulfoxaflor, the first marketed sulfoximine-based pesticide, has similar negative effects on bees, we fed either untreated sucrose solution (1.8M), or sucrose solution containing 5µg/dm^3 (5 ppb) of sulfoxaflor, to nascent *Bombus terrestris* colonies reared from wild-caught queens. We based this concentration on available estimates for sulfoxaflor residues in forager-collected nectar post-spray (United States Environmental Protection Agency 2016) (Extended Data Fig 2.1A), because spray application is currently the most common application procedure (although products containing sulfoxaflor have also been developed for seed-treatments and are already available for use on bee-pollinated crops within some markets (Pest Management Regulatory Agency (Canada) 2016)). After two weeks of laboratory-based exposure, size-matched colonies were placed in the field around a university parkland campus following a paired design, and no longer provided with additional resources. Staggered weekly nocturnal censuses revealed a clear difference in colony demographics between control and experimental colonies. The bumblebee colony cycle is characterised by an early growth phase in which worker numbers increase rapidly to create a large workforce, followed by a switch to production of reproductive brood later in the season. Between 2 and 3 weeks post-exposure, detectable differences in worker numbers between treated and control colonies began to emerge,

persisting until close to the end of the colony cycle (Figure 2.1A; Table S2.2D; glmer: treatment parameter estimate = -0.28, 95% CI: -0.48 to -0.01; treatment:week parameter estimate = -0.06, 95% CI: -0.11 to -0.01, treatment:week^2 parameter estimate: 0.11, 95% CI: 0.05 to 0.16).

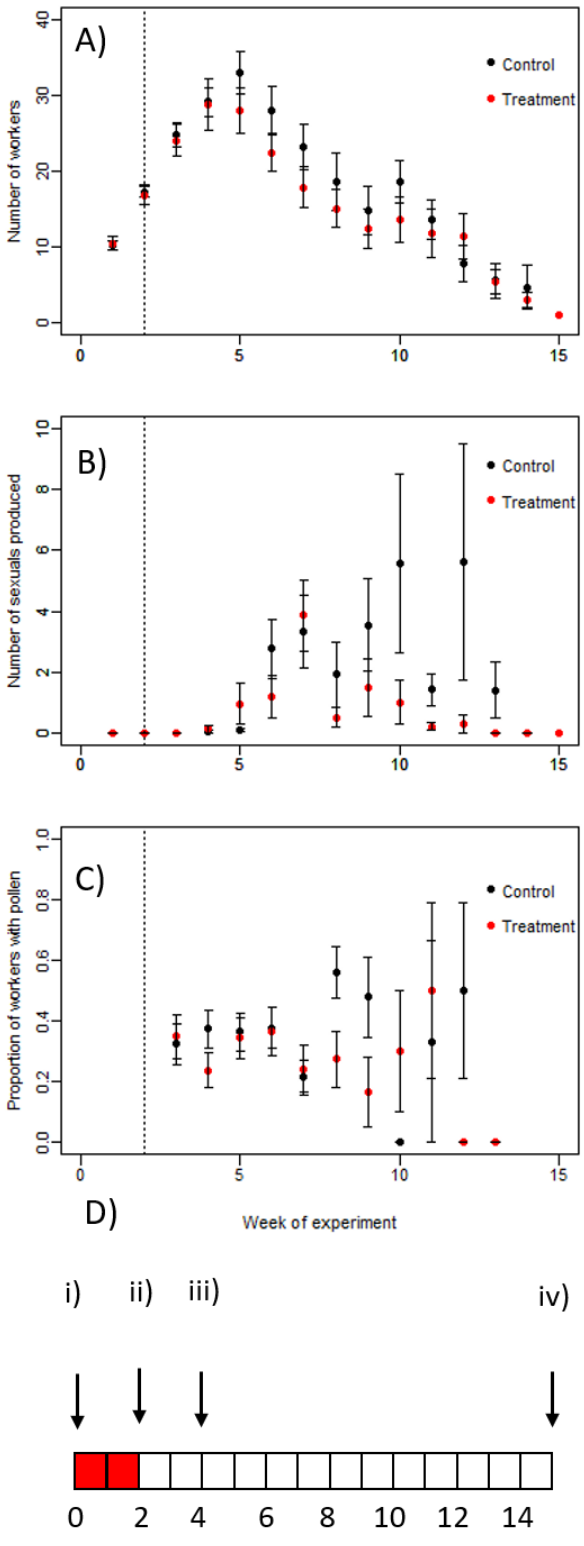

Figure 2. 1: **The impact of sulfoxaflor exposure on bumblebee colony life-history trajectories. Week-by-week colony field census data for (a) Number of workers, for treated (n = 26) and control colonies (n = 26) (b) Number of sexual offspring, and (c) Proportion of workers returning to the colony with pollen, for treated and control colonies (n = 25 and 26 respectively; reduced sample size for treated colonies reflects the death of one queen at week 2, see methods). Data presented show means ± SE. Demographic timeline (d) indicates the time points at which (i) laboratory-based exposure started (exposure period indicated in red), ii) colonies were moved into the field (iii) adults that encountered maximum exposure as larvae should begin to emerge (Alford 1975) and iv) maximum colony lifespan.**

As the colony cycle progressed, negative impacts upon colony reproductive output became apparent. Treated and control colonies were equally likely to produce male reproductive offspring, but treated colonies produced significantly fewer males in total (Zero-inflated count model, binomial section, treatment parameter estimate = 0.71, 95% CI = -0.67 to 2.09; count section, treatment parameter estimate = -0.54, 95% CI: -0.72 to -0.37; Figure 2.2). This difference became apparent from approximately week 9 onwards (Figure 2.1B). The dry mass of these males was no different to those produced by control colonies (w_i (null model) = 0.974), indicating that our results cannot be explained by differential investment in reproductive biomass. Neither treated nor control colonies produced an abundance of queens, but control colonies produced more than treated colonies (total: 36 new gynes from 3 of 26 control colonies, none in any of 25 treated colonies) and so our findings hold when the total number of sexual offspring is analysed (Zero-inflated count model, binomial section, treatment parameter estimate = 0.71, 95% CI = -0.67 to 2.09; count section, treatment parameter estimate = -0.64, 95% CI: -0.81 to -0.46). The timing of reproductive onset, queen longevity and colony survival did not differ between control and treated colonies (Extended Data Fig. 2.2; Survival analyses, treatment parameter estimate (reproductive onset) = -0.05, 95% CI: -0.41 to 0.31; (colony longevity) = -0.03, -0.43 to 0.38); (queen survival) = -0.07, -0.47 to 0.33).

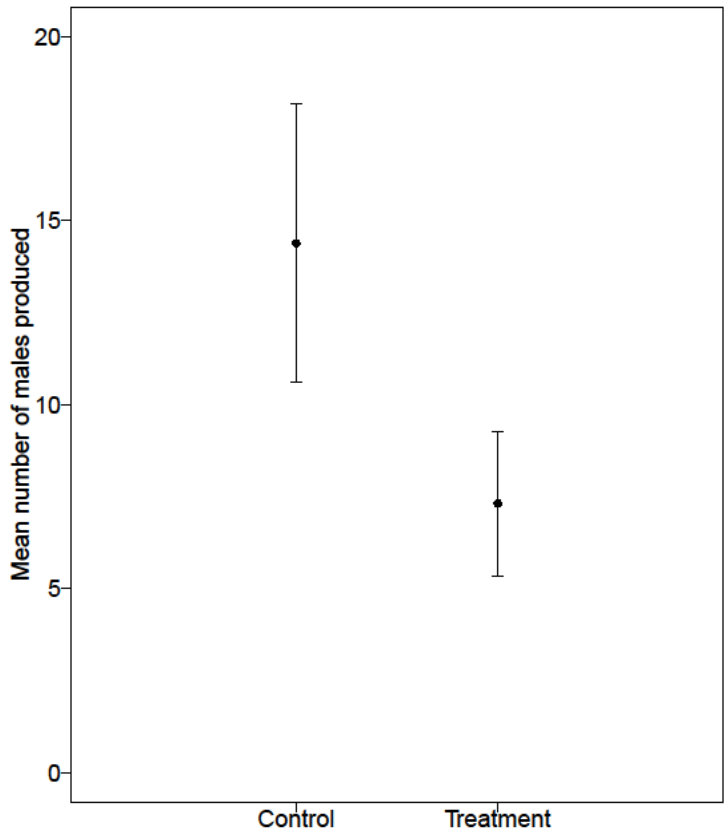

Figure 2. 2: Male offspring production. Mean (±SE) number of male sexual offspring produced by sulfoxaflor-treated (n=25) and control (n=26) colonies.

Based on the neonicotinoid literature, we considered the explanation that this difference in the production of sexual offspring might be mediated through poor provisioning of larvae by foraging workers (Gill *et al.* 2012; Feltham *et al.* 2014), at the time when sexual offspring were developing. However, daytime foraging censuses revealed no significant differences in the number of bees returning to control and treated colonies (generalized linear mixed model, treatment, parameter estimate = -0.07, 95% CI: -0.32 to 0.19). Similarly, although visual inspection of the data was suggestive of a lower proportion of workers returning with pollen to pesticide-treated vs. control colonies from week eight onwards (Fig. 2.1C), this effect did not receive statistical support (glm, week:treatment, parameter estimate = -0.14, CL: -0.29 to 0.001; treatment, parameter estimate = 0.46, CL:-0.38 to 1.31) and furthermore occurred too late in the colony cycle to explain differences in male production, which became apparent at approximately the same time. We also found no significant differences in the size of pollen loads collected between control and pesticide-treated colonies (Extended Data Fig

2.3). Instead, consideration of the timing of differences between control and treated colonies suggests that impacts of sulfoxaflor exposure on reproductive output were mediated by the early drop in worker numbers that began at weeks 2-3 post-exposure. Bumblebee worker pupae take approximately 14 days to develop (Alford 1975), so the onset of deceleration of colony workforce growth corresponds to the eclosion of individuals that had encountered maximum exposure as larvae (Figure 2.1D). It remains unclear whether this failure to eclose was driven by direct effects on exposed larvae (Wu *et al.* 2011) , or indirect effects, perhaps mediated by poor provisioning (Gill *et al.* 2012; Feltham *et al.* 2014) by exposed workers (although note that colonies were provided with pollen and sucrose in the laboratory during this time). In either case, the resultant drop in worker numbers led to deviation in the life-history trajectories of control and sulfoxaflor-treated colonies, with consequent effects on colony reproductive output (Bryden *et al.* 2013). These knock-on effects of early exposure to a small cohort of colony members are entirely consistent with the results of mathematical explorations of stress impacts on bee colonies, which predict that chronic stress at an early stage can push bee colonies beyond a 'tipping point', increasing the likelihood of colony failure (Bryden *et al.* 2013).

Sulfoxaflor is a systemic pesticide that is soluble in water and thus is transported around plant tissues following foliar or seed application. The likely exposure trajectory of pesticide crop treatments differs between seed treatments, which deliver prolonged exposure, and spray applications, which deliver a short-term dose that is initially high but typically declines rapidly. Sulfoxaflor, like neonicotinoid-based pesticides, can be administered using both methods, and sulfoxaflor-based products that are used as a seed-treatment have recently been developed for bee-attractive crops (including oilseed crops) (Syngenta Canada 2018). However, most currently marketed preparations are spray applications. The dosage used in this study is below US EPA estimates for field-realistic immediate post-spray concentrations of sulfoxaflor in forager-collected nectar, and remains below residual concentrations estimated at 10 days post-spray (the maximum period for which data are available; concentration range over whole period: 5.41 to 46.97µg a.i./kg, application rate: 2 x 0.045 lb active ingredient /A; Extended Data Fig 2.1A & B)(United States Environmental Protection Agency 2016). Note that our treatment protocol is particularly conservative in that our nascent colonies were fed untreated pollen in addition to the syrup provided, potentially producing significant underestimates of effects on larvae. Post-spray sulfoxaflor residues in pollen have been documented to be more than tenfold higher than those in forager-collected nectar (Extended data Fig. 2.1A & 2.1B), ranging from 510.95 to 50.12 µg a.i./kg over the same post-spray period (United States Environmental Protection Agency 2016) . Mitigation measures can be used to reduce bee exposure to sulfoxaflor when used as

spray treatments (for example, spray application to bee-attractive crops during bloom is precluded by law in the USA) (Centner *et al.* 2018), but globally, under current usage such measures are often either absent (Dow AgroSciences South Africa 2018) or limited to product label recommendations to avoid spray 6 days prior to bloom (Dow AgroSciences Ireland). No such measures are possible for those products that have been developed as a seed treatment (Syngenta Canada 2018).

The impact of sulfoxaflor identified here can be compared with previous experiments that focused upon exposure to neonicotinoids. For example, bumblebee colonies placed next to oil seed rape fields that were treated with neonicotinoids exhibited a 71% reduction in the mean number of queen cocoons found within the nest (Rundlöf *et al.* 2015) and a 32-36% reduction in the mean number of males/workers produced (Woodcock *et al.* 2017). Similarly, colonies foraging next to thiacloprid-treated raspberry crops had a 46% reduction in reproductive output (Ellis *et al.* 2017) and commercial bumblebee colonies exposed to imidacloprid for a period of two weeks had an 85% reduction in the number of new queens produced (Whitehorn *et al.* 2012). Here, we found that sulfoxaflor exposed colonies had a 54% reduction in the total number of sexual offspring produced compared with control colonies, suggesting that from the perspective of wild pollinators, sulfoxaflor exposure could lead to similar environmental impacts to neonicotinoids if used on bee-attractive crops in the absence of evidence-based legislation.

Sulfoximine-based pesticides are a newly-emerging class of product, but are already licensed in many countries worldwide, including China (Simon-Delso *et al.* 2015), Canada (Pest Management Regulatory Agency (Canada) 2016) and Australia (APVMA 2013). Within the European Union, where the use of certain neonicotinoids is now banned for open-field crops, substances containing sulfoxaflor as an active ingredient have been assessed by the European Food Safety Authority(EFSA 2014) and approval has been granted for use in 5 member states, with applications from seven more member states currently in progress (European Commission). Our results provide pre-emptive evidence that, if exposure at equivalent dosages to those used in our study occurs via bee-attractive crops pre- or during bloom, either through spray or seed treatment applications, these products could pose a significant risk to pollinators. The effects that we identified were the longer-term outcome of initial short-term exposure, and were only detected by monitoring of the full colony cycle. Bans and restrictions on neonicotinoid-based pesticides have largely been implemented to protect important pollinators such as bees, following years of widespread use with potential long-term population-level consequences. To avoid a situation whereby pesticides such as neonicotinoids are replaced with products that are similarly contentious, regulatory bodies should move towards an evidence-based approach that assesses both the lethal and sub-lethal consequences of novel insecticides such as

sulfoxaflor on non-target organisms, and incentivises integrated pest management approaches, before products are licenced for use (Sanchez-Bayo & Tennekes 2017).

Methodology

Exposure regime

Sulfoxaflor-based preparations have been developed for use on a wide range of bee-attractive crops that flower at varying times of the year. The regime used in our study most closely mimics spring-flowering crops in temperate environments, allowing comparison with similar neonicotinoid-based studies (Rundlöf *et al.* 2015; Woodcock *et al.* 2017; Baron *et al.* 2017a) that also exposed colonies for a short period during the early growth phase.

Preparations containing sulfoxaflor as an active ingredient are currently most commonly applied as a foliar spray. We thus based our pesticide concentrations on the best available information from a realistic and bee-relevant spray experiment reported by the USA EPA, in which sulfoxaflor was applied to a cotton crop at an application rate of 2 x 0.045 pounds of active ingredient per acre. Under this application regime, mean sulfoxaflor residue levels in honeybee-collected nectar did not drop below 5µg a.i./kg over an 11-day period (the maximum period for which data are available; Extended Data Fig 2.1A) (United States Environmental Protection Agency 2016). We are confident that our exposure is conservative because a) in the same experiment, pollen residue levels did not drop below 50µg a.i./kg[3] (United States Environmental Protection Agency 2016) (Extended Data Fig 2.1B), while we provided all colonies with untreated pollen *ab libitum*, and b) this application rate is similar to label recommendations for at least some sulfoxaflor-based products (Dow AgroSciences South Africa 2018). A second study has also measured residues (in cucumber), but application rates were 1.5 times above recommended usage, and the relevance of this experiment for bees is unclear as the cucumber tissue sprayed and sampled was not described (Xu *et al.* 2012).

In terms of current usage, our data are most relevant to sulfoxaflor preparations when sprayed on crops immediately prior to or during bloom (note that this practice has recently been reviewed and prohibited in the USA(United States Environmental Protection Agency 2016)). While some product labels recommend avoidance of spraying 6 days before bloom (Dow AgroSciences Ireland), this ignores experimental data showing that residues could remain present in pollen at levels which we show to have sub-lethal impacts after this 6-day period (United States Environmental Protection Agency 2016) (Extended Data Fig 2.1D). Other labels allow spraying during bloom at night (Dow AgroSciences South Africa 2018). To the best of our knowledge, no data are currently available on field-realistic residues for seed-treatment preparations that have been developed for use on oilseed crops and are already available in some markets (Pest Management Regulatory Agency (Canada) 2016).

Queen rearing

332 bumblebee (*Bombus terrestris audax*) queens were caught between the 28th of February and the 23rd of March 2017, from Windsor Great Park, Surrey, UK. Chilled queens were transported to the laboratory, where their faeces were microscopically examined for parasites (*Nosema* spp, *Apicystis bombi, Sphaerularia bombi* and *Crithidia bombi*; x400 magnification). Parasitized individuals (N = 54) were removed from the experiment. A second parasite screening was repeated after one week (29 further queens removed, remaining N=249).

Queens were placed in rearing boxes (W 67 x L 127 x D 50; Allied Plastics, Kingston, UK) and were provided with a gravity feeder containing an *ad libitum* supply of 1.8M sucrose solution (changed weekly; Thorne, Windsor, UK) and a pollen ball (changed twice-weekly, unless the queen was laying eggs in which case more pollen was added; Biobest, Westerlo, Belgium). Each queen was housed in a dark/red-lit room maintained at 26°C and 50-60% relative humidity. Queens that did not produce eggs after eight weeks were removed from the experiment (N=107). Once a queen had produced at least 6 workers, the colony was moved into a wooden nest box (W 280 x L 320 x D 160mm) and randomly assigned to a treatment group (see below). The time taken to reach this stage varied but on average was 7.2 weeks (± sd: 1.5 weeks). On transfer, the queens underwent a final parasite screening (2 queens removed). 2 queens died prior to transfer, thus 52 colonies reached this stage. The use of colonies from wild-caught queens is a key feature of our experimental design that enabled us to a) have a complete overview of the lifecycle of these colonies (both in the laboratory and the field, see below), and b) use colonies with a life-history that was adapted to the local environment.

Pesticide exposure

Prior to pesticide exposure, colonies were allocated to control and treatment groups and paired for size according to the number of workers present (mean = 8.43 ± SD 1.87). Each colony was then provided with an *ab libitum* supply of either 1.8M sucrose solution containing 5µg/dm^3 (5ppb) sulfoxaflor (derived from a stock solution of 1g/dm^3 in acetone; Greyhound chromatography and allied chemical, Merseyside, UK), or 1.8M sucrose containing equivalent acetone concentration but no sulfoxaflor, for a two-week period. Sucrose solution was weighed on placement in and removal from the colony; no differences in consumption were found between treatment groups (w_i (null model) = 0.985). During the exposure period we recorded the number of workers produced, colony mass and the number of dead workers, on a weekly basis. One queen died during the exposure period, thus 51 colonies were present at the start of the field experiment (control N = 26 and pesticide N = 25).

Field placement

After two weeks of exposure in the laboratory, colonies were moved into the field. Nest boxes were placed within plastic field boxes (W 440 x L 710 x D 310mm; Really Useful Box, Kingston, UK) containing insulation wrap (Thermawrap, Creswell, UK) and aluminium foil, and placed at locations around the Royal Holloway University of London campus, Egham, UK (45ha; Extended Data Fig 2.4). Paired colonies were matched for location within the campus, and were positioned at least 20 metres from one another to reduce drifting. Each colony entrance was demarcated by a distinctive visual pattern. Colonies were placed in discreet, shaded and south-east facing locations, and secured with a ratchet strap to avoid badger damage. To prevent usurpation attempts from other queens and social parasite species *(Bombus vestalis*)*,* queen excluders were placed on each colony. Upon initial placement in the field the colonies were supplied with a gravity feeder containing 46g 1.8M sucrose solution, after which they received no further food supplements. The process of field placement was staggered over six weeks (10/04/2017- 21/05/2017) owing to variation in the date at which queens were initially caught. The week of placement was included as a predictor in each statistical analysis (see below).

Data collection

We combined methodological approaches from previous studies of the effects of neonicotinoids on bumblebees (Gill *et al.* 2012; Whitehorn *et al.* 2012) , as well as studies of bumblebee life-history (Baer & Schmid-Hempel 1999) to maximise our measurement of both impacts and potential mechanisms. We conducted censuses every night such that each colony was visited once per week, between the hours of 21:30-04:00. Using a red-light torch, we recorded the number of live workers (average of three counts), dead workers, males, and new queens. We also recorded the state of the original queen (dead or alive), the presence of gyne larvae and/or pupae, the presence of worker larvae and/or pupae, the number of pollen and nectar pots containing stores, and the mass of the colony (average of three recordings; EM-30KAM balance, A&D instruments). In cases where the wax covering prevented observation, we peeled it back in order to conduct the count. Weekly censuses continued until moribundity, defined as either a live queen and 3 or fewer workers, or no queen and 10 workers or fewer (Samuelson *et al.* 2018). After the experiment, all sexual offspring that had been found in the colonies (N = 600) were dried for 72 hours and weighed (accuracy = $\pm$ 0.001g).

All 51 colonies were also visited during daylight hours twice per week. Colony traffic (number of bees entering and leaving the nest) was recorded during 10-minute counts, once between 9:00 & 13:00 and once between 14:00 & 18:00. We also recorded whether returning workers had large (pollen basket was over-flowing) or small (pollen enclosed within pollen basket) pollen loads relative to their body size. Control and pesticide pairs were always observed directly after one another, in a random order.

The average daily temperature, humidity and total rainfall were obtained from a local weather station (www.wunderground.com).

Statistical analyses

We employed an information theoretic model selection approach. For each response variable, the initial candidate set included a full model and all subsets, including a null model. Reported parameter estimates and confidence intervals are based upon full-set averaging of the 95% confidence set (i.e. the set of models with cumulative Akaike weight $\geq$ 0.95). Model types, error structuring, a list of parameters included within each model and parameter estimates are provided in tables S2.1 & S2.2. Briefly, to analyse the number of workers produced per week, we used a generalized linear model (glmer; Poisson error structure) with colony nested within pair as a random factor, and the week of initial field placement (week started), treatment, week of experiment and a two-way interaction between treatment and week of experiment as fixed factors. Since the number of workers increased to a maximum and then decreased for each colony, "week of experiment" was modelled as a quadratic factor (ΔAIC between full linear and full quadratic model: 1206.40). Many colonies did not produce sexual offspring, so we used zero-inflated generalized linear models (zeroinfl) to analyse differences in both the overall number of sexual offspring and the number of males produced by colonies, with the week of initial field placement, treatment and their interaction as predictors. The number of workers returning to the nest was analysed using a zero-inflated generalised linear model (glmmadmb; negative binomial error structure) in which treatment, week started, colony week and temperature were included as fixed factors and colony as a random factor. The proportion of workers returning with pollen was also analysed using a generalised linear model (glmmadmb: binomial error structure) with treatment, colony week and their interaction, week started, temperature, and time of day included as fixed factors and colony/pair included as a random factor. Week of reproductive onset and queen survival were analysed using a Cox proportional hazards survival analysis that contained treatment and week started as fixed factors. All analyses were conducted in R studio (Version 1.0.136) using the r packages*, pscl* (Jackman 2011)*, lme4* (Bates *et al.* 2015), *glmm* (Geyer 1994)*, MuMin* (Burnham & Anderson 2002) *survival* (Therneau & Grambsch 2002) and *glmmadmb* (Kristensen *et al.* 2016).

Chapter 3: No evidence for negative impacts of acute sulfoxaflor exposure on bee olfactory conditioning or working memory

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Abstract

Systemic insecticides such as neonicotinoids and sulfoximines can be present in the nectar and pollen of treated crops, through which foraging bees can become acutely exposed. Research has shown that acute, field realistic dosages of neonicotinoids can negatively influence bee learning and memory abilities, with potential consequences for bee behaviour. As legislative reassessment of neonicotinoid use occurs globally, there is an urgent need to understand the potential risk of other systemic insecticides. Sulfoxaflor, the first branded sulfoximine-based insecticide, has the same mode of action as neonicotinoids, and may potentially replace them over large geographical ranges. Here we assessed the impact of acute sulfoxaflor exposure on performance in two paradigms that have previously been used to illustrate negative impacts of neonicotinoid pesticides on bee learning and memory. We assayed whether acute sulfoxaflor exposure influences (a) olfactory conditioning performance in both bumblebees (*Bombus terrestris*) and honeybees (*Apis mellifera*), using a proboscis extension reflex assay, and (b) working memory performance of bumblebees, using a radial-arm maze. We found no evidence to suggest that sulfoxaflor influenced performance in either paradigm. Our results suggest that despite a shared mode of action between sulfoxaflor and neonicotinoid-based insecticides, widely-documented effects of neonicotinoids on bee cognition may not be observed with sulfoxaflor, at least at acute exposure regimes.

Introduction

Bees provide vital pollination services for both wild flowers and commercial crops (Rader *et al.* 2016; Fijen *et al.* 2018), so localised declines in domestic honey bee populations and both localised and global range reductions of certain bumblebee species have led to suggestions that a global pollination crisis could be imminent (Biesmeijer *et al.* 2006; Colla & Packer 2008; Aizen & Harder 2009; Williams & Osborne 2009; Potts *et al.* 2010b; Cameron *et al.* 2011; Kerr *et al.* 2015; Goulson *et al.* 2015). Although the intensification of agriculture, habitat loss, global warming and pathogen exposure have all been linked with bee declines (Brown & Paxton 2009; Winfree *et al.* 2009; Cameron *et al.* 2011; Kerr *et al.* 2015; Goulson *et al.* 2015; Samuelson *et al.* 2018), particular attention has focused on the impact of agrochemicals. A key focus of research has been to understand the impact of neonicotinoid-based insecticides on bees (Whitehorn *et al.* 2012; Godfray *et al.* 2014, 2015; Stanley *et al.* 2015a; Rundlöf *et al.* 2015; Goulson *et al.* 2015; Kessler *et al.* 2015; Woodcock *et al.* 2016, 2017; Baron *et al.* 2017b; a; Tsvetkov *et al.* 2017; Main *et al.* 2018; Arce *et al.* 2018; Siviter *et al.* 2018b), leading to controversy worldwide and in some cases, legislative reassessment of their use. Sulfoximine-based insecticides, which share a mode of action with neonicotinoids as selective agonists of Nicotinic Acetyl Choline Receptors (NAChRs) (Zhu *et al.* 2011; Sparks *et al.* 2013), are a more recent entry to the insecticide market, and are currently approved for use in 81 countries around the world. In a recent horizon-scanning exercise involving 72 pollination biologists, sulfoximines were highlighted as an emerging potential threat to pollinators, based on a lack of knowledge regarding their sub-lethal effects (Brown *et al.* 2016).

Sulfoxaflor, the first branded sulfoximine-based insecticide, can negatively impact bumblebee colony fitness, reducing worker production and subsequent reproductive output (Siviter, Brown & Leadbeater 2018a), with the effects comparable to those observed with neonicotinoids (Whitehorn *et al.* 2012; Rundlöf *et al.* 2015). A plethora of research on neonicotinoids has linked small sub-lethal effects on bee behaviour at the individual level to major impacts at the colony level, with neonicotinoid exposure influencing bee foraging success and motivation, (Gill *et al.* 2012; Feltham *et al.* 2014; Gill & Raine 2014; Arce *et al.* 2017; Lämsä *et al.* 2018; Muth & Leonard 2019), homing success (Henry *et al.* 2012; Fischer *et al.* 2014), brood care and thermoregulation (Crall *et al.* 2018). One way in particular that neonicotinoids may influence bee behaviour is through impacts on bee cognition, with a recent meta-analysis confirming detrimental effects of insecticide exposure on learning and memory at acute and field realistic regimes (Siviter *et al.* 2018b). As a systemic insecticide, sulfoxaflor, like neonicotinoids, can be present in the nectar and pollen of plants following treatment, meaning that foraging bees may be exposed either via the crop itself or through flowering weeds present in

fields or orchards during spray (Botias *et al.* 2015; Kyriakopoulou *et al.* 2017). However, despite the similarity in mode of action between sulfoxaflor and neonicotinoids, the potential impact of sulfoxaflor exposure on bee learning and memory has not been tested.

In this study, we assay the impact of acute sulfoxaflor exposure on learning and memory in bees based on two paradigms through which previous authors have identified adverse effects of neonicotinoid exposure: a Proboscis Extension Reflex (PER) experiment (Stanley *et al.* 2015b; Siviter *et al.* 2018b) and a Radial Arm Maze-based assay (RAM;Samuelson *et al.* 2016). These paradigms are assays of (i) classical conditioning of olfactory stimuli and (ii) working memory (also known as short-term memory) respectively, and thus they may capture different aspects of foraging, although are unlikely to be mutually exclusive. For example, learning to discriminate between olfactory stimuli in a PER task may emulate learning to discriminate between rewarding and non-rewarding flower species, while RAM performance assays retention of short-term task-relevant information such as the location of flowers that a bee has recently visited (Foreman & Ermakova 1998; Lihoreau, Chittka & Raine 2010; Collett, Chittka & Collett 2013; Samuelson *et al.* 2016). Exposure to certain neonicotinoids, and other non-neonicotinoid insecticides, has been shown to influence PER performance in both *Apis* and *Bombus* (Williamson, Baker & Wright 2013; Stanley *et al.* 2015b; Piiroinen & Goulson 2016; Siviter *et al.* 2018b), while impacts on RAM performance have only been tested in bumblebees (Samuelson *et al.* 2016). Given that sulfoxaflor and neonicotinoids both act as agonists of nicotinic acetylcholine receptors (NAChRs) (Sparks *et al.* 2013), we predicted that sulfoxaflor exposure would have similar negative impacts on PER performance in *Apis* and *Bombus*, and RAM performance for *Bombus*.

Methods: PER- Experiment 1

Subjects and harnessing

Five bumblebee (*Bombus terrestris audax*) colonies with approximately 150 workers were purchased (Koppert Ltd, Haverhill, UK) and moved into a wooden colony box (28 x 10 x 18cm) connected to a flight arena (100 x 70 x 50cm) that contained an *ad libitum* supply of sucrose solution (50° Brix) and pollen. Only individuals that had been observed foraging on the feeder within the flight arena were subsequently used in the experiment (Martin, Fountain & Brown 2018). Previous studies suggest that bumblebees are more responsive when starved for a period of time (Stanley *et al.* 2015b), and consequently prior to all PER experiments involving bumblebees, we collected and harnessed all potential subjects before leaving them overnight, and tested them the following morning.

Returning foraging honey bees (*Apis mellifera*) were collected from the entrance of five hives from a research apiary at Royal Holloway University of London. Honeybee mortality is high when individuals are harnessed for a sustained period of time, and as a result we collected and harnessed honeybees in the same day, leaving them for one hour after harnessing, before randomly assigning them to a treatment group (see below) and conducting the experiment. Bumblebees and honeybees were tested on different days, and on any single test day, sixteen to forty bumblebees and honeybees were collected and harnessed.

Insecticide exposure

Sulfoxaflor has been developed for a range of different crops, including as a seed treatment for bee attractive crops, but its most common application is currently as a foliar spray (Centner *et al.* 2018). Foliar spray applications result in short-term bursts of high insecticide residues in the nectar of sprayed crops (United States Environmental Protection Agency 2016) and any concurrently flowering weeds. We thus based our estimates for acute exposure on data for the residue levels found in honeybee-collected nectar of cotton sprayed with sulfoxaflor from an Environmental Protection Agency (EPA) study, which demonstrated that over an 11 day period nectar concentrations ranged from 5.41- 46.97 ppb (United States Environmental Protection Agency 2016; application rate: 0.045 pounds (0.020 kg) of active ingredient per acre, applied twice). We derived our sulfoxaflor treatments from a stock solution of 1 g dm^{-3} sulfoxaflor (Greyhound Chromatography and Allied Chemicals) in acetone, which was combined with sucrose solution (50° Brix) to make three treatment groups: 2.4 µg dm^{-3} (2.4ppb), 10 µg dm^{-3} (10ppb), 250 µg dm^{-3}(250ppb; positive control) and the negative control (sucrose with acetone only). Before training, we placed each bee horizontally (held in place with modelling clay) and pipetted a 10µl droplet of sucrose solution containing the randomly-assigned treatment solution onto a plastic surface, from which the bees could feed. Bees that did not immediately drink were encouraged to extend their proboscis by antennal stimulation with sucrose. Bees that did not consume the full quantity of sucrose solution were not used in the experiment (excluded bumblebees N = 55, control = 13, 2.4ppb = 16, 10ppb = 16, 250ppb = 10; honeybees N = 17, control = 2, 2.4ppb = 6, 10ppb = 5, 250ppb = 4). After feeding, the bees were placed upright and left for an hour (Stanley *et al.* 2015b).

Training protocol

We used an absolute conditioning proboscis extension reflex (PER) procedure in which lavender scent (conditioned stimulus; CS) was forward paired with antennal stimulation by sucrose solution (unconditioned stimulus; US; 50° Brix). The subjects were placed 3 cm away from the odour tube that contained filter paper soaked in 4 µl of the lavender essential oil. A programmable logic controller computer was used to blow a constant stream of air containing the odours towards the subjects from

the odour tube. The odour tube was replaced every 20-30 trials to ensure that the odour was consistently strong throughout conditioning. Bees were exposed to 5 seconds of clean airflow (no odour), followed by 10 seconds of the odour. Six seconds after the start of odour exposure, the subject was presented with 0.8 µl of untreated sucrose solution (50° Brix) from a syringe. A positive response was recorded if the bee extended its proboscis in the first six seconds of odour presentation, before antennal stimulation with the US, and was always rewarded by immediate delivery of the sucrose solution. In the event of a negative response, we additionally recorded whether the bee responded to the antennal stimulation (to ascertain that the subject was motivated to extend its proboscis). Each subject received fifteen trials with an inter-trial interval of approximately 12 minutes. To ensure that the bees were learning about the odour and not other aspects of the experimental protocol, three non-scented probe trials were randomly distributed between the 5^{th} and 15^{th} learning trials. Bees that responded to the unscented stimulus in any probe trial were not included in the analysis (excluded bumblebees n = 10; honeybees n = 1). Each animal thus received 18 trials in total (15 test trials and 3 probe trials).

Medium- and long-term memory tests, whereby the subjects were presented with the conditioned odour in isolation for a single trial, were conducted with the same subjects 3 hours and 24 hours after the last learning trials, respectively. Once the experiment was finished, bees were frozen and their size recorded by measuring thorax width with electronic callipers (Mitutoyo), three times, from which a mean value was taken. We recorded size because it may influence the rate at which the insecticide is absorbed; larger bees empty their gut at a faster rate (Fournier *et al.* 2014) and previous studies have correspondingly found size-dependent effects of acute insecticide exposure on cognition (Samuelson *et al.* 2016).

In total, we tested 240 bumblebees and 174 honeybees. Bees that did not extend their proboscis in response to antennal sucrose stimulation in at least 5 learning trials were not used (bumblebee N = 64, control = 17, 2.4ppb = 14, 10ppb = 16, 250ppb = 17; honeybee N = 6, control = 1, 2.4ppb = 1, 10ppb = 2, 250ppb = 1). A further 3 bumblebees were removed from the experiment because they extended their proboscis before the odour was presented. Five bumblebees died, as did 46 honeybees. One bumblebee was harnessed poorly and thus not included, as were 10 honeybees. This resulted in final sample sizes of 102 bumblebees and 94 honeybees (bumblebees: control = 23, 2.4ppb = 26, 10ppb = 24, 250ppb = 29: honeybees: control = 29, 2.4ppb = 22, 10ppb = 22, 250ppb = 21).

Statistical analysis

We followed an information theoretic model selection approach. The initial model set included a full model and all subsets, including a null model that contained solely the intercept and “Colony” as a

random factor. We selected a 95% confidence set of models based on Akaike weights derived from AICc values. In cases where the 95% confidence set contained more than one model, the models were averaged (Burnham & Anderson 2002) (including the null if it was included within the confidence set) to produce parameter estimates and 95% confidence intervals. Data collected for bumblebees and honeybees were analysed separately due to potential differences between the species (see Siviter *et al.* 2018b).

Following Stanley *et al.* (2015b), we analysed three dependent variables to identify sulfoxaflor effects on PER performance: (i) whether the bee responded to the CS in the absence of antennal stimulation (hereafter: "positive response") in at least one trial overall (ii) the total number of positive responses (hereafter learning level) from bees that learnt the association, and (iii) the trial that the bee first exhibited a positive response. We used generalised linear mixed effect models with binomial or poisson error structures, or mixed effect Cox models, respectively, where treatment, bee size and their interaction were specified as fixed factors, and colony as a random factor (see tables S3.1 & S3.2). For medium- and long-term memory, we analysed whether or not the bee exhibited a positive response to the CS following the same method (binomial error structure). We used the packages, lme4 (Bates *et al.* 2015), MuMin (Barton 2016), Coxme (Therneau 2018), Hmisc (Harrell & Dupont 2018) and pscl (Jackman 2017).

Methods: RAM- Experiment 2

Subjects

Seven bumblebee colonies (*B. terrestris audax)* with approximately 150 workers were obtained from Biobest (Agralan Ltd, Swindon, Wiltshire, UK) and upon arrival transferred into a plastic bipartite nest box (28 x 16 x 10.5 cm, with a central divider that allowed access between compartments). When transferring bees into the nest box individual bees were tagged with unique number disks, allowing the identification of individuals. The nest box was attached to the radial arm maze (RAM; description below), with access controlled using sliding trap-doors. When the bees were not being tested, gravity feeders were placed in the RAM with an *Ad libitum* supply of 43°Brix sucrose solution. Colonies were provided with approximately 7 grams of pollen in the nest box 3 times a week. Colonies were used in succession rather than simultaneously, and newly emerged bees were tagged daily throughout the experiment.

Radial arm maze

A radial arm maze classically consists of 8 arms, each of which contains a hidden food reward (Foreman & Ermakova 1998). Animals forage within the maze and search for the food rewards, whilst avoiding

re-visiting arms that they have already depleted, and Samuelson *et al.* (2016) have previously confirmed that bumblebees use working memory to minimize such revisits. We based our design on the set-up used by Samuelson *et al.* (2016) but modified their original vertical design to create a horizontal version. The aim of this modification was to reduce reliance on learnt movement rules by forcing subjects to return to the centre of the maze between choices, as is usually the case for rodent versions of the RAM (Olton & Samuelson 1976; Foreman & Ermakova 1998). Our horizontal maze was constructed from acrylic plastic, sealed together with non-toxic grey silicone (Bondit). Each of the 8 arms contained a removable platform (7.2 x 2.6 x 0.5mm) upon which the bees could land to access a small hole in the wall. By extending the proboscis through this hole, bees could access a sucrose reward (43° Brix) that was not visible from the platform (volume varied between stages; see below). After visitation, the platform could be rapidly replaced with a clean alternative to prevent the use of scent marks to identify visited arms. The availability of visual global landmarks (often a view of the laboratory) has been shown to contribute to performance in a RAM for rodents and other animals (Foreman & Ermakova 1998; Wilkinson, Chan & Hall 2007), but a) our laboratory regularly changes in appearance and b) light control was important for our video software. Thus, our maze walls were opaque, but papered with a black and white panoramic photo of the laboratory to allow bees to orientate.

Stage 1- Group training

The objective of this stage was to identify motivated foragers. Each morning before testing, the RAM was set up with 10 µl 43°Brix sucrose solution on each landing platform. All bees were then allowed into the RAM to forage on the landing platforms (platforms were continuously reloaded with sucrose solution when drained). Only bees that were observed foraging within the maze at this stage (by inserting the proboscis into the holes at the end of each arm) proceeded to Stage 2 (Individual training).

Stage 2 – Individual training

The objective of the individual training stage was for bees to learn the win-shift nature of the RAM task, over the course of 10 training bouts. During each bout, bees were required to visit all 8 artificial platforms and then return to the nest box to empty their crop. At the outset of each bout, each platform contained 10µl of sucrose solution (20 µl for the first bout, to increase motivation). Rewards were not refilled after visitation, but landing platforms were replaced with identical but clean replacements. Once the bee found the final reward, we increased the amount of sucrose solution in that arm (from outside the maze) so that the bee's crop was full, encouraging her to return to the nest

box. Choices in the RAM were recorded as either: Correct – feeding from platforms that had not yet been visited, or Incorrect- attempting to feed from platforms that had already been depleted.

If a bee attempted to return to the nest box 3 times prior to visiting all landing platforms, or if the trial exceeded 20 minutes, the bee was permitted to return to the nest box. As with Samuelson *et al.* (2016) each bee completed 10 training bouts.

Stage 3- Pesticide exposure

Our pesticide exposure regime differed from that used in the PER regime because our RAM experiment was designed to allow direct comparison with the results described by Samuelson *et al* 2016, for thiamethoxam. Samuelson *et al* (2016) aimed to mimic the dosage received during one hour of foraging for nectar, whilst overcoming the problem that feeding on a large volume of sucrose may reduce a bee's motivation to participate in the RAM. To that end, bees were only fed half of the volume of nectar that would normally be consumed during such a foraging bout (0.5 x 37.7mg), with a doubled concentration of sulfoxaflor. To allow for direct comparison, we followed the same approach here (and bumblebees thus received a higher dosage than those in the PER treatment groups described above). Each test subject was intercepted as it was returning to the RAM after emptying its crop following the 10th training bout. They were placed into a plastic beaker, and fed 18.85 µl of sucrose solution from the randomly assigned treatment group. We included four treatment groups, intended to mimic foraging on crops with nectar containing either 0ppb (control), 5 µg dm^{-3} (5ppb), 10 µg dm^{-3} (10ppb) or 250 µg dm^{-3} (250ppb or positive control) of sulfoxaflor, with bees from each treatment groups receiving 0, 0.045, 0.091 & 2.5ng respectively. After consumption, the bees were held in the plastic beaker for 45 minutes before being returned to the nest (Samuelson *et al.* 2016). 60 bees were originally trained on the RAM but 2 failed to re-commence foraging after the exposure stage (N values, control = 14, 5ppb = 15, 10ppb = 15, 250ppb n = 14).

Stage 4- Test trial

Following exposure, the bees were presented with the exact set up they had experienced in the training phase of the experiment (stage 2) and tested one final time. After completing the task bees were collected and frozen and, at a later date, we measured their thorax width.

Statistical Analysis

As with experiment 1, we used an information theoretic model selection approach when analysing each dependent variable and, as in previous work (Olton & Samuelson 1976; Foreman & Ermakova

1998; Samuelson *et al.* 2016), three different measures were chosen to assess performance; (i) total revisits to platforms which have been previously visited, (ii) the number of correct choices made before making a revisit and (iii) the proportion of correct choices in the first 8 visits. For all dependent variables, treatment, bee size and the interaction between them were included as fixed factors with colony included as a random factor. To account for overdispersion, we used a generalised linear model with a negative binomial distribution error structure (glm.nb) to analyse total revisits, and a generalised linear model (glm) with a poisson distributed error structure to analyse the number of correct choices in the first 8 visits. A mixed effect cox model (coxme) was used to analyse correct choices before first revisit. All analyses were conducted in R studio (version 1.1.419) using the R packages lme4 (Bates *et al.* 2015), MuMin (Barton 2016), Coxme (Therneau 2018), AER (Kleiber & Zeileis 2008), MASS (Ripley & Venables 2002), Hmisc (Harrell & Dupont 2018).

