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Effects of pesticides on bees in agricultural landscapes

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Abstract

Bee declines, driven by a combination of stressors including pesticides, pathogens and lack of flowers, may exacerbate biodiversity loss and food insecurity. Beekeepers, scientists and policy-makers have particularly implicated neonicotinoid insecticides, which are frequently found in the pollen and nectar of crops and weeds. However, the effects of pesticides have rarely been assessed on free-foraging bees in agricultural landscapes.

Therefore, we examine, in this thesis, how organic farming impacts honeybee colonies to see whether they benefit from a farming system without synthetic pesticides. Then, we assess how exposure to the neonicotinoid clothianidin in oilseed rape affects the performance and pathogen susceptibility of honeybee and bumblebee colonies. Lastly, we examine neonicotinoid residues in oilseed rape nectar to determine conditions that favour neonicotinoid spread and uptake as well as potential risks for foraging bees.

Our results suggest that organic farming benefits honeybee colonies, possibly through a continuous provision of flowers partially resulting from reduced herbicide use. Clothianidin exposure had no detectable negative impacts on honeybee colonies but severe effects on the reproduction of bumblebees. Clothianidin exposure barely affected the pathosphere of honeybees and bumblebees, suggesting no major long-lasting immunosuppression. Finally, we determined that, despite being banned in bee-attractive crops, imidacloprid contamination in oilseed rape poses a risk for foraging bees. Therefore, we reaffirm the recent decision to ban imidacloprid, clothianidin and thiamethoxam in all outdoor crops in the EU.

Keywords: Pesticides, honeybees, bumblebees, neonicotinoids, bee pathogens, organic farming, weeds, agro-ecology

Résumé

Les abeilles sont en déclin, menacés par une combinaison de facteurs tels que les pesticides, les pathogènes et le manque de fleurs. Les apiculteurs, scientifiques et politiques ont particulièrement incriminé les insecticides néonicotinoïdes, fréquemment retrouvés dans le pollen et le nectar des plantes cultivées ou sauvages. Cependant, leurs impacts sont rarement évalués sur des abeilles butinant en plein champ.

Dans cette thèse, nous regardons si la surface en agriculture biologique autour des ruches affecte des abeilles domestiques, pour vérifier si un système de culture sans pesticide de synthèse leur est bénéfique. Puis, nous testons comment les colzas traités au clothianidine, un néonicotinoïde, affectent les performances et la sensibilité aux pathogènes des abeilles domestiques et des bourdons. Enfin, nous identifions les conditions qui modifient la quantité de néonicotinoïdes dans le nectar de colza et si cette quantité peut potentiellement menacée les butineuses.

Nos résultats suggèrent que l'agriculture biologique peut être bénéfique aux colonies d'abeilles, probablement grâce à une quantité continue de fleurs dans le temps venant partialement de l'absence de désherbants. L'exposition à la clothianidine affectait fortement la reproduction des bourdons, mais peu les performances des colonies d'abeilles domestiques et la pathosphère des deux espèces. Enfin, de quantités d'imidaclopride, avec un risque considérable pour les butineuses, ont été retrouvées dans le nectar malgré l'interdiction dans les cultures mellifères. Ces résultats confortent la récente décision d'interdire l'imidaclopride, clothianidine et thiamethoxame en plein champ sur les cultures en Europe.

Mot clés : Pesticides, abeilles domestiques, bourdons, néonicotinoïdes, pathogènes d'abeilles, agriculture biologique, adventices, agroécologie

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General introduction

Global agricultural challenges

Global agriculture must meet the twin challenge of increasing production, while reducing environmental impacts. Continuing human population growth and a shift in dietary habits in developing countries towards the consumption of more caloric foods such as meat and dairy products challenge global food systems (Crist, Mora, & Engelman, 2017; C. H. M. Godfray et al., 2010; Popp, Peto, & Nagy, 2013). To make matters worse, food production may increasingly compete with the production of biofuels for arable land, as the depletion of fossil fuels and efforts to restrict climate change could further promote their demand (C. H. M. Godfray et al., 2010; Murphy, Woods, Black, & McManus, 2011; Yin, Xiong, Jiang, & Pang, 2010). Agricultural production gains must, however, be attained on largely existing farmland, as any further expansion would exacerbate deforestation (Crist et al., 2017; Gasparri, Grau, & Gutiérrez Angonese, 2013; Grau, Gasparri, & Aide, 2005), climate change (Forster et al., 2007; Lawrence & Vandecar, 2015; Myhre et al., 2013) and consequently the dramatic worldwide biodiversity loss (Steffen et al., 2015). Biodiversity ensures the functioning of ecosystems, which provide several services that are essential for agricultural production such as nutrient cycling, crop pollination and natural pest control (Bommarco, Kleijn, & Potts, 2013; Mace, Norris, & Fitter, 2012). Modern agriculture achieves high productivity by limiting pre-harvest losses through the use of pesticides (Cerda et al., 2017; Oerke, 2006; Popp et al., 2013). These pesticides will therefore likely remain an indispensable component of farming systems in the foreseeable future, but global agriculture cannot rely on them to increase productivity, as their use further threatens biodiversity (Beketov, Kefford, Schafer, & Liess, 2013; L. Pisa et al., 2017). Hence, farmers need to use selected pesticides in moderation and explore alternative, more environmentally friendly ways of closing yield gaps (Bommarco et al., 2013; Foley et al., 2011; C. H. M. Godfray et al., 2010; Kovács-Hostyánszki et al., 2017; Petit et al., 2015; Popp et al., 2013).

Importance of bees as pollinators

Pollinators benefit both biodiversity and crop production (Potts et al., 2016). Nearly 90% of wild plant species depend at least in part on animal pollination (Ollerton, Winfree, & Tarrant, 2011). Pollinators contribute to the yield and quality of 75% of globally important crop species (C. G. Cutler & Scott-Dupree, 2014), some of which are main sources of several micronutrients (Eilers, Kremen, Greenleaf, Garber, & Klein, 2011; Potts et al., 2016). Even though over 60% of global crop production volumes are of species that do not benefit from pollinators, 5-8% of global crop volumes are directly ascribed to animal pollination (Mayberry & Elle, 2010) and in some world regions up to 25% of crop volumes would be lost without pollinators (Potts et al., 2016).

Bees are considered the most important pollinators (Potts et al., 2016) and the Western honeybee (*Apis mellifera* L.) the species with the largest impact on crop pollination (Aizen & Harder, 2009; Geldmann & González-Varo, 2018). Nevertheless, other pollinators are needed to maximize fruit set and to supplement honeybees (Garibaldi et al., 2013), as honeybees avoid flying during bad weather conditions and collecting the pollen of certain crops (Javorek, Mackenzie, & Vander Kloet, 2002). For instance, tomatoes, peppers and berries need their anthers to be vibrated to release their pollen. Bumblebees do this effectively and are therefore commonly used in greenhouses (Button & Elle, 2014; Cameron et al., 2011).

Bee declines

In recent decades, the stock of managed honeybee colonies declined markedly in Europe and the United States. Stocks declined in Central Europe by 25% between 1985 and 2005 (Potts, Roberts, et al., 2010) and in the United States by 46% in a similar timeframe (1980-2008) (van Engelsdorp, Hayes, Underwood, & Pettis, 2008). In the United States, the decline has already been documented since the end of World War II with a 59% loss between 1947 and 2005. However, beekeeping gained popularity in other parts of the world, leading to a 45% rise in the global stocks of managed honeybee colonies between 1961 and 2008 (Aizen & Harder, 2009; Potts, Biesmeijer, et al., 2010). The number of managed honeybee (*Apis mellifera*) colonies depends largely on socio-economic factors

(K. M. Smith et al., 2013), but feral honeybees experienced much more severe declines than managed honeybees in Europe and North America (Potts, Biesmeijer, et al., 2010). In addition, beekeepers around the world reported exceptionally high seasonal colony losses in the last decade (Neumann & Carreck, 2010; Potts et al., 2016). Data on wild pollinator trends are limited to some regions and taxa, but there is clear evidence for declines in local abundance, geographical range or even extinctions of several bee species (Biesmeijer et al., 2006; Potts et al., 2016; Vanbergen et al., 2013). Particularly in North America and Europe, reductions in wild bee diversity are well-documented. In Africa, Asia and Latin America yield gaps due to pollination deficits have been identified (Garibaldi et al., 2016).

Drivers of bee declines

Bee declines have been attributed to a combination of stressors including parasites, pathogens, pesticides, habitat loss, invasive species and climate change (Goulson, Nicholls, Botías, & Rotheray, 2015; Potts et al., 2016). Human activity has given rise to bee population declines. The transport of honeybee colonies for their hive products and pollination services around the globe has spread bee parasites and pathogens such as *Nosema* spp, the *Varroa* mite (*Varroa destructor*) and the viruses it transmits including *Deformed wing virus, Acute bee paralysis virus, Kashmir bee virus* and *Israeli acute bee paralysis virus* (Goulson et al., 2015; Mondet, de Miranda, Kretzschmar, Le Conte, & Mercer, 2014). In Europe and North America most feral honeybees disappeared after the introduction of the *Varroa* mite and beekeepers have to treat periodically against the mite to limit colony losses (Rosenkranz, Aumeier, & Ziegelmann, 2010). The *Varroa* mite shifted from its original host, the Asian honeybee (*Apis cerana*) to the Western honeybee, which is far less effective in combatting the mite, despite using similar behavioural defence mechanisms (Locke, 2016). Fortunately, the mite is restricted to honeybees unlike the viruses it vectors or the microsporidians *Nosema* spp. Nonetheless, the *Varroa* mite may indirectly threaten wild bees, since dramatically increased virus titres in honeybees have been suggested to facilitate virus transmission to bumblebees (Fürst, McMahon, Osborne, Paxton, & Brown, 2014; Mcmahon et al., 2015). The commercial mass-breeding of bumblebees, which are often fed with pollen collected by honeybees,

may further promote pathogen transmission and proliferation (Goulson et al., 2015; Velthuis & Van Doorn, 2006). In addition, the global transport of bumblebees for their pollination services has facilitated the spread of invasive species and bee pathogens as well as precipitated range restrictions and regional extinctions of several native bumblebee species (Goulson et al., 2015; Mayberry & Elle, 2010; Schmid-Hempel et al., 2014). Invasive species that endanger bees include also predators, most prominently the yellow-legged hornet, which has recently been introduced into Europe from Asia (Monceau, Bonnard, & Thiéry, 2014).

Agriculture is not only a major beneficiary of the pollination services provided by bees, but also one of the largest drivers of their declines. Long-term declines of wild bees have been linked to the conversion of often flower-rich natural and semi-natural habitat to arable land (Goulson et al., 2015). The consequent loss and fragmentation of habitat and reduction in flower diversity harms particularly specialist foragers (Goulson et al., 2015; Kennedy et al., 2013), but also generalist foragers like honeybees suffer from lack of flowers (Goulson et al., 2015; Requier, Odoux, Henry, & Bretagnolle, 2016). Particularly declines in the abundance and diversity of collected pollen in the absence of mass-flowering crops, semi-natural elements or melliferous catch crops were directly or indirectly linked to winter honeybee colony mortality (Alaux et al., 2017; Requier et al., 2016). Both honeybees and wild bees forage intensively on weeds (Bretagnolle & Gaba, 2015; Requier et al., 2015), which are often removed in farmland either mechanically or through the application of herbicides.

Pesticides have, however, mostly received attention because of their direct toxic effects to bees (Goulson et al., 2015). Bees are exposed to mixtures of numerous pesticides throughout their lifetime of which insecticides pose the greatest direct threat to bees (Sanchez-Bayo & Goka, 2014). However, also fungicides, herbicides and acaricides can be toxic to bees (Sanchez-Bayo & Goka, 2014). In addition, fungicides and certain substances added to pesticide formulations can synergistically magnify the toxicity of insecticides by inhibiting their metabolism, detoxification and excretion (Collison, Hird, Cresswell, & Tyler, 2016; Goulson et al., 2015; Sanchez-Bayo & Goka, 2014; Tsvetkov et al., 2017).

Pesticides do not only interact among themselves but they interact also with other stressors. For instance, they have been implicated in the accelerated spread of bee diseases in recent decades (Collison et al., 2016; Sánchez-Bayo et al., 2016). Pesticides were shown to weaken the immune function of bees (Alaux et al., 2010; Brandt, Gorenflo, Siede, Meixner, & Büchler, 2016; Brandt et al., 2017; Di Prisco et al., 2013; Tsvetkov et al., 2017; Wu-Smart & Spivak, 2016) and give consequently rise to pathogens and parasites (Aufauvre et al., 2012; Di Prisco et al., 2013; Doublet, Labarussias, de Miranda, Moritz, & Paxton, 2015; Nazzi et al., 2012; Pettis, Vanengelsdorp, Johnson, & Dively, 2012; Vidau et al., 2011). Pesticide-induced impairment of the foraging ability of bees can cause or exacerbate nutritional stress (Feltham, Park, & Goulson, 2014; Gill & Raine, 2014; Gill, Ramos-Rodriguez, & Raine, 2012; Stanley, Russell, Morrison, Rogers, & Raine, 2016), which may in turn render bees more susceptible to the adverse effects of pesticides (Tosi, Nieh, Sgolastra, Cabbri, & Medrzycki, 2017) and pathogens (M. J. F. Brown, Loosli, & Schmid-Hempel, 2000; DeGrandi-Hoffman & Chen, 2015; Moret & Schmid-Hempel, 2000).

The effect of pesticide use on bees depends on the landscape context

Pesticide-free flowers in the landscape can dilute exposure levels. Diversion from contaminated to pesticide-free floral resources is a possible explanation as to why the negative effect of pesticide use on wild bee abundance, diversity and pollination services can be buffered by increased availability of semi-natural habitat in the surroundings of treated crops (Carvalheiro, Seymour, Nicolson, & Veldtman, 2012; Park, Blitzer, Gibbs, Losey, & Danforth, 2015). Due to widespread insecticide contamination, small isolated flower strips set apart for the conservation of pollinators do, however, not necessarily decrease the risk of pesticide exposure (Mogren & Lundgren, 2016). It is also conceivable that natural habitat provides dietary benefits, which increase the resilience of bees against negative pesticide effects. Pollen abundance, diversity and quality can affect bee physiology directly (Alaux et al., 2017; Di Pasquale et al., 2016, 2013) and the diet of bees may affect their microbiome (J. C. Jones et al., 2018). Social bees transmit specific microorganisms through feeding (Koch & Schmid-Hempel, 2011; Martinson et al.,

2011), which can protect them from pathogens (Cariveau, Elijah, Koch, Winfree, & Moran, 2014; Kaltenpoth & Engl, 2014; Koch & Schmid-Hempel, 2011; Kwong, Mancenido, & Moran, 2017) and help them live on suboptimal diets (Zheng et al., 2016; Zheng, Powell, Steele, Dietrich, & Moran, 2017). For example, the bee gut symbiont *Gilliamella apicola* engages in the detoxification of xenobiotics (Zheng et al., 2016, 2017). The extent to which bees are impacted by intoxication depends also on abiotic factors and the physical structure of the landscape. Weather conditions and landmarks that serve as orientation affect how strongly neonicotinoid ingestion impairs the homing ability of honeybees (Henry et al., 2014).

Landscape-scale studies on the overall effect of pesticides on bees

The overall effect of pesticides can be measured by continuous toxicity indices. Studies relating bee variables to toxicity indices showed, however, varying results. For instance, one study (Kremen, Williams, Bugg, Fay, & Thorp, 2004) failed to link to pollination services by insecticide use, while another one (Mineau, Harding, Whiteside, Fletcher, & Knopper, 2008) could explain honeybee mortality incidents at hives by a pesticide index. Pesticide use explained also a reduction in the abundance and diversity of wild bees, but effects varied between seasons and bee taxa (Mallinger, Werts, & Gratton, 2015; Park et al., 2015; Tuell, 2010; Williams et al., 2010). This underlines the need for long-term studies and shows that each bee taxon can only serve to some extent as a surrogate for other bees due to differences in life history traits (Tuell, 2010) and in sensitivity to pesticides (Arena & Sgolastra, 2014; Cresswell, Robert, Florance, & Smirnoff, 2014). Besides, toxicity indices are typically based on acute lethal doses and may therefore fail to accurately represent the harmful effects of chronic exposure to sublethal dosages, which may affect the functioning of social bee colonies more than the premature death of a few individual foragers (Bryden, Gill, Mitton, Raine, & Jansen, 2013). In addition, indices do usually not take into account potential synergistic effects among pesticides.

Comparisons between organic and conventional farming can provide insight into the general effect of the ensemble of conventionally applied synthetic pesticides. Organic

farming is an agricultural approach that aims at limiting negative ecological consequences by restricting pesticide and fertilizer use to selected natural products. There is clear evidence that organic farming benefits the diversity (Holzschuh et al. 2007; Holzschuh et al. 2008; Kennedy et al. 2013; Schneider et al. 2014; Forrest et al. 2015) and abundance (Forrest et al., 2015; Holzschuh et al., 2007, 2008; Kennedy et al., 2013; Morandin, Winston, Franklin, & Abbott, 2005) of wild bees and enhances pollination rates (Morandin & Winston, 2005; H. G. Smith, Andersson, Rundlo, Rundlöf, & Smith, 2012). However, organic farming differs often from conventional agriculture in several respects that are only indirectly linked to the ban of synthetic pesticides and fertilizers, such as the crop choice or the amount of semi-natural elements in the surroundings. Two studies that used – unlike other studies - organic fields that were similar to the conventional controls in floral resources and the land use of the surroundings failed to detect effects on wild bees (Brittain, Bommarco, Vighi, Settele, & Potts, 2010) and pollination rates (Brittain et al., 2010; Winfree, Williams, Gaines, Ascher, & Kremen, 2008), which indicates the importance of deciphering direct pesticide effects from effects of resource availability. It is also unclear how organic farming affects honeybees, which have different habitat requirements than wild bees. Beekeepers provide honeybees with hives and honeybees have a broader foraging spectrum in terms of distance and flower choice than many wild bees. In addition, there is no evidence that honeybees forage preferentially on organic land (Couvillon, Schürch, & Ratnieks, 2014).

Neonicotinoids and other insecticides

In recent years, the number of studies on insecticide effects on bees has substantially increased with a clear focus on insecticides belonging to the class of neonicotinoids (Collison et al., 2016; Lundin, Rundlöf, Smith, Fries, & Bommarco, 2015; L. Pisa et al., 2017). Neonicotinoids, as other common insecticide classes, such as organophosphates, pyrethroids, carbamates and phenylpyrazoles act on the nervous system of insects (Belzunces, Tchamitchian, & Brunet, 2012). Neonicotinoids were introduced on the market in the 1990s when many pest insects had developed resistances against the prevailing insecticides at the time (N. Simon-Delso et al., 2015). They quickly gained

popularity among farmers, as they provide long-lasting crop protection from a wide range of insect pests at comparably low application rates due to high persistence and efficacy of the active substances (Jeschke, Nauen, Schindler, & Elbert, 2011). Today, they are considered the most widely used class of insecticides with a market share of around 30% (Casida, 2018). Neonicotinoids are used in a broad range of globally important cash crops including insect-pollinated ones, such as oilseed rape, maize and cotton as well as on garden plants (Jeschke et al., 2011). Exceptionally high versatility in application methods contributed to the rise of neonicotinoids (Jeschke et al., 2011). They are most often used as seed coatings, but can also be applied as foliar sprays or soil treatments. Due to their systemic nature, they are taken up through the seed, roots or leaves and translocate then to all parts of the plant including the pollen and the nectar, which bees feed on (N. Simon-Delso et al., 2015). The consequent chronic exposure of bees to neonicotinoids has been suspected to be a major driver of bee declines (Blacquière, Smagghe, Van Gestel, & Mommaerts, 2012; C. J. Godfray et al., 2015; H. C. J. Godfray et al., 2014; Goulson et al., 2015; L. Pisa et al., 2017; L. W. Pisa et al., 2015). Neonicotinoids are not the only systemic pesticides. In fact, systemic pesticides have been used long before the invention of neonicotinoids and also many organophosphates, some carbamates and the phenylpyrazole fipronil are systemic (Sanchez-Bayo, Tennekes, & Goka, 2013). However, neonicotinoids combine systemic properties with high persistence, exceptionally high toxicity to bees and a particular widespread use, which has alarmed beekeepers, scientists and policy-makers alike.

Elevated honeybee colony losses that coincided with the introduction of imidacloprid on the market in the early 1990s caused suspicions among French beekeepers (Maxim & van der Sluijs, 2007). However, some point out that the then described syndrome resembles symptoms of *Varroa* infestation (Carreck, 2017), which may or may not be linked to neonicotinoid exposure (Sánchez-Bayo & Desneux, 2015). Nonetheless, about a decade later, severe bee poisoning incidents from neonicotinoid dust released during seed drilling were reported in several countries (Carreck, 2017; Pistorius, Bischoff, Heimbach, & Stähler, 2010). In 2012, artificial feeding experiments showed sublethal effects of neonicotinoids on the reproduction of bumblebees (Whitehorn, O'Connor, Wackers, & Goulson, 2012) and the foraging behaviour (Gill et al., 2012) and homing success (Henry

et al., 2012) of honeybees. In light of this, the European Union passed in 2013 a moratorium on the three most common neonicotinoids - imidacloprid, clothianidin, thiamethoxam - for use in bee-attractive crops. In the following years, numerous laboratory-based studies showed sublethal effects of neonicotinoids (reviewed in (Blacquière et al., 2012; C. J. Godfray et al., 2015; Goulson, 2013; L. Pisa et al., 2017)) and some suggested interactions with other bee stressors (Goulson et al., 2015). In particular, neonicotinoid-induced impairments of the innate (Brandt et al., 2016, 2017; Di Prisco et al., 2013) and social immune system (Alaux et al., 2010; Tsvetkov et al., 2017; Wu-Smart & Spivak, 2016) of bees were shown to increase pathogen loads (Di Prisco et al., 2013; Doublet et al., 2015; Pettis et al., 2012; Vidau et al., 2011) and pathogen-induced bee mortality (Alaux et al., 2010; Doublet et al., 2015; Fauser-Misslin, Sadd, Neumann, & Sandrock, 2014; Vidau et al., 2011).

Nonetheless, the moratorium was heatedly debated and criticized for being based on laboratory studies, which may overstate exposure (Carreck, 2017; Carreck & Ratnieks, 2014; Klatt, Rundlöf, & Smith, 2016; Walters, 2013). It was argued that in real-world agricultural landscapes bees may be exposed to doses lower than those used in laboratory studies, including those labelled 'field-realistic' (Carreck & Ratnieks, 2014; Walters, 2013). Pesticide concentrations vary vastly between regions and some residue levels have been determined under conditions that may not be representative for Europe as recommended application rates and timeframes between sowing and flowering differed from the typical European practice (Carreck & Ratnieks, 2014). In addition, sometimes doses representative for longer durations of exposure were administered as one-time doses - neglecting the bees' ability to detoxify (Carreck & Ratnieks, 2014). It was also suspected that bees might sense neonicotinoids or other pesticides and actively avoid foraging on treated crops. However, choice experiments failed to determine a preference of bumblebees for untreated to imidacloprid-treated (Gels et al., 2002) or clothianidin-treated (Larson et al., 2013) white clover. More recent choice experiments showed, however, avoidance of neonicotinoids by honeybees in a laboratory trial with pure and pesticide-laced sucrose solution, but less so in a field trial with apple orchards (Kang & Jung, 2017). Another study found that both bumblebees and honeybees even prefer nectar laced with the neonicotinoids imidacloprid and thiametoxam to insecticide-free nectar

(Kessler et al., 2015). As bees could not taste these insecticides, the preference was to the activation of the targeted nicotinic acetyl-choline receptors (nAChRs) in the bees' brains.

Recently, large-scale studies confirmed neonicotinoid effects on free-foraging bees, but results varied with spatial location and bee species. Surveys across more than a decade and wide regions in the UK could link neonicotinoid seed treatment in oilseed rape to honeybee colony mortality (Budge et al., 2015) and long-term population changes in wild bees (Ben A. Woodcock et al., 2016). In Sweden, a well-replicated field study with matched and paired landscapes found strong effects of clothianidin seed dressing in oilseed rape on the reproduction and colony growth of bumblebees and the nesting success of the solitary mason bee *Osmia bicornis*, but no effects on honeybee colonies. Due to their large size, honeybee colonies may be particularly capable of buffering adverse neonicotinoid effects on individual bees. In fact, a French study (Henry et al., 2015) found thiamethoxam field exposure increased losses of honeybee foragers, which did, however, not result in smaller colonies, as they were ameliorated by an increased production of workers at the expense of energetically more costly males. Several other European and North American field trials with varying degrees of site replication showed no major effects on mason bees (Peters, Gao, & Zumkier, 2016), bumblebees (Sterk, Peters, Gao, & Zumkier, 2016) or honeybee (M. Alburaki et al., 2016; Mohamed Alburaki et al., 2015; G. C. Cutler & Scott-Dupree, 2007; G. C. Cutler, Scott-Dupree, Sultan, McFarlane, & Brewer, 2014; Pilling, Campbell, Coulson, Ruddle, & Tornier, 2013; Rolke, Fuchs, Grünewald, Gao, & Blenau, 2016). A study conducted in three European countries found country-specific effects of exposure to neonicotinoid seed treated oilseed rape (B. A. Woodcock et al., 2017). During crop flowering, both positive and negative effects on honeybees and bumblebees were found, depending on the country where the experiment was conducted. However, in honeybees only a negative effect on worker numbers in Hungarian colonies persisted over winter and in bumblebees and mason bees reproduction was negatively correlated to neonicotinoid residues in nests, which consisted also of neonicotinoids not applied in the study. Country-specific differences were in part explained by differences in the extent by which bees foraged on oilseed rape and in parasite prevalence. In an attempt to disentangle effects of pesticides from confounding factors, (Tsvetkov et al., 2017) matched the duration and magnitude of

honeybee colony exposure to neonicotinoids in the lab to exposure levels determined in colonies placed in Canadian maize growing regions. They showed that clothianidin and thiamethoxam exposure increased bee mortality and decreased social immunity, which may render colonies more susceptible to pathogens and parasites. Impairment of immune defenses is a potential mechanism of neonicotinoid action and differences in pathogen pressure may explain varying results between countries. This remains, however, still to be confirmed, as there is in particular a distinct lack of studies assessing the effect of neonicotinoids on immunocompetence under field conditions.

Effects of experimental neonicotinoid treatment may have also been confounded by the persistence of neonicotinoids in the landscape (B. A. Woodcock et al., 2017). Widespread neonicotinoid contamination of non-target flowers suggests that exposure is not limited to the flowering periods of treated crops and that bees can be exposed to substances banned in bee-attractive crops. Neonicotinoids have been frequently detected in wild flowers and bee-collected pollen consisting mostly of uncultivated flowers (Alaux et al., 2017; Botías et al., 2015; David et al., 2016; Mogren & Lundgren, 2016; Rundlöf et al., 2015; Tsvetkov et al., 2017). Even after the enactment of the EU moratorium, restricted neonicotinoids were detected in honeybees (Calatayud-Vernich, Calatayud, Simó, Suarez-Varela, & Picó, 2016; Daniele, Giroud, Jabot, & Vulliet, 2017), bee-collected pollen (Daniele et al., 2017; Tosi, Costa, Vesco, Quaglia, & Guido, 2018; B. A. Woodcock et al., 2017) and honey (Mitchell et al., 2017; Ben A. Woodcock et al., 2018). In the year after the partial ban came into effect, maximum prevalence of the restricted neonicotinoids in UK honey samples coincided with oilseed rape flowering and their concentrations increased with the area of oilseed rape surrounding the regarded honeybee hives (Ben A. Woodcock et al., 2018). In fact, several studies detected substantial neonicotinoid levels in oilseed rape fields that were intended as control fields and/or sown when the neonicotinoid was already banned in bee-attractive crops (Henry et al., 2015; Thompson H et al., 2013; B. A. Woodcock et al., 2017). Nonetheless, there are indications that the partial ban has decreased contaminations of bee-collected plant residues (Daniele et al., 2017; Ben A. Woodcock et al., 2018), but studies assessing neonicotinoid contamination in insect-pollinated flowers across multiple years under the EU restrictions are missing.

Post-moratorium contaminations may originate mainly from winter cereals or sugar beets, for which neonicotinoid use is currently not restricted. Neonicotinoid seed coated crops take up only a small portion of the active substance, leaving the greatest amount of applied neonicotinoid in soil and the wider environment (Hladik, Main, & Goulson, 2018; Sánchez-Bayo, 2018; Wood & Goulson, 2017). Consequently, neonicotinoids are frequently detected in soil and sometimes even several years after applications ceased (Hladik et al., 2018; A. Jones, Harrington, & Turnbull, 2014), as half-lives in soil can exceed 1000 days (Bonmatin et al., 2015; A. Jones et al., 2014; Krupke, Hunt, Eitzer, Andino, & Given, 2012). It is therefore unsurprising that neonicotinoids were found in untreated crops grown on soil where previously neonicotinoid treated crops were cultivated (Wood & Goulson, 2017). Neonicotinoids further spread in the environment through wind and water. Contaminated soil can be transported by wind erosion (Limay-Rios et al., 2016; Schaafsma, Limay-Rios, Baute, Smith, & Xue, 2015) and contaminated dust during sowing disperses neonicotinoids to non-target plants (Girolami et al., 2013; Greatti, Barbattini, Stravisi, Sabatini, & Rossi, 2006; Greatti, Sabatini, Barbattini, Rossi, & Stravisi, 2003; Krupke et al., 2012; Pistorius et al., 2010; Pochi, Biocca, Fanigliulo, Pulcini, & Conte, 2012; Tapparo et al., 2012). Although seed drills have been improved, dust drift can still be a source of contamination (Bonmatin et al., 2015; Wood & Goulson, 2017). To be systemic neonicotinoids have to be relatively water-soluble, which makes them prone to leaching and transportation via run-off (Huseth & Groves, 2014; Kurwadkar, Wheat, McGahan, & Mitchell, 2014). Leaching potential of neonicotinoids depends on the texture of soils. Neonicotinoids are very mobile in sandy soils and least mobile in loams, where the highest residual concentrations were found (Wood & Goulson, 2017). In general, it remains, however, poorly understood what conditions facilitate the spread of neonicotinoids in the environment. Neonicotinoids contaminations in crops can also originate from commercial seeds that contain neonicotinoids they were not treated with, possibly because the machinery used to dress or count the seeds gets in contact with treated seeds or because the seeds are harvested from crops that were treated with neonicotinoids (Botías et al., 2015; Sánchez-Hernández, Higes, Martín, Nozal, & Bernal, 2016).

Landscape-scale studies identifying specific pesticides that harm bees

To identify specific pesticides whose use causes harmful effects on bees, several observational studies used molecular methods to determine pesticide residues in bees or hive matrices. The residues were then compared to concentrations known to be toxic to bees and/or linked to observed bee mortality or health degradation. Sanchez-Bayo & Goka 2014 combined data from several surveys on pesticide residues in pollen and honey/nectar and assessed their risk to bumblebees and honeybees based on their acute lethal toxicity and known synergistic effects of pesticide mixtures. The authors found frequently a large number of pesticides, but only few of them posed a considerable risk to bees. The risk of ingesting contaminated plant residues was considered concerning for some systemic pesticides, particularly the neonicotinoids thiamethoxam and imidacloprid. By contact exposure, pollen contaminated with neonicotinoid or pyrethroid insecticides presented the largest direct threat and synergism with ergosterol inhibiting fungicides increased their risk strongly. The number of fungicide residues could also be linked to honeybee colony disorders, such as colony or queen death (Noa Simon-Delso et al., 2014).

In another study, maximum concentrations of several systemic pesticides (imidacloprid, fipronil, clothianidin and thiamethoxam) in dead bees from incidents of exceptionally high honeybee mortality were found to reach LD_{50} values for oral toxicity (Kasiotis, Anagnostopoulos, Anastasiadou, & Machera, 2014). However, in the majority of cases, the concentrations of all 115 pesticides screened for were far below lethal levels. This suggests that pesticides were likely not the main cause of death, although it could not be excluded that toxic metabolites of pesticides induced mortality. Among a range of risk factors, *Varroa* mite numbers followed by acetamiprid and thiacloprid presence in hive matrices were the best predictors of winter colony loss in another observational study (Van Der Zee, Gray, Pisa, & De Rijk, 2015). Presence of these neonicotinoids in honey explained honeybee colony losses better than their presence in bees or pollen.

Restricted neonicotinoids were also detected in bees and bee-collected plant residues after the initial moratorium came into force. In a study (Tosi et al., 2018) that analysed honeybee-collected pollen sampled from Italian apiaries between 2012-2014, i.e. ranging

from before to after the moratorium took effect, imidacloprid and thiamethoxam were detected in all three years with high maximum hazard quotients (HQ), which represented the ratio of the maximum residue level (µg kg^{-1}) of each pesticide to its respective oral median lethal dose (µg bee^{-1}). In fact, imidacloprid was the pesticide that exceeded most often the threshold that indicates an elevated risk (HQ>1000).

Imidacloprid was also the third most frequently detected agrochemical after the organophospates chlorpyrifos and dimethoate in dead bees collected in traps at hives in Spanish citrus plantations (Calatayud-Vernich et al., 2016). Imidacloprid was detected at concentrations known to cause at least sublethal effects. However, due to presence of other pesticides, imidacloprid could, unlike the two organophospates, not be linked to changes in mortality rate, which was measured by time-series of dead bee counts. Kiljanek *et al.* 2017 compared residues of a vast range of pesticides and their metabolites in honeybees sampled across Poland either alive from healthy colonies or dead from colonies that exhibited symptoms of poisoning. The bees from poisoned colonies contained clearly more different pesticides and higher pesticide concentrations. All restricted neonicotinoids, some pyrethroids, fipronil, abamectin and spinosyn were only detected in bees from poisoned colonies. Correspondingly, in dead bees, hazard quotients based on acute toxicity and pesticide loads exceeded in 92% the threshold that indicates a relevant risk (HQ>50) and in 60% the threshold that shows an elevated risk (HQ>1000). Hazard quotient indicated that organophosphates posed the greatest threat to bees. This was, however, not only the case in poisoned bees, but also in bees from healthy colonies. Other important insecticide classes reached relevant levels only in dead bees. Apart from organophosphates, neonicotinoids were the pesticides that reached most often relevant levels. However, pyrethroids and fipronil and its metabolites were more often found in levels characterized as elevated risk.

Aims

Laboratory studies are needed to establish causal relationships, but they may fail to depict realistic exposure situations. They often examine the effect of single pesticides in single

doses over a limited amount of time, neglecting possible long-term or cocktail effects. Administered doses do often not reflect the prevalence and concentrations of pesticides in the landscape over time. It is a trivial finding that pollinating insects are not exempt from the adverse effects of insecticides if administered in sufficiently high concentrations. The crucial question is how real-world pesticide use affects bee populations and the vitality of managed honeybee colonies. Pesticide exposure is largely determined by the spatio-temporal distribution of pesticides in the environment and the foraging behaviour of bees, which depends on the landscape context. Therefore, studies on the landscape scale are needed to accurately assess the risk of pesticides to bees.

In this thesis, we address several knowledge gaps concerning the effects of real-world pesticide use on bees. In a first step, we estimate the effect of organic farming on honeybee colony performance. Organic farming is a counter-model to conventional agriculture in which synthetic pesticides and mineral fertilizers are banned. The comparison of organic to conventional farming should therefore give insights into the combined effect of conventionally used pesticides or at least show how these compare to organic pesticides. We also attempt to disentangle direct effects of pesticides on honeybee colonies from effects of organic farming that result from differing floral resources. For this purpose, we relate life history traits of colonies placed in agricultural settings to a gradient of organic farmland as well as to gradients of field cover types whose prevalence differs between organic and conventional agriculture.

Afterwards, we focus on neonicotinoids. We extend a large-scale study on the effect of clothianidin seed treatment in oilseed rape on bees (Rundlöf et al., 2015) to identify potential long-term effects and mechanisms through which neonicotinoids act on bees. We study both honeybees and bumblebees to reveal potential genus-specific differences in susceptibility. In particular, we aim at verifying laboratory-based studies that suggest neonicotinoid-induced impairments of the innate and social immune system of bees and at identifying whether gut symbionts that protect from pathogens (Cariveau et al., 2014; Kaltenpoth & Engl, 2014; Koch & Schmid-Hempel, 2011; Kwong et al., 2017) are affected by exposure to the insecticide. Therefore, we analyse an additional season of exposure as well as new data on colony development, neonicotinoid residues, immune

gene expression, gut symbionts and pathogens/parasites in honeybees. In bumblebees, we examine levels of gut bacteria and pathogens as well as their relation to bee performance. In addition, we examine numbers of bees per caste and the size of bees in exposed and unexposed bumblebee colonies, as this may reveal trade-offs in the production of reproductives (Henry et al., 2015) and/or between number of bees per colony and the size of individuals (Cueva del Castillo, Sanabria-Urbán, & Serrano-Meneses, 2015).

Finally, we evaluate neonicotinoid residues from a multi-season survey on oilseed rape nectar to determine potential contaminations of neonicotinoids of which some are banned in bee-attractive crops. We examine whether there is spill-over of neonicotinoids from treated winter cereals to oilseed rape and if so, which environmental conditions favour this. In addition, we assess the risk to foragers of neonicotinoids detected and quantified in the analysed nectar based on typical sugar consumption of several bee taxa to determine whether the European Union-wide moratorium on three neonicotinoids has effectively eliminated the risk of neonicotinoid-induced mortality.

General materials and methods

Study sites

a) Southern Sweden

The effect of clothianidin exposure on honeybee (Chapter II) and bumblebee (Chapter III) colonies placed by spring-sown, seed-treated oilseed rape was studied in southern Sweden. The region is characterized by a warm temperate climate with 650-800 mm of mean annual precipitation and a mean annual air temperature of approximately 7-8 °C. In 2013, 20 fields were paired based on the land use within 2 km based on data for 2011 and geographic proximity; then one field per pair was randomly assigned to be sown with 25 mL Elado (Bayer; 400 g L^{-1} clothianidin &180 g L^{-1} β-cyfluthrin) per kg and the fungicide thiram, while the other was sown with only and thiram. All experimental fields were sown with the hybrid oilseed rape cultivar Majong at a seeding rate of 150 plants per square metre. In the end of May 2013, fields were inspected for the presence of other flowering crops in a 2 km radius and consequently four fields were excluded due to the presence of large fields with flowering oilseed rape or red clover. Two experimental oilseed rape fields were kept despite the presence of single other oilseed rape fields at 0.9 km (6.5 ha) and 1.0 km (4.4 ha) distance. The four fields that lost their pair after the determination of flowering crops were paired among themselves. This yielded a total of 16 spatially separated (> 4 km) fields (8 control & 8 clothianidin-treated) along a gradient of the proportion of agricultural land, ranging from 6-88%, but with similar land use in the two treatments.

In 2014, 10 different fields of the same farms were used, but the treatment was reversed, i.e. the farms with control fields in 2013 were sown with clothianidin-treated seeds in 2014 and vice-versa (treated: 6; control: 4). In 2014, farmers in the region reported the area of their oilseed rape fields to the Swedish Board of Agriculture, whose records suggest that half of the focal oilseed rape fields had another oilseed rape field (1-13 ha) within 2 km distance.

b) Zone Atelier Plaine & Val de Sèvre, France

The effect of organic farming on honeybee colony performance (Chapter I) and the neonicotinoid contamination of oilseed rape nectar and its associated risk for bees (Chapter IV) were studied in the *Zone Atelier Plaine & Val de Sèvre* in central western France (Figure 1b). The 435 km^2-large Long-Term Social-Ecological Research (LTSER) site is used to assess agricultural and conservational practices. The site is located in region with warm temperate climate with 820 mm annual precipitation and a mean annual air temperature of 12 °C. In the LTSER site, approximately 29000 inhabitants live in 23 municipalities (*communes*), excluding the town Niort whose outskirts partially overlap with the study site (Bretagnolle et al., 2018). The study site is covered by 10% urban areas and 3% woodland and is bordered to the south by a large forest reserve, which is protected under the NATURA 2000 network. The LTSER is primarily used for arable and mixed farming and the land cover contains 39% cereals (including 31% wheat), 10% sunflower 9% maize, 6% oilseed rape and 8% grassland.

In the LTSER site land use has been recorded since 1994. In 2003, more than half of the study site was designated as NATURA 2000 site under the Birds Directive (Bretagnolle et al., 2018). In 2008, the site obtained the status of a *'Zone Atelier'*, making it part of a network of areas dedicated to interdisciplinary research of the anthropogenic impact on the environment funded by the (French) National Center for Scientific Research (CNRS). In the same year, the ECOBEE monitoring program was initiated (Odoux et al., 2014).

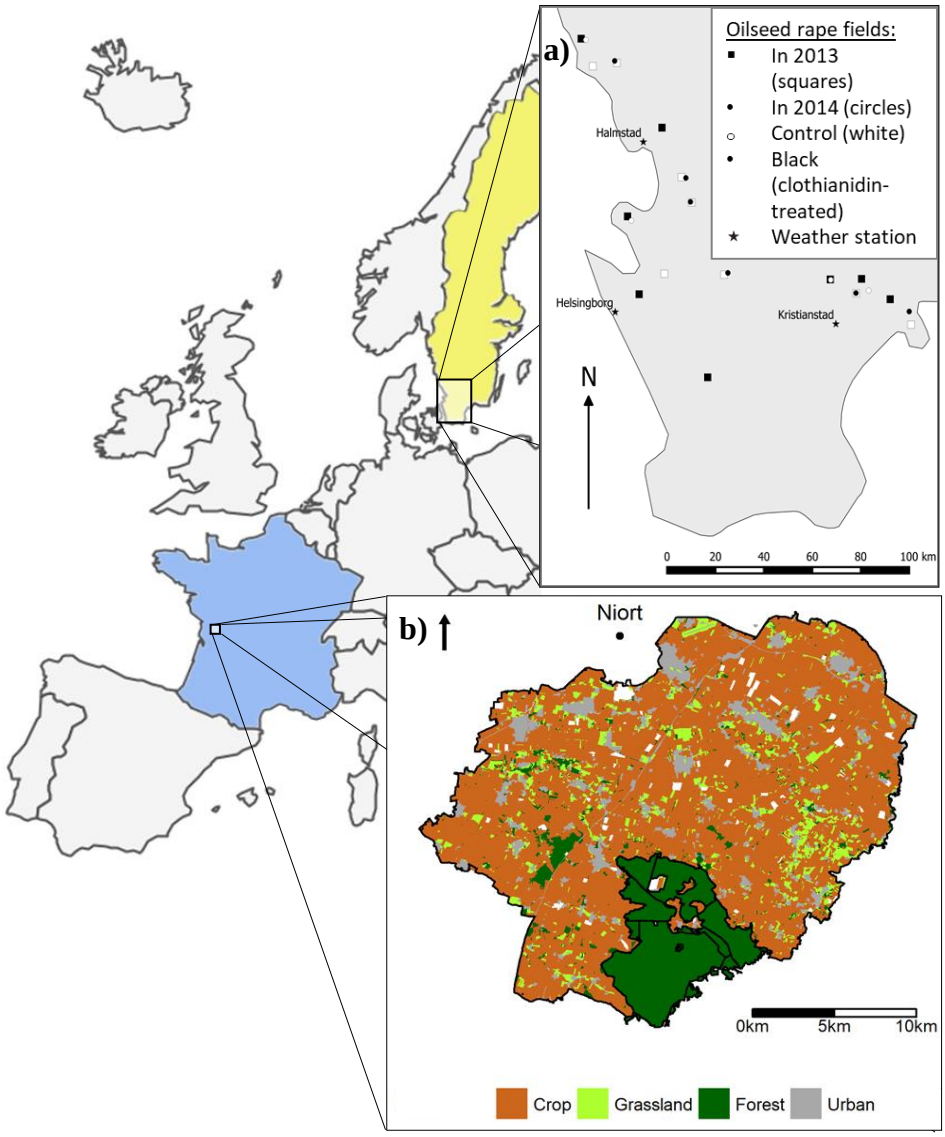

Figure 1 | Location of study sites in Sweden (a; yellow) and France (b; blue). a) Oilseed rape fields in southern Sweden were paired on land use and geographical proximity (but separated by >4 km) and then assigned to be sown with clothianidin-treated or clothianidin-free oilseed rape seeds. Honeybee (2013 & 2014) and bumblebee (2013) colonies were placed next to the fields during oilseed rape flowering. **b)** The Long-Term Social-Ecological Research (LTSER) site '*Zone Atelier Plaine & Val de Sèvre'* and a forest reserve bordering to the south. The map is colour-coded according to land use in 2018. The honeybee monitoring program ECOBEE has been set up in the site since 2008 and nectar from selected oilseed rape fields has been sampled for neonicotinoid screening since 2014.

Study designs & monitoring programs

a) Honeybee and bumblebee colonies next to spring-sown oilseed rape fields in southern Sweden

To determine the effect of clothianidin exposure through seed-treated oilseed rape on free-foraging bees, 96 honeybee (*Apis mellifera* L.) colonies and 96 bumblebee (*Bombus terrestris* L.) colonies were placed next to 16 selected oilseed rape fields (8 clothianidin-treated & 8 control) in 2013, resulting in 6 colonies of each species per field. After the oilseed rape flowering period, the bumblebee colonies were freeze-killed and the honeybee colonies were moved to an overwintering apiary. In 2014, 40 out of the 96 honeybee colonies were reused and placed by spring-sown oilseed rape fields (6 clothianidin-treated and 4 control fields) in apiaries consisting of 4 colonies. To ensure that control colonies in 2014 were not affected by clothianidin-exposure in the previous year, only colonies placed by control fields in 2013 were placed by control fields in 2014. Except for two control colonies, colonies placed by clothianidin-treated fields in 2014 had also been placed by treated fields in 2013.

Before placement at the focal oilseed rape fields, honeybee colonies were equalized in a 60 ha-large organically managed winter-sown oilseed rape field. At the start of the experiment the colonies had two combs with mainly capped brood (with bees), two full honeycombs (with bees), one drawn out empty comb, five combs with wax formation, bees from two additional combs and a 1-2 year old queens. Queen age and lineage was matched between field pairs, but otherwise colonies were randomly distributed to the fields. In June 2014, honeybee colonies were selected that had not swarmed earlier in the year and had an egg-laying queen that had been one year old at the start of the experiment. These colonies were reused and equalized within treatment groups. To avoid swarming during the experiment, relatively small colonies were used at the start of the experiment and their size was intentionally reduced before exposure to the second oilseed rape flowering period but after an assessment of colony strength in spring. Each honeybee colony was treated against the *Varroa* mite twice (10 August: 20 mL 60% formic acid & 4 December: 60 g oxalic acid per litre of water) and fed with 20 kg sugar (in a 55-60% water solution) over three occasions in August and September (Chapter II).

Pre- and post-exposure assessments of honeybee colonies included measurements of the number of adult bees, the number of capped brood cells, colony weight, *Varroa* abundance as well as sampling of 100 bees for microbial analyses. Honey production over the oilseed rape flowering period was estimated from the colony weight measures before and after the oilseed rape bloom. In April 2014, honeybee colonies were assessed for the number of capped brood cells and number of adults to determine their condition after over-wintering.

When the commercially reared bumblebee colonies were placed by the focal oilseed rape fields, they were approximately 10 weeks old and had around 50 workers, one queen, and both pupae and larvae (Chapter III). The colonies, including adult bees, brood and nesting material were weighed when placed by the focal fields and thereafter every two weeks. All 12 colonies of a field pair were killed simultaneously when new queens were determined in any of these. Afterwards, the colonies were weighed and the number of bees per caste (males, queens, workers) was counted. The number of cocoons was also counted, but only queen cocoons were differentiated from the smaller cocoons of males and workers. Cocoons were opened and ten pupae of either males or workers were sampled for microbial analyses and weighed. In colonies in the transition from worker to male production, samples of both male and worker pupae were obtained. In addition, ten adult workers per colony were weighed, measured (for their intertegular span) and stored for microbial analyses.

Four out of the six bumblebee colonies per site and all honeybee colonies were screened for the presence and abundance of microorganisms. Microorganisms were quantified in bumblebee pupae and adults and in honeybee adults using quantitative Polymerase Chain Reaction (qPCR) with a preceding Reverse Transcription step for RNA viruses (RT-qPCR). Microbial analyses included 13 RNA viruses, two *Nosema* species and two beneficial gut bacteria (*Snodgrassela alvi* & *Gilliamella apicola*) for honeybees and seven RNA viruses, the DNA virus *Apis mellifera* filamentous virus, three *Nosema* species, the trypanosomatid *Crithidia bombi* and two beneficial gut bacteria for bumblebees. Bumblebee pupae were, however, only screened for the RNA viruses.

b) Honeybee colonies in the ECOBEE program in France

The ECOBEE program was initiated in 2008 to facilitate research on the impact of farming practices and landscape elements on the intra- and inter-annual dynamics of honeybee colony performance. For ECOBEE, the *Zone Atelier Plaine & Val de Sèvre* was divided into 50 square plots. Each year, ten of these are randomly sampled without replacement for the installation of an experimental apiary. After five years, when apiaries have been installed in all squares, a new 5-year cycle starts.