Results: PER - Experiment 1

For our first measure of learning (production of at least one conditioned response to the stimulus), we found no evidence that acute sulfoxaflor exposure influenced bumblebees (Figure 3.1A, glmer, 2.4 ppb parameter estimate (PE) = -0.00, 95% confidence intervals (CI) = -0.34 to 0.33; 10 ppb PE = 0.00, 95% CI = -0.35 to 0.36; 250 ppb PE= 0.05, 95% CI = -0.43 to 0.53) or honeybees (Figure 3.2A; glmer, 2.4 ppb PE = -1.30, 95% CI = -14.19 to 11.60; 10 ppb PE = -1.26, 95% CI = -14.82 to 12.31; 250 ppb PE = -7.32, 95% CI = -53.10 to 38.45). Learning level (number of positive responses) was also not influenced by sulfoxaflor exposure (bumblebees, Figure 3.1B; glmer; *wi* (treatment) = 0.017; honeybees, Figure 3.2B; glmer, 2.4 ppb PE = 1.18, 95% CI = -8.23 to 10.59; 10 ppb PE = 1.05, 95% CI = -6.93 to 9.04; 250 ppb PE = 0.31, 95% CI = -4.11 to 4.72). Finally, there was no evidence to suggest that sulfoxaflor exposure influenced the speed at which either bumblebees or honeybees learnt the olfactory association (bumblebees, Figure 3.1C, coxme, 2.4 ppb PE = -0.00, 95% CI = -0.93 to 0.78; 10 ppb PE= -0.00, 95% CI = -0.91 to 0.87; 250 ppb PE = 0.03, 95% CI = - 0.39 to 1.22; honeybees, Figure 3.2C; coxme, 2.4 ppb PE = -0.11, 95% CI = -0.72 to 0.51; coxme, 10 ppb PE = -0.02, 95% CI = -0.34 to 0.29; coxme, 250 ppb PE = -0.01, 95% CI = -0.30 to 0.28), suggesting no influence of acute sulfoxaflor exposure on olfactory conditioning performance in either species.

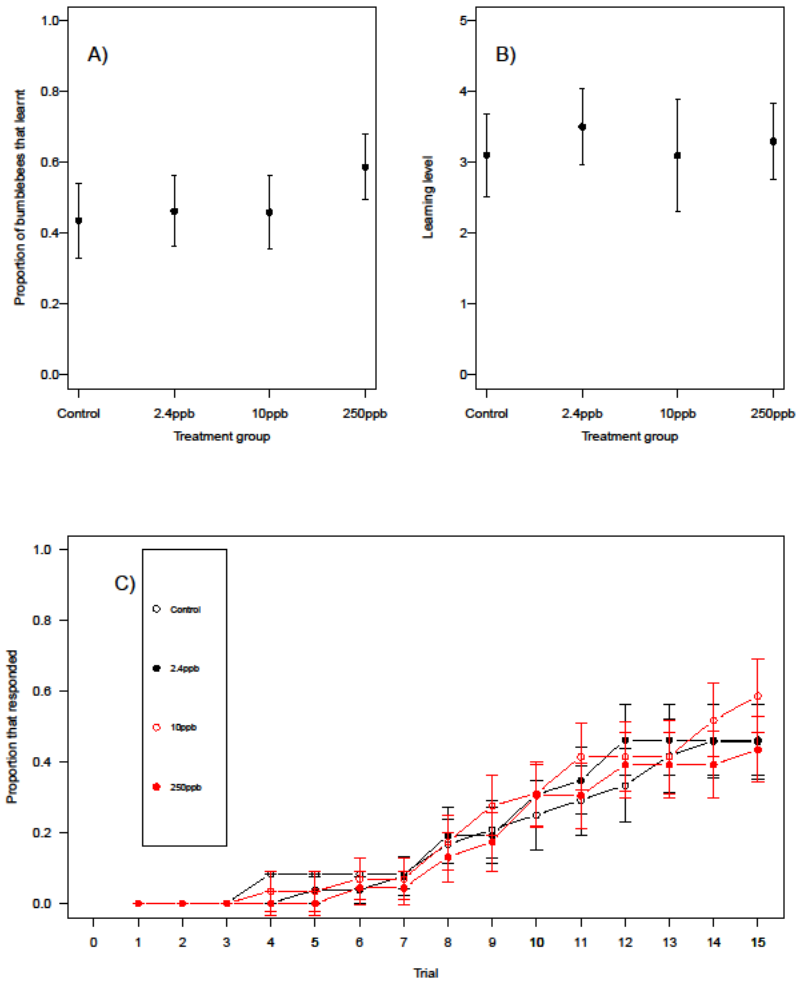

Figure 3. 1: **The performance of bumblebees in an olfactory learning task: (a) The proportion (± SEM) of bumblebees that learnt the olfactory association (b) the learning level (± SEM) of the bees that did learn the association and (c) the trials in which bees learnt the association (± SEM) in reference to trial number. (Control n = 23, 2.4ppb n = 26, 10ppb n = 24, 250ppb n = 29).**

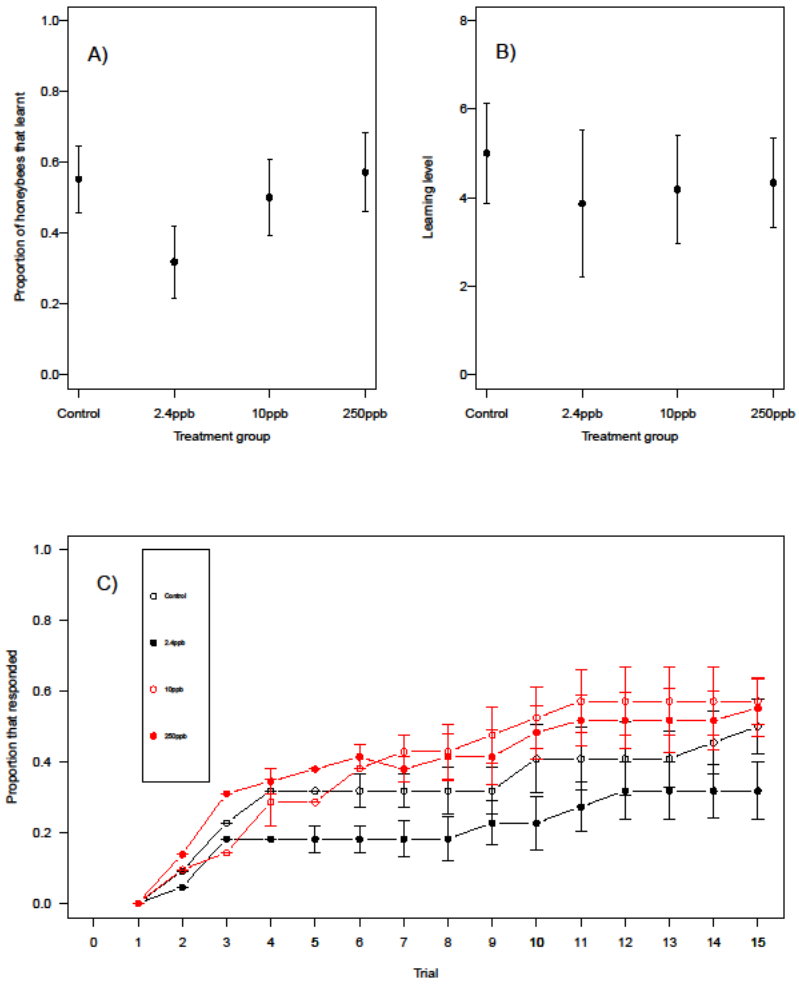

Figure 3. 2: The performance of honeybees in an olfactory learning task: (a) The proportion (± SEM) of honeybees that learnt the olfactory association (b) the learning level (± SEM) of the bees that did learn the association (b) and (c) the trials in which bees learnt the association (± SEM) in reference to trial number. (Control n = 29, 2.4ppb n = 22, 10ppb n = 22, 250ppb n = 21).

Similarly, there was no impact of sulfoxaflor exposure on either bumblebee or honeybee memory at 3 hours after training (bumblebee; Figure 3.3A; glmer, 2.4 ppb PE = 0.02, 95% CI = -0.59 to 0.63; 10 ppb PE = -0.07, 95% CI = -0.98 to 0.83; 250 ppb PE= 0.06, 95% CI = -0.62 to 0.75; honeybee; Figure 3.3C; *wi* (treatment) = 0.033) or at 24 hours after training (bumblebee; Figure 3.3B; *wi* (treatment) = 0.042; honeybee ; Figure 3.3D; glmer, 2.4 ppb PE = -0.39, 95% CI = -1.79 to 1.02; 10 ppb PE = -0.36, 95% CI = -1.66 to 0.94; 250 ppb PE = 0.04, 95% CI= -0.79 to 0.88).

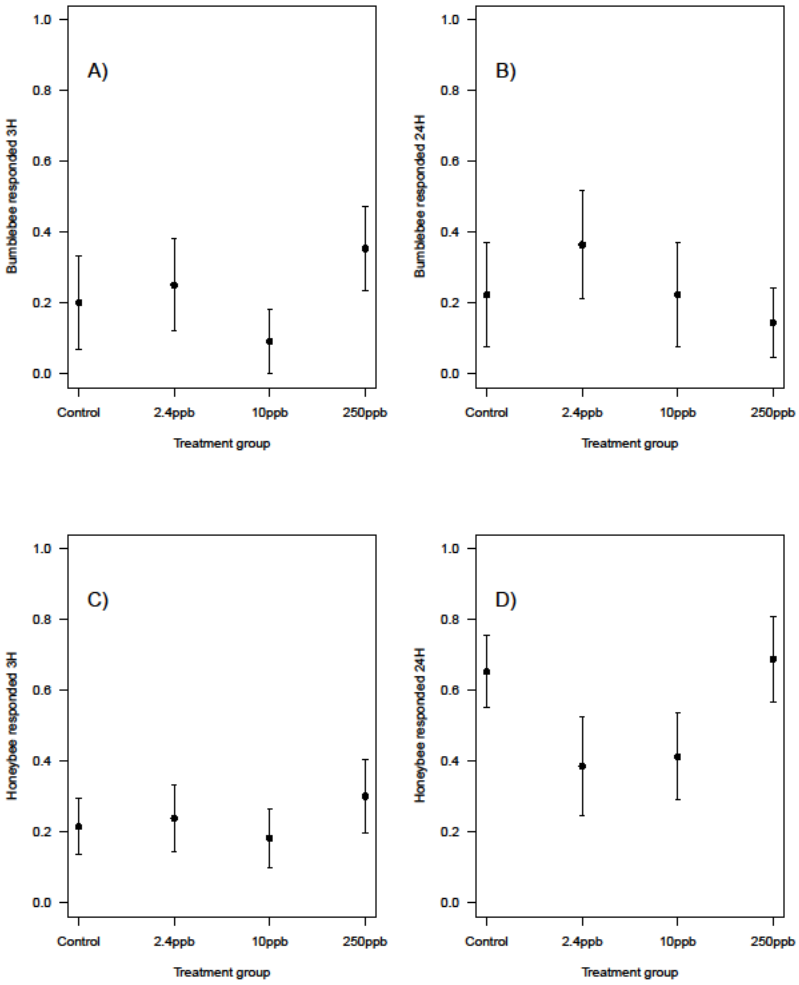

Figure 3. 3: Bumblebee and honeybee olfactory memory test: The proportion of bumblebees and honeybees (±SEM) responding to the conditioned stimuli 3 hours (A & B) and 24 hours (C & D) after training had finished. (Bumblebee 3H, Control n = 10, 2.4 ppb n = 12, 10 ppb n = 11, 250ppb n = 17; bumblebee 24H Control n = 9, 2.4 ppb n = 11, 10 ppb n = 9, 250ppb n =14; Honeybee 3H, Control n = 28, 2.4 ppb n = 21, 10 ppb n = 22, 250ppb n = 20; honeybee 24H Control n = 23, 2.4 ppb n = 13, 10 ppb n = 17, 250ppb n = 16).

Results: RAM – Experiment 2

We found no statistical support for an effect of sulfoxaflor exposure on total revisits (Figure 3.4A; glm.nb, 5ppb treatment PE = 0.24, 95% CI = -0.56 to 1.05; 10ppb PE = 0.16, 95% CI = -0.46 to 0.79; 250ppb PE = 0.23, 95% CI = -0.55 to 1) or on the proportion of correct choices in the first 8 visits of bumblebees following sulfoxaflor exposure (Figure 3.4B; glm, (*wi* (treatment) = 0.038). Similarly, we found no statistically significant effect of sulfoxaflor exposure on the number of correct choices before

the first revisit (Figure 3.5; coxme, 5ppb PE = 0.55, 95% CI = -0.54 to 1.64; 10ppb PE = 0.25, 95% CI = -0.48 to 0.98; 250ppb PE = 0.49, 95% CI = -0.52 to 1.51), suggesting no impact of acute sulfoxaflor exposure on bumblebee working memory. Further analysis also suggested no impact on bumblebee behaviour (see supplementary material).

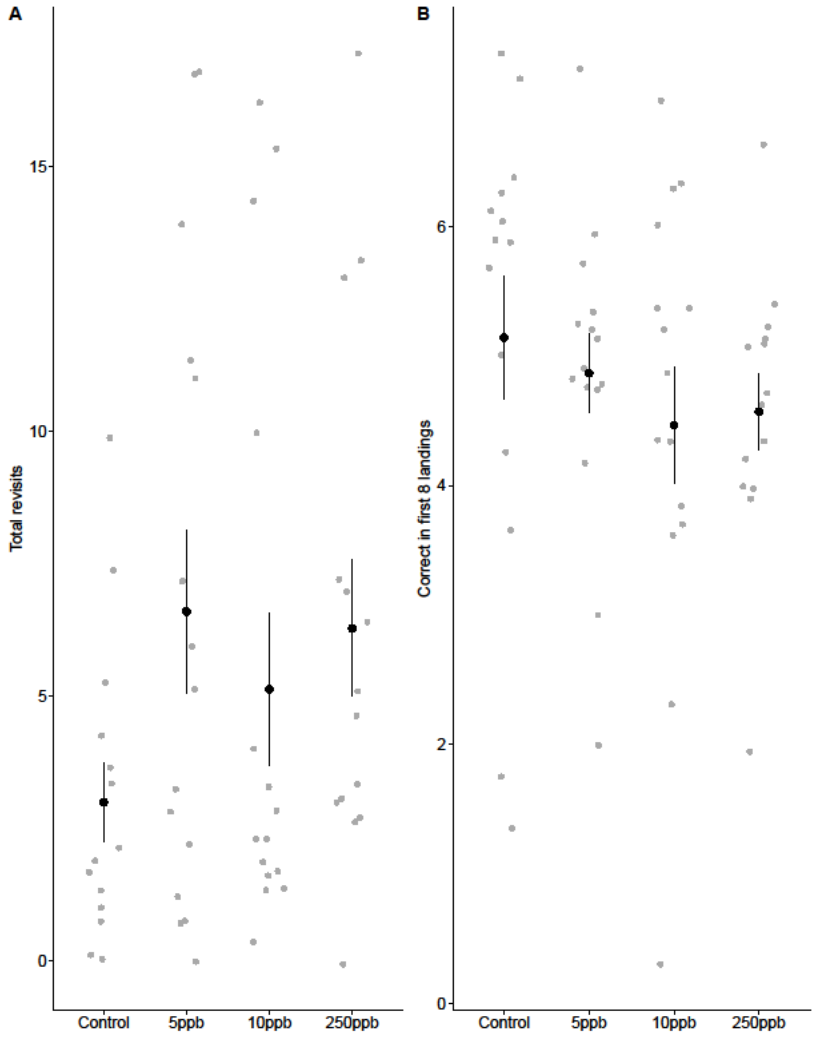

Figure 3. 4: Bumblebee performance on the radial arm maze: (a) the total number of revisits (± SE) to already depleted landing platforms and, (b) the mean number of correct landing (± SE) in the bees first 8 landings.

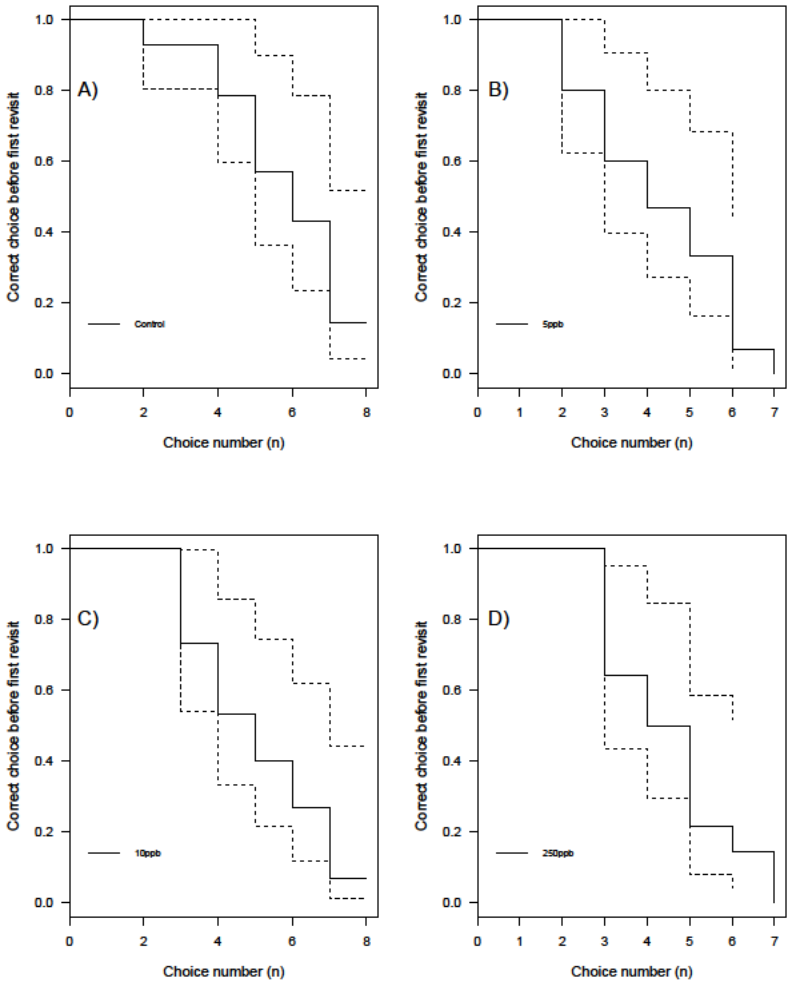

Figure 3. 5: Kaplan-Meier curves of bees visiting landing platforms until a revisit to an already depleted resource occurs. A = Control (n=14), B = 5ppb (n=15), C = 10ppb (n=15) and D = 250 ppb (n=14).

Discussion

We found no evidence to suggest that acute sulfoxaflor exposure influenced bumblebee or honeybee olfactory conditioning or bumblebee working memory, even at the highest concentrations of exposure tested (250ppb). Given the range of dosages we tested, which included positive controls that far exceeded levels likely to be found in the field, it is unlikely that acute sulfoxaflor exposure in adult bees will influence cognition after environmental exposure, at least with regard to olfactory conditioning and working memory performance.

We used two experimental paradigms to investigate the impact of acute sulfoxaflor exposure on bee learning and memory. Although a wide variety of different paradigms can be used to assess bee cognition (Bernadou *et al.* 2009; Zhang & Nieh 2015; Lämsä *et al.* 2018) we chose to use both PER and the RAM, in combination, as these paradigms allow us to consider the impact of sulfoxaflor exposure on working memory (also known as short-term memory), medium-term and long-term memory (Menzel 2012). Interestingly, in both of these paradigms, the neonicotinoid thiamethoxam, one of the three neonicotinoids insecticides banned from outdoor agricultural use within the European Union, has been shown to influence performance at comparable dosages (Stanley *et al.* 2015b; Samuelson *et al.* 2016). Both neonicotinoids and sulfoximine-based insecticide share the same mode of action, acting as selective agonists of Nicotinic Acetyl Choline Receptors (NAChRs) (Zhu *et al.* 2011; Sparks *et al.* 2013). Acute neonicotinoid exposure can inactivate the mushroom bodies of bee brains (Palmer *et al.* 2013), which are essential for learning and memory in bees (Menzel 2012). The effects of sulfoxaflor exposure on bee neurology have not been explored, but could provide useful information in understanding why neonicotinoids, but not sulfoximine-based insecticides, influence bee cognition, at least under these experimental paradigms and dosages. Ultimately, sulfoxaflor could be used as a reference substance to understand why some insecticides, which act on nicotinic acetyl choline receptors (NAChRs), have a negative impact on bee cognition, while others do not.

We tested the impact of acute sulfoxaflor exposure (rather than chronic exposure) on bee learning and memory. A recent meta-analysis showed that chronic insecticide exposure can have larger effects on bee memory than acute exposure for adult bees, and so we cannot rule out that more prolonged exposure would have identified an effect of sulfoxaflor exposure. However, an acute dosage potentially mimics the exposure regime of a foraging adult bee in the field more closely, because individuals may forage on a range of different crops and flowers in addition to the treated crop, over an extended period of time. Chronic exposure is nonetheless clearly relevant for larval brood, and the same meta-analysis highlighted that exposure as a larva is more likely to have a negative impact on bee learning than adult exposure (Siviter *et al.* 2018b). Larval exposure to thiamethoxam has been shown to influence synaptic density in the mushroom bodies of bee brains (Peng & Yang 2016) and increase neural vulnerability to mitochondrial dysfunction (Moffat *et al.* 2015), which may be linked to documented effects of exposure on cognitive function (Klein *et al.* 2017). Thus, although our results show no effect of acute sulfoxaflor exposure on bumblebee or honeybee cognition, further research needs to be conducted to understand the potential impact of chronic exposure, both in adults and larvae. Furthermore, given the dearth of data on non-*Apis*/*Bombus* bees (Siviter *et al.* 2018b), researchers should prioritise assessing the impact of sulfoxaflor on non-social bees.

The hypothesis that negative effects of neonicotinoid exposure on bees are mediated in part by the widely-documented sub-lethal effects on learning and memory described above, which may impact upon bee foraging behaviour and thus potentially colony productivity, has received much attention (Klein *et al.* 2017; Siviter *et al.* 2018b). However, neonicotinoid insecticides have many other sub-lethal effects on bee behaviour and physiology (Wu *et al.* 2012; Laycock *et al.* 2012; Baron *et al.* 2017b; a; Crall *et al.* 2018) and any causal link between reduced cognitive performance and foraging efficiency remains to be established, because data linking bee cognitive traits and foraging efficiency are difficult to collect. The evidence that does exist is contradictory. Raine & Chittka (2008) found a positive association between the nectar collection rate of workers allowed to forage outdoors, and the visual learning performance of their sisters from the same colony, but Evans, Smith & Raine (2017) found no correlation between individual visual learning performance and nectar collection rate. A better understanding of the relationship between bee cognitive traits and foraging efficiency is clearly important if we are to identify and mitigate against the sub-lethal impacts that underlie negative impacts of neonicotinoid insecticide exposure on bumblebee colony reproductive output (Whitehorn *et al.* 2012; Rundlöf *et al.* 2015; Woodcock *et al.* 2017). In contrast, our findings suggest that sub-lethal effects on learning and memory are unlikely to underlie the negative impacts of sulfoxaflor on colony reproductive output in bumblebees.

If memory and learning are unaffected by exposure, what other mechanisms might underlie the impact of sulfoxaflor on bumblebee colony fitness (Siviter et al. 2018a)? While previous work on impacts of neonicotinoids on learning and memory (Samuelson *et al.* 2016; Siviter *et al.* 2018b) inspired the work reported here, these insecticides have been demonstrated to produce a range of sublethal impacts, beyond cognitive effects. These include reductions in food intake, foraging motivation, thermoregulatory activity, nursing behaviour, ovary development, and egg laying (Laycock *et al.* 2012; Baron *et al.* 2017b; Lämsä *et al.* 2018; Crall *et al.* 2018). Impacts of sulfoxaflor on bumblebee colony fitness appear to be driven by reduced worker production at the early stage of colony development (Siviter et al. 2018a), but our results here suggest that this is unlikely to be due to impacts on worker learning or memory in food-related tasks. Consequently, we suggest that future work should focus on examining potential sub-lethal impacts on ovary development and egg laying, which could directly relate to reductions in worker production.

Conclusions

Sulfoximine-based insecticides are becoming globally important, with sulfoxaflor now registered for use in 81 countries, including a number of European Union member states (European Commission). Although mitigation measures can reduce the likelihood of pollinator exposure (Centner *et al.* 2018), uptake of such measures varies widely across legislative regimes. Previous work with neonicotinoids demonstrated the importance of understanding sub-lethal effects of insecticides on bee health. Here we find no evidence for an impact of acute sulfoxaflor exposure on bee olfactory conditioning or bumblebee working memory, despite the occurrence of such impacts when using the same protocols with neonicotinoid exposure. This suggests that the impacts of sub-lethal exposure in learning and memory are unlikely to be the mechanism behind impacts of sulfoxaflor on colony reproductive success (Siviter *et al.* 2018a). Further studies are needed to understand how, and under what conditions, sulfoxaflor may impact bee health. Such data will enable more informed regulatory and policy decisions on the future use of this insecticide in crops that attract bees.

Chapter 4: Quantifying the impact of pesticides on learning and memory in bees

Harry Siviter, Julia Koricheva, Mark J F Brown, Ellouise Leadbeater

Siviter, H., Koricheva, J., Brown, M.J.F. & Leadbeater, E. (2018) Quantifying the impact of pesticides on learning and memory in bees. *Journal of Applied Ecology*, **55**, 2812–2821.

Abstract

1) Most insecticides are insect neurotoxins. Evidence is emerging that sublethal doses of these neurotoxins are affecting the learning and memory of both wild and managed bee colonies, exacerbating the negative effects of pesticide exposure and reducing individual foraging efficiency.
2) Variation in methodologies and interpretation of results across studies has precluded the quantitative evaluation of these impacts that is needed to make recommendations for policy change. It is not clear whether robust effects occur under acute exposure regimes (often argued to be more field-realistic than the chronic regimes upon which many studies are based), for field-realistic dosages, and for pesticides other than neonicotinoids.
3) Here we use a meta-analyse to examine the impact of pesticides on bee performance in proboscis extension-based learning assays, the paradigm most commonly used to assess learning and memory in bees. We draw together 104 (learning) and 167 (memory) estimated effect sizes across a diverse range of studies.
4) We detected significant negative effects of pesticides on learning and memory (i) at field realistic dosages, (ii) under both chronic and acute application, and (iii) for both neonicotinoid and non-neonicotinoid pesticides groups.
5) We also expose key gaps in the literature that include a critical lack of studies on non-*Apis* bees, on larval exposure (potentially one of the major exposure routes), and on performance in alternative learning paradigms.
6) *Policy implications.* Procedures for the registration of new pesticides within EU member states now typically require assessment of risks to pollinators if potential target crops are attractive to bees. However, our results provide robust quantitative evidence for subtle, sublethal effects, the consequences of which are unlikely to be detected within small-scale pre-licensing laboratory or field trials, but can be critical when pesticides are used at a landscape scale. Our findings highlight the need for long-term post-licensing environmental safety monitoring as a requirement within licensing policy for plant protection products.

1. Introduction

A wealth of empirical evidence for global pollinator decline has driven unprecedented interest in the mechanisms by which anthropogenic changes influence both domestic honey bees (*Apis* spp.) and native wild bees (e.g. *Bombus* spp.; Aizen & Harder 2009; Potts *et al.* 2010; Goulson *et al.* 2015). Habitat loss, an increase in the prevalence of bee pathogens, the spread of invasive species, and climate change have all been implicated as potential drivers (Brown & Paxton 2009; Winfree *et al.* 2009; Potts *et al.* 2010b; Cameron *et al.* 2011; Kerr *et al.* 2015; Goulson *et al.* 2015; Woodcock *et al.* 2016). Recently, considerable attention has also been devoted to the contribution of agricultural pesticides, and particularly neonicotinoids, which are present in the nectar and pollen of treated crops and nearby wildflowers, and thus in colony food-stores (Simon-Delso *et al.* 2015; Mitchell *et al.* 2017).

There is strong evidence to associate pesticide use with bee population decline (Woodcock *et al.* 2016) and consequently with potential losses to pollination services and crop yields (Stanley *et al.* 2015a; Stanley & Raine 2016). At the colony level, pesticide exposure is associated with negative impacts on fitness-determining traits that include colony initiation, colony growth and reproductive output (Whitehorn *et al.* 2012; Baron, Raine & Brown 2014; Baron *et al.* 2017b; a; Rundlöf *et al.* 2015; Tsvetkov *et al.* 2017; Woodcock *et al.* 2017; Arce *et al.* 2017). The mechanisms that underlie these effects remain unclear, but pesticides have been shown to negatively impact key aspects of worker performance including foraging efficiency and navigation ability (Gill *et al.* 2012; Henry *et al.* 2012; Feltham *et al.* 2014; Gill & Raine 2014; Stanley *et al.* 2016). Models of colony growth predict that such small negative impacts on a limited cohort of workers can have severe negative consequences downstream in the colony cycle (Bryden *et al.* 2013).

Many insecticides are neurotoxins that alter synaptic function within the insect central nervous system (Goulson *et al.* 2015). For example, neonicotinoids and sulfoximines bind to nicotinic acetylcholine receptors (NAChRs), disrupting cholinergic transmission, which can lead to neural cells failing to develop or being inactivated (Palmer *et al.* 2013; Peng & Yang 2016), while fipronil (a phenylpyrazole) inhibits GABA signalling (El Hassani *et al.* 2009; Moffat *et al.* 2015, 2016) and can increase neural cell death (Boitard *et al.* 2015). The mushroom bodies are a neural region specifically associated with olfactory learning and memory in bees (Hourcade *et al.* 2010; Devaud *et al.* 2015), and there is now strong evidence that mushroom body development and function can be directly impaired through chronic or acute exposure to NAChR agonists, respectively (Palmer *et al.* 2013; Peng & Yang 2016). The potential consequences for learning and memory are of concern because cognitive abilities are integral to bee foraging. Bees are one of the few taxonomic groups in which there is empirical evidence that directly links cognitive abilities with foraging efficiency, a fitness-determining trait (Raine &

Chittka 2008). The nectar and pollen rewards offered by floral resources change over time (Heinrich 2004), and individuals must not only remember which flower species are currently rewarding, but also their location, how to handle different flower types, which inflorescences have just been visited, and where the nest is located (Chittka & Thomson 2001; Gegear & Laverty 2001; Heinrich 2004). Consequently, numerous studies have set out to examine the effects of pesticides on cognitive traits (Klein *et al.* 2017).

Narrative reviews have highlighted the challenge of drawing general conclusions about pesticide impacts on bees (Godfray *et al.* 2014, 2015; Goulson *et al.* 2015; Wood & Goulson 2017). This is largely due to considerable variation in methodologies. Pesticide dosage, for instance, varies across experiments, as does the definition of a field-realistic dose (Godfray *et al.* 2014). Studies also follow alternative exposure-regime strategies in attempts to mimic field realistic scenarios. While foraging bees may be acutely exposed through consumption during one foraging bout, chronic exposure may occur through repeated foraging on a large pesticide-treated food source that flowers over a prolonged period, such as oil seed rape, and may be extended by the presence of pesticides within honey and pollen stores (Mitchell *et al.* 2017). Impacts might also vary across bee genera. For instance, some evidence now suggests that pesticides could differentially affect honey bees (*Apis*) and bumblebees (*Bombus*), with honey bees appearing to be more vulnerable to pesticides in relation to their cognitive abilities than bumblebees under some circumstances (Piiroinen & Goulson 2016). Finally, effects of pesticides on bee cognition may vary across classes of pesticides, reflecting different modes of action (Klein *et al.* 2017). Such variation is important as certain neonicotinoids (imidacloprid, clothianidin and thiamethoxam) are now under a total ban in the EU with respect to agricultural use outside of permanent greenhouse structures (to be implemented by December 2018)(European Commission 2018a), which is likely to create market demand for other pesticides as replacements (Campbell 2013; Brown *et al.* 2016).

Sub-lethal effects are more difficult to detect than direct effects on pollinator mortality in small-scale laboratory and field trials, but may have critical impacts on pollinator health at the landscape scale. There is thus an urgent need to synthesize the literature assessing sub-lethal effects in order to provide robust evidence-based conclusions for policy makers. Here, we quantitatively explore the evidence for sub-lethal effects of pesticides on bee cognition through meta-analysis. This enables us to measure the magnitude of the effects of pesticides on bee learning and memory, to explore the sources of heterogeneity underlying these effects (Koricheva, Gurevitch & Mengersen 2013), and to identify evidence gaps in the current literature. Specifically, our analysis aimed to answer five questions:

1) Do pesticides negatively affect the learning ability and memory of bees?
2) Do field realistic dosages of pesticides significantly affect bee learning and memory?
3) Do chronic and acute exposure differentially affect learning and memory?
4) Are honey bees and bumblebees differentially affected by pesticides?
5) Do neonicotinoids affect bee learning and memory more than other pesticides?

2. Materials and Methods

2.1) Scope and search strategy

We focused upon olfactory learning and memory, which are typically assessed in bees through an olfactory proboscis extension reflex paradigm (hereafter PER). During a PER experiment, a harnessed bee learns to associate a previously unrewarded scent with sucrose. Bees initially exhibit proboscis extension as an unconditioned response (UR) to antennal contact with sucrose (the unconditioned stimulus; US). When this contact is paired with a scent (the conditioned stimulus; CS), the bee learns to extend its proboscis in response to the scent alone (a conditioned response; CR). Typically, PER-based experiments that relate to pesticides use an absolute conditioning paradigm (where bees learn to associate only one scent with sucrose) rather than differential conditioning (where one scent is rewarded and an alternative is not; Stanley, Smith & Raine 2015b). Although other paradigms to test learning and memory (e.g. free-flying association, spatial learning, aversive learning, or tactile learning (Bernadou *et al.* 2009; Tan *et al.* 2014; Zhang & Nieh 2015; Samuelson *et al.* 2016)) are available and widely used in the cognitive literature, only a very small number of studies have used such methods to assay how pesticides influence performance (see Discussion; Bernadou *et al.* 2009; Zhang & Nieh 2015; Samuelson *et al.* 2016). In contrast, the PER paradigm is the most commonly used methodology to assess bee learning and memory and thus provides an obvious target for our study.

We used Web of Science and Google Scholar as search databases (search performed in April 2018). The search criteria used in Web of Science were ("pesticide*" OR "insecticide*" OR "neonicotinoid*") AND ("bumblebee*" OR "bumble bee*" OR "honey bee*" OR "honeybee*" OR "bee*" OR "*apis*" OR "*bombus*") AND ("learning" OR "memory" OR "PER" OR "cognition" OR "proboscis extension reflex" OR "proboscis extension response"). After the Web of Science search we used the same key words in Google Scholar and checked the first 200 results, which yielded 3 additional papers (Figure S4.1). Twenty-three papers remained eligible after title and abstract screening, and applying inclusion criteria (see below and Table S4.1). All 23 papers had their reference lists examined and we did not find any additional data.

2.2) Inclusion criteria, data extraction, and final database

To be included in the meta-analysis, a study had to involve oral exposure of bees to a pesticide followed by an assay of learning and/or memory via a PER conditioning paradigm. Studies were excluded if they did not contain a control group (no pesticide exposure) or if we were unable to extract the means, the standard deviations and the sample sizes for both the control and the treatment groups. Some raw data were available online (N = 3), but in most cases (N = 17) the means and standard deviations could be extracted from graphs using WebPlotDigitizer (https://automeris.io/WebPlotDigitizer/). In cases where information was not available, some authors were successfully contacted (N = 3). We excluded experimental groups where the bees had been exposed to multiple stressors (for example, both parasites and pesticides), as we could not be sure which stressor was potentially causing an effect. In all studies included in the analysis, bees were tested either directly or 24 hours after pesticide exposure. We excluded one study where the post-exposure testing period varied (with delays of up to 11 months; table S4.1). After sensitivity analysis (see below) the 23 papers included in the final database (see Table S4.1) yielded 104 effect sizes for the influence of pesticides on learning ability from 23 papers and 167 effect sizes from 19 papers for the influence of pesticides on memory. These studies were published between 2009 and 2017.

PER experiments use varying criteria to assess learning performance, including the number of trials in which the bee responded to the CS, the first trial in which it responded, or mean performance in a specified batch of trials. For example, Stanley *et al*. (2015b) used 15 learning trials (trials in which the UR and the CS are paired) per condition, while Piiroinen *et al*. (2016) tested their bees over 10 trials. To enable direct comparison, we redefined learning across studies as the proportion of bees that responded positively to the CS by the final learning trial (inter-trial interval; mean = 8.17 ± 5.6). Similarly, we collated memory data (the number of bees responding to the CS) from all reported time lengths (range: 10 minutes – 48 hours) into two categories that approximate short- and long-term memory (see below). Note that these timings reflect neurologically distinct processes in bees, the transition from short- to long-term memory being translation-dependent (reviewed in Menzel 2012).

2.3) Potential moderators

Moderators are used in meta-analysis to investigate the sources of variation in effect sizes between studies (Koricheva *et al.* 2013). Our meta-analysis included the following as potential moderators of the size of the effect that pesticide exposure had on learning and memory: pesticide exposure regime (chronic or acute), dosage (field realistic or above), pesticide type (neonicotinoid or other) and genus (*Apis* or *Bombus*). For the memory data, we also included short (<24 hours) and long-term (≥24 hours) memory retention as a potential moderator (see below for full models). The treatment was considered

acute when the bees were exposed to one dosage of pesticide and chronic when the bees were repeatedly exposed over a sustained period of time, which varied between experiments from 4 days (Yang *et al.* 2012; Williamson & Wright 2013) to 24 days (Stanley *et al.* 2015b).

The definition of a field-realistic dose is highly contentious and the toxicity of different pesticides varies. To standardise this, we categorized dosages as field-realistic or above based on pesticide concentrations in nectar, pollen, honey, and bee-bread extracted from (Glaberman & White 2014; Sanchez-Bayo & Goka 2014; Bonmatin *et al.* 2015). Where more than one estimate was available for a given pesticide we took the mean value (see Table S4.2 for individual pesticides). For the acute dosages, the nectar pesticide concentration data were further combined with the mean amount of nectar that bees are able to ingest in one foraging bout (40 ng for honey bees; 37.7ng for bumblebees; Table S4.3) to calculate the field realistic dose (Cresswell 2011; Samuelson *et al.* 2016). Dosages higher than the above thresholds were considered not field realistic.

2.4) Meta-analysis

All analyses were conducted in R (version 1.0.136) using the package *metafor* (Viechtbauer 2010). Data for learning and memory were analysed separately. We used standardized mean difference in bee learning ability or memory between the control groups and the treatment groups (Hedges' d) as a measure of effect size (calculated using escalc function in *metafor*). For both data sets, we fitted random effects models to calculate the grand mean effect as well as the group means (e.g. effects of acute *vs* chronic exposure). The restricted maximum likelihood approach (REML) was used to estimate the parameters of the meta-analysis models. For each of the two datasets, meta-regression was then used to explore the sources of variation in effect sizes by including all the moderators (see above) within a single model. Pesticide type was not included in these models because a subset of studies simultaneously exposed bees to more than one pesticide (Williamson & Wright 2013; Williamson *et al.* 2013), which would have led to these studies being dropped from the analyses (for full list of pesticides in meta-analysis see Table S4.2). Consequently, we analysed pesticide-type in a sub-model that excluded these studies. 'Study' was included as a random factor in all the models to control for potential non-independence of multiple effect sizes from the same study.

We initially included in the analysis results from studies where bees were exposed to pesticides as larvae. However, there were very few of these (three studies for learning data and two studies for the memory data) and we found that the overall effect of pesticides on bee learning when these studies were included in the overall analysis was much stronger (d = -0.60, 95% CI = -0.90 to -0.30) while the overall effect of pesticides on bee memory was similar (d = -0.24, 95% CI = -0.28 to -0.20) compared

to the effects based on the analysis when larval data were excluded from the analysis (see Results section for comparison). Thus, to preclude bias, we removed these studies from subsequent analyses. Furthermore, given the small number of studies conducted on bumblebees compared to honey bees, we conducted sensitivity analysis with studies that used honey bees only (see Figure S4.2). Within this analysis we also compared the impact of pesticides between the European (*A. mellifera)* and the Asian honey bee (*A. cerana*) (see, Figure S4.2). We also re-ran the overall analysis without studies that used multiple pesticides (learning n = 2 and memory n = 2) and the results did not change (see supplementary material). We tested whether the number of learning trials undergone by the bees influenced the results and found no significant effect (p = 0.15) and thus we did not include this factor in the overall model. To test for any potential publication bias, a trim-and -fill technique was used on both the learning and memory data Duval & Tweedie (2000).

3. Results

Overall, pesticide exposure had a significant negative effect on both learning score (d= -0.28, 95% CI = -0.36 to -0.20; Figure 4.1A) and memory (d = -0.24, 95% CI = -0.28 to -0.20; Figure 4.1B). The proportion of between-study heterogeneity for the learning data was high (I^2 = 75.61%) but lower for the memory data (I^2 = 31.51%). When mean effects were recalculated after adjusting for a possible publication bias with a trim-and-fill technique, the effect size estimates did not change for the learning results (d = -0.28, 95% CI = -0.36 to -0.20; Figure S4.3) and also showed no bias for the memory data (d = -0.28, 95% CI = -0.32 to -0.24; Figure S4.4).

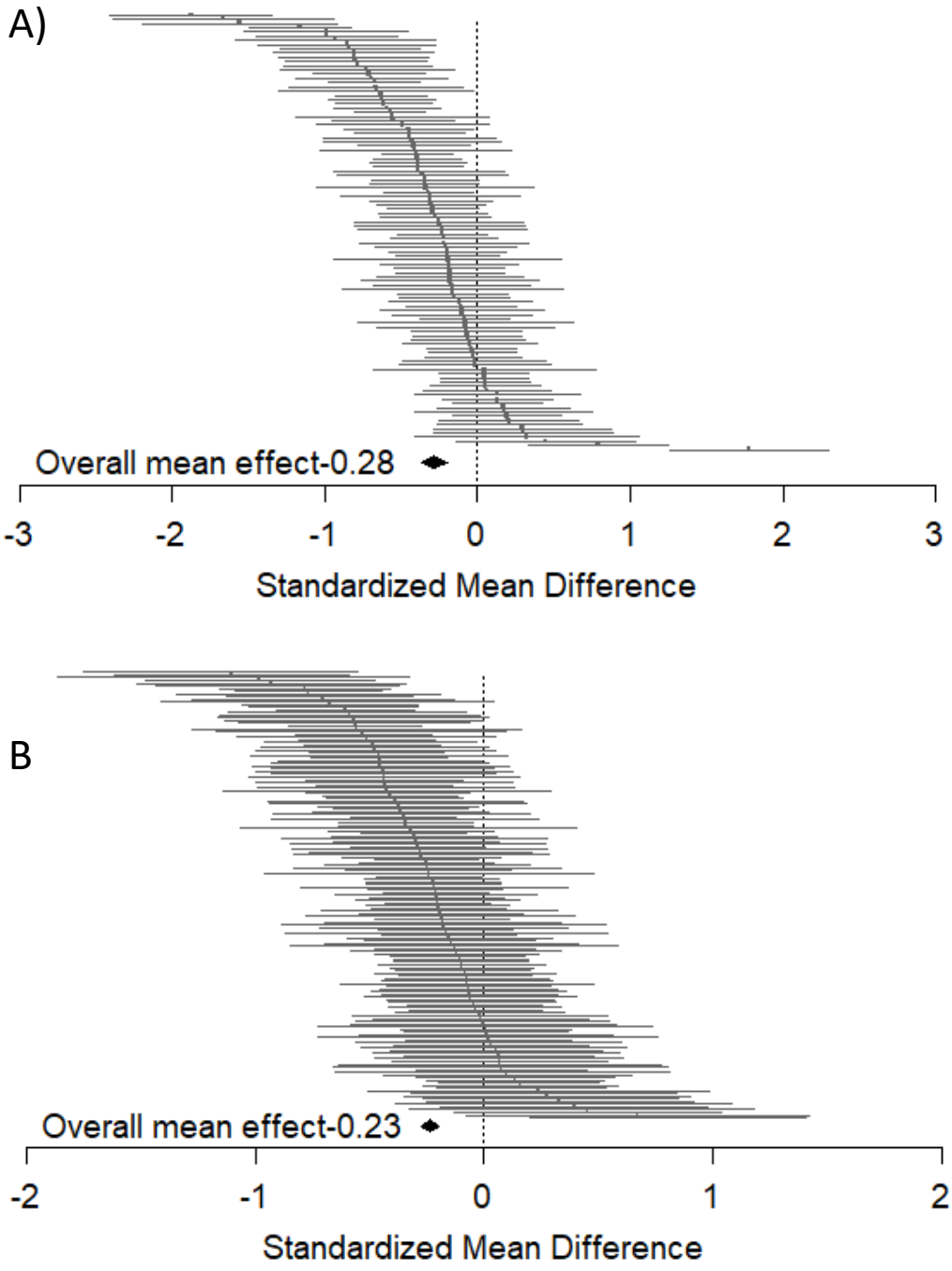

Figure 4. 1: Hedges' d values ± 95% confidence intervals for effects of pesticides on (a) learning ability (b) memory.

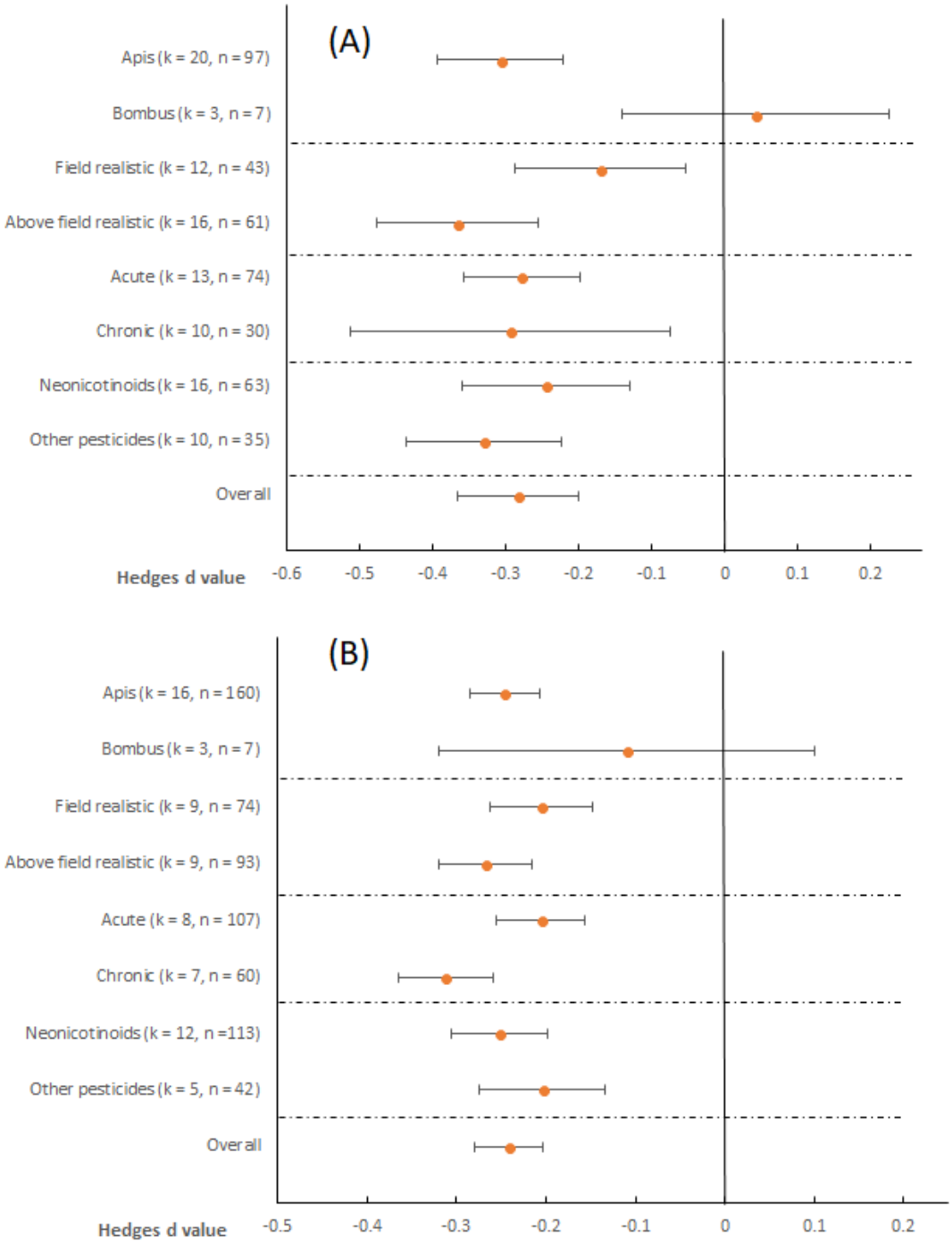

Figure 4. 2: Mean effect size estimates (± 95% confidence intervals) for subsets of the data on the effects of pesticides on (a) learning and (b) memory. Number of studies (k) and number of effect sizes (n) are given for each subgroup

While both field realistic and higher doses of pesticide had significant negative effects on learning and on memory, as expected, effects were significantly larger at higher doses ($p < 0.05$ in both cases; Figures 4.2A & 4.2B). While both chronic and acute pesticide exposure had significant negative effects on learning score (Figure 4.2A), there was no significant difference between their effects ($p = 0.08$). In contrast, chronic exposure had a significantly stronger negative impact than acute exposure on memory ($p < 0.05$, Figure 4.2B). We also found that learning scores of honey bees were more negatively affected by pesticides than those of bumblebees ($p < 0.05$), but these results need to be interpreted with caution given that the majority of studies focused on honey bees. In contrast, while the same trend was present for the effects of pesticides on memory, there was no significant difference between bee species ($p > 0.05$). We found no difference between the effects of neonicotinoids and other pesticides on learning score ($p = 0.29$) or on memory ($p = 0.14$). Finally, there were no differences between effects of pesticides on long-term (24 hours and longer) and short-term (less than 24 hours) memory retention ($p = 0.47$).

4. Discussion

Our findings draw together a body of evidence to produce quantitative estimates of the magnitude of pesticide effects on bee learning and memory, across a range of dosage regimes and pesticide treatments. Importantly, our results confirm that pesticide exposure has a significant negative impact on bee learning and memory at field-realistic doses. Chronic pesticide exposure had a stronger effect on bee memory than acute exposure, although the same effect was not found in relation to learning score. Despite their different modes of action, there were no detectable differences between neonicotinoids and other insecticides in their impacts on learning and memory.

Narrative reviews of pesticide impacts on bees have struggled to draw general conclusions, highlighting the need for a meta-analytical approach (Godfray *et al.* 2014, 2015; Goulson *et al.* 2015; Wood & Goulson 2017). This tool is particularly valuable when studies show a range of significant and non-significant effects. Meta-analytic assessments of the effects of pesticides on bee biology are currently limited to an analysis of the LD50 paradigm (Arena & Sgolastra 2014), or a focus on individual pesticides and a specific species (Cresswell 2011), while one recent meta-analysis showed that neonicotinoids have a negative impact on performance of beneficial arthropods (Main *et al.* 2018). The current study provides a significant step forward in our understanding of pesticide impacts on learning and memory, and as such makes progress towards resolving a number of issues in this field.

Firstly, pesticide research has been criticised on the basis that experimental dosages are not field-realistic (Campbell 2013; Carreck & Ratnieks 2014; Godfray *et al.* 2014, 2015). Here we systematically re-classified studies based on up-to-date estimates of field-realistic exposure and found significant negative impacts of field-realistic pesticide doses on learning and memory. Secondly, it has been suggested that chronic pesticide exposure is unrealistic, because wild flowers offer an alternative to pesticide treated crops (Godfray *et al.* 2014, 2015; Garbuzov *et al.* 2015). Here we have shown that even short-term (acute) exposure during one foraging bout can significantly impair learning and memory in bees. Chronic exposure had a stronger effect than acute exposure for the memory dataset, potentially because bodily pesticide residues from acute doses may be more likely to have been metabolized before the memory trial than chronic doses, but both chronic and acute doses significantly impaired both learning and memory. Chronic pesticide exposure is increasingly likely to occur in the field as water-soluble systemic pesticides have been found to occur in wild flowers on field margins (Botias *et al.* 2015), and in flowers sold in garden centres (Lentola *et al.* 2017), while pesticide products are freely available for gardeners to purchase, and bees preferentially feed on sucrose solutions that have been treated with pesticides (Kessler *et al.* 2015). Our results draw together a body of evidence that in combination suggests the rising prevalence of pesticides in the environment (Mitchell *et al.* 2017) is increasingly likely to influence the cognitive abilities of bees.

The studies used in the analysis assayed the effects of pesticides on learning and memory in adult bees. Pesticides are regularly found in the honey and pollen stores of honey bees, with a recent global study finding neonicotinoids in 75% of all honey samples (Mitchell *et al.* 2017). Consequently, bee larvae are likely to be exposed to pesticides while developing. Such larvae can take longer to develop, and adult bees show reduced longevity (Wu *et al.* 2011). Prior to the removal of larval-exposure experiments, our results showed a stronger effect of pesticides on bee learning, making our current estimates conservative. This suggests that bees could be more sensitive to pesticide exposure when exposed as larvae. Given that the impacts of larval exposure are relatively unexplored (Yang *et al.* 2012; Tan *et al.* 2015, 2017; Peng & Yang 2016), future research should test whether exposure of bee larvae to field realistic levels of pesticides has a stronger effect on the cognitive abilities of bees than exposure of adults, which could subsequently lead to stronger sub-lethal effects in the field (Klein *et al.* 2017).

Our systematic search highlighted a knowledge gap that results from a heavy focus on *Apis*, with a dearth of studies on bumblebees and other wild bees. We found no evidence for an effect of pesticide exposure on bumblebee cognition, but the small dataset available for *Bombus* lacks power, and should be interpreted with caution. There is evidence to suggest that feeding rates drop following pesticide

exposure in *Bombus* but not *Apis* (Cresswell *et al.* 2014) which could lead to reduced exposure for *Bombus* over the longer term in chronic experiments. However, the same study found that metabolic breakdown of pesticides was quicker in *Apis* than *Bombus*, with bumblebees maintaining much higher bodily residues than honeybees that were fed the same dose (Cresswell *et al.* 2014). It is also possible that robust differences exist in target-site sensitivity, as have been reported in other insects (Lind *et al.* 1998; Liu *et al.* 2005), but such effects are yet to be investigated in *Bombus* and *Apis.* It is too early to draw conclusions about species differences in the impact of pesticides on bee cognitive abilities, and this knowledge gap is important given that wild bee flower visits can enhance the fruit set of crops regardless of the presence of honey bees (Garibaldi *et al.* 2013), and are thought to offer an important buffer in the case of a domesticated honey bee collapse (Greenleaf & Kremen 2006). Research on non-*Apis* species, such as bumblebees (including species other than *Bombus terrestris*) and solitary bees, is sorely needed, and the development of non PER-based paradigms for testing the effects of pesticides on cognition is welcome in this respect (Tan *et al.* 2014; Samuelson *et al.* 2016).

The results also provide no support for differential effects of neonicotinoids and other pesticides on bee learning and memory. Neonicotinoids have been a particularly controversial pesticide group because they are typically applied as a seed treatment, resulting in contamination of the pollen and nectar of exposed plants, which are then consumed by bees (Bonmatin *et al.* 2015). Despite restrictions on their use within Europe, neonicotinoids are the most widely used type of insecticide worldwide (Simon-Delso *et al.* 2015), which has driven an abundance of pesticide research focussing on their use. Currently, however, there is not enough available data on other, non-neonicotinoid pesticide groups (pyrethroids, phosphorothioates, etc.) to make more specific comparisons between effects of neonicotinoids and other classes of neurotoxins. One possible consequence of the European moratorium, and now the total ban of certain neonicotinoids, is the creation of a gap in the market for alternative products to achieve the same effect (Campbell 2013; Klatt, Rundlöf & Smith 2016). Thus, in order for policy makers to make conclusive comparisons between neonicotinoids and other pesticides, future research should focus on generating more data on how other pesticides, including novel pesticides such as sulfoximines (Brown *et al.* 2016), influence bee cognition.

One limiting factor in the literature to date is that almost all the available data collected so far has derived from a PER paradigm. This paradigm is extraordinarily useful in providing a sensitive means to exclude confounding variables and experimental noise, but several alternative methodologies are available that potentially mimic an ecologically realistic scenario more closely (e.g., Samuelson *et al.* 2016) as they involve free-flying bees foraging for the colony. Such paradigms may lend themselves more fruitfully to non-*Apis* species than is the case for PER. Furthermore, pesticide exposure has been

shown to influence olfactory processing (Andrione *et al.* 2016) suggesting that exploration of alternative visual and/or spatial modalities will be critical if researchers are specifically interested in how pesticides influence bee learning and memory at the level of neural processing, rather than stimulus perception. Initial exploration of these methodologies has provided evidence for cognitive effects of pesticides outside of olfactory paradigms, and should be further explored (Samuelson *et al.* 2016).

A final, and important, knowledge gap that remains is quantification of the link between worker cognitive performance and fitness. Detecting long-term colony-level consequences of sublethal stress on pollinators is time- and resource-intensive. In contrast, PER is quick, repeatable, widely used and accessible on a large scale. As such, it could provide a valuable addition to current LD50 methodologies to test effects of pesticides on bees (OECD 2017). However, linking cognitive traits with fitness measures, such as foraging success, is a major outstanding challenge in the literature (Rowe & Healy 2014), because it is difficult to control for confounding variables when assaying cognition in a natural environment. However, as central-place foragers, bee colonies lend themselves to laboratory-based cognitive testing followed by fitness assays in the wild. Using this type of methodology, bumblebee colony foraging intake has been shown to increase with the proportion of fast learning-workers (Raine & Chittka 2008), although more recent research failed to find the same relationship at an individual level (Evans *et al.* 2017). Conversely, there is evidence to suggest that bees that are poor learners come across novel resources more frequently, potentially increasing foraging performance (Burns 2005; Evans & Raine 2014). The relationship between investment in cognitive performance and colony foraging success is likely to be multifaceted, and is a clear avenue for further exploration.

5. Conclusions

Current interest in the effects of pesticides on pollinators is based upon the need to understand the nature of negative effects in order that they can be reduced via policy change. To this end, the results of this meta-analysis provide the evidence that pesticides have a significant negative influence on the learning and memory of bees at field realistic exposure levels, confirming that classical ecotoxicological tests are failing to assess the sub-lethal consequences of pesticide exposure. Our results also highlight evidence gaps that should be addressed in order to move forward. Future research needs to focus on (1) testing how larval pesticide exposure influences cognition, (2) understanding how pesticides influence non-*Apis* bee species, and (3) generating data on how potential replacements for neonicotinoid pesticides influence bee cognition.

This study demonstrates that meta-analyses can be used to quantify how pesticides influence bee biology, an approach that could ultimately aid in pollinator conservation. In recognition of the fact

that pesticide exposure poses potential risks to pollinators, plant protection product licensing protocols often require evidence of risk assessment to be included with application dossiers. While these policies may promote detection of direct mortality risks, they are unlikely to uncover subtle sub-lethal effects (such as those demonstrated here) that may have major environmental consequences when pesticides are applied at the landscape scale post-licensing. Our findings thus highlight the need for policies promoting post-licensing environmental safety monitoring for plant protection products, mirroring that which is in place for pharmaceutical products and food safety (Milner & Boyd 2017).

Chapter 5: Sulfoxaflor exposure reduces egg laying in bumblebees (*Bombus terrestris*)

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Siviter, H., Horner J., Brown, M.J.F. & Leadbeater, E. (Accepted) Sulfoxaflor exposure reduces egg laying in bumblebees (*Bombus terrestris*) *Journal of Applied Ecology*

Abstract

1. Sulfoximine-based insecticides, such as sulfoxaflor, are of increasing global importance and have been registered for use in 81 countries, offering a potential alternative to neonicotinoid insecticides.
2. Previous studies have demonstrated that sulfoxaflor exposure can have a negative impact on the reproductive output of bumblebee colonies, but the specific life-history variables that underlie these effects remain unknown.
3. Here, we used a microcolony-based protocol to assess the sub-lethal effects of chronic sulfoxaflor exposure on egg laying, larval production, ovary development, sucrose consumption, and mortality in bumblebees. Following a pre-registered design, we exposed colonies to sucrose solutions containing 0, 5, 10 and 250ppb of sulfoxaflor. Exposure at 5ppb has been previously shown to negatively impact colony reproductive success.
4. Our results showed that sulfoxaflor exposure at 5ppb (lowest exposure tested) reduced the number of eggs found within the microcolonies (Hedge's d = -0.37), with exposed microcolonies also less likely to produce larvae (Hedge's d = -0.36). Despite this, we found no effect of sulfoxaflor exposure on ovarian development. Sulfoxaflor-exposed bumblebees consumed less sucrose solution, potentially driving the observed reduction in egg laying.
5. *Policy direction*: Regulatory bodies such as the European Food Safety Authority (EFSA) are under increasing pressure to consider the potential impact of insecticides on wild bees, such as bumblebees, but sublethal effects can go undetected at lower-tier testing. In identifying just such an effect for bumblebees exposed to sulfoxaflor, this study highlights that microcolony-based protocols are a useful tool that could be implemented within an ecotoxicology framework. Furthermore, the results provide evidence for potentially negative consequences of pollinator exposure to an insecticide that is currently undergoing the licensing process in several EU member states.

Introduction

Neonicotinoids are the most commonly used insecticides worldwide (Simon-Delso *et al.* 2015), but evidence demonstrating their negative sub-lethal impacts on important pollinators, such as bees (Rundlöf *et al.* 2015; Tsvetkov *et al.* 2017; Woodcock *et al.* 2017; Siviter *et al.* 2018b), has resulted in legislative re-assessment globally. Most noticeably, within the European Union, 3 commonly used neonicotinoids (thiamethoxam, imidacloprid, and clothianidin) are now banned from agricultural use outside of commercial greenhouses. In contrast to neonicotinoids, sulfoxaflor, the first branded sulfoximine-based insecticide, is an increasingly important insecticide product that is now registered for use in 81 countries, offering an alternative to neonicotinoid-based insecticides (Brown *et al.* 2016). However, the legislative re-assessment of neonicotinoid-based insecticides was driven by research that demonstrated the potential sub-lethal consequences of neonicotinoid exposure on pollinators (European Commission 2018b). The regulatory process by which novel agrochemicals are licensed for use is changing in Europe and North America, but in its current form, is largely reliant on tier-based toxicity tests that can fail to detect sub-lethal effects at lower tiers. Therefore, despite sulfoximine-based insecticides and neonicotinoids having a similar biological mode of action, as selective agonists of Nicotinic Acetyl Choline Receptors (NAChRs) (Zhu *et al.* 2011; Sparks *et al.* 2013), we still have a limited understanding of the potential sub-lethal effects of sulfoxaflor on bee colonies.

Siviter *et al.* (2018a) recently demonstrated that chronic exposure to sulfoxaflor at a concentration of 5ppb had negative consequences for the worker production and reproductive output of bumblebee (*Bombus terrestris audax*) colonies. Colony level impacts of neonicotinoid exposure on bees are thought to be driven in part by impaired bee foraging behaviour and cognition (Gill *et al.* 2012; Feltham *et al.* 2014; Stanley *et al.* 2015b; Samuelson *et al.* 2016; Klein *et al.* 2017; Lämsä *et al.* 2018; Siviter *et al.* 2018b; Muth & Leonard 2019). Interestingly, in both Siviter *et al.* (2018a), and a follow-up study (Siviter *et al.* 2019), no significant effect of sulfoxaflor exposure on either bumblebee foraging behaviour or cognition was observed, and consequently, the mechanism behind the sub-lethal colony-level effects of sulfoxaflor remains unknown. An alternative explanation, again based on previous work with neonicotinoids (Laycock *et al.* 2012; Baron *et al.* 2017b; a), is that exposure to sulfoxaflor early in the colony life cycle could reduce egg laying, or impair larval development, with downstream consequences for reproductive output (Siviter *et al.* 2018a).

Neonicotinoid insecticides can negatively influence bumblebee ovary development and fecundity (Laycock *et al.* 2012; Laycock & Cresswell 2013; Baron *et al.* 2017b; a). For example, Laycock *et al.* (2012) demonstrated that queenless microcolonies exposed to field realistic concentrations of imidacloprid had a one third reduction in the total amount of brood produced. These effects occurred

in the absence of impacts on ovary development at field realistic concentrations, with the reduced reproductive output instead most likely mediated through lower feeding rates in exposed microcolonies. Baron *et al.* (2017b) showed that exposure to field realistic concentrations of thiamethoxam reduced the average length of terminal oocytes in the ovaries of queens in 4 wild bumblebee species, (*Bombus lucorum, B pascuorum*, *B. pratorum, B. terrestris*), with knock-on consequences for colony initiation and egg laying (Baron *et al.* 2017b; a).

Bumblebee workers are able to produce male offspring if the queen dies or is deposed, and will show signs of ovarian development approximately 7 days after being removed from the colony (Alaux *et al.* 2007; Amsalem *et al.* 2009). We manipulated this reproductive plasticity and, following Laycock *et al.* (2012), created queenless microcolonies that were subsequently exposed to varying dosages of sulfoxaflor within sucrose. We monitored the sucrose consumption, mortality and egg laying of bumblebee workers (*Bombus terrestris*) chronically exposed to sulfoxaflor over a 14-day period. After the 14 days of exposure, we recorded the number of eggs/larvae produced by each microcolony and dissected and measured the ovaries of each surviving worker. Based on Siviter *et al.* (2018a) we hypothesised that sulfoxaflor exposure may have a negative impact on bumblebee ovarian development, with knock on effects for egg laying, and larval development.

Methods

Insecticide exposure

Sulfoxaflor-based insecticides have been developed for a range of different crops and are most commonly used as a spray application. The residue levels of systemic insecticides vary from crop to crop (Bonmatin *et al.* 2015; Kyriakopoulou *et al.* 2017), but despite sulfoxaflor being licenced for use in 81 countries, there is still a limited understanding of the likely post-spray residue levels that are to be expected in the nectar and pollen of sulfoxaflor treated crops. We based our dosages on Environmental Protection Agency (EPA) data that showed that the residue levels of sulfoxaflor range between 5.41- 46.97ppb in the nectar of sulfoxaflor sprayed cotton across an eleven day period (United States Environmental Protection Agency 2016; application rate: 0.045 pounds (0.020 kg) of active ingredient per acre applied twice). It is worth noting that in the same EPA study, pollen residue levels were higher than nectar levels (ranging between 50.12 – 510.95ppb) (United States Environmental Protection Agency 2016) and that while spraying bee-attractive crops during flowering is prohibited in Europe and North America (Dow AgroSciences Ireland; Dow AgroSciences USA 2018), this is not the case globally (Dow AgroSciences Australia Limited 2018; Dow AgroSciences New Zealand 2018; Dow AgroSciences South Africa 2018). Our sulfoxaflor treatments were derived from a stock solution of 1 g dm^{-3} sulfoxaflor (Greyhound Chromatography and Allied Chemicals) in acetone, which

was combined with sucrose solution (50°Brix) to make four treatment groups: 5 µg dm^{-3} (5ppb), 10 µg dm^{-3} (10ppb) & 250 µg dm^{-3}(250ppb; positive control). These were compared to a control solution containing just acetone 0 µg dm^{-3} (0ppb).

Microcolonies

Seven bumblebee colonies of approximately 100 workers were ordered (Biobest, Westerlo, Belgium) and, upon arrival, 5 workers from each colony were collected and screened for common bee parasites through faecal examination (*Apicystis bombi, Crithidia* spp., and *Nosema* spp) (Rutrecht & Brown 2009). All colonies were unparasitized and workers were returned to the colony. From these original colonies, we created 120 queenless microcolonies by randomly placing groups of four workers from the same queen-right colony in small Perspex boxes (67 x 127 x 50mm; Allied Plastics). The age of individual workers was not known, although there was no difference in the size of workers between different treatment groups (mean size of workers; Control = 5.24 ± 0.34mm, 5ppb = 5.21 ± 0.42mm, 10ppb = 5.21 ± 0.42mm, 250ppb = 5.23 ± 0.40). Each microcolony contained a gravity feeder with an *ad libitum* supply of untreated sucrose solution (50° Brix). Microcolonies were kept in darkness at 26°C and 50-60% humidity and then left overnight (workers that died overnight were replaced with workers from the same original colony; N = 4).

Insecticide exposure began the following day, when the untreated sucrose solution was replaced with weighed sucrose solution (50° Brix) containing either 0, 5, 10 or 250ppb sulfoxaflor according to the randomly assigned treatment group. Workers that died after exposure began were not replaced. Sucrose remaining in the feeder was measured daily, when the bees were first fed and on the following day, to get a recording of daily feeding (OHAUS advanced portable balance scout STX) by a researcher who was blind to treatment. Pollen balls (1.66 g ± SD 0.14) were added to the microcolonies on days 1, 4, 8 & 11. Following Siviter *et al.* (2018a), pollen balls were only replaced if eggs had not been laid; in cases when eggs had been laid, more pollen was added. Mortality and egg laying were recorded daily via visual inspection. Seven boxes containing just sucrose and no bees were also included as evaporation controls. The experiment ran for a total of 15 days (1-day pre-exposure and 14 days of exposure). The total sample size of 120 microcolonies initially contained a total of 480 bees, with 30 microcolonies in each treatment group.

Fecundity and ovary development

At the end of the experiment individual bees were frozen at minus 20°C for later dissection. Pollen balls from the nests were also frozen and examined for the presence of eggs and larvae. Pollen balls that contained brood were dissected, and the number of eggs and larvae counted, with a reference photo taken for each microcolony. Bees were dissected in distilled water to remove their ovaries,

using a Nikon (SM2800) dissecting microscope. Bumblebee workers each have two ovaries, containing four ovarioles, with each ovariole containing several oocytes. Following Brown *et al.* (2000), Laycock *et al.* (2012) and Baron *et al.* (2017b), we (i) recorded the presence or absence of developed ovarioles and (ii) used an ocular graticule to measure the length of each intact terminal oocyte. The mean of all intact oocytes per bee (mean oocyte size per bee), and the largest oocyte length (maximum per bee) were used as our measure of ovarian development/investment. We successfully dissected and examined the ovaries of 373 bees (control = 102: 5ppb = 110: 10ppb = 105: 250ppb = 56). Thorax width was also recorded using digital callipers (Mitutoyo).

Statistical analysis

The statistical analysis described below was pre-registered prior to the experiment, as was the experimental design (https://aspredicted.org/vw63q.pdf). Any deviations from the pre-registration document are noted in the text below.

We followed an information theoretic model selection approach. For each analysis we considered a full model, other models containing the same random factors (specified below) and all subsets of the fixed factors, and a null model containing just the random factors and the intercept. Parameter estimates and confidence intervals are based on model averaging across the 95% confidence set (i.e. the smallest set of models for which the cumulative Akaike weight (w_i) was equal to or greater than 0.95, where models are added to the set-in decreasing order of w_i).

We used a hurdle model to analyse both the number of eggs and larvae (analysed separately) produced per microcolony, with treatment included as a fixed factor and colony of origin included as a random factor. Hurdle models handle excess zeros by incorporating two processes: a binomial process that models the occurrence of zero *vs* non-zero values, and a truncated count process that only fits positive (i.e. non-zero) counts. The estimates thus provide two types of information: a) whether there is variation across treatments in the likelihood of producing eggs/larvae at all (termed zero-count process; note that a positive parameter estimate here implies that a zero count is more likely) b) if eggs/larvae are produced, whether there is variation in the number produced (termed positive-count process).

A mixed effects Cox proportional hazards model was used to analyse latency to lay eggs, with treatment included as a fixed factor and original colony and microcolony included as random factors.

Our results (see below) showed that multiple bees within each microcolony showed signs of ovarian development, with no obvious dominant individual in most cases, and we therefore conducted our analysis on all bees. Ovarian development (whether a bee had developed ovaries or not) was analysed

using a generalized linear mixed effects model (binomial error distribution, link function = "logit")), with treatment and bee size included as fixed factors, and microcolony nested within colony of origin as a random factor (*n.b.* in our pre-registered document, we stated that we intended to consider the interaction between treatment and bee size, but when included, the model failed to converge). The mean and maximum oocyte length per bee were analysed using linear mixed effect models (Poisson error distributions) with treatment, thorax width, and their interaction included as fixed factors and original colony and microcolony included as random factors. As only 7 workers from the positive control had developed ovaries, this treatment was excluded from this analysis.

A linear mixed effects model was also used to analyse sucrose consumption per worker ([sucrose consumed per microcolony – evaporation control] / number of workers in microcolony), which also included treatment, day and their interaction as fixed factors, and microcolony (nested within colony of origin) as random factors. In our pre-registration document, we failed to consider the possibility of spillage, but spillages did occur during the experiment. Thus, we removed all data points where spillages had been recorded by the experimenter (n= 64; control n=12, 5ppb n=15, 10ppb n=15, 250ppb n=21) or where apparently negative consumption occurred, implying spillage of the evaporation control (n=19). This left a final sample size of 1416 for this section of the analysis.

Our analysis included one additional deviation from our pre-registration document. We observed higher mortality during the experiment than envisaged and thus chose to additionally analyse individual survival data (time-to-death). We used a mixed effects Cox proportional hazards model, with treatment included as a fixed factor, and original colony and microcolony included as random factors. Throughout the analyses we used the packages Hmisc, lme4, coxme, MuMIn, ggplot2, glmmTMB (Wickham 2009; Bates *et al.* 2015; Barton 2016; Brooks *et al.* 2017; Harrell & Dupont 2018; Therneau 2018).

Results

Sulfoxaflor exposure did not significantly influence the binary likelihood of microcolonies producing eggs at 5 & 10ppb, although there was an effect at 250ppb, (Figure 5.1A, hurdle (zero-count output), 5ppb parameter estimate relative to negative control (PE) = 0.89, 95% confidence intervals (CI) = -0.45 to 2.23; 10ppb PE = 0.47, 95% CI = -0.88 to 1.84, 250ppb PE = 4.87, 95% CI = 2.81 to 6.93). However, we found that in the microcolonies that produced eggs, sulfoxaflor exposure reduced the total number of eggs laid at 5 & 250ppb, although there was no significant difference at 10ppb (Figure 5.2A, same hurdle model (positive-count output), 5ppb parameter estimate relative to negative control (PE) = -0.16, 95% CI = -0.31 to -0.01; 10ppb PE = -0.10, 95% CI = -0.24 to 0.04, 250ppb PE = -1.30, 95% CI = -1.91 to -0.70; note however that there was no evidence for an increase between 5ppb & 10ppb; PE

(5 vs 10ppb) = 0.06, 95% CI = -0.09 to 0.21). In other words, for colonies that produced eggs, the number produced was lower than the control for 5ppb and 250ppb, and at 250ppb, more colonies also failed to produce eggs at all.

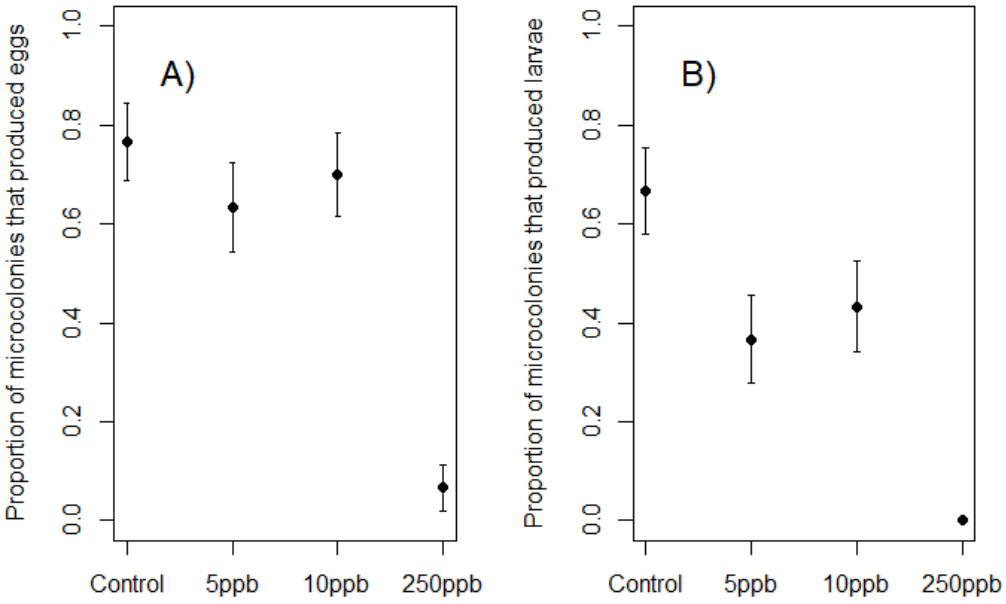


Figure 5. 1: The mean (± SD) number of microcolonies that produced eggs (A) or larvae (B). Data analysed in the zero part of the hurdle model (see statistical analysis)

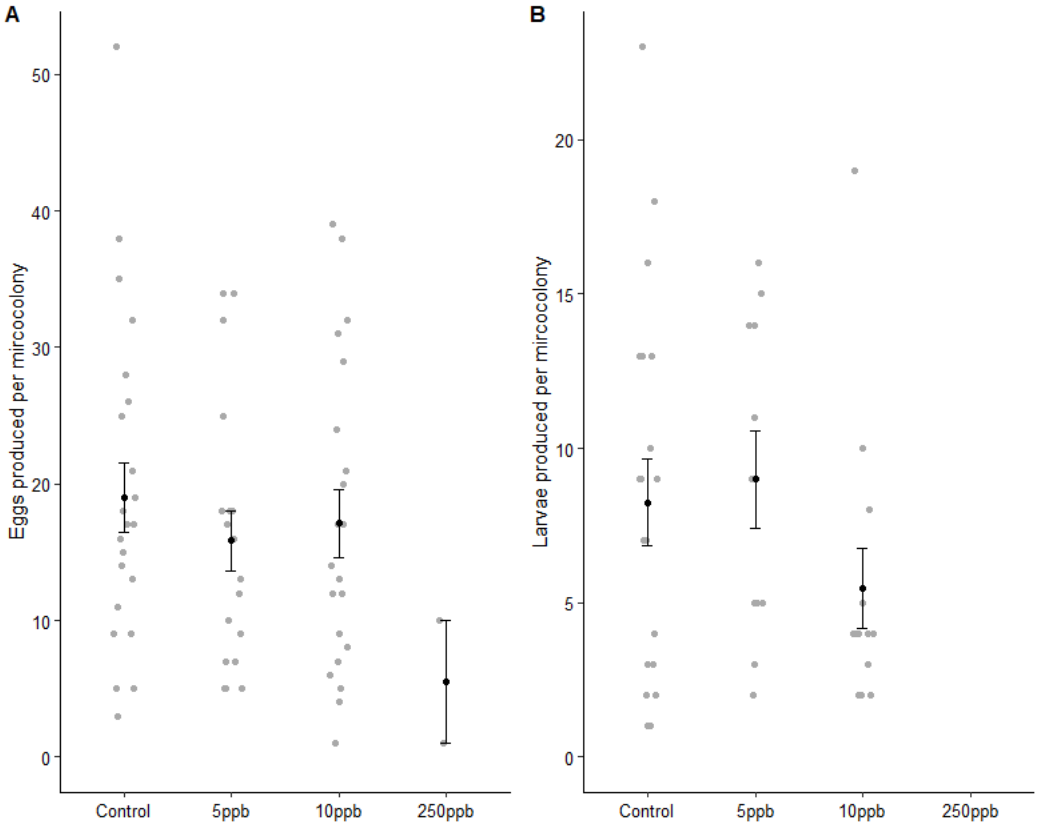


Figure 5. 2: The proportion (± SE) of eggs (A) and larvae (B) produced per microcolony that contained eggs. Data analysed in the count part of the hurdle model (see statistical analysis)

We found a similar but more dose-dependent pattern for the presence of developing larvae (Figure 5.1B). In this case, sulfoxaflor-exposed microcolonies were less likely than the control to contain any developing larvae at all at 5ppb, but not at 10ppb (Figure 5.1B, hurdle (zero-count output), 5ppb, parameter estimate (PE) = 1.24, 95% CI = 0.16 to 2.32; 10ppb, PE = 0.96, 95% CI = -0.10 to 2.02; 250ppb produced no larvae at all and were excluded from the analysis to aid model fit). However, at 10ppb, those colonies that did produce larvae produced significantly fewer than the control, which was not the case at 5ppb (Figure 5.2B, hurdle (positive-count output), 5ppb, parameter estimate (PE) = 1.24, 95% CI = 0.16 to 2.32; 10ppb, PE = 0.96, 95% CI = -0.10 to 2.02).

The latency to lay eggs (time to when a microcolony first laid eggs) did not differ between control groups and 5 & 10ppb treatment groups (Figure 5.3, coxme, 5ppb parameter estimate (PE) = -0.41, 95% confidence intervals (CI) = -1.02 to 0.19; 10ppb, PE = -0.45, 95% CI = -1.04 to 0.14; 250ppb, PE = -4.40, 95% CI = -6.42 to 2.38), suggesting that the speed at which ovaries developed, and eggs laid, did not drive the observed differences in fecundity.

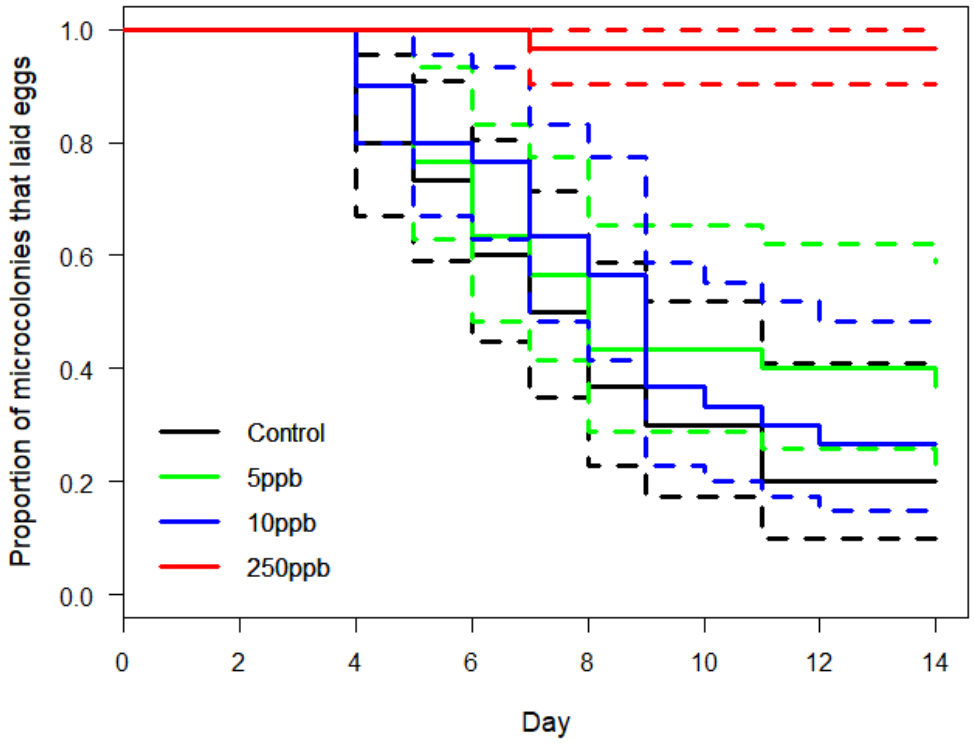


Figure 5. 3: The latency with which microcolonies first laid eggs.

Of the 373 bees we dissected, 254 had developed ovaries (Control group: 83/102 (81%) developed: measured; 5ppb: 88/110 (80%); 10ppb: 76/105 (72%); 250ppb: 7/56 (12%)) with only the positive control differing significantly from the control group (Figure S5.1, glmer, binomial distribution, 5ppb, PE = 0.15, 95% CI = -0.72 to 1.02; 10ppb, PE = -0.42, 95% CI = -1.25 to 0.42; 250ppb, PE = -4.74, 95% CI = -6.13 to -3.34). Furthermore, we found no significant effect of sulfoxaflor exposure on mean or maximum oocyte size per bee (mean oocyte size per bee; Figure 5.4, lmer, 5ppb parameter estimate (PE) = -0.73, 95% CI = -1.81 to 0.36; 10ppb, PE = -0.42, 95% CI = -1.73 to 0.89; maximum oocyte size per bee; Figure S5.2, Table S5.2; lmer, 5ppb parameter estimate (PE) = -0.46, 95% CI = -1.29 to 0.37; 10ppb, PE = -0.31, 95% CI = -1.44 to 0.82). Despite no overall effect of sulfoxaflor exposure on mean oocyte size per bee at the 95% confidence level, the model containing both treatment and bee size was strongly supported (*wi* (treatment+ bee size) = 0.847) with the null model, and models containing just treatment and bee size in isolation receiving no support (*wi* (treatment) = 0.00), (*wi* (bee size) = 0.00)), (*wi* (null model) = 0.00), (Table S5.1).

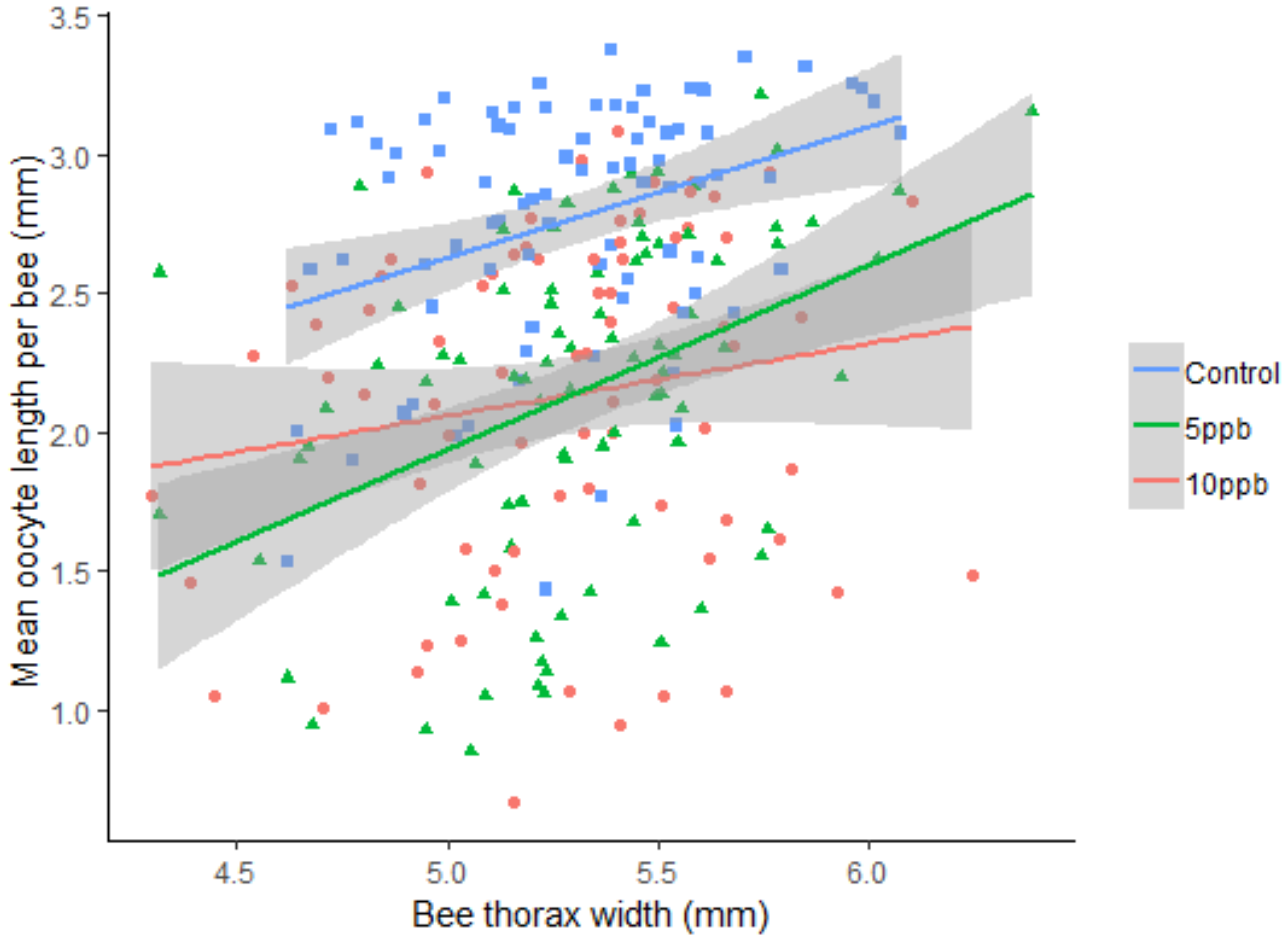


Figure 5. 4: The mean oocyte length per bee plotted against bee thorax width

Another potential driver of lower fecundity is a reduction in feeding, as a consequence of insecticide exposure (Laycock *et al.* 2012). Sulfoxaflor-exposed microcolonies had reduced sucrose consumption per bee at 5 and 250ppb, but not significantly so at 10ppb (Figure 5.5, lmer, 5ppb, PE = -0.09, 95% CI = -0.17 to -0.02; 10ppb PE = -0.06, 95% CI = -0.13 to 0.02; 250ppb, PE = -0.23, 95% CI = -0.31 to -0.16).