Before placement in the study site, colonies are assessed in a common overwintering site and then grouped to overall equally strong sets of five, which are then randomly distributed to the selected plots. Three of the five colonies per apiary are examined every two weeks during the beekeeping season, while the other two serve as replacements in case of queen or colony failure and as controls to determine whether the intrusive measures affect colony performance (Odoux et al., 2014). The colonies are examined for the number of adult bees, the amount of brood, honey reserves, *Varroa* abundance and signs of diseases (Odoux et al., 2014). In addition, every 10 days a pollen trap is installed for 24 hours. The collected pollen is stored for palynological analyses and for sporadic pesticide screenings. The honeybee colonies were managed in accordance with typical local beekeeping practices, including treatment against the *Varroa* mite and syrup supply in periods of low resource availability. Queen cells are generally removed to prevent swarming and if needed colonies were re-queened with queens of the same lineage.

c) Neonicotinoid concentrations in winter-sown oilseed rape in France

In the five years of the EU moratorium, a total of 536 nectar samples from 291 winter-sown oilseed rape fields were obtained. The oilseed rape samples were selected based on the previous land use to obtain gradients of the number of oilseed rape and winter cereal cultivations and on soil type. Using micro-capillaries nectar was sampled on 1-6 dates per field. The sampled nectar was then analysed for its sugar content and the concentrations

of the five neonictinoids approved in the European Union: acetamiprid, clothianidin, imidacloprid, thiacloprid, thiamethoxam. Neonicotinoid analyses were done using liquid chromatography with electrospray tandem mass spectrometry.

Chapter I: Effects of organic farming on the seasonal dynamics of honeybee life-history traits

(Manuscript in review in *Journal of Applied Ecology*)

Preface and summary

Conventional farming has been implicated in biodiversity losses and bee declines. Organic farming is an alternative farming approach that aims at limiting ecological repercussions by abstaining from the use of synthetic pesticides and mineral fertilizers. Indeed, studies show that organic farming benefits wild bees and weeds, but its effects on honeybees are largely unknown, partially because their assessment is complicated. Unlike wild bee abundance, honeybee abundance is a relatively poor indicator for the impact of organic farming since honeybees are typically managed by beekeepers who choose their hive location. And, unlike pesticide effects, effects of organic farming cannot be studied in the laboratory. Organic farming may act on bees at different scales. For instance, organic farming may reduce direct pesticide inputs through spray drift into honeybee hives at the local scale and may affect resource availability and dietary pesticide exposure at the landscape scale. Therefore, we made use of the ECOBEE program and studied the effects of organic farming on honeybee colony performance in agricultural landscapes at two spatial scales (300 m & 1500 m) with the intention to disentangle direct pesticide effects from effects of resource availability.

We found evidence that organic farming mitigated the floral dearth between the blooms of oilseed rape and sunflower, as suggested by a larger brood production, which later on translated into larger colonies and more honey reserves, although not in all years. We suspect that organic farming acts on honeybee colonies mainly through a more continuous provision of floral resources due to a higher diversity of crops, more abundant semi-natural elements and more weeds.

Résumé

Effet de l'agriculture biologique sur la dynamique des traits d'histoire de vie de l'abeille mellifère au cours des saisons

Les abeilles peuvent tirer profit de l'agriculture biologique par le fait d'une exposition réduite aux pesticides mais aussi par un approvisionnement plus régulier en ressources florales. De nombreuses études ont montré des effets d'insecticides, en particulier les néonicotinoïdes sur les abeilles, mais l'ampleur de ces effets sur des butineuses en plein champs reste à éclaircir car nombre d'entre elles ont été réalisées dans des contextes simplifiés souvent limitées à des applications de pesticides ponctuelles dans la nourriture. En réalité, les expériences cherchant à lier les performances de l'abeille à des indices linéaires de toxicité montrent des résultats variés.

L'agriculture biologique peut aussi bénéficier aux abeilles par une offre continue en ressources florales tout au long de la saison sur l'ensemble du paysage environnant. L'interdiction des herbicides de synthèse et des engrais minéraux augmente la diversité des plantes adventices en système biologique. Les pratiques biologiques emblavent en général une plus grande variété de cultures qu'en culture conventionnelle et intègrent de plus grandes surfaces d'éléments semi-naturels tels que les haies, qui procurent le gîte et le couvert à l'ensemble des abeilles.

Les effets positifs de l'agriculture biologique sur les abeilles sauvages n'impliquent pas des effets positifs pour les abeilles mellifères. Les apiculteurs fournissent la ruche à leurs abeilles, leur évitant de chercher des matériaux nécessaires pour la construction d'un nid. De plus, comparativement aux abeilles sauvages, d'importantes réserves de nourriture et des distances de butinage supérieures permettent aux abeilles mellifères de compenser les disettes de proximité immédiate. En fait, ces dernières pourraient être désavantagées par les faibles surfaces de colza dans les paysages cultivés en agriculture biologique car elles butinent les cultures oléagineuses à floraison massive de façon intensive. Néanmoins, les abeilles peuvent y bénéficier d'un approvisionnement plus régulier. Comparé à un environnement d'agriculture conventionnelle, l'agriculture biologique offre plus de prairies et de plantes adventices dans les cultures annuelles, sur lesquelles les butineuses

peuvent compter dans les périodes où les ressources sont plus rares en particulier entre les floraisons du colza et du tournesol.

Afin d'examiner les effets de l'agriculture biologique sur les dynamiques des colonies d'abeille, nous avons analysé six années de données, recueillies sur une étude à grande échelle où 50 colonies dispersées en 10 ruchers par an ont été mesurées toutes les deux semaines durant la saison apicole (mars-septembre). Nous avons estimé la surface du couvain d'ouvrières, le nombre d'adultes et les réserves et les avons reliées aux proportions de surfaces conduits en agriculture biologique à l'échelle locale (300m de rayon) ou à l'échelle du paysage (1500m de rayon). Ces échelles ont été choisies afin d'identifier l'effet direct des pratiques agricoles sur les ruches, telles que les pulvérisations par exemple et leur effets différentiels sur les ressources disponibles ou encore l'exposition aux pesticides. Pour déterminer si le type de couverture du sol altère la relation entre l'agriculture biologique et les performances des colonies, nous avons également inclus une interaction entre les jours Julien et les proportions spécifiques de colza, de prairie ou de tournesol dans des modèles additifs généralisés à effets mixtes (GAMM). Nous avons aussi exploré si la récolte de miel après la floraison du colza ou du tournesol était affectée ou non par la proportion d'agriculture biologique ou par les cultures mellifères.

Nous avons trouvé une preuve que l'agriculture biologique diminue la rareté des ressources entre les floraisons de colza et de tournesol, suggérée par une baisse atténuée de la production de couvain. Bien que terminant la période de floraison du colza avec une plus faible quantité de couvain qu'en milieu conventionnel, les colonies bénéficiant d'une large quantité de champs en agriculture biologique au niveau local (300 m) tant qu'au niveau du paysage (1500 m) ont eu clairement plus de couvain pendant la période de disette. Cette relation a été relativement constante dans le temps, bien que l'ampleur de l'effet ait varié d'une année à l'autre et entre les échelles. Cette augmentation de couvain se traduisait généralement tout aussi bien dans les colonies les plus grosses, mais pas toutes les années. De plus, lors de la floraison massive du colza au moment où l'exposition aux pesticides est la plus forte, lorsque le colza est à la fois la culture la plus traitée et la plus attractive, nombre d'abeilles adultes profitent des parcelles biologiques

quand bien même il n'y aurait pas eu d'effet sur les surfaces de couvain. La production de miel a révélé des réponses variées – bien qu'essentiellement positives – apparaissant sujette à un mélange d'effets directs et indirects, incluant des compromis et des effets cascades.

De façon générale, les performances des colonies ont été plus fortement corrélées à l'agriculture biologique à l'échelon de proximité plutôt qu'à l'échelle du paysage. Cela suggère que les champs en biologique à proximité des ruches ont un plus grand impact sur les abeilles, vraisemblablement parce qu'ils réduisent davantage les efforts de butinage ou de résistance à la prise de nourriture contaminée aux pesticides, les colonies butinent donc plus intensément à proximité de leurs ruches.

Nous concluons que l'agriculture biologique peut accroître les performances de la colonie d'abeille dans une période de faibles ressources florales, par un approvisionnement continu en ressources florales généré par les adventices, les couverts végétaux ou les habitats semi-naturels. La surface de couvain d'ouvrières, dont le lien avec la survie hivernale a déjà été avéré, était particulièrement augmentée pendant cette période de disette, ce qui suggère que les terres agricoles biologiques peuvent atténuer les répercussions de l'agriculture intensive sur la vitalité des colonies. Par conséquent, l'agriculture biologique profite à un pollinisateur crucial avec des implications positives potentielles pour l'agriculture dans un paysage plus large.

PhD student's contribution

I led the data analysis and the writing of the article and I contributed to the collection of honeybee data in 2016 and 2017.

Effects of organic farming on seasonal dynamics of honeybee life-history traits

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Summary

1. Conventional farming has been implicated in global biodiversity and pollinator declines and organic farming is often regarded as a more ecological alternative. However, the effect of organic farming on honeybees remains elusive, despite their importance as pollinators of crops and wild plants.
2. Using six years of data from a large-scale study in France with fortnightly measurements of honeybee colony performance traits (10 apiaries per year), we related worker brood area, number of adult bees and honey reserves to the proportions of organic farmland in the surroundings of the hives at two spatial scales (300 m & 1500 m).
3. We found evidence that at the local scale organic farming increased both worker brood production and number of adult bees in the dearth period between the blooms of oilseed rape and sunflower. At the landscape scale, organic farming increased honey reserves during the dearth period and the beginning of the sunflower bloom.
4. The results may suggest that worker brood development benefitted from organic farming mostly through a more diverse diet due to the availability of diverse pollen sources in close proximity of their hives. Reduced pesticide drift may have additionally improved bee survival. Honey reserves were possibly mostly affected by increased availability of melliferous flowers at the foraging scale.
5. *Synthesis and applications.* Organic farming can increase honeybee colony performance in a period of low resource availability, likely through a continuous supply of floral resources including weeds, cover crops and semi-natural elements. Particularly worker brood area was increased in the critical dearth period, which has previously been linked to winter colony survival, suggesting that organic farmland may mitigate repercussions of intensive farming on colony vitality. We conclude that organic farming benefits a crucial crop pollinator with potential positive implications for agriculture in the wider landscape.

Introduction

Modern farming has been questioned because of its effect on public health (O'Kane, 2012), climate change (Conway, 2012) and biodiversity (Stoate et al., 2009). Biodiversity decline causes losses of ecosystem functions, such as biological pest control and insect pollination (Thompson et al., 2014). A radical alternative to conventional agriculture is organic farming that bans the use of synthetic inputs. Organic farming aims at providing healthy food (Forman & Silverstein, 2012), conserving species richness and maintaining ecosystem functioning (Sandhu, Wratten, & Cullen, 2010). Indeed, organic farming increases biodiversity on-site (Hole et al., 2005; Tuck et al., 2014) and in adjacent fields (Henckel, Borger, Meiss, Gaba, & Bretagnolle, 2015). This holds particularly true for pollinators, which show a greater increase in diversity than other functional groups (Tuck et al., 2014). Organic farming enhances bee species richness (Holzschuh, Steffan-Dewenter, & Tscharntke, 2008; Kennedy et al., 2013), the abundance of solitary bees and bumblebees (Holzschuh et al., 2008; Kennedy et al., 2013; Morandin & Winston, 2005) and pollination rates (Morandin & Winston, 2005; Smith, Andersson, Rundlo, Rundlöf, & Smith, 2012). Wild bees benefit from organic farming on both the local (Kennedy et al., 2013) and the landscape scale (Holzschuh et al., 2008).

The reasons why wild bees benefit from organic farming are less clear, however. Positive effects may result from lower pesticide exposure and therefore a reduced intoxication risk. Numerous laboratory and field studies showing toxic effects of single pesticides, particularly the neonicotinoids, suggest that bees may profit from the ban of synthetic pesticides in organic farming, but the extent to which this would occur remains unclear (Mallinger, Werts, & Gratton, 2015). Indeed, studies assessing the impact of pesticide use along a continuous toxicity index showed varying results. Mineau *et al.* (2008) could link reported honeybee mortality incidents at hives to pesticide use intensity, while Kremen *et al.* (2004) failed to relate delivered pollination services to insecticide use. Intensive pesticide use reduces the abundance and species richness of wild bees, but impacts vary across seasons and taxa (Mallinger et al., 2015; Park, Blitzer, Gibbs, Losey, & Danforth, 2015; Tuell, 2010). Varying impacts may result from differing landscape composition (Carvalheiro, Seymour, Nicolson, & Veldtman, 2012; Mallinger et al., 2015; Park et al.,

2015) or from differences between species in life-history traits (Tuell, 2010) or the sensitivity to pesticides (Arena & Sgolastra, 2014). In particular honeybees may be less susceptible than wild bees to pesticide effects as they live in large colonies that can compensate for individual forager losses (Henry et al., 2015; Osterman et al., 2019; Rundlöf et al., 2015).

Boosted bee populations in organic farms are therefore not necessarily due to reduced pesticide exposure. In fact, the risk of intoxication can in some instances be higher in organic than in conventional agricultural land (Mallinger et al., 2015). Alternatively, organic farming may outperform conventional agriculture in maintaining large diverse pollinator communities by provisioning floral resources continuously across the landscape and throughout the season (Brittain, Bommarco, Vighi, Settele, & Potts, 2010; Winfree, Williams, Gaines, Ascher, & Kremen, 2008). The ban of synthetic herbicides and mineral fertilizers increases the diversity (Ekroos, Hyvönen, Tiainen, & Tiira, 2010; Gabriel & Tscharntke, 2007) and density (Bengtsson, Ahnström, & Weibull, 2005; Ponce, Bravo, de León, Magaña, & Alonso, 2011) of weeds in organic farms. In addition, organic farmland is often sown with a greater variety of crops than conventionally farmed land (Barbieri, Pellerin, & Nesme, 2017; Hole et al., 2005) and comprises larger areas of semi-natural elements (Gibson, Pearce, Morris, Symondson, & Memmott, 2007), such as hedgerows, which provide forage and nesting opportunities to bees (Hannon & Sisk, 2009).

However, how organic farming affects honeybees (*Apis mellifera* L.) cannot necessarily be inferred from positive effects on wild bees. Evidence for preferential honeybee foraging on organic farmland is lacking (Couvillon, Schürch, & Ratnieks, 2014) and honeybees differ from wild bees in many respects such as nesting requirements, foraging behavior and the extent of human management. Honeybees forage particularly intensively on mass-flowering oilseed crops (Rollin et al., 2013) and may therefore be disadvantaged by the low amount of oilseed rape in organic land in Europe (Barbieri et al., 2017). In addition, naturally larger food reserves and greater foraging distances (Gathmann & Tscharntke, 2012; Steffan-Dewenter & Kuhn, 2003) allow honeybees to

better compensate local or temporary food shortages as compared to wild bees. Nevertheless, honeybees may benefit from a more continuous provision of flowers in organic farmland. Compared to conventional agricultural land, organic farmland contains more grassland and weeds in annual crops (European Commission, 2018), which honeybees rely on in periods of low resource availability, e.g. between the blooms of oilseed rape and sunflower (J. F. Odoux et al., 2012; Requier et al., 2015). To sum up, potential benefits of reduced pesticide exposure may be offset in spring by less forage due to a lower availability of oilseed rape in organic than in conventional agriculture, but over the course of the season honeybees should profit from a more continuous supply of wild flowers in organic agriculture.

Here, we use empirical data collected during six years from 60 apiaries (326 hives) located in landscapes varying in the proportion of organically farmed land to quantify how organic farming affects honeybee colony performance. We predicted that during the oilseed rape bloom, organic farming benefits particularly adult bees through reduced pesticide exposure, but potentially harms honey or brood production through reduced availability of oilseed rape. However, afterwards organic farming should mitigate the dearth between the blooms of oilseed rape and sunflower through a more continuous supply of resources. Despite potential trade-offs with worker brood area, we predict that organic farming will increase honey reserves towards the end of the dearth period due to enhanced availability of melliferous weeds or a prior positive effect on number of adults and therefore the number of available foragers. We test these hypotheses and assess more generally (i) how honeybee colonies respond to organic farming (ii) at what spatial scale responses are the largest and (iii) what proportion of organic farmland in the landscape is required to observe an effect on honeybee colony performance. Finally, we aim at gaining insight into the characteristics of organic farming (crop choice, weeds, insecticide risk) that affect honeybee colonies the most.

Materials & methods

THE STUDY SITE

The study was conducted in the *'Zone Atelier Plaine & Val de Sèvre',* a 435 km^2 large Long-Term Social-Ecological Research (LTSER) site in central western France (46°23'N, 0°41'W; Fig. 1). The region is characterized by a warm temperate climate with c. 820 mm of annual precipitation and a mean annual temperature of 12.0 °C. Since 1994, the land use within the LTSER site has been recorded and mapped on vector-based shapefiles (Bretagnolle et al., 2018). Within the study period (2012-2017), the area was covered on average by 40.4% with cereals (mainly winter wheat: 33.8%), 9.9% maize, 9.7% sunflower, 7.9% grassland, 7.7% oilseed rape, 3.5% alfalfa and 7.5% other crops. The site contains also 9.8% of urban areas and 3.1% of fragmented woodlands and is bordered in the north by the town Niort and in the south by a large forest reserve (Fig. 1).

Organic land could be accurately mapped on shapefiles, because the director of the LTSER site contracts farmers on behalf of regional and national authorities for the application of Agri-Environmental-Measures (AEM; half of the LTSER site is designated as a Natura 2000 site under the Birds Directive), including organic farming. Farmers receive payments for both the conversion to and the maintenance of organic farming practices. Here, we merged organic farmland in the conversion (three years) and the maintenance period. Within the study period, the organic farmland in the study site was covered on average to 34.7% with cereals (mainly winter wheat: 22.7%), 13.7% grassland, 17.7% legumes (mostly alfalfa: 9.5%), 9.1% sunflower, 6.0% maize, 1.3% oilseed rape.

THE STUDY DESIGN

In 2008, ECOBEE, a monitoring program of experimental apiaries was launched in the LTSER site. ECOBEE aims at correlating honeybee colony performance metrics with landscape composition and farming practices. For this purpose, the LTSER site was divided into 50 square plots, of which 10 are randomly selected without replacement each

year for the installation of an apiary. After all plots have once been occupied with an experimental apiary (i.e. after five years), a new random sampling cycle starts.

The apiaries, consisting of five colonies, are installed in semi-natural habitat near the center of the 10 km^2-large plots, which encompass the mean foraging distance (c. 1.5 km) in such agricultural landscapes (Steffan-Dewenter & Kuhn, 2003). After each beekeeping season (March-September), colonies are assembled to overwinter outside the study site.

The colonies are managed using common practices of local beekeepers, including control treatment against the *Varroa* mite and syrup supply in periods of low resource availability. In the beginning of the season, hives consist of only a 10-frame-Dadant-Blatt brood box; as the colonies grow, honey supers are added (Odoux et al., 2014). Honey is harvested after the sunflower bloom, and from 2008 to 2012, also after the oilseed rape bloom. When needed, colonies are re-queened with queen cells of the same lineage.

Due to the colony placement scheme and the heterogeneous distribution of organic land, colonies were exposed to different amounts of organic land. In the LTSER site, the proportion of organic farmland increased gradually from 0.6% to 7.1% between 2008 and 2017, because several conventional farmers converted to organic farming, while no organic farmers switched to conventional agriculture.

In 2008-2011, the number of apiaries exposed to high amounts of organic farmland was too low to allow for meaningful inferences of how honeybee colony performance is affected by an organic farmland gradient and in 2008 honeybee data were only collected in June and July. Therefore, we restricted our analyses to 2012-2017, but presented results from analyses of the dataset for 2009-2017 as Supporting Information (Fig. S1 & S2).

MEASURED PARAMETERS

Monitoring of colonies in ECOBEE is described in detail in Odoux *et al.* (2014). We used three colony performance traits that are major components of a colony's temporal

dynamic: worker brood area, number of adults and honey reserves. These parameters were recorded in three colonies every two weeks during the beekeeping season (two additional colonies are used as controls or as substitutes in case of queen or colony failure (Odoux et al., 2014)). On both sides of the hive frames, the lengths and widths of the area covered by eggs, larvae or pupae were measured to estimate the elliptic brood area, which was then accumulated for each hive. Drone brood area was equally estimated and deducted from the total brood area to obtain worker brood area. Hive frames, honey supers and hive bottoms were weighed with and without adult bees. The difference was then divided by 0.1 g bee^{-1} to obtain an estimate of number of adults. This estimate does not account for the bees that were foraging during monitoring. To estimate honey reserves, the weights of honey supers and frames without bees were summed up; then, the estimated brood weight and the initial weight of empty supers and frames were deducted from this. The brood weight was derived from the brood area and an estimated brood surface density of 3.91 kg m^{-2} (Odoux et al., 2014). The weights of pollen and wax were neglected, as they are largely surpassed by the weights of nectar and honey.

STATISTICAL ANALYSES

Plant phenology varies between years due to differences in meteorological conditions, particularly the accumulation of heat (Miller, Lanier, & Brandt, 2001). To be able to compare years, Julian dates were, therefore, standardized through adjustment according to growing degree days (GDDs) for oilseed rape (Appendix S1).

In a first step, we examined how honeybee colony performance traits (i.e. worker brood area, number of adults and honey reserves) evolved over spring and summer, i.e. from GDD-adjusted Julian day number (hereafter 'Julian day') 70 to 220. The colony performance traits were fitted by generalized additive mixed models (GAMMs) using the 'gamm' function of the 'mgcv' package in R with a 's' smooth term (i.e. a penalized thin-plate regression spline) for Julian days. To obtain homoscedasticity and normally distributed residuals, honey reserves were fitted using GAMMs with a gamma distribution and a logarithmic link function, while for worker brood area and number of adults could a Gaussian distribution was used. Smoothness selection was done via

maximum likelihood (ML) for GAMMs with Gaussian distribution and via penalized quasi-likelihood (PQL) for GAMMS with Gamma distribution. All GAMMs containing data of multiple years included colony identity nested in apiary identity nested in year as random factors, while GAMMs on individual years included colony identity nested in apiary identity as random factors. Confidence intervals of GAMM fits were calculated by non-parametric bootstraps with 1100 simulations, whereby apiaries were randomly selected.

In a second step, the relation between organic farming and honeybee colony performance was evaluated at two *a priori* defined spatial scales (300 m & 1500 m). The smaller spatial scale (hereafter 'local scale') was chosen to cover the fields directly neighboring the apiaries (mean field size = 5 ha), while the larger one (hereafter 'landscape scale') was chosen in regard to the average foraging distance of honeybees in farmland landscapes (mean=1300-1800 m, median=1100-1300 m, (Steffan-Dewenter & Kuhn, 2003)). For this purpose, the proportion of organic farmland in 300 m and 1500 m circular buffers around the hives was obtained from shapefiles. GAMMs used to evaluate the effect of organic farming on colony performance, included a smooth term for the main effects, and the interaction of Julian days and the proportion of organic farmland in the surroundings of the hives at either of the spatial scales (fixed effects smooth-term: s(Julian days, proportion of organic farmland)). Finally, a third set of GAMMs was run, that included also two-way interactions between Julian days and the proportion of either oilseed rape, sunflower or grassland as predictor variables (fixed effects smooth-terms: s(Julian days, proportion of organic farmland) + s(Julian days, proportion of a field cover type)). These were used to test whether differences between colonies with different extents of exposure to organic farming were simply due to differences in field cover rather than due to differences in farming practices. Unlike organic farmland, the three field cover types (oilseed rape, grassland, sunflower) were only mapped in the LTSER site; therefore, when calculating their proportion in the surroundings of apiaries at the edge of the study site, only the land area within the LTSER site and the neighboring forest reserve was considered (Fig. 1). This is based on the assumption that the

percentage of these field cover types in the LTSER site is largely the same as in the directly neighboring area outside the LTSER site, except where the forest reserve is.

Before fitting GAMMs containing interaction terms, all predictor variables were mean-centered and scaled to allow for isotropic smoothing. GAMMs on the whole study period (2012-2017) were fit to 326 colonies from 60 apiaries (a new apiary identity was attributed each year). A grand total of 2506 observations were used for worker brood area and number of adults. GAMMs on honey reserves were fit to fewer observations (1792), as we excluded data that were collected after the sunflower honey harvest. For colonies without honey harvest, we considered only data that were obtained before the date of the last honey harvest of the year in any apiary. We did not account for differences in honey harvest after the oilseed rape bloom, as within the study period, oilseed rape honey was only harvested in 2012.

Using the GAMMs, colony performance traits were estimated in 5% intervals within 0-15% organic farmland at 1500 m and 10% intervals within 0-30% at 300 m and in 5-day intervals of the timeframe between the beginning of the oilseed rape period, shortly after colonies were placed in the study site, to the end of the sunflower bloom, before the harvesting of honey. Estimation was done in smaller ranges of dates and organic farmland proportions than the ranges of the data used to fit the models to ensure high estimation accuracy at boundaries.

To estimate the effect of organic farming independently of field cover, estimation at different dates and organic farmland proportions was done using models incorporating the proportion of a field cover type, which was set to its mean.

Because the seasonal effect was very pronounced, the effect of organic farming (*OF effect*) was highlighted by expressing estimates at any proportion of organic farmland (*OF estimate)* as a percentage difference to the mean of the estimate itself and the estimate for no organic farmland at the same Julian day (*CONV estimate*):

OF effect = 2 × 100% × (*OF estimate* - *CONV estimate*) / (*OF estimate* + *CONV estimate*) (eqn. 1).

Taking the mean across both the OF and the CONV estimate ensured equal weighting. *P*-values were obtained from bootstraps with 1100 simulations, whereby apiaries were randomly selected. *P*-values under the null hypothesis that *OF effect* does not differ from zero were computed as the fraction of simulated mean-centered *OF effect* values that are greater than or equal to the estimate of *OF effect.*

The effect of organic farming on honey harvest was evaluated using two different parameters. First, we tested how organic farming affected the probability that honey could be harvested from a colony using generalized linear mixed-effects models (GLMM) with a logit link function; second, we analyzed the effect on harvested amounts only in those colonies with honey harvest by linear mixed-effects models (LMM) with a normal error distribution. Models on the honey harvest after the oilseed rape bloom in 2012 contained apiary identity as a random factor and (G)LMMs on the honey harvest after the sunflower bloom contained year and apiary identity as random factors. Amounts of honey harvest after the sunflower bloom were square-root transformed to obtain normally distributed model residuals. *P*-values of (G)LMMs were calculated by likelihood ratio tests. Absence of considerable spatial autocorrelation was visually determined as exemplarily shown for honey harvest after the sunflower bloom (Fig. S3).

The ‘lmer’ and ‘glmer’ functions of the ‘lme4’ package were used to fit (G)LMMs. Shapefiles were prepared in QGIS and proportions of land use were calculated in R using the ‘raster’ package. All analyses were done in R version 3.5.0.

Results

The amount of organic farmland varied strongly over space, which resulted in very different exposure levels between apiaries (Fig. 1 & S1). The proportions of organic land at the landscape and the local scale strongly correlated (r_S=0.67, P<0.001, N=60), but this was due to apiaries without any organic farmland at the local scale; when removed there was no correlation anymore (r_S=0.23, P=0.41, N=15). All apiaries were exposed to oilseed rape, grassland and sunflower at the landscape scale. Proportion of grassland correlated negatively with oilseed rape at both spatial scales and positively with sunflower at the local scale (Table S1). At neither scale, the proportions of these field cover types correlated with the proportion of organic farmland around the apiaries (Table S1).

All three colony traits varied along the season showing peaks in both spring and summer (Fig. 2, Fig. S5, Table S2). Worker brood production was highest in the second half of April, declined in May, and peaked again at the end of June. Number of adults exhibited a similar but less marked seasonal pattern, peaking approximately 10 days later than worker brood area in spring, whereas the summer peaks coincided. Honey reserves showed a first peak at the end of the oilseed rape flowering period and a much more pronounced one at the end of the sunflower bloom.

HONEYBEE COLONY RESPONSES TO ORGANIC FARMING

Honey reserves and worker brood area varied more strongly with organic farming and time than number of adults (Fig. 2, Table S2).

In the dearth period (between the blooms of oilseed rape and sunflower), colonies with organic farmland in their local environment had up to 37% more worker brood than colonies without organic farmland exposure at the same spatial scale. In fact, at the local scale (300 m), worker brood area tended to be positively related to organic farmland in almost all years (Fig. S6). The effect size varied, however, between years and was largest in 2012 and 2015, years in which all colonies exposed to organic farming at the local scale were exposed to at least 25% organic farmland. At the landscape scale, no effect of organic farming on worker brood area was detected (Fig. 2).

Number of adults followed generally a similar pattern to worker brood area, but effects tended to be weaker (Fig. 2) and statistically significant differences were detected in fewer years (Fig. S7). Largest positive differences between colonies with and without organic farmland in their surroundings were, as for worker brood area, detected at the local scale during the dearth period (~+20% at 10-25% organic farmland), which was particularly the case in 2014 when the estimated effect was even larger and occurred over a longer period than for worker brood area (Fig. 2 & S6). As for worker brood, no effect of organic farming on number of adults was observed at the landscape scale.

Contrary to worker brood area and number of adults, honey reserves was not related to organic farming at the local scale but at the landscape scale. Honey reserves tended to be larger in colonies with organic farming exposure at the landscape scale throughout the dearth period until shortly before the peak of the sunflower bloom (Fig. 2; +53% at 5% organic farmland). We only determined for colonies exposed to little amounts of organic farmland a positive effect on reserves, but this is only because strong positive effects in colonies with high organic farmland exposure in 2013 and 2014 (Fig. S8) were partly offset by non-significant negative effects in 2016.

Most consistent positive effects were observed at the landscape scale at the beginning of the sunflower bloom (Fig. 2 & Fig. S8). At the local scale, strong contrasting effects offset themselves (Fig. S8) so that no overall effect could be detected (Fig. 2).

We observed only relatively subtle effects on the estimated relation between organic farming and colony performance, when accounting for differences in field cover (Fig. S9, S10 & S11). Including the proportion of grassland reduced the positive effects of organic farming on worker brood area and number of adults (Fig. S9 & S10).

HONEY HARVEST

In 2012, honey was harvested from 62% of colonies after the oilseed rape bloom and the probability of harvest increased with the amount of organic farmland in a 300 m radius (Fig. 3; χ^2=4.39, P=0.036). Incorporating the proportion of oilseed rape in 300 m distance as a covariate into the model increased statistical significance (χ^2=6.74, P=0.009). At the landscape scale, no effect could be determined (χ^2=0.81, P=0.37), as confidence intervals

were wider. Among colonies with harvest after the oilseed rape bloom, there was no relationship between organic farming and the amount of honey harvest in a 300 m (χ^2=0.47, *P*=0.49) or 1500 m radius (χ^2=0.78, *P*=0.46). In all years, honey was harvested after the sunflower bloom. The proportion of colonies with harvest varied, however, strongly between years from 6% in 2015 to 64% in 2012, but was unaffected by the proportion of organic farmland in 1500 m (χ^2=1.14, *P*=0.29) or 300 m distance (χ^2=0.31, *P*=0.58). Among colonies with harvest after the sunflower bloom, the amount of harvest was not affected by organic farming at the landscape scale (χ^2=1.14, *P*=0.29) or at the local scale (χ^2=2.69, *P*=0.10).

Discussion

Intensive agriculture has been blamed for low vitality and survival rates of honeybee colonies and organic farming is often regarded as a more bee-friendly alternative. However, how organic farming affects honeybee colony performance has, to our knowledge, not been studied yet.

We expected the effect of organic farming to vary with the period of the year and between colony traits, either in relation to reduced pesticide intoxication risk during mass-flowering of oilseed crops or in relation to increased availability of floral resources, such as weeds, meadows and semi-natural elements, during the dearth period (between the blooms of oilseed rape and sunflower). In the oilseed rape flowering period, we suspected, however, that honeybee colonies in landscapes rich in organic farmland may have fewer resources available, since oilseed rape, a crop that honeybees forage on extensively for nectar and moderately for pollen (Requier et al., 2015), is less commonly cultivated in organic agriculture.

We found, however, no negative relationship between honeybee colony performance and organic farming during the oilseed rape bloom. Oilseed rape was about seven times more common in conventional than in organic farmland in our study site, but due to dilution in the surrounding landscape, the correlation between the proportions of organic land and oilseed rape was not significant and barely negative (r_s ~ -0.13). Accounting for the

proportion of oilseed rape in the surroundings of the bee hives did not affect the estimated organic farming effect, suggesting that differences in oilseed rape availability were not a major driver of colony performance, possibly because negative effects of reduced oilseed rape availability may have been offset by positive effects due to reduced pesticide exposure (Balfour et al., 2017), particularly since oilseed rape is typically the most heavily treated insect-pollinated crop in France (AGRESTE, 2013).

After the oilseed rape bloom, worker brood area declined less in colonies exposed to organic farming at the local scale compared to colonies without organic farming exposure, so that they had substantially more brood in the dearth period. Although effect sizes varied, this positive effect was fairly consistent across years. Worker brood production requires pollen supply and pollen resources are rare in the dearth period (Odoux et al., 2012; Requier et al., 2015; Requier, Odoux, Henry, & Bretagnolle, 2017). Organic farming may provide floral resources, including pollen sources, more continuously throughout the season and therefore prevent worker brood production from plummeting in periods of low resource availability. Higher weed availability, resulting from the ban of synthetic herbicides in organic farming (Bengtsson et al., 2005; Henckel et al., 2015; Tuck et al., 2014) and more perennial or legume cover crops for nitrogen fixation (Decourtye, Mader, & Desneux, 2010) may increase floral abundance in periods when no major cash crop is flowering. More abundant grassland in organic farming may further increase the temporal continuity of resource availability (Bengtsson et al., 2005), which is supported by the finding that the size of the estimated organic farming effect on worker brood area during the dearth period decreased when incorporating the proportion of grassland in the model. As expected, positive effects on worker brood area translated into positive effects on number of adults (Requier et al., 2016), although with a lower effect size, possibly because worker brood area fluctuates more than adult number. In addition, positive effects on number of adults may have been in part offset by a trade-off between colony size and individual bee longevity, as honeybees in larger colonies tend to forage at a younger age, which in turn reduces their lifespan (Rueppell, Kaftanouglu, & Page Jr., 2009).

Positive relationships between organic farming and worker brood area or number of adults were only observed at the local scale suggesting that organic fields impact colony size especially when they are in a short distance. Fields in proximity of hives are more likely to be foraged on (Couvillon et al., 2014), since honeybees attempt to minimize their energy consumption (Stabentheiner & Kovac, 2016). Therefore, organic fields in close proximity to hives may reduce foraging efforts of honeybees more strongly than fields at greater distance. Honeybee colonies next to organic fields may be less impacted by pesticide drift and forage on a wider diversity of pollen sources and suffer therefore from fewer micro-nutrient deficiencies (Filipiak et al., 2017). During the sunflower bloom, no relationship between organic farming and worker brood area or number of adults could be observed. In this period, organic farming may provide fewer benefits to bees as sunflower is approximately equally used in organic and conventional agriculture and less intensively treated than oilseed rape (AGRESTE, 2013).

Honey reserves is the colony trait that has the most complex relationship to organic farming. Organic farming can directly affect honey reserves through the availability of melliferous flowers or indirectly through effects on worker brood area and number of adults, which then affect honey reserves through trade-offs or cascading effects (Requier et al., 2016). In the dearth period and at the beginning of the sunflower bloom, colonies exposed to organic farmland at the landscape scale tended to have larger honey reserves, suggesting that colonies in landscapes rich in organic farmland benefitted from an increased availability of melliferous flowers after the oilseed rape bloom. It is also conceivable that colonies with access to organic farming could satisfy their pollen demands more easily, which allowed them to forage more intensively on nectar sources.

At the local scale, strong positive effects in some years offset similarly strong negative effects in other years. This may potentially be due to trade-offs between worker brood and honey production, as e.g. suggested by the finding that the most pronounced negative effects on honey reserves occurred with a short delay but in the same year as the strongest positive effects on worker brood area (2015; Fig. S6 & S8).

Conclusions

Our study presents strong evidence that organic farming affects honeybee colony performance. Several pathways through which organic farming may act on honeybee colonies, including insecticide reduction, herbicide reduction, crop choice and provision of semi-natural elements and cover crops, need to be studied in isolation or in fully crossed experiments, because they may counteract each other. In our study, we found, however, that positive effects (wild flower resources, pesticide ban) prevailed over negative ones (reduced oilseed rape occurrence). We suspect that organic farming may provide benefits to beekeepers by increasing colony survival. Winter colony mortality has previously been linked to reduced pollen collection and brood production in the period between the blooms of oilseed rape and sunflower, which is characterized by low resource availability (Requier et al., 2016). Our results suggest that organic farming may counteract declines in worker brood production in this period and therefore potentially increase long-term colony survival. We, therefore, conclude that organic farming can buffer adverse effects of intensive agriculture on honeybee colonies. Increased vitality of honeybee colonies, which forage at a large scale and are crucial pollinators of natural vegetation and cropland (Potts et al., 2016), suggest that organic farming may enhance pollination not only on field but also in the wider landscape. This remains to be confirmed, but such an effect would suggest that organic farming could provide benefits to both biodiversity conservation and agricultural production.

Authors' contributions

J-FO and VB designed the monitoring scheme; J-FO and DW engaged in data collection; VB, DW and J-FO defined the research questions and hypotheses; DW, VB and JC conducted the statistical analysis; DW and VB led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Data accessibility

After publication, we will make the data available on request.

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Figures

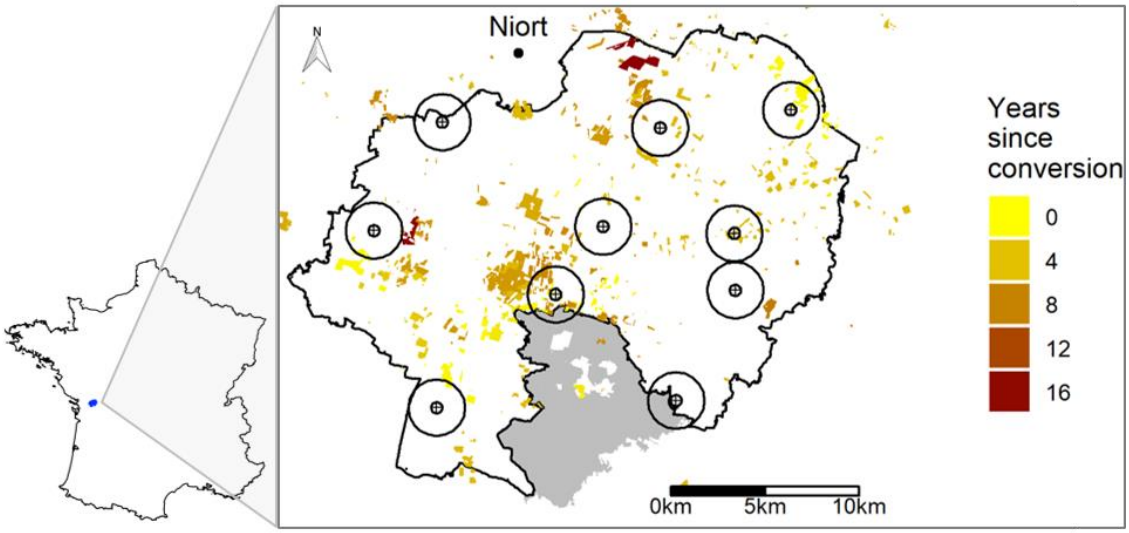

Fig. 1. Location of the Long-Term Social-Ecological Research (LTSER) site '*Zone Atelier Plaine & Val de Sèvre'* within France and a map extract showing the LTSER site, the bordering forest reserve (in grey) and organic fields in 2016, which are color-coded according to the number of years since conversion to organic farmland. Crosses indicate locations of experimental apiaries in 2016. The small circles touching the crosses indicate 300 m buffer areas and large circles show 1500 m buffer areas.

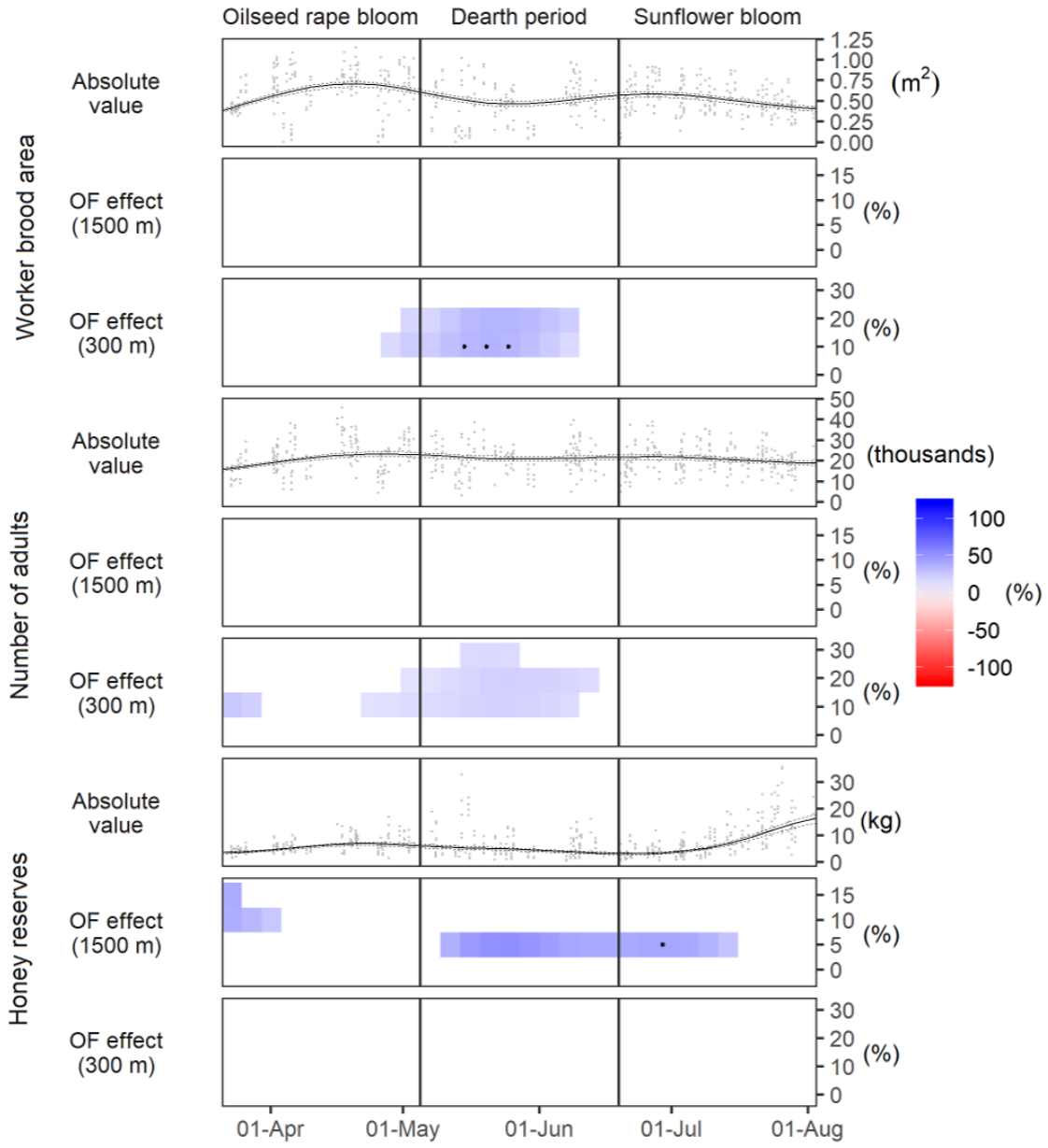

Fig. 2. Variation of worker brood area, number of adults and honey reserves across spring and summer. The solid lines denote estimates of generalized additive mixed models, the dashed lines bootstrapped 95% confidence intervals and the dots mean values per apiary and day. The seasonal variation of the relation between colony performance traits and the proportion of organic farmland in a 1500 m or 300 m radius around the hives is illustrated as a color-coded percentage difference between colonies with and without exposure to organic farmland (*OF effect*, equation. 1). The color gradient shows positive differences (i.e. higher values in colonies exposed to organic farmland) in blue

and negative ones in red. Cells in white indicate that $P>0.05$ and dots that $P<0.001$. *OF effect* has been calculated for 5-15% organic farmland at the landscape scale (1500 m) and 10-30% organic farmland at the local scale (300 m). The *P*-values of different point estimates are not independent and have not been corrected for multiple testing.

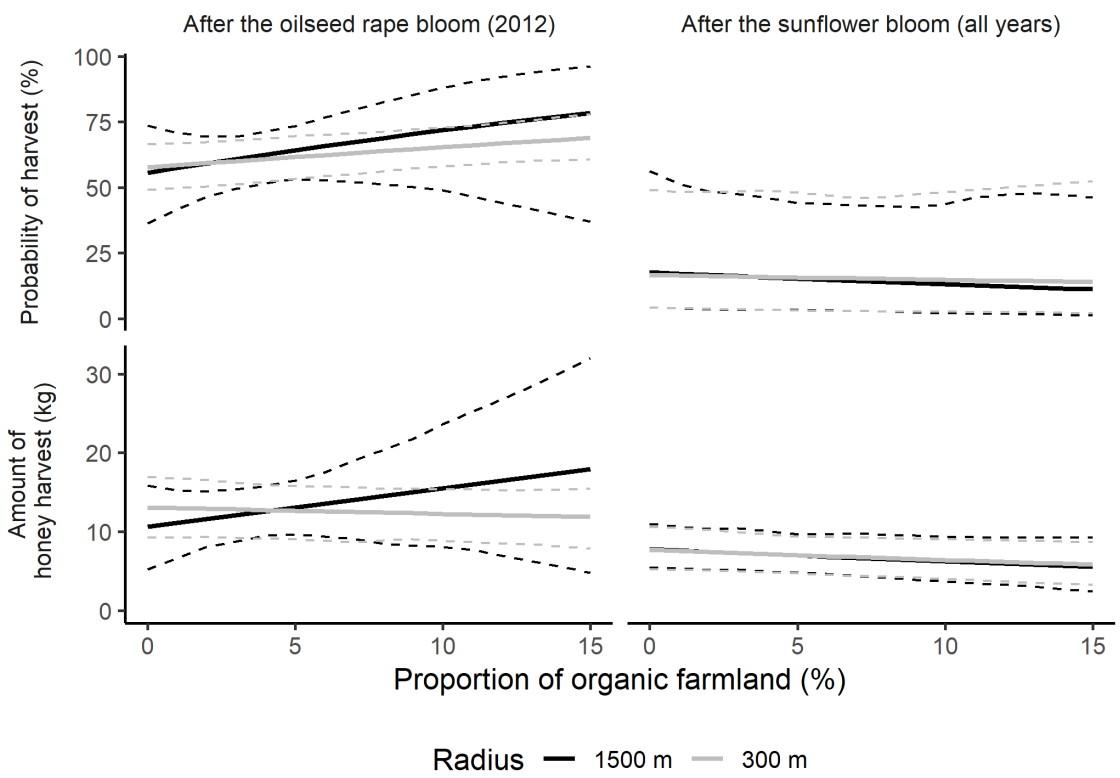

Fig 3. Honey harvest after the oilseed rape bloom in 2012 and after the sunflower bloom in all years (2012-2017) in relation to the proportion of organic farmland in a 1500 m and a 300 m radius around the honeybee hives. Honey harvest is characterized by two parameters: the probability that honey could be harvested from a colony & the amount of honey harvest among those colonies with harvest.

Supporting Information

Appendix S1. Adjustment of Julian days according to growing degree days.

To correct for inter-annual differences in ambient temperature, Julian days were adjusted according to growing degree days (GDDs) for oilseed rape (base temperature = 5 °C). GDDs were calculated by subtracting the base temperature from the mean of the daily minimum and maximum ambient temperature. Negative values were set to zero, as no (oilseed rape) plant growth occurs below the base temperature. GDDs were then accumulated from the first day of the year to each other day. Afterwards, Julian days between 2009 and 2017 were linked to their cumulative GDDs by a locally weighted regression (LOESS). Adjusted Julian days were then obtained by predicting them based on the LOESS fit and the measured cumulative GDDs of each regarded date.

Table S1. Spearman correlations between the proportions of organic farmland and oilseed rape, grassland and sunflower in a 1500 m and 300 m radius around 60 apiaries.

Field cover types		**r_s (1500 m)**	***P*** **(1500 m)**	**r_s (300 m)**	***P*** **(300 m)**
Organic land	Oilseed rape	-0.13	0.324	-0.12	0.336
Organic land	Grassland	-0.03	0.847	0.11	0.420
Organic land	Sunflower	0.08	0.554	0.13	0.339
Oilseed rape	Grassland	**-0.36**	**0.005**	**-0.26**	**0.046**
Oilseed rape	Sunflower	0.04	0.761	0.06	0.650
Grassland	Sunflower	**-0.28**	**0.033**	-0.19	0.143

Table S2. Model statistics of generalized additive mixed-effects models (GAMMs). Julian day numbers have been adjusted to cumulative oilseed rape growing degree days (see Appendix S1). Effective degrees of freedom (e.d.f.) were selected based on maximum likelihood without setting an upper limit (k).

Response	**Spatial scale**	**Predictor**	***F***	***e.d.f.***	***P***
Worker brood area	n/a	s(Julian day)	**145.1**	**8.8**	**<0.001**
Number of adults	n/a	s(Julian day)	**40.9**	**8.0**	**<0.001**
Honey reserves	n/a	s(Julian day)	**177.4**	**8.6**	**<0.001**
Worker brood area	1500 m	s(Julian day, organic farmland)	**47.6**	**27.5**	**<0.001**
Worker brood area	300 m	s(Julian day, organic farmland)	**50.0**	**26.2**	**<0.001**
Number of adults	1500 m	s(Julian day, organic farmland)	**15.6**	**24.4**	**<0.001**
Number of adults	300 m	s(Julian day, organic farmland)	**17.3**	**22.4**	**<0.001**
Honey reserves	1500 m	s(Julian day, organic farmland)	**58.2**	**26.9**	**<0.001**
Honey reserves	300 m	s(Julian day, organic farmland)	**60.5**	**24.9**	**<0.001**
Worker brood area	1500 m	s(Julian day, organic farmland)+	**11.0**	**27.5**	**<0.001**
		s(Julian day, oilseed rape)	**2.7**	**18.7**	**<0.001**
Worker brood area	300 m	s(Julian day, organic farmland)+	**12.8**	**26.8**	**<0.001**
		s(Julian day, oilseed rape)	**2.7**	**12.6**	**0.001**
Worker brood area	1500 m	s(Julian day, organic farmland)+	**9.0**	**27.3**	**<0.001**
		s(Julian day, grassland)	**3.5**	**20.3**	**<0.001**
Worker brood area	300 m	s(Julian day, organic farmland)+	**8.9**	**25.4**	**<0.001**
		s(Julian day, grassland)	**6.0**	**23.4**	**<0.001**
Worker brood area	1500 m	s(Julian day, organic farmland)+	**9.0**	**27.0**	**<0.001**
		s(Julian day, sunflower)	**5.0**	**22.1**	**<0.001**
Worker brood area	300 m	s(Julian day, organic farmland)+	**10.8**	**26.5**	**<0.001**
		s(Julian day, sunflower)	**2.8**	**15.4**	**<0.001**
Number of adults	1500 m	s(Julian day, organic farmland)+	**6.2**	**23.9**	**<0.001**
		s(Julian day, oilseed rape)	**2.2**	**10.5**	**0.009**
Number of adults	300 m	s(Julian day, organic farmland)+	**5.1**	**20.6**	**<0.001**
		s(Julian day, oilseed rape)	1.2	14.5	0.259
Number of adults	1500 m	s(Julian day, organic farmland)+	**15.6**	**24.5**	**<0.001**
		s(Julian day, grassland)	0.9	1.0	0.339
Number of adults	300 m	s(Julian day, organic farmland)+	**5.7**	**18.3**	**<0.001**
		s(Julian day, grassland)	**3.6**	**22.5**	**<0.001**
Number of adults	1500 m	s(Julian day, organic farmland)+	**4.8**	**21.4**	**<0.001**
		s(Julian day, sunflower)	**6.0**	**22.1**	**<0.001**
Number of adults	300 m	s(Julian day, organic farmland)+	**5.8**	**16.9**	**<0.001**
		s(Julian day, sunflower)	**6.5**	**20.0**	**<0.001**
Honey reserves	1500 m	s(Julian day, organic farmland)+	**21.2**	**26.5**	**<0.001**

		s(Julian day, oilseed rape)	**3.0**	**10.8**	**0.001**
Honey reserves	300 m	s(Julian day, organic farmland)+	**21.1**	**2.0**	**<0.001**
		s(Julian day, oilseed rape)	**41.1**	**25.1**	**<0.001**
Honey reserves	1500 m	s(Julian day, organic farmland)+	**9.1**	**25.1**	**<0.001**
		s(Julian day, grassland)	**3.5**	**17.2**	**<0.001**
Honey reserves	300 m	s(Julian day, organic farmland)+	**4.7**	**4.5**	**<0.001**
		s(Julian day, grassland)	**24.6**	**24.9**	**<0.001**
Honey reserves	1500 m	s(Julian day, organic farmland)+	**11.4**	**25.4**	**<0.001**
		s(Julian day, sunflower)	**4.0**	**19.4**	**<0.001**
Honey reserves	300 m	s(Julian day, organic farmland)+	**12.1**	**23.9**	**<0.001**
		s(Julian day, sunflower)	**2.2**	**14.0**	**0.006**

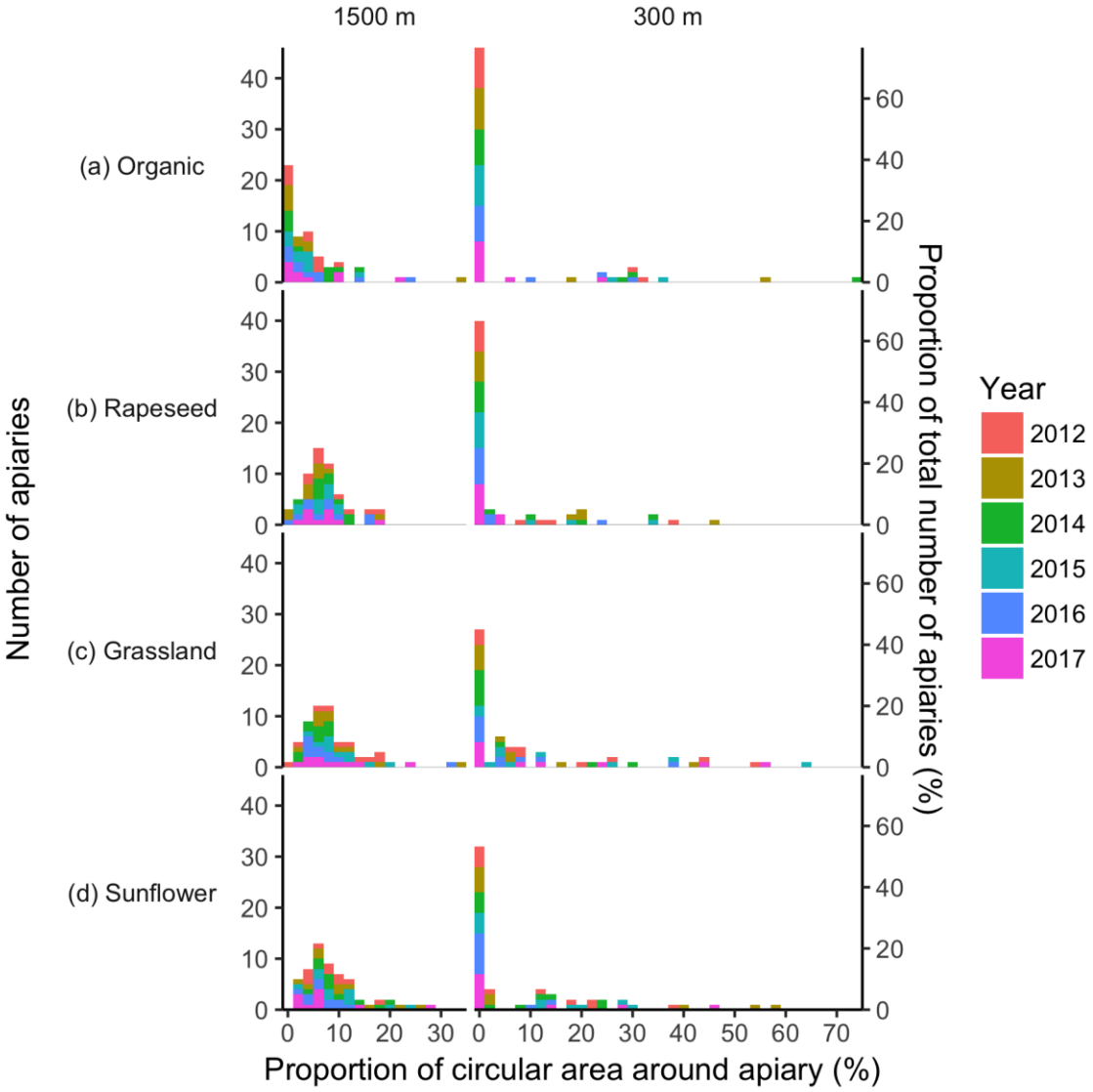

Fig. S1. Histograms of the percentage of (a) organic farmland, (b) oilseed rape (c) grassland and (d) sunflower in 300 m and 1500 m circular buffers around the apiaries expressed in absolute numbers and as a share of the total number of apiaries.

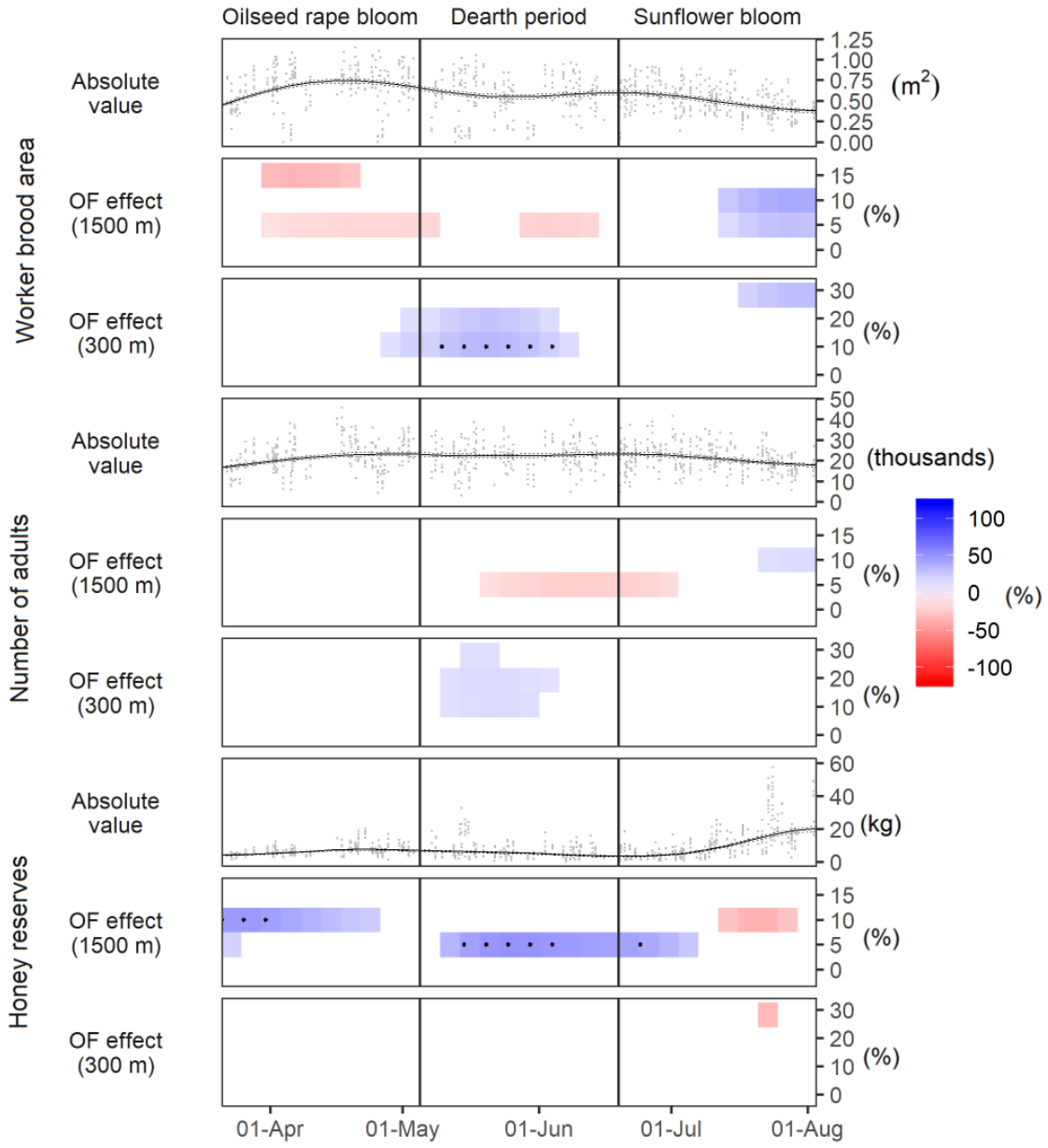

Fig. S2 Variation of worker brood area, number of adults and honey reserves across spring and summer for the years 2009-2017. The solid lines denote estimates of generalized additive mixed models, the dashed lines bootstrapped 95% confidence intervals and the dots measured mean values per apiary and day. The seasonal variation of the relation between life-history traits and the proportion of organic farmland in a 1500 m or 300 m radius around the hives is illustrated as a color-coded percentage difference

between colonies with and without exposure to organic farmland (*OF effect*, equation. 1). The color gradient shows positive differences (i.e. higher values in colonies exposed to organic farmland) in blue and negative ones in red. Cells in white indicate that $P>0.05$ and dots that $P<0.001$. The *P*-values of different point estimates are not independent and have not been corrected for multiple testing.

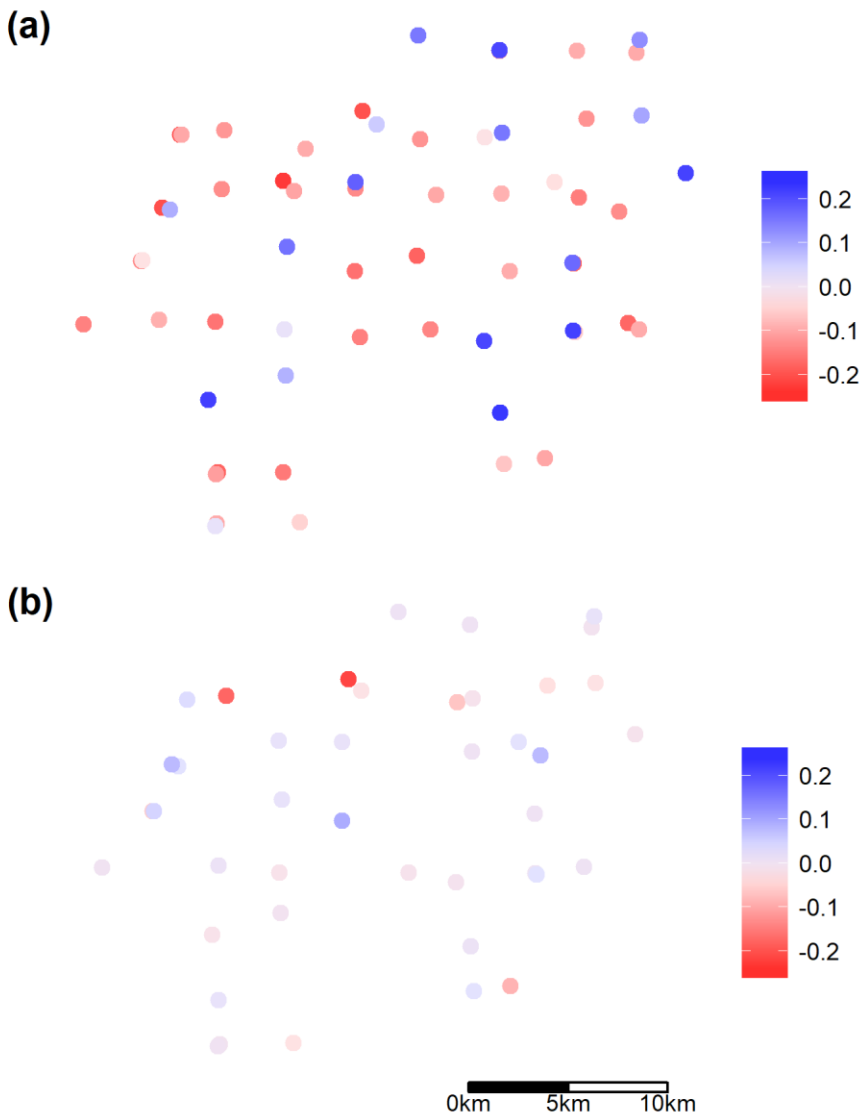

Fig. S3. Mean model residuals per apiary of models on **(a)** the probability of honey harvest after the sunflower bloom (in log odds ratios) and **(b)** the amount of honey harvest among those colonies with honey harvest after the sunflower bloom (in $kg^{0.5}$) in the years between 2012-2017.

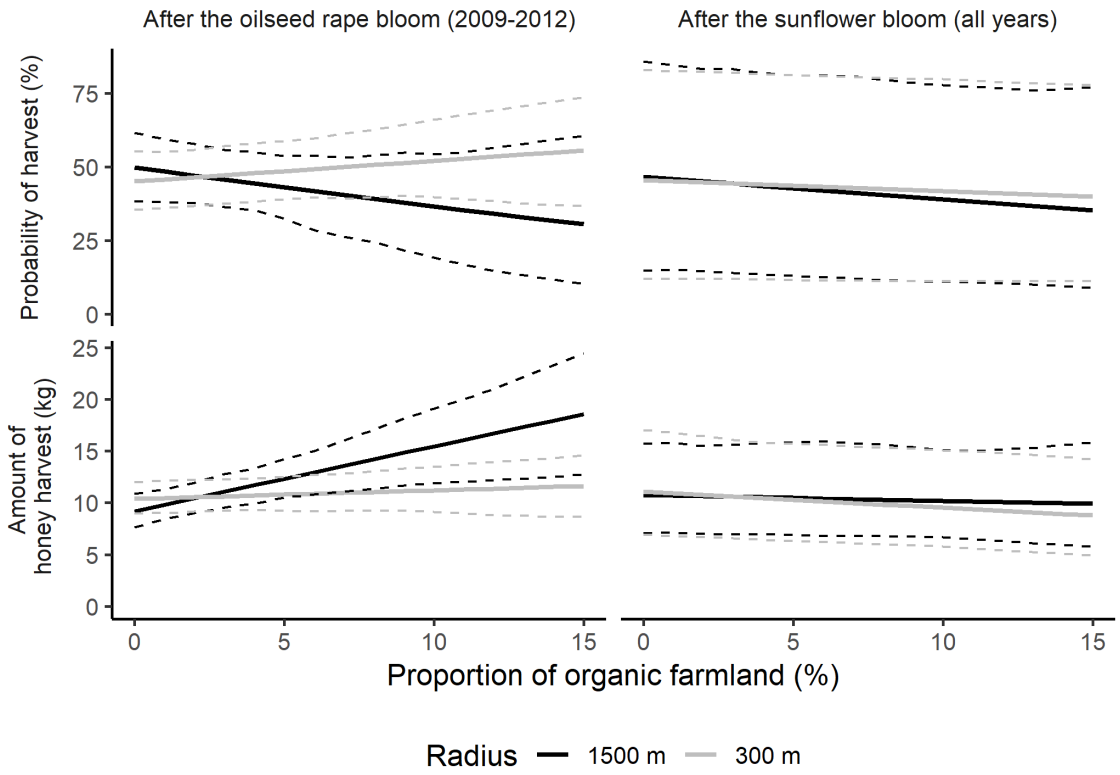

Fig. S4. Honey harvest after the oilseed rape bloom in 2009-2012 and after the sunflower bloom in all years (2009-2017) in relation to the proportion of organic farmland in a 1500 m and a 300 m radius around the honeybee hives. Honey harvest is characterized by two parameters: the probability of honey harvest per colony & the amount of honey harvest among those colonies with harvest.

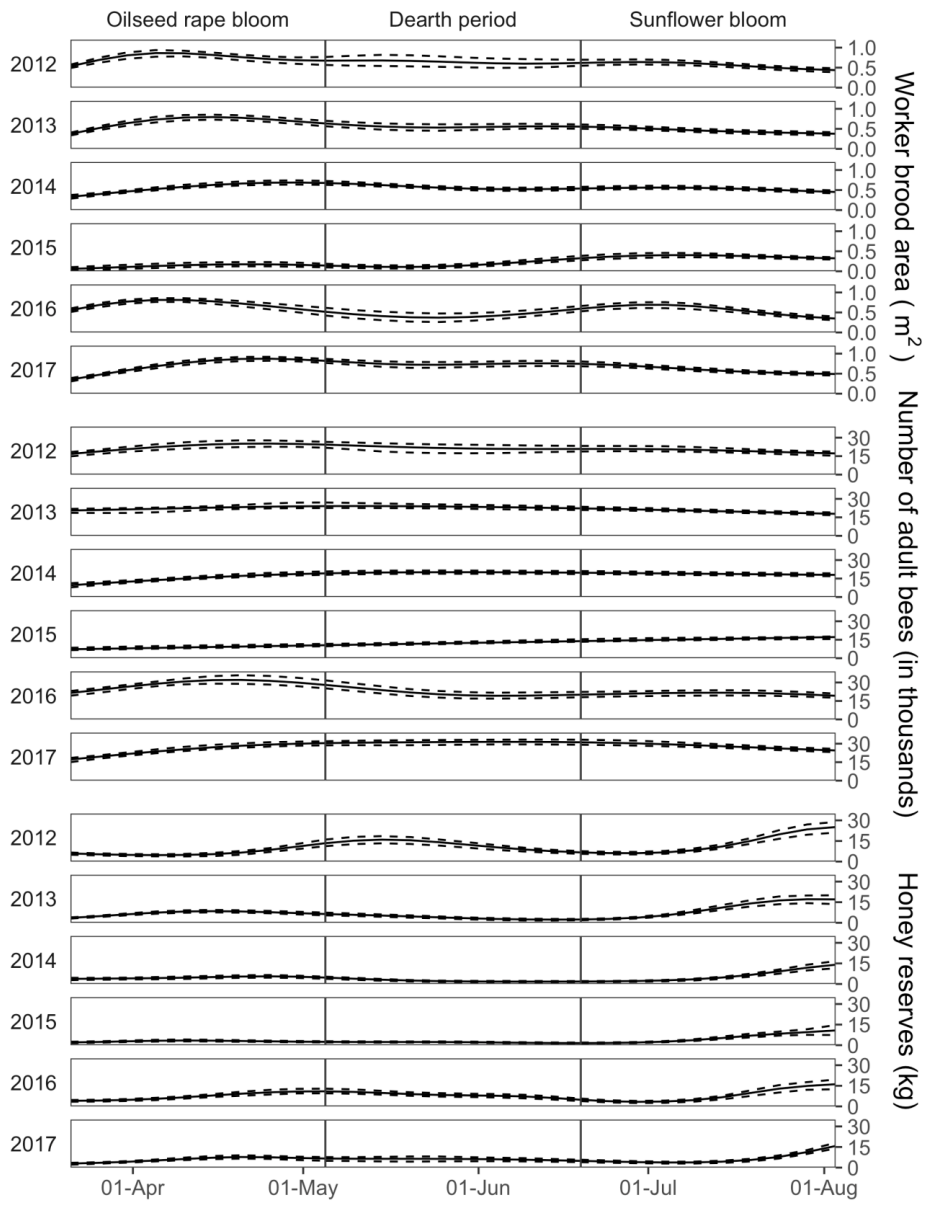

Fig. S5. Seasonal variation of colony performance traits separately for each year. The solid lines denote predictions of generalized additive mixed models, the dashed lines indicate bootstrapped 95% confidence intervals and the dots show measured mean values per apiary and day. Confidence intervals were calculated by 1100 bootstrap simulations.

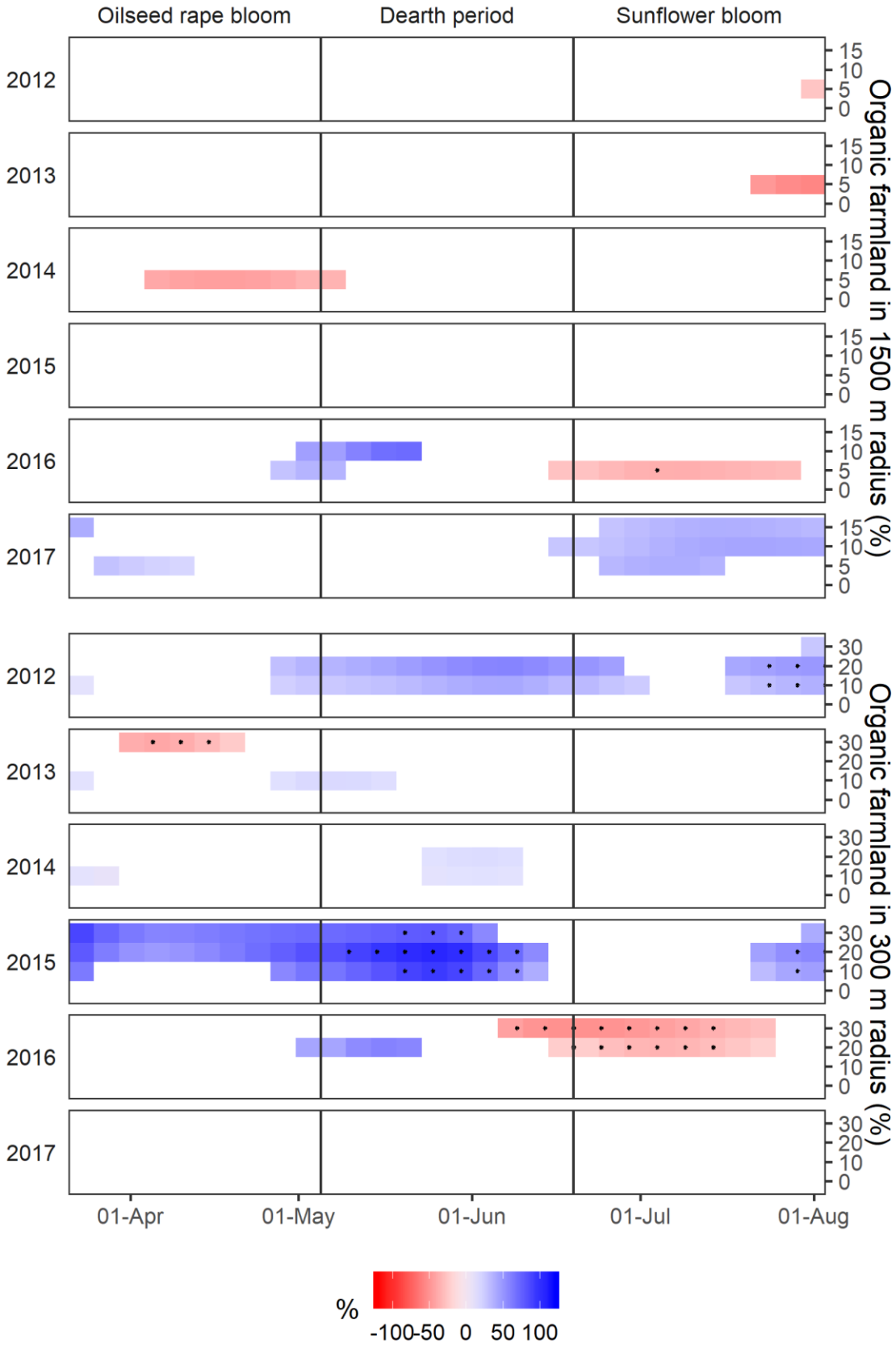
Oilseed rape bloom
Dearth period
Sunflower bloom
2012
2013
2014
2015
2016
2017
Organic farmland in 1500 m radius (%)
Organic farmland in 300 m radius (%)
01-Apr
01-May
01-Jun
01-Jul
01-Aug
%
-100 -50 0 50 100

Fig. S6. Seasonal variation of the relation between worker brood area and the proportion of organic farmland in a 1500 m or 300 m radius around the hives separately for each year. The size of the organic farming effect (*OF effect*) is color-coded with higher values in colonies with organic farmland exposure shown in blue (and the reverse in red). *OF effect* represents the weighted percentage difference in GAMM predictions of worker brood area at the same (growing degree day-adjusted) Julian day between colonies with and without exposure to organic farmland (see equation 1). Cells in white indicate that $P>0.05$ and dots that $P<0.001$. *P*-values were calculated under the null from 1100 bootstrap simulations. The *P*-values of different point estimates are not independent and have not been corrected for multiple testing.

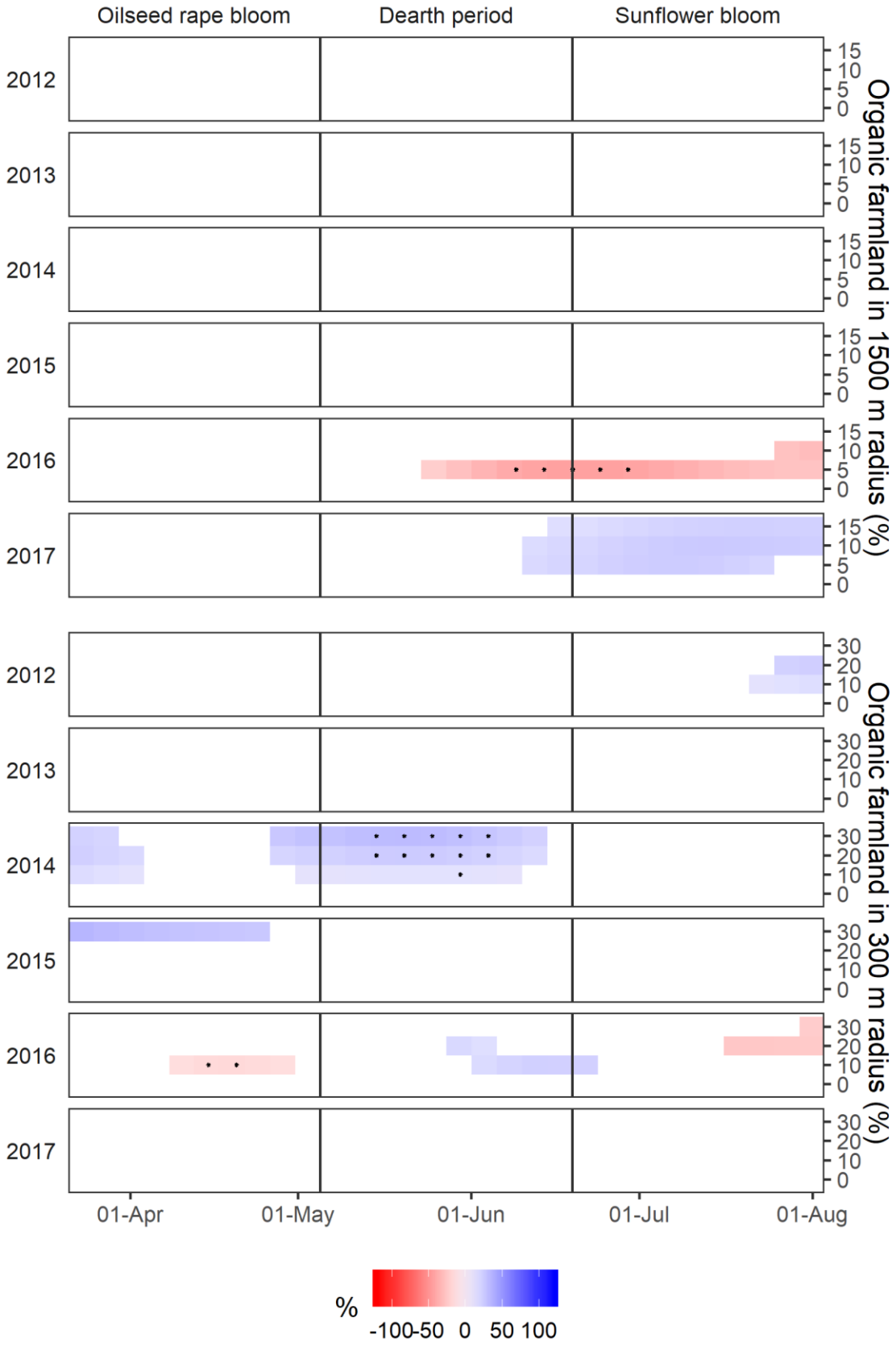
Oilseed rape bloom
Dearth period
Sunflower bloom
2012
2013
2014
2015
2016
2017
Organic farmland in 1500 m radius (%)
Organic farmland in 300 m radius (%)
01-Apr
01-May
01-Jun
01-Jul
01-Aug
%
-100 -50 0 50 100

Fig. S7. Seasonal variation of the relation between number of adults and the proportion of organic farmland in a 1500 m or 300 m radius around the hives separately for each year. The size of the organic farming effect (*OF effect*) is color-coded with higher values in colonies with organic farmland exposure shown in blue (and the reverse in red). *OF effect* represents the weighted percentage difference in GAMM predictions of number of adults at the same (growing degree day-adjusted) Julian day between colonies with and without exposure to organic farmland (see equation 1). Cells in white indicate that $P>0.05$ and dots that $P<0.001$. *P*-values were calculated under the null from 1100 bootstrap simulations. The *P*-values of different point estimates are not independent and have not been corrected for multiple testing.

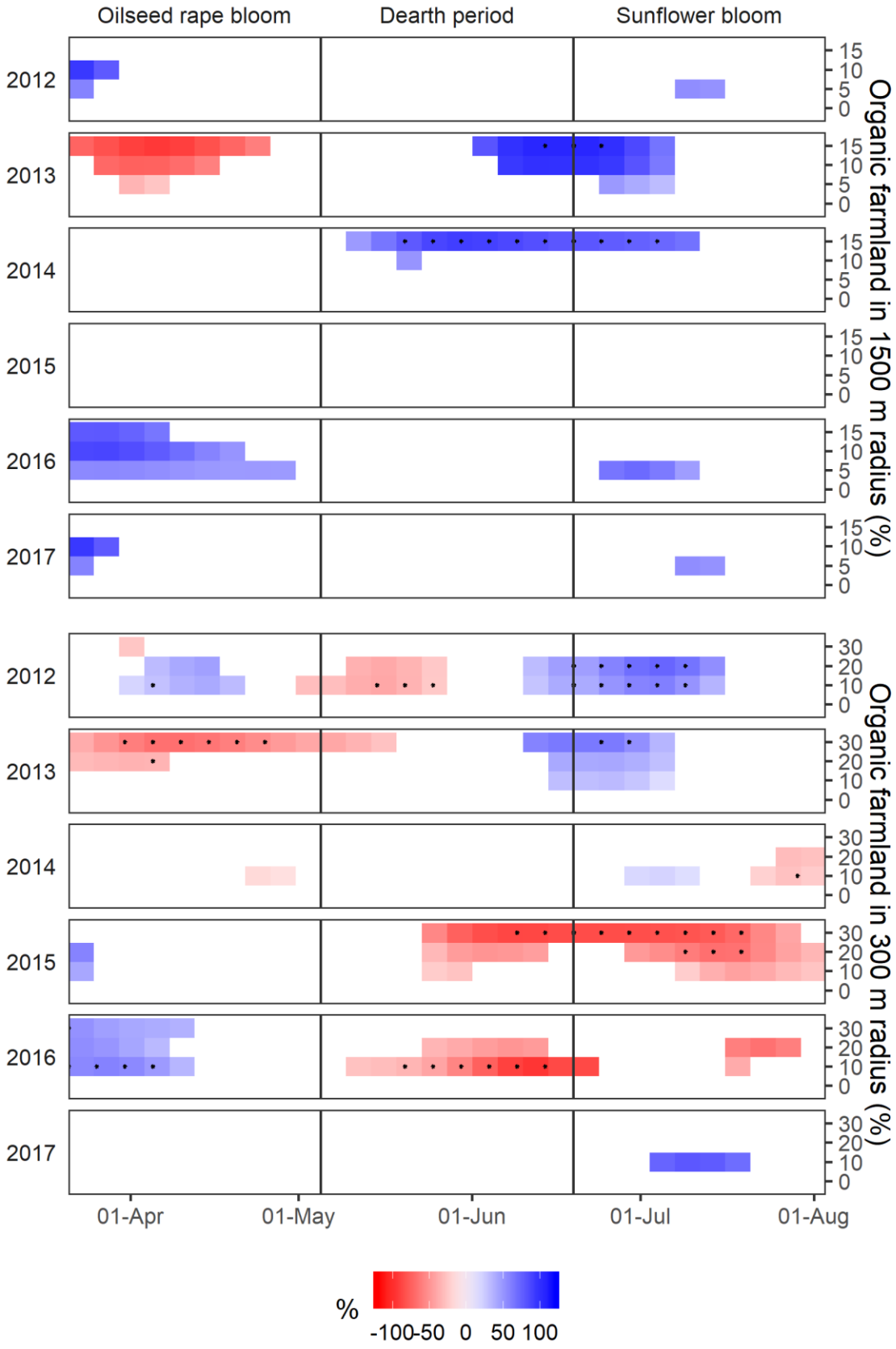
Oilseed rape bloom
Dearth period
Sunflower bloom
2012
2013
2014
2015
2016
2017
Organic farmland in 1500 m radius (%)
Organic farmland in 300 m radius (%)
01-Apr
01-May
01-Jun
01-Jul
01-Aug
%
-100 -50 0 50 100

Fig. S8. Seasonal variation of the relation between honey reserves and the proportion of organic farmland in a 1500 m or 300 m radius around the hives separately for each year. The size of the organic farming effect (*OF effect*) is color-coded with higher values in colonies with organic farmland exposure shown in blue (and the reverse in red). *OF effect* represents the weighted percentage difference in GAMM predictions of colony honey reserves at the same (growing degree day-adjusted) Julian day between colonies with and without exposure to organic farmland (see equation 1). Cells in white indicate that $P>0.05$ and dots that $P<0.001$. *P*-values were calculated under the null from 1100 bootstrap simulations. The *P*-values of different point estimates are not independent and have not been corrected for multiple testing.

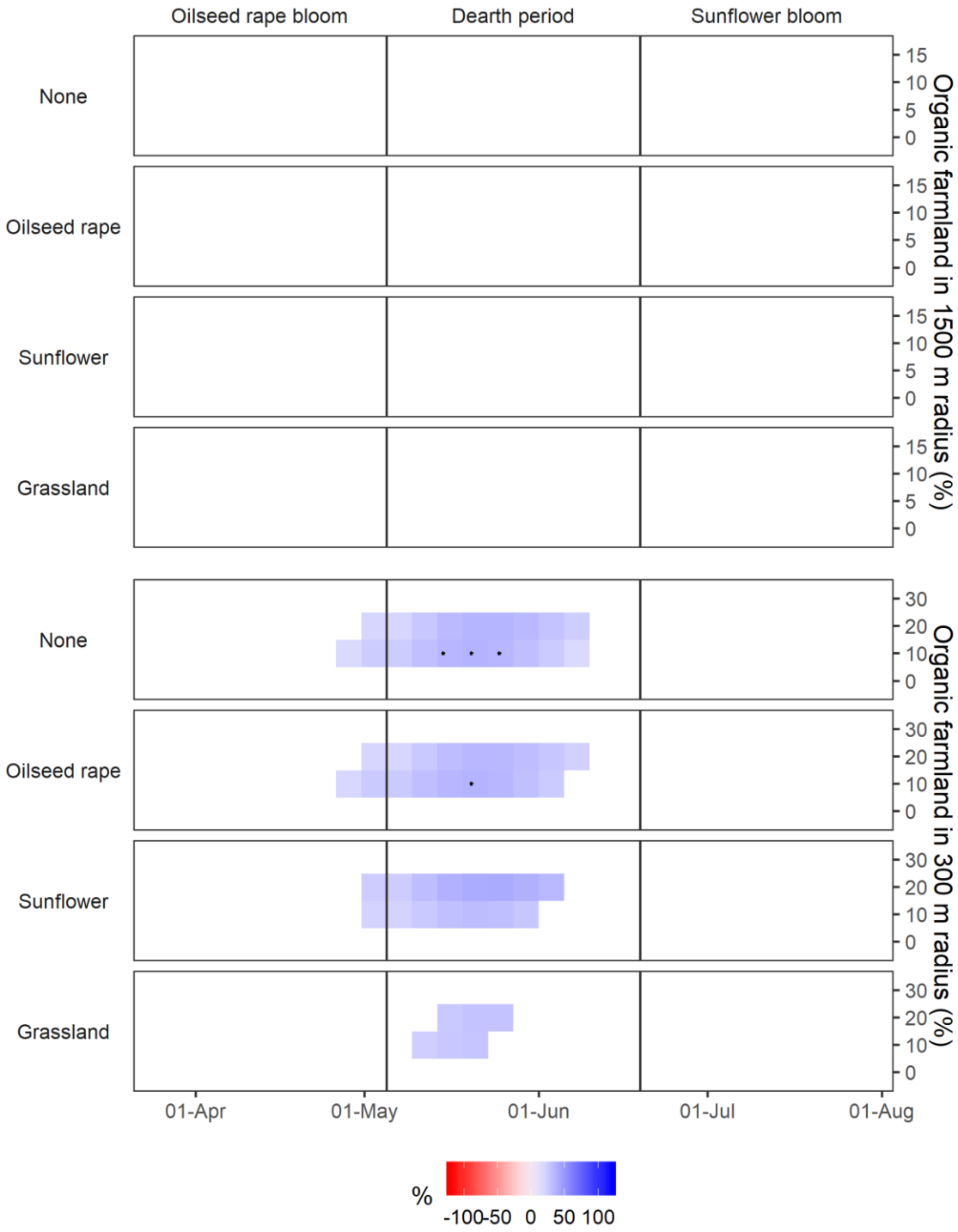

Fig. S9. Seasonal variation of the effect of organic farming on worker brood area, across spring and summer when incorporating in addition to an interaction between (growing degree day-adjusted) Julian days and the proportion of organic land either no field cover

variable or an interaction between Julian days and the proportion of oilseed rape, sunflower or grassland in circular areas around the hives. The size of the organic farming effect (*OF effect*) is color-coded with higher values in colonies with organic farmland exposure shown in blue (and the reverse in red). *OF effect* represents the weighted percentage difference in Generalized Additive Mixed Model (GAMM) predictions of worker brood area at the same Julian day between colonies with and without exposure to organic farmland (see equation 1). Cells in white indicate that $P>0.05$ and dots that $P<0.001$. *P*-values were calculated under the null from 1100 bootstrap simulations. The *P*-values of different point estimates are not independent and have not been corrected for multiple testing.

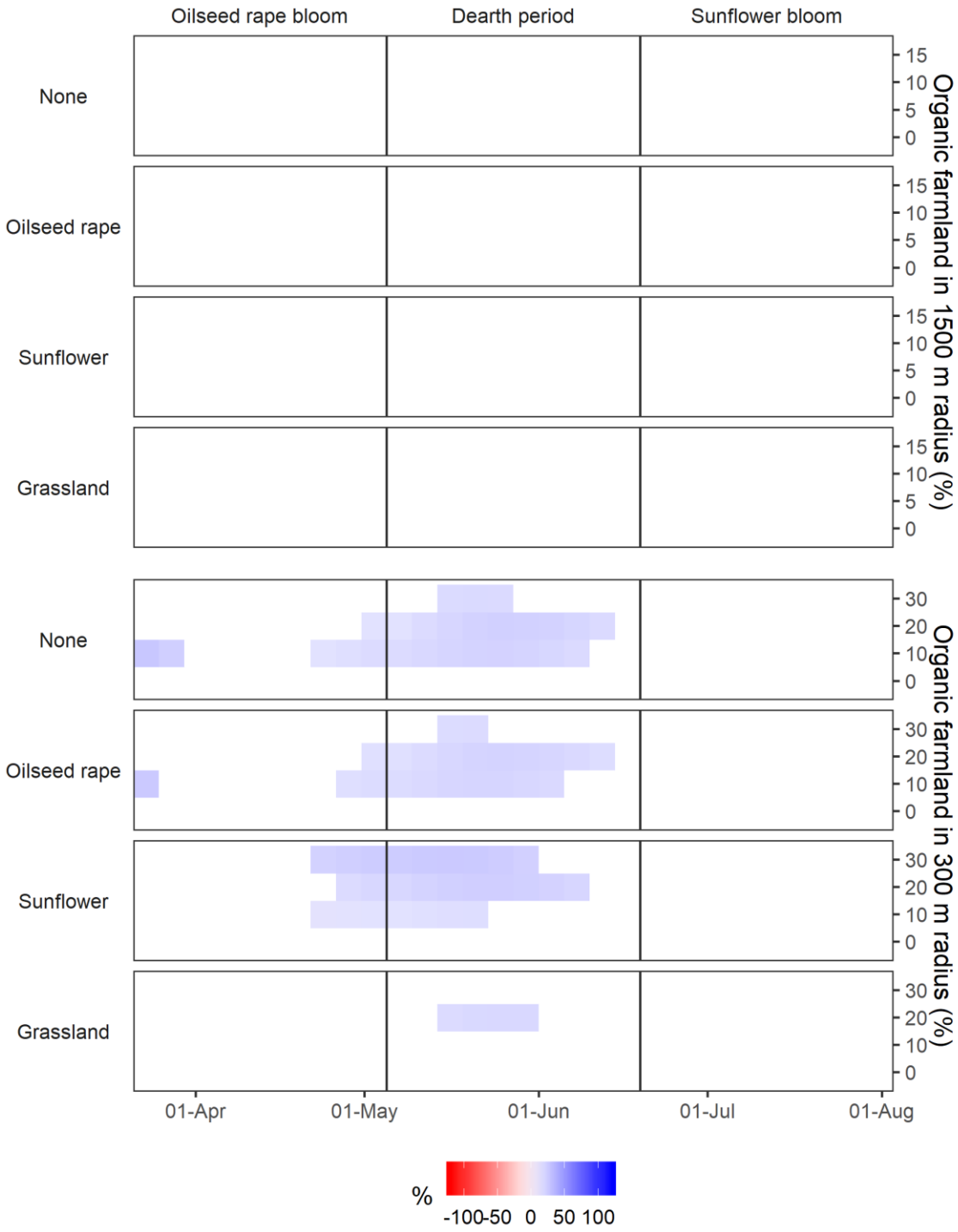

Fig. S10. Seasonal variation of the effect of organic farming on number of adults, across spring and summer when incorporating in addition to an interaction between (growing degree day-adjusted) Julian days and the proportion of organic land either no field cover

variable or an interaction between Julian days and the proportion of oilseed rape, sunflower or grassland in circular areas around the hives. The size of the organic farming effect (*OF effect*) is color-coded with higher values in colonies with organic farmland exposure shown in blue (and the reverse in red). *OF effect* represents the weighted percentage difference in Generalized Additive Mixed Model (GAMM) predictions of number of adults at the same Julian day between colonies with and without exposure to organic farmland (see equation 1). Cells in white indicate that $P>0.05$ and dots that $P<0.001$. *P*-values were calculated under the null from 1100 bootstrap simulations. The *P*-values of different point estimates are not independent and have not been corrected for multiple testing.

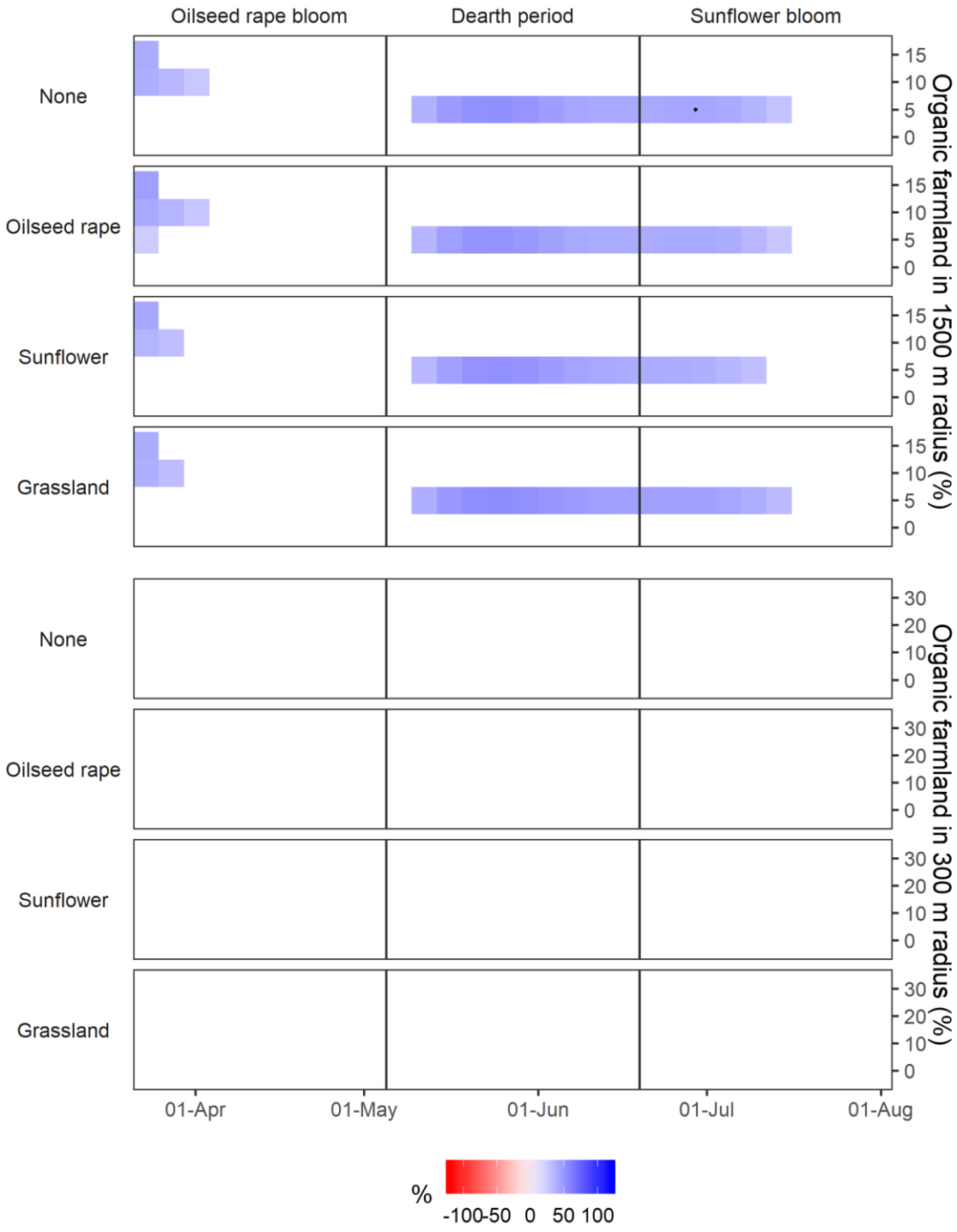

Fig. S11. Seasonal variation of the effect of organic farming on honey reserves, across spring and summer when incorporating in addition to an interaction between (growing degree day-adjusted) Julian days and the proportion of organic land either no field cover

variable or an interaction between Julian days and the proportion of oilseed rape, sunflower or grassland in circular areas around the hives. The size of the organic farming effect (*OF effect*) is color-coded with higher values in colonies with organic farmland exposure shown in blue (and the reverse in red). *OF effect* represents the weighted percentage difference in Generalized Additive Mixed Model (GAMM) predictions of honey reserves at the same Julian day between colonies with and without exposure to organic farmland (see equation 1). Cells in white indicate that $P>0.05$ and dots that $P<0.001$. *P*-values were calculated under the null from 1100 bootstrap simulations. The *P*-values of different point estimates are not independent and have not been corrected for multiple testing.