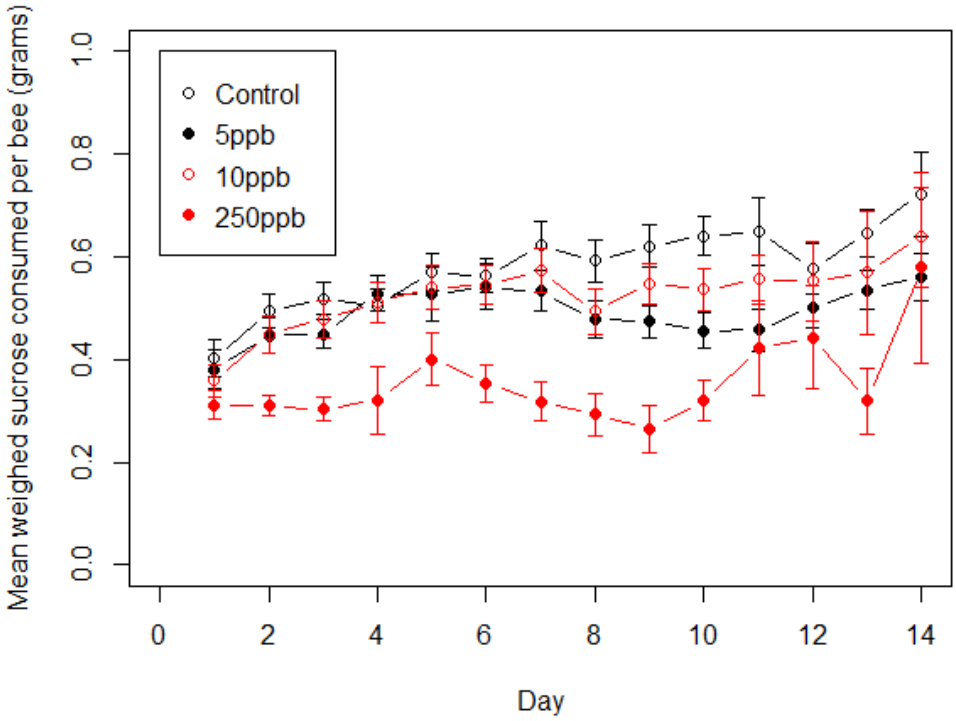


Figure 5. 5: The mean (± SE) amount of sucrose consumed (grams) per bee.

We further found no effect of sulfoxaflor exposure on worker survival at 5 or 10ppb (Figure S5.3, coxme, 5ppb parameter estimate (PE) = -0.46, 95% confidence intervals (CI) = -1.44 to 0.52; 10ppb PE = 0.13, 95% CI = -0.75 to 1.01) but microcolonies exposed to 250ppb had fewer workers surviving throughout the experiment (Figure S5.3, coxme, 250ppb parameter estimate (PE) = 2.07, 95% confidence intervals (CI) = 1.35 to 2.79).

Discussion

Our results showed that sulfoxaflor exposure can negatively impact bumblebee egg laying, at least for workers in a queenless environment, with subsequent consequences for the number of larvae found within microcolonies. Sulfoxaflor exposure also resulted in a reduction in sucrose consumption per bee, which could be a possible driver of the observed differences in egg laying. Ultimately our results confirm that sulfoxaflor exposure at the levels we tested could be hazardous to bumblebees and suggest that reduced egg laying is a possible mechanism driving previously described effects of sulfoxaflor exposure on bumblebee colony reproductive output (Siviter *et al.* 2018a).

We previously found that sulfoxaflor exposure early in the colony life cycle influences the reproductive output of bumblebee colonies, although the mechanism that drove these effects remained unknown

as we found no impact of sulfoxaflor exposure on bee foraging, and, in subsequent experiments, bee cognitive performance (Siviter *et al.* 2018a; Siviter *et al*. in press). Here we show that sulfoxaflor exposure at 5ppb and 250ppb can influence the egg laying of worker bumblebees, although we found no evidence at the 95% confidence level to support the same effect at 10ppb. It is not clear whether the lack of effect at 10ppb reflects true biological differences or statistical power. Nevertheless, the sub-lethal consequences of insecticides are not always dose-dependent (Samuelson *et al.* 2016), and so further research would be necessary to establish the true shape of this relationship. Ultimately, our study provides evidence for an effect of sulfoxaflor exposure on egg laying at 5ppb. If similar effects to those observed in this experiment occur when queen bumblebees are exposed to sulfoxaflor, this has the potential to drive previously observed differences in colony reproductive output (Siviter *et al.* 2018a).

Despite these effects on egg laying, we found no detectable effect of sulfoxaflor exposure at 5 & 10ppb on both the likelihood of bees showing evidence of ovarian development, nor on terminal oocyte size. Interestingly, Laycock *et al.* (2012) also demonstrated that imidacloprid exposure, despite not influencing ovarian development, reduced brood production, with the authors suggesting that the reduction in fecundity was driven by reduced feeding rates in exposed colonies. In insects, both carbohydrate intake (sucrose) and protein intake (pollen) are essential for brood development (Murphy, Launer & Ehrlich 1983; Boggs 1997; Laycock *et al.* 2012; Rotheray, Osborne & Goulson 2017). In our experiment, egg laying in the pollen balls provided occurred in the first week of the experiment, so we were unable to determine whether sulfoxaflor exposure influenced pollen consumption. Our results did however show that sulfoxaflor-exposed microcolonies consumed less sucrose than controls, suggesting that lower nutritional intake here could be a potential driver for the observed differences in egg laying.

Sulfoxaflor exposed microcolonies contained fewer larvae than control colonies. Impacts were evident at 5ppb on the likelihood of producing larvae, and at 10ppb on the number of larvae. During the experiment we observed no evidence of dead or discarded larvae in any of the microcolonies, suggesting that reduced egg laying resulted in lower larval numbers, although we cannot rule out that sulfoxaflor exposure led to fewer eggs hatching. Sulfoxaflor exposure, however, could still influence larval growth & development and there are two alternative influencing factors that have yet to be explored: (i) the potential impact of sulfoxaflor exposure on brood care and (ii) the direct impact of sulfoxaflor consumption on bumblebee larvae. In a recent experiment, Crall *et al.* (2018) demonstrated that bumblebee colonies exposed to the neonicotinoid imidacloprid have reduced nursing behaviour and thermoregulation, which could potentially impact larval development.

Furthermore the nectar and pollen stores of both honeybees and bumblebees frequently contain numerous agrochemicals (Wu *et al.* 2012; Mitchell *et al.* 2017; Nicholls *et al.* 2018) and yet, despite evidence in honeybees suggesting that insecticide exposure can influence larval development (Wu *et al.* 2012), no studies to date have investigated the direct impact of insecticide exposure on bumblebee larval development. Bumblebee larvae have blind guts, and do not excrete waste material until pupation begins (Chapman 1998), and thus while acute exposure might not have direct impacts on larval mortality or growth, chronic exposure over a prolonged period of time could result in bioaccumulation of insecticides, which could potentially influence larval mortality, development and emergence. Future research should focus on understanding the relationship between insecticide exposure and bumblebee larval development.

Bumblebee workers develop their ovaries when the founding queen is absent (Alaux *et al.* 2007; Amsalem *et al.* 2009) and microcolony based designs are therefore not a direct reflection of a healthy bumblebee colony. Typically, in cases when the queen is absent, one worker will dominate reproduction, and become a pseudo-queen (Blacquière *et al.* 2012). However, in contrast to our expectations, we found no evidence that one worker dominated, as several bees within each microcolony developed their ovaries. Without behavioural observations, or relating egg laying to individual workers, we cannot be sure whether one worker dominated the microcolonies or not. The microcolony dynamics, and in-turn egg laying are likely to be sensitive to the number of workers present (Larrere & Couillaud 1993; Babendreier *et al.* 2008; Mommaerts *et al.* 2010; Laycock *et al.* 2012). Having more workers within microcolonies could potentially increase egg laying, and reproduction, although it could also lead to greater competition (Reeve & Keller 2001) and this would be less representative of a healthy colony. For microcolony-based studies to become a ring-tested methodology, the number of workers housed within microcolonies needs to be standardised. However, whilst reproduction in microcolonies obviously differs from bumblebee colony reproduction, our results demonstrate that they are a useful proxy for understanding the potential sub-lethal impacts of agrochemicals on bumblebees (Laycock *et al.* 2012).

Insecticide residue levels vary widely between exposure regimes, crops and application rates (Bonmatin *et al.* 2015) and currently there is a dearth of data on the likely residue levels of sulfoxaflor found in the nectar and pollen of treated crops, particularly at field realistic application rates. The best available contemporary residue data is largely based on post-spray applications, applied during flowering (Xu *et al.*; United States Environmental Protection Agency 2016; Cheng *et al.* 2018), which is prohibited in Europe and North America, dramatically reducing the residue levels that bees are likely to encounter (Centner *et al.* 2018). Spraying flowering crops is however not prohibited in many other

geographical areas across the globe (Dow AgroSciences Australia Limited 2018; Dow AgroSciences New Zealand 2018; Dow AgroSciences South Africa 2018), and additionally, even pre- or post-bloom spraying could lead to direct spray of non-target plants if their flowering period does not coincide with the target crop (e.g. wildflowers/weeds; particularly in orchard strips) (EFSA 2013). Results in this experiment showed that chronic sulfoxaflor exposure can negatively influence the egg laying of bumblebees, confirming that sulfoxaflor can be hazardous to bumblebees. Future studies however should focus on understanding the potential risk that sulfoxaflor exposure poses and focus on generating sulfoxaflor residue data from a range of crops at field realistic application rates. A robust understanding of the residue levels of sulfoxaflor in various crops will allow regulators and policy-makers to offer clear advice on mitigation (Centner *et al.* 2018) and legislation that can reduce the risk of sulfoxaflor on pollinators.

Regulators are increasingly considering the potential impact of insecticides on wild bees such as bumblebees and solitary bees (Gradish *et al.* 2019; Sgolastra *et al.* 2019). Large scale field experiments (e.g., Rundlöf *et al.* 2015; Woodcock *et al.* 2017) are a means to detect sub-lethal effects of insecticide exposure on non-target organisms and are vital for understanding the wider implications of pesticide use on wild pollinators, but they are often expensive and difficult to standardise across countries (Woodcock *et al.* 2017). When licencing insecticides for use, regulatory bodies such as EFSA use a tier-based system (EFSA 2013), whereby lower-tiered studies that assess the direct mortality consequences of insecticide exposure are conducted to determine whether higher-tier field-realistic testing is needed. Tier 1 currently consist of LD50 & LC50 experiments that determine toxicity over 96 hours on honeybees, but these experiments do not consider (i) the consequences of chronic exposure, (ii) the potential impact on non-*Apis* bees and (iii) the potential sub-lethal consequences of insecticide exposure (Sanchez-Bayo & Tennekes 2017; Gradish *et al.* 2019).

Our results here, along with others (Laycock *et al.* 2012), demonstrate that microcolony-based studies can be used to assay the potential sub-lethal impacts of chronic insecticide exposure on bumblebees. Given that bumblebees are potentially more vulnerable to insecticide exposure than honeybees (Rundlöf *et al.* 2015; Gradish *et al.* 2019) it is vital that they (and other wild bees (Sgolastra *et al.* 2019)) are represented in the regulatory process. We therefore recommend that regulatory bodies and policy-makers consider using and developing microcolony-based experiments for *Bombus* as standard within an ecotoxicology framework, alongside other ring-tested methodologies. Failure to design and implement experiments that consider the sub-lethal impacts of novel insecticides on bumblebees, and other wild pollinators, will results in insecticides being licenced for use without a true understanding of the potential hazard that they could have.

Chapter 6: Sulfoxaflor and *Nosema bombi* exposure impair growth of early-stage bumblebee (*Bombus terrestris*) larvae

Harry Siviter, Arran J Folly, Mark J F Brown & Ellouise Leadbeater

Siviter, H., Folly, A.J., Brown, M.J.F. & Leadbeater, E. (*In prep*) Sulfoxaflor exposure reduces egg laying in bumblebees (*Bombus terrestris*) Planned submission to *Proceedings B*

Abstract

Beneficial insects, such as bees, face a plethora of anthropogenic and environmental stressors. Sulfoxaflor is an important insecticide globally, despite having negative impacts on bumblebee (*Bombus terrestris*) reproductive output. The life-history mechanism driving these effects remains unclear. One hypothesis is that sulfoxaflor exposure early in the colony's life cycle can impair bumblebee larval development, which reduces the number of workers produced, and ultimately lowers colony reproductive output. However, insecticide exposure is not the only stressor bumblebee colonies are faced with, and here we assess how both sulfoxaflor and the fungal parasite *Nosema bombi* influence bumblebee larval mortality and development when tested in insolation and combination. In experiments 1 & 2 we found that both sulfoxaflor and *N. bombi* can negatively influence bumblebee larval growth but found contradictory results between experiments. We therefore repeated these experiments (experiments 3 & 4) with a greater level of colony level replication, and confirmed that both sulfoxaflor and *N. bombi* impair larval growth. We found no synergistic interactions between sulfoxaflor and *N. bombi* but did find evidence of antagonistic interactions. Ultimately, our results show that sulfoxaflor exposure can impair bumblebee larval growth, which could be a potential mechanism driving colony level consequences of sulfoxaflor exposure on bumblebees. As sulfoxaflor is licenced for use globally, our results highlight the need to understand how novel insecticides impact beneficial insects at various stages of their development.

Introduction

Bees are vital for the pollination of both agricultural crops and wild flowers (Rader *et al.* 2016), so declines in bee numbers, both local and global, are a cause for concern (Biesmeijer *et al.* 2006; Aizen & Harder 2009; Cameron *et al.* 2011). Bee populations face many anthropogenic and environmental challenges, including the intensification of agriculture, habitat loss, agrochemical exposure, climate change, and pathogens (Brown & Paxton 2009; Winfree *et al.* 2009; Cameron *et al.* 2011; Kerr *et al.* 2015; Goulson *et al.* 2015; Samuelson *et al.* 2018). Neonicotinoids are the most commonly used insecticides around the world (Simon-Delso *et al.* 2015) but a plethora of research demonstrating negative impacts on bees (Gill *et al.* 2012; Henry *et al.* 2012; Whitehorn *et al.* 2012; Gill & Raine 2014; Rundlöf *et al.* 2015; Woodcock *et al.* 2016, 2017; Samuelson *et al.* 2016; Ellis *et al.* 2017; Baron *et al.* 2017b; a; Tsvetkov *et al.* 2017; Arce *et al.* 2017; Siviter *et al.* 2018b) has resulted in legislative re-assessment of their use. The most notable example of this is within the European Union, where a total ban on the outdoor agricultural use of three commonly used neonicotinoid insecticides (imidacloprid, clothianidin, thiamethoxam) has now been implemented. Whilst this has generally been well-received by conservationists and scientists alike (Goulson 2018), there is a growing concern about potential replacement agrochemicals (Brown *et al.* 2016).

Sulfoxaflor, the first branded sulfoximine-based insecticide, has the same biological mode of action as neonicotinoids, acting as an agonist of nicotinic acetylcholine receptors (NAChRs) in invertebrate nervous systems (Zhu *et al.* 2011; Sparks *et al.* 2013), and has been suggested as a likely replacement for neonicotinoids (Brown *et al.* 2016). We have recently shown that sulfoxaflor exposure can have a negative impact on bumblebee (*B. terrestris)* colony reproductive output and worker production. The drop in worker production occurred at the point in the colony cycle that larvae that had been maximally exposed to sulfoxaflor were emerging, leading us to hypothesise that sulfoxaflor exposure could have negative impacts on bumblebee larval development (Siviter *et al.* 2018a). We suggested that sulfoxaflor exposure could (i) directly impact larval mortality and/or growth (whereby consumption or contact with sulfoxaflor has a direct impact on larvae) or (ii) indirectly effect bumblebee larvae production through effects on adult bees (reducing brood care, egg laying etc). Follow up experiments found no effect of sulfoxaflor exposure on bee learning or memory (Siviter et al, in press) but, in a microcolony-based design, sulfoxaflor exposure did impair bumblebee egg laying and was associated with reduced numbers of larvae (Siviter et al, submitted).

Both bumblebee and honeybee colonies can contain a plethora of different agrochemicals in the nectar and/or pollen stores (Mitchell *et al.* 2017; Nicholls *et al.* 2018; Wood *et al.* 2019) and, while little is known about the sulfoxaflor residue levels in colonies, residue levels found in the pollen

collected by foraging honeybees can be up to ten times higher than that found in nectar (United States Environmental Protection Agency 2016). Given that pollen is collected to feed developing larvae, and that bumblebee exposure levels are thought to be greater than that those experienced by honeybees (Gradish *et al.* 2019) it seems likely that bumblebee larvae will be fed pollen containing sulfoxaflor in agricultural environments. These larvae develop into the future workers/sexuals, so negative impacts of insecticide use on bumblebee larval growth and/or mortality could have down-steam consequences for worker and sexual production (Siviter *et al.* 2018a). Sulfoxaflor is now registered for use in 81 countries around the world, so there is an urgent requirement to understand the potential consequences of sulfoxaflor use on bees (Brown *et al.* 2016; Siviter *et al.* 2018a).

Agrochemicals are not the only stressor that bees are likely to encounter, as they host a wide variety of different pathogens (Schmid-Hempel 1998) and so it is likely that bumblebee larvae can be exposed to agrochemicals and pathogens simultaneously. When environmental stressors, such as agrochemicals and parasites, interact, the outcome can be (i) antagonistic, whereby the impact of both stressors combined is less than would be predicted from adding the individual impacts of each stressor together, (ii) additive, where the impact of two stressors matches their combined individual impacts, or (iii) synergistic, where the impact of combined stressors is significantly higher than predicted additive effects (Folt *et al.* 1999; González-Varo *et al.* 2013). Synergistic effects on bees between agrochemicals and pathogens may potentially occur when insecticides, such as neonicotinoids, suppress the immune system of insects, increasing their vulnerability to pathogens (Pamminger *et al.* 2018). Alternatively, antagonistic interactions could occur when certain insecticides reduce parasite intensity and/or prevalence (Vidau *et al.* 2011). Previous studies investigating the relationship between pesticides and pathogens have largely focused on *Apis* adults/larvae (Alaux *et al.* 2010; Vidau *et al.* 2011; Pettis *et al.* 2012; Locke *et al.* 2012; Di Prisco *et al.* 2013; Fauser *et al.* 2017) (but see (Baron *et al.* 2017a)) and producing varied results, (Doublet *et al.* 2015; López *et al.* 2017; Papach *et al.* 2017; Fine, Cox-Foster & Mullin 2017; Grassl *et al.* 2018), with additive (Doublet *et al.* 2015), synergistic (Vidau *et al.* 2011; López *et al.* 2017; Grassl *et al.* 2018), and antagonistic (Vidau *et al.* 2011; Papach *et al.* 2017) interactions all documented (for review see (Collison *et al.* 2016)). Less is known about non-*Apis* larvae such as bumblebees.

Nosema bombi is a fungal parasite that is found in bumblebee colonies globally. *N. bombi*, a microsporidia parasite, is thought to be a major driver of bumblebee declines in North America (Cameron *et al.* 2011, 2016; Brown 2011, 2017). Cameron *et al.* (Cameron *et al.* 2011) reported that the range of four North American bumblebee species (*Bombus affinis*, *B. occidentalis*, *B. pensylvanicus, B. terricola*) had decreased between 23-87% within the last 20 years and that

populations that were in decline had a significantly higher level of *N. bombi.* Laboratory experiments have demonstrated that bumblebee colonies exposed to *N. bombi* have increased worker and male mortalities (Otti & Schmid-Hempel 2007) and that bumblebee queens exposed to *N. bombi* produced smaller colonies that have a lower reproductive output and reduced individual bee longevity (Otti & Schmid-Hempel 2008; Rutrecht & Brown 2009). However, our understanding of this potentially important pathogen is still limited (Brown 2017), and how it interacts with other stressors (if at all) remains unknown.

In this experiment we consider the potential impact of simultaneous sulfoxaflor exposure and *N. bombi* inoculation on bumblebee (*B. terrestris*) larvae. Our previous research suggests that sulfoxaflor exposure on bumblebee larvae could be an important underlying mechanism that drives the observed negative impacts on bumblebee reproduction (Siviter *et al.* 2018a). Given the historical impacts of *N. bombi* on bumblebees (Cameron *et al.* 2011, 2016; Brown 2017) there is an urgent need to evaluate the impact of these environmental stressors, in isolation and when in interaction on bumblebee larvae. We hypothesised that both sulfoxaflor exposure and *N. bombi* inoculation would influence bumblebee larval development. Our study followed a series of steps, each of which was pre-registered and built upon the results of the previous stage. In Experiment one, we assayed the impact of chronic sulfoxaflor exposure at various concentrations on bumblebee larval mortality and growth, and in Experiment 2 we investigated the combined impacts of sulfoxaflor and *N. bombi* exposure. However, we found contradictory results between experiment 1 & 2 for exposure to sulfoxaflor (alone) at 5ppb. We thus questioned the robustness of our results, and chose to repeat the experiment with increased colony-level replication (increase from 3 to 8 colonies per experiment; note that results from all experiments were analysed separately to avoid Type I error rate inflation).

Experiment 6.1: Does sulfoxaflor exposure influence bumblebee larval mortality and development?

Methods

Sulfoxaflor exposure

Sulfoxaflor has been registered for use in 81 countries around the world. Data from the United States Environmental Protection Agency (EPA) has shown that the sulfoxaflor residue levels in the nectar of a cotton crop sprayed twice with 0.45 pounds of sulfoxaflor per acre over an 11-day period did not fall below 5ppb, with pollen levels higher by a factor of approximately 10 (United States Environmental Protection Agency 2016). It should be noted that spraying flowering crops is prohibited in Europe (Dow AgroSciences Ireland; Dow AgroSciences USA 2018) but this is not the case globally (Dow AgroSciences Australia Limited 2018; Dow AgroSciences New Zealand 2018; Dow AgroSciences South Africa 2018)

and recent legislative changes in the USA means that sulfoxaflor can be now be sprayed on numerous bee attractive crops during flowering (including, with restrictions, cucurbits, strawberries and ornamental plants) (EPA 2019). Based on the EPA data above, we chose to expose the larvae to sulfoxaflor at a concentration of 5ppb, which is the same concentration as used in previous work (Siviter *et al.* 2018a). We also included a higher dose of 50ppb, to assess dose-dependency, together with a positive control of 500ppb. Fresh treatment solutions were made every 3-4 days to account for degradation of the active ingredient (EFSA 2014).

Experimental protocol

Three commercially-obtained bumblebee colonies (*Bombus terrestris audax;* Biobest, Belgium), with approximately 150 workers each, were housed in a room at 26°C (50-60% humidity) with *ad libitum* access to sucrose solution. 5 workers per colony were removed and faecally screened for common bumblebee parasites (*Apicystis bombi, Crithidia bombi, Nosema spp.)* (Rutrecht & Brown 2009; Folly *et al.* 2017). None of the colonies contained any of these parasites.

We removed 96 early larvae (instars 1 & 2) and 96 late larvae (instars 3 & 4) from the three colonies (range n = 57 to 75 per colony), and placed each one in an individual well lined with filter paper (24 wells per plate; 4 rows, 1 row per treatment). Plates were then incubated (Sanyo MIR-554; 32°C; approx. 60% humidity (Pereboom, Velthuis & Duchateau 2003)). Larvae were starved for an hour, and then fed untreated sucrose solution (50° Brix) before examination under a dissection microscope (Nikon SM2800) to confirm (through observation of movement) that the larva was still alive. Based on the results of a pilot experiment that aimed to establish a feeding regime that minimized mortality (Experiment S6.1; Figure S6.1), early larvae were then subsequently fed pollen dissolved in sucrose solution (35.12g pollen per litre of 50°Brix sucrose), containing the relevant concentration of sulfoxaflor, for 10 days (Cnaani *et al.* 1997) at 4x2 µl a day. Late larvae were fed the same solution, but for 5 days at 4x2 µl per day. After the last feed of each day we observed the larva under a dissection microscope (Nikon SM2800). If the larva did not respond with movement to (a) the feeding solution alone or (b) subsequent touch with forceps, it was assumed to have died. Otherwise, pictures (iPhone 7) of the larva were taken for image J analysis to analyse growth (days 1, 5 & 10 for early larvae, days 1 & 5 for the late larvae). After day 10, the early larvae were frozen at -20 degrees Celsius. The late larvae were left in the incubator to monitor pupation and emergence. Six late larvae that were dropped on the floor during the experiment were excluded from the analysis.

Statistical analysis

We used an information theoretic approach based on AICc values. For every response variable tested we created a full model containing all fixed and random measured factors, for comparison with all

subsets of that full model (retaining all the random factors in each case) and a null model containing just the intercept and random factors (see Table S5.1). We selected a 95% confidence set of models based on Akaike weights derived from AICc values. When models could not be rejected with a 95% certainty, we used model averaging across all remaining models to produce parameter estimates and confidence intervals.

Larval mortality was analysed with a survival analysis (mixed effects Cox model) with treatment, size at the start of the experiment and the interaction between them included within the model, and with colony of origin and plate included as random factors. For early larvae, growth (day 5 growth = surface area on day 5 – surface area on day 1; day 10 growth = surface area on day 10– surface area on day 5) was analysed with a liner mixed effects model (lmer) with treatment, day (day 5 or 10), size at the start of the experiment and the interactions between day and treatment, and between size and treatment, included within the model. Original colony, plate and individual ID were also included as random factors. For late larvae, growth (day 5 growth = surface area on day 5 – surface area on day 1) was also analysed with a liner mixed effect model (lmer) with treatment and size at the start of experiment and the interaction between the two included as fixed factors. Original colony and plate were also included as random factors. Larvae that started to pupate were not included (n = 36).

We made 3 deviations from the original pre-registered analysis plan (PDF attached with submission); (i) here and in all below mentioned experiments (1-4) we pre-registered that we would consider larval growth at day 10 as (larval growth = larval surface area on day 10 – larval surface area on day 1). However, we realised that this approach did not allow us to understand larval growth at different ages, and thus chose to analyse growth at Day 10 as (larval growth = larval surface area on day 10 – larval surface area on day 5). (ii) We did not include in our pre-registered design that we would include the interaction between day (the day the measurement was taken) and treatment within the analysis. However, we realised that including this interaction could provide information about differences in growth trajectories across treatments, and therefore in all growth analyses (experiments 1-4) we considered day of measurement and the interaction with treatment within the analysis. Note that excluding this interaction does not qualitatively change the main effects. (iii) We also had originally stated in our pre-registration (in experiments 1 & 2) that we would remove negative growth values from the analysis but post-experiment decided to include them as numerous larvae did not grow (experiment 1, early larvae n = 30, late larvae n = 55; experiment 2, early larvae n = 25, late larvae n = 17) suggested that larval shrinkage could be occurring.

We used the R packages Hmisc, lme4, coxme and MuMIn (Bates *et al.* 2015; Barton 2016; Harrell & Dupont 2018; Therneau 2018).

Results: Experiment 1

We found no significant effects of sulfoxaflor exposure on the mortality of either early-stage larvae (Figure 6.1A, Table S6.4; coxme: 5ppb parameter estimate (PE) relative to control = 0.60, 95% confidence intervals (CI) = -0.55 to 1.74; 50ppb PE = 0.25, 95% CI: -0.88 to 1.37; 500ppb PE = 0.93, 95% CI: -0.01 to 1.87) or late-stage larvae (Figure 6.1B, Table S6.4, coxme: 5ppb PE = -0.05, 95% CI: -0.89 to 0.78; 50ppb PE = 0.03, 95% CI: -0.66 to 0.73; 500ppb PE = 0.00, 95% CI: -0.71 to 0.71).

We further found no significant differences in larval growth between the control and the various treatment groups for early larvae (Figure 6.2A, Table S6.4; lmer: 5ppb PE = 0.71, 95% CI: -0.64 to 2.05; 50ppb PE = -0.24, 95% CI: -1.38 to 0.90; 500ppb PE -0.06, 95% CI: -1.24 to 1.11) or for late larvae (Figure 6.2B; Table S6.4, lmer: 5ppb PE relative to control= -0.05, 95% CI: -0.89 to 0.78; lmer: 50ppb PE = 0.03, 95% CI: -0.66 to 0.73; 500ppb PE = 0.00, 95% CI: -0.71to 0.71). There was also no significant interaction between treatment and day (only relevant for early larvae, suggesting the larvae followed similar growth trajectories (Figure 6.2A, Table S6.4; lmer: day*5ppb PE = 0.05, 95% CI: -1.61 to 1.71; day*50ppb PE = 0.07, 95% CI: -1.50 to 1.63; day*500ppb PE 0.73, 95% CI: -1.52 to 2.99). There was no effect of sulfoxaflor exposure on larval pupation or emergence (see supplementary material, experiment 6.1). All results are presented in table S6.4.

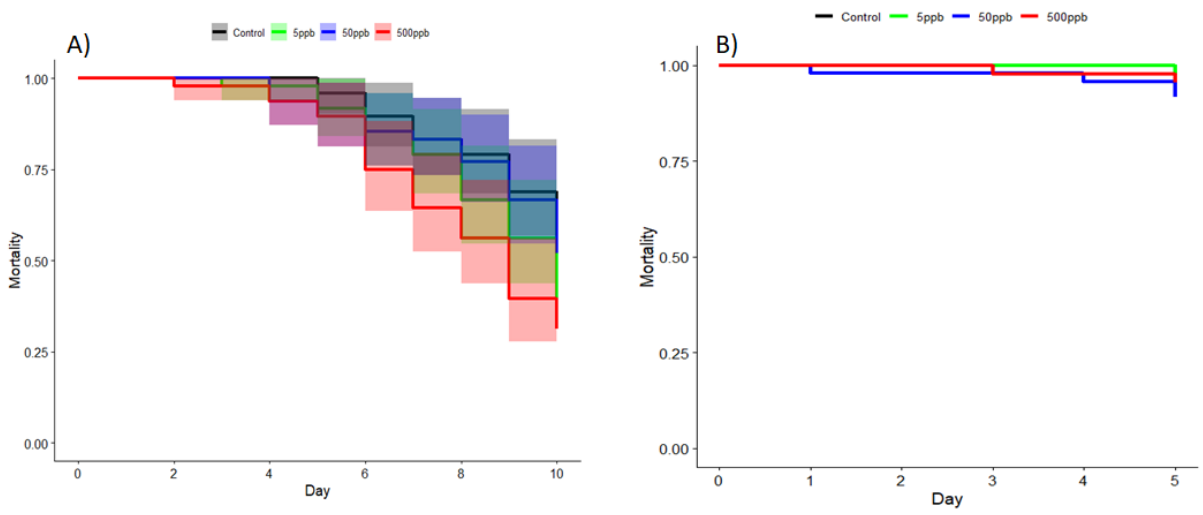


Figure 6. 1: **Kaplan-Meier survival curves for early larvae chronically exposed to varying dosages of sulfoxaflor for (A) early larvae and (B) late larvae.**

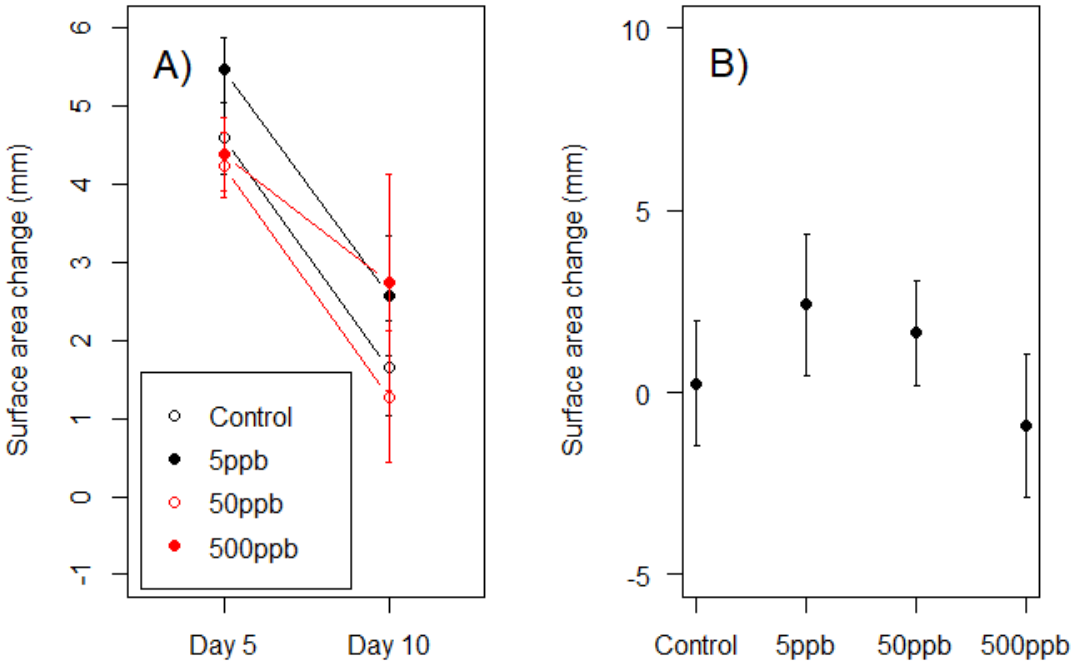


Figure 6. 2: Surface area change (mm^2 ± SE) of early larvae (A) at day 5 & 10. Day 5 surface area change = Individual larval surface area day 5 – surface area at the start of the experiment. Day 10 surface area change = Larval surface area day 10 – surface area at day 5. Late larvae (B) surface area change at day 5 (Individual larval surface area day 5 – surface area at the start of the experiment)

Experiment 6.2: Do Sulfoxaflor and N. bombi influence bumblebee larval mortality and growth in combination?

Methods:

Parasite preparation

A wild bumblebee queen (*Bombus terrestris)* infected with *N. bombi* (determined through faecal examination) was collected from Windsor Great Park in 2016. The infected queen was dissected, and the fat body and gut were homogenized in 0.01M NH_4Cl. Then, as described in Rutrecht & Brown (Rutrecht & Brown 2008), the spore solution was placed in a centrifuge set to 4°C and 5000 rpm ($2400G_n$) for 10 minutes to isolate and purify the spore pellet. The spore solution was then resuspended in 0.01M NH_4Cl and the concentration of *N. bombi* spores was calculated using a Neubauer improved haemocytometer. This inoculum was used to infect 3 bumblebee colonies (*Bombus terrestris audax)* from which we sampled bees to create the inoculum used in the present experiment.

Experimental protocol

The same basic experimental protocol was used as in experiment 1. We used a fully crossed design that included 4 treatment groups, (control (no sulfoxaflor or *N. bombi*), *N. bombi* alone, sulfoxaflor alone, *N. bombi* and sulfoxaflor). Larvae that were allocated to receive sulfoxaflor exposure were fed a 5ppb sulfoxaflor in sucrose/pollen solution (see experiment 1) throughout, and the control and *N. bombi* larvae were fed a sucrose/pollen solution containing just acetone.

Following Folly *et al. (*2017) we combined our *N. bombi* stock solution with 1000 µl of 0.01M NH_4Cl to make a stock solution of 50,000 per µl for larval inoculation. In the first feed of the experiment, each of the larvae in the parasite treatment groups were fed 2 µl of the *N. bombi* solution (paired with either control or sulfoxaflor laced sucrose/pollen solution respectability), and from this the bee received approximately 50,000 spores, a quantity that is known to infect 45 % of larvae (Folly 2018). The rest of the experiment used the exact same methodology described in experiment 1.

We were able to graft and incubate 191 early larvae and 123 late larvae from 3 commercial colonies (Biobest, Belgium). This was fewer than originally pre-registered (PDF provided with submission) because the colonies contained fewer larvae than envisaged.

The same statistical approach as described in experiment 6.1 was used.

Results and discussion

We found no significant differences in mortality between treatment groups for early larvae (Figure 6.3A, Table S6.4; coxme: parasite PE = -0.10, 95% CI: -0.24 to 0.45; sulfoxaflor PE = 0.11, 95% CI: -0.24 to 0.46; parasite & sulfoxaflor PE = 0.03, 95% CI: -0.26 to 0.32) or late larvae (Figure 6.3B, Table S6.4; coxme: parasite PE = -0.12, 95% CI: -0.85 to 1.08; sulfoxaflor PE = 1.60, 95% CI: -0.67 to 3.87).

Early larvae exposed to either sulfoxaflor or *N. bombi* in isolation grew less than control larvae, although interestingly, larvae exposed simultaneously to sulfoxaflor and *N. bombi* did not differ significantly from the control (Figure 6.4A, Table S6.4; lmer: *Nosema* PE = -2.69, 95% CI: -4.46 to -0.91; sulfoxaflor PE = -2.84, 95% CI: -4.66 to -1.03; sulfoxaflor & *Nosema* PE = -1.62, 95% CI: -3.54 to 0.30). Although this effect appeared to be driven by growth prior to day 5 (Figure 6.4A), we found no significant interaction between day and the various treatment groups (lmer: day**Nosema* PE = 3.21, 95% CI: -0.04 to 6.47; day*sulfoxaflor PE = 2.50, 95% CI: -0.77 to 5.77; day*sulfoxaflor & *Nosema* PE = 1.08, 95% CI: -2.40 to 4.56) and no impact of *N. bombi* or sulfoxaflor exposure on late larval growth (Figure 6.4B; lmer: *Nosema* PE = -0.52, 95% CI: -4.95 to 3.90; sulfoxaflor PE = -0.87, 95% CI: -5.42 to 3.69; sulfoxaflor & *Nosema* PE = -1.86, 95% CI: -7.03 to 3.31). We also found no effect of sulfoxaflor or *N. bombi* on probability of pupation or emergence (see supplementary material, experiment 6.2).

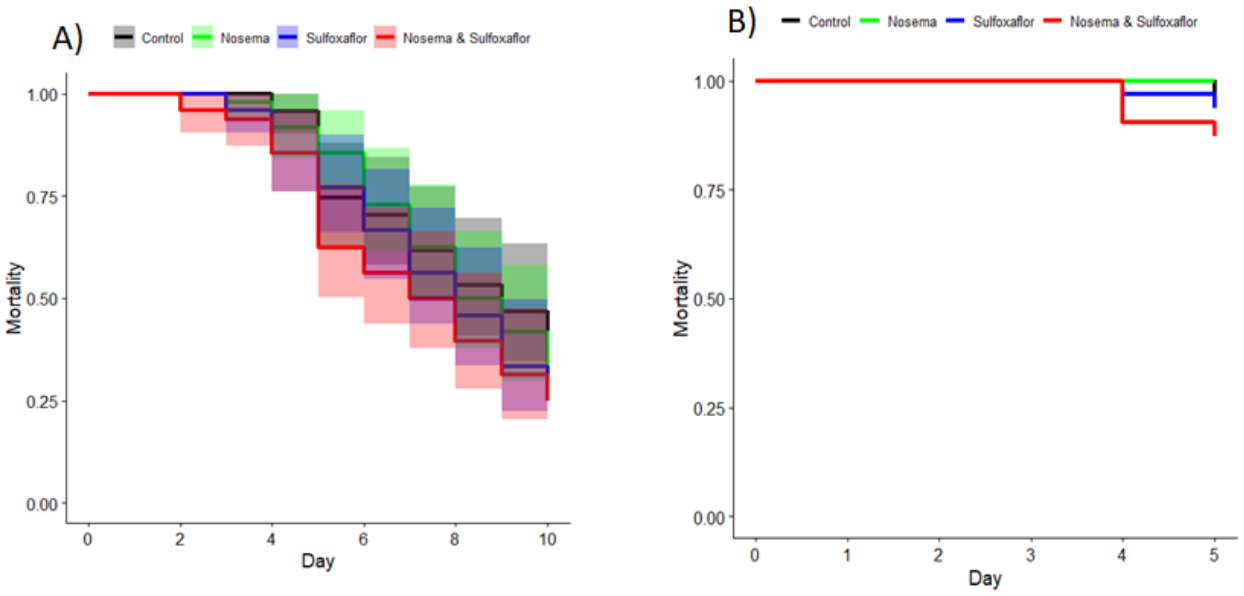


Figure 6. 3: The proportion of early larvae (A) and late larvae (B) that died during the experiment.

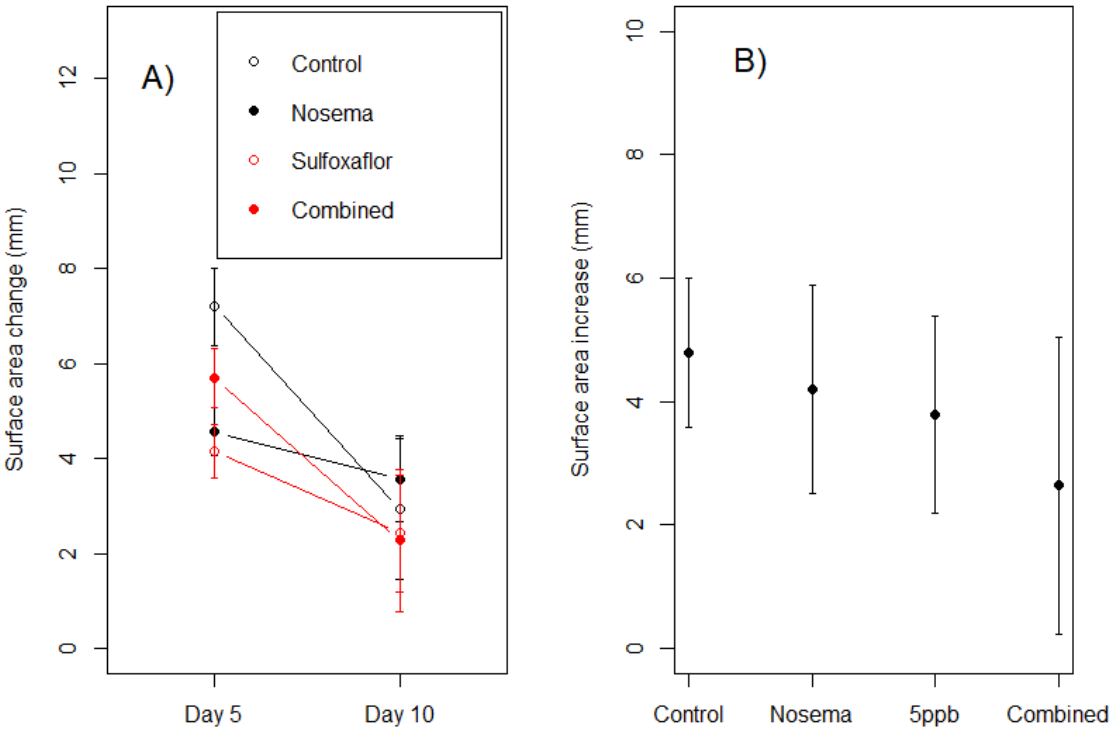


Figure 6. 4: Surface area change (mm^2 ± SE) of early larvae (A) at day 5 & 10. Day 5 surface area change = Individual larval surface area day 5 – surface area at the start of the experiment. Day 10 surface area change = Larval surface area day 10 – surface area at day 5. Late larvae (B) surface area change at day 5 (Individual larval surface area day 5 – surface area at the start of the experiment).

Experiment 6.3: Testing the impact of sulfoxaflor dose on early larval development with a greater level of colony replication

Our results in experiments 1 and 2 were contradictory with respect to the effect of sulfoxaflor exposure for early larvae. In experiment 2, sulfoxaflor exposure at 5ppb was detrimental to early larval growth, but we could not be confident in this result because experiment 1 failed to detect a similar effect in an identical treatment. We thus chose to repeat both experiments with much higher replication at the colony level, in order to ensure that our findings were robust and replicable. In experiments 6.3 & 6.4 we repeat experiments 6.1 & 6.2 but increase the colony-level replication from 3 to 8 colonies per experiment. Results were never pooled across experiments, and so these represent independent replicates.

Methods

Experiment 6.3 was identical to experiment 6.1, with three exceptions. Firstly, since the ambiguity in our results related only to early-stage larvae, we did not include late-stage larvae within the new study. Secondly, we collected larvae from 8 colonies rather than 3. Thirdly, we replaced our 50ppb treatment with a 0.28ppb treatment, based on data from the Pest Management Regulatory Agency Canada (Pest Management Regulatory Agency (Canada) 2016) which demonstrated that sulfoxaflor residue levels in the nectar of seed-treated crops may be significantly lower than in sprayed crops. Again, the design and analysis were pre-registered.

We removed all living larvae (n = 692, instar stages 1 & 2; fewer than planned on pre-registration because fewer were present in the colonies). Of 692, 28 died in transit. In contrast to experiment 1 & 2, the larvae were then left in the incubator overnight (due to increased sample size it was not possible to move all larvae into the well plates and feed them in the same day). 14 larvae died overnight and were removed from the experiment (surviving larvae (n = 650)).

The statistical approach was as described in experiment 6.1.

Results

We found no significant effect of sulfoxaflor exposure on larval mortality at either 0.28 or 5ppb, although larvae exposed to 500ppb died earlier than control larvae (Figure 6.5, Table S6.4; coxme, 0.28ppb PE = 0.26, 95% CI = -0.13 to 0.65; 5 ppb PE = 0.17, 95% CI = -0.34 to 0.68, 500 ppb PE = 0.42, 95% CI = 0.06 to 0.78).

In agreement with experiment 6.2, we found a significant negative effect of sulfoxaflor exposure on larval growth at both 5 and 500 ppb, although there was no detectable effect at 0.28ppb (Figure 6.5, Table S6.4; lmer, 0.28 ppb PE = -1.08, 95% CI = -2.18 to 0.02; 5 ppb PE = -1.03, 95% CI = -2.05 to -0.01, 500 ppb PE = -1.45, 95% CI = -2.62 to -0.28). There was also no interaction effect between day and treatment, suggesting that the growth trajectories did not differ between treatment groups (Figure 6.2, Table S6.4; lmer, day*0.28ppb PE = 1.41, 95% CI = -0.86 to 3.69; day*5ppb PE = 0.68 -, 95% CI = -1.05 to 2.40, day*500ppb PE = 1.07, 95% CI = -1.02 to 3.16).

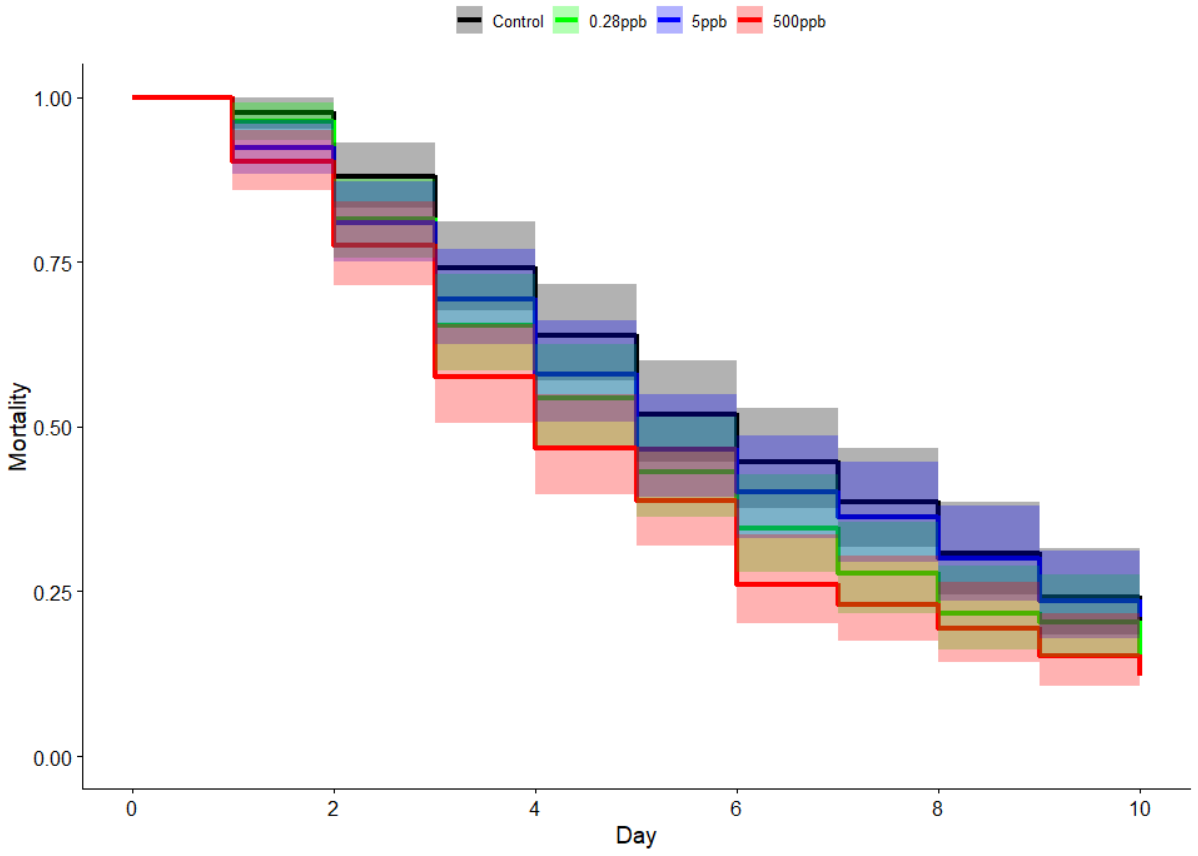


Figure 6. 5: Kaplan-Meier survival curves for early larvae chronically exposed to varying dosages of sulfoxaflor

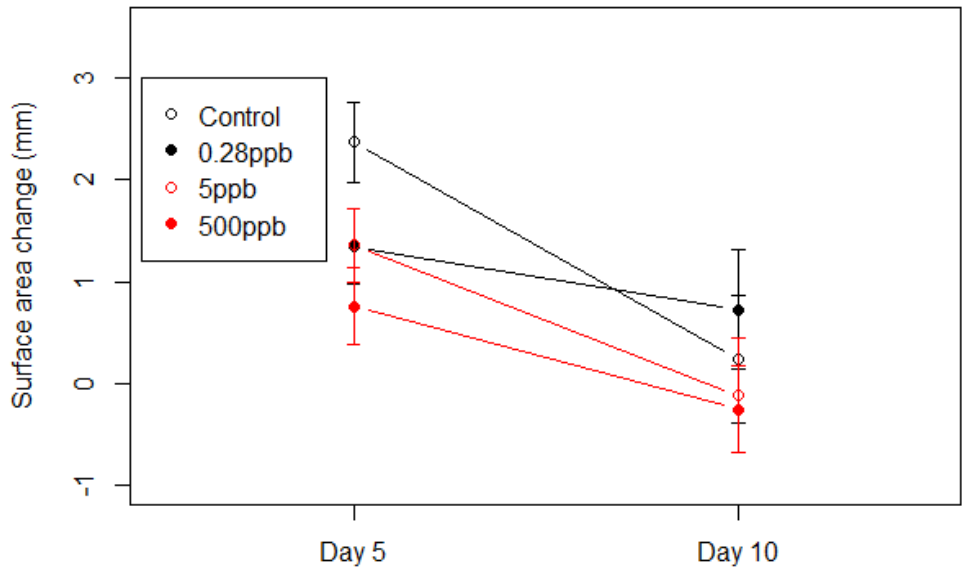


Figure 6. 6: Surface area change (mm^2 ± SE) of larvae at day 5 & 10. Day 5 surface area change = Individual larval surface area day 5 – surface area at the start of the experiment. Day 10 surface area change = Larval surface area day 10 – surface area at day 5.

Experiment 6.4: Testing the potential impact of sulfoxaflor and N. bombi on early bumblebee larvae development at a greater level of colony replication.

Methods

We followed the same methodology as described in Experiment 6.2, again with the exception that we focussed only on early-stage larvae, and that we used 8 colonies rather than 3.

We were able to graft 768 larvae from 8 colonies. Seven larvae died during the plating process and 15 died over-night and were not included in the experiment. 8 larvae were removed due to experimental error so our final sample size was 738.

Our statistical analysis followed the same approach used above and was pre-registered (PDF provided).

Results

We found no effect of sulfoxaflor or *N. bombi* exposure on bumblebee larval mortality, and no impact when used in combination (Figure 6.7, Table S6.4; coxme, *N. bombi* PE = 0.06, 95% CI = -0.16 to 0.28; sulfoxaflor 5 ppb PE = 0.22, 95% CI = -0.01 to 0.44, *N. bombi* & sulfoxaflor PE = 0.10, 95% CI = -0.21 to 0.41).

As in Experiment 6.2, both sulfoxaflor and *N. bombi* exposure, in isolation, negatively influenced bumblebee larval growth (Figure 6.8, Table S6.4; lmer, *N. bombi* PE = -2.45, 95% CI = -3.14 to -1.76; sulfoxaflor 5ppb PE = -3.35, 95% CI = -4.04 to -2.64), and in this case, the combined treatment also had a significant negative impact (combined PE = -3.29, 95% CI = -4.02 to -2.56). Interestingly, we found that the growth trajectories of larvae in some treatments differed from those observed previously. In Experiment 6.2, growth rates decreased as the larvae got older. This was also true for the control and *Nosema* treatments in this experiment, but for the sulfoxaflor and combined treatments, the trend was reversed (Figure 6.8, Table S6.4; lmer: day* *N. bombi* PE = 1.11, 95% CI: -0.31 to 2.53; day*sulfoxaflor PE = 4.15, 95% CI: 2.59 to 5.70; day*sulfoxaflor & *N. bombi* PE = 3.96, 95% CI: 2.28 to 5.64).

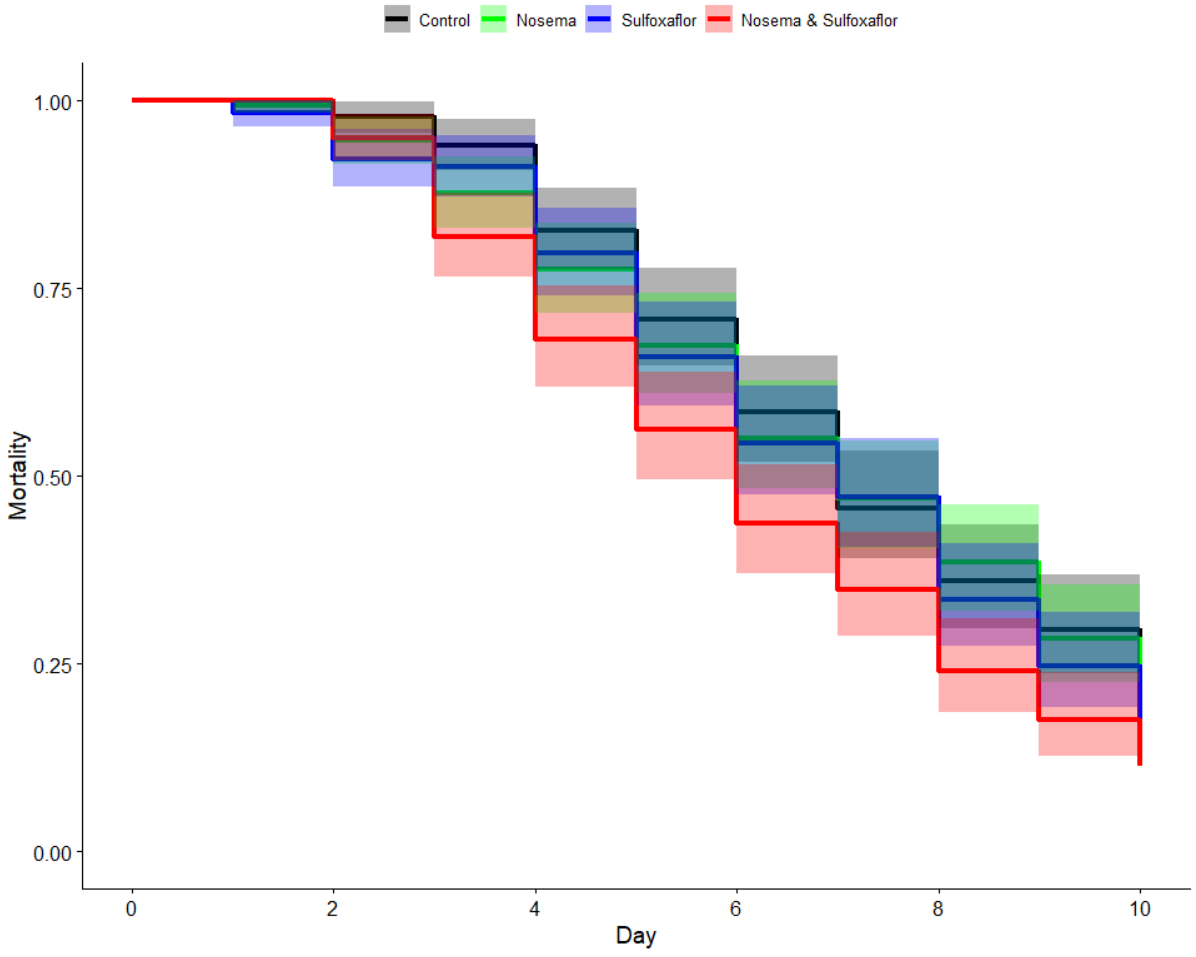


Figure 6. 7: Kaplan-Meier survival curves for early larvae exposed to sulfoxaflor and *N. bombi*

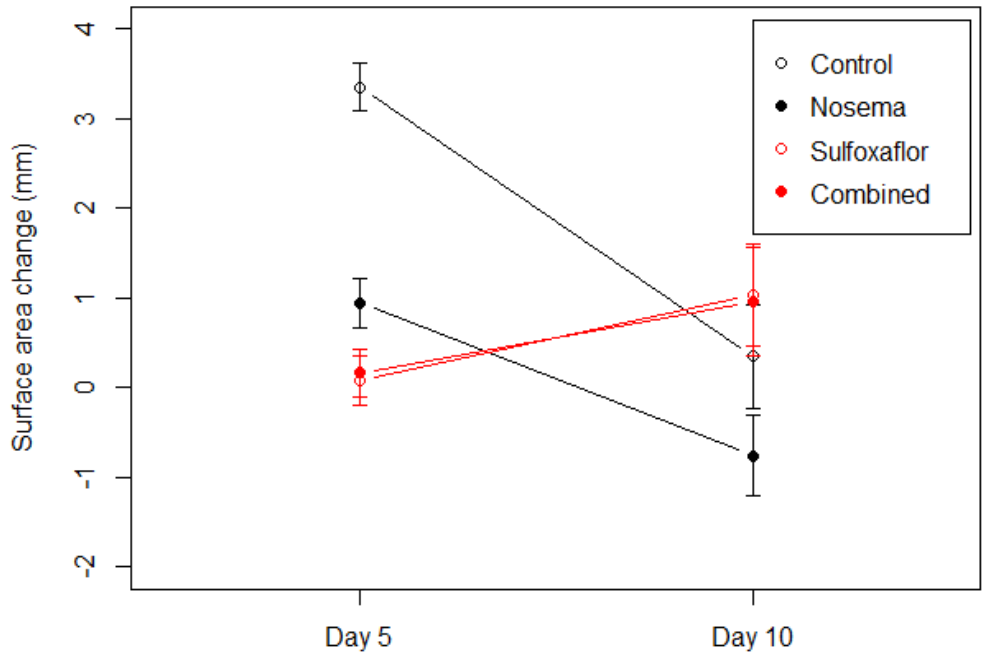


Figure 6. 8: Surface area change (mm^2 ± SE) of larvae at day 5 & 10 from the start of the experiment for larvae. Day 5 surface area change = Individual larval surface area day 5 – surface area at the start of the experiment. Day 10 surface area change = Larval surface area day 10 – surface area at day 5.

Discussion

In previous work (Siviter *et al.* 2018a), we observed that sulfoxaflor exposure early in the colony cycle was associated with a subsequent reduction in worker numbers, and a later reduction in reproductive offspring. We hypothesised that sulfoxaflor exposure might increase larval mortality, driving the observed downstream consequences on reproductive output. Here, in the present experiment, we find no evidence that sulfoxaflor exposure increased larval mortality, except at extremely high doses, but did find (in three of our four experiments) that sulfoxaflor exposure at potentially field realistic levels of 5ppb negatively influenced larval growth.

The time it takes for a bumblebee larva to develop varies (Cnaani *et al.* 1997) and impaired growth could result in (i) larvae taking longer to start pupating or (ii) larvae starting to pupate at a smaller size so emerging bees are smaller (Couvillon & Dornhaus 2009). In Siviter *et al*. (2018a) it was not possible to measure whether there were differences in size of workers between control and sulfoxaflor treatment groups, but the results from the present experiment suggest that colonies exposed to

sulfoxaflor may produce smaller bees. Similarly, if during sexual production larvae are exposed to sulfoxaflor, then it is possible that emerging males and gynes might be of a poorer quality than unexposed bees (Straub *et al.* 2016). Given that gyne larvae take longer to develop into adults than workers and males (Cnaani *et al.* 1997), it might be the case that gyne larvae are particularly vulnerable to sulfoxaflor exposure. Such potential knock on consequences for emerging adult bees requires urgent attention.

Our results also showed that *N. bombi* impairs larval development, which could in turn have downstream consequences on emerging adults and contribute to bumblebee declines (Cameron *et al.* 2011). A prolonged developmental period is arguably advantageous to the parasite, as it could potentially increase parasite intensity within emerging bees, possibly leading to higher rates of faecal transmission both in and outside the nest (Rutrecht, Klee & Brown 2007). Interestingly we found, in Experiment 2, that the larvae exposed to both sulfoxaflor and *N. bombi* in combination did not grow significantly less than control larvae, despite us finding that larvae exposed to sulfoxaflor and *N. bombi* in isolation grew less than control larvae (See Table S6.4). In experiment 6.4, with a larger sample size, we found no evidence for additive or synergistic interactions between *N. bombi* and sulfoxaflor, but rather, that exposure to sulfoxaflor and *N. bombi* in combination have a less severe effect on bumblebee larvae growth than when larvae are just exposed to *N. bombi* in isolation. This suggests, in contrast to our prediction, that sulfoxaflor and *N. bombi* may potentially have an antagonistic interaction in relation to larvae growth. Whether sulfoxaflor overwhelms the impact of this co-evolved parasite is unclear, but previous studies have shown that certain pesticides can reduce parasite intensity (for review see (Collison *et al.* 2016)). If sulfoxaflor has a similar effect on either *N. bombi* prevalence and/or intensity, this could explain our results, but future experiments would be required to determine if this is the case. More broadly, an understanding of why certain pesticides have a synergistic interaction with parasites, and other do not (Collison *et al.* 2016) could be invaluable in the future development of insecticides that are less harmful to beneficial insects, such as bees.

Regulators and governing bodies are under increasing pressure to consider the potential impact of agrochemicals on non-*Apis* bees so there is a need to develop new methodologies and frameworks that can be used in a standardised, ring-tested methodology (Sanchez-Bayo & Tennekes 2017; Dorigo *et al.* 2019; Sgolastra *et al.* 2019). While rearing honeybee larvae *in vitro* has been established over decades (Crailsheim *et al.* 2013) our results here are some of the first to demonstrate how to rear and monitor bumblebee larvae *in vitro* (Pereboom *et al.* 2003). Despite this, there are large gaps in our understanding of how to rear bumblebee larvae. For example, repeated experiments with honeybees have demonstrated the ideal humidity, nutrition and temperature for incubating honeybee larvae

(Crailsheim *et al.* 2013). Specific experiments are required aimed at understanding how all these varying factors impact bumblebee larval development. Our research can hopefully provide the basis of framework that we and other researchers can build upon.

In its current form, the insecticide licensing process focuses on how agrochemicals in isolation impact bees. However, as we allude to above, bees are likely to encounter a plethora of different anthropogenic, and co-evolved environmental stressors (Goulson, O'Connor & Park 2018; Nicholls *et al.* 2018). Previous research has shown that the interactions between pesticides and pathogens can impact honeybee mortality (Doublet *et al.* 2015), pathogen load (Pettis *et al.* 2012), behaviour (Blanken, van Langevelde & van Dooremalen 2015), and immune response (Di Prisco *et al.* 2013). Bees are also likely to come into contact with multiple agrochemicals, increasing the likelihood of both lethal (Tsvetkov *et al.* 2017) and sub-lethal consequences (Gill *et al.* 2012). Our results suggested antagonistic interactions between *N. bombi* and sulfoxaflor, which could potentially reduce parasite intensity and/or prevalence (Vidau *et al.* 2011). Given that bees, and other pollinators are likely to be exposed to a multitude of different anthropogenic and co-evolved environmental stressors, we suggest that regulatory bodies and policy-makers should increasingly consider how novel insecticides interact with other environmental and anthropogenic factors such as parasites/pathogens. While considering every potential interaction between stressors is likely to be impractical in the pre-approval period, improvements to the post-licensing assessment process (which is currently minimal (Milner & Boyd 2017)) achieve this aim by monitoring safety in real-world landscape scale applications.

While significant research has been conducted on the impact of environmental stressors on adult bumblebees, impacts on larvae remain under-researched. We show here that both sulfoxaflor exposure and *N. bombi* inoculation can negatively impact bumblebee larval growth. Given the growing global importance of sulfoxaflor, and the increasing prevalence and intensity of *N. bombi* in bumblebee populations (Cameron *et al.* 2016), such effects may provide a potential mechanism through which exposure to these stressors can reduce bumblebee colony fitness. Our results highlight the need to understand how novel insecticides influence beneficial insects, such as bumblebees, at various stages of their life cycle.

7. General discussion

The main aim of my PhD thesis was to determine whether sulfoxaflor exposure has sub-lethal impacts on *Bombus terrestris*, at the individual and colony levels.

In Chapter 2, I established that chronic sulfoxaflor exposure (5ppb) can reduce bumblebee colony reproductive output by 54% (see Figure 7.1). In chapter 3, I determined that acute sulfoxaflor exposure does not influence bumblebee olfactory learning/memory or working memory but in Chapter 4 I confirmed, with a meta-analysis, that other insecticides can negatively influence bee learning/memory. In Chapter 5, using a microcolony based design, I demonstrated that sulfoxaflor exposure can negatively influence bumblebee egg laying, offering a possible mechanism for the results observed in Chapter 2. I also found, in Chapter 6, that sulfoxaflor exposure can negatively influence bumblebee larval growth, although I found no evidence for synergistic effects on either larval growth or mortality when larvae were simultaneously exposed to sulfoxaflor and the fugal parasite *Nosema bombi*. Overall my results showed that sulfoxaflor can have negative sub-lethal impacts on bumblebees.

In this final chapter I synthesise what I believe my key findings are, and make suggestions for future research directions.

7.1 Key findings

7.1.1 What is the mechanism driving the negative impacts of sulfoxaflor on bumblebee reproductive output?

In Chapter 2 I demonstrated that sulfoxaflor can have a negative impact on bumblebee colony reproductive output, similar to those observed with neonicotinoids (Whitehorn *et al.* 2012; Ellis *et al.* 2017; Woodcock *et al.* 2017; See Figure 7.1). The rest of my thesis focused on determining what the underlying mechanism (s) that drove this effect were.

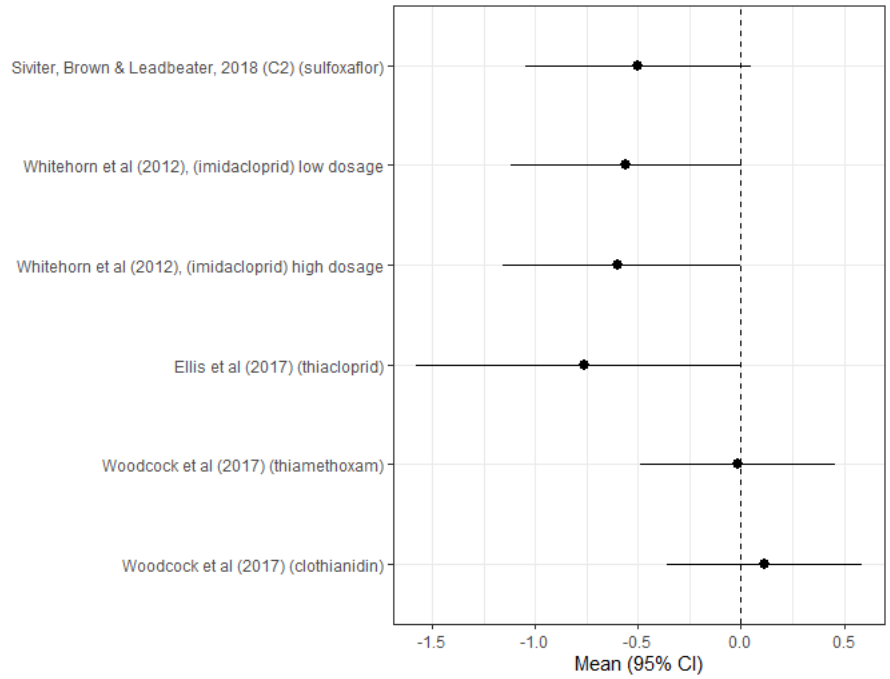


Figure 7. 1: Mean effect sizes (± 95% confidence intervals) for bumblebee colony reproductive output when colonies are exposed to different insecticides (results all compared with control colonies). Data from (Whitehorn *et al.* 2012; Ellis *et al.* 2017; Woodcock *et al.* 2017; Siviter *et al.* 2018a).

Bumblebee colonies that had been chronically exposed to sulfoxaflor produced fewer workers than those in control colonies (Chapter 2). This drop-in worker numbers first became apparent at the time point when individuals that received maximum exposure as larvae began to eclose, leading me to hypothesise that sulfoxaflor exposure, early in the colony's life cycle, could impact egg production and/or larval development. In Chapter 6 I tested this hypothesis by exposing bumblebee larvae to varying concentrations of sulfoxaflor (0.28, 5 & 500ppb). I found no effects of sulfoxaflor exposure on bumblebee larval mortality at 0.28 & 5ppb. I did however find an effect of sulfoxaflor exposure on bumblebee larvae growth. Furthermore, in Chapter 5, using a microcolony-based design, I also demonstrated that bumblebees exposed to sulfoxaflor (5ppb) produced fewer eggs and larvae than control bumblebees (for a summary of all results see Figure 7.2).

Similarly to my research with sulfoxaflor, the underlying mechanism that drives the observed negative impacts of neonicotinoids on bees can be debated. Impaired bee learning/memory and, relatedly, worker foraging efficiency have been suggested by some (Klein *et al.* 2017). Yet this hypothesis has been questioned by others, who highlight impacts on foraging motivation, flight duration and/or

metabolic rate (Leonard & Hochuli 2017; Lämsä *et al.* 2018; Muth & Leonard 2019). Neonicotinoids can also impair egg laying, reducing ovary development in both honeybees, and bumblebees (Brandt *et al.* 2017; Baron *et al.* 2017b; a), or reducing drone sperm count (Straub *et al.* 2016). Other researchers suggest that impaired immune function leave bees more vulnerable to naturally occurring pathogens and parasites (Goulson *et al.* 2015; Sánchez-Bayo *et al.* 2016). It, therefore, seems unlikely that the negative impacts of neonicotinoids have one underlying mechanism.

Similarly, I demonstrate in my thesis that sulfoxaflor can impact bumblebees in more than one way. Below I present a forest plot (Figure 7.1) depicting the outcomes of each experiment from my thesis. I only include my data from bumblebees, and specifically chose sub-lethal dependent variables (as opposed to mortality in Chapters 5 & 6). I conducted the meta-analysis (using the package *Metafor*) which determined the Hedges d values, and 95% confidence intervals for each output. In this thesis I find no evidence that sulfoxaflor exposure has significant impacts on bee foraging (Chapter 2) or working memory (Chapter 3) but I did find suggestive negative trends (Figure 7.1), which could warrant future research (see below). However, my overall results show (Figure 7.2) that sulfoxaflor exposed bumblebees consistently fair worse than unexposed bumblebees.

While studies attempting to understand the underlying mechanism that drive observed negative impacts of insecticides on bees are important, clearly insecticide use will influence beneficial insects in more than one way. Even if perceived sub-lethal effects are subtle, or small, the cumulative impact of these stressors is more likely to drive negative impacts on bumblebee fitness (Figure 7.2). So, while studies investigating potential mechanisms are vital for understanding how, and why certain insecticides differ from one another, I believe that research focused on understanding colony level consequences of insecticide exposure, namely reproductive output in bumblebees, should be the priority focus of future research with sulfoxaflor, and indeed other insecticides.

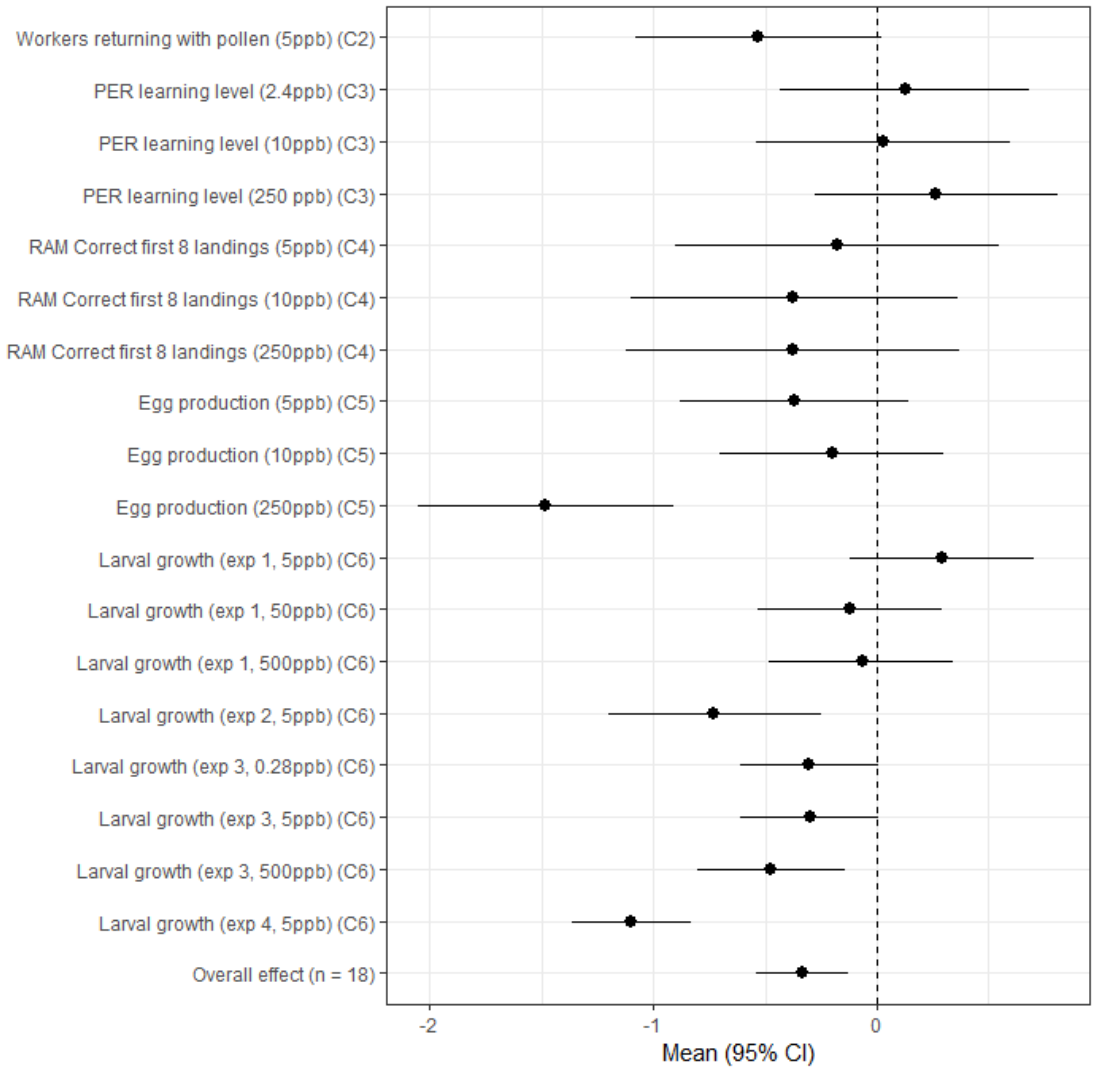


Figure 7. 2: Mean effect sizes (± 95% confidence intervals) for each dependant variable produced in thesis that examined the potential sub-lethal impact of sulfoxaflor exposure on bees. (One dependant variable presented for each group of bees tested).

7.1.2 Does sulfoxaflor impair bumblebee cognition and foraging?

The evidence demonstrating that neonicotinoids can negatively influence bee learning and memory (Decourtye *et al.* 2004a; b; Stanley *et al.* 2015b; Samuelson *et al.* 2016) has led some to suggest that impaired cognition drives reduced foraging efficacy and homing success (Gill *et al.* 2012; Feltham *et al.* 2014; Gill & Raine 2014), and ultimately influences colony fitness (Klein *et al.* 2017). For this reason, in Chapter 3 I set out to establish whether sulfoxaflor exposure impacts bumblebee learning and memory. I used two experimental designs, based on previous work by Stanley *et al.* (2015) and Samuelson *et al.* (2016), to test this. Stanley *et al.* (2015) demonstrated that acute exposure to the neonicotinoid thiamethoxam impaired olfactory learning in bumblebees in a proboscis extension

reflex test, while Samuelson *et al.* (2016) found that thiamethoxam impaired bumblebee spatial working memory. I found no evidence that sulfoxaflor exposure influenced bumblebee learning/memory despite using comparable doses and dosage regimes to those used in Stanley *et al.* (2015) and Samuelson *et al.* (2016). My meta-analysis (Chapter 4), suggested that *Apis* is perhaps more vulnerable to insecticide exposure in terms of impaired cognition than *Bombus*. I therefore also conducted olfactory learning tests (using the proboscis extension reflex test again) in honeybees and found no evidence that sulfoxaflor exposure influenced honeybee olfactory learning. In combination with my findings from (Chapter 2), in which sulfoxaflor did not influence the proportion of foragers returning to the nest with pollen, the findings from this thesis suggest that impaired foraging efficiency, after sulfoxaflor exposure, does not drive the observed reduction in bumblebee colony reproductive output (Chapter 2).

Nevertheless, before this can be confirmed two areas of future research must first be addressed. Firstly, the impact of chronic (as opposed to acute) sulfoxaflor exposure on bee foraging and cognition needs to be further examined. In Chapter 2 bees did not forage outside during the exposure period of the experiment, so comparing my results with other studies examining the impact of neonicotinoids on bee foraging efficiency is difficult (Gill *et al.* 2012; Feltham *et al.* 2014; Gill & Raine 2014; Stanley *et al.* 2016). Experiments that chronically expose bumblebees and record their foraging efficiency using detailed recording based on RFID tags are the next logical step. Secondly my meta-analysis (chapter 4) showed that honeybees and bumblebees chronically exposed to pesticides performed worse in the memory task than those exposed to an acute dose. Therefore, while my results in Chapter 3 suggest that acute sulfoxaflor exposure does not influence bee cognition, future research should focus on understanding whether chronic sulfoxaflor exposure influences bee learning and memory.

From a wider perspective, the assumption that learning and memory performance are closely associated with foraging efficiency may be an oversimplification. The idea that impaired cognition, after insecticide exposure, drives observed colony- and population- level bee declines has received a lot of attention (Decourtye, Lacassie & Pham-Delègue 2003; Samuelson *et al.* 2016; Klein *et al.* 2017; Siviter *et al.* 2018b). Indeed, in both popular science writing and journalism, impacts of insecticides on cognitive traits are often highlighted as the reasons that 'bees are declining' (Morelle 2013; McMillan 2018; Solly 2018). The idea that neonicotinoids impair bee cognition is an attractive and intuitive story, so much so that it was the inspiration behind the *Sunday Times* bestseller fictional book *Coffin Road,* that follows a scientist's quest to discover how neonicotinoid are 'killing bees'. In the book fictional scientist Christopher Connolly, (named after researcher Dr Christopher Connolly from the University of Dundee) states that honeybees exposed to neonicotinoids are unable to find their way to their nest,

and forage less efficiently, due to impaired cognition. While there is evidence to suggest the bees exposed to neonicotinoids are less likely to return to the colony, and indeed forage less efficiently (Gill *et al.* 2012; Henry *et al.* 2012; Gill & Raine 2014; Stanley *et al.* 2016), the underlying mechanism driving this could be one of several (Leonard & Hochuli 2017; Kenna *et al.* 2019; Muth & Leonard 2019).

The evidence linking colony level fitness and bee cognition is both limited and contradictory (Raine & Chittka 2008; Evans *et al.* 2017). Raine & Chittka (2008) found that bumblebees (*B. terrestris*) colonies that contained a higher number of 'quick' learner had workers that returned to the nest with more nectar, but in this study i) the individuals that underwent cogntive testing were not those that also foraged in the wild ii) the results may well effect confounding variables at the colony level, such as food store availability. In a follow-up study, indivudal foraging effciency and bee learning speed were not correlated (Evans *et al.* 2017). It is of course possible that enhanced bee learning/memory in certain environments will increase foraging efficiency, but the importance of bee cognition for colony level success is likely to be context dependent. For example, consider a bumblebee colony foraging within an environment that is plentiful in floral resources. In an environment where food is abundant, do bees that quickly learn how to find and extract nectar/pollen from flowers give the colony an advantage over other colonies, or does the shear abundance of resources mean that all bees forage at a similar level? Similar arguments could be made for bees foraging in a florally poor environment; do bees with poorer learning/memory, that forage less efficiently, stumble across more floral resources (Burns 2005; Evans & Raine 2014), or do bees with that are better at learning outcompete other conspecifics?

In order to truly understand the relationship between bee cognition and foraging efficiency/homing, experiments need to be conducted that determine how individual cognitive performance and bee behaviour are linked, across a range flower rich, and flower poor environments. Only then will we be able to understand whether insecticide impaired cognition is an important underlying mechanism driving colony level impacts of insecticide exposure or not.

7.2 Future research

7.2.1. Understanding the risk of sulfoxaflor exposure

When determining whether an agrochemical should be licensed for use or not, regulatory bodies consider both the risk of exposure (the likelihood of non-target organisms encountering the active ingredient) and the potential hazardous impact of that exposure, should it occur. My PhD has focussed on determining whether sulfoxaflor can be hazardous to bees at sub-lethal dosages. Our understanding of the likelihood, or risk, of bees being exposed to sulfoxaflor in the field, however, remains data deficient at the current time (see Chapter 1, section 1.6).

Sulfoxaflor has now been registered for use in 81 countries around the world and the spray recommendations/ guidelines vary between countries. In Europe, spraying during flowering is prohibited, as it was in the USA up until recently (EPA 2019). In most other countries spraying during flowering is not prohibited but label guidelines recommend against it (Dow AgroSciences Ireland; Dow AgroSciences Australia Limited 2018; Dow AgroSciences New Zealand 2018; Dow AgroSciences South Africa 2018) (although insecticide label recommendations are often misinterpreted by end users (Jallow *et al.* 2017)). However, even if spray treatments are used pre-or post-bloom, if non-target flowers are present in crop margins or between crop rows (in apple orchards for example) then the existing residue data (see Chapter 1, section 1.6) suggest that bees are likely to come into contact with sulfoxaflor at dosages comparable to those used as "field-realistic" throughout this thesis. At the current time, all publicly available residue data were collected from plants that were sprayed during flowering which means we do not have a good understanding of what expected residue levels are likely to be in treated crops under field applications (United States Environmental Protection Agency 2016; Cheng *et al.* 2018; Abdourahime *et al.* 2019).

In order to understand the potential risk of sulfoxaflor exposure in Europe, three areas of future research are required. Firstly, we need experiments designed to determine the likely residue levels of sulfoxaflor when crops are sprayed using label recommendations. Given that residue levels vary between application rate and crop (Bonmatin *et al.* 2015; Kyriakopoulou *et al.* 2017) it is vital that residue levels are determined across a range of crops and application regimes. Secondly, we need to understand what the residue levels are predicted to be when bees forage on non-target wild-flowers and weeds. A recent study demonstrated that 60.07% of pollen containing neonicotinoids returned to honeybee nests were from non-target crops such as weeds (Wood *et al.* 2019), suggesting exposure to non-target crops is a common route of exposure. Neonicotinoids in this experiment (Wood *et al.* 2019) were only used as a seed treatment, and the authors suggest that weeds and plants in crop margins were contaminated due to neonicotinoid persistence in soil (see section 1.5.2). Given that sulfoxaflor is potentially less persistent in soil that neonicotinoids (Pest Management Regulatory Agency (Canada) 2016), future research with sulfoxaflor should focus on determining the reside levels in non-target flowers and weeds when crops are sprayed.

Thirdly, while I have demonstrated that sulfoxaflor exposure can influence bumblebees in both laboratory and semi-field experiments, future research that determines whether bumblebees foraging in an environment with sulfoxaflor treated crop (in experiments similar to (Rundlöf *et al.* 2015; Woodcock *et al.* 2017; described in Chapter 1, section *1.5.3.3*) (i) come into contact with sulfoxaflor residues and (ii) whether exposure has colony level consequences. My research over the course of this

thesis has demonstrated that sulfoxaflor can be hazardous to bumblebees, and so future research is urgently required to understand the likelihood of bees being exposed.

7.2.2 Do other novel insecticides pose a threat to bees? Licensing process shortfalls.

I focused on the potential impact of sulfoxaflor on bumblebees for my PhD based on a horizon scanning exercise that identified sulfoxaflor as one of the greatest emerging threats to pollination services (Brown *et al.* 2016). However, other 'novel' insecticides could also replace neonicotinoids over large geographical ranges, and also pose a threat to pollinators.

Due to their lower toxicity to bees (Manjon *et al.* 2018), the neonicotinoids thiacloprid and acetamiprid are not included within the restrictions placed on other neonicotinoids (imidacloprid, thiamethoxam, clothianidin) within the European Union. Despite this, research has shown that thiacloprid can have sub-lethal impacts on individual bees, impairing honeybee (*A. mellifera*) navigation (Fischer *et al.* 2014), learning (Tison *et al.* 2017) and reducing the likelihood of the waggle dance behaviour (Tison *et al.* 2016). More importantly, thiacloprid exposure can have colony level consequences on bumblebee (*B. terrestris*) reproductive output and can reduce reproductive output by up to 46% (Ellis *et al.* 2017) (See figure 7.1). Less is known about the potential impact of acetamiprid but acetamiprid can reduce sucrose sensitivity, olfactory memory retention and can also impair locomotion in honeybees (*A. mellifera*) (El Hassani *et al.* 2008; Aliouane *et al.* 2009)

Similarly, flupyradifurone is a novel butanolide insecticide, which, as with neonicotinoids and sulfoxaflor, acts as an agonist of nicotinic acetylcholine receptors (Nauen *et al.* 2015). Flupyradifurone is also systemic and could also be used as a neonicotinoid replacement (Nauen *et al.* 2015). Laboratory experiments have shown that chronic flupyradifurone exposure can increase Asian honeybee (*A. cerana*) mortality, and can also impair honeybee learning, when exposed as a larvae or as an adult (Tan *et al.* 2017). In an experiment that considered how flupyradifurone interacts with other agrochemicals (the fungicide propiconazole), Tosi & Nieh (2019) demonstrated synergistic interactions between the two agrochemicals on honeybee (*A. mellifera*) behaviour and mortality. However, in one of the few studies to investigate the potential sub-lethal impacts of flupyradifurone on bees, Campbell *et al.* (2016) placed honeybee (*A. mellifera*) colonies next to buckwheat crops that have being sprayed with flupyradifurone or untreated control fields that had not been sprayed, finding no detectable effects of flupyradifurone on honeybee colonies (although the experiment only had four treated fields, so the results should be interpreted with caution, due to a small sample size).

The current available data suggests that thiacloprid, acetamiprid, and flupyradifurone could have similar impacts on bees to those observed with sulfoxaflor, and indeed other non-restricted neonicotinoids.

My results in this thesis, and those mentioned above, demonstrate that novel insecticides can be licenced for use despite the potential sub-lethal impacts they can have on bees. This is of concern because these sub-lethal effects can have severe consequences for colony reproductive output (Whitehorn *et al.* 2012; Rundlöf *et al.* 2015; Ellis *et al.* 2017; Siviter *et al.* 2018a); yet they go undetected by the regulatory risk assessment process.

When agrochemicals are licensed for use, Tier 1 assessments are initially conducted, to determine toxicity. Tier 1 assessments are based upon LD50 & LC50 exposure protocols and expose honeybees for a maximum of 96 hours. Based on these endpoints further (Tier 2) assessments (that consider sub-lethal impacts) will or will not be conducted (Sanchez-Bayo & Tennekes 2017). This means that insecticides can be licensed for use without regulators assessing (i) the potential impact of chronic insecticide exposure or (ii) understanding how agrochemicals influence non-*Apis* bees. Higher tiered field experiments, although vital for understanding the potential impact of agrochemicals on important pollinators, are expensive and difficult to standardise across countries. Indeed, experiments assessing the impact of neonicotinoids on honeybees have yielded varying results (Cutler *et al.* 2014; Rundlöf *et al.* 2015; Tsvetkov *et al.* 2017; Osterman *et al.* 2019), even within the same study (Woodcock *et al.* 2017). It is therefore vital that regulatory bodies and policy makers produce standard protocols for Tier 1 based assessments that are mandatory and assess the potential sub-lethal impacts of agrochemical exposure on non-*Apis* bees, at both acute and chronic exposure regimes, before agrochemicals are licensed for use.