Chapter II: Clothianidin seed-treatment has no detectable negative impact honeybee colonies and their pathogens

(Manuscript published in Nature Communications)

Preface and summary

In Chapter I, we examined the overall effect of commonly used pesticide combinations through a comparison of honeybee colonies that are exposed to different degrees to organic or conventional farming. Here, we focus on a single pesticide – clothianidin. Clothianidin is one of three neonicotinoids that have been banned in bee-attractive crops in the European Union due to implications in bee declines. Recent laboratory studies suggest synergism between neonicotinoids and another stressor of bee populations: diseases. We extended a well-replicated landscape-scale study by (Rundlöf et al., 2015) with an additional season of exposure of honeybees to oilseed rape that was either treated or not treated with clothianidin. The colonies were screened for 13 viruses, two *Nosema* species and the *Varroa* mite in both years of the experiment and the expression of immune genes was determined for the first year. Residue analyses of honeybees as well as honeybee-collected pollen and nectar revealed a clear difference in clothianidin exposure between colonies at treated and control fields. However, we found no negative effect on honeybee colonies. Clothianidin exposure was even associated with a slight increase in health. During the oilseed rape bloom, colonies at clothianidin-treated sites increased more in the number of adults and in the amount of brood. In addition, clothianidin exposure was positively associated with the abundance of the beneficial gut bacterium *Gilliamella apicola* and negatively with the abundances of *Aphid lethal bee virus* and *Black queen cell virus.* However, most of these differences between clothianidin-exposed and control colonies were only observed in the second year and they were minor compared to the large seasonal variations in colony development and microbial parameters. In addition, immune gene expression was unaffected be treatment. Therefore, we conclude that honeybee colonies are relatively robust to field-level clothianidin exposure under moderate disease pressure.

Résumé

Le traitement des semences à la clothianidine n'a pas d'impact négatif détectable sur les colonies d'abeilles domestiques et leurs agents pathogènes

L'abeille mellifère européenne (*Apis mellifera*), en contribuant à la pollinisation des cultures du monde entier, est le principal pollinisateur domestique. Cependant, ces dernières années, des taux élevés de mortalité des colonies d'abeille ont été mis en évidence, menaçant la production des cultures dépendantes des pollinisateurs. Le déclin des abeilles mellifères a été attribué à une combinaison de causes parmi lesquelles les maladies sont considérées comme le facteur majeur. Les abeilles sont soumis à un bouquet de pathogènes et parasites, dont l'ectoparasite *Varroa destructor*, de façon conjointe avec les virus qu'il porte sont clairement les plus dévastateurs. A vrai dire, les abeilles vagabondes sont devenues rares en Europe et en Amérique du Nord car les colonies survivent rarement sans traitement contre l'acarien *Varroa*. Cependant, les pertes de colonies durant cette dernière décennie ne peuvent pas être attribuées qu'aux maladies.

Les abeilles sont également menacées par l'exposition chronique aux pesticides, et en particulier aux insecticides néonicotinoïdes dont la responsabilité est avérée concernant leur déclin. Les néonicotinoïdes sont mondialement utilisés sur une large gamme de cultures. Ils peuvent être appliqués sous des formes variées mais sont le plus souvent utilisés en traitement de semences. Leur systémie leur permet de se distribuer dans toutes les parties de la plante, y compris le pollen et le nectar dont les abeilles se nourrissent. Des doses sublétales de néonicotinoïdes ont montré un effet négatif sur l'efficacité du butinage, les capacités cognitives et le développement de couvain et d'adultes chez l'abeille mellifère.

De récentes études en laboratoire ont aussi mis en évidence que les néonicotinoïdes ont des effets additifs ou synergiques sur la longévité de l'abeille et son immunocompétence lorsqu'ils sont combinés avec une pression de pathogènes ou parasites. Les néonicotinoïdes peuvent intensifier à la fois les effets létaux et sublétaux des pathogènes sur les larves ou les adultes et nuire au système immunitaire individuel et social des

abeilles. Les néonicotinoïdes peuvent également agir sur les bactéries intestinales des abeilles, cependant ceci reste encore à éclaircir. Les principaux symbiontes intestinaux *Gilliamella apicola* et *Snodgrassella alvi* ont montré leur implication dans la décomposition des nutriments et xénobiotiques comme dans les mécanismes de défense vis à vis des pathogènes des abeilles. Des essais aux champs sur les néonicotinoïdes ont révélé des résultats contrastés et il persiste encore un manque d'essais dans des conditions réelles sur le thème de la sensibilité aux pathogènes chez l'abeille.

Ici, nous avons prolongé une étude de Rundlöf *et al.* (2015) bien répliquée à l'échelle du paysage qui a révélé de forts effets négatifs de l'exposition à la clothianidine appliquée en traitement de semences sur colza, sur la reproduction des abeilles sauvages mais pas sur le développement des colonies d'abeilles mellifères. Ici, nous avons placé certaines des colonies expérimentales d'abeilles domestiques de cette étude comme dans la première année contre des champs de colza traité ou non. Les champs ne sont pas les même que dans la première année, mais ils mais ils appartiennent aux mêmes agriculteurs. Cependant nous avons inversé les traitements, c'est-à-dire que les agriculteurs qui ont appliqué la clothianidine la première année ne devaient pas le faire la seconde année et vice-versa.

Toutefois, des colonies d'abeille qui ont été placées près des champs traités la première année (2013) ont à nouveau été placées près de champs traités la deuxième année (2014) pour identifier les effets cumulatifs de l'exposition de clothianidine à long terme. En plus des performances des colonies (nombre d'adultes, quantité de couvain, quantité de miel produit), nous avons estimé sur les deux années les abondances de plusieurs virus, des deux pathogènes *Nosema*, l'acarien *Varroa* ainsi que deux symbiotes intestinales. La première année de l'essai, nous avons aussi examiné l'effet de l'exposition à la clothianidine sur l'expression du gène immunitaire. L'étude a globalement suivi un plan expérimental « Avant-Après-Contrôle-Impact », mesurant la différence de changement au cours des périodes de floraison du colza entre les colonies exposées à la clothianidine et les colonies témoins. De plus, nous avons comparé la mortalité et la force des colonies (quantité de couvain et nombre d'abeilles adultes) après l'hivernage pour les deux traitements. Les analyses de résidus montrent que les abeilles ont butiné les colzas de

façon intensive et la clothianidine a clairement été détectée plus fréquemment et à une plus forte concentration dans des échantillons d'abeilles et de pollen et de nectar collectés par des abeilles provenant de champs traités à la clothianidine par rapport aux champs témoins.

Cependant nous n'avons pas trouvé des effets négatifs de la clothianidine sur les colonies d'abeille. L'exposition à l'insecticide n'a pas influée l'expression des gènes immunitaires, mais elle a été associée à un léger accroissement du nombre d'abeilles adultes. Dans la seconde année, la production de couvain et l'abondance du symbionte intestinal bénéfique *Gilliamella picola* étaient également associées de façon positive à cette exposition de clothianidine. De plus, l'abondance du virus ALPV *Aphid lethal paralysis virus* a augmenté moins fortement dans les colonies exposées pendant la floraison du colza, et celle du virus BQCV *Black queen cell* virus était également associées de façon négative dans la deuxième année. Cette association positive entre l'exposition à la clothianidine et la santé des abeilles mellifères ne doit pas être surinterprétée, car elle pourrait résulter de légères différences avant la floraison du colza et d'une surcompensation ultérieure. De plus, les effets observés sont mineurs comparés aux importantes fluctuations saisonnières des paramètres au niveau des colonies.

La colonie d'abeille est considérée comme un super-organisme dont la structure sociale et l'organisation peut améliorer la résistance au stress. Des recherches précédentes ont démontré des pertes d'ouvrières induites par les néonicotinoïdes qui ont été compensées par une augmentation de la production d'ouvrières au détriment de la production de mâles, qui ne contribuent pas à la subsistance de la colonie mais qui s'accouplent avec les reines d'autres colonies. Les abeilles d'une colonie coopèrent aussi en combattant les effets négatifs des maladies sur le fonctionnement de la communauté. A la différence des études en laboratoire, nos résultats suggèrent qu'à la fois cette immunité sociale et le système immunitaire inné des abeilles ne sont pas altérés de façon marquée par l'exposition chronique venant des semences de colza traitées. Cela suggère que les effets négatifs peuvent être trop temporaires pour produire des effets majeurs sur le fonctionnement des colonies. Par exemple, il a été démontré que les effets sur l'expression des gènes diminuent progressivement après un jour et, dans notre expérience,

les abeilles ont potentiellement été échantillonnées plusieurs jours après l'exposition à la clothianidine.

Des colonies d'abeilles relativement saines, comme les nôtres, pourraient peut-être compenser les baisses temporaires de l'immunité et ainsi prévenir des maladies. Par conséquent, nous concluons que qu'en conditions de pleins champs, les colonies d'abeilles domestiques sont relativement résistantes aux effets négatifs potentiels de la clothianidine.

PhD student's contribution

I led the data analysis and the writing of the article. I ran most of the microbial assays and measured individual bee parameters.

Clothianidin seed-treatment has no detectable negative impact on honeybee colonies and their pathogens

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Abstract

Interactions between multiple stressors have been implicated in elevated honeybee colony losses. Here, we extend our landscape-scale study on the effects of placement at clothianidin seed-treated oilseed rape fields on honeybees with an additional year and new data on honeybee colony development, swarming, mortality, pathogens and immune gene expression. Clothianidin residues in pollen, nectar and honeybees were consistently higher at clothianidin-treated fields, with large differences between fields and years. We found large variations in colony development and microbial composition and no observable negative impact of placement at clothianidin-treated fields. Clothianidin treatment was associated with an increase in brood, adult bees and *Gilliamella apicola* (beneficial gut symbiont) and a decrease in *Aphid lethal paralysis virus* and *Black queen cell virus* - particularly in the second year. The results suggest that at colony level, honeybees are relatively robust to the effects of clothianidin in real-world agricultural landscapes, with moderate, natural disease pressure.

Introduction

Pollinating insects, mainly bees, provide an important ecosystem service through maintaining wild plant biodiversity and contributing to global food security as well as beekeeper and farmer livelihoods[1,2]. Recent global declines in wild bee abundance[3,4] and diversity[5,6] are threatening plant biodiversity as well as the production of pollinator dependent crops[2]. The European honeybee (*Apis mellifera*) is the most important managed pollinator and contributes to crop pollination worldwide[1,2]. However, high honeybee colony mortality rates have been reported[2] with disease being the main driver of honeybee colony losses[7,8]. Honeybees are plagued with a bouquet of pathogens and parasites, but the ectoparasitic mite, *Varroa destructor*, together with the viruses it vectors[9] are clearly the most devastating. *Varroa* mites require regular monitoring and mite control treatments by beekeepers to mitigate colony losses[10].

Another threat to honeybees is the chronic exposure to pesticides used in agriculture[11]. Neurotoxic neonicotinoids are a class of insecticides that are used globally[12] and are usually applied to arable agricultural crops as a seed dressing or a spray[13]. The active compounds are systemic and can be found in all parts of the plant including pollen and nectar[14], which is a route of exposure to foraging bees[15]. Sublethal doses of neonicotinoids have been found to affect honeybee foraging behaviour and success[16], impact memory and learning abilities of honeybees[17] and inhibit the development of brood, adults and queens[17,18].

Elevated winter colony mortality is probably best explained by the combination of several of these stressors[2,8]. There is laboratory evidence that neonicotinoids have additive or synergistic effects on honeybee longevity and immunocompetence when combined with pathogen or parasite pressure[19–25]. At sublethal doses, neonicotinoids can enhance the harmful effects of honeybee pathogens on larvae and adults bees, especially at high doses and infection levels[23], and the combination of neonicotinoid exposure and pathogen pressure is associated with higher individual honeybee mortality rates[20].

Furthermore, neonicotinoids can negatively affect individual immune competence, leading to greater susceptibility to opportunistic pathogens and parasites[11,19]. The alteration of the honeybee microbiota might contribute to the deterioration of honeybee health, but this is yet understudied[26,27]. There is evidence that the most prominent gut symbionts *Gilliamella apicola* and *Snodgrassella alvi* play an important role in honeybee nutrition and pathogen defense[28,29]. The ability to combat infections might be reduced through a negative effect of neonicotinoids on the honeybee microbial community[30].

Since December 2013, an EU moratorium has restricted the use of three common neonicotinoids, clothianidin, imidacloprid and thiamethoxam, in bee-attractive crops[31]. This moratorium was widely debated, since it had mostly been based on laboratory rather than field studies[32]. As further evidence of adverse effects of neonicotinoids has accrued, the European Union recently decided to ban all outdoor uses of these three neonicotinoids from December 2018 onwards[33], although they can still be used in permanent green houses and for outdoor use in countries outside of the European Union. The negative impact of neonicotinoids on wild bees under field exposure has been demonstrated by several studies[34–37]. However, the impact of field-level neonicotinoid exposure on honeybee health and survival has been less decisive, with the spectrum of conclusions ranging from negative impact[36,38,39] to no impact[34,40–42], sometimes even in the same study[36]. Also, clear evidence of synergistic interactions between real-world field-level exposure to pesticides and pathogens on colony performance is lacking, especially within the context of the general adaptability of honeybee colonies to environmental challenges.

In 2015, we published a well-replicated field study on effects of the neonicotinoid clothianidin on solitary bees, bumblebees and honeybees[34]. Here, we present new data on honeybees from a second consecutive year of this experiment, designed to uncover cumulative effects of placement at clothianidin-treated fields over two years, and analyses of honeybee samples from both years for symbiotic gut bacteria, several pathogens and immune gene expression levels. While most parameters remained unaffected by the clothianidin treatment, the impact that we do find is mostly positive. Placement at clothianidin-treated fields was associated with increased brood production

in the second year and with more adult bees across both years. In 2014, colonies at clothianidin-treated fields showed a lower decline rate in *Gilliamella apicola* abundance over the oilseed rape bloom than control colonies and clothianidin treatment was negatively associated with the abundance of *Black queen cell virus* in 2014 and *Aphid lethal paralysis virus* (both years). However, these effects are minor compared to the extensive natural seasonal fluctuations in colony-level parameters and pathogen abundance, suggesting that relatively healthy honeybee colonies have sufficient colony-level social and demographic plasticity to compensate for possible individual bee-level impacts of neonicotinoid exposure.

Results

Study design

Ninety-six honeybee colonies were placed at 16 spring sown oilseed rape fields (six colonies per field) in southern Sweden in 2013; eight fields were sown with seeds coated with clothianidin and a fungicide and eight control fields were sown with seeds coated only with the fungicide, with clothianidin-treated and control fields matched in pairs based on geographical proximity and land use in the surrounding landscape[34]. After overwintering at a common location, the colonies were in 2014 randomly re-assigned to six clothianidin-treated and four control spring-sown oilseed rape fields from the same study design and farmers as 2013, with four colonies per field (forty colonies in total), except for the following two main conditions that, first, colonies from clothianidin-treated fields in 2013 were again placed at clothianidin-treated fields in 2014 and second, that the treatment allocation on each farm was reversed relative to 2013, and different local fields were used, due to crop rotation (Fig. 1).

We examined honeybee colony development (number of adults, amount of brood), honey production, swarming/supersedure and colony mortality as well as the prevalence and abundance of 13 RNA viruses (Supplementary Table 1), two pathogenic microsporidian gut parasites, two non-pathogenic gut bacteria and the ectoparasitic mite *Varroa destructor* and, in the first year, the expression of eight genes related to the honeybee

innate immune response. We analysed each of these parameters individually in relation to the seed-treatment of the fields where the colonies were placed (clothianidin-treated, control), in relation to the oilseed rape bloom (before, after) and in relation to the interaction between seed-treatment and bloom, represented by a differential response during the oilseed rape bloom between colonies at treated and untreated fields, for both years combined and (where necessary) also separately for each year. The colonies were managed according to recommended beekeeping practices, including treatment against *Varroa* after the post-exposure assessment in 2013 (Supplementary Fig. 1).

Verification of clothianidin exposure

To verify use of the focal crop and clothianidin exposure in both years, we estimated the proportion of oilseed rape pollen collected by honeybees and quantified the clothianidin concentrations in bee tissue and in bee-collected pollen and nectar (Table 1). In 2013, the pollen collected during the mid-oilseed rape bloom consisted on average of 53% and 63% oilseed rape pollen at control and clothianidin-treated fields respectively[34]. In 2014, the pollen collected contained substantially higher proportions of oilseed rape than in 2013, both at clothianidin-treated fields (93%, 95% confidence interval (CI): 88-97%) and at control fields (91%, CI: 83-95%), with no significant difference between control and clothianidin-treated fields (generalized linear model (GLM), $F_{1,7} = 0.98$, $P = 0.36$). In 2014, the clothianidin levels detected in pollen (6.1 ± 2.0 ng g^{-1}; mean ± s.e.m.; n =6) and nectar (4.9 ± 1.1 ng ml^{-1}; n = 6) from honeybees foraging in the clothianidin-treated fields and honeybees (1.1 ± 0.20 ng g^{-1}; n = 6) from colonies by the same farms were approximately half of the levels detected in 2013[34] (pollen 13.9 ± 1.8 ng g^{-1}; nectar 10.3 ± 1.3 ng ml^{-1}; bee tissue 2.4 ± 0.5 ng g^{-1}; n = 8 for all matrices). In both years, concentrations at the control fields were mostly below the limit of detection (LOD), still we detected clothianidin residues in some samples in 2013 (honeybee tissue and nectar). However, exposure of clothianidin differed between clothianidin-treated and control fields (Table 1). Four other neonicotinoids were also detected, both in clothianidin-treated fields as well as in control fields, in both years of the experiment (Supplementary Table 2). In 2013, when we examined individual honeybees and their nectar loads, there was large variation between three different clothianidin-treated fields in the residue levels

in the honeybees (ANOVA, $F_{2,33} = 15.84$, $P < 0.001$) and the nectar collected from their honey stomachs (ANOVA, $F_{2,33} = 4.68$, $P = 0.016$; Supplementary Fig. 2), with a correlation of residues between the clothianidin concentration in bee tissue and nectar within each honeybee (multiple linear regression, $R^2 = 0.866$, $F_{1,32} = 90.03$, $P < 0.001$).

Colony development and honey production

Placement at clothianidin-treated fields was associated with an increase in colony strength (amount of brood and number of adult bees) particularly during the second year of the experiment, although this latter result should be interpreted with caution, since the colonies at clothianidin-treated fields were left slightly understrength relative to the control colonies by the beekeeper interventions in June 2014, and may have overcompensated as a result. The colonies also gained strength much more quickly during the 2013 bloom than during the 2014 bloom, for all colonies irrespective of field treatment (Fig. 2, Table 2). Since the analysis of the number of capped brood cells (amount of brood) showed an interaction between seed-treatment, bloom and year, this parameter was analysed separately for each year (Table 2). In 2013, colonies had less brood after the oilseed rape bloom than before, but this seasonal change in brood amount was not affected by the clothianidin treatment (Table 2), nor was there any delayed effect of the clothianidin treatment in 2013 on brood amounts after winter, during the first assessment in spring 2014 (Fig. 2, Table 2). During the 2014 oilseed rape bloom, the amount of brood in the control colonies decreased slightly while in the exposed colonies it increased, thus accounting for the interaction between bloom and seed-treatment, and the positive effect of the clothianidin treatment on brood amount (Fig. 2). Clothianidin treatment was positively related to the number of adults, as clothianidin-exposed colonies had on average more adults than control colonies after the oilseed rape bloom despite starting with fewer adults (Fig. 2). This pattern was more pronounced in 2014, but the seed treatment x bloom x year interaction was not statistically significant (Table 2). In contrast to brood amount, the number of adult bees more than doubled during the oilseed rape bloom in 2013, but showed a weak decline in 2014 (Fig. 2). Similar to the brood amounts, there was no delayed effect of the 2013 clothianidin treatment on the number of adult bees during the first spring 2014 colony assessments (Fig. 2, Table 2). Honey

production differed between years, with more honey produced in the first year than in the second year, but this was not affected by clothianidin treatment in either year (Fig. 3, Table 2).

Swarming/supersedure and colony mortality

During the 2013 oilseed rape bloom, 32 colonies (16 each at clothianidin-treated and control fields) prepared to replace their queen, with 27 eventually doing so: 15 at clothianidin-treated fields and 12 at control fields ($\chi^2_1 = 0.07$, $P = 0.795$), and about twice as often through swarming as through supersedure (Supplementary Table 3). The swarming/supersedure rates were higher for 2-year old queens (50%) than for the 1-year old queens (25%). Only 5 colonies prepared to swarm/supersede during the 2014 season (3 at treated and 2 at untreated fields) with 2 colonies eventually swarming (1 each at treated and untreated fields) all from 2-year old queens, since only colonies with 1-year old queens throughout 2013 were retained for the 2014 experiments. Swarming/supersedure in 2013 was a major factor for subsequent colony mortality (Supplementary Table 3), while placement at clothianidin-treated fields was not a factor ($\chi^2_1 = 0.10$, $P = 0.749$). Between May 2013 and April 2014, 28 experimental colonies died; 15 from clothianidin-treated fields and 13 from control fields. One colony was removed from the 2013 experiment after losing its queen during transport to its (clothianidin-treated) field. Of the remaining 27 fatalities, 22 colonies had swarmed or superseded during 2013, giving a mortality rate of 81% (22/27) for swarmed/superseded colonies, compared to 7% (5/68) for colonies that did not swarm/supersede. Sixteen colonies (all of which had swarmed/superseded) were removed already in September 2013 for being too small to overwinter, while the remaining 11 colonies (6 of which had swarmed/superseded) died during the winter 2013-2014.

Pathogen, parasite and microbe prevalence

We detected both symbiotic gut bacteria (*Gilliamella apicola* and *Snodgrassella alvi*), both *Nosema* species (*N. apis* and *N. ceranae*), the *Varroa* mite and all 13 viruses screened for (Fig. 4, Supplementary Table 1). The overall prevalence of pathogens and

parasites was higher in 2014 than in 2013, with often large differences between pre- and post-exposure assessments for individual microbes, which are furthermore not always consistent between years. However, placement at clothianidin-treated fields had generally no effect on the change in prevalence during the oilseed rape bloom for most pathogens and parasites (Supplementary Table 4, Fig. 4).

Several microbes (*G. apicola*, *S. alvi*, BQCV, LSV-1, SBV, and *N. apis* (in 2014)) were present in nearly all colonies, clothianidin-treated and control, throughout the oilseed rape bloom during both years, presenting insufficient variation for insightful analysis into the relative importance of various factors on their prevalence (Supplementary Table 1). Others (BSRV and LSV-2) were effectively absent throughout the experiment, in all colonies, and were therefore similarly uninformative. Many viruses (ABPV, CBPV, IAPV, KBV and SBPV) were largely absent in 2013 but moderately/highly prevalent in 2014, showing major differences between years. The viruses showed different patterns of prevalence over the oilseed rape bloom season, but their seasonal prevalence could not be linked to clothianidin treatment (Fig. 4, Supplementary Table 4). ALPV increased over the oilseed rape bloom seasons across both years, while changes in prevalence could not be statistically confirmed for ABPV, CBPV; KBV or IAPV (Fig. 4, Supplementary Table 4). DWV-A prevalence increased during the summers, which is a well-established seasonal pattern[9,43] (Fig. 4, Supplementary Table 4), probably attributable to its vector *Varroa*[9]. DWV-B prevalence decreased during the 2013 bloom, but increased during the 2014 bloom (Fig. 4, Supplementary Table 4).

Both *Nosema* species decreased in prevalence during summer, which corresponds to their known seasonal patterns[43], but this decrease was more drastic in 2013 than in 2014. The change in *Nosema* prevalence over the blooming season was not influenced by the placement at clothianidin-treated fields (Supplementary Table 4). In 2013, *N. ceranae* was less prevalent in colonies placed at clothianidin-treated fields than at control fields both before and after exposure, which is evident by the significant seed-treatment effect (Supplementary Table 4).

Parasite, pathogen and microbe abundance

For most pathogens, microbes and parasites, we also tested the effect of placement at clothianidin-treated fields on their abundance in colonies where they were detected (Fig. 4, Supplementary Table 1). Where possible, we included both years, in a full model, but we excluded years with a sample size ≤ 10 colonies (Supplementary Table 1). Placement at clothianidin-treated fields generally had relatively little detectable effect on pathogen, parasite or microbe abundance, but was in 2014 positively related to *G. apicola* abundance, and negatively related to BQCV abundance as well as negatively to ALPV abundance across both years.

Gilliamella apicola abundance in adult bees was explained by an interaction between seed-treatment, bloom and year, therefore the dataset was analysed separately for each year (Supplementary Table 5). During the oilseed rape bloom in 2013, *G. apicola* abundance declined, irrespective of the placement at clothianidin-treated fields (Supplementary Table 5, Fig. 5). In 2014, there was an interaction between bloom and seed-treatment, with the *G. apicola* abundance declining more quickly in control colonies than in clothianidin-exposed colonies (Supplementary Table 5, Fig. 5). The abundance of *S. alvi* remained stable in 2013, but increased over the bloom in 2014, irrespective of the placement at clothianidin-treated fields (Supplementary Table 5, Fig. 5). In contrast to *G. apicola*, ALPV abundance increased during the oilseed rape bloom and this increase was less pronounced in clothianidin-treated than in control colonies (Supplementary Table 5, Fig. 5). Similar to *G. apicola*, BQCV abundance was explained by an interaction between treatment, bloom and year, therefore the dataset was analysed separately for each year (Supplementary Table 5). In 2013, BQCV abundance decreased over the oilseed rape bloom, irrespective of the placement at clothianidin-treated fields. The abundance of BQCV decreased also in 2014 clothianidin-exposed colonies but not in control colonies (Supplementary Table 5, Fig. 5). In 2014, IAPV abundance showed a similar non-significant seasonal pattern as its prevalence (Supplementary Table 5, Fig. 5). The abundances of ABPV, CBPV and KBV were not affected by the clothianidin treatment and showed also no seasonal patterns (Supplementary Table 5). In contrast, SBPV abundance increased during the oilseed rape bloom in 2014, but was similarly not

affected by the clothianidin treatment (Supplementary Table 5). Just as for prevalence, DWV-A abundance increased generally during the oilseed rape bloom, but more strongly in 2014 then 2013, while the abundance of DWV-B showed contrasting seasonal patterns in the two years (Supplementary Table 5, Fig. 5). During the oilseed rape bloom in 2013, DWV-B abundance decreased, whereas in 2014 the abundance increased (Supplementary Table 5, Fig. 5). However, neither DWV strain was affected by clothianidin treatment. The abundance of LSV-1 was not related to the clothianidin treatment, but was explained by an interaction between bloom and year, with an increase in 2013 and a decrease in 2014 (Supplementary Table 5, Fig. 5). The abundance of SBV was relatively high and stable throughout the experiment (Supplementary Table 5, Fig. 5). The two *Nosema* species decreased during oilseed rape blooms, where we could statistically assess this (*N. ceranae* in both years and *N. apis* in 2013). In contrast, the adult bee *Varroa* infestation rate increased during oilseed rape blooms from, on average, around 0.3 to 1 mites per 100 bees in 2013 and from 0.8 to 3.6 mites per 100 bees in 2014. Abundances of neither species of *Nosema* nor *Varroa* were associated with placement at clothianidin-treated fields (Supplementary Table 5, Fig. 5).

Immune gene expression

The mRNA levels of several key honeybee immune genes involved in the honeybee molecular response to neonicotinoids[19,44,45] were tested in 2013 at the apiary level. The levels of the mRNAs of these genes relative to that of a standard internal reference gene were unaffected by the placement of colonies at clothianidin-treated sites, nor did they change over the oilseed rape bloom except for *Apidaecin* and SPH51, which were more abundant after the oilseed rape bloom than before (Supplementary Table 6, Fig. 6).

Discussion

In this extensive field experiment, we identified large fluctuations between and within years in honeybee colony development, (attempted) swarming/supersedure, colony mortality, microbial composition and *Varroa* infestation, but no verified negative effects of placement at the clothianidin-treated fields on these parameters. Both these

observations are keys to the interpretation of the results. It places the effects of neonicotinoid exposure on individual bees, as observed in laboratory[17] and (semi-)field studies[16,18] within the context of the high plasticity of the colony as a social unit in response to natural and anthropogenic environmental challenges. It therefore identifies sociality itself as a potent additional homeostatic mechanism, available to social bees but not to solitary bees, for compensating the negative individual effects of neonicotinoid exposure, with or without additional pathogen/environmental pressures. Similarly, while neonicotinoid exposure can increase pathogen-induced mortality of individual bees, especially at high levels of infection[23], the forces that drive the natural volatility in pathogen and microbial prevalence and abundance at colony level appear stronger than the effects of clothianidin exposure, as shown by the large fluctuations during the bloom periods and between years, relative to the placement at clothianidin-treated fields.

One of the most potent colony-level responses to internal and external cues is a colony's decision to replace the queen, either through swarming or supersedure. This is a complex process, involving extensive sensory perception, communication and decision making among the worker bees[46]. Supersedure is often a consequence of (perceived) dissatisfaction with the queen's attributes[25,47] whereas swarming is also triggered by (perceived) lack of space and seasonal cues[46]. All these factors (queen attributes, sensory perception, communication, decision making) can be affected by neurological agents such as neonicotinoids[25,39,44,47–49]. We previously documented as part of this landscape study the severe effects of placement at clothianidin-treated fields on reproduction in *Bombus terrestris* and *Osmia bicornis*[34], making its possible influence on queen acceptance in honeybee colonies particularly relevant. A large enough number of colonies swarmed/superseded during 2013 to establish that, in our experiment, the placement at clothianidin fields had minimal influence on the decision to swarm/supersede. This is supported by the low swarming/supersedure of the surviving colonies in 2014, under similar conditions. The high swarming/supersedure rate in 2013 is most likely due to the extensive splitting and forced re-queening when preparing the colonies, since it is not unusual for queens to be rejected under these circumstances. Also the high colony mortality during 2013-2014 can be largely attributed to

swarming/supersedure, rather than the placement at clothianidin-treated fields. Excluding the swarmed/superseded colonies, the 2013-2014 colony morality rate (7%) fits the average for the region and year (8%[50]), again with no difference between colonies at treated and untreated fields.

We also observed no delayed impact of placement at clothianidin-treated fields in 2013 on colony strength (brood amount or adult bees) after overwintering, during the spring 2014 assessments. We even observed a positive association between placement at clothianidin-treated fields and both adult bees (both years combined) and brood amount (2014). Placement at clothianidin-treated fields had no effect on the prevalence and no severe effect on the abundance of pathogens, parasites and beneficial gut symbionts and the few observed effects are typically positive for bee health. Clothianidin treatment was associated with a weaker increase in *Aphid lethal paralysis virus* abundance and in 2014, BQCV abundance declined only in colonies placed at clothianidin-treated fields during the oilseed rape bloom while not in control colonies. In addition, the abundance of the gut symbiont *G. apicola* declined less strongly in colonies at clothianidin-treated fields than in control colonies during the oilseed rape bloom in 2014. The abundances of all other pathogens, the *Varroa* mite and the gut symbiont *Snodgrassella alvi* were not related to the clothianidin treatment. Jones *et al*. (2018) demonstrated that although the gut microbial composition is influenced by the surrounding landscape, the relationship between environmental stressors, the gut symbiotic bacteria and its host is too complex and interactive for simple reductionist assessments[30]. Two other field studies also found no effect of neonicotinoids on *Varroa* abundance[41,51], while a third found a positive association[52]. In addition, in-hive feeding with imidacloprid-spiked pollen increased *Varroa* abundance, but only at a very high imidacloprid concentration[49].

Laboratory studies have shown that the expression of several immune genes can be influenced by pesticide exposure[19,53,54] including possible secondary effects on pathogen susceptibility[19,44,55–57]. However, a recent study by Collison *et al*. (2018) found weak and inconsistent effects of neonicotinoids on transcriptional responses, and no harmful impact on bee health through suppressed immunocompetence could be detected[58]. In our study

the mRNA levels of several key honeybee immune genes were not affected by the placement at clothianidin-treated fields or, generally, by the oilseed rape bloom in 2013. mRNA is a short-lived intermediate in the communication between an organism's genetic resources and its physiological needs, whose induced response is measured in minutes or hours, as is generally the case in laboratory studies, rather than weeks or months, as was the case in our study. As Collison *et al*. (2018) demonstrated, alterations in gene expressions changed over time often with a peak between 8 and 24 hours after exposure, followed by a gradual decline within the next 6 days[58]. At a longer time scale, a difference in mRNA levels would indicate a major biological shift in the constitutive expression rates of these genes, rather than the temporary induction most frequently investigated in laboratory studies. Both types of changes are important and necessary features of an organism's molecular-adaptive response to its environment, but the longer term type of change is more impactful, since it represents a fundamental change in the molecular baselines and norms on which the organism functions. The more fleeting response may very well also have occurred here, in individual bees immediately after their exposure to clothianidin (*i.e.* forages encountering neonicotinoids in the fields, or house bees when handling contaminated pollen or nectar) but our samples lack the resolution, both in time and sampling unit (colony/apiary), to detect this. We sampled bees at the common overwintering site, after the oilseed rape bloom and possibly several days after being in direct contact with the neonicotinoid. Task-based division of labour may also have diffused the levels of exposure of different bees of the same colony, and gene expression can be socially regulated in honeybees[59].

In both years, honeybee-collected pollen and nectar contained substantially higher concentrations of clothianidin from colonies at treated fields than in control colonies, verifying the exposure scenarios of the treated and control field conditions in this study. Interestingly, the clothianidin concentrations at the treated fields in 2013 were twice as high as in 2014, despite almost identical seed coatings, study farms and analysis methods. Neonicotinoid degradation and leaching are related to temperature and moisture conditions in the soil, with longer half-life under cool and dry conditions[60]. The spring of 2013 had a lower average temperature, more days with frost and less precipitation than

the spring of 2014 in our study region (Supplementary Table 7). We suspect that these differences in spring weather could have contributed to the variation in clothianidin residues in pollen and nectar between the two years. However, this does not imply a lower exposure of clothianidin to the honeybees in the second year compared to 2013, as they collected a higher proportion of oilseed rape pollen in 2014 than in 2013. The detection of clothianidin in honeybee-collected nectar and in honeybee tissue at control sites in 2013 demonstrates the difficulty of setting up control conditions in field experiments, since the wide flight range of honeybees means they can forage on other potentially treated fields. Still, this experiment adequately captured the exposure to clothianidin compared to control conditions, since we demonstrated large differences between the treated and control sites in measured clothianidin residues. Furthermore, the clothianidin concentrations in pollen from clothianidin-treated fields were higher in both years than what has been reported in other studies[36,40,41]. This is probably because the oilseed rape was sown in spring rather than autumn, as it was the case in the other studies. Furthermore, we demonstrated considerable variation within and among treated fields in clothianidin concentrations of honeybee-collected nectar and bee tissue sampled from both individual bees and pooled bee samples from fields. These variations might be the result of uneven foraging alternatives in the various landscapes or differences in clothianidin concentrations in the plants. This indicates that the neonicotinoid exposure risk for bees may differ depending on the cultivar, the time of sowing, the geographical location and possibly also the weather conditions.

There have now been several more or less well designed field studies that have failed to detect a major impact of field-level neonicotinoid exposure on honeybee colony development, performance and overwintering success[34,36,40,41,61]. At the same time several field-realistic studies have reported the impairment of individual and social bee life parameters due to chronic exposure to neonicotinoids[39,47–49]. The importance of the results presented here is that in fundamentally healthy colonies, like the ones studied here, the natural homeostatic health mechanisms mediating the colony's response to its environment are robust enough to overcome these impairments, even in undersized colonies, during two consecutive years of one month direct exposure, in the middle of the

short Swedish bee foraging season. These conclusions are compatible with a recent French study, where individual free-flying honeybees placed near fields treated with thiamethoxam disappeared faster than bees at untreated fields, due to a higher mortality rate, but that the total number of adult bees and the honey production of the colonies remained unaffected[48] due to effective colony-level compensating mechanisms. We were able to detect relatively subtle differences, as our experiment had sufficient power to comply with the requirements set by EFSA[32] (effect size of < 7% with a power of 80%) for the assessment of pesticide effects on colony size.

Our study provides insight into the interactions between two drivers of honeybee colony losses, pathogens and pesticides, by demonstrating that foraging on oilseed rape grown from clothianidin-coated seeds had no observable negative effects on honeybees at colony level on either constitutive immune gene expression, microbial composition (pathogens or symbiotic bacteria) or *Varroa* infestation under real-world field conditions. We acknowledge that detrimental effects could well have existed at individual bee level, but were effectively compensated for by colony-level social regulatory mechanisms, supported by the robust general health of these colonies, and that less robust colonies may well have yielded different results. We also contrast the large natural plasticity of honeybee colony performance and microbial composition with the insignificant negative influence of the placement of colonies at clothianidin-treated fields on these parameters, to highlight the importance of sociality as an additional adaptive mechanism for managing environmental challenges even in undersized colonies, confirming the need for separate exposure study models for social and solitary bees. The contrasting results from different field studies on honeybee health show the importance of the context of the exposure and the study system, as well as the need for more extensive research in multiple biogeographical areas and crop systems. Additionally, the large within and between-year differences in colony parameters demonstrate the importance of multiple sample points within a year and long-term studies of the cumulative effects the placement of colonies at clothianidin treated fields, including the general landscape context[3], which are currently lacking. Studying the effect of insecticides on honeybee colonies under field conditions is crucial to understand realistic effects of neonicotinoids for further policy

decisions, in addition to laboratory experiments, which seem a more sensitive system. To make informative decisions on pesticide use in natural, agricultural and urban landscapes, there is a need for improved understanding of the context-dependence of colony-level responses in social bees[36,62], as well as the as-yet largely understudied effects on wild and solitary bee species[35,36].

Methods

Study design

In 2013, a total of 16 fields (8.9 ± 5.4 ha; mean ± SD) in southern Sweden, intended for spring-sown oilseed rape (*Brassica napus L.*) were paired according to geographical proximity (but separated by >4 km) and land-use (Fig. 1, see above). The surrounding landscape was inspected for the absence of flowering crops. However in 2013, two fields remained in the study even though another oilseed rape field was present nearby, so as to retain as many farm-pairs as possible[34]. In each farm-pair, one field was randomly assigned to be sown with clothianidin-treated oilseed rape seed while the other field was sown with seed not treated with clothianidin (treated: 8; control: 8). The same paired farms were used in 2014 but with the treatments reversed, *i.e.* locations with treated fields in 2013 had untreated fields in 2014 and *vice versa* (treated: 6; untreated: 4). Due to crop rotation, different fields within each farm were used in 2013 and 2014 (Fig. 1, see above). In order to create Fig. 1, we downloaded a map from the World Borders Database (downloadable here: http://thematicmapping.org/downloads/world_borders.php).

Information on surrounding landscape variables for the different farms in 2014 is presented in Supplementary Table 8. In 2014, half of the focal fields had additional spring-sown oilseed rape (1-13 ha) within a 2 km radius. The clothianidin-treated seeds for the focal fields were coated with Elado, a trademarked blend of two active ingredients: clothianidin (400 g l^{-1}) and β-cyfluthrin (+ 80 g l^{-1}), chosen for this study because it was the predominant seed insecticide treatment in oilseed rape in Sweden and in other parts of Europe[63]. Clothianidin is taken up by the plant and systemically distributed to all its parts for protection against insects[64]. β-cyfluthrin is not considered to

be systemic and no residues were detected in samples collected in this study[34]. Both clothianidin-treated and control seeds were coated with the fungicide thiram in 2013.

The participating farmers were instructed not to use other neonicotinoids in the fields during the study, although other insecticide foliar sprays; primarily Plenum (pymetrozine), Avaunt/Steward (indoxacarb) and Mavrik (tau-fluvalinate; also used as *Varroa*cide in beekeeping) were used for pest control (Supplementary Table 9). However, Biscaya, tradename for a spray formulation containing the neonicotinoid thiacloprid, was applied to one control field in 2013, followed by a Mavrik spray one week later, and to one treated field in 2014, on both occasions at 0.3 L ha^{-1}. Thiacloprid has a considerably lower acute toxicity for bees than clothianidin[65] and only trace amounts of thiacloprid were detected in the pollen, nectar and bee samples in 2013 and none in 2014. While Rundlöf *et al*. (2015) did not observe any change in results when fields where Biscaya was applied were excluded from the analyses[34], we detected some qualitative changes (Supplementary Table 10). These changes could be due to the higher thiacloprid residues detected in 2014, but may just as well not relate to Biscaya but rather to the difference between the Biscaya/Mavrik and the alternative insecticide spray combinations used[34] or be due to reduced statistical power.

Honeybee colonies

One hundred and sixteen honeybee colonies were prepared at the end of May 2013 by a professional beekeeper in single full-size Langstroth hives containing two combs with mainly sealed brood (with bees), two full honeycombs (with bees), one drawn out empty comb, five combs with wax foundation, bees shaken from two combs and either a 1-year old (84 experimental colonies) or 2-year old (12 experimental colonies plus 20 reserve colonies) queen of known descent, to produce relatively small, equally-sized (3,418 ± 123 adult bees; mean ± s.e.m.; n = 96 colonies) colonies with plenty of room for growth, that could become strong enough to survive the coming winter, but not outgrow their space during the summer. Six experimental colonies were placed along the field edge in each of the 16 oilseed rape fields (96 colonies in total) between 14th and 28th of June 2013, at the onset of oilseed rape flowering (Supplementary Fig. 1). Queen lineage and

age was matched between farm-pairs, but colonies were otherwise distributed randomly. Colonies were kept at a 60 ha organically managed winter oilseed rape field in full bloom before placement at the 16 experimental fields (Supplementary Fig. 1), to ensure pre-experiment colony growth was based as much as possible on pesticide-free foraging.

When the oilseed rape bloom in the experimental field had ceased, the colonies were moved between the 2nd and the 31st of July (Supplementary Fig. 1) to a common apiary to overwinter. On the 10th of August, the colonies were given a formic acid vapour treatment against *Varroa* mites, consisting of 20 ml 60% formic acid soaked into a flat household sponge placed under the inner cover on top of the frames. The colonies were fed a total of 20 kg of sugar per colony in the form of a 55-60% v/v sucrose solution, provided in a feeding box across three occasions during August-September 2013. An additional light *Varroa* treatment was carried out on the 4th of December by sprinkling 30 ml 2.6% oxalic acid in 60% sucrose in between the frames, directly onto the bee cluster. In spring 2014, colonies were moved to an organically managed oilseed rape field before placement at the ten spring-sown oilseed rape fields (Supplementary Fig. 1). Colonies were considered for inclusion in the 2014 part of the study if they had a two-year old, egg-laying queen in April 2014 (excluding colonies that died, re-queened or had three-year old queens in April 2014) and had not swarmed by the beginning of June 2014. These restrictions, in addition to the requirement that colonies would be exposed/unexposed to clothianidin for both years of the experiment, meant that in 2014 only four colonies could be allocated to each field. Colonies placed by treated fields in 2013 were again placed by treated fields in 2014, so as to assess the cumulative effects of multi-year clothianidin exposure, but were otherwise re-randomized prior to placement to minimize unintended biases. Even so, two control colonies from 2013 had to be placed by a clothianidin-treated field in 2014, due to insufficient qualifying exposed colonies for the six clothianidin-treated fields. Enough colonies were available for the four control fields. The strength of colonies was equalized as described for 2013, but only within each treatment group. The colonies were reduced and equalized a second time (8 June 2014), after some of them grew too large and attempted to swarm (Supplementary Fig. 1). Each reduced colony included 1 full honey comb (with bees), 3 combs with mainly

sealed brood (with bees), and the original queen from 2013 and 6 combs with wax foundation. Colonies were moved to the spring oilseed rape fields between June 16th and 25th 2014 and brought back to the common overwintering site between July 14th and 22nd 2014 (Supplementary Fig. 1).

Residue analyses

To confirm clothianidin exposure, 24 adult honeybees per field caught at the hive entrance, pollen pellets collected from five honeybees foraging in the oilseed rape fields and nectar removed from the stomachs of five nectar-foraging honeybees in the oilseed rape fields were analysed from each site for clothianidin residues. Pollen (>25 ml) was collected using pollen traps, which were installed for one day on three colonies per site and analysed for plant species origin. Samples were handled and analysed as in Rundlöf *et al.* (2015)[34] and collected during the peak bloom assessments in both years (Supplementary Fig. 1), with the concentrations of clothianidin and four other neonicotinoids used in Sweden (Supplementary Table 2) quantified using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and pollen identified to oilseed rape type using light microscopy and a pollen reference library (see Supplementary Table 2 for limits of detection and quantification). For further analyses of the variation in neonicotinoid exposure of honeybees in different sites, we collected 12 honeybees per site from the hive entrance. This sampling was done at three clothianidin-treated sites in 2013. Nectar was extracted from the honey stomach of the collected bees. The concentrations of clothianidin was thereafter quantified in both nectar and bee tissue for each bee individual. More details on the sample treatment for different matrices, LC-MS/MS method and quality controls are given in Supplementary Methods.

Colony development, re-queening and honey production

Honeybee colony development was assessed by the same trained observer and one assistant. The presence of a laying queen was established, as well as the presence of queen cells. If a re-queening event was accompanied by a large loss of adult bees, it was deemed to have swarmed. If no loss of adult bees was observed, the colony was deemed to have re-queened through supersedure. Colony honey production and development was

determined by weighing the colonies and by assessing colony strength using the Liebefeld method[66], as the total number of adult bees and the area of capped brood over all frames. The number of adult bees was estimated by counting honeybees on both sides of the 10 frames. The number of capped brood cells (amount of brood) was determined by multiplying the proportion of closed brood coverage by 2700, which is the number of cells on one side of the frames used. The colonies were weighed during pre-exposure and post-exposure assessments (using a Mettler Toledo bench scale able to weigh up to 32 kg with 1 g precision), to estimate honey production. Full honey frames were replaced by empty frames during the oilseed rape bloom, to allow the colony to grow and reduce swarming. Both the full and the empty frames were weighed, for inclusion in the calculating of honey production. During post-exposure assessment, as many honey frames as possible were removed (max 10% of the area covered with covered brood), to simulate the beekeepers honey harvest. Pre-exposure assessments were done at the organically managed winter-sown oilseed rape field on 6-17 June 2013 and 9-11 June 2014, and post-exposure assessments at the common overwintering apiary on 29 July to 9 August 2013 and 28-31 July 2014 (Supplementary Fig. 1). Furthermore, a spring colony strength assessment was performed in April 2014, by estimating the total number of adult bees and the number of capped brood (Supplementary Fig. 1). The colony assessor and assistants were blinded during data collection with respect to the treatment regimen of the fields.

Pathogen and parasite sample collection and processing

Samples of around 100 adult honeybees were taken from each colony during pre- and post-exposure assessments in the clothianidin-treated and control experimental oilseed rape fields in both 2013 and 2014 (Supplementary Fig. 1). Bees were taken from the outer comb of each colony and consisted therefore of a mixture of house bees and forager bees[67]. All bee samples were stored at -20 °C until the laboratory work was performed. The *V. destructor* infestation rates for each colony were determined by washing the adult bee samples with soapy water to dislodge and count the mites[68]. The abdomens of 60 adult honeybees per colony (for individual colony analyses) or per apiary (for the 2013 pooled colony analyses, 10 bees per colony) were removed and placed in a polyethylene

bag with an inner mesh (BioReba). The abdomens were ground in the bag using a pestle and 30 ml of nuclease-free (Milli-Q) water (0.5 ml bee^{-1}) was mixed thoroughly with the sample to create a homogenous suspension. Several 1 ml aliquots of this suspension were removed and frozen immediately at -80 °C, for DNA and RNA extraction, and as future reference material.