In this thesis, I have recommended that microcolony-based designs should be used in tier 1 assessments to determine whether insecticides have sub-lethal impacts on bumblebees (Chapter 5). Prior to this thesis, no studies have considered the potential impact of agrochemicals on bumblebee larvae. What is perhaps more surprising is that only a few studies have attempted to rear bumblebee larvae *in vitro*, despite decades of research with honeybees (Crailsheim *et al.* 2013). My results in Chapter 6 showed that sulfoxaflor exposure, although not lethal to bumblebee larvae, can reduce bumblebee larval growth, demonstrating for the first time that insecticide exposure can have a negative impact on bumblebee larvae. Baseline larval mortality was high in my experiment, so this protocol needs further development to become a standardised methodology for the regulatory process. Bumblebee larvae are the future workers and sexuals of the colony however and it is vital the regulatory process should consider them. Given this, I recommend that future research build on my

methodology (Chapter 6) in order to determine how best to rear bumblebee larvae *in vitro*, with the aim of producing a standardised protocol that can be used within the regulatory framework.

7.2.3 Does sulfoxaflor impact other wild bees and beneficial insects?

Prior to this thesis there was no information available on the potential sub-lethal effects of sulfoxaflor exposure on bees. We now know that sulfoxaflor exposure can have negative sub-lethal impacts on bumblebee reproductive output. Consideration of the potential impacts of sulfoxaflor on other beneficial insects such as wasps, beetles, butterflies and hoverflies are also crucial and, as of yet, are not considered.

Two obvious candidate groups are solitary bees, such as mason bees (*Osmia lignaria* in North America and *O.bicornis* in Europe) and hoverflies. Previous research has demonstrated that insecticides, such as neonicotinoids, can have both lethal and sub-lethal consequences for solitary bees and hoverflies (Moens, De Clercq & Tirry 2011; Rundlöf *et al.* 2015; Sgolastra *et al.* 2018, 2019). Given their importance for commercial crop and wild-flower pollination (Jauker & Wolters 2008; Garibaldi *et al.* 2013; Rader *et al.* 2016; Horth & Campbell 2018) the arguments for developing the methodologies that safeguard bumblebees can also be applied to hoverflies and solitary bees. Evidence has shown that neonicotinoids can have sub-lethal impacts on individual butterflies (Gilburn *et al.* 2015; Basley & Goulson 2018; Whitehorn *et al.* 2018) (although including butterflies within the regulatory process would be controversial, given that certain butterfly species are considered to be pest species). While this is by no means an exhaustive list, there is clearly a need to develop protocols to assess the potential impact of insecticides on other beneficial insects.

7.3 General comments

7.3.1. Should sulfoxaflor be banned?

In 1962 Rachel Carson published her famous book *Silent Spring,* in which she outlined how organochlorine chemicals, such as DDT, were devastating wildlife (Carson 1962). Organochlorine pesticides were subsequently banned from agricultural use globally and now, as a result of Carson (1962), when an agrochemical is licenced for use, its potential lethal consequences on wildlife are considered.

In more recent times, researchers have demonstrated that neonicotinoids can have sub-lethal impacts on pollinators (Goulson *et al.* 2015), and that these sub-lethal impacts can have significant consequences at the population level. As a result, certain neonicotinoids (imidacloprid, clothianidin & thiamethoxam) are banned from outside agricultural use in the European Union. Bans and restrictions on insecticides, however, will only be effective if replacement insecticides such as

sulfoxaflor, do not have similar sub-lethal impacts on pollinators. The results from my thesis suggest that sulfoxaflor does have sub-lethal impacts on bumblebees.

Asking 'should sulfoxaflor be banned from agricultural use' is an obvious question, given my results, but I believe that a more urgent question we should consider is whether sulfoxaflor should have been licenced for use in the first place?

The first neonicotinoid that was licensed for agricultural use was imidacloprid in 1991, and it was widely used for more than two decades before its use was restricted (Bass *et al.* 2015). Other novel insecticides such as flupyradifurone can also be hazardous to bees (see section 7.2.2) but are being registered for use globally (Tosi & Nieh 2019). Likewise, sulfoxaflor can be potentially hazardous to bumblebees, despite now being registered for use in 81 countries globally.

The regulatory process by which insecticides are licenced for use is changing, at least in Europe, but, in its current form, is largely based on toxicity tests such as LD50 experiments. In order to safeguard pollination services experimental protocols such as those outlined in Chapters 5 & 6, need to be used and developed to assess the potential sub-lethal impacts of chronic insecticides exposure on non-*Apis* bees, prior to licencing. Furthermore, while worst case scenario experiments (spraying at higher than field realistic applications, during bloom) are important in determining the maximum sulfoxaflor residue levels found in treated crops, research that considers the residue levels in crops sprayed at field realistic applications should be mandatory. These data can then be used to determine if agrochemicals are hazardous at field realistic applications.

Ultimately, my results show that sulfoxaflor can be hazardous to bumblebees but data on the potential impacts of sulfoxaflor on bees, or indeed most novel agrochemicals, is limited, making comparisons between insecticides difficult (Figure 7.1). Therefore, instead of discussing whether individual chemicals should be banned from agricultural use or not, I believe we should focus on improving the regulatory process by which agrochemicals are licensed for use. Only then will we be able to safeguard pollinators from the unintended impacts of agrochemical use.

7.3.2. Can we have insecticide-free agriculture?

Ever since the second world war, and the development of organochlorine pesticides, farmers have been advised to use insecticides to protect their crops (Kogan 1998). Many agronomists are employed by insecticide companies, which will often recommend using agrochemicals. Previous research however, has shown (UN 2017; Lechenet *et al.* 2017) using insecticides is not only costly, but does not guarantee increased yields. An idea often suggested is that pesticides are required to feed our growing global population, and that pesticides are essential for reducing human hunger (UN 2017). Despite

this, in a damming report commissioned by the UN, it was stated that it was a 'myth' that agriculture is dependent on pesticides to feed the worlds growing population (UN 2017). Within the report the authors state that social-economic issues, such as distribution routes and poverty are more likely to increase human hunger than reducing pesticide use, as similar yields are achievable whilst still reducing pesticide use (UN 2017). The report also highlights the lobbying power that agrochemical companies have, particularly in developing countries (UN 2017).

So, can we sustain a growing global population without the insecticides that intensive agriculture is currently dependent on? Below I briefly review alternatives to intensive agriculture.

Organic food has been suggested as an alternative to intensive agriculture, and existing data suggest that organic food production can be more profitable than conventional farming (Crowder & Reganold 2015; Reganold & Wachter 2016). For example, a meta-analysis that used data collected over 40 years, from 55 countries across 5 continents found that organic farm production was 22-35% more profitable than conventional farming (Crowder & Reganold 2015). However, despite this, and the growing demand for organic food (Crowder & Reganold 2015), there is scepticism as to whether organic food production, without intensive agriculture, would suffice to feed a growing population (de Ponti, Rijk & van Ittersum 2012). Famously, in 1971, US Secretary of Agriculture Earl Butz is quoted as saying 'Before we go back to organic agriculture in this country, somebody must decide which 50 million Americans we are going to let starve or go hungry'. Indeed, a recently published paper that used a food systems approach, demonstrated that if agriculture was to become purely organic, more land would be required for agriculture, increasing deforestation (Muller *et al.* 2017). Despite this, the authors also highlight that organic food production could produce enough food without increasing the amount of land required for agriculture, if humans reduced animal product consumption, and also, more importantly, reduced food wastage (Muller *et al.* 2017). For example, in US alone the average person is predicted to waste 422g of food each day (Conrad *et al.* 2018). However, scepticism about organic farming is clearly deep-rooted, and while this idea is changing, it seems unlikely that organic farming can replace intensive agriculture on a large scale (de Ponti *et al.* 2012) in the absence of significant technological innovation and/or a change in the behaviour and preference of consumers. That being said, there is clearly a market for organic food (Crowder & Reganold 2015) and organic food production could be used to reduce pesticide use, alongside other farming strategies (Muller *et al.* 2017).

One way that pesticide use could be reduced is by breeding crops that are repellent or tolerant to plant pests (Mitchell *et al.* 2016). Despite public concern about the impact of genetically modified food, GM crops can increase crop yields and also reduce the quantity of insecticides used in agriculture

(Klümper & Qaim 2014; Pellegrino *et al.* 2018). A recent meta-analysis confirmed that in genetically modified soybean, maize, and cotton crops there was a 37% reduction in the quantity of insecticide used (Klümper & Qaim 2014). Bt crops are a well-known example of pest-resistant crops. In Bt crops, crops are modified to produce Bt toxins when attacked by pest-species. A meta-analysis that considered the impact of Bt cotton and maze on non-target invertebrates found that, when compared to fields that did not have Bt crops and were manged with pesticides (pyrethroids), Bt crops had a greater abundance of non-target, beneficial insects (although it should be noted that both groups had a lower abundance than the control groups where no treatment was made) (Marvier *et al.* 2007). A similar meta-analysis that specifically analysed the impact of Bt crops on non-target arthropods in cotton, maze and potato also found that arthropod abundance was greater in Bt treated crops (Wolfenbarger *et al.* 2008). Currently however, the majority of seed companies are owned by insecticide companies which focus on breeding crops with increased yields, so less attention is given to pest resistance, as pesticides can be used to control pests (UN 2017). For example, the development of genetically modified, herbicide resistant crops such as Roundup Ready crops actually promote increased herbicide use. Despite this, breeding and developing crops that are resistance or tolerant to unwanted pests is undoubtably a way of reducing insecticide use in farming (Mitchell *et al.* 2016).

Another suggested alternative to intensive agriculture is agroecology. In intensive agriculture, crops are often planted in unsuitable environments, making them more vulnerable to pests, which farmers control with agrochemicals (Dewar & Walters 2016). In agroecology both the environment and the biology of the plant are considered, so crops are planted in suitable environments, reducing the need for insecticides (Wezel *et al.* 2009). Agroecological pest management relies on promoting the crop's natural enemies for pest management, as a form of biological control, and habitat restoration is essential for promoting this (UN 2017). Agroecology relies heavily on understanding ecological processes and uses this to improve farmland biodiversity and ultimately increase the number of natural predators on a farm. For example, one of the problems with biological control is that predatory insects are more likely to reside in crop margins next to fields, so target pests might be free from predators in the centre of the field. Ongoing experiments at the Centre for Ecology and Hydrology involve sowing wild flowers strips through the middle of fields to increase the number of predatory insects within fields (see https://assist.ceh.ac.uk/). An alternative biological insect control mechanism is using the sterile insect technique (SIT) (Knipling 1955). In SIT sterile males of the unwanted pests are released into the environments whereby they compete with fertile males for reproduction, reducing the reproductive potential of fertile females. The birth of CRISPR-based technology could

improve the efficiency of SIT and it's possible that this form of biological control could become cost-effective, and prominent in the future (Kandul *et al.* 2019).

Integrated pest management (IPM) seems like an obvious way that consistent yields can be achieved whilst still reducing insecticide use. IPM uses above-mentioned control mechanisms such as biological control to control pests but monitors crops for pests much more frequently than in intensive agriculture. IPM uses an economic injury level (EIL) whereby the cost of using an insecticide and loss of crop as a result of a pest out-break are both considered. EIL levels vary between crops and location, but only when pest levels get higher than the EIL will insecticides bee used. An IPM approach reduces the amount of insecticide used in farms and so targets pests are less likely to develop resistance and farmers can also make significant savings from purchasing less insecticides (Bass *et al.* 2015).

Based on the above, I would argue that we already have a good understanding of how to reduce insecticide use in agriculture while still maintaining crop yields. However, reducing insecticide use in farming is as much an economic and cultural issue as it a biological one.

In my opinion IPM, and changing the culture around insecticide use, is the easiest way of reducing insecticide use in agriculture. By increasing crop monitoring with an IPM approach, the quantity of agrochemicals used in farming could fall. I demonstrated in this thesis and numerous other researchers have before done so before me, that agrochemical use is continually harming wildlife (Carson 1962; Goulson *et al.* 2015; Pisa *et al.* 2017). With bees it is easy to see the economic cost of this (Garibaldi *et al.* 2013; Rader *et al.* 2016; Horth & Campbell 2018; Martin *et al.* 2019). Pollination by animals is valued at an estimated US$361 billon (Lautenbach *et al.* 2012), and the cost of replacing these freely-provided services would be great (Potts *et al.* 2016).

While the cost of moving towards a pesticide reduced future might be greater in the short-term, the opportunity cost of not reducing pesticide use could be great. Ultimately, for long-term food security it is clear that insecticide use can and needs to be reduced within agriculture.

Conclusion

The original aim of this thesis was to determine whether sulfoxaflor exposure has sub-lethal impacts on bumblebees. The results demonstrate that sulfoxaflor can be hazardous to *B. terrestris,* influencing colony reproductive output, worker production, egg laying and larvae development. Bumblebees are crucial for both crop and wildflower pollination (Garibaldi *et al.* 2013; Rader *et al.* 2016; Martin *et al.* 2019) and any agrochemicals that are hazardous to bees could have direct impacts on pollination services, risking food security (Potts *et al.* 2016). Bumblebees are also in decline across large geographical ranges (Williams 1982; Biesmeijer *et al.* 2006; Fitzpatrick *et al.* 2007; Cameron *et al.*

2011; Schmid-Hempel *et al.* 2014; Powney *et al.* 2019) and my results suggest that sulfoxaflor application could contribute to bee declines, a grave concern for bumblebee conservation moving forward.

Given the economic and social importance of this charismatic insect group, my results highlight the urgent need to re-evaluate and change how insecticides are licenced for use. Failure to do so could result in the continual process by which insecticides are licenced for use without a true understanding of the potential environmental impact they can have.

Appendix Chapter 2: Sulfoxaflor exposure reduces bumblebee reproductive success

Harry Siviter, Mark J F Brown, Ellouise Leadbeater

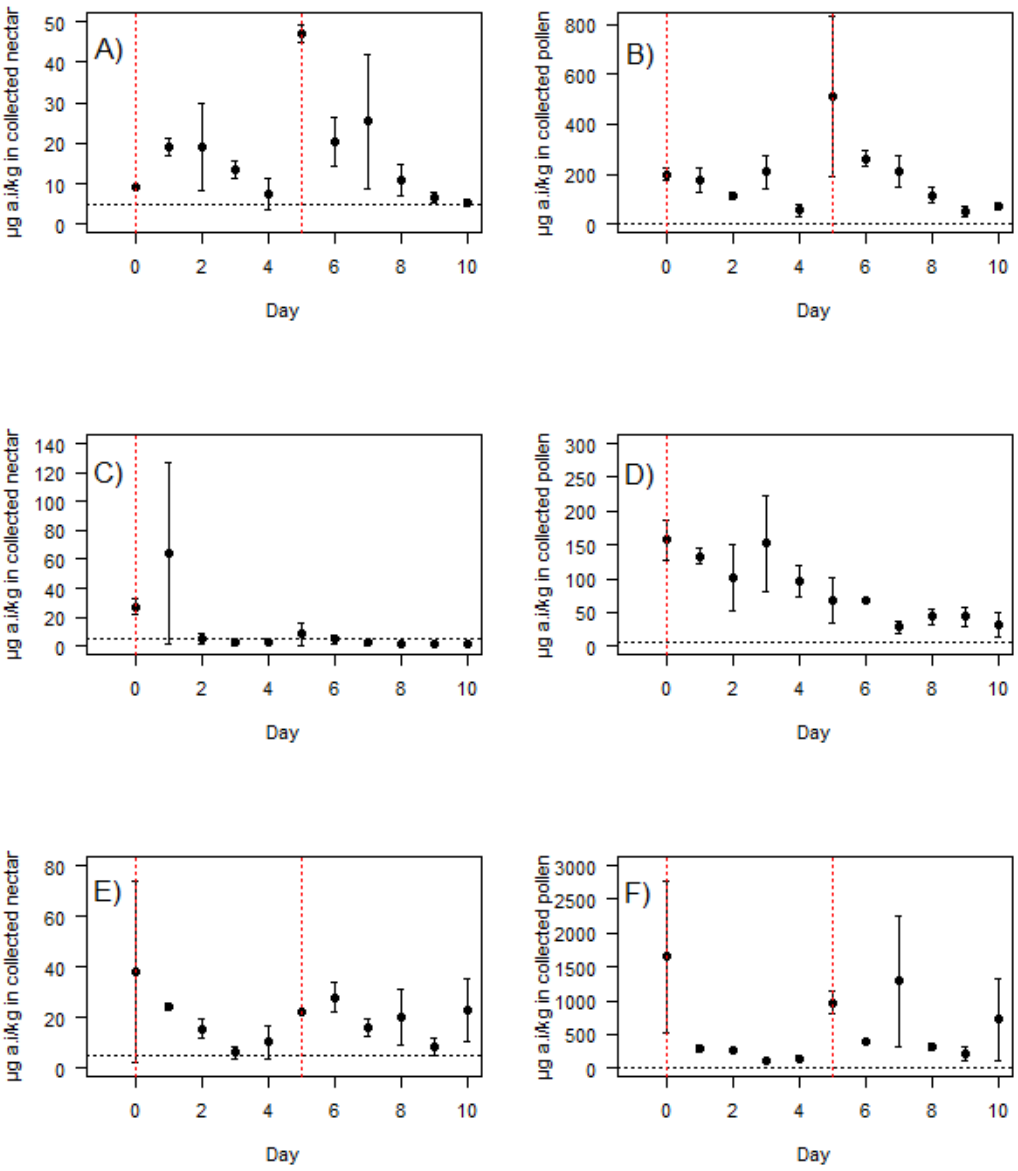
Siviter, H., Brown, M.J.F. & Leadbeater, E. (2018) Sulfoxaflor exposure reduces bumblebee reproductive success. *Nature*, **561**, 109–112.

Supplemental Analyses

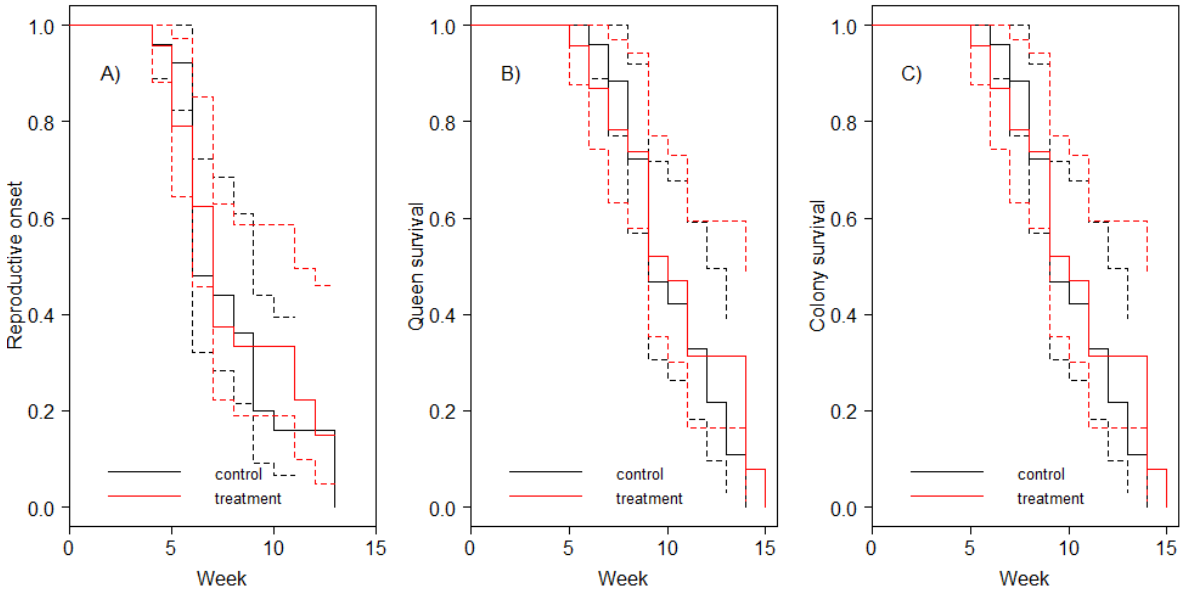
Colony mass: We found no evidence to suggest an overall effect of sulfoxaflor exposure on colony mass (lme: treatment parameter estimate = -0.00, 95% CI: -0.02 to 0.01; Figure S2.1), or on the number of nectar stores and pollen stores found within the nest (Nectar: glmmadmb: treatment: week parameter estimate = -0.01, 95% CL: -0.06 to 0.04; Pollen: glmmadmb: treatment:week parameter estimate = -0.00, 95% CL: -0.04 to 0.03); Figure S2.2 A & B). When observing returning workers, we recorded whether they carried small pollen loads or large pollen loads relative to the size of their body and found no differences in the proportion of large (glmer: treatment: week parameter estimate = -0.02, 95% CL: -0.19 to 0.16; Extended Data Fig.2.3) and small pollen loads (glmer: treatment: week parameter estimate = 0.02, 95% CL: -0.18 to 0.21) being brought back to the nest by workers.

In contrast to other pesticide studies, we used wild-caught queens, so colonies were placed in the field over a six-week period according to colony development. We included the date of placement ("week started") within all analyses, and found that week started significantly influenced the number of sexual offspring produced (Zero-Inflated Count Model: week started, parameter estimate = 0.08, 95% CL: 0.04 to 0.13; Figure S2.3). Colonies moved into the field during weeks two and three of the experiment produced fewer sexual offspring, most likely due to poor weather conditions during these placement weeks. Note that colonies were always moved into the field in pairs, and so treatments were balanced across weeks. There was no effect of week started on the number of nectar stores contained within the nest (glmmadmb: week started, parameter estimate = -0.06, 95% CL: -0.03 to 0.14;Figure S2.4A) but there was a significant effect on pollen stores (glmmadmb: week started, parameter estimate = 0.16, 95% CL: 0.08 to 0.24; Figure S2.4B) with colonies put out during weeks 3, 4 & 5 containing on average more pollen stores.

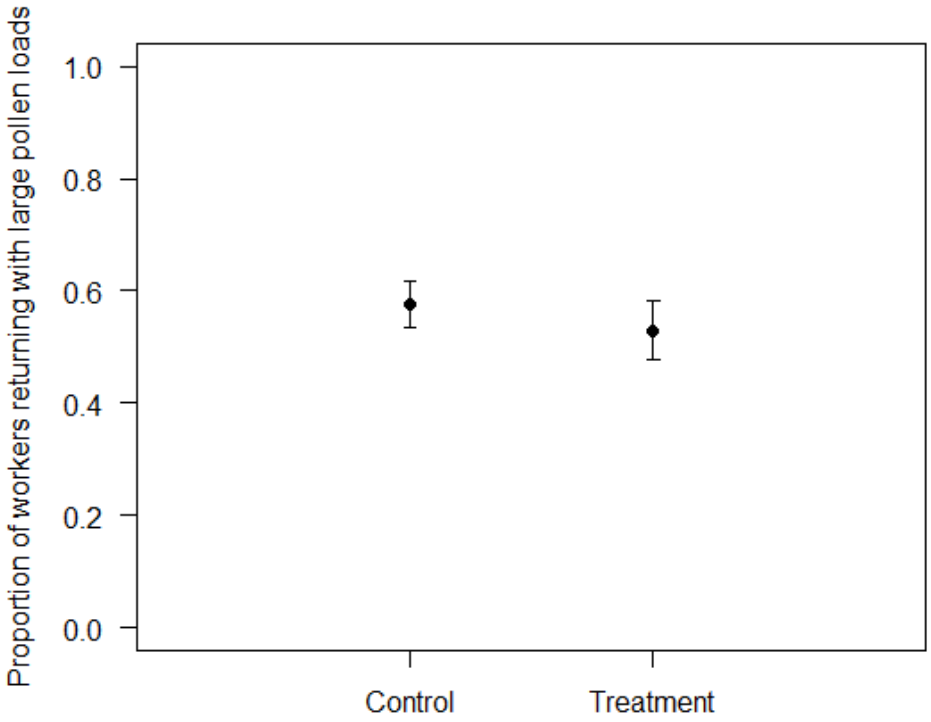
Our results also showed that a greater proportion of workers returned to the colony with pollen in the morning than in the afternoon (glm, time of day, parameter estimate = -0.36, CL: -0.65 to -0.08; Figure S2.5). Temperature also had a significant effect on the proportion of workers returning with pollen (glm, temperature, parameter estimate = 0.10, CL: 0.05 to 0.15).



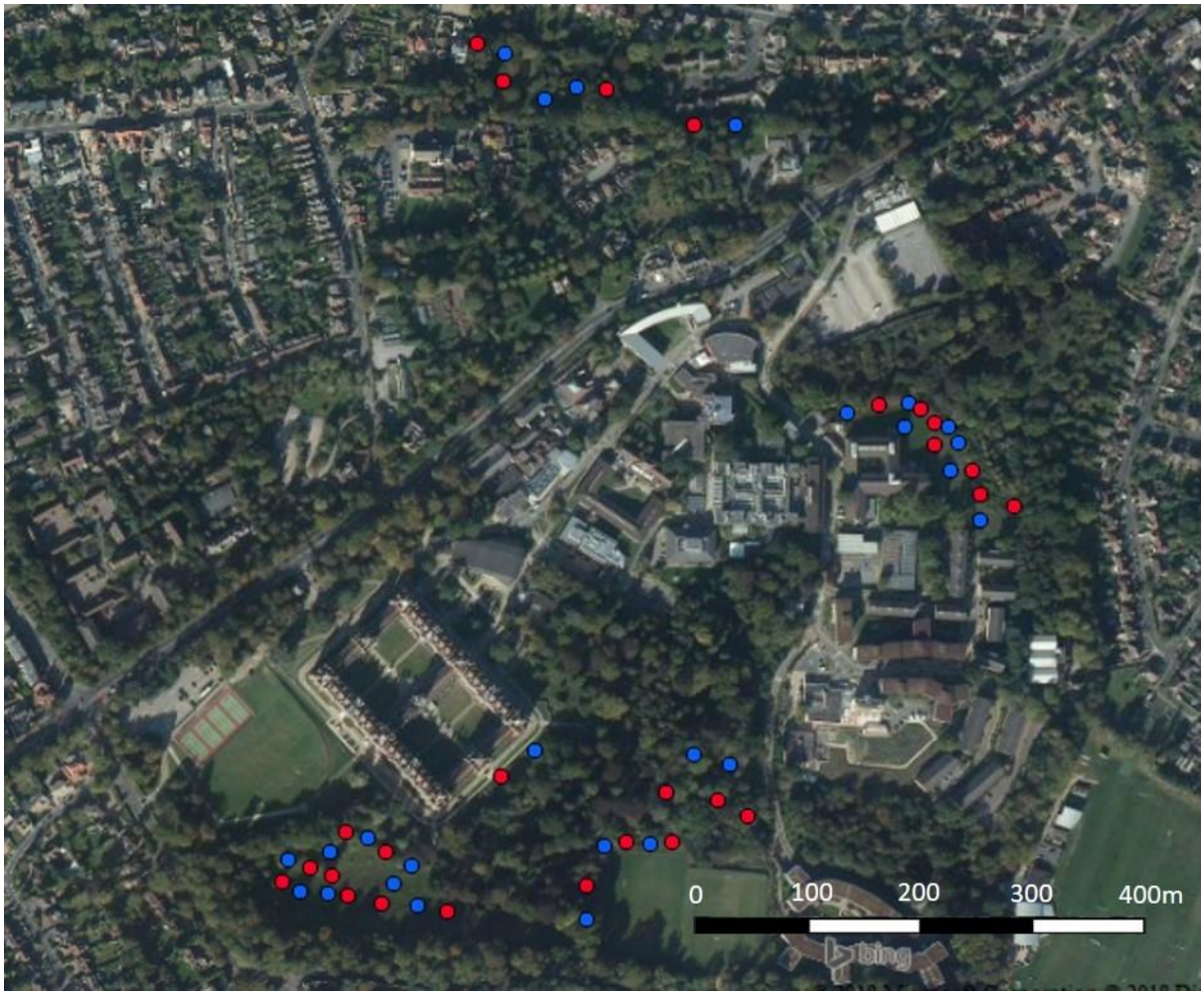
Extended data Fig.2.1: Concentrations of sulfoxaflor in forager-collected resources from a USA EPA cotton study(United States Environmental Protection Agency 2016)**. Mean µg of active ingredient (a.i.)/kg (± SE) found in the (A, C, E) nectar and (B, D, F) pollen of honeybees foraging on cotton crops sprayed with sulfoxaflor. Note the differences in y-axis scale between graphs, due to considerably higher concentrations in pollen. Red lines indicate spray application. Dosage: (A & B) twice over ten days at 0.045 lb a.i. per acre; (C & D) once over ten days at 0.045 lb a.i. per acre; (E & F) twice over ten days at 0.089 lb a.i. per acre. The black horizontal line indicates the equivalent amount of sulfoxaflor (5 ppb) that was fed to sulfoxaflor-treated colonies in sucrose, within our experiment. Data are means from two hives; number of individual bees sampled is not published.**



Extended data Fig.2.2: Timing of colony life-history events. The probability of (a) reproductive onset, (b) queen survival and (c) colony survival for control (n = 26) and sulfoxaflor-treated (n = 25) colonies (± confidence intervals).



Extended data Fig.2.3: Pollen foraging. The mean proportion (± SE) of foragers returning to the nest with large pollen loads, for control (n = 25) and pesticide-treated (n = 22) colonies (note that not all of the colonies in the experiment had pollen foragers).



Extended data Fig.2.4) Distribution of colonies across the Royal Holloway Campus. Blue dots indicate control colonies, red dots indicate treated colonies (grid reference; TQ000706; Imagery ©Google, Map data©2018 Google).

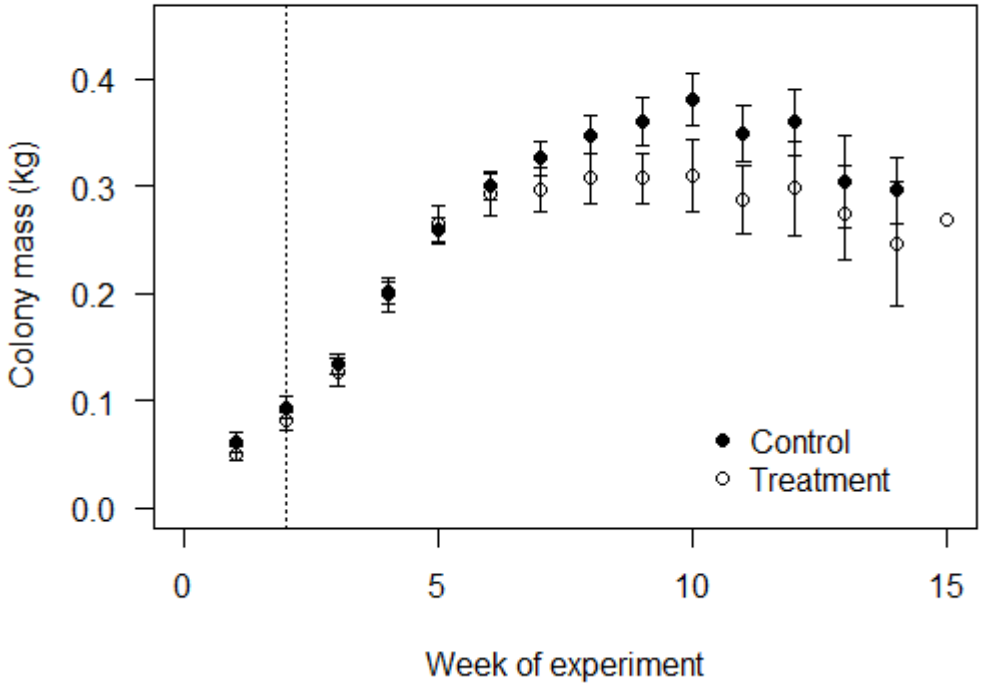


Figure S2.1: Changes in colony mass across the experimental period. Mean mass in kilograms (± SE) of control (n = 26) and treated colonies (n = 26).

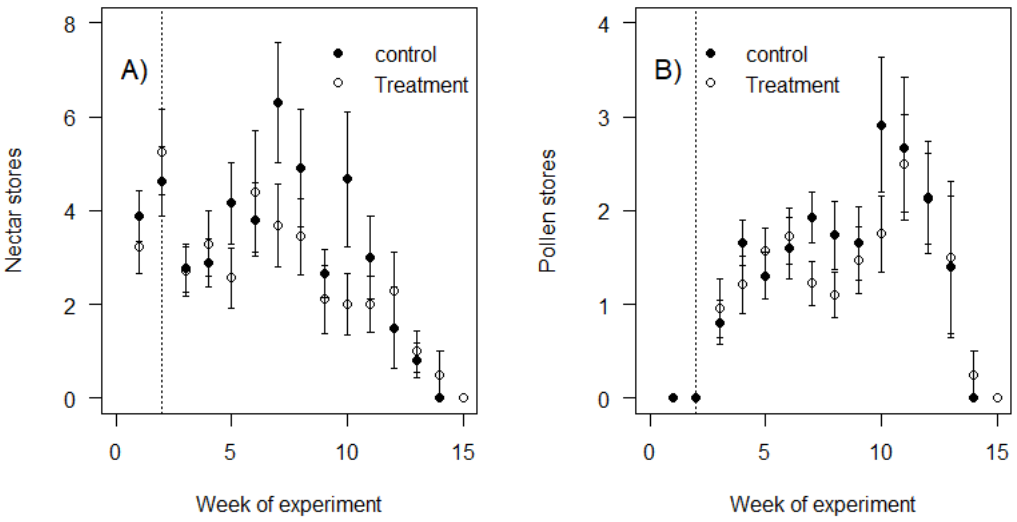


Figure S2.2: Nectar and pollen stores. The number (mean ± SE) of (A) nectar and (B) pollen pots in the nests of control (n =26) and treated colonies (n = 26).

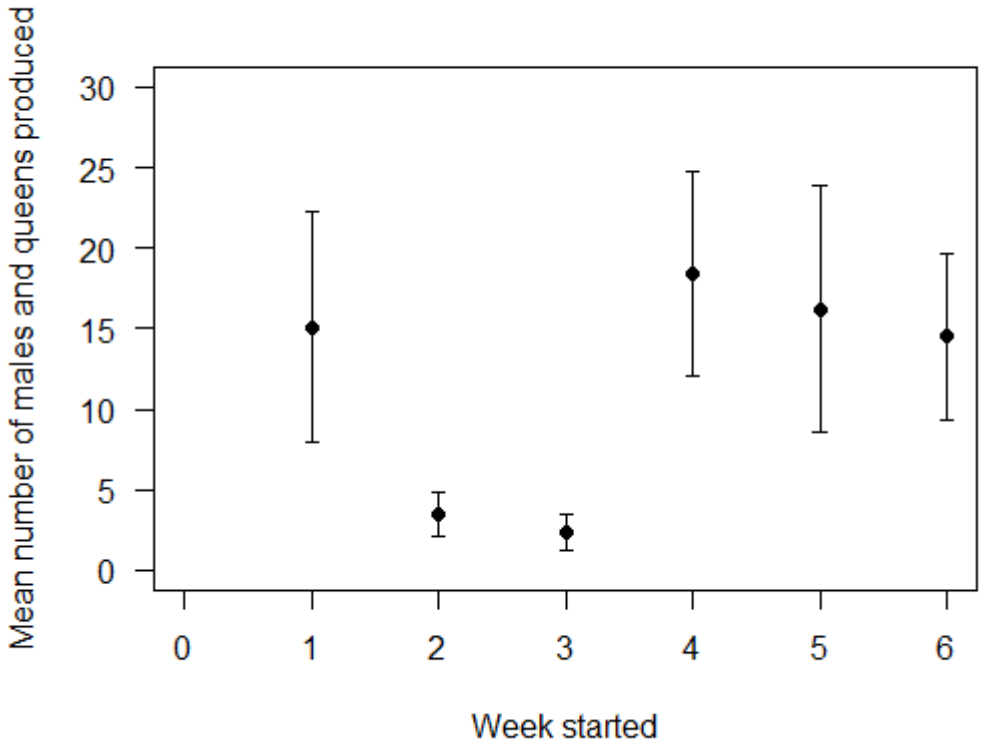


Figure S2.3: Number of males and queens produced in relation to experimental timing: Impact of the week in which the colony was first placed in the field on the mean number (± SE) of sexual offspring produced by colonies (Week started 1, 2, 3, 4 & 5, n = 12, 12, 6, 9, 5 & 7 respectively).

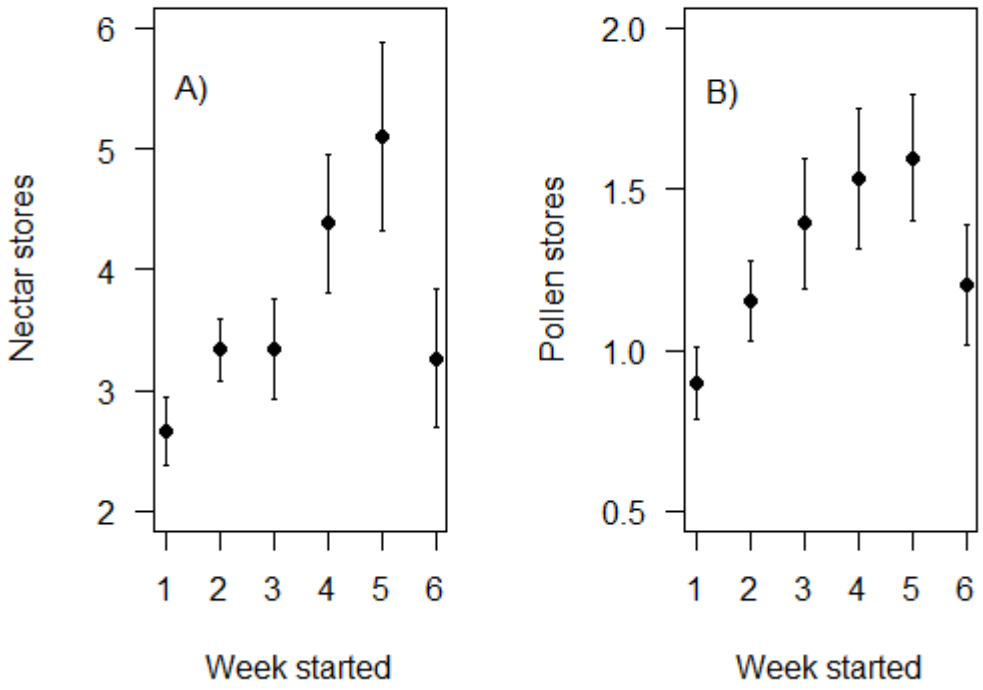


Figure S2.4: Nectar and pollen stores in relation to experimental timing. Mean number (± SE) of (A) nectar and (B) pollen pots produced, in relation to the week in which the colony was first placed in the field (Week started 1, 2, 3, 4 & 5, n = 12, 12, 6, 9, 5 & 8 respectively).

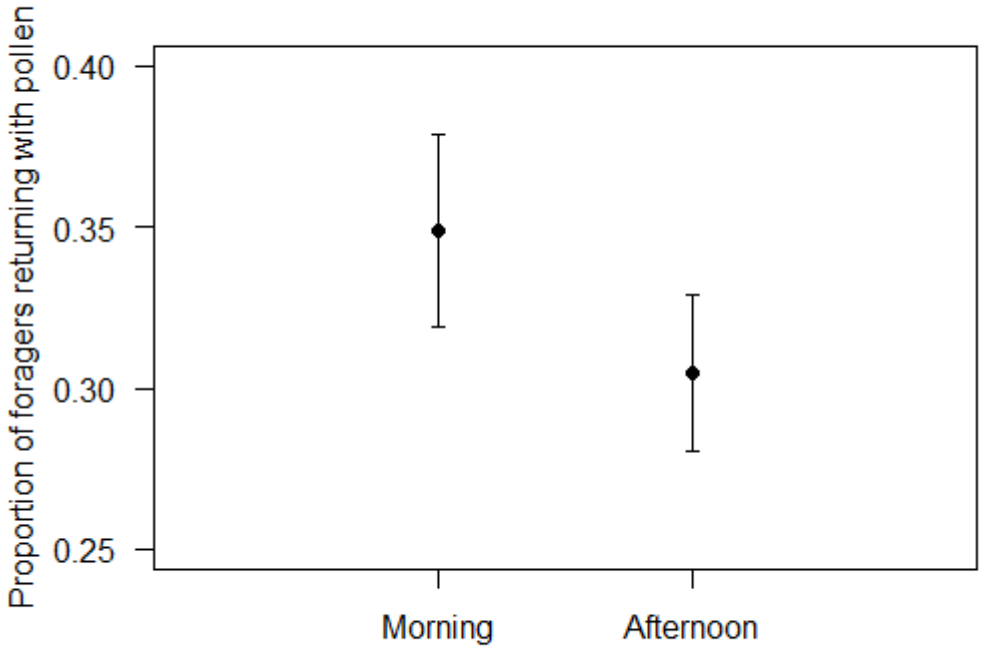


Figure S2.5: Pollen foraging across the day: Mean proportion (± SE) of worker bees returning to the nest with pollen in the morning (n = 552) compared to the afternoon (n = 390).

Table S2.1) All model sets included the full model (below), all subsets and a basic model (that included the intercept and random factors).'Week colony' refers to the week of experiment (eg week 4 represents the fourth week of the experiment, four weeks after exposure started and two weeks after it ended) and week^2 was included when

A) male size (biomass); analysed with a linear mixed model; colony and pair included as random factors
Full model = week + treatment + week started + treatment:week + treatment:week started

B) colony growth rates (weight gained); fitted linear model; colony and pair included as random factors
Full model = week colony + treatment + week started + treatment:week colony + treatment:week started + week^2 + week^2:treatment

C) sucrose consumed during exposure period; fitted linear model with pair included as a random factor
Full model = treatment + week started

D) number of workers; generalized linear mixed model with colony and pair included as random factors, poisson distribution
Full model = Treatment + scale(week started) + scale(week colony) + week^2 + treatment:week started + treatment:week colony + treatment:week^2

E) proportion of pollen foragers; generalized linear mixed model with colony and pair included as random factors, binomial distribution
Full model = treatment + week colony + week started + time of day + temperature + treatment*week colony

F) total reproductive output (males & queens); zero inflated count regressions
Full model = treatment + week started

G) males produced; zero inflated count regressions
Full model = treatment + week started

H) number of returning workers; generalized linear mixed model, using AD model builders with colony included as a random factor, negative binomial distribution
Full model = treatment + week colony + week started + temperature

I) nectar stores counted within nest; generalized linear mixed model, using AD model builders with colony and pair included as random factors, negative binomial distribution
Full model = treatment + week started + week colony + treatment:week colony + treatment:week started

J) pollen stores counted within nest; generalized linear mixed model, using AD model builders with colony included as a random factor, poisson distribution
Full model = treatment + week started + week colony + treatment:week colony + treatment:week started

K) reproductive onset (week colonies first produced sexuals); coxph survival analysis
Full model = treatment + week started

L) number of weeks queen survived; coxph survival analysis
Full model = treatment + week started

M) number of weeks colony survived; coxph survival analysis
Full model = treatment + week started

N) proportion of pollen foragers returning with large pollen loads; generalized linear mixed model with colony and pair included as random factors, binomial distribution
Full model = treatment + week colony + week started + time of day + average temperature + treatment*week colony

o) proportion of pollen foragers returning with small pollen loads; generalized linear mixed model with colony and pair included as random factors, binomial distribution
Full model = treatment + week colony + week started + time of day + average temperature + treatment*week colony

Table S2.2 Parameter estimates and 95% confidence intervals derived by model averaging across the confidence set of models. Parameters highlighted in bold have 95% confidence intervals that do not cross zero.

A) male size (biomass)	Estimate	Std. Error		
Intercept	0.29	0.01		
B) colony growth rate (weight gained)	Estimate	Std. Error	Lower CI	Upper CI
(Intercept)	0.12	0.01	0.09	0.14
week colony	**0.10**	**0.00**	**0.09**	**0.11**
week colony^2	**-0.01**	**0.00**	**-0.01**	**0.00**
treatment	0.00	0.01	-0.02	0.01
C) sucrose consumed during exposure period	Estimate	Std. Error		
(Intercept)	0.15	0.01		
D) number of workers	Estimate	Std. Error	Lower CI	Upper CI
(Intercept)	**3.37**	**0.15**	3.11	3.44
week colony	**-0.12**	**0.02**	**-0.15**	**-0.08**
week^2	**-0.46**	**0.02**	**-0.50**	**-0.42**
week started	-0.03	0.04	-0.18	0.08
treatment	**-0.28**	**0.17**	**-0.48**	**-0.01**
treatment:week started	0.01	0.04	-0.11	0.15
treatment:week colony	**-0.06**	**0.03**	**-0.11**	**-0.01**
treatment:week^2	**0.11**	**0.03**	**0.05**	**0.16**
E) proportion of pollen foragers	Estimate	Std. Error	Lower CI	Upper CI
(Intercept)	-2.10	0.43	-2.94	-1.26
treatment	0.46	0.43	-0.38	1.31
week colony	-0.02	0.05	-0.12	0.08
time of day	**-0.36**	**0.15**	**-0.65**	**-0.08**
average temperature	**0.10**	**0.03**	**0.05**	**0.15**
treatment:week colony	-0.14	0.07	-0.29	0.00
week started	0.01	0.03	-0.05	0.06
F) total reproductive output (males & queens)	Estimate	Std. Error	Lower CI	Upper CI
count (Intercept)	2.66	0.09	2.48	2.84
count treatment	**-0.64**	**0.09**	**-0.81**	**-0.46**
count week started	**0.08**	**0.02**	**0.04**	**0.13**
zero (Intercept)	-1.30	0.80	-2.86	0.27
zero treatment	0.71	0.70	-0.67	2.09
zero week started	-0.14	0.20	-0.53	0.26
G) males produced	Estimate	Std. Error	Lower CI	Upper CI
count (Intercept)	2.66	0.12	2.42	2.89
count treatment	**-0.54**	**0.09**	**-0.72**	**-0.37**
count week started	0.05	0.03	-0.01	0.12
zero (Intercept)	-1.37	0.77	-2.89	0.15
zero treatment	0.71	0.70	-0.67	2.09
zero week started	-0.11	0.19	-0.49	0.26
H) number of returning foragers	Estimate	Std. Error	Lower CI	Upper CI
(Intercept)	1.52	0.25	1.03	2.01
week colony	**-0.19**	**0.02**	**-0.23**	**-0.15**
temperature	-0.01	0.01	-0.04	0.02
week started	-0.02	0.04	-0.11	0.06
treatment	-0.07	0.13	-0.32	0.19
I) nectar stores counted within nest	Estimate	Std. Error	Lower CI	Upper CI
(Intercept)	1.46	0.20	1.07	1.86
treatment	-0.10	0.20	-0.50	0.30
week started	0.06	0.04	-0.03	0.14
week colony	-0.02	0.02	-0.06	0.02
treatment:week colony	-0.01	0.02	-0.06	0.04
treatment:week started	0.01	0.04	-0.06	0.08
J) pollen stores counted within nest	Estimate	Std. Error	Lower CI	Upper CI
(Intercept)	-0.97	0.21	-1.39	-0.55
week started	**0.16**	**0.04**	**0.08**	**0.24**
week colony	**0.14**	**0.02**	**0.11**	**0.18**
treatment	-0.03	0.18	-0.38	0.33
treatment:week colony	0.00	0.02	-0.04	0.03
treatment:week started	-0.01	0.03	-0.08	0.06
K) reproductive onset (week colonies first produced sexuals)	Estimate	Std. Error	Lower CI	Upper CI
week started	0.14	0.12	-0.10	0.38
treatment	-0.05	0.18	-0.41	0.31
L) number of weeks queen survived	Estimate	Std. Error	Lower CI	Upper CI
week started	0.23	0.12	0.00	0.46
treatment	-0.07	0.20	-0.47	0.33
M) number of weeks colony survived	Estimate	Std. Error	Lower CI	Upper CI
week started	0.15	0.15	-0.14	0.44
treatment	-0.03	0.21	-0.43	0.38
N) proportion of pollen foragers returning with large pollen load	Estimate	Std. Error	Lower CI	Upper CI
Intercept	-0.94	0.54	-2.00	0.13
average temperature	0.02	0.03	-0.04	0.09
week colony	0.01	0.02	-0.04	0.05
time of day	-0.01	0.08	-0.17	0.15
treatment	-0.02	0.09	-0.19	0.16
week started	0.00	0.02	-0.05	0.05
O) proportion of pollen foragers returning with a small pollen loa	Estimate	Std. Error	Lower CI	Upper CI
Intercept	-0.04	0.64	-1.29	1.22
average temperature	-0.05	0.04	-0.13	0.03
treatment	0.02	0.10	-0.18	0.21
time of day	0.01	0.09	-0.16	0.19
week started	0.00	0.03	-0.05	0.05
week colony	-0.01	0.03	-0.07	0.05

Appendix Chapter 3: No evidence for negative impacts of acute sulfoxaflor exposure on bee olfactory conditioning or working memory

Harry Siviter[†], Alfie Scott[†], Grégoire Pasquier, Christopher D Pull, Mark J F Brown & Ellouise Leadbeater

[†]These authors contributed equally

Siviter, H., Scott A, Pasquier G, Pull C D, Brown, M.J.F. & Leadbeater, E. (Accepted) No evidence for negative impacts of acute sulfoxaflor exposure on bee olfactory conditioning or working memory. *Peer J*

Impacts of sulfoxaflor exposure on bee behaviour

As in Lämsä *et al.* (2018) the time it took the bees to start flying once in the arena was used as a proxy of foraging motivation. Time to start flying was analysed used a linear mixed effect model, with treatment, bee size and their interaction included as fixed factors and covariates respectably. Colony was included as a random factor. There was one outlier in the 250ppb treatment (See Figure S3.1), which was removed, to improve model fit. The dependant variable was also square rooted to improve model fit.

As a proxy for foraging speed we analysed the time to drink from the first landing platform. As above, we analysed this using a linear mixed effect model, with treatment, bee size, their interaction as fixed factors and covariates respectably with colony included as a random factor. The dependant variable was also square rooted to improve model fit.

Results

We found no effect of sulfoxaflor exposure on time to start flying (Figure S3.1; lmer, 5ppb treatment PE = -0.24, 95% CI = -1.96 to 1.34; 10ppb PE = 0.24, 95% CI = -1.42 to 2.05; 250ppb PE = 1.16, 95% CI = -0.19 to 3.16) or foraging speed (Figure S3.2; lmer, 5ppb treatment PE = -2.40, 95% CI = -15.55 to 10.74; 10ppb PE = -3.37, 95% CI = -20.03 to 13.28; 250ppb PE = -3.18, 95% CI = -19.19 to 12.81).

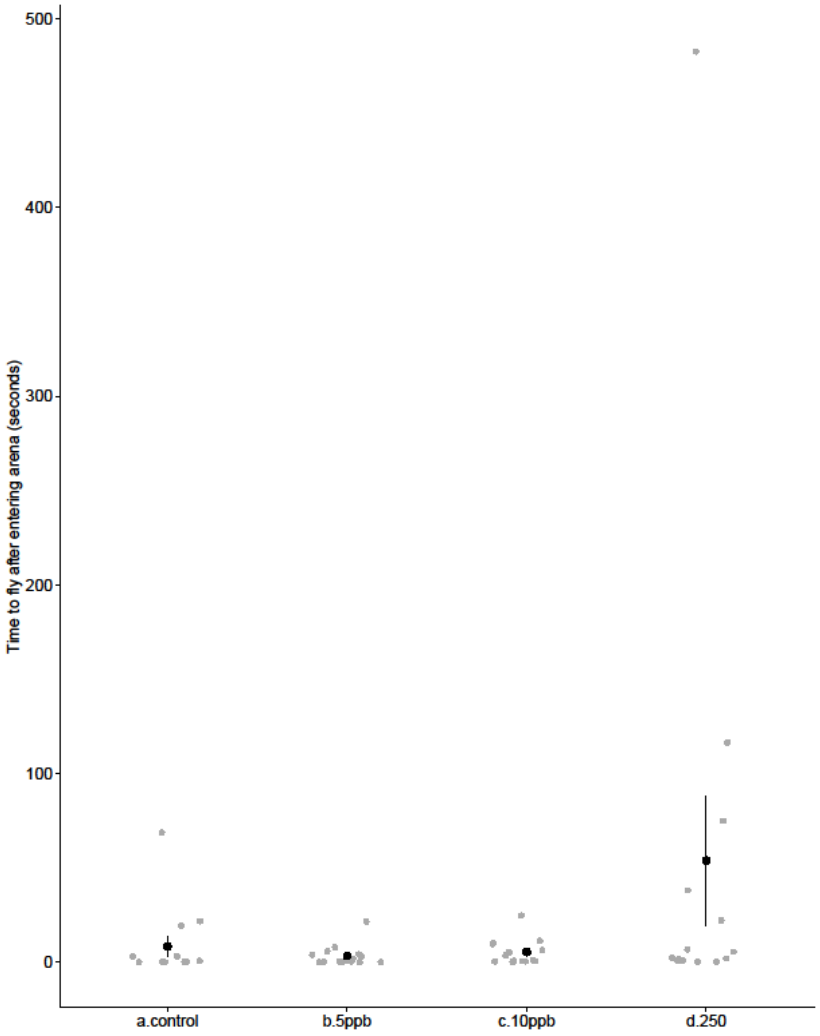


Figure S3.1: Bumblebee performance on the radial arm maze: Time (seconds) that is took bees to drink from the first flower.

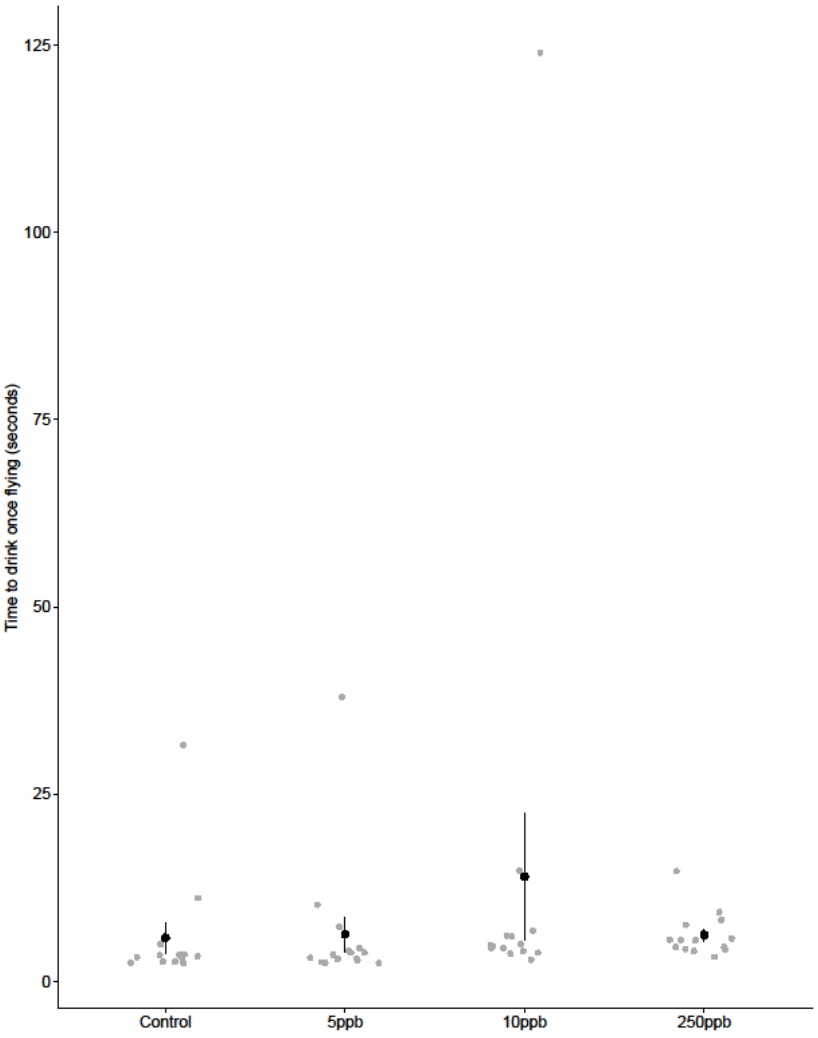


Figure S3.2: Bumblebee performance on the radial arm maze: Time (seconds) that is took bees to drink once they started flying.

Table S3.1: Table of candiate models used for each analysis.

A) Bumblebee PER, binomial learning; analysed with a generalized linear mixed effect model (binomial); colony included as random factor	AICc	ΔAICc	*w* i
Null model	**129.7**	**0**	**0.658**
Bee size	**131.7**	**1.94**	**0.249**
Treatment	**134.3**	**4.6**	**0.066**
Treatment + Bee size	136.2	6.5	0.026
Treatment + Bee size + Treatment:Bee size	142.4	12.65	0.001
B) Honeybee PER, binomial learning; analysed with a generalized linear mixed effect model (binomial); colony included as random factor	AICc	ΔAICc	*w* i
Null model	**134.4**	**0**	**0.54**
Bee size	**136.5**	**2.1**	**0.189**
Treatment	**137.3**	**2.91**	**0.126**
Treatment + Bee size + Treatment:Bee size	**137.7**	**3.34**	**0.102**
Treatment + Bee size	139.4	5.06	0.043
C) Bumblebee PER, learning level; analysed with a generalized linear mixed effect model (poisson); colony included as random factor	AICc	ΔAICc	*w* i
Null model	**215.8**	**0**	**0.533**
Bee size	**216.2**	**0.4**	**0.437**
Treatment	222.7	6.85	0.017
Treatment + Bee size	223.3	7.47	0.013
Treatment + Bee size + Treatment:Bee size	229.9	14.12	0
D) Honeybee PER, learning level; analysed with a generalized linear mixed effect model (poisson); colony included as random factor	**AICc**	**ΔAICc**	***w* i**
Bee size	**284**	**0**	**0.492**
Null model	**285.1**	**1.11**	**0.283**
Treatment + Bee size	**287.6**	**3.61**	**0.081**
Treatment + Bee size + Treatment:Bee size	**287.7**	**3.66**	**0.079**
Treatment	**288.1**	**4.06**	**0.065**
E) Bumblebee PER, Speed to learn	**AICc**	**ΔAICc**	***w* i**
Null model	**413.3**	**0**	**0.587**
Bee size	**414.6**	**1.28**	**0.309**
Treatment	**417.8**	**4.46**	**0.063**
Treatment + Bee size	418.8	5.5	0.038
Treatment + Bee size + Treatment:Bee size	423.2	9.9	0.004
F) Honeybee PER, Speed to learn	**AICc**	**ΔAICc**	***w* i**
Null model	**391.7**	**0**	**0.579**
Bee size	**393.7**	**2.05**	**0.208**
Treatment	**394.6**	**2.94**	**0.133**
Treatment + Bee size	396.8	5.11	0.045
Treatment + Bee size + Treatment:Bee size	397.3	5.66	0.034
G) Bumblebee PER, Memory 3H	**AICc**	**ΔAICc**	***w* i**
Null model	**58.9**	**0**	**0.64**
Bee size	**60.8**	**1.87**	**0.251**
Treatment	**63.2**	**4.27**	**0.075**
Treatment + Bee size	65	6.13	0.03
Treatment + Bee size + Treatment:Bee size	69.1	10.25	0.004
H) Honeybee PER, Memory 3H	**AICc**	**ΔAICc**	***w* i**
Null model	**101.5**	**0**	**0.526**
Bee size	**102**	**0.47**	**0.416**
Treatment	107.1	5.55	0.033
Treatment + Bee size	107.8	6.22	0.023
Treatment + Bee size + Treatment:Bee size	113.8	12.27	0.001
I) Bumblebee PER, Memory 24H	**AICc**	**ΔAICc**	***w* i**
Null model	**50.9**	**0**	**0.716**
Bee size	**53.2**	**2.27**	**0.23**
Treatment	56.6	5.65	0.042
Treatment + Bee size	59.3	8.36	0.011
Treatment + Bee size +Treatment:Bee size	67.3	16.32	0
J) Honeybee PER, Memory 24H	**AICc**	**ΔAICc**	***w* i**
Null model	**96.5**	**0**	**0.453**
Bee size	**97.9**	**1.4**	**0.225**
Treatment	**98**	**1.43**	**0.222**
Treatment + Bee size	**99.6**	**3.08**	**0.097**
Treatment + Bee size +Treatment:Bee size	106.5	9.94	0.003
K) RAM, total revisits	**AICc**	**ΔAICc**	***w* i**
Null model	**321.7**	**0**	**0.468**
Treatment	**323**	**1.37**	**0.236**
Bee size	**323.2**	**1.51**	**0.22**
Treatment + Bee size	**325.4**	**3.75**	**0.072**
Treatment + Bee size + Treatment:Bee size	330.5	8.8	0.006
L) RAM , correct in first 8	**AICc**	**ΔAICc**	***w* i**
Null model	**229**	**0**	**0.706**
Bee size	**231.1**	**2.13**	**0.244**
Treatment	234.8	5.84	0.038
Treatment + Bee size	237.2	8.23	0.012
Treatment + Size + Treatment:Bee size	244.7	15.74	0
M) RAM , correct before revisit	**AICc**	**ΔAICc**	***w* i**
Treatment	**356.6**	**0**	**0.437**
Null model	**357.3**	**0.72**	**0.306**
Treatment + Bee size	**358.9**	**2.27**	**0.141**
Bee size	**359.4**	**2.76**	**0.11**
Treatment + Bee size + Treatment:Bee size	365.2	8.57	0.006

Table S3.2. Parameter estimates and 95% confidence intervals derived by model averaging across the confidence set of models. Parameters highlighted in bold have 95% confidence intervals that do not cross zero.

A) Bumblebee PER, binomial learning	Estimate	Std. Error	Lower CI	Upper CI
Intercept	-0.50	1.65	-3.78	2.77
Bee size	0.06	0.31	-0.55	0.68
Treatment (2.4 ppb)	0.00	0.17	-0.34	0.33
Treatment (10 ppb)	0.00	0.18	-0.35	0.36
Treatment (250 ppb)	0.05	0.24	-0.43	0.53
B) Honeybee PER, binomial learning	Estimate	Std. Error	Lower CI	Upper CI
(Intercept)	1.06	6.13	-11.06	13.19
Bee size	-0.27	1.57	-3.38	2.84
Treatment (2.4 ppb)	-1.30	6.51	-14.19	11.60
Treatment (10 ppb)	-1.26	6.85	-14.82	12.31
Treatment (250 ppb)	-7.32	23.30	-53.10	38.45
Bee size:Treatment (2.4 ppb)	0.28	1.64	-2.98	3.53
Bee size:Treatment (10 ppb)	0.31	1.76	-3.18	3.81
Bee size:Treatment (250 ppb)	1.91	6.05	-9.98	13.79
C) Bumblebee PER, learning learning	Estimate	Std. Error	Lower CI	Upper CI
(Intercept)	0.37	1.11	-1.84	2.57
Bee size	0.14	0.21	-0.29	0.56
D) Honeybee PER, learning learning	Estimate	Std. Error	Lower CI	Upper CI
(Intercept)	4.46	3.89	-3.25	12.18
Bee size	-2.37	2.67	-7.66	2.92
Treatment (2.4 ppb)	1.18	4.77	-8.23	10.59
Treatment (10 ppb)	1.05	4.05	-6.93	9.04
Treatment (250 ppb)	0.31	2.20	-4.11	4.72
Bee size	0.04	0.28	-0.53	0.61
Bee size:Treatment (2.4 ppb)	-0.33	1.26	-2.81	2.15
Bee size:Treatment (10 ppb)	-0.29	1.08	-2.42	1.83
Bee size:Treatment (250 ppb)	-0.09	0.57	-1.23	1.06
E) Bumblebee PER, Speed to learn	Estimate	Std. Error	Lower CI	Upper CI
Bee size	0.11	0.28	-0.41	1.12
Treatment (2.4 ppb)	0.00	0.11	-0.93	0.78
Treatment (10 ppb)	0.00	0.12	-0.91	0.87
Treatment (250 ppb)	0.03	0.15	-0.39	1.22
F) Honeybee PER, Speed to learn	Estimate	Std. Error	Lower CI	Upper CI
Bee size	0.01	0.56	-1.09	1.12
Treatment (2.4 ppb)	-0.11	0.31	-0.72	0.51
Treatment (10 ppb)	-0.02	0.16	-0.34	0.29
Treatment (250 ppb)	-0.01	0.15	-0.30	0.28
G) Bumblebee PER, Memory 3H	Estimate	Std. Error	Lower CI	Upper CI
(Intercept)	-2.38	3.14	-8.65	3.90
Bee size	0.16	0.58	-1.00	1.33
Treatment (2.4 ppb)	0.02	0.30	-0.59	0.63
Treatment (10 ppb)	-0.07	0.45	-0.98	0.83
Treatment (250 ppb)	0.06	0.34	-0.62	0.75
H) Honeybee PER, Memory 3H	Estimate	Std. Error	Lower CI	Upper CI
(Intercept)	-5.93	7.58	-20.89	9.03
Bee size	2.71	2.13	-2.65	5.05
I) Bumblebee PER, Memory 24H	Estimate	Std. Error	Lower CI	Upper CI
(Intercept)	-1.45	2.42	-6.34	3.45
Bee size	0.05	0.48	-0.92	1.02
J) Honeybee PER, Memory 24H	Estimate	Std. Error	Lower CI	Upper CI
(Intercept)	2.70	5.76	-8.72	14.12
Bee size	-0.61	1.49	-3.57	2.36
Treatment (2.4 ppb)	-0.39	0.71	-1.79	1.02
Treatment (10 ppb)	-0.36	0.66	-1.66	0.94
Treatment (250 ppb)	0.04	0.42	-0.79	0.88
K) RAM, total revisits	Estimate	Std. Error	Lower CI	Upper CI
(Intercept)	1.96	1.43	-0.88	4.79
Treatment (5 ppb)	0.24	0.41	-0.57	1.05
Treatment (10 ppb)	0.16	0.31	-0.46	0.79
Treatment (250 ppb)	0.23	0.39	-0.55	1.00
Bee size	-0.08	0.23	-0.53	0.37
L) RAM correct in first 8	Estimate	Std. Error	Lower CI	Upper CI
(Intercept)	1.53	0.51	0.51	2.54
Bee size	0.01	0.09	-0.16	0.18
M) RAM , correct before revisit	Estimate	Std. Error	Lower CI	Upper CI
Treatment (5 ppb)	0.55	0.56	-0.54	1.64
Treatment (10 ppb)	0.25	0.37	-0.48	0.98
Treatment (250 ppb)	0.49	0.52	-0.52	1.51
Bee size	0.00	0.19	-0.38	0.38

Appendix Chapter 4: Quantifying the impact of pesticides on learning and memory in bees

Harry Siviter, Julia Koricheva, Mark J F Brown, Ellouise Leadbeater

Siviter, H., Koricheva, J., Brown, M.J.F. & Leadbeater, E. (2018) Quantifying the impact of pesticides on learning and memory in bees. *Journal of Applied Ecology*, **55**, 2812–2821.

Sensitivity analysis

When the analysis was re-run using just data collected on honey bees, effects of pesticides on learning were significantly stronger for above field realistic dosages ($p < 0.001$) than for field realistic dosages (Figure S4.2). There were also significant difference between the effects of pesticide dosages on bee memory ($p = 0.04$) although both field realistic dosages still had an impact (Figure S4.2). There was no detectable difference between effects of chronic and acute exposure of pesticide on bee learning ($p = 0.11$) although there were differences in effects of chronic and acute exposure to pesticides on memory ($p < 0.01$) with chronic exposure resulting in stronger negative effects. We found no statistical differences in the learning score of ($p = 0.09$) or memory of ($p = 0.47$) *A. mellifera* and *A. cerana* and no differences between neonicotinoid pesticides and others (learning, $p = 0.53$; memory, $p = 0.1$). The results also showed no difference between short-term and long-term memory ($p = 0.47$).

We also re-ran the analysis excluding results that used a combination of pesticides (learning n = 2, memory n = 2). There were no significant differences in the learning data between bees that had been acutely and chronically exposed to pesticides ($p = 0.73$). There were significant differences between field realistic and above field realistic exposure ($p < 0.01$) with field realistic dosages having a stronger impact (field realistic, d = -0.16, 95% CL = -0.28 to -0.04; above field realistic, d = -0.36, 95% CL = - 0.47 to -0.25) and significant differences between *Apis* and *Bombus* ($p < 0.05$). The memory results did not change, as there were significant differences between pesticide effects on memory of acutely and chronically exposed bees ($p < 0.05$), significant differences between field realistic and non-field realistic exposure ($p < 0.05$), but not *Apis* and *Bombus* ($p = 0.15$), or short term and long-term memory ($p = 0.67$).

Figure S4.1: Modified PRISMA flowchart.

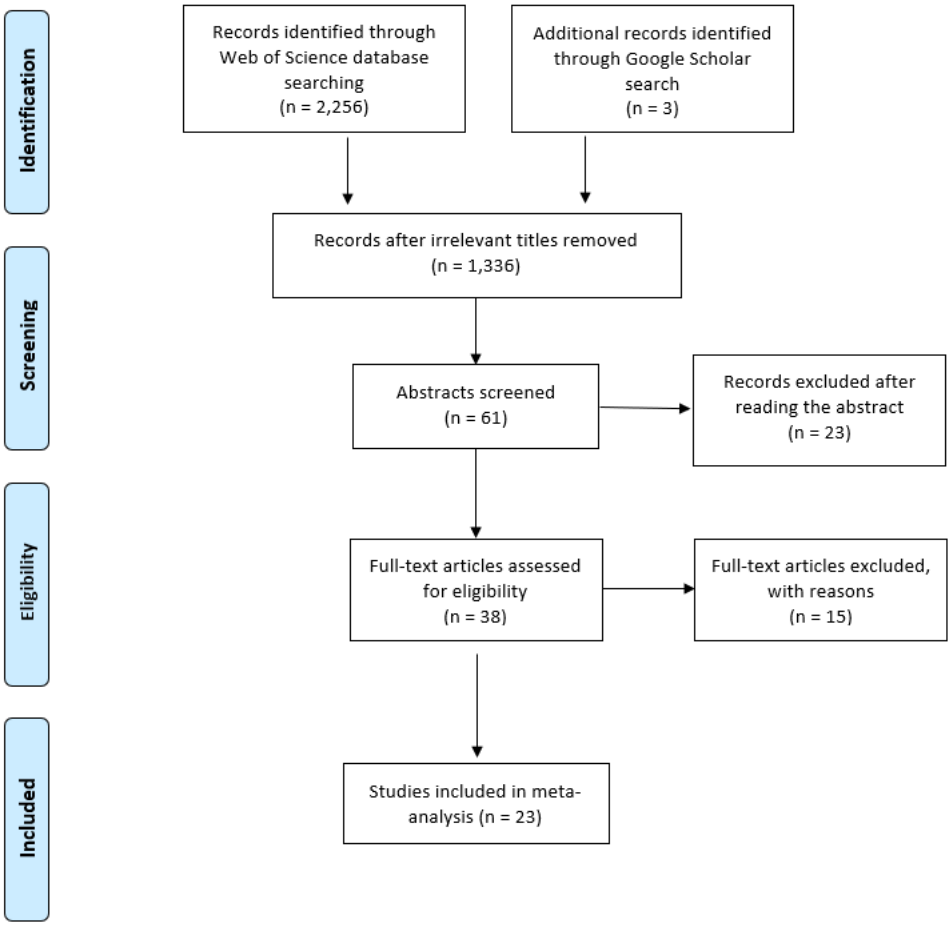


Table S4.1. Full list of the papers and reasons why some were not included.

Study number	Reference	Included	Reason for exclusion
1)	(Alkassab & Kirchner 2016)	✓	
2)	(Piiroinen *et al.* 2016)	✓	
3)	(Urlacher *et al.* 2016)	✓	
4)	(Stanley *et al.* 2015b)	✓	
5)	(Goñalons & Farina 2015)	✓	
6)	(Wright, Softley & Earnshaw 2015)	✓	
7)	(Chakrabarti *et al.* 2015)	X	N values not specific and acquisition results not reported
8)	(Tan *et al.* 2015)	✓	
9)	(Frost, Shutler & Hillier 2013)	✓	
10)	(Tan *et al.* 2013)	✓	
11)	(Williamson *et al.* 2013)	✓	
12)	(Williamson & Wright 2013)	✓	
13)	(Yang *et al.* 2012)	✓	
14)	(Ciarlo *et al.* 2012)	✓	
15)	(Schneider, Eisenhardt & Rademacher 2012)	X	Not a pesticide
16)	(Han *et al.* 2010)	✓	
17)	(Aliouane *et al.* 2009)	✓	
18)	(Ramirez-Romero *et al.* 2008)	X	No N values
19)	(El Hassani *et al.* 2008)	✓	
20)	(Ramirez-Romero, Chaufaux & Pham-Delegue 2005)	X	Results for effects of pesticides on learning are not clear
21)	(Decourtye *et al.* 2005)	X	Not clear which n values go with which treatment group
22)	(Abramson *et al.* 2004)	X	N values are not clear
23)	(Decourtye *et al.* 2004b)	X	N values are not clear and no SD
24)	(Decourtye *et al.* 2003)	X	N value are a range – not specific
25)	(Weick & Thorn 2002)	X	Injection not oral
26)	(Decourtye *et al.* 2001)	X	Could not gain access
27)	(Abramson *et al.* 1999)	X	No SD
28)	(Burden *et al.* 2016)	X	Metal not pesticides
29)	(Piiroinen & Goulson 2016)	✓	
30)	(Thany *et al.* 2015)	✓	
31)	(Abramson *et al.* 2012)	✓	
32	(Tan *et al.* 2017)	✓	

33	(Tison *et al.* 2017)	✓	
34	(Papach *et al.* 2017)	✓	
35	(Hesselbach & Scheiner 2018)	✓	
36	(Li *et al.* 2017)	X	N values given as a range
37	(Bonnafe *et al.* 2017)	X	Bees tested 11 months after pesticide exposure or up to 21 days after. Not comparable to the other studies
38	(Rix & Christopher Cutler 2017)	X	Topically exposed not orally

Table S4.2. The average and maximum concentrations of pesticides found in the pollen and nectar of crops and plants and in the nectar and pollen content found in bee colonies (Glaberman & White 2014; Sanchez-Bayo & Goka 2014; Bonmatin *et al.* 2015)

Pesticide	Pesticide Type	Average (ppb)	Maximum (ppb)	Reference
Acetamiprid	Neonicotinoid	12.266	112.8	(Sanchez-Bayo & Goka 2014)
Chlorpyrifos	Organophosphate	18.25	830	(Sanchez-Bayo & Goka 2014)
Clothianidin	Neonicotinoid	6.61	319	(Sanchez-Bayo & Goka 2014; Bonmatin *et al.* 2015)
Coumaphos	Phosphorothioate	105.5	5917	(Sanchez-Bayo & Goka 2014)
Deltamethrin	Pyrethroid	4 .6	91	(Sanchez-Bayo & Goka 2014)
Fipronil	Phenylpyrazole	33.6	70	(Bonmatin *et al.* 2015)
Flumethrin	Pyrethroid	6.7	158	(Sanchez-Bayo & Goka 2014)
Flupyradifurone	Butenolide	113.6	1800	(Glaberman & White 2014)
Imidacloprid	Neonicotinoid	8.43	912	(Sanchez-Bayo & Goka 2014; Bonmatin *et al.* 2015)
Tau-fluvalinate	Pyrethroid (synthetic)	15.9	2670.0	(Sanchez-Bayo & Goka 2014)
Thiacloprid	Neonicotinoid	41.86	187.6	(Sanchez-Bayo & Goka 2014; Bonmatin *et al.* 2015)
Thiamethoxam	Neonicotinoid	9.584	162.1	(Sanchez-Bayo & Goka 2014; Bonmatin *et al.* 2015)

Table S4.3 The average and maximum concentrations of pesticide (ppb) residue found in the nectar content of flowers/crops and in nectar found in bee colonies. Field realistic acute exposure for both honey bees and bumblebees were worked out using the average amount of nectar ingested while a bee foraged. Values are based on Sanchez-Bayo & Goka (2014) and Bonmatin et al. (2015)

Pesticide	Pesticide Type	Average (ppb)	Maximum (ppb)	Honey bee average (ng/40mg)	Honey bee maximum (ng/40mg)	Bumble bee average (ng/37.7 mg)	Bumble bee maximum (ng/37.7 mg)
Acetamiprid	Neonicotinoid	2.4	2.4	0.096	0.096	0.090	0.090
Clothianidin	Neonicotinoid	7.765	319	0.310	12.76	0.292	12.026
Chlorpyrifos	Organophosphate	3.9	15	0.156	0.6	0.147	0.565
Coumaphos	Phosphorothioate	105.5	2020	4.22	80.8	3.977	76.154
Deltamethrin	Pyrethroid	4.6	6.7	0.184	0.268	0.173	0.252
Fipronil	Phenylpyrazole	33.6	100	1.344	4.0	1.266	3.77
Flumethrin	Pyrethroid	6.7	158	0.268	6.32	0.252	5.956
Flupyradifurone	Butenolide	131.5	1500	5.26	60	4.95	56.55
Imidacloprid	Neonicotinoid	5.226	95.2	0.209	3.808	0.197	3.589
Tau-fluvalinate	Pyrethroid (synthetic)	15.9	750	0.636	30	0.599	28.275
Thiacloprid	Neonicotinoid	4.15	6.5	0.166	0.26	0.156	0.245
Thiamethoxam	Neonicotinoid	4.054	20	0.162	0.8	0.152	0.754

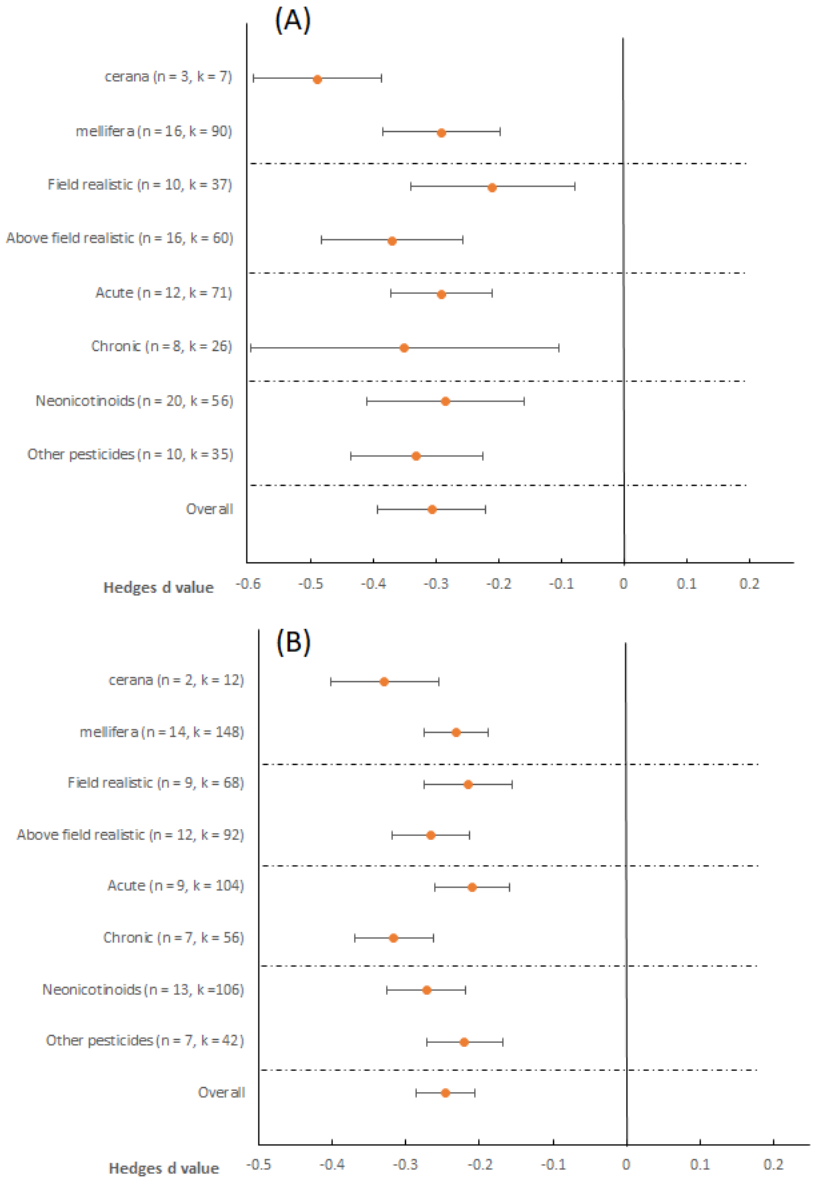


Figure S4.2. Mean effect size estimates (± 95% confidence intervals), for subsets of the data when only the *Apis* data were analysed, for impacts of pesticides on (a) learning and (b) memory. Number of studies (n) and number of effect sizes (k) are given for each subgroup.

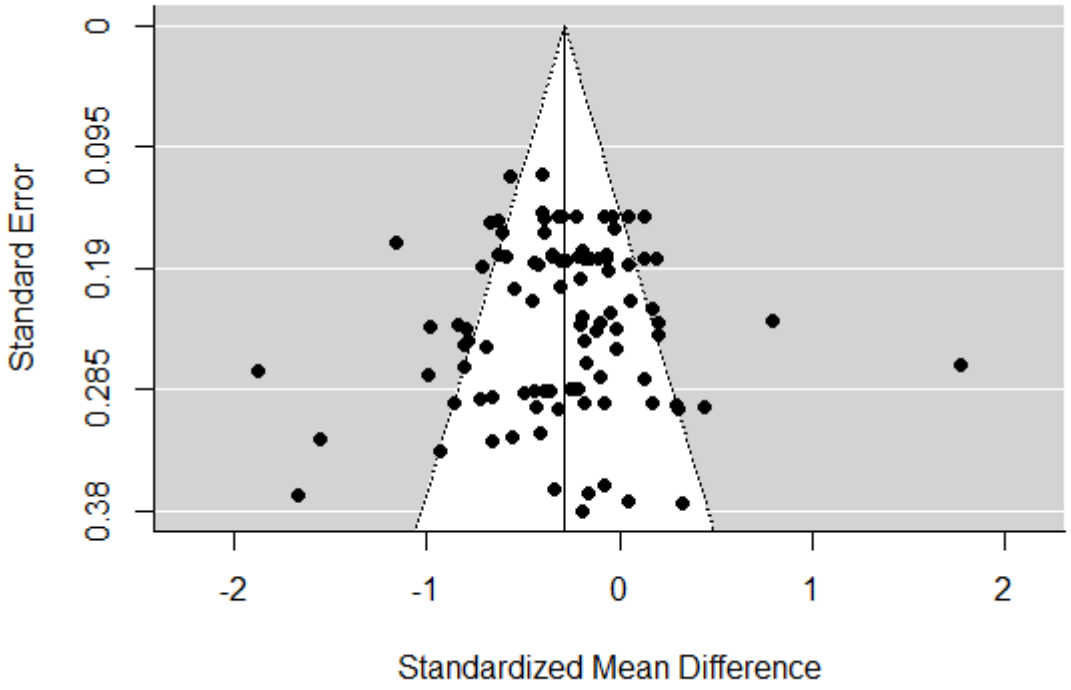


Figure S4.3: Funnel plot for the learning data.

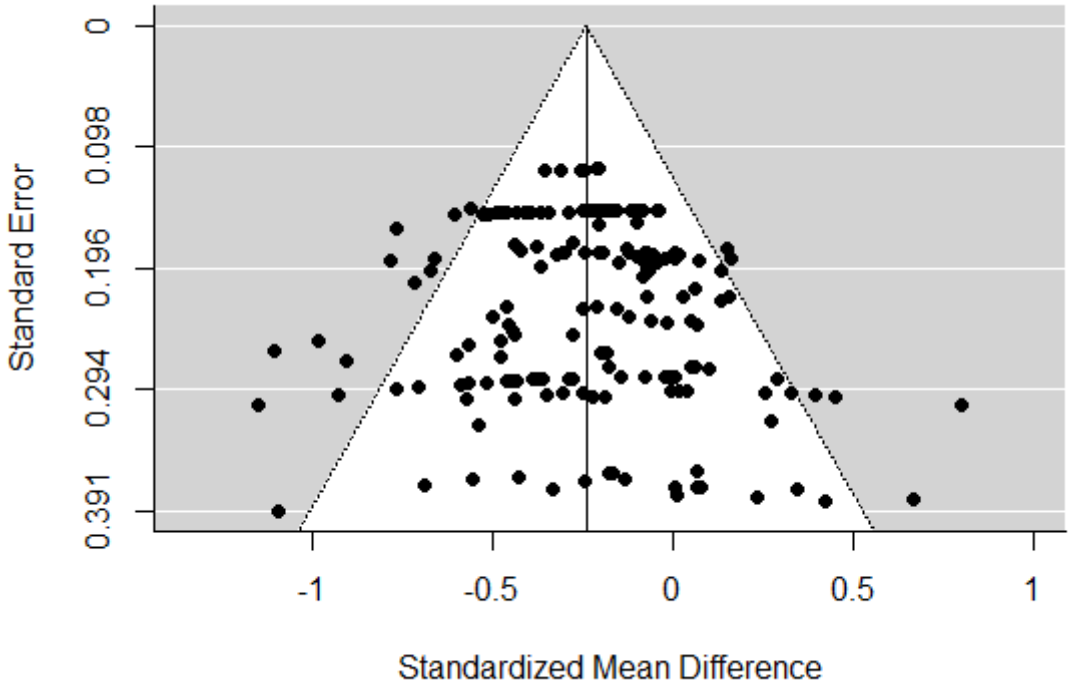


Figure S4.4 Funnel plot for the memory data

Appendix Chapter 5: Sulfoxaflor exposure reduces egg laying in bumblebees (*Bombus terrestris*)

Harry Siviter[†]*, Jacob Horner*[†]*, Mark J F Brown, Ellouise Leadbeater*

[†]These authors contributed equally

Siviter, H., Horner J., Brown, M.J.F. & Leadbeater, E. (Under review) Sulfoxaflor exposure reduces egg laying in bumblebees (*Bombus terrestris*) *Journal of Applied Ecology*

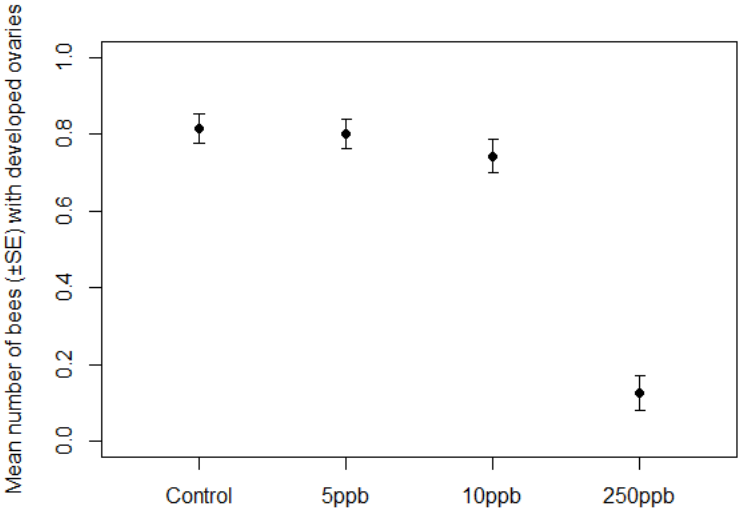


Figure S5.1: The mean proportion (± SE) of bees which had developed ovaries.