Parasites, pathogens, symbiotic microbes and immune genes

The collected bee samples were assessed for a variety of pathogenic and non-pathogenic parasites and microbes in order to study the impact placement of colonies at clothianidin-treated fields on their prevalence and abundance. The organisms included the ubiquitous ectoparasite *Varroa destructor*, 13 viruses: *Acute bee paralysis virus* (ABPV), *Aphid lethal paralysis virus* (ALPV), *Big Sioux River virus* (BSRV), *Black queen cell virus* (BQCV), *Chronic bee paralysis virus* (CBPV), *Deformed wing virus* type-A (DWV-A), *Deformed wing virus* type-B (DWV-B), *Israeli acute paralysis virus* (IAPV), *Kashmir bee virus* (KBV), *Lake Sinai virus* strain 1 (LSV-1) and strain 2 (LSV-2), *Sacbrood virus* (SBV), *Slow bee paralysis virus* (SBPV); two common honeybee microsporidian gut parasites (*Nosema apis* and *Nosema ceranae*) and two symbiotic gut-bacteria (Gammaproteobacterium: *Gilliamella apicola* and Betaproteobacterium: *Snodgrassella alvi*). For the 2013 samples we also analysed at apiary level the mRNA levels of eight honeybee genes (*Amel*\LRR, *Apidaecin*, cSP33, *Dorsal*-1A, *Eater*-like, NimC2, PGRP-S2 and SPH51), whose expression had previously been linked to pesticide, pathogen and/or parasite exposure[19,44] and (social) immunity in honeybees[45].

Nucleic acid extraction

DNA was extracted from the bee homogenates using the protocol for extracting DNA from *Nosema* spores[69], which is sufficiently robust to also extract DNA from bacteria and other microorganisms. Five hundred µl primary bee homogenate was centrifuged for 5 min in a microfuge at 13000 rpm. The pellet was repeatedly frozen-thawed with liquid nitrogen and ground with a sterile Teflon micropestle until pulverized. The pulverized pellet was resuspended in 400 µl Qiagen Plant tissues DNeasy AP1 lysis buffer containing 4 µl RNAse-A (10 mg ml^{-1}) and incubated and shaken for 10 min at 65 ºC,

after which 130 µl P3 neutralization buffer (3.0 M potassium acetate pH 5.5) was added, followed by 5 min incubation on ice and centrifugation for 5 min at 14000 rpm to remove the lysis debris. DNA was purified from 500 µl of the supernatant by the Qiagen automated Qiacube extraction robot, following the plant DNeasy protocol and eluting the DNA into 100 µl nuclease-free water. RNA was extracted by the Qiacube robot directly from 100 µl primary honeybee homogenate using the Qiagen Plant RNeasy protocol (including the Qia-shredder for additional homogenization[70]) and the RNA was eluted into 50 µl nuclease-free water. The approximate nucleic acid concentration was determined by NanoDrop, after which the samples were diluted with nuclease-free water to a uniform 10 ng μl^{-1} (DNA and LSV-1(RNA)) or 20 ng μl^{-1} (for all other RNA samples) and stored at -80 °C.

RT-qPCR and qPCR

The various microorganisms and host mRNA targets were detected and quantified by either One-Step Reverse Transcription-quantitative PCR (RT-qPCR), for pathogens with an RNA genome and the immune and internal reference gene mRNA targets, or by quantitative PCR (qPCR) for organisms with a DNA genome. Details of the assays are shown in Supplementary Table 11, Supplementary Table 12 and Supplementary Table 13. The reverse primer for *Amel*\LRR was slightly re-designed from Di Prisco *et al.* (2013)[19] because the extremely high complementarity between the original forward and reverse primers resulted in high levels of PCR artefacts dominating the quantitative signal. The reactions were conducted in duplicate, in 20 µL (DNA) or 10 µL (RNA) reaction volumes containing 2 µL (DNA) or 1.5 µL (RNA) template, 0.4 µM (DNA) or 0.2 µM (RNA) of forward and reverse primer and either the Bio-Rad Eva Green qPCR mix (DNA) or the Bio-Rad One-Step iTaq RT-qPCR mix with SYBR Green detection chemistry (RNA). The reactions were incubated in 96 well optical qPCR plates in the Bio-Rad CFX connect thermocycler, using the following amplification cycling profiles:
10 min at 50 °C for cDNA synthesis (RT-qPCR only): 5 min at 95 °C (to inactivate the reverse transcriptase and activate the Taq polymerase) followed by 40 cycles of 10 s at 95 °C for denaturation and 30 s at 58 °C for primer annealing, extension, and data collection. For DNA assays the following amplification cycle profiles were used: 2 min at 98 °C for

the initial denaturation followed by 40 cycles of 5 s at 98 °C for denaturation and 10 s at 60 °C for primer annealing, extension and data collection. The amplification cycles were followed by a melting curve analysis to determine the specificity of the amplification by reading the fluorescence at 0.5 °C increments from 65 °C to 95 °C. Included on each reaction plate were positive and negative (non-template) assay controls. For each type of assay (Supplementary Table 11, Supplementary Table 12 and Supplementary Table 13) a calibration curve was prepared through a 10-fold dilution series of a positive control of known concentration covering 6 orders of magnitude, for quantitative data conversion, establishing the reference melting curve profile of the amplicon and estimating the reaction performance statistics.

Data conversion and normalization

The melting curves of individual reactions were evaluated visually in order to separate out non-specific amplifications, which differ in melting temperature profiles from true target cDNA/DNA amplicons. Non-specific amplifications were deleted from the data set. All assays were run in duplicate, with the mean value of these two duplicates used in further calculations. Both duplicates had to yield a positive quantitative value and pass the melting curve analysis for the data to be included in the data set. The raw RT-qPCR data of all confirmed amplifications were subsequently converted to estimated copy numbers of each target RNA, using the corresponding calibration curve for the assay. These data were multiplied by the various dilution factors throughout the procedure to calculate the estimated copies of each target per bee[69]. Since RNA is easily degraded there is a risk that differences between individual samples in RNA quality (*i.e.* degradation) can affect the results[70]. To correct for this, RT-qPCR assays for the mRNA of a common honeybee internal reference gene (RP49) were run on all samples. The data for the RNA targets of interest were then normalized to the average value for RP49 mRNA, thus correcting the data for sample-specific differences in RNA quality with respect to RT-qPCR performance[70].

Statistical analyses

The proportions of honeybee-collected pollen that originated from oilseed rape type plants were compared between treatments (clothianidin seed-treatment / untreated) using a generalized linear model assuming binominal distribution and correcting for over-dispersion. The clothianidin concentrations in nectar and pollen collected by honeybees and in bee tissue were compared between treatments using Wilcoxon-Mann-Whitney tests. To compare the concentrations of clothianidin in the bee tissue and nectar in individual bees between fields, we used analyses of variance (ANOVA), with field identity as predictor. Furthermore, the concentrations of clothianidin in the tissue and nectar stomach content of individual bees were related using a multiple linear regression with field identity and concentration of clothianidin in nectar as explanatory variables and concentration of clothianidin in bee tissue as response variable.

The study followed in general a Before-After-Control-Impact (BACI) design, with a paired field structure, repeated for two consecutive years at colony level for data on colony development as well as the prevalence and abundance of parasites, pathogens and gut bacteria. The years, 2013 and 2014, were analysed together in one full model, with seed-treatment, bloom, year and their interactions as fixed factors. The effect of the clothianidin treatment was assessed by the interaction between bloom and seed-treatment, as this term reflects the difference in change between treatments over the oilseed rape bloom(s). If the three-way interaction (bloom x seed-treatment x year) was significant (i.e. if the variable responded differently to the clothianidin treatment from one year to the next), the dataset was split by year and year was dropped as a fixed factor. Furthermore, the dataset was analysed for only one year if the data consisted of a sample size ≤ 10 in one year both for microbiota prevalence and abundance (Supplementary Table 1). Colonies that swarmed (eight at control fields and ten at clothianidin-treated fields in 2013; one at a control field and two at clothianidin-treated fields in 2014) were excluded from the analysis, since swarming has a large effect on colony development. Also excluded is the single colony that lost its queen during transport before field placement in 2013 (treated field). Excluding colonies that swarmed from the analysis qualitatively altered some results (see Supplementary Table 14). Changes in significance level might be due to reduced statistical power, random chance or biological effects.

Linear mixed-effects models (LMM) were used to test the effect of the clothianidin treatment on colony development measured as the number of capped brood cells (amount of brood) and the number of adult bees. Seed-treatment (clothianidin or control), bloom (before or after oilseed rape bloom), year (2013 or 2014) and their interactions were fixed factors. Farm pair identity, farm identity and colony identity were included as random factors. Honey production was compared between treatments using a LMM with farm pair identity and farm identity as random factors. GLMMs were used to test the influence of the clothianidin treatment on re-queening and mortality of the colonies with farm identity as a random factor.

The influence of the clothianidin treatment on spring colony development, measured as the number of adult bees and amount of brood, was tested using a LMM and a GLMM, respectively, with seed-treatment as fixed factor and farm pair identity and farm identity as random factors. For the number of capped brood cells we used a negative binomial error distribution and logarithmic link function.

The microbiome and *Varroa* mite data were analysed both on their binomial (presence/absence) and quantitative (abundance) character, using generalized linear mixed models (GLMM; with binomial error distributions and a logit link function) and LMMs (with normal error distributions), respectively, with seed-treatment, bloom, year and their interactions as fixed factors. GLMMs on microorganism or *Varroa* mite prevalence included only colony identity as random factor, as the effective sample size (i.e. the less frequent outcome of the presence/absence data) did not allow for the inclusion of more random factors. Only organisms and years with an (effective) sample size > 10 were analysed for both the prevalence and the abundance data. In addition, colonies that did not at least once test positive for a particular microorganism were excluded from the analysis of abundance. Bee pathogen and bacterial abundance were logarithmically ($\log_{10}$) transformed, as they are generally exponentially distributed. LMMs on target organism abundance contained farm pair identity, farm identity and colony identity as random factors. *Varroa* mite numbers per 100 bees and colony weight

were square-root transformed to avoid non-normally distributed residuals. Confidence intervals were calculated based on profile likelihood. For the square-root transformed data, estimates were back-transformed to the original scale for graphical illustrations.

The immune gene transcripts were only available for 2013 and at apiary level but also followed the BACI design. LMMs on gene expression contained seed-treatment and bloom as fixed factors and farm identity as random factors.

Statistical data analyses were performed using R except for analyses addressing the verification of clothianidin exposure and land use, for which SAS 9.4 for Windows (SAS Institute Inc.) was used. LMMs were fit using the *lmer* function of the *lme4* package and GLMMs were fit using the *glmmTMB* function of the *glmmTMB* package in R. *P*-values from GLMMs were calculated by likelihood ratio tests. *P*-values from LMMs were calculated using the *Anova* function of the *car* package, whereby type-III F-tests were used for models containing interactions and type-II F-tests for models without interactions (i.e. spring assessment and honey production). Effects of fixed factors were estimated using sum-to-zero contrasts in all models except those on neonicotinoid residues. Sum-to-zero contrasts allow for the determination of main effects/interactions (*i.e.* estimation independently of other independent variables) and show the effects of factors as deviations from the grand mean (intercept). For factors with two levels the magnitude of the deviation of each level from the grand mean is the same but the direction differs. We represent effects of factors (seed-treatment, bloom, year), as the deviance of the second level (clothianidin, after, 2014) from the grand mean. This was also the case for interactions, so that for example the seed-treatment × bloom interaction indicates to what extent clothianidin-exposed colonies differed in change over the oilseed rape bloom from the mean change of both treatments.

Power analysis

We performed power analyses for number of adult bees and the number of capped brood cells and honey production, to investigate the effect size we could potentially detect given our design, replication and model choice. Power was determined for a range of effect

sizes at a nominal confidence level of $\alpha = 0.05$ by 1000 Monte Carlo simulations per effect size using the powerSim function of the simr package. Power was calculated for a range of effect sizes, expressed as the change in number of adult bees, number of capped brood cells or honey production. By dividing the effect size with the mean number of bees, the mean number of brood cells or honey production of all control colonies, we obtained effect size expressed as the percentage change of those matrices (Supplementary Fig. 3). This power analysis made it possible to compare our effect size with the effect size presented by Rundlöf *et al*. (2015)[34]. Using the full model, we could detect an effect size for the number of adult bees of below 5% with a power of 80%, compared to the effect size below 20% presented by Rundlöf *et al*. (2015)[34]. This is even lower than the requirements of an effect size < 7% set by EFSA[32]. As a result of a significant interaction of seed-treatment, bloom and year, the dataset for the number of capped brood cells were analysed separately for each year. Therefore, we present here the power analysis for each year. The effect size at which 80% was reached increased from below 10% in 2013 to slightly below 11% in 2014 (Supplementary Fig. 3), likely due to the reduced replication in 2014. We also performed a power analysis of honey production (amount of honey per colony in kg) using the dataset of both years, showing that an effect size of below 20% could be detected with a power of 80% (Supplementary Fig. 3).

Data availability

The authors declare that the data supporting the findings of this study are available within the paper, its supplementary information files and/or Rundlöf *et al*. (2015)[34]. The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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AUTHOR CONTRIBUTIONS

Conceptualization, JM, MR, RB, HS, TRP; Methodology, JM, JO, DW, EF, ES, PO, MR, OJ; Validation, JM, MR, ES, PO, OJ; Investigation, JO, DW, ES, PO; Chemical analysis, OJ; Formal analysis, JO, DW, MR, RB; Data Curation, JM, MR; Writing, BL, JO, DW, MR; Editing, All; Visualization, BL, JO, DW, MR; Supervision JM, MR, PR; Project Administration, EF, MR, TRP; Funding Acquisition, EF, JM, MR, OJ, RB, TRP, HS.

Competing interests

The authors declare no competing interests.

Figures & Tables

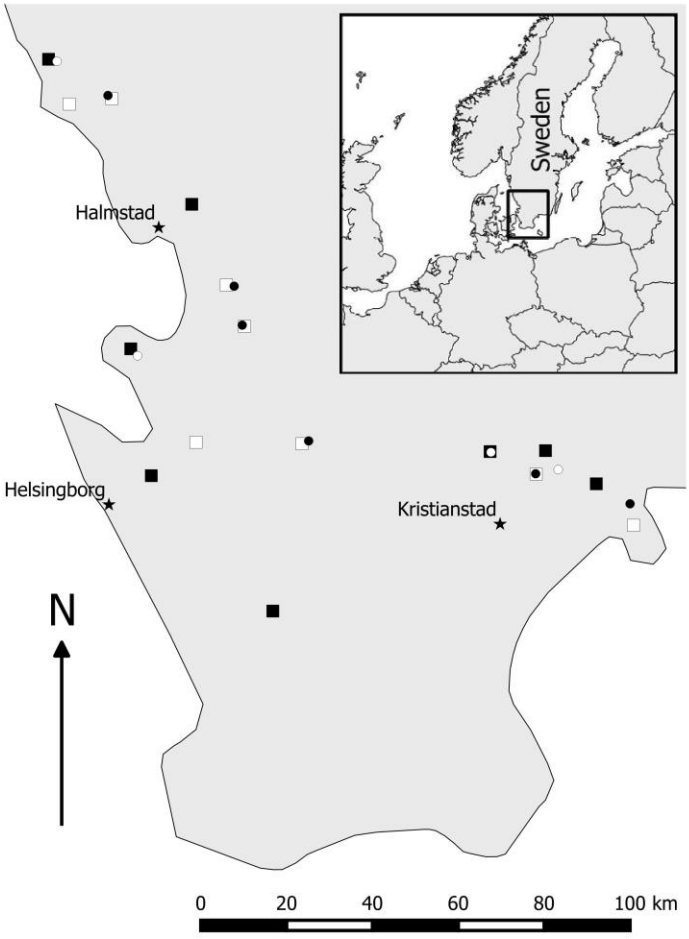


Figure 1 | Study design with replicated landscapes in southern Sweden. In 2013 (squares) 16 oilseed rape fields and in 2014 (circles) 10 oilseed rape fields were either sown with seeds coated with clothianidin and a fungicide (black) or only a fungicide (control fields, white). Stars represent locations of the three weather stations in Supplementary Table 7. The map is based on the World Borders Database (downloadable here: http://thematicmapping.org/downloads/world_borders.php).

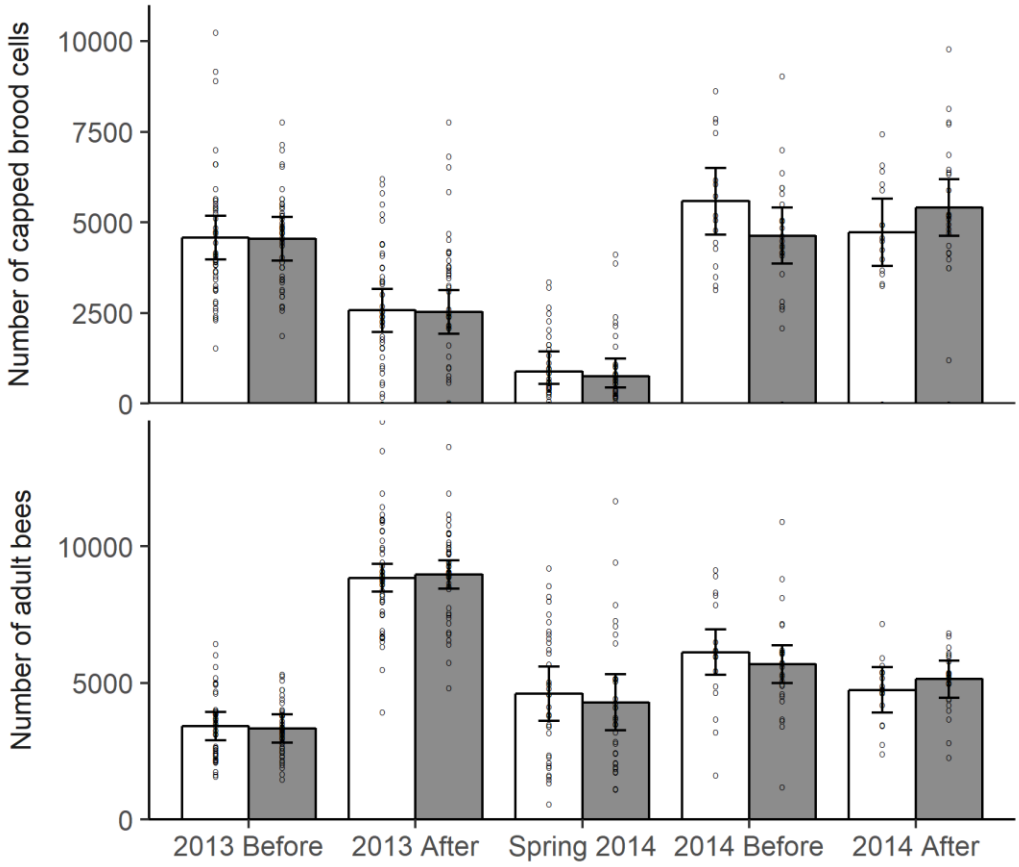


Figure 2 | Honeybee colony development. The mean (± 95% confidence limits) number of adult bees and the number of capped brood cells per colony in relation to seed-treatment (control = white bars; clothianidin-treated = grey bars) in oilseed rape fields before and after the oilseed rape bloom in two years (2013 and 2014) and at the 2014 spring inspection. n = 8 fields per treatment in 2013 and n = 4 control fields and 6 clothianidin-treated fields in 2014. Circles indicate measured values per colony.

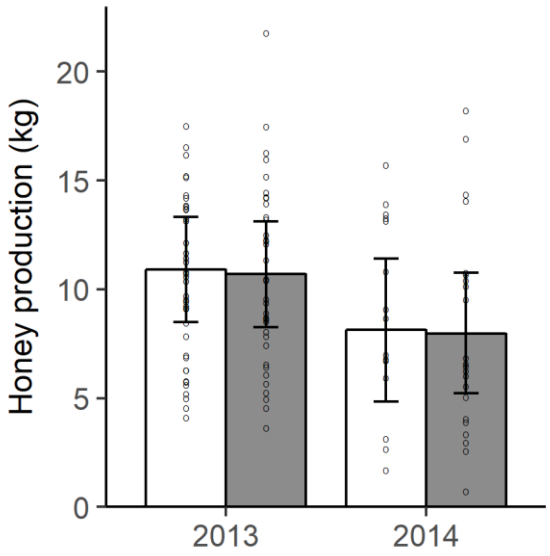


Figure 3 | Honey production. Mean (± 95% confidence limits) honey production measured in kilogram in relation to seed-treatment (control = white bars; clothianidin-treated = grey bars) during the oilseed rape bloom in two years (2013 and 2014). n = 8 fields per treatment in 2013 and n = 4 control fields and 6 clothianidin-treated fields in 2014. Circles indicate measured values per colony.

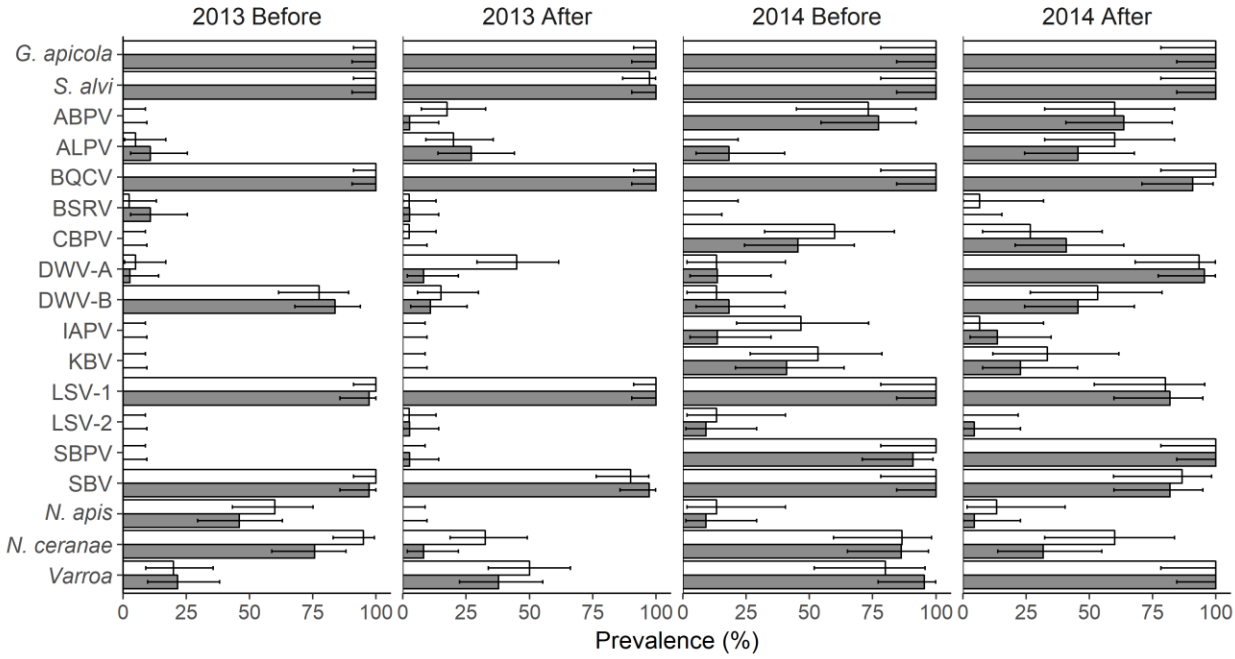


Figure 4 | Microorganism/parasite prevalence. Percentage (± 95% confidence limits) of honeybee colonies infected with the gut bacteria *Gilliamella apicola* and *Snodgrassella alvi*, the viruses *Acute bee paralysis virus* (ABPV), *Aphid lethal paralysis virus* (ALPV), *Black queen cell virus* (BQCV), *Big Sioux River virus* (BSRV), *Chronic bee paralysis virus (CBPV)*, *Deformed wing virus* type-A (DWV-A), *Deformed wing virus* type-B (DWV-B), *Israeli acute paralysis virus (IAPV)*, *Kashmir bee virus* (KBV), *Lake Sinai virus* type-1 (LSV-1), *Lake Sinai virus* type-2 (LSV-2), *Slow bee paralysis virus* (SBPV), *Sacbrood virus* (SBV), the microsporidians *Nosema apis*, *Nosema ceranae* or infested with the *Varroa* mite (*Varroa destructor*) in relation to treatment (control = white bars; clothianidin-treated = grey bars) in oilseed rape fields (n = 8 fields per treatment in 2013; n = 4 control and n = 6 treated fields in 2014) before and after the oilseed rape bloom in two years (2013 and 2014).

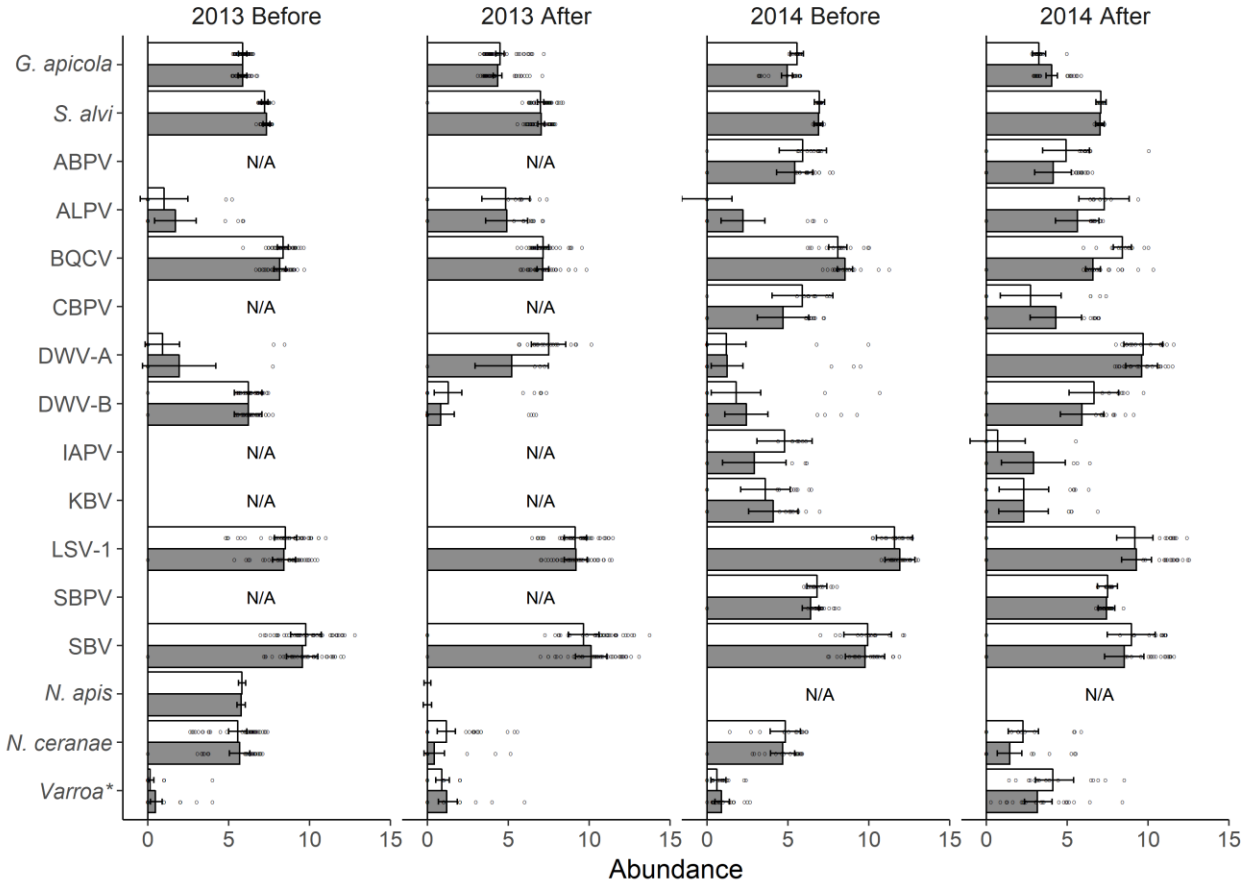


Figure 5 | Microorganism/parasite abundance. The abundance (± 95% confidence intervals) of frequently detected target organisms (*Gilliamella apicola*, *Snodgrassella alvi*, *Acute bee paralysis virus* (ABPV), *Black queen cell virus* (BQCV), *Deformed wing virus* type-A (DWV-A), *Deformed wing virus* type-B (DWV-B), *Israeli acute paralysis virus* (IAPV), *Kashmir bee virus* (KBV), *Lake Sinai virus 1* (LSV-1), *Slow bee paralysis virus* (SBPV), *Sacbrood virus* (SBV), *Nosema ceranae* and *Varroa destructor*) in honeybee colonies in relation to the treatment (control = white bars; clothianidin-treated = grey bars) in oilseed rape fields (n = 8 fields per treatment in 2013; n = 4 control and n = 6 treated fields in 2014) before and after the oilseed rape bloom in two years (2013 and 2014). *The abundance of *V. destructor* was represented by the number of mites per 100 bees, while the microorganisms were expressed as $\log_{10}$ [units] per bee[70]. Microorganism abundance was not analysed (N/A) if it was detected in less than 11 samples per year. Circles indicate measured values per colony.

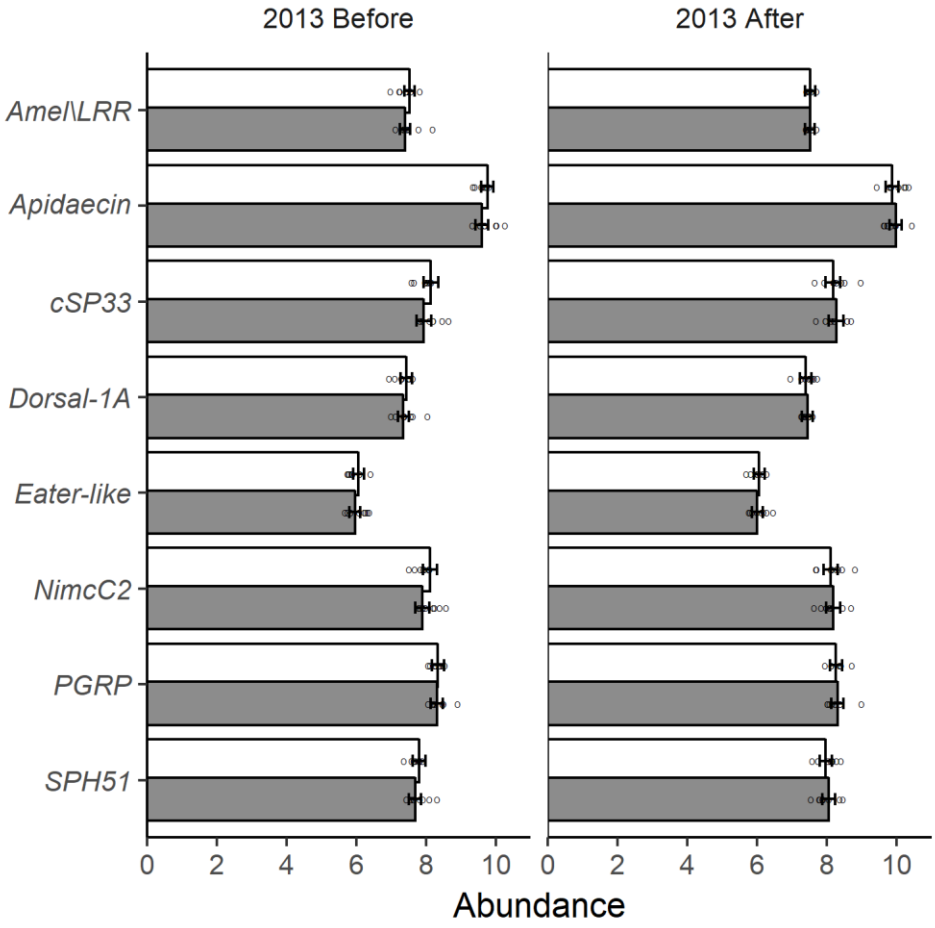


Figure 6 | Immune gene expression at apiary level in 2013. The abundance (± 95% confidence intervals) of expressed immune genes in honeybees at apiary level in 2013 in relation to the treatment (control = white bars; clothianidin-treated = grey bars) in oilseed rape fields (n = 8 fields per treatments) before and after their bloom. Immune gene expression data are presented as the $\log_{10}$ number of estimated mRNA copies per bee. Circles indicate measured values per colony.

Table 1 | Clothianidin residues in honeybees, pollen and nectar during 2013-2014.

	Control fields*		Treated fields**				
	Range#	**Mean±s.e.m#**	**Range#**	**Mean±s.e.m#**	***Z*§**	***P*§**	**n(T,C)‡**
2013							
Honeybees	<LOD-0.89	0.13±0.11	0.35-4.90	2.4±0.50	-3.29	**0.001**	8/8
Pollen	<LOD	<LOD	6.60-23.00	13.9±1.80	-3.16	**0.002**	6*/8
Nectar	<LOD-0.61	0.11±0.08	6.70-16.00	10.30±1.30	-3.40	**<0.001**	8/8
2014							
Honeybees	<LOD	<LOD	0.15-1.50	1.10±0.20	-2.53	**0.011**	4/6
Pollen	<LOD	<LOD	2.40-16.00	6.10±2.00	-2.53	**0.011**	4/6
Nectar	<LOD	<LOD	2.60-9.80	4.90±1.10	-2.53	**0.011**	4/6

* Sample size: 2013, n = 8; 2014, n = 4. No honeybees with pollen could be found at two of the control fields.

** Sample size: 2013, n = 8; 2014, n = 6.

Concentrations of clothianidin in honeybees (ng g-1) and in pollen (ng g-1) and nectar (ng ml-1) sampled from foraging honeybees in clothianidin-treated oilseed rape fields (T) and control fields (C) during 2013-2014.

§ Wilcoxon-test for differences between treatments. P values < 0.05 are highlighted in bold.

‡ Sample size. T, clothianidin-treated oilseed rape fields; C, control fields.

Table 2 | Colony development* and honey production in relation to fixed effects**.

Model	Model type‡	Effect measure‡	Estimates§	Degrees of freedom	*F*§	*P*§
Colony development#						
Number of capped brood cells	Full model	Intercept	4445.953			
		Seed-treatment	-23.591	1, 16	0.04	0.837
		Bloom	-271.905	1, 110	6.06	**0.015**
		Year	783.702	1, 19	42.12	**<0.001**
		Seed-treatment x Bloom	171.907	1, 110	2.42	0.122
		Seed-treatment x Year	-41.545	1, 17	0.13	0.719
		Bloom x Year	466.244	1, 110	17.83	**<0.001**
		Seed-treatment x Bloom x Year	276.231	1, 110	6.26	**0.014**
	2013	Intercept	3656.020			
		Seed-treatment	20.252	1, 7	0.22	0.872
		Bloom	-738.149	1, 75	46.74	**<0.001**
		Seed-treatment x Bloom	-104.324	1, 75	0.10	0.380
	2014	Intercept	5098.070			
		Seed-treatment	-60.730	1, 4	0.08	0.790
		Bloom	194.339	1, 35	0.94	0.338
		Seed-treatment x Bloom	448.139	1, 35	5.02	**0.032**
Number of adult bees	Full model	Intercept	5866			
		Seed-treatment	-61	1, 17	0.24	0.629
		Bloom	1274	1, 110	161.40	**<0.001**
		Year	-439	1, 20	11.26	**0.003**
		Seed-treatment x Bloom	206	1, 110	4.22	**0.042**
		Seed-treatment x Year	-17	1, 17	0.02	0.893
		Bloom x Year	-1647	1, 110	269.79	**<0.001**
		Seed-treatment x Bloom x Year	-140	1, 110	1.94	0.166
Spring assessment						
Number of capped brood cells	2014	Intercept	6.684			
		Seed-treatment	0.029	1	$X^2 = 0.82$	0.821
Number of adult bees	2014	Intercept	4421.691			
		Seed-treatment	-164.356	1, 7	0.19	0.674
Honey production	Full model	Intercept	9.425			
		Seed-treatment	-0.095	1, 16	0.02	0.880
		Year	-1.378	1, 18	4.47	**0.049**
		Seed-treatment x Year	0.009	1, 16	<0.01	0.989

* Colony development during pre- and post- exposure assessment and during spring assessment.
** Full model: n = 14, clothianidin-treated; n= 12, control fields; repeated measurements. 2013: n = 8 per treatment. 2014: n = 6, clothianidin-treated; n = 4 control fields.
‡ The number of capped brood cells and the number of adult bees in relation to clothianidin seed-treatment, bloom (before or after the oilseed rape flowering period) and year (2013 and 2014) using linear mixed effect models.
§ Main effects/interactions were estimated using sum-to-zero contrasts and the deviation of the second level (after, clothianidin, 2014) of each factor (Bloom: before/after, Seed-treatment: control/clothianidin and Year: 2013/2014) from to the grand mean (intercept) is presented. P values < 0.05 are highlighted in bold.
# Measurements were taken during pre- and post- exposure assessment.

Supplementary Information

Supplementary Tables

Supplementary Table 1 | Number of positive and negative samples per year and target organism

Target Organism*	Year**	Number of samples‡	Number of positive samples	Number of negative samples	Statistical analysis prevalence§	Statistical analysis abundance§§
Gilliamella apicola	2013	154	154	0	No	Yes
	2014	74	74	0	No	Yes
Snodgrassella alvi	2013	154	153	1	No	Yes
	2014	74	74	0	No	Yes
Acute bee paralysis virus (ABPV)	2013	154	8	146	No	No
	2014	74	51	23	Yes	Yes
Aphid lethal paralysis virus (ALPV)	2013	154	24	130	Yes	Yes
	2014	74	23	51	Yes	Yes
Black queen cell virus (BQCV)	2013	154	154	0	No	Yes
	2014	74	72	2	No	Yes
Big Sioux River virus (BSRV)	2013	154	7	147	No	No
	2014	74	1	73	No	No
Chronic bee paralysis virus (CBPV)	2013	154	1	153	No	No
	2014	74	32	42	Yes	Yes
Deformed wing virus type-A (DWV-A)	2013	154	24	130	Yes	Yes
	2014	74	40	34	Yes	Yes
Deformed wing virus type-B (DWV-B)	2013	154	72	82	Yes	Yes
	2014	74	24	50	Yes	Yes
Israeli acute paralysis virus (IAPV)	2013	154	0	154	No	No
	2014	74	14	60	Yes	Yes
Kashmir bee virus (KBV)	2013	154	0	154	No	No
	2014	74	27	47	Yes	Yes
Lake Sinai virus type-1 (LSV-1)	2013	154	153	1	No	Yes
	2014	74	67	7	No	Yes
Lake Sinai virus type-2 (LSV-2)	2013	154	2	152	No	No
	2014	74	5	69	No	No
Slow bee paralysis virus (SBPV)	2013	154	1	153	No	No
	2014	74	72	2	No	Yes
Sacbrood virus (SBV)	2013	154	148	6	No	Yes
	2014	74	68	6	No	Yes
Nosema apis	2013	154	42	113	Yes	Yes
	2014	74	7	67	No	No
Nosema ceranae	2013	154	82	72	Yes	Yes
	2014	74	48	26	Yes	Yes
Varroa destructor	2013	154	50	104	Yes	Yes
	2014	74	70	4	No	Yes

* Species names are spelled in italics.

** Target organism was analysed in a full model (2013 and 2014) of sample size was >9 in both years for prevalence.

‡ Number of samples in 2013 (38 samples were excluded from the 192 (96 before and 96 after oilseed rape bloom) samples due to swarming (2 x 18) or loss of queen (2 x 1)) and in 2014 (6 samples were excluded from the 80 samples (2 x 40) due to swarming (2 x 3)).

§ If the data consisted of a sample size < 10 (positive or negative sample size) it was not statistically analysed for prevalence.

§§ If the data consisted of <10 positive samples it was not statistically analysed for abundance.

Supplementary Table 2 | Residues of neonicotinoids in honeybees, pollen and nectar*

	Control (2013: n = 8**; 2014: n = 4)		Clothianidin treated (2013: n = 8; 2014: n = 6)			
	Detected in n samples	**Highest concentration**	**Detected in n samples**	**Highest concentration**	**LOD†**	**LOQ†**
2013						
Honeybee pollen (ng g^{-1})						
Acetamiprid	1	0.34	0		0.080	0.24
Clothianidin	0		8	23	0.50	1.5
Imidacloprid	1	0.23††	0		0.30	0.90
Thiacloprid	3	1.4§	4	0.29	0.070	0.21
Thiametoxam	0		0		0.10	0.30
Honeybee nectar (ng ml^{-1})						
Acetamiprid	0		0		0.033	0.10
Clothianidin	2	0.61	8	16	0.17	0.50
Imidacloprid	3	0.35	0		0.17	0.50
Thiacloprid	2	0.35§	2	0.044	0.033	0.10
Thiametoxam	1	0.19	0		0.17	0.50
Honeybee tissue (ng g^{-1})						
Acetamiprid	1	0.012††	0		0.020	0.060
Clothianidin	2	0.89	8	4.9	0.080	0.25
Imidacloprid	0		0		0.040	0.12
Thiacloprid	2	0.058§	2	1.1	0.030	0.090
Thiametoxam	1	0.19	0		0.070	0.20
2014						
Honeybee pollen (ng g^{-1})						
Acetamiprid	1	0.18	0		0.1	0.3
Clothianidin	0		6	16	0.25	0.75
Imidacloprid	0		1	0.39	0.2	0.6
Thiacloprid	3	1.3	2	358§§	0.025	0.075
Thiametoxam	0		0		0.06	0.2
Honeybee nectar (ng ml^{-1})						
Acetamiprid	0		1	0.68	0.033	0.1
Clothianidin	0		6	9.8	0.17	0.5
Imidacloprid	0		1	1.3	0.17	0.5
Thiacloprid	1	0.06	2	15§§	0.033	0.1
Thiametoxam	0		1	0.2	0.17	0.5
Honeybee tissue (ng g^{-1})						
Acetamiprid	0		1	0.026	0.02	0.06
Clothianidin	0		6	1.5	0.08	0.25
Imidacloprid	1	0.57	0		0.04	0.12
Thiacloprid	1	0.043	3	6.9§§	0.03	0.09
Thiametoxam	0		0		0.07	0.2

* Residues of neonicotinoids in honeybee- collected pollen and nectar and honeybee tissue from control fields and fields sown with clothianidin treated seeds in 2013 and 2014.
** n = 6 for pollen collected by honeybees at control fields, because no such bees with pollen could be found at two fields.
† LOD, limit of detection; LOQ, limit of quantification.

†† Sample weight of 0.091 g explains reported value slightly below the estimated limit of detection, based on a 0.056 g sample weight.
§ One oilseed rape field sprayed with Biscaya (12 June 2013), where thiacloprid is the active ingredient (Supplementary Table 8).
§§ One oilseed rape field sprayed with Biscaya (29 June 2014), where thiacloprid is the active ingredient (Supplementary Table 8).

Supplementary Table 3 | Honeybee colony mortality and re-queening for 2013 and 2014

		2013*				† 2013 - 2014				2014*			
		Clothianidin Treated		Control		Clothianidin Treated		Control		ClothianidinTreated		Control	
		No.	%	No.	%	No.	%**	No.	%**	No.	%	No.	%
MORTALITY	**Autumn decision‡**					**9**	**19**	**7**	**15**				
	re-queened					9	60	7	58				
	1-yr queen‡‡					0	0	0	0				
	2-yr queen‡‡					0	0	0	0				
	Winter loss§					**5**	**13**	**6**	**15**				
	re-queened					3	50	3	60				
	1-yr queen‡‡					2	7	3	9				
	2-yr queen‡‡					0	0	0	0				
	Total loss					**14**	**30**	**13**	**27**				
	swarmed/superseded					12	80	10	83				
	original queen					2	6	3	8				
REQUEENING§§	**Queen-cells**	**16**	**34**	**16**	**33**	**12**	**75**	**10**	**63**	**3**	**13**	**2**	**13**
	1-yr queen‡‡	13	32	13	31	9	69	8	62	n.a.#	n.a.#	n.a.#	n.a.#
	2-yr queen‡‡	3	50	3	50	3	100	2	67	3	13	2	13
	Swarmed	**10**	**21**	**8**	**17**	**8**	**80**	**7**	**88**	**2**	**8**	**1**	**6**
	1-yr queen‡‡	8	20	8	19	6	75	7	88	n.a.#	n.a.#	n.a.#	n.a.#
	2-yr queen‡‡	2	33	0	0	2	100	0	n.a.	2	8	1	6
	Superseded	**5**	**11**	**4**	**8**	**4**	**80**	**3**	**75**	**0**	**0**	**0**	**0**
	1-yr queen‡‡	4	10	1	2	3	75	1	100	n.a.#	n.a.#	n.a.#	n.a.#
	2-yr queen‡‡	1	17	3	50	1	100	2	67	0	0	0	0

* Sample size: n = 8 fields per treatment in 2013, with 6 colonies per field; n = 4 control and n = clothianidin-treated fields in 2014, with 4 colonies per field.

** Mortality percentages are calculated with respect to the total number of available colonies at the point of time in the season for the main categories (**bold**), and with respect to the number of available colonies for each queen age-group for the sub-categories.

‡ Autumn decision (04 September 2013) refers to the number/proportion of 2013 experimental colonies deemed too weak to survive winter by the assessing beekeeper.

‡‡ 1-yr queen, one-year old queen; 2-yr queen, two-year old queen.

§ Winter loss (between 05 September 2013 and April 2014) refers to number/proportion of colonies lost out of those deemed strong enough to overwinter by the assessing beekeeper.

§§ For the 2013 and 2014 re-queening events, the proportions for the main category (**bold**) were calculated as a function of the total available treated or untreated colonies, whereas for the sub-categories it was calculated with respect to the number of available colonies in the specific queen-age subcategory. The 2013-2014 mortality data for this section refers exclusively to those colonies that had re-queened during 2013 that were no longer alive in April 2014, either through winter loss or autumn beekeeper assessment.

Only colonies with one-year old queens throughout 2013 (i.e. two-year old queens during 2014) were used for the 2014 experiment.

Supplementary Table 4 | Pathogen and parasite prevalence in relation to fixed effects (seed treatment, bloom and year)*

Pathogen or Parasite**	**Model type#**	**Effect measure**	**Estimate‡**	**DF**	***χ2***	***P*‡**
Acute bee paralysis virus	2014	Intercept	0.960			
		Seed-treatment	0.104	1	0.10	0.751
		Bloom	-0.375	1	1.85	0.174
		Seed-treatment x Bloom	-0.013	1	<0.01	0.964
Aphid lethal paralysis virus	Full model	Intercept	-3.935			
		Seed-treatment	2.737	1	0.97	0.324
		Bloom	3.396	1	21.36	**<0.001**
		Year	-2.076	1	7.11	**0.008**
		Seed-treatment x Bloom	-2.786	1	2.56	0.109
		Seed-treatment x Year	2.430	1	0.27	0.601
		Bloom x Year	2.727	1	1.24	0.265
		Seed-treatment x Bloom x Year	-2.676	1	2.83	0.093
Chronic bee paralysis virus	2014	Intercept	-0.328			
		Seed-treatment	0.013	1	<0.01	0.989
		Bloom	-0.457	1	2.18	0.140
		Seed-treatment x Bloom	0.351	1	1.76	0.185
Deformed wing virus type-A	Full model	Intercept	-1.135			
		Seed-treatment	-0.387	1	8.32	**0.004**
		Bloom	2.020	1	72.29	**<0.001**
		Year	1.666	1	55.66	**<0.001**
		Seed-treatment x Bloom	-0.188	1	0.56	0.455
		Seed-treatment x Year	0.477	1	1.98	0.159
		Bloom x Year	0.881	1	5.97	**0.015**
		Seed-treatment x Bloom x Year	0.338	1	1.16	0.280
Deformed wing virus type-B	Full model	Intercept	-0.549			
		Seed-treatment	0.010	1	<0.01	0.969
		Bloom	-0.425	1	30.12	**<0.001**
		Year	-0.307	1	4.86	**0.028**
		Seed-treatment x Bloom	-0.183	1	1.11	0.292
		Seed-treatment x Year	0.003	1	<0.01	0.980
		Bloom x Year	1.256	1	57.60	**<0.001**
		Seed-treatment x Bloom x Year	0.012	1	<0.01	0.947
Israeli acute paralysis virus	2014	Intercept	-1.616			
		Seed-treatment	-0.230	1	2.03	0.154
		Bloom	-0.626	1	3.35	0.067
		Seed-treatment x Bloom	0.626	1	3.37	0.066
Kashmir bee virus	2014	Intercept	-0.538			
		Seed-treatment	-0.258	1	1.06	0.304
		Bloom	-0.421	1	2.92	0.087
		Seed-treatment x Bloom	-0.0607	1	<0.01	0.977

Nosema apis	2013	Intercept	-11.959			
		Seed-treatment	-0.130	1	1.53	0.216
		Bloom	-12.080	1	72.61	**<0.001**
		Seed-treatment x Bloom	-0.154	1	<0.01	1.000
Nosema ceranae	Full model	Intercept	0.535			
		Seed-treatment	-0.587	1	13.82	**<0.001**
		Bloom	-1.414	1	90.22	**<0.001**
		Year	0.305	1	6.32	**0.012**
		Seed-treatment x Bloom	-0.129	1	0.28	0.597
		Seed-treatment x Year	0.289	1	1.56	0.211
		Bloom x Year	0.395	1	3.30	0.069
		Seed-treatment x Bloom x Year	-0.157	1	0.60	0.437
Varroa destructor	2013	Intercept	-0.878			
		Seed-treatment	-0.114	1	0.50	0.481
		Bloom	0.600	1	10.38	**0.001**
		Seed-treatment x Bloom	-0.165	1	0.75	0.388

* Pathogen and parasite prevalence in honeybee colonies in relation to clothianidin seed treatment, bloom (before and after oilseed rape bloom) and year (2013 or 2014).

** Species names are spelled in italics.

Full model: n = 14, clothianidin-treated; n = 12, control fields, repeated measurements. 2013: n 0 8 per treatment. 2014: n = 6, clothianidin-treated; n = 4, control.

‡ Main effects/interactions were estimated using sum-to-zero contrasts and the deviation of the second level (After, Clothianidin, 2014) of each factor (Bloom: before/after, Seed-treatment: control/clothianidin, Year: 2013/2014) from to the grand mean (intercept) is presented. P values < 0.005 are highlighted in bold.

Supplementary Table 5 | Pathogen and parasite prevalence in honeybee colonies in relation to clothianidin seed-treatment, bloom (before or after oilseed rape bloom) and year (2013 or 2014)

Pathogen or Parasite*	**Model type**	**Effect measure**	**Estimates§**	**Degrees of freedom**	***F§***	***P*‡**
Gilliamella apicola	Full model	Intercept	4,791			
		Seed-treatment	0,003	1, 17	<0.01	0,955
		Bloom	-0,764	1, 110	176,63	**<0.001**
		Year	-0,344	1, 21	30,81	**<0.001**
		Seed-treatment x Bloom	0,158	1, 110	7,52	**0,007**
		Seed-treatment x Year	0,042	1, 18	0,5	0,488
		Bloom x Year	-0,044	1, 110	0,58	0,448
		Seed-treatment x Bloom x Year	0,191	1, 110	11,07	**0,001**
	2013	Intercept	5,135			
		Seed-treatment	-0,044	1, 7	0,37	0,564
		Bloom	-0,716	1, 75	150,38	**<0.001**
		Seed-treatment x Bloom	-0,028	1, 75	0,33	0,569
	2014	Intercept	4,447			
		Seed-treatment	0,037	1, 4	0,11	0,755
		Bloom	-0,808	1, 35	61,85	**<0.001**
		Seed-treatment x Bloom	0,349	1, 35	11,54	**0,002**
Snodgrassella alvi	Full model	Intercept	7,067			
		Seed-treatment	0,003	1, 17	<0.01	0,95
		Bloom	-0,035	1, 110	0,61	0,437
		Year	-0,078	1, 21	2,53	0,126
		Seed-treatment x Bloom	-0,009	1, 110	0,04	0,834
		Seed-treatment x Year	-0,032	1, 18	0,48	0,498
		Bloom x Year	0,097	1, 110	4,69	**0,033**
		Seed-treatment x Bloom x Year	0,009	1, 110	0,04	0,844
Acute bee paralysis virus	2014	Intercept	5,102			
		Seed-treatment	-0,324	1, 4	0,78	0,426
		Bloom	-0,579	1, 30	2,94	0,097
		Seed-treatment x Bloom	-0,078	1, 30	0,05	0,819
Aphid lethal paralysis virus	Full model	Intercept	3,447			
		Seed-treatment	0,164	1,7	0,14	0,615
		Bloom	2,214	1, 40	65,88	**<0.001**
		Year	0,333	1, 9	0,77	0,333
		Seed-treatment x Bloom	-0,567	1, 40	3,8	**0,039**
		Seed-treatment x Year	-0,023	1, 7	0,01	0,941

		Bloom x Year	0,453	1, 40	3,49	0,096
		Seed-treatment x Bloom x Year	-0,406	1, 40	2,87	0,135
Black queen cell virus	Full model	Intercept	7,799			
		Seed-treatment	-0,196	1, 18	5,25	**0,034**
			-0,48	1, 110	35,71	**<0.001**
		Year	0,103	1, 22	1,36	0,225
		Seed-treatment x Bloom	-0,261	1, 110	10,56	**0,002**
		Seed-treatment x Year	-0,142	1, 19	2,73	0,116
		Bloom x Year	0,074	1, 110	0,84	0,361
		Seed-treatment x Bloom x Year	-0,305	1, 110	14,36	**<0.001**
	2013	Intercept	7,695			
		Seed-treatment	-0,058	1, 7	0,57	0,474
		Bloom	-0,554	1, 75	70,79	**<0.001**
		Seed-treatment x Bloom	0,043	1, 75	0,44	0,511
	2014	Intercept	7,888			
		Seed-treatment	-0,326	1, 4	2,58	0,185
		Bloom	-0,407	1, 35	4,76	**0,035**
		Seed-treatment x Bloom	-0,566	1, 35	9,21	**0,005**
Chronic bee paralysis virus	2014	Intercept	4,407			
		Seed-treatment	0,089	1, 4	0,03	0,88
		Bloom	-0,898	1, 22	3,91	0,061
		Seed-treatment x Bloom	-0,691	1, 22	2,31	0,143
Deformed wing virus type-A	Full model	Intercept	4,661			
		Seed-treatment	-0,163	1, 13	0,3	0,592
		Bloom	3,34	1, 53	149,79	**<0.001**
		Year	0,774	1, 15	6,39	**0,023**
		Seed-treatment x Bloom	-0,435	1, 53	2,54	0,117
		Seed-treatment x Year	0,15	1, 15	0,23	0,637
		Bloom x Year	0,87	1, 53	10,17	**0,002**
		Seed-treatment x Bloom x Year	-0,395	1, 53	2,09	0,154
Deformed wing virus type-B	Full model	Intercept	3,912			
		Seed-treatment	-0,069	1, 23	0,1	0,759
		Bloom	-0,251	1, 83	1,34	0,251
		Year	0,285	1, 25	1,57	0,221
		Seed-treatment x Bloom	-0,23	1, 83	1,13	0,292
		Seed-treatment x Year	0,047	1, 22	0,05	0,833
		Bloom x Year	2,33	1, 83	115,5	**<0.001**
		Seed-treatment x Bloom x Year	-0,113	1, 83	0,27	0,604

Israeli acute paralysis virus	2014	Intercept	2,827			
		Seed-treatment	0,086	1, 2	0,02	0,897
		Bloom	-1,029	1, 12	4,42	0,057
		Seed-treatment x Bloom	1,02	1, 12	4,34	0,059
Kashmir bee virus	2014	Intercept	3,084			
		Seed-treatment	0,12	1, 3	0,08	0,798
		Bloom	-0,777	1, 22	3,8	0,064
		Seed-treatment x Bloom	-0,127	1, 22	0,1	0,753
Lake Sinai virus type-1	Full model	Intercept	9,651			
		Seed-treatment	0,048	1, 18	0,09	0,771
		Bloom	-0,459	1, 110	8,12	**0,005**
		Year	0,841	1, 23	24,73	**<0.001**
		Seed-treatment x Bloom	-0,013	1, 110	0,01	0,935
		Seed-treatment x Year	0,064	1, 19	0,15	0,7
		Bloom x Year	-0,81	1, 110	25,23	**<0.001**
		Seed-treatment x Bloom x Year	-0,046	1, 110	0,08	0,775
Slow bee paralysis virus	2014	Intercept	7,032			
		Seed-treatment	-0,115	1, 4	0,51	0,512
		Bloom	0,425	1, 35	9,36	**0,004**
		Seed-treatment x Bloom	0,08	1, 35	0,33	0,568
Sacbrood virus	Full model	Intercept	9,534			
		Seed-treatment	-0,044	1, 17	0,03	0,867
		Bloom	-0,221	1, 110	1,48	0,226
		Year	-0,239	1, 21	0,8	0,38
		Seed-treatment x Bloom	0,051	1, 110	0,08	0,78
		Seed-treatment x Year	-0,106	1, 18	0,17	0,687
		Bloom x Year	-0,338	1, 110	3,46	0,065
		Seed-treatment x Bloom x Year	-0,122	1, 110	0,45	0,504
Nosema apis	2013	Intercept	2,894			
		Seed-treatment	-0,016	1, 5	0,06	0,82
		Bloom	-2,894	1, 39	2248,31	**<0.001**
		Seed-treatment x Bloom	0,016	1, 39	0,07	0,79
Nosema ceranae	Full model	Intercept	3,257			
		Seed-treatment	-0,202	1, 18	1,74	0,203
		Bloom	-1,931	1, 96	242,15	**<0.001**
		Year	0,048	1, 21	0,09	0,763
		Seed-treatment x Bloom	-0,193	1, 96	2,42	0,123
		Seed-treatment x Year	-0,045	1, 18	0,09	0,773
		Bloom x Year	0,479	1, 96	14,9	**<0.001**
		Seed-treatment x Bloom x Year	0,023	1, 96	0,04	0,851

Varroa destructor‡‡	Full model	Intercept	1,077			
		Seed-treatment	0,044	1, 14	0,67	0,426
		Bloom	0,381	1, 74	72,44	**<0.001**
		Year	0,307	1, 17	31,05	**<0.001**
		Seed-treatment x Bloom	-0,069	1, 74	2,4	0,126
		Seed-treatment x Year	-0,068	1, 14	1,62	0,224
		Bloom x Year	0,137	1, 74	9,39	**0,003**
		Seed-treatment x Bloom x Year	-0,032	1, 74	0,52	0,472

*Species names are spelled in italics.

§Estimates and test statistics of generalized linear mixed effects models on pathogen and parasite abundance in honeybee colonies in relation to clothianidin seed-treatment and bloom (before or after placement at flowering oilseed rape fields) in 2013 and 2014. Main effects/interactions were estimated using sum-to-zero contrasts and the deviation of the second level (after, clothianidin, 2014) of each factor (Bloom: before/after, Seed-treatment: control/clothianidin, Year: 2013/2014) from to the grand mean (intercept) is presented.

‡ P values < 0.05 are highlighted in bold.

‡‡The abundance of *V. destructor* was modelled as the square-root transformed number of mites 100 bees^{-1}, while the microorganisms were expressed as $\log_{10}$ [units] bee^{-1} [70].

Supplementary Table 6 | mRNA transcript levels of various immune defense genes in relation to clothianidin seed seed-treatment and boom (before or after oilseed rape bloom) at apiary level in 2013.

Gene expression*	Effect measure		Estimates**	Degrees of freedom	*F*	*P*‡
Amel\LRR	Intercept		6.649			
	Seed-treatment		0.013	1, 14	0.14	0.716
	Bloom		-0.024	1, 14	0.56	0.466
	Seed-treatment	x	0.039	1, 14	1.48	0.243
	Bloom					
Apidaecin	Intercept		9.802			
	Seed-treatment		-0.015	1, 14	0.10	0.758
	Bloom		0.120	1, 14	6.51	**0.023**
	Seed-treatment	x	0.065	1, 14	1.93	0.187
	Bloom					
cSP33	Intercept		8.133			
	Seed-treatment		-0.029	1, 14	0.26	0.617
	Bloom		0.092	1, 14	2.75	0.119
	Seed-treatment	x	0.073	1, 14	1.71	0.211
	Bloom					
Dorsal-1A	Intercept		7.406			
	Seed-treatment		-0.010	1, 14	0.05	0.823
	Bloom		0.013	1, 14	0.10	0.755
	Seed-treatment	x	0.032	1, 14	0.56	0.468
	Bloom					
Eater-like	Intercept		6.022			
	Seed-treatment		-0.039	1, 14	0.88	0.365
	Bloom		0.010	1, 14	0.06	0.813
	Seed-treatment	x	0.013	1, 14	0.10	0.754
	Bloom					
NimcC2	Intercept		8.078			
	Seed-treatment		-0.037	1, 14	0.46	0.510
	Bloom		0.073	1, 14	1.80	0.201
	Seed-treatment	x	0.073	1, 14	1.80	0.200
	Bloom					
PGRP-S2	Intercept		8.306			
	Seed-treatment		0.001	1, 14	0.00	0.987
	Bloom		-0.022	1, 14	0.23	0.640
	Seed-treatment	x	0.019	1, 14	0.18	0.682
	Bloom					
SPH51	Intercept		7.880			
	Seed-treatment		-0.008	1, 14	0.03	0.876
	Bloom		0.133	1, 14	8.02	**0.013**
	Seed-treatment	x	0.005	1, 14	1.18	0.296
	Bloom					

* Species names are spelled in italics.
** Main effects/interactions were estimated using sum-to-zero contrasts and the deviation of the second level (After, Clothianidin) of each factor (Bloom: before/after, Seed-treatment: control/clothianidin) from to the grand mean (intercept) is presented. N = 8 fields per treatment.

‡ P values < 0.05 are highlighted in bold.

Supplementary Table 7 | Average monthly temperature (ºC), days with frost and precipitation (mm) at three weather stations (Helsingborg, Halmstad, Kristianstad) in the study region (see Fig. 1: Map) during April-June in 2013 and 2014[1]

		Helsingborg		Halmstad		Kristianstad	
		2013	**2014**	**2013**	**2014**	**2013**	**2014**
Temperature	April	6.0	9.1	5.2	9.0	5.4	8.1
	May	13.2	12.5	13.5	12.4	12.8	12.0
	June	15.4	15.4	15.2	15.3	15.7	15.0
Days with frost	April	11	1	13	5	13	6
	May	3	0	2	4	4	3
	June	0	1	0	1	0	0
Precipitation	April	25	42	29	50	24	23
	May	65	95	49	78	35	89
	June	76	51	111	64	73	70

Supplementary Table 8 | Field size and land use in the landscapes (radius = 2 km) surrounding the focal oilseed rape fields for 2014

	Untreated (n = 4)		Clothianidin treated (n = 6)		Test of difference between treatments		Correlation matrix (Pearson correlation coefficient, r)									
	mean ± s.e.m.	min-max	mean ± s.e.m.	min-max	F_{df}	P	Agricultural land	Annually tilled arable land	Semi-natural grassland	Length of permanent field borders	Maize cultivation	Spring sown oilseed rape	Winter sown oilseed rape	Mass-flowering crops*	Forest	Urban
Size of focal oilseed rape field (ha)	10.0 ± 3.4	5.0-20.0	8.7 ± 1.7	4.0-14.0	$0.15_{1,8}$	0.71	0.610	0.740	-0.583	0.477	0.314	0.882	0.398	0.666	-0.562	-0.330
Agricultural land (%)	59.1 ± 11.2	27.2-76.9	52.8 ± 5.8	11.5-86.8	$0.29_{1,8}$	0.73		0.896	-0.062	0.922	0.692	0.702	0.483	0.693	-0.992	-0.313
Annually tilled arable land (%)	26.5 ± 7.4	9.4-45.0	33.2 ± 12.5	2.7-65.7	$0.24_{1,8}$	0.64			-0.441	0.684	0.589	0.856	0.553	0.851	-0.847	-0.376
Semi-natural grassland (%)	5.4 ± 1.4	1.9-8.5	2.9 ± 0.9	1.0-6.6	$2.35_{1,8}$	0.16				0.225	0.159	-0.522	-0.084	-0.371	-0.021	0.265
Length of permanent field borders (km)	15.2 ± 2.0	1.0-18.5	12.3 ± 1.9	4.4-16.4	$1.04_{1,8}$	0.34					0.625	0.500	0.269	0.489	-0.950	-0.244
Maize cultivation (%)	2.0 ± 0.8	0.8-4.1	1.5 ± 0.6	0-3.8	$0.24_{1,8}$	0.64						0.539	0.544	0.512	-0.730	0.316
Spring sown oilseed rape (%) – including the focal field	0.9 ± 0.3	0.4-1.9	0.9 ± 0.3	0.2-2.1	$0.01_{1,8}$	0.92							0.678	0.782	-0.648	-0.376
Winter sown oilseed rape (%)	1.2 ± 0.2	0.6-1.7	0.9 ± 0.8	0-4.8	$0.09_{1,8}$	0.78								0.659	-0.426	-0.264
Mass-floweing crops* (%)	6.5 ± 0.5	5.4-7.8	6.2 ± 2.2	0.2-13.6	$0.01_{1,8}$	0.93									-0.641	-0.234
Forest (%)	22.5 ± 11.9	4.4-56.2	31.6 ± 11.4	2.8-69.0	$0.28_{1,8}$	0.61										0.221
Urban (%)	2.0 ± 1.4	0-5.9	1.0 ± 0.8	0-4.9	$0.45_{1,8}$	0.52										

*Mass-flowering crops include potato (35%), oilseed rape (31%), pea (22%), fruit and berry cultivation (85%), bean (4%), herbs and seeds (<1%).

Supplementary Table 9 | Insecticide spray treatments in the oilseed rape fields during the 2013 and 2014 growing seasons

		Spray treatment 1			Spray treatment 2			Spray treatment 3				
Pair	**Seed treatment ***	**Date**	**Product**	**Dose**	**Date**	**Product**	**Dose**	**Date**	**Product**	**Dose**	**Swarm /Supers §**	**Winter loss‡**
		2013										
P01	untr	04 June	Mavrik	0.25 l ha^{-1}							3	3
P01	treat	06 June	Plenum	150 g ha^{-1}	15 June	Steward	85 g ha^{-1}				3	3
P02	untr	31 May	Plenum	160 g ha^{-1}	10 June	Mavrik	0.20 l ha^{-1}				0	1
P02	treat	04 June	Plenum	150 g ha^{-1}	10 June	Steward	85 g ha^{-1}				0	0
P03	untr	No treatment									3	2
P03	treat	12 June	Avaunt	170 g ha^{-1}							0	0
P04	untr	16 June	Avaunt	160 g ha^{-1}							2	3
P04	treat	07 June	Plenum	150 g ha^{-1}							0	0
P05	untr	12 June	Plenum	150 g ha^{-1}							0	0
P05	treat	30 May	Plenum	150 g ha^{-1}							4	3
P06	untr	12 June	Biscaya	0.30 l ha^{-1}	19 June	Mavrik	0.25 l ha^{-1}				1	2
P06	treat	07 June	Avaunt	170 g ha^{-1}							5	5
P07	untr	04 June	Avaunt	170 g ha^{-1}	08 June	Plenum	150 g ha^{-1}				3	2
P07	treat	31 May	Plenum	150 g ha^{-1}							3	2
P08	untr	30 May	Avaunt	170 g ha^{-1}							0	0
P08	treat	04 June	Plenum	150 g ha^{-1}	14 June	Avaunt	120 g ha^{-1}				0	1
		2014										
P01	treat	15 June	Mavrik	0.25 l ha^{-1}							1	x
P01	untr	31 May	Mavrik	0.30 l ha^{-1}	6 June	Avaunt	0,20 l ha^{-1}				1	x
P02	treat	29 June	Biscaya	0.30 l ha^{-1}							0	x
P04	untr	28 May	Plenum	150 g ha^{-1}	9 June	Mavrik	0.20 l ha^{-1}				0	x
P04	treat	14 June	Avaunt	170 g ha^{-1}							0	x
P05	untr	24 May	Mavrik	0.25 l ha^{-1}	2 June	Plenum	150 g ha^{-1}	10 June	Mavrik	0.25 l ha^{-1}	0	x
P05	treat	5 June	Plenum	150 g ha^{-1}	8 June	Mavrik	0.20 l ha^{-1}				0	x
P06	treat	10 June	Avaunt	170 g ha^{-1}	18 June	Mavrik	0.20 l ha^{-1}				0	x
P07	untr	24 May	Plenum	150 g ha^{-1}	31 May	Avaunt	0.20 l ha^{-1}	9 June	Plenum	150 g ha^{-1}	0	x
P07	treat	30 May	Avaunt	170 g ha^{-1}	8 June	Plenum	150 g ha^{-1}	17 June	Mavrik	0.25 l ha^{-1}	0	x

*untr, untreated; treat, clothianidin treated.
§swarm, swarmed; supers, superseded. Refers to the number of colonies per field that swarmed or superseded during the experiment.
‡Winter loss (between 05 September 2013 and April 2014) refers to number of colonies lost per field out of those deemed strong enough to overwinter by the assessing beekeeper.

Supplementary Table 10 | Observed changes in significance level and test statistics when excluding fields spray-treated with Biscaya from the analyses

Model*	**Model type****	**Effect measure**	**Biscaya field**	**Estimate#**	**Degrees of freedom**	***χ2* or *F* value**	***P*§**
Colony development							
Number of adult bees	Full model	Seed-treatment x Bloom	Included	206	1, 110	*F*=4.22	**0.042**
			Excluded	148	1, 101	*F*=2.12	0.149
Honey production	Full model	Year	Included	-1.378	1, 18	*F*=4.47	**0.049**
			Excluded	-1.408	1, 16	*F*=3.68	0.073
Pathogen prevalence							
Nosema ceranae	Full model	Bloom x Year	Included	0.395	1	$\chi 2$=3.30	0.069
			Excluded	0.486	1	$\chi 2$=4.48	**0.034**
Pathogen abundance							
Aphid lethal paralysis virus	Full model	Seed-treatment x Bloom	Included	-0.567	1, 40	*F*=3.80	**0.039**
			Excluded	-0.434	1, 39	*F*=2.72	0.107
		Bloom x Year	Included	0.453	1, 40	*F*=3.49	0.096
			Excluded	0.983	1, 39	*F*=4.96	**0.032**
Chronic bee paralysis virus	2014	Seed-treatment x Bloom	Included	-0.691	1, 22	*F*=2.31	0.143
			Excluded	-0.983	1, 20	*F*=4.32	**0.050**

* Species names are spelled in italics.

** Full model: n = 14, clothianidin-treated; n =12, control fields; repeated measurements. 2014: n = 6, clothianidin-treated; n = 4 control fields.

Main effects/interactions were estimated using sum-to-zero contrasts and the deviation of the second level (after, clothianidin) of each factor (Bloom: before/after, Seed-treatment: control/clothianidin) from to the grand mean (intercept) is presented.

§ P values < 0.05 are highlighted in bold.

Supplementary Table 11 | Virus primers. Details of the qPCR assays (RNA) for the pathogenic viruses screened in this study.

Pathogen/Parasite*	Abbreviation	Primers	Sequence '5-'3	Reference
Acute bee paralysis virus	ABPV	ABPV-F6548 KIABPV-B6707	TCATACCTGCCGATCAAG CTGAATAATACTGTGCGTATC	[2]
Aphid lethal paralysis virus	ALPV	qALP-R6046 qALP-F5854	GCAGCACCGGAAACGTTTTTATGG ACACCATAGTTCGCGAAGAACGCA	[2]
Black queen cell virus	BQCV	BQCV-qF7893 BQCV-qB8150	AGTGGCGGAGATGTATGC GGAGGTGAAGTGGCTATATC	[2]
Big Sioux River virus	BSRV	qBSRV-R6134 qBSRV-F5853	CCCGCGATATAATTGCGTTTGTGAGC GCGCCTATTTTCTGCAGCGCC	[3]
Chronic bee paralysis virus	CBPV	CBPV1-qF1818 CBPV1-qB2077	CAACCTGCCTCAACACAG AATCTGGCAAGGTTGACTGG	[2]
Deformed wing virus type-A	DWV-A	DWV-F8668 DWV-B8757	TTCATTAAAGCCACCTGGAACATC TTTCCTCATTAACTGTGTCGTTGA	[4]
Deformed wing virus type-B	DWV-B	VaDV-F1409 DWV-B1806	GCCCTGTTCAAGAACATG CTTTTCTAATTCAACTTCACC	[2]
Israeli acute paralysis virus	IAPV	IAPV-F6627 KIABPV-B6707	CCATGCCTGGCGATTCAC CTGAATAATACTGTGCGTATC	[2]
Kashmir bee virus	KBV	KBV-F6639 KIABPV-B6707	CCATACCTGCTGATAACC CTGAATAATACTGTGCGTATC	[2]
Lake Sinai virus strains 1	LSV-1	qLSV1-R2743 qLSV1-F2569	GGGACGCAGCACGATGCTCA AGAGGTTGCACGGCAGCATG	[3]
Lake Sinai virus strains 2	LSV-2	qLSV2-R1947 qLSV2-F1722	GCGGTGTCGATCTCGCGGAC CGTGCTGAGGCCACGGTTGT	[3]
Slow bee paralysis virus	SBPV	SPV-F3177 SPV-B3363	GCGCTTTAGTTCAATTGCC ATTATAGGACGTGAAAATATAC	[5]
Sac brood virus	SBV	SBV-qF3164 SBV-qB3461	TTGGAACTACGCATTCTCTG GCTCTAACCTCGCATCAAC	[2]

* Species names are spelled in italics.

Supplementary Table 12 | Parasite and microbe primers. Details of the qPCR assays (DNA) for the pathogenic and non-pathogenic organisms screened in this study.

Pathogen/Parasite	Abbreviation	Primers	Sequence '5-'3	Reference
Gilliamella apicola	*Gilliamella*	Gilliam-16S-F	GTAACATGAGTGCTTGCACT	**This study**
		Gilliam-16S-R	CGCATGGCCCGAAGG	
Snodgrassella alvi	*Snodgrassella*	Snodgras-16S-F	ACGGAGAGCTTGCTCTC	**This study**
		Snodgras-16S-R	AAATAACGCGAGGTCTTTCGA	
Nosema apis	*N. apis*	forward	CTAGTATATTTGAATATTGTTTACAATGG	[6]
		reverse	GTCGCTATGATCGCTTGCC	
Nosema ceranae	*N. ceranae*	forward	TATTGTAGAGAGGTGGGAGATT	[6]
		reverse	GTCGCTATGATCGCTTGCC	

Supplementary Table 13 | Primers of the RT-qPCR assays for the immune defense and internal reference genes screened for the study

Immune defense gene	Abbreviation	DNA/RNA analysis	Primers	Sequence '5-'3	Reference
	Amel\LRR	RNA	forward	TAGTGAAATCTAGACCTC	This study
			reverse	ATGCAAAGAGCTATCATCA	
	Apidaecin	RNA	forward	TTTTGCCTTAGCAATTCTTGTTG	[7]
			reverse	GAAGGTCGAGTAGGCGGATCT	
	cSP33	RNA	forward	CGTCGGTGGTAAAGCGGCGA	[8]
			reverse	AACGGCGACCAACGTTGCCA	
	dorsal-1A	RNA	forward	TCGGATGGTGCTACGAGCGA	[8]
			reverse	AGCATGCTTCTCAGCTTCTGCCT	
	Eater-like	RNA	forward	GGCGAGTGCACCGGCTTGAA	[8]
			reverse	GCGCCATCGCGTCATAGCCA	
	NimC2	RNA	forward	GCGTGGAGGACGGGAAACCG	[8]
			reverse	ACATCGATGGCAGAGCGGCG	
	PGRP-S2	RNA	forward	GGCCACACACCAAATGCAGCAG	[8]
			reverse	CGAGGACCAGTGTGGCCATGT	
	SPH51	RNA	forward	TGGCAATTGTCTTTGCGGGCG	[8]
			reverse	TACTTCCGCCGCCGTTACGC	

Supplementary Table 14 | Observed changes in significance level and test statistics when including colonies that lost their queen or swarmed

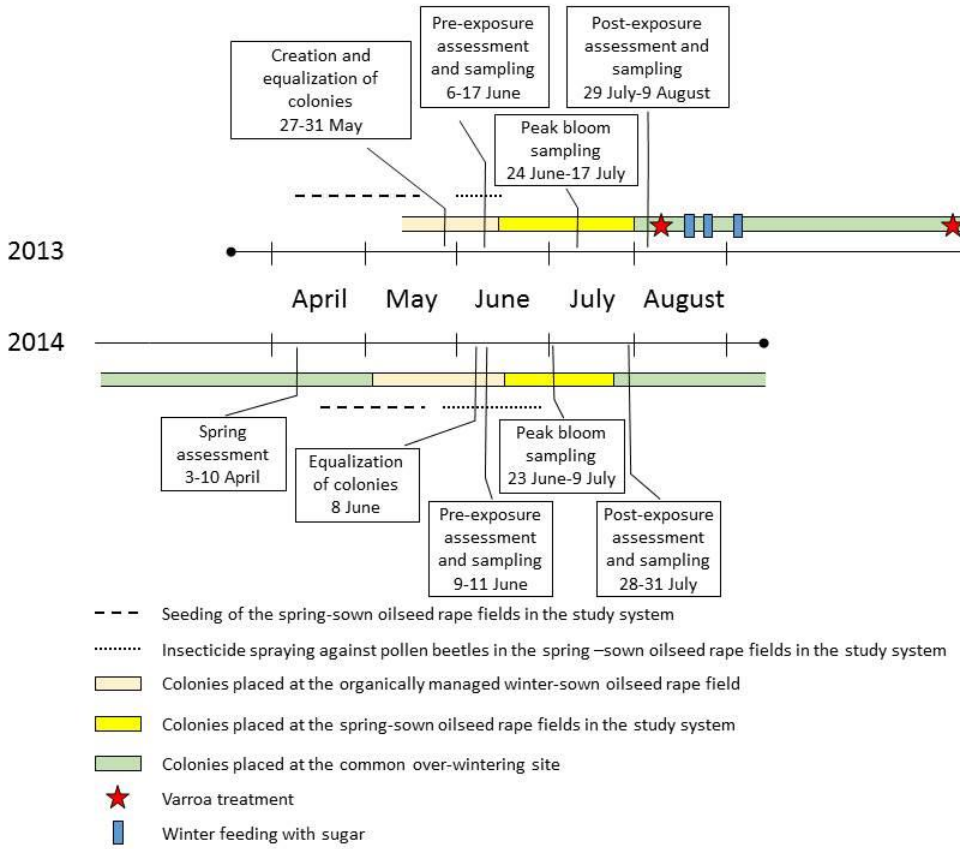
Model*	Model type**	Effect measure	Swarmed/Queen loss	Estimate#	Degrees of freedom	χ^2 or F value	P§
Colony development							
Number of capped brood cells	Full model	Seed-treatment x Bloom x Year	Excluded	276	1, 110	F=6.26	**0.014**
			Included	206	1, 131	F=3.07	0.084
	2014	Seed-treatment x Bloom	Excluded	448	1, 35	F=5.02	**0.032**
			Included	410	1, 38	F=3.59	0.066
Number of adult bees	Full model	Seed-treatment x Bloom	Excluded	206	1, 110	F=4.22	**0.042**
			Included	130	1, 131	F=1.69	0.195
Pathogen prevalence							
Nosema ceranae	Full model	Bloom x Year	Excluded	0.395	1	χ^2=3.30	0.069
			Included	0.513	1	χ^2=6.11	**0.013**
Pathogen abundance							
Black queen cell virus	Full model	Seed-treatment	Excluded	-0.196	1, 18	F=5.25	**0.034**
			Included	-0.172	1, 20	F=3.66	0.070
Chronic bee paralysis virus	2014	Bloom	Excluded	-0.898	1, 22	F=3.91	0.061
			Included	-1.013	1, 25	F=5.79	**0.024**
Kashmir bee virus	2014	Bloom	Excluded	-0.777	1, 22	F=3.80	0.064
			Included	-0.838	1, 23	F=4.72	**0.040**
Snodgrassella alvi	Full model	Bloom x Year	Excluded	0.097	1, 110	F=4,69	**0.033**
			Included	0.086	1, 131	F=3.03	0.084
Varroa destructor	Full model	Seed-treatment x Bloom	Excluded	-0.069	1, 74	F=2.40	0.126
			Included	-0.101	1, 85	F=5.51	**0.021**

* Species names are spelled in italics

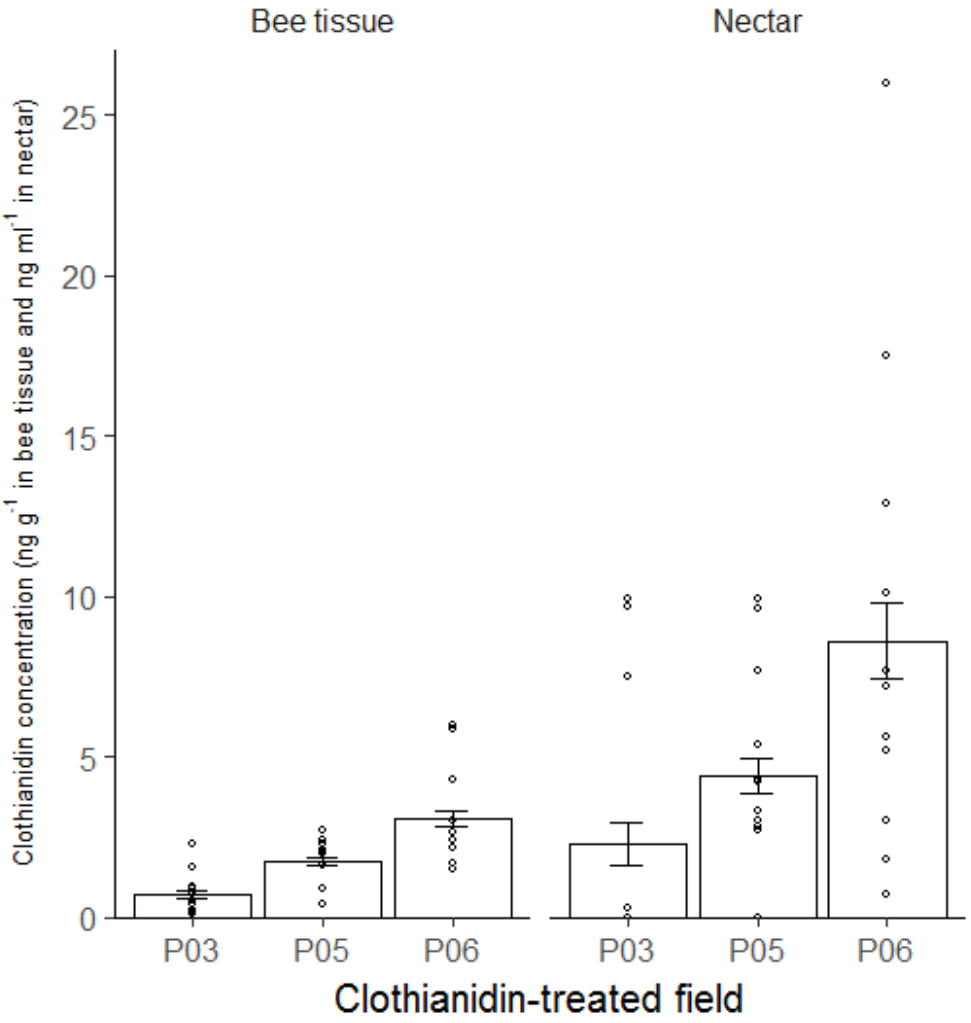
** Full model: n = 14, clothianidin-treated; n =12, control fields; repeated measurements. 2014: n = 6, clothianidin-treated; n = 4 control fields.

Main effects/interactions were estimated using sum-to-zero contrasts and the deviation of the second level (after, clothianidin) of each factor (Bloom: before/after, Seed-treatment: control/clothianidin) from to the grand mean (intercept) is presented.

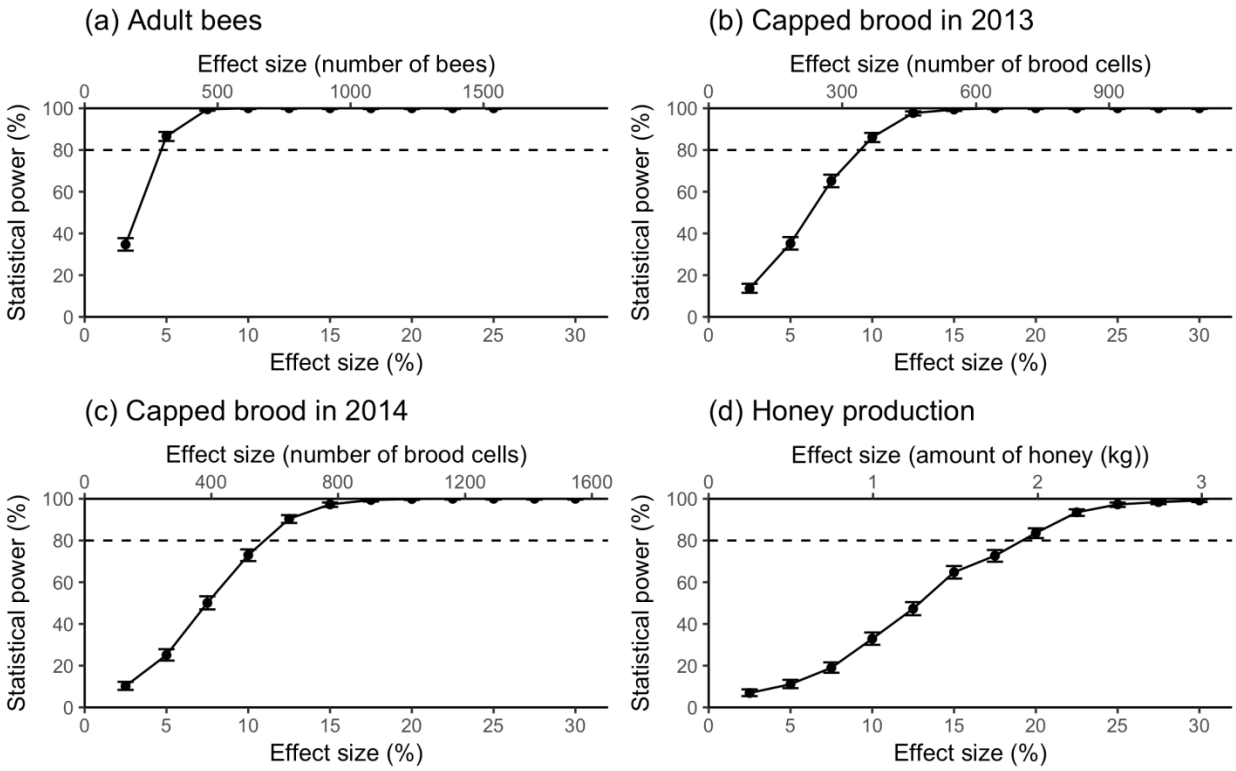
§ P values < 0.05 are highlighted in bold.



Supplementary Figure 1 | Experimental timeline. Overview of events within the study in both years (2013 and 2014), including honeybee colony creation and equalization, movement, treatment against *Varroa* mite and winter feeding of colonies with sugar, as well as seeding and insecticide spraying of the focal spring-sown oilseed rape fields. Information on dates of creation and equalization of the colonies and assessments are noted. Pre- and post-exposure assessments include measurements of colony strength (number of brood cells and adult bees per colony), hive weight to measure honey production and sampling of 100 bees for microbial assessments. Peak bloom sampling included collection of honeybees foraging in the oilseed rape fields, honeybees from the colonies and pollen from pollen traps on the hive entrance for clothianidin residue analysis and determination of pollen plant species origin.



Supplementary Figure 2 | Clothianidin residues in individual bees. Mean clothianidin concentration (± 95 confidence limits) in honeybee tissue and nectar from their honey stomach in bees collected from hives adjacent to three 2013 oilseed rape fields (clothianidin-treated fields from P03, P05 and P05 pairs). Circles represent individual bees (N = 12 per field).



Supplementary Figure 3 | Power curves for honeybee colony strength and honey production. Relationship between statistical power and effect size estimated for interactive effects between clothianidin seed-treatment, bloom (before or after the oilseed rape bloom) and year (2013 and 2014) for the number of adult honeybees (full model) and the interactive effect between seed-treatment and bloom for the number of capped brood cell (separate models for the years). The effect size is expressed as the difference between treatments in the change in honeybee colony strength during the oilseed rape bloom in absolute terms and as a percentage of the mean number of adult honeybees or the number of capped brood cells in the control group before the oilseed rape bloom. For honey production, the relationship between statistical power and effect size estimated for amount of honey in kg in relation to seed-treatment is presented. The dashed horizontal line indicates a power of 80%.

Supplementary Methods

In order to determine neonicotinoid residues, pollen and nectar as well as honeybee tissue was analysed, pooled for each field (Supplementary Table 2). From each bee sample collected from the hive entrances during the peak bloom sampling (Supplementary Fig. 1), a subsample of 24 bees was weighed and homogenized with drying agent using a glass rod. From this homogenate a fraction corresponding to four bees was analysed. After addition of internal standard (IS) solution the homogenate was extracted twice using a 70:30 mixture of acetone and ethyl acetate (6 ml followed by 3 ml) under strong sonication (Vibra-Cell VCX 130, Sonics, USA). Extracts were further cleaned by dispersive solid phase extraction (SPE, using C18 and PSA) and evaporated to dryness at 40 °C under nitrogen gas flow. The extract residue was dissolved in 150 µl acetonitrile and analysed using liquid chromatography tandem mass spectrometry (LC-MS/MS) with positive electrospray ionization.

Pollen sample weights ranged between 0.025 and 0.104 g with an average of 0.057 g. Since the sample amounts were very small the entire sample was used for analysis. The extraction and clean-up steps were as for the pooled bee samples but in a downscaled format.

Honey stomachs were dissected in the field, pooled within each field and stored frozen pending analysis. Nectar samples were handled according to the Capillary Microsampling technique[9,10]. At the analytical laboratory nectar was transferred from the dissected honey stomachs, pooled, and then collected in glass capillaries with an exact volume of 16 µl. For three of the samples the nectar volume available was below 16 µl and an 8 µl capillary was used instead and a dilution factor applied. The capillaries were placed in 1 ml polypropylene tubes to which 32 µl IS solution in acetonitrile, and an extra 32 µl acetonitrile, were added. The total dilution of the nectar sample was thus five times. After vigorously mixing of the tube followed by centrifugation, a volume of approximately 50-60 µl was transferred to an LC injection vial. Calibration samples at seven concentration levels (N = 2) and quality control samples at two levels (N = 3) were prepared in the same way by adding blank nectar (collected by bees and free from neonicotinoids) with a

16 µl capillary, 32 µl IS solution and finally a suitable amount of neonicotinoids via the extra 32 µl acetonitrile volume, i.e. a matrix match procedure.
Furthermore, to identify possible variation in neonicotinoid exposure of honeybees in different sites, we collected 12 honeybees per site from the entrance from three clothianidin-treated fields. Nectar was extracted from these honeybees and each bee and the nectar was analysed individually thereafter. Pesticide concentrations in bee tissue from single bees were determined after first removing the nectar from the honey stomach. The bee tissue was treated and extracted in the same way as for the pooled bee samples, but in a downscaled format and without the dispersive SPE clean-up step.

In the analytical laboratory, frozen bees were thawed and the abdomen containing the honey stomach was separated from the thorax with a scalpel. Nectar was collected after gently applying pressure with two fingers to the sides of the abdomen. Nectar coming out of the esophagus was collected in an 8 µl glass capillary and treated in the same way as described for the pooled nectar samples, but with an IS-volume of 40 µl, giving a total dilution of ten times. After filling the analytical capillary any surplus nectar from the honey stomach was gently pressed out and the remaining bee tissue was transferred to a sample tube for determination of neonicotinoid concentrations.

The LC-MS/MS (ESI+) method used for determination of the five neonicotinoids acetamiprid, imidacloprid, clothianidin, thiacloprid and thiamethoxam in extracts from bees, pollen and nectar samples was a modification of an accredited multi residue method for pesticides in water[11]. The main modifications was that 10 µl of acetonitrile extract was injected instead of 500 µl water, and that the stable isotope labelled internal standards clothianidin-D_3 (Teknolab AB, Kungsbacka, Sweden) and imidacloprid-D_4 (Dr Ehrenstorfer, Augsburg, Germany) were used. For the quantification of acetamiprid, thiacloprid and thiamethoxam D6-isoproturone was used as IS. Stock solutions of the investigated pesticides were certified by the manufacturers and dilutions from these stock solutions were made according to current standard operating procedures.

For the quantification of neonicotinoids in bee and pollen samples calibration curves in acetonitrile (seven concentration levels, duplicate injections; before and after samples) were used together with spiking experiments in blank bee and pollen matrix (two levels, N = 3). A factor for each compound and matrix, based on the measured and nominal concentrations of the spiked samples together with the sample weight, was used to calculate the concentrations in the study samples. The precision (RSD) in spiked bee samples was 2.4 – 19 % and in spiked pollen samples 0.9 – 18 %, for both concentration levels and all five neonicotinoids.
To confirm that the homogenates from the 24 bees were uniform, extra analyses were performed on subsamples (N = 4) from bee samples from two treated fields. The results showed a RSD of 4.6 and 8.5 %, respectively, for clothianidin.

For the nectar samples the concentrations were achieved directly from the calibration curve since study samples and calibration samples were both in nectar matrix and treated in exactly the same way. The quality control samples showed accuracies of 89-101 % and precisions (RSD) of 1.1 - 8.2 % for both concentration levels and all five neonicotinoids.
In all analytical runs matrix blanks and solvent blanks were analysed to confirm selectivity, and to check for carry-over between samples.

In a study separate from the field studies, the stability of neonicotinoids, as well as several other pesticides was investigated in frozen bees (-20 °C), living bees (for 1 h) and dead bees stored in room temperature (22 h), after individual feeding of bees with a sugar solution containing a pesticide mix[12]. All five neonicotinoids were stable (defined as degradation less than 20 %) in the freezer for up to 21 months and at room temperature for 22 hours. In living bees clothianidin was stable for 1 h while acetamiprid, imidacloprid, thiacloprid and thiamethoxam showed degradation with 44, 44, 43 and 22 %, respectively. Each estimate was based on triplicate samples and four bees per sample.

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Chapter III: Field-level clothianidin exposure affects bumblebees but generally not their pathogens

(Manuscript published in *Nature Communications*)

Preface and summary of Chapter III

In Chapter II, we showed that honeybee colonies are only mildly affected by field-level clothianidin exposure both in terms of colony development and pathogen susceptibility. Here, we examine the bumblebee colonies of the field study by (Rundlöf et al., 2015) in detail to determine potential discrepancies between free-foraging bumblebees and honeybees in the sensitivity to clothianidin exposure at colony level. We analyse the size of individual bees as well as the numbers of bumblebees per caste to determine potential trade-offs between colony size and individual bee size and between the production of reproductives and workers. In addition, we analyse how clothianidin exposure through seed-treated oilseed rape affects the prevalence and abundance of several pathogens and beneficial bacteria as well as their effect on bumblebee performance.

We find strong effects on bumblebee performance, particularly on the size and number of reproductives. However, clothianidin-exposed and control colonies had similar levels of viruses and beneficial bacteria and we observed no decrease in parasite tolerance in clothianidin-exposed colonies. Therefore, we conclude that field-level clothianidin exposure affects bumblebee colonies, potentially trough an impaired pollen foraging ability, but does generally not affect susceptibility to pathogens in bumblebees.

Résumé

Le niveau d'exposition au champ de clothianidine affecte les bourdons mais globalement pas leur pathogènes

Le déclin mondial des abeilles est attribué à la destruction des habitats, à l'usage des pesticides, aux agents pathogènes, au changement climatique, ou à des combinaisons entre ces différents facteurs. On pense aujourd'hui que l'histoire de l'agriculture a provoqué ce déclin de la diversité des abeilles par la conversion d'habitats (semi-) naturels, souvent riches en fleurs, vers des terres arables. De plus, l'exposition chronique aux pesticides agricoles menace les abeilles, en particulier les néonicotinoïdes neurotoxiques, qui ont été désignés parmi les causes de ce récent déclin des abeilles, en raison de leur utilisation à grande échelle, de leur nature systémique et de leur grande toxicité pour les abeilles. Des expériences avec une exposition aux néonicotinoïdes au moyen d'alimentation artificielle, aux niveaux comparables à ceux des résidus trouvés dans le pollen et le nectar de cultures ou de fleurs sauvages, ont montré toute une variété d'effets sublétaux sur les abeilles. Ces effets ont été partiellement confirmés par des études de terrain à l'échelle du paysage, mais les résultats variaient selon la localisation spatiale et les espèces d'abeilles, et quelques études ne montrent pas d'effet du tout.

Afin de comprendre ces sources de variation, il est important d'identifier les mécanismes par lequel les néonicotinoïdes influent sur les performances des abeilles en condition de plein champ. La mortalité chez les abeilles sociales peut être masquée par des mécanismes de compensation de colonies, tels que le compromis entre la production d'ouvrières et des mâles plus coûteux en énergie, ou entre la taille de la colonie et la taille individuelle de toutes les castes d'abeilles. Les effets spécifiques de l'exposition des néonicotinoïdes dans un lieu donné sur les abeilles ont été en partie attribués aux différences géographiques des niveaux de parasite, puisqu'il a déjà été démontré que l'impact des néonicotinoïdes sur les abeilles interagit avec les agents pathogènes et les parasites.