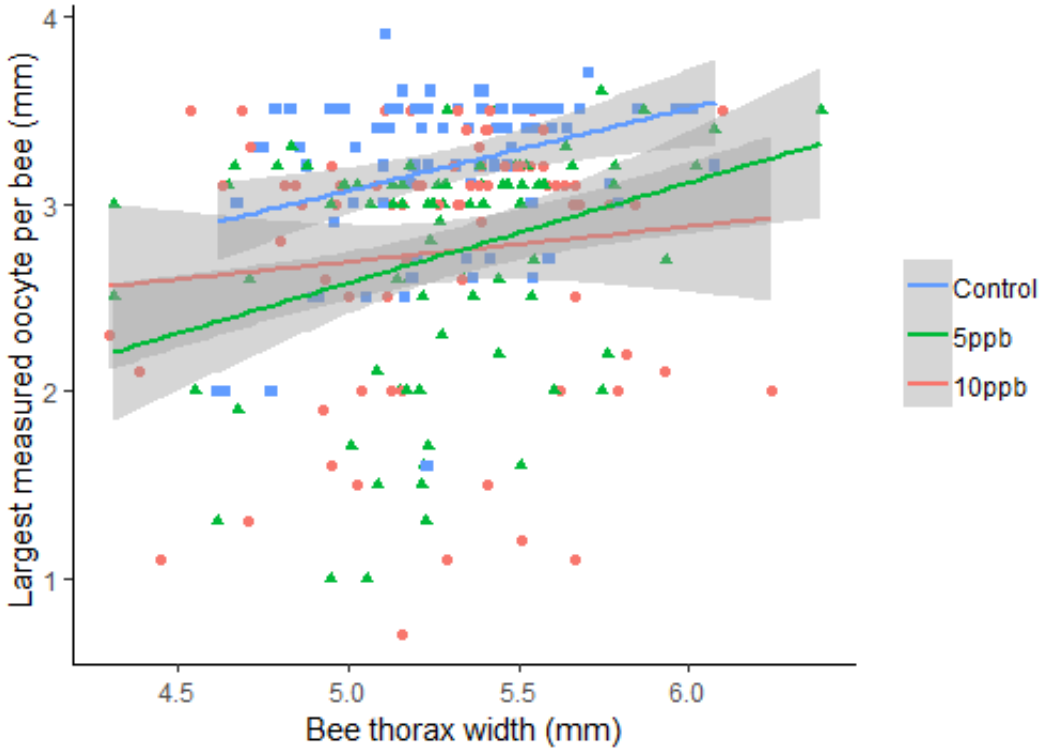


Figure S5.2: The maximum oocyte length per bee plotted against bee thorax width.

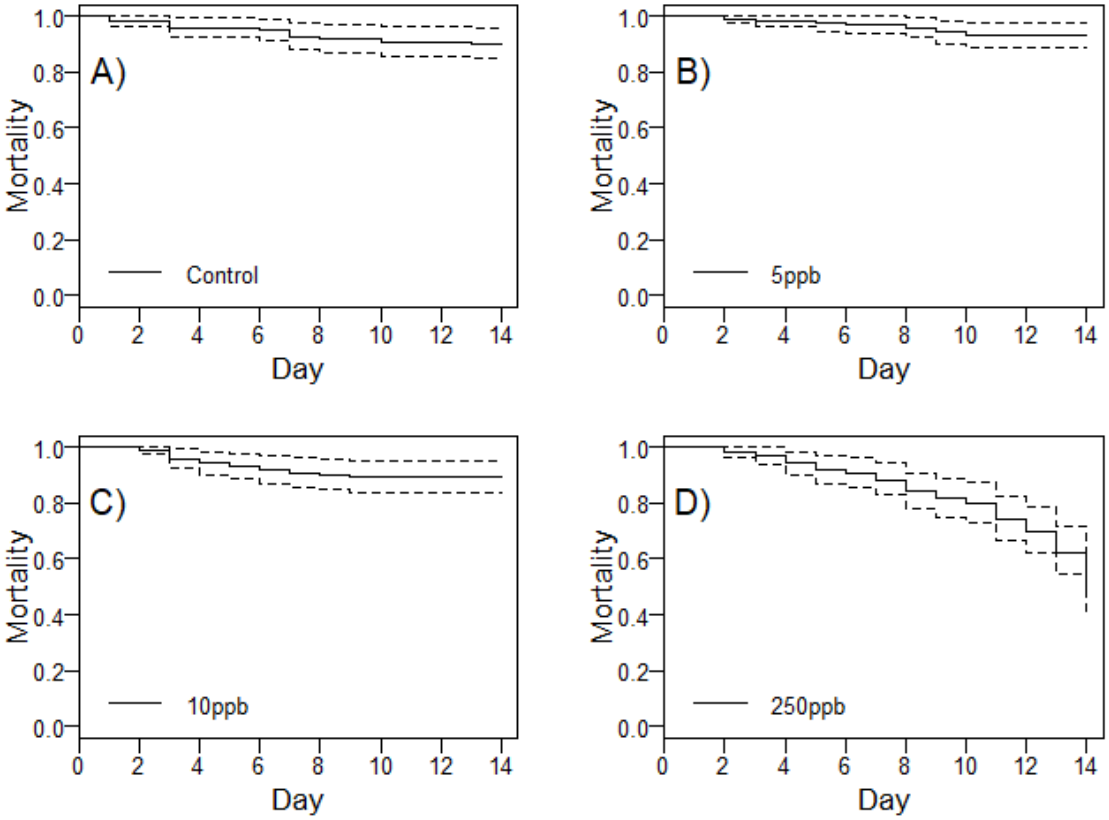


Figure S5.3: Kaplan-Meier curves for indiviudal bee mortality in each treatment group (A = control, B = 5ppb, C = 10ppb, D = 250ppb).

Table S5.1: Table of candiate models used for each analysis. Bold indicates that the model was selected within the anlaysis (wi ≥ 0.05)

A) Latency to lay eggs: Mixed effect cox model (coxme). Colony of origin and microcolony included as random factors	**AICc**	**ΔAICc**	***w*i**
null model	**509.10**	**0.00**	**1.00**
treatment	601.60	92.50	0.00
B) Sucrose drank per bee (lmer): Colony of origin and microcolony included as random factors	**AICc**	**ΔAICc**	***w*i**
treatment + day	**-1303.90**	**0.00**	**1.00**
treatment + day + treatment:day	-1279.50	24.40	0.00
day	-1275.50	28.33	0.00
treatment	-1250.70	53.16	0.00
null model	-1221.80	82.11	0.00
C) Egg production (glmmTMB hurdle model): Colony of origin included as a random factor	**AICc**	**ΔAICc**	***w*i**
treatment	**849.20**	**0.00**	**1.00**
null model	871.90	22.67	0.00
D) Larvae production (glmmTMB hurdle model): Colony of origin included as a random factor	**AICc**	**ΔAICc**	***w*i**
treatment	**439.90**	**0.00**	**0.94**
null model	**445.50**	**5.56**	**0.06**
E) Ovary development (glmer, binomial distribution): Colony of origin and microcolony included as random factors	**AICc**	**ΔAICc**	***w*i**
treatment + bee size	**323.20**	**0.00**	**1.00**
treatment	362.60	39.38	0.00
bee size	405.70	82.50	0.00
null model	436.50	113.32	0.00
F) Mean oocyte size per bee (lmer): Colony of origin and microcolony included as random factors	**AICc**	**ΔAICc**	***w*i**
treatment + bee size	**397.10**	**0.00**	**0.85**
treatment + bee size + treatment:bee size	**400.50**	**3.42**	**0.15**
treatment	414.70	17.55	0.00
bee size	443.20	46.11	0.00
null model	462.10	65.01	0.00
G) Maximum ooyte size per bee (lmer) : Colony of origin and microcolony included as random factors	**AICc**	**ΔAICc**	***w*i**
treatment + bee size	**443.30**	**0.00**	**0.89**
treatment + bee size + treatment:bee size	**447.80**	**4.52**	**0.09**
treatment	451.50	8.20	0.02
bee size	461.70	18.41	0.00
null model	470.20	26.93	0.00
H) Worker survival (coxme) : Colony of origin and microcolony included as random factors	**AICc**	**delta**	**weight**
treatment	**1034.30**	**0.00**	**1.00**
null model	1110.70	76.39	0.00

Table S5.2. Parameter estimates and 95% confidence intervals derived by model averaging across the confidence set of models. Parameters highlighted in bold have 95% confidence intervals that do not cross zero. Results correct to 2 decimal places.

A) Latency to lay eggs	Estimate	Std. Error	Lower CI	Upper CI
5ppb	-0.41	0.31	-1.02	0.19
10ppb	-0.45	0.30	-1.04	0.14
250ppb	**-4.40**	**1.03**	**-6.42**	**-2.38**
B) Sucrose drank per bee	**Estimate**	**Std. Error**	**Lower CI**	**Upper CI**
(Intercept)	0.49	0.04	0.41	0.57
5ppb	**-0.09**	**0.04**	**-0.17**	**-0.02**
10ppb	-0.06	0.04	-0.13	0.02
250ppb	**-0.23**	**0.04**	**-0.31**	**-0.16**
day	**0.01**	**0.00**	**0.01**	**0.02**
C) Egg production	**Estimate**	**Std. Error**	**Lower CI**	**Upper CI**
(Intercept)	2.92	0.06	2.80	3.04
5ppb (count)	**-0.16**	**0.08**	**-0.31**	**-0.01**
10ppb (count)	-0.10	0.07	-0.24	0.04
250ppb (count)	**-1.30**	**0.31**	**-1.91**	**-0.70**
(Intercept zero)	-1.53	0.77	-3.03	-0.03
5ppb (zero)	0.89	0.69	-0.45	2.24
10ppb(zero)	0.48	0.70	-0.89	1.84
250ppb (zero)	4.88	1.05	2.82	6.93
D) Larvae production	**Estimate**	**Std. Error**	**Lower CI**	**Upper CI**
(Intercept count)	2.00	0.16	1.68	2.31
5ppb (count)	0.04	0.13	-0.22	0.30
10ppb(count)	**-0.38**	**0.17**	**-0.69**	**-0.12**
(Intercept zero)	-0.69	0.39	-1.46	0.08
5ppb (zero)	**1.24**	**0.54**	**0.16**	**2.32**
10ppb(zero)	0.96	0.53	-0.10	2.02
E) Ovary development	**Estimate**	**Std. Error**	**Lower CI**	**Upper CI**
(Intercept)	-11.88	2.34	-16.47	-7.28
5ppb	0.15	0.44	-0.72	1.02
10ppb	-0.42	0.43	-1.25	0.42
250ppb	**-4.74**	**0.71**	**-6.14**	**-3.34**
bee size	**2.61**	**0.46**	**1.72**	**3.51**
F) Mean oocyte size per bee	**Estimate**	**Std. Error**	**Lower CI**	**Upper CI**
(Intercept)	0.31	0.59	-0.85	1.47
5ppb	-0.73	0.55	-1.81	0.36
10ppb	-0.42	0.67	-1.73	0.89
bee size	**0.46**	**0.11**	**0.24**	**0.67**
bee size:5ppb	0.02	0.10	-0.18	0.23
bee size:10ppb	-0.04	0.13	-0.28	0.21
G) Maximum oocyte size per bee	**Estimate**	**Std. Error**	**Lower CI**	**Upper CI**
(Intercept)	1.14	0.62	-0.09	2.36
5ppb	-0.46	0.42	-1.29	0.37
10ppb	-0.31	0.57	-1.44	0.82
bee size	**0.38**	**0.12**	**0.15**	**0.61**
bee size:5ppb	0.01	0.08	-0.15	0.16
bee size:10ppb	-0.02	0.11	-0.24	0.19
H) Worker survival	**Estimate**	**Std. Error**	**Lower CI**	**Upper CI**
5ppb	-0.46	0.50	-1.44	0.52
10ppb	0.13	0.45	-0.75	1.01
250ppb	**2.07**	**0.37**	**1.35**	**2.79**

Appendix Chapter 6: Sulfoxaflor and *Nosema bombi* exposure impair growth of early-stage bumblebee (*Bombus terrestris*) larvae

Harry Siviter, Arran J Folly, Mark J F Brown & Ellouise Leadbeater

Siviter, H., Folly, A.J., Brown, M.J.F. & Leadbeater, E. (*In prep*) Sulfoxaflor exposure reduces egg laying in bumblebees (*Bombus terrestris*) Planned submission to *Proceedings B*

Experiment S6.1: Designing the bumblebee larvae feeding protocol

Methodology

Three bumblebee colonies were purchased from Biobest (Westerlo, Belgium) and left in the laboratory for five days with access to syrup solution and pollen. The evening prior to grafting the larvae we removed the majority of the workers, leaving approximately 20 with the queen in each colony (we left some workers to tend to the brood). The following day we removed all workers and the queen. After this the same protocol for moving the larvae into the well plates was used, as described in experiments 6.1, 6.2, 6.3 & 6.4. Six larvae perished during this process and were removed from the analysis.

Feeding regime

Pereboom (2000) demonstrated that bumblebee larvae are fed on average 0.88µl when fed by a worker with a pollen/protein ratio of 35.12g/l, and that this did not change for males/queens and worker larvae. Pendrel & Plowright (Pendrel & Plowright 1981) showed that on average bumblebee larvae are fed at intervals of 20.24 minutes. If we combine the results of Pereboom (2000) and Pendrel & Plowright (1981) this suggests that bees are typically fed 0.88µl 3 times an hour . This suggests that larvae are fed 63.36µl a day and therefore 760µl over the course of development. Therefore, to emulate a field-realistic scenario and establish a robust feeding regime for larvae, we fed both early (instar stage 1 & 2) and late (instar stage 3 & 4) larvae development stages for a total of 6 days, and based our feeding regime on the combined data of (Pendrel & Plowright (1981) and Pereboom (2000) (see tables S6.1 & S6.2).

Table S6.1) Feeding regimes for 'early' stage larvae.

Regime	Feeding amount (amount given at each feeding)	Number of times fed in day	Total amount consumed in one day	Total amount consumed across 6 days
1)	2µl	4	8µl	48µl
2)	1µl	8	8µl	48µl
3)	4µl	4	16µl	96µl
4)	2µl	8	16µl	96µl
5)	8µl	4	32µl	192µl
6)	4µl	8	32µl	192µl

Table S6.2) Feeding regimes for 'late' stage larvae.

Regime	Feeding amount (amount given at each feeding)	Number of times fed in day	Total amount consumed in one day	Total amount consumed across 6 days
1)	4µl	4	16µl	96µl
2)	2µl	8	16µl	96µl
3)	8µl	4	32µl	192µl
4)	4µl	8	32µl	192µl
5)	16µl	4	64µl	384µl
6)	8µl	8	64µl	384µl

Fresh food was made each day by combining 35.12 grams of pollen (Biobest, Westerlo, Belgium) into 1 litre of 50% (°Brix) sucrose solution (Pereboom 2000). Once combined, the solution was then mixed with a vortex until pollen was dissolved.

Mortality was recorded as in experiment 6.1 & 6.2 and pictures of each well plate were taken on day 1, 3 & 6 (at the end of the experiment) for image J analysis. Some of the late larvae started to pupate during the experiment and we therefore also recorded the day at which this occurred. 22 pupae successfully hatched into adult bees.

Statistical analysis

As in experiment 6.1 & 6.2 we based our analysis on an information theoretic approach, whereby parameter estimates were based on averaging of the 95% confidence set of models, identified by Aikaike weight.

We used a fitted mixed effect cox model to analysis mortality with well and plate nested within colony as random factors. The amount of food fed to the larvae and the number of feeds (4 or 8) were included as covariates. We used a fitted linear mixed-effects model with the same random and fixed factors to analysis larval growth (larvae growth = larval size day 6 – larval size day 1) and a generalised linear model (binomial error distribution, link function = "logit")) to analyse adult emergence.

Results: Experiment S6.1

Mortality.

We were able to successfully graft and feed 282 larvae in total (138 early larvae and 144 late larvae). Of the 138 early development larvae included in the experiment, 49 died before the end of the experiment and mortality was not evenly distributed between different feeding regimes (Figure S6.1A, coxme, daily feeding amount early larvae, parameter estimate = 0.05, 95% CI: -0.02 to 0.08), with larvae fed the smallest quantity of food having the lowest mortality rate (Figure S6.1). The number of feeds received by the larvae (4 or 8 a day) did not influence survival (coxme, number of feeds, parameter estimate = 0.10, 95% CI: -0.09 to 0.20). We also found a significant effect of daily feeding amount on the survival of the late stage development larvae, (Figure S6.1B, coxme, daily feeding amount late larvae, parameter estimate = 0.07, 95% CI: 0.04 to 0.10) despite only 32 of the 144 larvae dying before the end of the experiment, with larvae fed the highest amount of food more likely to die before the end of the experiment. The number of feeds did not influence survival (coxme, number of feeds, parameter estimate = -0.18, 95% CI: -0.36 to 0.08).

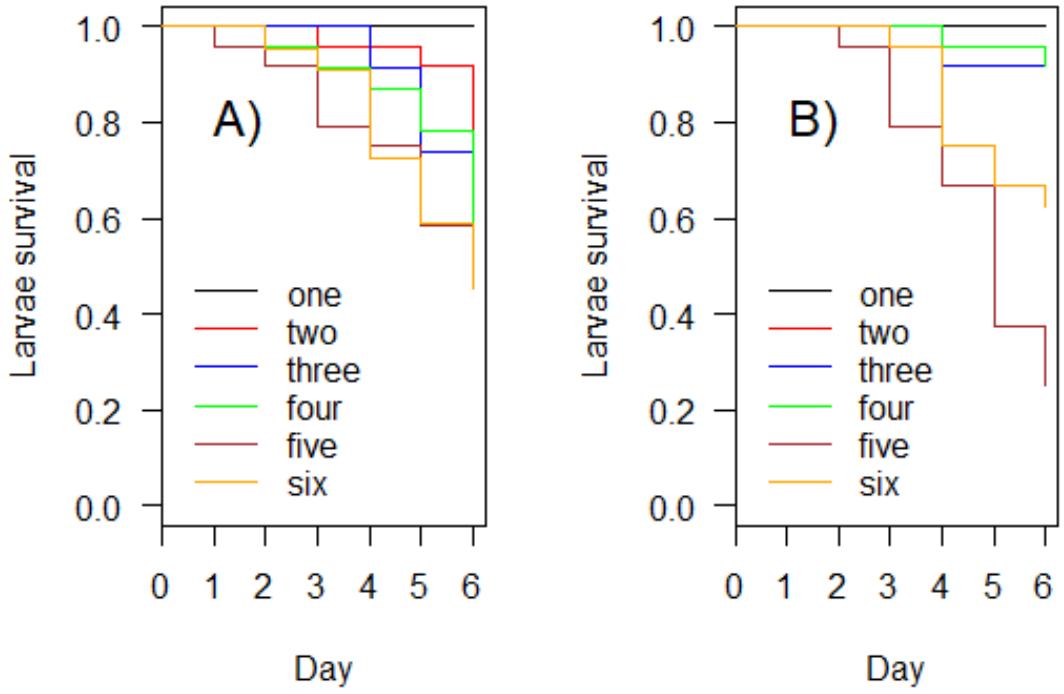


Figure S6.1: Kaplan-Meier curves for early (a) and late (b) stage larvae in different feeding regimes. Figure S6.1A, feeding regimes; one = 2µl*4, two = 1µl*8, three = 4µl*4, four = 2µl*8, five = 8µl*4, six = 4µl*8; Figure S6.1B, feeding regimes; one = 4µl*4, two = 2µl*8, three = 8µl*4, four = 4µl*4, five = 16µl*4, six = 8µl*8)

Growth

Of the 282 larvae in the experiment we were able to record the growth of 173 larvae. As mentioned above, 81 larvae died in the experiment (and we could not record their growth), and there were 4 larvae we were unable to measure as the photos taken did not show all of the larvae. We also had to remove 24 larvae from the growth data as they had started to pupate. Unsurprisingly, the more food fed to the larvae, the bigger they grew for both early (lm, early larvae, daily feeding amount, parameter estimate = 0.23, 95% CI: 0.16 to 0.30; Figure S6.3A) and late larvae (lm, late larvae, daily feeding amount, parameter estimate = 0.16, 95% CI: 0.08 to 0.24; Figure S6.3B). There was however no impact of the number of feeds on early larvae growth (lm, early larvae, number of feeds, parameter estimate = 0.12, 95% CI: -0.22 to 0.47) or late larvae (lm, late larvae, number of feeds, parameter estimate = -0.26, 95% CI: -0.98 to 0.46).

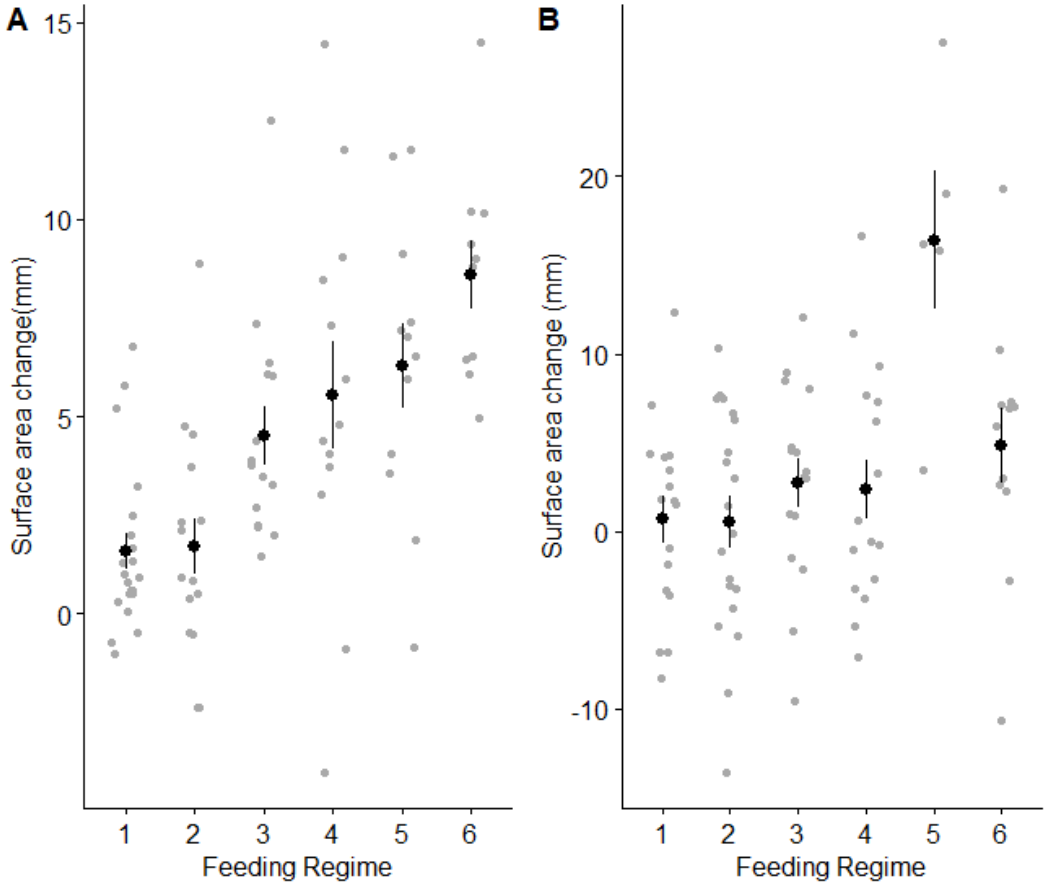


Figure S6.2: Surface area change mm^2 ± SE for early stage larvae (a) and for late stage larvae (b) from different feeding regimes.

Experiment 6.1: Supplementary results

Pupation and adult emergence

Pupation rates did not significantly differ between treatment groups (Figure S6.3A; coxme: 5ppb parameter estimate = 0.10, 95% CI: -1.12 to 1.32; 50ppb parameter estimate = 0.13, 95% CI: 0.83 to 1.08; 500ppb parameter estimate: 0.09, 95% CI: -0.78 to 0.96) with 76 larvae having started to pupate by day 10. The results also showed no significant difference between the number of adults emerging, with 53 emerging in total (Figure S6.3B; glmer: 5ppb parameter estimate = -0.02, 95% CI: -0.76 to 0.72; 50ppb parameter estimate = -0.20, 95% CI: -1.31 to 0.91; 500ppb parameter estimate: --0.13, 95% CI: -0.98 to 0.73).

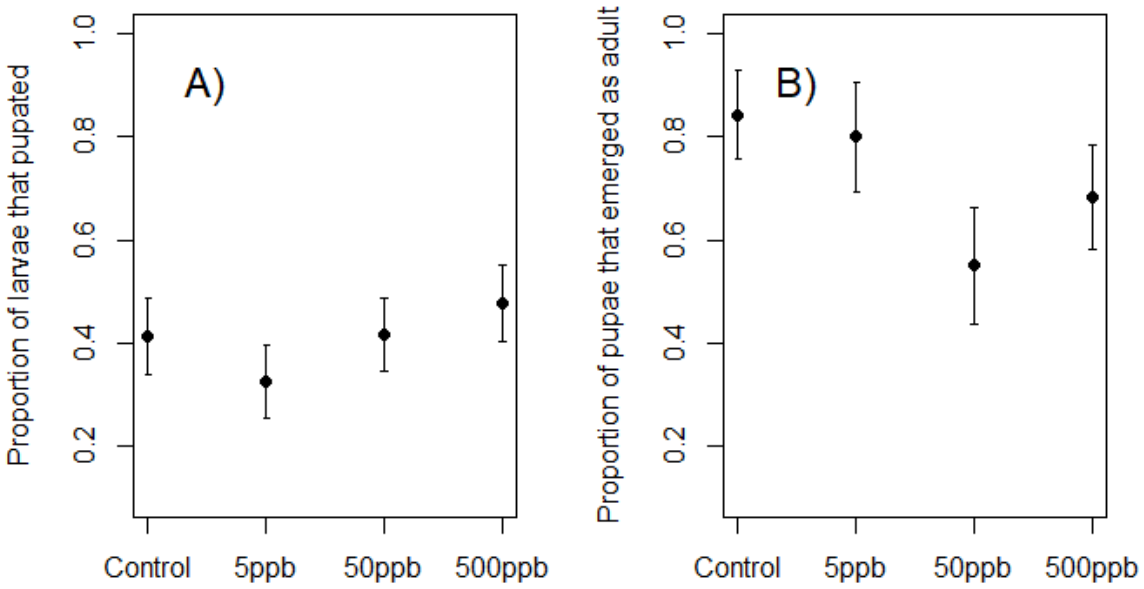


Figure S6.3: Experiment 6.1; The proportion of late larvae (mean ± SE) that pupated (a) and emerged as adults (b).

Experiment 6.2: Supplementary results

Pupation and adult emergence

Of 108 late larvae that we were able to monitor (experimental error meant that we could not use 15 larvae), 43 larvae pupated with no differences between treatment groups (Figure S6.4A; coxme: *Nosema* larvae pupation parameter estimate = 0.0, 95% CI: -0.48 to 0.48; sulfoxaflor larvae pupation parameter estimate = -0.11, 95% CI: -0.73 to 0.50; sulfoxaflor & *Nosema* larvae pupation parameter estimate = 0.13, 95% CI: -0.49 to 0.75) with 10 adults successfully emerged from pupation, with no differences between treatment groups (Figure S6.4B; coxme: (*wi* (full model) = 0.035)).

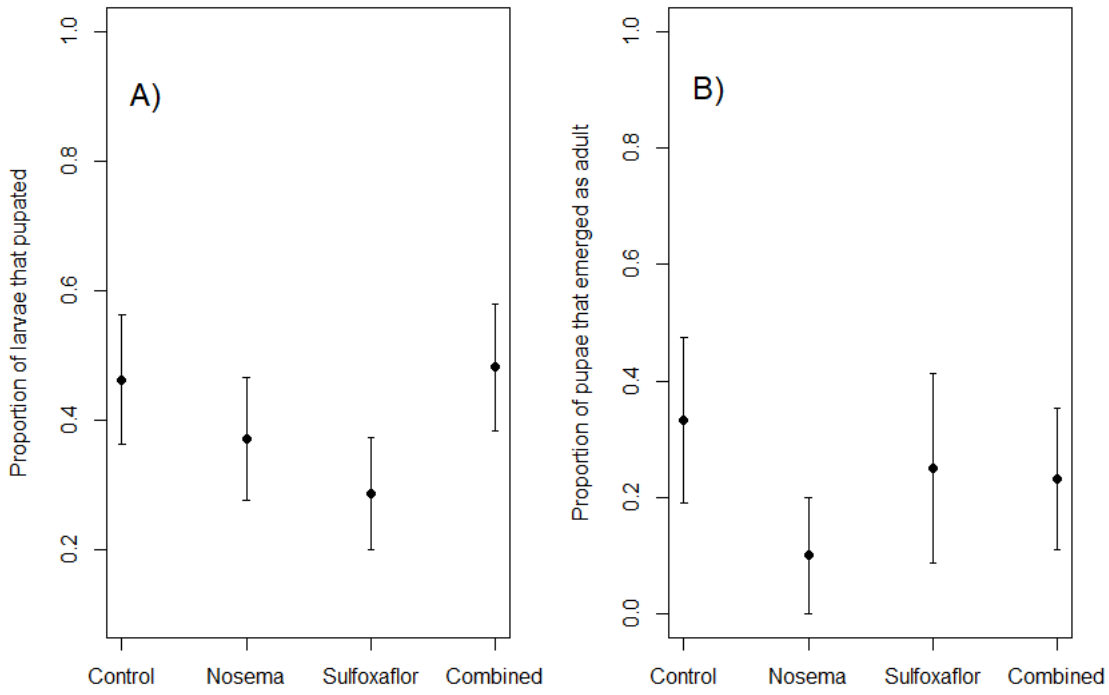


Figure S6.4: Experiment 6.2; The proportion of late larvae (mean ± SE) that pupated (a) and emerged as adults (b).

Table S6.3: Table of candiate models used for each analysis.

A) Experiment 1: Early larvae mortality; Colony and Plate included as random factors	AICc	ΔAICc	*w i*
Treatment + Larva size	**1006.3**	**0**	**0.579**
Treatment	**1007.4**	**1.12**	**0.332**
Treatment + Larva size + Treatment:Larva size	**1010.8**	**4.47**	**0.062**
Larva size	1013.2	6.89	0.018
Null model	1014.7	8.44	0.009
B) Experiment 1: Late larvae mortality; Colony and Plate included as random factors. (interaction not included as model failed to converge)	AICc	ΔAICc	*w i*
Larva size	**73.6**	**0**	**0.916**
Larva size + Treatment	**78.5**	**4.85**	**0.081**
null model	85.3	11.7	0.003
Treatment	89.3	15.66	0
C) Experiment 1; Early larval growth; Colony, larva & plate included as random factors	AICc	ΔAICc	*w i*
Treatment + Day + Treatment:Day	1387	0	0.393
Treatment + Day	1388.1	1.07	0.229
Day	1388.3	1.3	0.205
Treatment + Day + Treatment:Day + Larva size	1391	3.96	0.054
Treatment + Day + Larva size + Treatment:Day + Larva size + Treatment:Larva size	1391.4	4.36	0.044
Day + Larva size	1391.7	4.62	0.039
Treatment + Larvae size + Day + Treatment:Larvae size	1391.9	4.83	0.035
Treatment	1423.6	36.53	0
Null model	1426	39	0
Treatment + Larvae size	1427.4	40.34	0
Treatement + Larvae size +Treatment:Larvae size	1427.8	40.75	0
Larvae size	1429.1	42.06	0
D) Experiment 1; Late larval growth	**AICc**	**ΔAICc**	***w i***
Larva size	**790.5**	**0**	**0.646**
Treatment + Larva size	**791.7**	**1.2**	**0.354**
Treatment + Larva + Treatment:Larvae size	806.8	16.27	0
Treatment	1045.8	255.3	0
Null model	1051.4	260.85	0
E) Experiment 1; late larvae pupation	**AICc**	**ΔAICc**	***w i***
Larvae size	**673**	**0**	**0.802**
Treatment + Larvae size	**676.9**	**3.92**	**0.113**
Treatment + Larva size + Treatment:Larva size	**677.4**	**4.47**	**0.086**
Null model	760.9	87.96	0
Treatment	765.7	92.75	0
F) Experiment 1; late larvae emergence	**AICc**	**ΔAICc**	***w i***
Larva size	**90.8**	**0**	**0.752**
Treatment + Larva size	**94**	**3.19**	**0.152**
Null model	**95.6**	**4.78**	**0.069**
Treatment	97.5	6.65	0.027
G) Experiment 2: Early larvae mortality; Colony and Plate included as random factors	**AICc**	**ΔAICc**	***w i***
Sulfoxaflor	**1225.5**	**0**	**0.338**
N. bombi	**1225.6**	**0.03**	**0.334**
Sulfoxaflor + N.bombi	**1226.4**	**0.87**	**0.219**
N. bombi + Sulfoxaflor + N. bombi:Sulfoxaflor + Larva size	**1227.8**	**2.28**	**0.108**
null model	1237.9	12.34	0.001
H) Experiment 2: Late larvae mortality	**AICc**	**ΔAICc**	***w i***
Sulfoxaflor + Larva size	**61.8**	**0**	**0.614**
N. bombi + Sulfoxaflor + Larva size	**63.6**	**1.82**	**0.247**
N. bombi + Larva size	**65.5**	**3.75**	**0.094**
Null model	67	5.27	0.044
I) Experiment 2; Early larval growth; Colony, larva & plate included as random factors	**AICc**	**ΔAICc**	**wi**
Treatment + Day + Treatment:Day	**1137.5**	**0**	**0.861**
Treatment + Day + Larva size +Treatment:Day	**1142.1**	**4.59**	**0.087**
Treatment + Day	1143.3	5.81	0.047
Day	1148.5	10.96	0.004
Day + Larva size	1152.2	14.73	0.001
Treatment + Day + Larva size +Treatment:Day + Treatment:Larva size	1153.9	16.36	0
Treatment + Day + Larva size + Treatment:Larvae size	1159.3	21.77	0
Treatment	1164.5	27.02	0
Null model	1168.2	30.68	0
Treatment + Larva size	1169.9	32.4	0
Larva size	1173	35.46	0
Treatment + Larva size + Treatment:Larva size	1180.7	43.23	0
J) Experiment 2; Late larval growth	**AICc**	**ΔAICc**	**wi**
Treatment	**467.4**	**0**	**0.862**
Null model	**471.1**	**3.67**	**0.138**
K) Experiment 2; Late Larval pupation	**AICc**	**delta**	**weight**
Larva size	**301.5**	**0**	**0.736**
Treatment + Larva size	**303.9**	**2.39**	**0.223**
Treatment + Larva size + Treatment:Larva size	307.3	5.81	0.04
Treatment	332	30.48	0
Null model	333.5	32.02	0
L) Experiment 2; late larvae emergence	**AICc**	**ΔAICc**	**wi**
Larva size	**66.5**	**0**	**0.943**
Treatment + Larva size	73.1	6.59	0.035
Null model	74.4	7.88	0.018
Treatment + Larva size + Treatment:Larva size	78.4	11.84	0.003
Treatment	79.8	13.25	0.001
M) Experiment 3: Early larvae mortality; Colony and Plate included as random factors	**AICc**	**ΔAICc**	**wi**
Treatment + Larva size	**6157.2**	**0**	**0.795**
Treatment + Larva size + Treatment:Larva size	**6160.1**	**2.86**	**0.19**
Larva size	6165.1	7.92	0.015
Treatment	6251.3	94.12	0
Null model	6260.4	103.2	0
N) Experiment 3: Early larvae growth	**AICc**	**ΔAICc**	**wi**
Treatment + Day + Treatment:Day	**2038.5**	**0**	**0.684**
Treatment + Day	**2041**	**2.52**	**0.194**
Treatment + Day + Larva size + Treatment:Day	**2043.1**	**4.63**	**0.067**
Day	2043.7	5.23	0.05
Day + Larva size	2048.6	10.07	0.004
Treatment	2056.6	18.12	0
Treatment + Day + Larva size + Treatment:Day + Treatment:Larva size	2058.2	19.75	0
Null model	2059.2	20.72	0
Treatment + Larve size +Treamt:Larva size + Day	2060.6	22.12	0
Treatment + Larva size	2061.2	22.65	0
Larva size	2064.1	25.56	0
Treatment +Larva size + Treatment:Larva size	2076.1	37.57	0
O) Experiment 4: Early larvae mortality	**AICc**	**ΔAICc**	**wi**
N. bombi + Sulfoxaflor + N. bombi:Sulfoxaflor + Day	**7192.1**	**0**	**0.395**
N. bombi + Sulfoxaflor + Day	**7192.2**	**0.11**	**0.373**
Sulfoxaflor	**7193.2**	**1.12**	**0.225**
N. bombi	7200.2	8.09	0.007
Null model	7224.8	32.71	0
P) Experiment 4: Early larvae growth	**AICc**	**ΔAICc**	**wi**
Treatment + Day + Treatment:Day	**3042.9**	**0**	**0.973**
Treatment + Day + Larva size + Treatment:Day	3050.1	7.14	0.027
Treatment + Day + Larva size + Treatment:Day + Treatment:Larvae size	3066.6	23.67	0
Treatment + Day	3077.7	34.78	0
Treatment	3089.4	46.48	0
Treatment + Larva size	3096.5	53.55	0
Treatment + Day + Larva size + Treatment:Larva size	3101.1	58.21	0
Treatment + Larvae size + Treatment:Larva size	3112.7	69.78	0
Day	3147.6	104.67	0
Day + Larva size	3155.1	112.14	0
Null model	3156.1	113.17	0
Larva size	3163.5	120.61	0

Table S6.4. Parameter estimates and 95% confidence intervals derived by model averaging across the confidence set of models. Parameters highlighted in bold have 95% confidence intervals that do not cross zero.

A) Experiment 1: Early larvae mortality	**Estimate**	**Std. Error**	**Lower CI**	**Upper CI**
5ppb	0.60	0.58	-0.55	1.74
50ppb	0.25	0.57	-0.88	1.37
500ppb	0.93	0.48	-0.01	1.87
Larval size	-0.03	0.04	-0.10	0.04
Larva size:5ppb	0.01	0.03	-0.05	0.06
Larva size:50ppb	0.01	0.03	-0.05	0.06
Larva size:500ppb	0.00	0.02	-0.04	0.05
B) Experiment 1: Late larvae mortality	**Estimate**	**Std. Error**	**Lower CI**	**Upper CI**
Larva size	**-0.18**	**0.06**	**-0.30**	**-0.05**
5ppb	-0.05	0.43	-0.89	0.78
50ppb	0.03	0.35	-0.66	0.73
500ppb	0.00	0.36	-0.71	0.71
C) Experiment 1: Early larvae growth	**Estimate**	**Std. Error**	**Lower CI**	**Upper CI**
(Intercept)	4.68	0.69	3.32	6.03
5ppb	0.71	0.69	-0.64	2.05
50ppb	-0.24	0.58	-1.38	0.90
500ppb	-0.06	0.60	-1.24	1.11
Day	**-2.89**	**0.63**	**-4.13**	**-1.64**
Day:Treatment 5ppb	0.05	0.84	-1.61	1.71
Day:Treatment 50ppb	0.07	0.80	-1.50	1.63
Day:Treatment 500ppb	0.73	1.15	-1.52	2.99
Larva size	0.00	0.02	-0.04	0.04
D) Experiment 1: Late larvae growth	**Estimate**	**Std. Error**	**Lower CI**	**Upper CI**
Larva size	**-0.18**	**0.06**	**-0.30**	**-0.05**
5ppb	-0.05	0.43	-0.89	0.78
50ppb	0.03	0.35	-0.66	0.73
500ppb	0.00	0.36	-0.71	0.71
E) Experiment 1: Late larvae pupation	**Estimate**	**Std. Error**	**Lower CI**	**Upper CI**
Larva size	0.09	0.01	0.06	0.11
5ppb	0.10	0.62	-1.12	1.32
50ppb	0.13	0.49	-0.83	1.08
500ppb	0.09	0.44	-0.78	0.96
Larva size:5ppb	0.00	0.01	-0.03	0.02
Larva size:50ppb	0.00	0.01	-0.02	0.02
Larva size:500ppb	0.00	0.01	-0.02	0.02
F) Experiment 1; Late larvae adult emergence	**Estimate**	**Std. Error**	**Lower CI**	**Upper CI**
(Intercept)	-1.26	1.25	-3.74	1.21
Larva size	**0.05**	**0.03**	**0.00**	**0.10**
5ppb	-0.02	0.37	-0.76	0.72
50ppb	-0.20	0.56	-1.31	0.91
500ppb	-0.13	0.43	-0.98	0.73
G) Experiment 2: Early larvae mortality	**Estimate**	**Std. Error**	**Lower CI**	**Upper CI**
Sulfoxaflor	0.11	0.18	-0.24	0.46
Larva size	**-0.08**	**0.02**	**-0.12**	**-0.04**
N. bombi	0.10	0.18	-0.24	0.45
N. bombi:Sulfoxaflor	0.03	0.15	-0.26	0.32
H) Experiment 2: Late larvae mortality	**Estimate**	**Std. Error**	**Lower CI**	**Upper CI**
Sulfoxaflor	1.60	1.16	-0.67	3.87
Larva size	-0.08	0.04	-0.16	0.00
N. bombi	0.12	0.49	-0.85	1.08
I) Experiment 2: Early larvae growth	**Estimate**	**Std. Error**	**Lower CI**	**Upper CI**
(Intercept)	7.31	1.24	4.87	9.74
N. bombi	**-2.69**	**0.90**	**-4.46**	**-0.91**
5ppb	**-2.84**	**0.92**	**-4.66**	**-1.03**
5ppb & N. bombi	-1.62	0.97	-3.54	0.30
Day	**-4.78**	**1.16**	**-7.07**	**-2.48**
Day:N. bombi	3.21	1.65	-0.04	6.47
Day:5ppb	2.50	1.66	-0.77	5.77
Day:5ppb & N.bombi	1.08	1.77	-2.40	4.56
Larva size	0.01	0.03	-0.05	0.06
J) Experiment 2: Late larvae growth	**Estimate**	**Std. Error**	**Lower CI**	**Upper CI**
(Intercept)	4.68	1.72	1.25	8.11
N. bombi	-0.52	2.21	-4.95	3.90
5ppb	-0.87	2.28	-5.42	3.69
N. bombi & 5ppb	-1.86	2.59	-7.03	3.31
K) Experiment 2: Late larvae pupation	**Estimate**	**Std. Error**	**Lower CI**	**Upper CI**
Larva size	**0.11**	**0.02**	**0.07**	**0.16**
N. bombi	0.00	0.24	-0.48	0.48
5ppb	-0.11	0.31	-0.73	0.50
N. bombi & 5ppb	0.13	0.32	-0.49	0.75
L) Experiment 2; Late larvae adult emergence	**Estimate**	**Std. Error**	**Lower CI**	**Upper CI**
Larva size	**0.12**	**0.04**	**0.03**	**0.21**
M) Experiment 3; Early larvae mortality	**Estimate**	**Std. Error**	**Lower CI**	**Upper CI**
0.28ppb	0.26	0.20	-0.13	0.65
5ppb	0.17	0.26	-0.34	0.68
500ppb	**0.42**	**0.19**	**0.06**	**0.78**
Day	**-0.10**	**0.01**	**-0.13**	**-0.07**
Day:0.28ppb	0.00	0.01	-0.03	0.02
Day:5ppb	-0.01	0.02	-0.04	0.03
Day:500ppb	0.00	0.01	-0.02	0.02
N) Experiment 3: Early larval growth	**Estimate**	**Std. Error**	**Lower CI**	**Upper CI**
(Intercept)	2.25	0.55	1.16	3.34
0.28ppb	-1.08	0.56	-2.18	0.02
5ppb	**-1.03**	**0.52**	**-2.05**	**-0.01**
500ppb	**-1.45**	**0.60**	**-2.62**	**-0.28**
Day	**-2.23**	**0.73**	**-3.65**	**-0.80**
Day:0.28ppb	1.41	1.16	-0.86	3.69
Day:5ppb	0.68	0.88	-1.05	2.40
Day:500ppb	1.07	1.06	-1.02	3.16
Larva size	0.00	0.02	-0.03	0.04
O) Experiment 4 : Early larva mortality	**Estimate**	**Std. Error**	**Lower CI**	**Upper CI**
N. bombi	0.06	0.11	-0.16	0.28
Sulfoxaflor	0.22	0.11	-0.01	0.44
Day	**-0.05**	**0.01**	**-0.07**	**-0.03**
N. bombi:Sulfoxaflor	0.10	0.16	-0.21	0.41
P) Experiment 4 : Early larva growth	**Estimate**	**Std. Error**	**Lower CI**	**Upper CI**
(Intercept)	3.32	0.39	2.54	4.09
N. bombi	**-2.45**	**0.35**	**-3.14**	**-1.76**
5ppb	**-3.35**	**0.36**	**-4.04**	**-2.64**
N. bombi & Sulfoxaflor	**-3.29**	**0.37**	**-4.02**	**-2.56**
Day	**-3.08**	**0.52**	**-4.10**	**-2.05**
Day:N. bombi	1.11	0.73	-0.31	2.54
Day:5ppb	**4.15**	**0.80**	**2.59**	**5.70**
Day:N. bombi & Sulfoxaflor	**3.96**	**0.86**	**2.28**	**5.64**

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