Il a été démontré que l'exposition aux néonicotinoïdes a augmenté l'abondance des agents pathogènes chez les abeilles mais pas chez les bourdons et a agi en synergie avec les pathogènes en augmentant la mortalité chez les deux espèces. Les fonctions immunitaires des individus peuvent être affaiblies par l'exposition aux néonicotinoïdes, soit en supprimant les gènes immunitaires, soit en réduisant l'immunité cellulaire. L'exposition aux néonicotinoïdes peut également nuire au comportement hygiénique et à la production de composés antiseptiques qui aident à préserver les réserves de nourriture. De plus, les bourdons exposés aux néonicotinoïdes collectent moins de pollen, ce qui risque de provoquer une sous-alimentation et donc d'affaiblir l'immunocompétence des abeilles.

Nous avons prolongé l'étude présentée par Rundlöf et al. (2015) à l'échelle du paysage comprenant des couples de sites répliqués, qui avait clairement démontré les effets nocifs d'un néonicotinoïde, la clothianidine, sur des bourdons et abeilles solitaires en conditions réelles agricoles. Conduite sur 96 colonies de bourdons (*Bombus terrestris* L.) à côté de 16 champs de colza cultivés à partir de graines traitées ou non à la clothianidine, elle a montré que les colonies situées dans des sites traités à la clothianidine grossissaient moins et produisaient moins de cocons que celles situées sur les sites non traités. Cette fois-ci, nous avons examiné en détail quatre des six colonies par site en comptant les adultes de toutes les castes, en mesurant la taille des corps de bourdons aux stades nymphe et adulte, et analysant la composition microbienne, comprenant à la fois des agents pathogènes et des symbiotes intestinaux bénéfiques.

Les microorganismes ont été quantifiés en utilisant la réaction en chaîne de polymérase quantitative (qPCR) après avoir préalablement réalisé une transcription inverse pour les virus à ARN (RT-qPCR). Les essais microbiens comprenaient sept virus à ARN pour les nymphes et les adultes, ainsi que le virus filamenteux à ADN d'*Apis mellifera*, trois espèces de *Nosema*, la trypanosomatide *Crithidia bombi* et deux bactéries intestinales bénéfiques seulement pour les adultes. Nous avons testé si l'exposition au champ par la clothianidine avait une incidence sur les performances des bourdons au niveau individuel ou au niveau des colonies, ainsi que sur la prévalence (c'est-à-dire la proportion de

colonies infectées) et l'abondance de microorganismes pathogènes et non pathogènes. Nous avons également examiné les effets interactifs potentiels entre l'exposition aux néonicotinoïdes et différents micro-organismes en vérifiant si l'abondance des micro-organismes co-variait de manière différente selon le traitement avec les paramètres de performance du bourdon. Pour cela, nous avons combiné les données de l'étude initiale sur le poids des colonies et le nombre de cocons d'ouvrières/mâles avec de nouvelles données sur la taille corporelle et le nombre d'abeilles par caste.

Nous avons constaté des effets importants sur la reproduction, car les colonies exposées à la clothianidine comptaient environ 70% de reines et de mâles adultes en moins, ainsi que plus de 20% de nymphes mâles plus légères. Nous avons aussi constaté un effet modéré sur la largeur du thorax, mais pas sur la masse corporelle ni sur le nombre d'ouvrières adultes. La prévalence et l'abondance des micro-organismes ne différaient guère entre les colonies des sites traités et non traitées. En fait, seul le parasite *Crithidia bombi* était plus répandu parmi les colonies placées dans les champs traités. L'exposition à la clothianidine n'avait en général que peu d'effet sur la relation entre l'abondance des microorganismes et la performance des bourdons, mais le nombre de mâles adultes et la masse corporelle des ouvrières ont augmenté avec l'abondance de la bactérie *Gilliamella apicola* chez les ouvrières adultes dans les colonies exposées à la clothianidine uniquement. Cela suggère que *G. apicola* pourrait avoir partiellement atténué les effets néfastes de la clothianidine, peut-être parce que *Gilliamella* spp aide à digérer les xénobiotiques et peut favoriser la prise de poids des bourdons. Ces niveaux d'infection similaires entre les deux traitements suggèrent qu'il n'y avait pas de suppression durable du système immunitaire. Au lieu de cela, l'exposition à la clothianidine peut avoir causé la malnutrition des abeilles, en particulier des larves, en raison de la perte de la capacité de butinage du pollen ou de l'insuffisance des soins au couvain par les nourrices. Cela pourrait expliquer le report tardif de la production de sexués et la masse corporelle inférieure des nymphes mâles et la taille réduite des ouvrières adultes. La production de reproducteurs (reines, mâles) peut n'avoir été que suspendue plutôt que réduite de façon permanente. Comparativement aux colonies témoins, les colonies exposées à la clothianidine présentaient un ratio mâles/ouvrières plusieurs fois inférieur au niveau des nymphes, mais le couvain d'ouvrières était nettement plus avancé que le couvain mâle

lorsque les colonies ont été supprimées par congélation après la floraison du colza, indiquant alors que les colonies étaient justes en train de passer de la production d'ouvrières à la production sexuée de mâles.

Nous concluons que l'exposition au colza issu de semences traitées à la clothianidine affecte les bourdons à la fois au niveau de la colonie et des individus, mais n'augmente pas leur sensibilité aux agents pathogènes. Des effets graves sur la reproduction suggèrent que la clothianidine peut avoir un effet délétère sur la taille des populations de bourdons, mais des études à long terme au niveau de la population sont nécessaires pour déterminer si les colonies de bourdons peuvent se rétablir d'une production de couvain temporairement réduite.

PhD student's contribution

I led the data analysis and the writing of sections on the results and the description of the statistical analyses. I also contributed extensively to the writing of all other sections.

Field-level clothianidin exposure affects bumblebees but generally not their pathogens

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Abstract

Neonicotinoids are implicated in bee declines and laboratory studies imply that they impair the bee immune system, thereby precipitating a rise in pathogen levels. To establish whether such synergisms impair bee performance in real-world agricultural landscapes, we analysed the microbial composition of the bumblebee (*Bombus terrestris*) samples from our recent landscape study on the impacts of field-level clothianidin exposure. We related clothianidin exposure and microbial composition to both individual- and colony-level performance parameters, to better understand the direct and indirect mechanistic effects of neonicotinoid exposure on bumblebees. We show that exposure to clothianidin from seed coated oilseed rape reduces bumblebee size and numbers, particularly of reproductives. However, exposure does not affect the levels of non-pathogenic bacteria or viruses, nor induce rises in the levels or virulence of intracellular parasites. We conclude that field exposure to the neonicotinoid clothianidin affects bumblebee performance but generally not their pathogenic or beneficial microbiota.

Introduction

Bees are essential pollinators and their recent declines may have adverse effects on both natural plant biodiversity and the production of crops that depend on insect pollination[1]. Global declines of bees have been attributed to habitat destruction, pesticide use, pathogens, climate change, or some combination of these factors[1,2]. The conversion of often flower-rich natural or semi-natural habitat to arable land is thought to play a major role in the long-term decreases in bee diversity through habitat loss and fragmentation[2]. Moreover, chronic exposure to agricultural pesticides, particularly the neurotoxic neonicotinoids, has recently been implicated in bee declines (reviewed in [2,3]).

Neonicotinoids are used worldwide to control insect pests of economically important crops[4]. They are taken up systemically to all parts of the plant including pollen and nectar - the major foods for bees. Artificial feeding experiments with neonicotinoid exposure that are comparable to residue levels found in pollen and nectar of crops and wild flowers[3,5] showed a variety of sublethal effects on bee reproduction[6,7], homing success[8,9], foraging behaviour[9–11], crop pollination[12] and immune function[13], all of which may ultimately cause colony failure[14]. Field studies[15–17] and UK-wide surveys[18,19] confirmed adverse effects of neonicotinoid seed-dressings in oilseed rape on bees, but results varied with spatial location[17] and bee species[16] and some studies found no effects[20–22].

To understand the sources of this variation, it is important to identify the mechanisms by which neonicotinoids impact bee performance under field conditions. Mortality in social bees can be masked by colony compensation mechanisms, such as the trade-off between worker production and more energy-costly males[15] or between colony size and individual body size of all bee castes[23]. Regional differences in the effects of field-level neonicotinoid exposure on wild and managed bees have been partly ascribed to regional differences in parasite levels[17], since the impact of neonicotinoids on bees had previously been shown to interact with pathogens and parasites[7,13,24–29] (but see [29–32]). Neonicotinoid exposure was shown to increase pathogen abundance in honeybees[13,24–26] but not in bumblebees[7,32] and to act synergistically with pathogens in increasing mortality of honeybees[25,27,28] and bumblebees[7]. Immune functions in individual bees can be weakened

by neonicotinoid exposure, either by suppressing immune genes, which has been shown to stimulate virus replication[13,33], or by impairing individual immunocompetence through a reduction in the number of hemocytes, wound-healing, the antimicrobial activity of the hemolymph and levels of phenoloxidase, an enzyme involved in the melanisation of pathogens[34–36]. Neonicotinoid exposure can also impair hygienic behaviour[37,38] and the production of antiseptic compounds that help preserve food stores[27], both major components of social immunity. In addition, bumblebees exposed to neonicotinoids collect less pollen[9–11], risking undernourishment and consequently weakening the bees' immunocompetence[39,40]. Bumblebees socially transmit distinct microbiota[41], which can enhance the ability to live on suboptimal diets[42,43] and protect against pathogens[41,44,45]. However, it remains unclear whether neonicotinoid exposure affects the gut bacteria that potentially contribute to or alter the bees' immunocompetence.

Most studies examining interactions between neonicotinoids and pathogens in bees have been laboratory-based and focused mainly on honeybees and occasionally on bumblebees[7,32,36,46]. Moreover, there remains a distinct deficit of landscape-scale field studies involving real-world neonicotinoid exposure[24]. We recently demonstrated clear harmful effects of the neonicotinoid clothianidin on wild bees in real agricultural landscapes, using a landscape-scale study design with free-flying bumblebees (*Bombus terrestris*) from colonies placed next to fields that were spring-sown with either clothianidin-treated or insecticide-free oilseed rape (*Brassica napus*) seeds[16]. The study showed that bumblebee colonies at clothianidin-treated sites grew less in weight and produced fewer cocoons than those at non-treated sites. Here, we extend this study by counting adult bees of all castes, measuring the body size of premature and adult bumblebees and analysing the bumblebee microbial composition, including both pathogens and beneficial gut symbionts. We test whether clothianidin field exposure affects individual-level or colony-level bumblebee performance and the prevalence (i.e. proportion of infected colonies) and abundance of pathogenic and non-pathogenic microorganisms. We also examine potential interactive effects between neonicotinoid exposure and different microorganisms by testing whether microorganism abundance co-varies differently between treatments with bumblebee performance parameters. For this,

we combine the data published in Rundlöf *et al.* (2015) on colony weight and the number of worker/male cocoons with new data on body size and numbers of bees per caste.

Our results confirm that field-level neonicotinoid exposure impairs reproduction in bumblebee colonies, as shown by fewer queens and males. The negative effects of clothianidin exposure on colony-level performance are supported by negative effects at the individual scale, with clothianidin-exposed colonies producing smaller bees. We find, however, no major effect of the neonicotinoid on pathogens, beneficial gut bacteria or their relationship to the host's performance, suggesting that the mechanisms by which clothianidin affects bumblebees in agricultural landscapes are largely independent of the bumblebee microbiota.

Results

Number and size of bee pupae and adults

Colonies at clothianidin-treated fields had on average 234 (41%) fewer total bees (intact cocoons + adults) than colonies at control fields (LRT, $P<0.001$), despite a similar number of adult workers in the two groups (LRT, $P=0.53$, Table 1; Fig. 1). The production of reproductives (queens and males) was markedly reduced in colonies at clothianidin-treated fields, as indicated by 32.8 or 66% fewer adult males (LRT, $P<0.001$) and 71.1 or 74% fewer queens (intact cocoons + adults; $P<0.001$; see also Rundlöf *et al.*[16].

Because the control colonies tended to be further along in their development than the exposed colonies, we were able to obtain male pupal samples from 28 of 32 colonies at untreated fields, but from only 16 of 32 colonies at clothianidin-treated fields. Similarly, we were able to obtain samples of at least 7 worker pupae more often from clothianidin-exposed (18) than control colonies (4). Samples of both male and worker pupae could be obtained from four clothianidin-exposed colonies that were in the transition from worker to male production, while two exposed colonies had neither worker nor male pupae. Generally, the male pupae were at an earlier developmental stage (LRT, $P<0.001$, Table

2) and had a larger body mass than the worker pupae (LRT, $P<0.001$). Only the male pupae data were further analysed, because there were too few unexposed colonies (4) with enough worker pupae to allow meaningful data analyses and interpretation. The male pupae did not differ in developmental stage between treatments (LRT, $P=0.96$), but were 21.5-23.9% (depending on developmental stage) lighter at clothianidin-treated fields than similar pupae at control fields (LRT, $P<0.001$; Fig. 2a, Table 2). Overall, the male pupal body mass decreased about 8 mg per developmental stage (corresponding to approximately 2 days; LRT, $P<0.001$).

The adult workers at clothianidin-treated and control fields had similar body mass (LRT, $P=0.18$, Table 2; Fig 2b), but those at treated fields had on average 4.8% (0.26 mm) smaller thoraxes (intertegular distance), than those at control fields ($P=0.015$).

Microorganism prevalence

The two principal symbiotic gut bacteria of adult bees (*Gilliamella apicola* and *Snodgrasella alvi*) were detected at all fields. In each treatment group, *G. apicola* was detected in adult worker bumblebee samples from 91% of the colonies. *Snodgrasella alvi* tended to be more prevalent in colonies at clothianidin-treated fields than in colonies at control fields (LRT, $P=0.057$, Fig. 3, Supplementary Table 1). The most prevalent pathogen was *Crithidia bombi*, which in contrast to *S. alvi* was less frequently detected in colonies at clothianidin-treated fields than in control colonies ($P=0.037$). *Apicystis bombi* was the only other pathogen that was detected in a majority of the colonies, but its prevalence did not differ between treatments (LRT, $P=0.40$). *Nosema bombi*, *Sacbrood virus* (SBV), *Slow bee paralysis virus* (SBPV) and *Acute bee paralysis virus* (ABPV) were sporadically detected. *Deformed wing virus* type A (DWV-A), *Black queen cell virus* (BQCV), *Chronic bee paralysis virus* (CBPV), *Lake Sinai virus* types 1 and 2 (LSV-1, LSV-2), *Apis mellifera filamentous virus* (AmFV) and the microsporidians *Nosema apis* and *Nosema ceranae* were not detected in any of the 64 bumblebee colonies. With the exception of two ABPV-infected control colonies, the pupal samples were free of viruses. Of the five colonies with ABPV-infected adults, two had also ABPV-infected pupae.

Microorganism abundance

Quantitative analyses of microorganism abundance were restricted to the positive samples of the four most prevalent microorganisms (*G. apicola, C. bombi, S. alvi, A. bombi*), in order to test the effects of clothianidin exposure on microorganism abundance independently of any effects on prevalence. Datasets from low-prevalence microorganisms were not analysed, since these were either too small (if non-detections are excluded) or too distorted by excess zero values (if non-detections were included) for meaningful analysis. Clothianidin exposure did not affect the abundance of any of these microorganisms in the infected bumblebee colonies (LRT, $P \geq 0.1$, Supplementary Table 2; Fig. 4). Neither did the abundance of any of these microorganisms co-vary with the abundance of any of the other microorganisms (Supplementary Table 3).

Interaction between clothianidin exposure and microorganisms

Here, we assessed the interactive effect of clothianidin exposure and the abundance of the four most frequently detected microorganisms on the body size and the numbers of bees (of different castes) as well as on the previously in Rundlöf *et al.*[16] reported colony weight and number of worker/male cocoons. Clothianidin exposure affected how *G. apicola* abundance in adult workers co-varied with the body mass of adult workers ($P=0.006$) and the number of adult males ($P=0.027$, Supplementary Table 4). In the clothianidin-exposed colonies, increase in *G. apicola* abundance was associated with an increase in worker body mass (LRT, $P<0.001$; ± 47 mg between mean-max $\log_{10}$ *G. apicola* abundance, n = 32 colonies) and in the number of adult males (LRT, $P=0.001$; ± 28.2 bees between mean-max $\log_{10}$ *G. apicola* abundance), while no such co-variance was found for the control colonies (LRT, worker body mass: $P=0.97$; number of adult males: $P=0.92$).

No other interactions between treatment and microorganism abundance could be identified, although there was a weak indication that the relationship between the number

of adult workers and *A. bombi* abundance differed between treatments (LRT, P=0.057; Supplementary Table 4).

Independently of treatment, the number of adult males declined with *S. alvi* abundance in adult workers (LRT, P=0.022; ± 6.9 bees between mean-maximum $\log_{10}$ *S. alvi* abundance, n = 64 colonies). We observed no other treatment-independent co-variation between microorganism abundance and bee performance parameters except for a non-significant tendency of the number of worker/male cocoons to decline with the abundance of *A. bombi* in adult worker bees (LRT, P=0.054).

Discussion

In this study, we confirm the previously reported negative effects of field exposure to clothianidin seed-treated oilseed rape on bumblebees (*B. terrestris*) at the colony level[16] and show that this is also reflected at the individual bumblebee level, by a reduction in body size. In addition, we provide further evidence for reduced production of reproductives[6,16,17] in colonies next to clothianidin-treated fields compared to colonies next to control fields and find that these adverse effects are unrelated to differences in microbiome composition. The shift from the production of workers to reproductives, which typically occurs after a colony growth phase, seemed to be delayed in colonies at clothianidin-treated fields. Colonies at treated fields had about 70% fewer queens and adult males compared to colonies at control fields, although the number of adult worker bees was comparable between clothianidin-exposed and control colonies. Although, the number of workers may have been affected by the proportion of bumblebees that were not in their nest when these were removed, clothianidin-exposed colonies exhibited also a several-times lower ratio of males to workers among the examined pupae than the control colonies. This reduced, or delayed, production of reproductives may be due to colony compensation mechanisms for worker losses[15], impaired ovary development[32,47] and/or undernourishment due to reduced pollen foraging or insufficient brood care[9–11]. When the colonies were terminated, the developmental stage of the worker brood was clearly more advanced than that of the male brood, suggesting that the colonies were switching from worker production to male/queen production. This suggests that the differential between exposed and control colonies in the preponderance of worker or male brood may represent a delay in, rather than an abandonment of, the production of reproductives.

We observed that colonies at clothianidin-treated fields had not only fewer, but also smaller bees. Lighter male pupae and smaller worker bees suggest that clothianidin exposure may directly or indirectly interfere with the development of individual bees. The size of bumblebees as adults is strongly influenced by the amount of food provisioned at the larval stage[48], suggesting that pupae in clothianidin-exposed colonies may have been undernourished as larvae, as a result of impaired pollen foraging success or deficient brood care by the adult bees[9–11]. Such reduced brood care may reflect a

quantitative shift by the colony towards foraging to compensate for a perceived deficit in foraging success, either through insufficient food intake or neuronal changes[11]. *Bombus terrestris* and honeybee workers can be attracted to neonicotinoid contaminated food, even though its consumption can reduce the bees' overall food intake[49]. However, this effect was not observed for clothianidin[49]. As smaller bumblebees are less efficient foragers[50,51], decreased worker size may exacerbate undernourishment, although our previous results showed that clothianidin exposure did not affect the number of food storage cells[16]. Larger body size is also thought to be advantageous for the mating ability of males[52,53], therefore the drastic reduction in body mass of male pupae may have implications for their future reproductive success.

The adverse effects on body size and the production of reproductives are likely not due to increased pathogen susceptibility. Clothianidin-exposed and control colonies showed only little difference in the prevalence or the abundance of symbiotic and pathogenic microbiota. Of the eight RNA viruses tested, only three were detected in the adult bee samples, and furthermore only in a minority of colonies, while only one (ABPV) was detected in pupal samples. Of the six DNA pathogens tested, only the three bumblebee specific parasites (*A. bombi, C. bombi*, *N. bombi*) were detected. The prevalences of these pathogens were generally comparable to other studies on commercially reared bumblebees[54,55], although *C. bombi* was detected more frequently in our study. This parasite showed a higher prevalence in the control than in the clothianidin-exposed group, even though previous laboratory-based research could not detect an effect of neonicotinoid exposure on *C. bombi*[7,32]. This may be due to reduced *C. bombi* proliferation, which then leads to lower transmission or detection rates of the parasite. Clothianidin exposure may directly reduce *C. bombi* proliferation or indirectly by inducing changes in bumblebee size demographics, nourishment and foraging activity that also affect *C. bombi* proliferation[56]. Beta-proteobacteria in the bee gut have previously been suggested to protect bumblebees against *C. bombi* infection[41,45] and in our study *S. alvi* tended to be, contrary to *C. bombi*, more prevalent in colonies at clothianidin-treated fields. However, we did not find a relationship between the abundances of *S. alvi* and *C. bombi.* As those bees that failed to return to their nests or

were removed by their nest mates after dying could not be sampled, it is conceivable that a decreased survival rate or homing success of *C. bombi* infected bees that lacked *S. alvi* or were additionally immune-challenged by the neonicotinoid[7] caused a lower detection rate of *C. bombi* and masked a negative relation between *C. bombi* and *S. alvi*.

The absence of interactive effects between clothianidin exposure and the abundance of intracellular parasites suggests that clothianidin did not affect the virulence of the parasites or the tolerance of the host to parasite infection. *Gilliamella apicola* was the only microorganism whose relationship with the bumblebee performance parameters depended on clothianidin exposure. In clothianidin-exposed colonies, the body mass of adult worker bees and the number of adult males increased with the abundance of *G. apicola* in adult workers, while no co-variation was observed in the control group. *Gilliamella* spp. promote weight gain in bees through the decomposition of otherwise indigestible or toxic carbohydrates[42,43], which may be one possible explanation as to why adult worker bees of the two treatment groups did not significantly differ in body mass, but did differ in intertegular distance. In contrast, to *G. apicola*, *S. alvi* abundance in adult workers showed a negative co-variation with the number of adult males independently of treatment. We are aware that caution has to be applied when interpreting marginally significant effects (such as the effects of microorganisms in interaction or independently of treatment on the number of adult males or the treatment effect on *C. bombi* prevalence), since the probability of a false positive finding increases with the number of parameters tested and can be much greater than the probability of falsely rejecting the null hypothesis evaluated by *P*-values[57].

The exposure of bumblebees to clothianidin from seed-coated oilseed rape during one flowering season, which was confirmed by residue analysis of bumblebee-collected nectar[16], did not affect the levels of symbiotic gut bacteria or viruses, nor induce rises in the levels or virulence of intracellular parasites. This suggests that the bumblebees' combined individual, adaptive and social immune defences[7,13,33–36] were not sufficiently affected to impair colony-level pathogen susceptibility during this time interval, and that the mechanisms by which exposure to clothianidin affects bumblebee colonies are largely

independent from those affected by biological pathogens or diseases. Two comparable field-level studies, but with free-foraging honeybees instead of bumblebees (and lacking adequate site replication), obtained contrasting results for the effects of neonicotinoids on parasite and pathogen levels in honeybees. One study found no impact of clothianidin on *Varroa* and virus levels[58], while the other detected in the first year of the experiment an increase in physiological stress, BQCV and *Varroa* abundance in honeybee colonies placed by neonicotinoid-treated maize fields relative to colonies at untreated maize fields, even though neonicotinoid exposure could not be confirmed[23]. The surviving honeybee colonies were placed for an additional season by the maize fields of the same treatment (control / clothianidin or thiamethoxam) and *Varroa* abundance was again higher in colonies placed by neonicotinoid-treated fields with low levels of neonicotinoid exposure confirmed for apiaries at treated sites and for one out of control apiaries[59]. Experimentally-induced exposure of honeybees to both *Varroa* and clothianidin spiked syrup showed no interactive effect between the two pressures[31]. In another study, long-lasting in-hive feeding of thiacloprid to honeybee colonies did not affect colony performance or the levels of parasites, pathogens and expressed immunity-related genes[30]. Thiacloprid belongs, however, to the group of cyano-substituted neonicotinoids, which are substantially less toxic to bees than nitro-substituted neonicotinoids, such as clothianidin or imidacloprid[3]. Pettis *et al.* (2012)[26] conducted two trials with emerging workers taken from colonies fed with imidacloprid to investigate synergism between the neonicotinoid and *Nosema* spp. They found that imidacloprid increased spore counts when the pathogens were administered with food, but decreased spore counts when *Nosema* was naturally acquired. Pathogen-pesticide interaction in bumblebees has been studied under laboratory conditions with the pyrethroid λ-cyhalothrin and *C. bombi*[60]. Chronic exposure to the pyrethroid did not affect *C. bombi* prevalence or abundance but the body mass of *B. terrestris* workers. Other individual-level or colony-level performance parameters were unaffected by the treatment[60].

Neonicotinoids and certain pathogens (predominantly bee viruses) share a common target: the bee nervous system[61]. This is a plausible causative explanation for the synergism observed between neonicotinoids and pathogens in laboratory studies,

particularly at high levels of infection and pesticide exposure[13,25,35]. In the field, however, pesticide–pathogen synergism may be masked by potentially more potent drivers of pathogen prevalence and abundance, such as population dynamics of bee communities, nutrient availability or adapted foraging behaviour[56,62].

We conclude that exposure to clothianidin seed-treated oilseed rape impacts bumblebees at both the colony and the individual level but does not increase their susceptibility to pathogens. The strong effects of clothianidin exposure on the production of queens and males suggest that neonicotinoids may deleteriously influence bumblebee population sizes[19], which may further be exacerbated by a neonicotinoid-induced reduction in colony initiation after hibernation[32]. Long-term studies at the population level are needed to investigate whether bumblebee colonies can recover from temporarily reduced brood production during periods when they are no longer exposed to neonicotinoids, as shown under laboratory conditions[63].

Methods

Study sites

In 2013 a total of 16 fields (field size = 8.9 ± 1.4 ha (mean ± s.e.m)) in southern Sweden, intended for the production of spring-sown oilseed rape (*Brassica napus* L.), were selected by the absence of other oilseed rape fields within a 2 km radius and paired based on geographical proximity and the land use in the surrounding landscapes (r = 2 km; for details see Rundlöf *et al.*[1]). For each field pair, one field was randomly assigned to be sown with clothianidin-treated oilseed rape seeds (25 mL Elado (Bayer; 400 g L^{-1} clothianidin + 180 g L^{-1} b-cyfluthrin) per kg seed and the fungicide thiram) while the paired field was sown with insecticide-free oilseed rape seeds treated only with thiram.

Farmers at both treated and untreated fields used non-neonicotinoid insecticide sprays but were instructed not to use other neonicotinoids for pest control (see Rundlöf *et al.*[16]). However, one control field was accidently sprayed with 0.3 L ha^{-1} Biscaya, which contains the neonicotinoid thiacloprid as active ingredient. Thiacloprid has a considerably

lower acute toxicity to bees than clothianidin[3]. Residue analyses of bee-collected pollen and nectar revealed that the overwhelming insecticide exposure, and greatest differential between control and treated fields, was from clothianidin, with minor traces of the spray insecticides distributed equitably between control and treated fields[16].

Bumblebee colonies

Six commercially reared *Bombus terrestris* colonies (Naturpol beehives, Koppert Biological Systems) were placed in triplets in two wooden, ventilated houses placed in shaded areas along the field edge in each of the 16 fields between June 14-28, 2013, at the onset of oilseed rape flowering in each field (for details see Rundlöf *et al.*[16]). Field allocation was randomized and there was no difference in weights between colonies at treated (723 ± 19 g (mean ± s.e.m), n = 32 colonies) and control (733 ± 18 g, n = 32) fields at placement[17]. The colonies were approximately 10 weeks old at the time of placement, containing roughly 50 workers, one queen, and both pupae and larvae. All 12 bumblebee colonies at a field-pair were freeze-killed simultaneously at -20 °C between July 7th and August 5th 2013, at first sighting of new queens in any one of those 12 colonies, one pair after closing nests with check valves for >24 hours and the rest at night when most bees were assumed to be in the nest. Since oilseed rape flower phenology influenced placement time and the switch to queen production determined termination time, the duration of field placement varied between 23-38 days for different sets of colonies.

Bee performance parameters

The two outer bumblebee colonies in each housing unit (total of four from each field) had been assessed previously for the number and weight of queen cocoons, the number and weight of worker/male cocoons, the number of pollen and nectar cells, and the weight of the nest structure[16]. These samples were stored frozen at -20 ºC. In this study, we determined the following additional parameters for these same colonies: the total number of bees (adults and pupae), the total number of queens (adults and pupae), the number of adult workers and the number of adult males, as well as the caste, weight and developmental stage of individual pupal cocoons, and the body mass and intertegular

distance of individual adult workers. We were unable to categorize ~0.7% of the adult bees, which were not used in analyses. Intertegular distance is the distance between the insertion points of the wings[64] and a standard measure of adult body size in bees. Intertegular distance was measured using a digital caliper and individual body mass of adults and pupae was measured using a balance with 0.1 mg resolution. Only intact bees were analysed. The developmental stage of individual pupae was rated into 6 categories based on eye colour (white = 1, pink = 2, brown = 3), body colour (white = 1-3, brown = 4, black = 5) and the presence of wings (6).

Pathogens and beneficial bacteria

The colonies that were assessed for bee performance parameters were also examined for the presence and abundance of the most common and important pathogens and beneficial microbes, including the RNA viruses *Deformed wing virus* type A (DWV-A), *Acute bee paralysis virus* (ABPV), *Black queen cell virus* (BQCV), *Sacbrood virus* (SBV), *Slow bee paralysis virus* (SBPV), *Chronic bee paralysis virus* (CBPV), *Lake Sinai virus* types 1 and 2 (LSV1 & LSV2), the DNA virus *Apis mellifera filamentous virus* (AmFV), the microsporidian gut parasites *Nosema apis*, *N. ceranae* and *N. bombi*, two other common internal parasites (*Apicystis bombi*, *Crithidia bombi*) and the non-pathogenic gut bacteria *Gilliamella apicola* and *Snodgrassella alvi* (Supplementary Table 5).

Sample processing and homogenization

For each colony, pooled samples of ten adult worker bumblebees, ten worker pupae and/or ten male pupae were prepared. Two bumblebee colonies (both from treated fields) did not contain any pupae and not all of the remaining colonies contained both worker and male pupae, so that the final sample set consisted of 64 adult samples, 22 worker pupae samples and 44 male pupae samples. Furthermore, five of the worker pupae samples contained only 7-9 individuals.

The 10 bees in each pooled sample were placed in a polyethylene bag with an inner mesh (BioReba). The bees were finely ground with a pestle. One mL nuclease-free water per

bee was added and the slurry was mixed thoroughly until the suspension was homogenous. The homogenates were stored at -80 °C in 1 mL aliquots until nucleic acid extraction.

Nucleic acid extraction

DNA was extracted from the adult bumblebee homogenates using the protocol for extracting DNA from *Nosema* spores[65], which is sufficiently robust to also extract DNA from bacteria and other microorganisms. One mL primary bee homogenate was centrifuged for 5 min in a microfuge at 13000 rpm. The pellet was repeatedly frozen-thawed with liquid nitrogen and ground with a sterile teflon micro-pestle until pulverized. The pulverized pellet was re-suspended in 400 µL Qiagen Plant tissues DNeasy AP1 lysis buffer containing 4 µL RNAse-A (10 mg mL^{-1}) and incubated and shaken for 10 min at 65 °C, after which 130 µL P3 neutralization buffer (3.0 M potassium acetate pH 5.5) was added, followed by 5 min incubation on ice and centrifugation for 5 min at 14000 rpm to remove the lysis debris. DNA was purified from 500 µL of the supernatant by the Qiagen automated Qiacube extraction robot, following the plant DNeasy protocol and eluting the DNA into 100 µL nuclease-free water. RNA was extracted by the Qiacube robot directly from 100 µL of both the adult and pupal bumblebee homogenates, using the Qiagen Plant RNeasy protocol (including the Qia-shredder for additional homogenization[66]), eluting the RNA into 50 µL nuclease-free water. The approximate nucleic acid concentration was determined by NanoDrop, after which the samples were diluted with nuclease-free water to a uniform 2 ng μL^{-1} (DNA) or 5 ng μL^{-1} (RNA) and stored at -80 °C.

RT-qPCR and qPCR

The RNA pathogens were quantified by Reverse Transcription-quantitative PCR (RT-qPCR). Two technical assays were also included in the RNA analyses: one for RNA250, a passive exogenous reference RNA of known concentration included in the RT-qPCR reaction mixture, for correcting sample-specific differences in assay performance[67], and one for the mRNA of the bumblebee internal reference gene Bt-RPL23[68], for correcting sample-specific differences in RNA quality[69]. The DNA pathogens were quantified by

qPCR. Novel qPCR assays were designed, experimentally optimized and confirmed through bidirectional Sanger sequencing of representative PCR products for *Crithidia bombi* (based on the GADH gene), *Nosema ceranae* and *N. bombi* (based on the small subunit ribosomal RNA gene), *Snodgrassella alvi* and *Gilliamella apicola* (based on the 16S ribosomal RNA gene). All sequences matched 100% their intended target.

The PCR reactions were run in duplicate and conducted in 20 µL volumes containing 2 µL template, 0.2 µM (RNA) or 0.4 µM (DNA) of forward and reverse primer (Supplementary Table 6) and either the Bio-Rad EvaGreen qPCR mix (DNA) or the Bio-Rad iScript One-Step RT-qPCR mix (RNA), both with SYBR Green detection chemistry. The reactions were incubated in 96-well optical qPCR plates in the Bio-Rad CFX connect thermocycler, using the following amplification cycling profiles for the RNA assays: 10 min at 50 °C for cDNA synthesis (RT-qPCR only): 5 min at 95 °C (to inactivate the reverse transcriptase and activate the Taq polymerase) followed by 40 cycles of 10 s at 95 °C for denaturation and 10 s at 58 °C for primer annealing, extension, and data collection. For the DNA assays the following amplification cycling profiles were used: 2 min at 98 °C for the initial denaturation followed by 40 cycles of 5 s at 98 °C for denaturation and 10 s at 60 °C for primer annealing, extension, and data collection. The amplification cycles were followed by a melting curve analysis to determine the specificity of the amplification by holding the temperature for 10 s at 95 °C and then reading the fluorescence at 0.5 °C increments from 65 °C to 95 °C. Included on each reaction plate were positive and negative (template-free) assay controls. For each type of assay (Supplementary Table 3) a calibration curve was prepared through a 10-fold dilution series of a positive control of known concentration covering seven to eight orders of magnitude, for quantitative data conversion, establishing the reference melting curve profile of the amplicon and estimating the reaction performance statistics.

Data conversion and normalization

The melting curves of individual reactions were evaluated visually in order to separate out non-specific amplifications, which differ in melting temperature profiles from true target cDNA/DNA amplicons. Non-specific amplifications were deleted from the data

set. All assays were run in duplicate, with the mean value of these two duplicates used in further calculations. Both duplicates had to yield a positive quantitative value and pass the melting curve analysis for the data to be included in the data set. The Cq-values (quantification cycle) of all confirmed amplifications were subsequently converted to estimated SQ-values (Starting Quantity) in copy numbers of each target DNA/RNA, using the corresponding calibration curves for the different assays. These data were multiplied by the various dilution/conversion factors incurred throughout the extraction, cDNA synthesis and amplification procedures, to calculate the estimated copies of each target per bee. The data for the RNA targets were adjusted with the sample-specific data for the two technical assays: the exogenous (RNA250) and internal (Bt-RPL23) reference RNAs. The data for RNA250 was used to calculate the individual cDNA conversion efficiency for each sample, *i.e.* the ratio of the amount of RNA250 estimated by RT-qPCR (output) versus the known amount of RNA250 added to the reaction (input). Since RNA is easily degraded there is a risk that differences between individual samples in RNA quality (*i.e.* degradation) can affect the results[69]. The data for the RNA targets of interest were therefore normalized to the average value for Bt-RPL23 mRNA, thus correcting the data for sample-specific differences in RNA quality with respect to RT-qPCR performance.

Statistical analyses

The data consisted of a range of quantitative biological and pathological parameters, which were measured at colony level (bee numbers, microbiome composition) or at individual level (body mass and intertegular distance). The microbial data was analysed on both their binary (presence/absence) and quantitative (abundance) characters. Since no samples were taken prior to exposure, the statistical analyses consisted largely of straight comparisons between colonies at clothianidin-treated fields and control fields. All analyses were done both including and excluding the field where Biscaya (containing the neonicotinoid thiacloprid) was sprayed, to determine if this influenced the results. Except in two cases, excluding the field sprayed with Biscaya from the data analysis did not

move *P*-values from below above the 0.05 threshold or vice versa (Supplementary Table 4).

The impact of treatment on the total numbers of bees (cocoons + adults) and the number of adult workers was assessed using linear mixed-effects models (LMM; with normal error distributions), while treatment effects on the total numbers of queens (cocoons + adults) and the number of adult males were analysed using generalized linear-mixed effects models (GLMM) with negative binomial error distributions and log links. All models on bee numbers contained treatment as a fixed factor and field pair identity and field identity as random factors.

To test whether the developmental stage of male pupae differed from worker pupae a cumulative link mixed model (CLMM; with logistic error distribution and logit link) was used. Differences in body mass between the two castes were examined using LMMs. All (C)LMMs for data on individual bees contained the field pair identity, field identity and colony identity as nested random effects. Treatment effects on pupae were only tested for males, due to the low incidence of worker pupa in control colonies. To identify whether the developmental stage of male pupae differed between treatments, a CLMM was used. Effects of treatment and developmental stage on male pupal body mass were examined with LMMs that contained both predictors as fixed factors. Body mass estimates for each stage and treatment were predicted while setting random effects to zero. Effects of clothianidin exposure on the body mass and intertegular distance of adult workers were analysed using LMMs with treatment as a fixed factor.

To test whether or not clothianidin exposure affected microorganism prevalence generalized linear mixed effects models with binomial error distribution and logit link with treatment as a fixed factor and field identity as a random factor were used for *A. bombi*, *C. bombi*, *S. alvi* and SBV. For all other microorganisms, the effective sample size (i.e. the less frequent outcome of the presence/absence variable) was too small for the use of random effects. Therefore two-sided tests of equal proportions were conducted using the prop.test function in R to test whether prevalences differed between treatments.

Hereby, a Yates' continuity correction was applied to avoid overestimation of statistical significance in small datasets.

Only four microorganisms (*A. bombi*, *C. bombi*, *G. apicola* and *S. alvi*) were prevalent enough for meaningful analysis of their quantitative levels. Microorganism abundances were logarithmically (log_{10}) transformed, because microorganism titres are exponentially distributed as a result of their growth dynamics. The impact of clothianidin on microorganism abundance was only tested among colonies infected with the microorganism to test effects on abundance independently of differences in prevalence and to avoid data distributions that are skewed by zero values. LMMs with normal error distributions containing treatment as a fixed effect and field pair identity and field identity as random effects were used to examine whether clothianidin impacted the levels of each microorganism in infected colonies. The co-variation between the abundance of the four prevalent microorganisms was tested on the data of all colonies, including those that were not infected by one or more of these microorganisms. LMMs predicting the abundance of a microorganism by the abundance of another were conducted for the whole dataset as well as separately for each treatment group.

The relation between bee performance parameters and the abundance of the four most prevalent microorganisms was analysed for all 64 colonies. Bee performance parameters included in addition to the bee size and bee number parameters from this study, colony weight and the number of worker/male cocoons reported in Rundlöf *et al.*[16]. The latter two parameters were analysed using LMMs with a normal error distribution and the other parameters were analysed with (G)LMMs with the same error distribution and link function as described in above. (G)LMMs with an interaction term of treatment and the abundance of one of the four prevalent microorganisms were used to examine whether bee performance parameters were differently related to microorganism abundance in the two treatment groups. These (G)LMMs contained field pair identity and field identity as random factors for colony-level parameters and additionally colony identity for individual-level parameters. The treatment × microorganism abundance interaction was removed from the model if $P>0.05$. In contrast, if $P<0.05$, the relation between bee

performance parameter and microorganism abundance was examined with separate (G)LMMs for each treatment group. (G)LMMs for only one treatment group contained field identity as random factor for colony-level parameters and field identity and colony identity for individual-level parameters.

Throughout this study, we calculated *P*-values of model estimates by likelihood ratio tests (LRT), because they, in contrast to conventional Wald tests, make no assumptions about the likelihood surface/curve and are therefore considered more reliable[70]. All analyses were conducted using the R Version 3.3.4. The lmer and glmer functions of the R package lme4 were used for LMMs and GLMMs with binomial error distribution, respectively, while the glmmTMB function of the R package glmmTMB was used for GLMMs with negative binomial error distribution and the clmm function of the ordinal package was used for CLMMs.

Power analysis

We performed a power analysis for treatment effects or interactive effects between microorganism abundance and treatment where $0.05>P<0.1$, to assess the effect size we could potentially detect given our study design, replication and model choice. Power was determined for a range of effect sizes at a nominal confidence level of $\alpha = 0.05$ by 1000 Monte Carlo simulations per effect size using the powerSim function of the simr package in R. For graphical illustration, effect sizes were transformed to percentages. For interactive effects between treatment and microorganism abundance on bee performance, effect size of a $\log_{10}$ unit increase in microorganism abundance was shown relative to the estimated response value of a control colony with average microorganism $\log_{10}$ abundance. For microorganism prevalence, effect size was shown as a percentage change of infected colonies relative to the total number of colonies.

The power analysis suggested that 80% power was reached for effect sizes ranging from under 15% (number of adult workers by *A. bombi* × treatment) to over 25% (*S. alvi* prevalence by treatment) (Supplementary Fig. 1).

Data availability

The data supporting the findings of this study are available within the paper, its supplementary information files and/or Rundlöf *et al.*[16]. The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

Conceptualization, JM, MR, RB, HS, TRP, JO, DW; Methodology, JM, DW, EF, ES, MR, GA; Investigation, DW, ES, GA; Formal analysis, DW, MR; Data curation, JM, MR; Writing, DW, BL; Editing, All; Visualization, DW; Supervision JM, MR; Project Administration, EF, TRP, RB, MR, HS; Funding Acquisition, JM, MR, RB, HS, TRP

Competing interests

The authors declare no competing interests.

Figures and tables

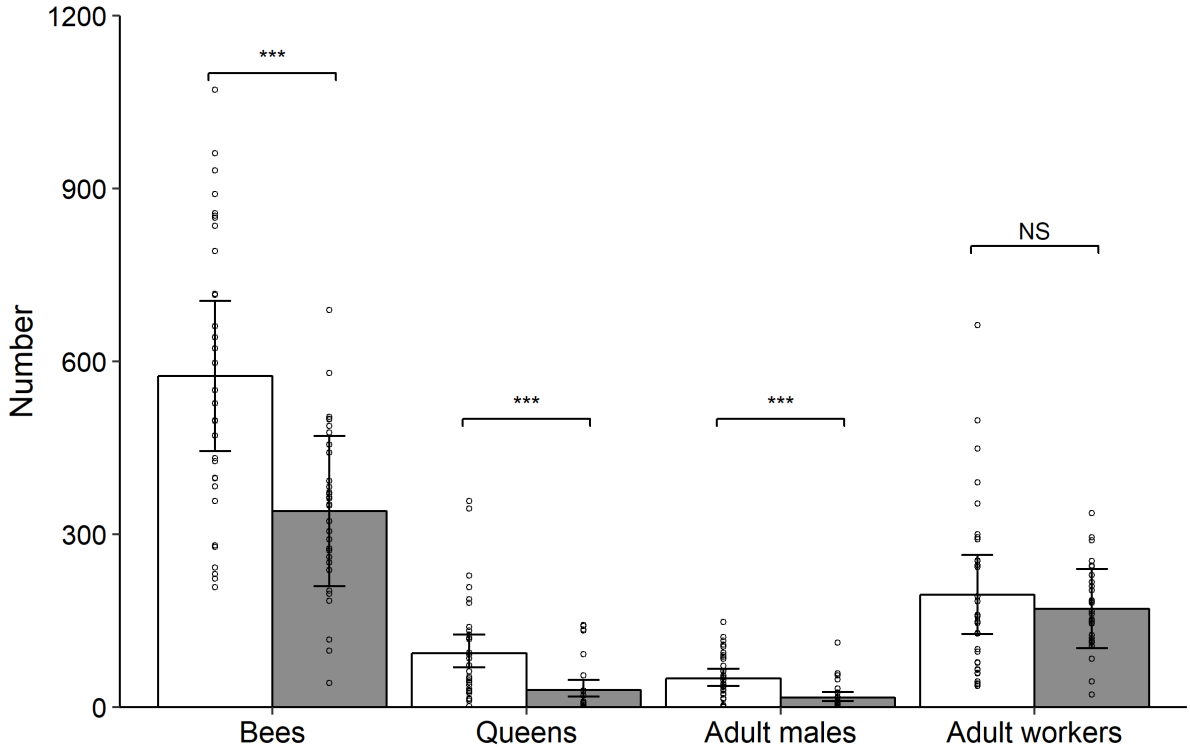


Figure 1 | Bee numbers. Number of bees (adults + cocoons), queens (adult queens + queen cocoons), adult males and adult workers per bumblebee colony (32 per treatment) in relation to treatment (white, control; grey, clothianidin seed coating) in oilseed rape fields (8 per treatment). The error bars represent 95% profile confidence intervals of linear mixed effects model estimates. Circles indicate measured values (per colony). NS: not significant ($P > 0.05$), ***$P < 0.001$. *P*-values were calculated by likelihood ratio tests on (generalised) linear mixed-effects models.

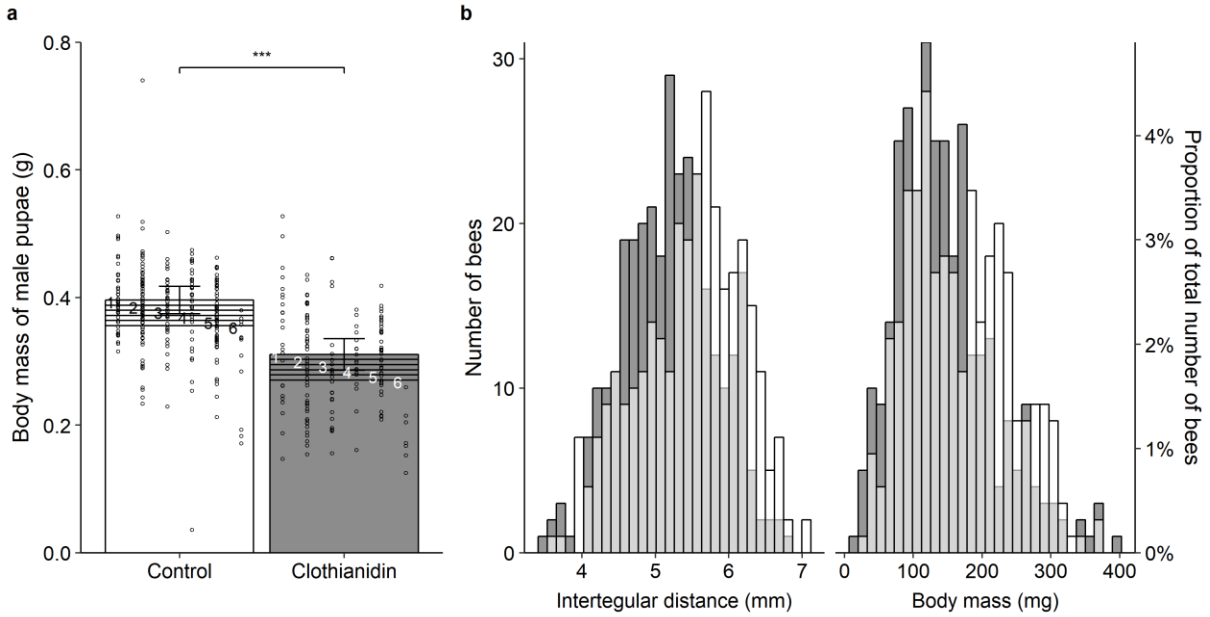


Figure 2 | Size of pupae and adult bumblebees. (**a**) Body mass of male pupae in relation to treatment (control or clothianidin seed coating) and pupal developmental stage (1-6). Error bars represent 95% profile confidence intervals of linear mixed effects model estimates at the earliest developmental stage (1). Circles indicate raw data on measured body mass (per bee). ***$P < 0.001$. *P*-values were calculated by likelihood ratio tests on a linear mixed-effects model. (**b**) Histograms of the intertegular distance and the body mass of adult worker bumblebees from colonies (32 per treatment) placed in oilseed rape fields (8 per treatment) sown from clothianidin-treated (dark grey; n = 320 bees) or insecticide-free seeds (white; n = 313). Overlaps between the two treatment groups are shown in light grey and values are expressed in absolute terms and percentages of the total number of measured bees.

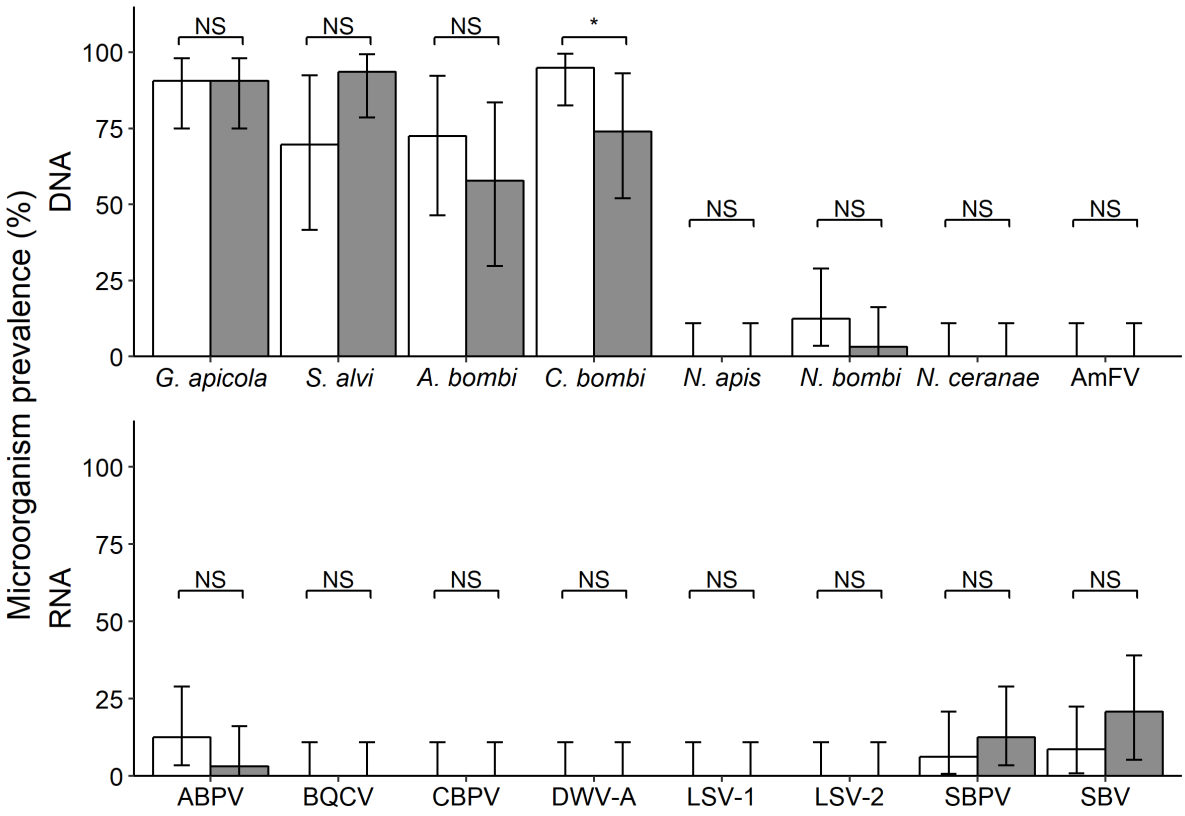


Figure 3 | Microorganism prevalence. Percentage of bumblebee colonies (32 per treatment) infected with microorganisms in relation to treatment (white, control; grey, clothianidin seed coating) in oilseed rape fields (8 per treatment). For *S. alvi*, *A. bombi*, *C. bombi* and SBV generalized mixed effects model estimates and their 95% confidence intervals are shown. For all other microbiota controlling for non-independence of colonies placed by the same field was not feasible. Therefore, the actual proportions of infected colonies per treatment and 95% confidence intervals calculated by two-sided binomial tests are illustrated. *P*-values were calculated based on likelihood ratio tests for model estimates and two-sided tests of equal proportions for measured proportions. NS: not significant ($P > 0.05$), *$P < 0.05$.

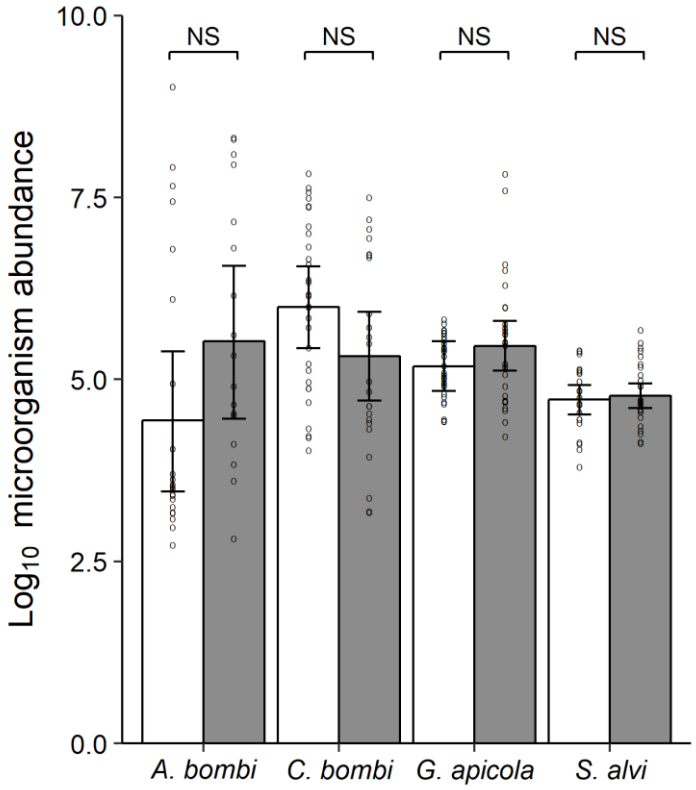


Figure 4 | Microorganism abundance. The $\log_{10}$ DNA copy numbers per bee and colony of the most frequently detected microorganisms (*Apicystis bombi* (n=40 colonies in N=15 fields), *Crithidia bombi* (n=53, N=16), *Gilliamella apicola* (n=58 N=16), *Snodgrassella alvi* (n=50, N=16)) in relation to treatment (white, control; grey, clothianidin seed coating) in oilseed rape fields (8 per treatment). The error bars represent 95% profile confidence intervals of linear mixed effects model estimates. Circles indicate measured values (per colony). NS: not significant ($P > 0.05$). *P*-values were calculated by likelihood ratio tests on linear mixed-effects models.

Table 1 | Bee numbers in relation to clothianidin seed treatment.

Response	**Model**	**Predictor**	**Estimate (number)**	**Estimate (%)** [a]	χ^2_1	***P*** [b]
Bees (adults + cocoons)	LMM[c]	Treatment	- 234.4	- 40.8	11.05	**<0.001**
Adult workers	LMM[c]	Treatment	- 24.4	- 12.5	0.04	0.526
Queens (adults + cocoons)	GLMM[d]	Treatment	- 71.1	- 73.5	17.52	**<0.001**
Adult males	GLMM[d]	Treatment	- 32.8	- 65.7	17.63	**<0.001**

[a] Effect sizes in % were calculated in reference to the control group
[b] *P*-values were calculated by likelihood ratio tests with 1 degree of freedom and $P<0.05$ is highlighted in bold
[c] Linear mixed-effects models (LMM; with normal error distribution)
[d] Generalized linear mixed-effects models (GLMM; with negative binomial error distribution and log link)

Table 2 | Bee size in relation to clothianidin seed treatment, caste and developmental stage.

Sample	Model	Response	Predictor	Estimate	χ^2_1	*P* [a]	N Fields	N Colonies	N Bees
All pupae	CLMM[b]	Stage	Caste[d]	-1.32 mg	34.09	**<0.001**	16	62	678
	LMM[c]	Body mass	Caste[d]	45.0 mg	18.52	**<0.001**			
Male pupae	CLMM[b]	Stage	Treatment	-0.02	0.00	0.959	16	47	456
	LMM[c]	Body mass	Treatment	-85.1 mg	20.40	**<0.001**			
			Stage	-8.0 mg	17.10	**<0.001**			
Adult workers	LMM[c]	Body mass	Treatment	-21.9 mg	1.76	0.184	16	64	633
	LMM[c]	Intertegular distance	Treatment	-0.26 mm	5.95	**0.015**			

[a] *P*-values were calculated by likelihood ratio tests with 1 degree of freedom and $P<0.05$ is highlighted in bold

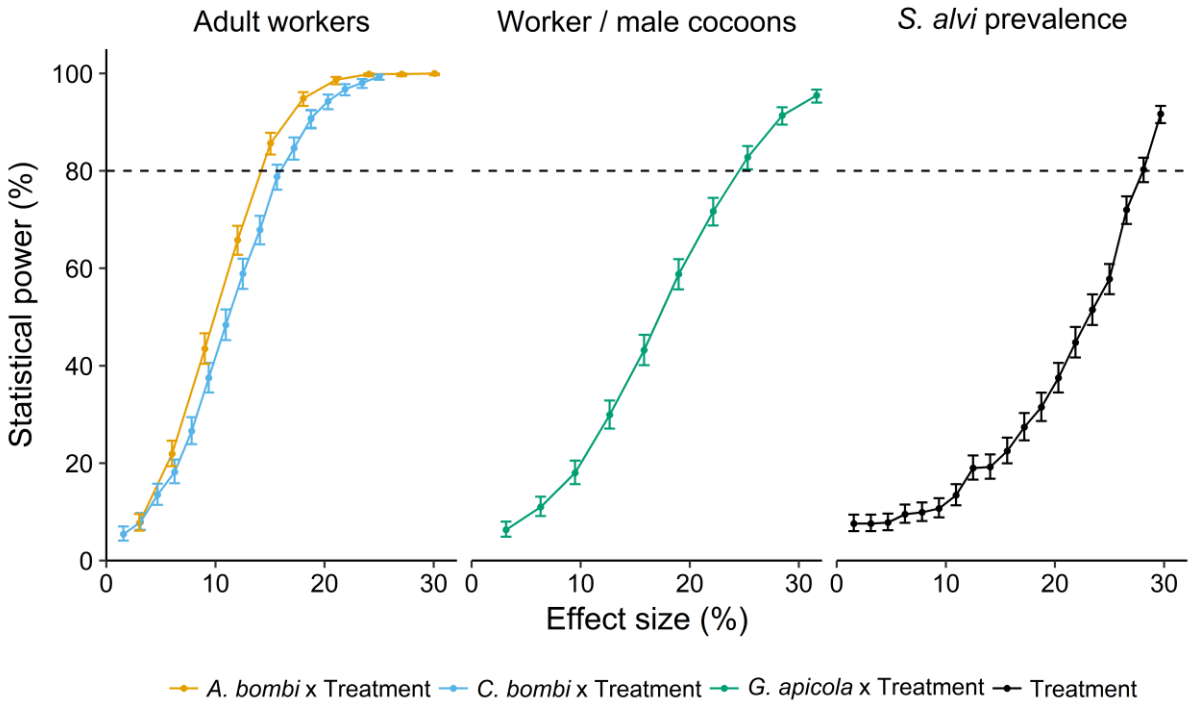
[b] CLMM = Cumulative link mixed model (with logistic error distribution and logit link)

[c] LMM = Linear mixed-effects model (with normal error distribution)

[d] Differences between castes are shown in reference to worker pupae.

Supplementary Information

Supplementary Figures



Supplementary Figure 1 | Statistical power. Power in relation to effect size for treatment effects or interactive effects between treatment and microorganism abundance where 0.05<*P*<0.1. For interactive effects on the numbers of adult workers and worker/male cocoons, effect size represents the effect of a $\log_{10}$ unit increase in microorganism abundance expressed as a percentage of the estimated value of a control colony with average microorganism $\log_{10}$ abundance. For *S. alvi* prevalence effect size represents an increase in the number of infected colonies illustrated as a percentage of all colonies. The dashed line indicates a power of 80%.

Supplementary Tables

Supplementary Table 1 | Microorganism prevalence. The prevalences of detected microbiota in adult worker bees were related to clothianidin seed treatment using two different kinds of tests: tests of equal proportions for straight comparisons of the proportions of infected colonies between treatments and analyses of variance (ANOVA) based on the number of infected colonies per field to control for non-independence of colonies placed by the same field.

Response	Predictor	Test	Estimate [c]	χ^2_1	*P* [d]
Acute bee paralysis virus	Treatment	Equal proportions [a]	-9.4%	0.87	0.352
Slow bee paralysis virus	Treatment	Equal proportions [a]	6.3%	1.84	0.668
Sacbrood virus	Treatment	LRT on GLMM [b]	12.2%	1.74	0.189
Apicystis bombi	Treatment	LRT on GLMM [b]	-14.7%	0.71	0.400
Crithidia bombi	Treatment	LRT on GLMM [b]	-20.8%	4.33	**0.037**
Nosema bombi	Treatment	Equal proportions [a]	-9.4%	0.87	0.352
Gilliamella apicola	Treatment	Equal proportions [a]	0%	0	1
Snodgrassella alvi	Treatment	LRT on GLMM [b]	23.8%	3.63	0.057

[a] Two-sided test of equal proportions using the prop.test function in R
[b] Likelihood ratio test on a generalized linear mixed effects model with field identity as random effect.
[c] Effect sizes are expressed as absolute differences between treatments in the percentage of infected colonies.
[d] *P*-values < 0.05 are highlighted in bold

Supplementary Table 2 | Microorganism abundance. Log_{10} abundance of prevalent microorganisms in worker bees of infected colonies (i.e. excluding colonies in which the target organism was not detected) in relation to clothianidin seed treatment [a].

Response	Predictor	Estimate (log_{10} units)	Estimate (%) [a]	χ^2_1	P [b]	N Fields	N Colonies
A. bombi	Treatment	1.09	24.5	2.39	0.122	15	40
C. bombi	Treatment	- 0.68	- 11.3	2.70	0.100	16	53
G. apicola	Treatment	0.28	5.39	1.39	0.239	16	58
S. alvi	Treatment	0.05	1.10	0.13	0.720	16	50

[a] Effect sizes in % were calculated in reference to the control group

[b] *P*-values were calculated by likelihood ratio tests with 1 degree of freedom on linear mixed effects models containing field identity and field pair identity as random factors.

Supplementary Table3 | Co-variation between microorganisms [a].

Response	Predictor	Control group			Clothianidin group			Whole dataset		
		Estimate	χ^2_1	P [b]	Estimate	χ^2_1	P [c]	Estimate	χ^2_1	P [c]
A. bombi	*C. bombi*	-0.056	0.05	0.825	-0.119	0.44	0.509	-0.101	0.53	0.465
C. bombi	*A. bombi*	0.020	0.06	0.811	-0.160	1.16	0.282	-0.078	0.51	0.474
A. bombi	*G. apicola*	0.007	0.00	0.976	-0.194	0.43	0.510	-0.084	0.19	0.666
G. apicola	*A. bombi*	0.007	0.01	0.938	-0.025	0.01	0.924	-0.013	0.01	0.911
A. bombi	*S. alvi*	0.226	1.27	0.259	-0.268	0.75	0.387	0.040	0.05	0.817
S. alvi	*A. bombi*	0.175	1.23	0.268	-0.115	1.96	0.161	0.020	0.04	0.841
C. bombi	*G. apicola*	0.374	3.34	0.067	0.228	0.58	0.448	0.292	2.59	0.107
G. apicola	*C. bombi*	0.268	3.41	0.065	0.090	0.31	0.575	0.127	1.71	0.191
C. bombi	*S. alvi*	-0.008	0.00	0.981	0.134	0.20	0.651	-0.056	0.14	0.706
S. alvi	*C. bombi*	-0.036	0.03	0.865	0.052	0.29	0.592	-0.033	0.11	0.737
G. apicola	*S. alvi*	0.174	1.87	0.171	-0.087	0.18	0.673	0.096	0.83	0.363
S. alvi	*G. apicola*	0.152	0.45	0.501	-0.064	0.20	0.652	0.061	0.21	0.647

[a] Co-variation between microorganism abundance of adult worker bees was measured by linear mixed effects models (LMM). Linear mixed effects models (LMM) on one treatment group contained field identity as random factor; LMMs on the whole dataset contained field identity and field pair identity as random factors.

[b] *P*-values were calculated by likelihood ratio tests with one degree of freedom.

Supplementary Table 4 | Interactive effect of clothianidin exposure and microorganisms. Bee performance parameters were related to an interaction of microorganism abundance and clothianidin seed treatment or only microorganism abundance a using (generalized) linear mixed effects models ((G)LMM [b]).

Response (Sample; Model)	Predictor	Estimate	χ^2_1	P [c]
Body mass of adult workers (all colonies; LMM)	*Apicystis bombi* × treatment	-0.082 √mg	0.19	0.665
	Apicystis bombi	-0.010 √mg	0.42	0.518
	Crithidia bombi × treatment	0.061 √mg	0.11	0.739
	Crithidia bombi	0.127 √mg	2.26	0.132
	Gilliamella apicola × treatment	0.696 √mg	7.45	**0.006**
	Snodgrassella alvi × treatment	0.007 √mg	0.00	0.992
	Snodgrassella alvi	-0.162 √mg	1.47	0.226
Body mass of adult workers (control group; LMM)	*Gilliamella apicola*	0.011 √mg	0.00	0.969
Body mass of adult workers (clothianidin group; LMM)	*Gilliamella apicola*	0.651 √mg	11.42	**0.001**
Intertegular distance (all colonies; LMM)	*Apicystis bombi* × treatment	0.007 mm	0.03	0.859
	Apicystis bombi	-0.022 mm	1.49	0.222
	Crithidia bombi × treatment	0.015 mm	0.25	0.619
	Crithidia bombi	0.011 mm	1.06	0.304
	Gilliamella apicola × treatment	-0.034 mm	0.40	0.525
	Gilliamella apicola	-0.014 mm	1.68	0.195
	Snodgrassella alvi × treatment	-0.022 mm	0.17	0.680
	Snodgrassella alvi	-0.012 mm	0.47	0.493
Number of bees (cocoons + adults; all colonies; LMM)	*Apicystis bombi* × treatment	-12.997	1.41	0.234
	Apicystis bombi	8.135	0.40	0.529
	Crithidia bombi × treatment	9.799	0.85	0.355
	Crithidia bombi	-4.490	0.05	0.822
	Gilliamella apicola × treatment	-8.126	0.60	0.439
	Gilliamella apicola	27.315	1.79	0.181
	Snodgrassella alvi × treatment	-13.400	0.06	0.805
	Snodgrassella alvi	24.154	1.29	0.256
Number of queens (cocoons + adults; all colonies; GLMM)	*Apicystis bombi* × treatment	0.012	0.08	0.779
	Apicystis bombi	0.089	1.14	0.286
	Crithidia bombi × treatment	0.095	0.43	0.510
	Crithidia bombi	0.000	1.08	0.299
	Gilliamella apicola × treatment	0.283	1.79	0.181
	Gilliamella apicola	0.041	1.54	0.214
	Snodgrassella alvi × treatment	0.310	1.08	0.299
	Snodgrassella alvi	0.156	3.36	0.067
Worker/male cocoons (all	*Apicystis bombi* × treatment	-4.596	0.17	0.676

colonies; LMM)	*Apicystis bombi*	-8.946	3.70	0.054
	Crithidia bombi × treatment	12.053	0.75	0.386
	Crithidia bombi	-9.627	0.06	0.805
	Gilliamella apicola × treatment	32.978	3.17	0.075
	Gilliamella apicola	-13.012	0.47	0.495
	Snodgrassella alvi × treatment	26.885	1.91	0.166
	Snodgrassella alvi	-8.670	0.07	0.792
Number of adult workers (all colonies; LMM)	*Apicystis bombi* × treatment	17.313	3.63	0.057
	Apicystis bombi	-12.617	0.29	0.592
	Crithidia bombi × treatment	-19.716	3.29	0.070
	Crithidia bombi	13.005	0.00	0.980
	Gilliamella apicola × treatment	-7.337	0.27	0.600
	Gilliamella apicola	2.747	0.04	0.838
	Snodgrassella alvi × treatment	-17.570	1.83	0.176
	Snodgrassella alvi	6.009	0.00	0.949
Number of adult males (all colonies; GLMM)	*Apicystis bombi* × treatment	-0.084	0.75	0.388
	Apicystis bombi	0.030	0.00	0.969
	Crithidia bombi × treatment	0.054	0.17	0.681
	Crithidia bombi	0.064	2.11	0.146
	Gilliamella apicola × treatment	0.449	4.88	**0.027**
	Snodgrassella alvi × treatment	0.272	2.62	0.106
	Snodgrassella alvi	-0.178	5.22	**0.022**
Number of adult males (control group; GLMM)	*Gilliamella apicola*	-0.010	0.01	0.916
Number of adult males (clothianidin group; GLMM)	*Gilliamella apicola*	0.467	6.65	**0.010**
Colony weight (all colonies; LMM)	*Apicystis bombi* × treatment	-2.197 g	0.02	0.898
	Apicystis bombi	-0.864 g	0.04	0.835
	Crithidia bombi × treatment	2.631 g	0.02	0.883
	Crithidia bombi	8.701 g	0.69	0.408
	Gilliamella apicola × treatment	42.055 g	1.97	0.161
	Gilliamella apicola	-16.857 g	0.26	0.611
	Snodgrassella alvi × treatment	41.242 g	1.80	0.179
	Snodgrassella alvi	-20.327 g	0.09	0.765

[a] If P (microorganism abundance × treatment) < 0.05, the relation between bee performance parameter and microorganism abundance was analysed separately for each treatment group. Otherwise, the co-variation between bee performance parameter and microorganism abundance was estimated in a (G)LMM that contained in addition to microorganism abundance treatment as a fixed factor.

[b] LMMs (with normal error distribution) and GLMMs (with negative binomial error distribution and log link) on one treatment group predicting colony-level parameters contained field identity as random factor; (G)LMMs on all colonies contained field identity and field pair identity as random factors except for GLMMs on adult males, which contained only field identity as random factor to avoid model convergence failures. LMMs on individual-level parameters included additionally colony identity as fixed factor.

[c] P-values were calculated based on likelihood ratio tests with one degree of freedom. P values < 0.05 are highlighted in bold.

Supplementary Table 5 | Primers. Forward (F) and reverse (R) primers used in RNA/DNA assays with a literature reference to previous usage.

Target	Primers	Sequence '5-'3
Acute bee paralysis virus[1]	ABPV-F6548 (F) KIABPV-B6707 (R)	TCATACCTGCCGATCAAG CTGAATAATACTGTGCGTATC
Black queen cell virus[1]	BQCV-qF7893 (F) BQCV-qB8150 (R)	AGTGGCGGAGATGTATGC GGAGGTGAAGTGGCTATATC
Chronic bee paralysis virus[1]	CBPV1-qF1818 (F) CBPV1-qB2077 (R)	CAACCTGCCTCAACACAG AATCTGGCAAGGTTGACTGG
Deformed wing virus[1]	DWV-F8668 (F) DWV-B8757 (R)	TTCATTAAAGCCACCTGGAACATC TTTCCTCATTAACTGTGTCG
Lake Sinai Virus type-1[1]	qLSV1-F2569 (F) qLSV1-R2743 (R)	AGAGGTTGCACGGCAGCATG GGGACGCAGCACGATGCTCA
Lake Sinai virus type-2[1]	qLSV2-F1722 (F) qLSV2-R1947 (R)	CGTGCTGAGGCCACGGTTGT GCGGTGTCGATCTCGCGGAC
RNA250[2]	RNA250 (F) RNA250 (R)	TGGTGCCTGGGCGGTAAAG TGCGGGGACTCACTGGCTG
Bt-RPL23[3]	Bt-RPL23 (F) Bt-RPL23 (R)	GGGAAAACCTGAACTTAGGAAAA ACCCTTTCATTTCTCCCTTGTTA
Slow bee paralysis virus[4]	SBPV-F3177 (F) SBPV-B3363 (R)	GCGCTTTAGTTCAATTGCC ATTATAGGACGTGAAAATATAC
Sacbrood virus[1]	SBV-qF3164 (F) SBV-qB3461 (R)	GCTCTAACCTCGCATCAAC TTGGAACTACGCATTCTCTG
Apis mellifera filamentous virus[5]	AmFV-BroN (F) AmFV-BroN (R)	TTATTAACACCGCAGGCTTC CATGGTGGCCAAGTCTTGCT
Gregarine spp.[6, b]	Gregarine (F) Gregarine (R)	CCAGCATGGAATAACATGTAAGG GACAGCTTCCAATCTCTAGTCG
Crithidia bombi [a,b]	Cbombi-GADH-F2 (F) Cbombi-GADH-R2 (R)	CAAGAGCTCGCCGGGT GGACGCGTTCGACACC
Nosema apis [7]	Napis-qF3 (F) NosUniv-qR3 (R)	TAGTATATTTGAATATTTGTTTACAATGG CGCTATGATCGCTTGCC
Nosema ceranae [a, b]	Nceranae-qF3 (F) NosUniv-qR3 (R)	GTATGTTTGAATAATTATTTATTTTATTG CGCTATGATCGCTTGCC
Nosema bombi [a, b]	Nbombi-qF3 (F)	TAGTATGTTTGAATATTTATTTATTACGA

	NosUniv-qR3 (R)	CGCTATGATCGCTTGCC
Gilliamella apicola [a,b]	Gilliam 16S (F) Gilliam 16S (R)	GTAACATGAGTGCTTGCACT CGCATGGCCCGAAGG
Snodgrassella alvi [a,b]	Snodgras 16S (F) Snodgras 16S (R)	ACGGAGAGCTTGCTCTC AAATAACGCGAGGTCTTTCGA

[a] The primers were first used in this study.
[b] Identity of target organism confirmed by bidirectional Sanger sequencing of selected PCR products. In all cases, the PCR product sequences matched 100% with the intended target.

Supplementary Table 6 | Data analysis excluding the Biscaya sprayed field. Tests that resulted in a change of the level of significance (based on $\alpha = 0.05$) if the field that was sprayed with Biscaya (containing thiacloprid) was removed from the data analysis.

Response	**Predictor**	**Sample**	**Estimate**	χ^2_1	***P*** [a]
Crithidia bombi prevalence	Treatment	All colonies	-20.8%	4.33	**0.037**
Crithidia bombi prevalence	Treatment	Excluding Biscaya sprayed field	-20.1%	3.63	0.057
Worker/male cocoons	*Apicystis bombi*	All colonies	-8.946	3.70	0.054
Worker/male cocoons	*Apicystis bombi*	Excluding Biscaya sprayed field	-14.174	5.08	**0.024**

[a] *P*-values are based on likelihood ratio test on a generalized linear mixed effects model with field identity as random effect.

Supplementary References

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Chapter IV: EU-wide restrictions of neonicotinoids in bee-attractive crops have not eliminated the risk for bees

(Manuscript in preparation)

Preface and summary

In the previous two chapters, we studied the impact of clothianidin on honeybee and bumblebee colonies and determined strong effects on the reproduction of bumblebees and identified residues of various neonicotinoids in honeybees and the pollen and nectar they collected from oilseed rape plants that were not treated with those. In fact, clothianidin, imidacloprid and thiamethoxam are banned in bee-attractive crops, but are frequently detected in bee-collected plant residues or in untreated crops. Two other neonicotinoids are still authorized for use on bee-attractive crops in the European Union.

Therefore, we study here neonicotinoid residues in oilseed rape nectar, relate them to environmental conditions and determine whether they pose a lethal risk to foraging bees. For this, we repeatedly sampled nectar from almost 300 oilseed rape fields over the five years, in which neonicotinoid use in bee-attractive crops has been restricted. We analysed the concentrations of sugar and the five neonicotinoids authorized for plant protection in the European Union and coupled them with theoretical sugar consumption amounts and acute and chronic lethal doses to estimate the mortality risk at individual fields for honeybee, bumblebee and solitary bee foragers.

We detected four out of the five neonicotinoids at least once within the five years. Imidacloprid was the most prevalent neonicotinoid followed by thiacloprid. Neonicotinoid contamination showed no clear declining trend since they have been restricted in bee-attractive crops. Neonicotinoid residues tended to increase with precipitation in the days before sampling and to be higher on red than on calcareous soil. In the study period, agronomic imidacloprid use was practically restricted to winter cereals and imidacloprid prevalence but not concentrations increased with cereal

cultivation in the preceding year. At some of the fields we found exceptionally high imidacloprid concentrations, which posed a considerable mortality risk to foragers. We speculate that neonicotinoids contaminate the environment in a diffuse manner through mainly water.

We conclude that the neonicotinoid restrictions in bee-attractive crops have not eliminated the risk for foraging bees and that the risk of imidacloprid-induced mortality may be particularly high after a few rainy days when high imidacloprid residues may coincide with intensive foraging. Our results confirm the appropriateness of the recent decision by the European Union to ban all outdoor use of imidacloprid, clothianidin and thiamethoxam.

Résumé

Les restrictions sur l’usage des néonicotinoïdes dans les cultures mellifères n'ont pas éliminé le risque pour les abeilles qui butinent le nectar de colza

L'exposition chronique des abeilles à des doses sublétales de néonicotinoïdes dans le pollen et le nectar a été dénoncée dans le déclin des abeilles, ce qui a amené la Commission européenne à interdire l'utilisation de trois néonicotinoïdes dans des cultures attractives pour les abeilles. Cependant, les néonicotinoïdes sont encore utilisés dans les céréales d'hiver et la betterave à sucre et sont fréquemment détectés à des niveaux substantiels dans les fleurs sauvages ou les cultures n'ayant pas été traitées avec ceux-ci. Les modes d’application des néonicotinoïdes sont divers, mais les utilisations en traitement des semences sont les plus courantes. Les cultures à partir de semences enrobées ne prennent seulement qu’une petite partie de la substance active (~5%); l’excédent reste dans le sol ou est transporté ailleurs. Les néonicotinoïdes peuvent être disséminés par le vent lors du semis. Ils sont très persistants et relativement solubles dans l’eau. Ces propriétés assurent la protection systémique des cultures traitées mais facilitent également le transport dans l'eau et l'absorption par les cultures suivantes ou les adventices de proximité. Cependant, les conditions environnementales qui favorisent le transfert des cultures traitées aux autres plantes entomophiles ne sont pas encore connues.

Dans cette étude, nous cherchons à identifier les conditions de contamination les plus défavorables et à évaluer l'efficacité des restrictions imposées à l’usage de néonicotinoïdes dans l'Union européenne. Pour déterminer la prévalence et les concentrations de néonicotinoïdes dans le colza, une culture à floraison massive et attractive pour les abeilles, nous avons à plusieurs reprises quantifié les résidus de néonicotinoïdes dans le nectar de fleurs de colza provenant de 291 champs au cours des cinq années, alors que l'utilisation de ces substances n’était pas autorisées sur ces cultures. Ces parcelles ont été par ailleurs suivies dans le cadre d’une zone atelier, un site

de recherche sur le socio-écosystème à long terme, permettant de connaître précisément l'historique des cultures depuis 1994. Nous avons alors relié les résidus de néonicotinoïdes dans le nectar de colza aux cultures de céréales d'hiver et de colza des années précédentes. De plus, nous avons déterminé comment les résidus de néonicotinoïdes variaient avec le temps, le type de sol et les conditions météorologiques. Enfin, nous avons évalué le risque de mortalité induite par les néonicotinoïdes pour les butineuses d'abeilles mellifères, les bourdons et les abeilles solitaires.

Nous avons détecté quatre néonicotinoïdes sur cinq homologués sur cultures agricoles dans l'Union Européen. Malgré son interdiction dans le colza, l'imidaclopride était le néonicotinoïde le plus répandu. La prévalence et les concentrations d'imidaclopride ont fortement varié d'une année à l'autre, mais n'ont pas réellement diminué alors même que ce pesticide était interdit dans les cultures attractives pour les abeilles. En effet, en 2015, 5% des échantillons et champs étaient positifs à l'imidaclopride avec une concentration maximale de 0,7 µg L^{-1}, alors qu'en 2016, la concentration maximale était 100 fois supérieure, où cette substance était détectée dans près des deux tiers des échantillons et sur plus de 90% des parcelles. Le thiaclopride, utilisé quant à lui en pulvérisation sur le colza, a également été détecté fréquemment et à des concentrations élevées toutes les années sauf en 2018. Nous n'avons trouvé aucune relation claire entre la prévalence de ces néonicotinoïdes dans le nectar de colza et les conditions météorologiques sur la saison ou la culture des céréales pendant les campagnes antérieures. Cependant, la prévalence et les concentrations de néonicotinoïdes avaient tendance à augmenter avec les précipitations les jours précédant l'échantillonnage et à être plus élevées sur un brunisol rouge que sur un sol argilo-calcaire.

La prévalence de l'imidaclopride était plus grande dans les champs où des céréales semées en hiver avaient été cultivées l'année précédente, mais les concentrations d'imidaclopride n'étaient pas liées à la culture de céréales d'hiver des années précédentes. Les concentrations de thiaclopride, mais non la prévalence, ont augmenté avec le nombre d'années sans colza dans le même champ. L'évaluation des risques basée sur les concentrations de néonicotinoïdes, déterminées par les valeurs de la littérature sur la toxicité aiguë et chronique des néonicotinoïdes ainsi que sur le comportement de butinage

du nectar a montré que l'imidaclopride posait le plus grand risque pour les abeilles butineuses. Nous avons trouvé que dans de nombreuses parcelles, l'espérance de vie des abeilles domestiques, bourdons ou abeilles solitaires serait raccourcie et que, dans quelques cas, la majorité des abeilles mourraient dans les 10 jours suivant la recherche de nourriture. Au total, dans 6 des 291 champs examinés, 85% des butineuses d'abeilles mourraient dans les 10 jours suivant la recherche de nectar de colza. Pour les bourdons et les abeilles solitaires, un tel risque était estimé respectivement à 24 et 28 champs.

On ne sait pas pourquoi les concentrations de thiaclopride ont diminué alors que la culture du colza était plus fréquente les années précédentes sur le même champ. Une explication possible pourrait être que les agriculteurs qui cultivent fréquemment le colza oléagineux utilisent moins couramment cette stratégie de pulvérisation. Le lien relativement faible entre la présence d'imidaclopride dans le colza et sa source probable que représentent les céréales d'hiver, suggère que les néonicotinoïdes contaminent l'environnement de manière diffuse. La principale voie de propagation des néonicotinoïdes dans l'environnement est probablement l'eau, ce que conforte notre constatation selon laquelle la prévalence et la concentration de néonicotinoïdes ont eu tendance à augmenter avec les précipitations des jours précédents. Les néonicotinoïdes peuvent être mieux retenus sur un sol rouge en raison de leur plus grande capacité de rétention d'eau et être moins dégradés en raison d'une moins bonne aération qu'en sol calcaire.

Nous avons déterminé un risque important pour les butineuses de nectar avec des conséquences délétères probables pour les populations d'abeilles mellifères et sauvages. Pour les abeilles solitaires, les pertes élevées de butineuses se traduisent directement un déclin de la population. En revanche, les colonies d'abeilles domestiques peuvent compenser les pertes d'ouvrières individuelles, en raison de la capacité d'adaptation de leur structure sociale. Cependant, dans les champs où l'on a estimé que la plupart des butineuses meurent prématurément à cause de l'imidaclopride, des conséquences négatives sur le fonctionnement de la colonie et éventuellement sur la survie à long terme, peuvent être attendues.

Nous reconnaissons que l'exposition aux néonicotinoïdes pendant la durée de vie des abeilles peut varier par rapport à nos estimations car le nombre de jours de recherche de nourriture, de sélection de butinage et de contamination d'autres résidus végétaux varie. Cela peut augmenter ou diminuer le risque. Pour les abeilles sauvages, l'Autorité européenne de sécurité des aliments a extrapolé certaines doses létales depuis les valeurs pour l'abeille mellifère. Celles-ci pourraient représenter des surestimations possibles de la toxicité des néonicotinoïdes, mais pour les abeilles mellifères, la toxicité peut être supérieure à celle supposée, les doses létales variant entre les études et les sous-espèces.

Nos résultats suggèrent également que certaines conditions environnementales, en particulier les précipitations, peuvent augmenter le risque pour les abeilles butineuses. Les abeilles évitent généralement de butiner pendant les jours de pluie mais peuvent avoir besoin de compenser cette carence lorsque les précipitations cessent. Dans les jours qui ont suivi les fortes pluies, la prévalence et la concentration d'imidaclopride ont augmenté, ce qui suggère qu'une recherche intensive de nourriture coïnciderait avec une concentration élevée de résidus d'imidaclopride.

Par conséquent, nous concluons que le moratoire européen interdisant l'utilisation d'imidaclopride, de thiaméthoxame et de clothianidine dans des cultures attractives pour les abeilles n'a pas éliminé le risque pour les abeilles qui butinent du nectar de colza. Nos conclusions réaffirment la décision récente de l'Union européenne d'interdire complètement l'utilisation à l'extérieur de ces trois néonicotinoïdes à partir de décembre 2018. Les néonicotinoïdes sont toutefois encore largement utilisés en dehors de l'Union européenne, ainsi que dans les cultures sous serres au sein même de l'Union européenne. Par conséquent, il reste nécessaire de déterminer les facteurs qui régissent la propagation des néonicotinoïdes dans l'environnement et leur absorption par les plantes. La grande variabilité de la prévalence et de la concentration de néonicotinoïdes que nous avons déterminée suggère que les pesticides devraient être évalués de manière approfondie dans des conditions de terrain différentes, avant que des conclusions sur leur sécurité ne puissent être tirées.

PhD student’s contribution

I led the data analysis and the writing of the article. I also contributed to the collection of nectar samples in 2016, 2017 and 2018.

EU-wide restrictions of neonicotinoid use in bee-attractive crops have not entirely eliminated the risk for bees foraging on oilseed rape nectar

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Abstract

Chronic exposure of bees to sublethal dosages of neonicotinoids in pollen and nectar has been implicated in recent declines of bees, which led the European commission to ban the use of three neonicotinoids in bee-attractive crops in 2013. However, neonicotinoids are frequently detected in substantial levels in wild flowers or crops that had not been treated with these neonicotinoids. Neonicotinoids are highly persistent and relatively water-soluble. These properties ensure systemic protection of treated crops but also facilitate transport in water and the uptake by succeeding crops. However, it remains elusive what conditions favour carryover from treated crops to insect-pollinated plants. Therefore, we repeatedly quantified neonicotinoid residues in nectar of winter-sown oilseed rape flowers from 291 fields within a 435 km^2-large Long-Term Socio-Ecological Research site in western France with documented land use over the five years in which the EU moratorium on neonicotinoids has been in effect. We detected four out of the five neonicotinoids that are approved for plant protection in the EU including all three banned substances. Imidacloprid was the most prevalent neonicotinoid, but its prevalence differed strongly between years with about 5% of fields being positive in 2015 and over 90% in 2016. Similarly, imidacloprid concentration varied among years with maxima in 2016 exceeding concentrations typically found in treated crops, even though the insecticide has been banned in oilseed rape. Neonicotinoid prevalence and concentrations increased with rainfall in the days before sampling and tended to be higher on red soil than on calcareous soil. Based on the determined neonicotinoid concentrations, literature values on the acute and chronic toxicity of neonicotinoids as well as the foraging behaviour of nectar foragers, we estimated the mortality risk to bees foraging on oilseed rape nectar. We found that at many fields the life expectancy of some honeybees, bumblebees or solitary bees would be shortened and at a few fields the majority of bees would die within 10 days. Our findings reaffirm, therefore, the recent decision to ban the outdoor use of imidacloprid, clothianidin and thiamethoxam in all crops in the European Union.

Introduction

Neonicotinoids comprise the dominant class of insecticides in the world with a market share of almost 30% (Casida, 2018; Simon-Delso et al., 2015). At their launch in the 1990s, they were considered more environmentally friendly than the prevailing insecticides, due to lower application rates and higher specificity to insects (Tomizawa & Casida, 2011). Neonicotinoids ensure efficient and lasting protection from insect pests, because the persistent active compounds translocate throughout the plant and then overstimulate neurotransmitter receptors in the brains of feeding insects (Fishel, 2014; Van der Sluijs et al., 2013). Relative specificity to insects results from a higher affinity of the active compounds to the nicotinic acetylcholine receptors of insects than those of vertebras (Tomizawa & Casida, 2005).

Paradoxically, the characteristics that contributed to the economic rise and environment-friendly image of neonicotinoids are also the cause of environmental concerns. High persistence and water solubility do not only ensure systemic protection but also cause neonicotinoids to accumulate in the environment and contaminate ground and surface waters (Limay-Rios et al., 2016; Sánchez-Bayo, Goka, & Hayasaka, 2016; Schaafsma, Limay-Rios, Baute, Smith, & Xue, 2015; Van der Sluijs et al., 2013). The chronic exposure of bees to sublethal doses in pollen and nectar has, however, caused most concern and led, therefore, in 2013, to a European Union-wide moratorium on the use of three neonicotinoids – imidacloprid, thiamethoxam and clothianidin – in bee-attractive crops.

After the moratorium came into effect, it was criticized for being entirely based on artificial feeding experiments, which may overstate exposure (Carreck & Ratnieks, 2014). However, since then, field studies (Henry et al., 2015; Rundlöf et al., 2015; B. A. Woodcock et al., 2017) and UK-wide surveys (Budge et al., 2015; Ben A. Woodcock et al., 2016) have shown negative effects of neonicotinoid seed treatment in oilseed rape on bees. In addition, a laboratory study showed that bees do not actively avoid neonicotinoid contaminated nectar as presumed, but rather exhibit a preference, possibly due to an addictive effect (Kessler et al., 2015).

Widespread contamination of wild flowers suggests that the duration of exposure may have even been underestimated (Botías et al., 2015; David et al., 2016; Tsvetkov et al., 2017). Concentrations in wild flowers at field borders may exceed those of the treated crop (David et al., 2016) and be the main source of neonicotinoid in bee-collected pollen (David et al., 2016; Tsvetkov et al., 2017). Hazardous neonicotinoid concentrations were also found in flowers and honeybee-collected nectar of untreated oilseed rape (Henry et al., 2015; Thompson H et al., 2013; B. A. Woodcock et al., 2017). In fact, three quarters of the samples of a worldwide survey of neonicotinoids in honey were tested positive for at least one neonicotinoid (Mitchell et al., 2017). In the year after the moratorium came into effect, maximum neonicotinoid prevalence in UK honey samples coincided with oilseed rape flowering and concentrations increased with the area of oilseed rape surrounding the regarded honeybee hives (Ben A. Woodcock et al., 2018).

Contamination of non-target plants can originate from previous cultivations on the same field (Henry et al., 2015; Wood & Goulson, 2017) or treated crops in the surroundings (Bonmatin et al., 2015; Wood & Goulson, 2017). Neonicotinoid seed-dressed crops take up only a small portion of the active ingredient (~5%); the remainder stays on the field unless it is transported by wind or water (Wood & Goulson, 2017). Neonicotinoids are persistent in the environment with half-lives in aerobic soil conditions (Van der Sluijs et al., 2013) ranging from a few months to years (Bonmatin et al., 2015). Consequently, neonicotinoids are often found in soil and sometimes even several years after applications ceased (Hladik, Main, & Goulson, 2018; Jones, Harrington, & Turnbull, 2014).

To determine the effectiveness of the EU restrictions of neonicotinoid use in bee-attractive crops, we examine the temporal and spatial variability of the prevalence (presence/absence) and concentration of neonicotinoid residues in winter-sown oilseed rape fields under the EU moratorium which came into effect in December 2013. We link neonicotinoid residue levels to weather conditions (precipitation and temperature), soil type and the previous land use on the same field to gain insight into the pathways by which neonicotinoids travel from treated crops to oilseed rape. Finally, we assess for

honeybees, bumblebees and solitary bees foraging on oilseed rape nectar the risk of the five neonicotinoids that are currently authorized as plant protection products in the European Union.

Methods

The study site

The study was conducted in the 435 km^2-large Long-Term Socio-Ecological Research (LTSER) Zone Atelier "Plaine & Val de Sèvre" site, central western France (46°23'N, 0°41'W), which is characterized by a high proportion of arable land and an oceanic climate with mean monthly temperatures ranging from 5-20 °C and 820 mm of precipitation well distributed over the year. In the LTSER site, the broad soil type (Fig. 1) and the precise land use have been documented and mapped on vector-based GIS shapefiles since 1994 (Bretagnolle et al., 2018). Within the study period, ranging from 2014 to 2018, the land area was covered on average to 33.0% with wheat 6.1% barley, 1.4% other cereals, 9.7% maize, 9.3% sunflower, 7.2% oilseed rape, 8.0% grassland, 7.1% legumes (mostly alfalfa: 3.0%) and 4.6% other crops. Organic farmland covered 6.5% of the area.

Oilseed rape nectar sampling

Between 2014 and 2018, a total of 291 winter-sown oilseed rape fields were selected for nectar sampling with respect to land use in previous years to cover a diversity of crop succession combinations with varying frequencies of cereal and oilseed rape cultivation. To estimate repeatability and temporal variation in neonicotinoid residue concentrations, fields were sampled 1-6 times per year (Supplementary Fig. 1) on different days between 23 March and 10 June (Supplementary Table 1) and between 9 a.m. and 7 p.m. (Supplementary Fig. 2), yielding a grand total of 536 nectar samples. Approximately 25 µL of nectar per sample was collected directly from randomly selected open flowers that were at least 10 m from field margins to avoid edge effects using 5.0 µL capillaries (Drummond Scientific, Broomall, PA, USA).

Neonicotinoid residue analysis

Around 2 µL of nectar were used to determine the sugar content by hand-held refractometers (BS Eclipse BS 45-81 / 45-82, Bellingham + Stanley Ltd., UK). The rest was analysed using liquid chromatography with electrospray tandem mass spectrometry by the EU reference laboratory for neonicotinoid multi-residual analyses (ANSES, Sophia-Antipolis, France) to identify (limit of detection (LOD) = 0.1 µg L^{-1}) and quantify (limit of quantification (LOQ) = 0.3 µg L^{-1}) five common neonicotinoids: acetamiprid, clothianidin, imidacloprid, thiacloprid, thiamethoxam. Samples < LOD were set to zero in risk assessments and to 0.05 µg L^{-1} (LOD/2) in analyses of repeatability. For both statistical analyses of repeatability and risk assessments 0.2 µg L^{-1} ((LOD+LOQ)/2) was assigned to samples < LOQ but > LOD.

Weather data

Daily minimum and maximum air temperature and precipitation data were obtained from a weather station in the town Niort, which borders the study site to the north, by the US National Oceanic and Atmospheric Administration. The temperature data were used to calculate growing degree days for winter wheat (base temperature = 0 °C (Ruiz Castillo & Gaitán Ospina, 2016)) and oilseed rape (base temperature = 5 °C). Growing degree days and the amount of precipitation were accumulated over the growing seasons of winter wheat and oilseed rape. As they were put in relation to sampling year, the wheat growing season of the year before the oilseed rape cultivation from which nectar was sampled was regarded. The wheat growing season was estimated to range from 1 October two years prior to nectar sampling to 1 July of the year before nectar sampling and the oilseed rape growing season from 1 August of the previous year to the sampling date. When sampling dates differed within a year, a mean value of all samples was used.

Statistical analyses

Analysis of repeatability of neonicotinoid prevalence and concentration of different measures on the same field were restricted to imidacloprid in 2016, 2017 and 2018 as well as thiacloprid in 2016, as these were the only neonicotinoids with sufficiently high detection frequency in years with multiple measures in a majority of fields. Repeatability

was estimated by the intraclass coefficient (ICC) using the *rpt* function of the *rptR* package in *R* (Stoffel, Nakagawa, & Schielzeth, 2017). ICCs were estimated on the logit link scale for prevalence and on the normal scale for logarithmically transformed (log_{10}) concentration. In this case, concentrations <LOD were included in the analysis. In contrast, in all other analyses attempting to explain variability of neonicotinoid (imidacloprid or thiacloprid) concentrations, samples <LOD were excluded to analyse effects on concentration independently on effects on presence and to avoid zero inflated datasets. The relation between neonicotinoid residues and sugar content, temporal parameters (sampling year, sampling Julian day number, sampling time of day) or environmental parameters (soil type, precipitation, mean air temperature, previous land use) was analysed for both the binary (presence/absence) prevalence data and the (quantitative) concentration data of thiacloprid and imidacloprid. Generalized linear models (GLMs; thiacloprid) or generalized linear mixed-effects models (GLMMs; imidacloprid) with a binomial error distribution and a logit link were used for the prevalence data. For concentrations >LOD, analyses of variance (ANOVA; thiacloprid) or linear mixed-effects models (LMMs; imidacloprid) with a Gaussian error distribution were used. Models on imidacloprid prevalence/concentration contained field identity as random factor, while the detection frequency for thiacloprid was too low to include random effects. The analyses followed a stepwise procedure in which land use (oilseed rape & cereal cultivations in previous years) and meteorological variables (mean air temperature & precipitation in days before sampling) were pre-selected and then included in a full model that was compared to all reduced models (with the same random effect). Model selection was based on the Akaike information criterion (AIC). Four variables were pre-selected: (i) precipitation, (ii) mean temperature, (iii) cereal cultivation and (iv) oilseed rape cultivation. For this purpose, a range of univariate models (i.e. with only one fixed effect) related neonicotinoid prevalence/concentration to (i) the amount of precipitation in periods of 1-10 days that ended 0-4 days before sampling day (hereafter '*lag'*), (ii) mean air temperature in periods of 1-5 days with a lag of 0-3 days (iii) the numbers of years passed since the last cereal cultivation (iv) the number of cereal cultivations in the previous 1-5 years, (v) the number of years passed since the last oilseed rape cultivation and (vi) the number of oilseed rape cultivations in the previous 2-

5 years. In the final step of the model selection procedure, all four pre-selected variables were included, if their univariate models (only one fixed effect) yielded a yielded a lower AIC than the null model (i.e. without a fixed effect). In addition to the pre-selected meteorological and land use variables, sugar content, time of day, year, Julian day number and soil type were included in the final model selection step, in which this full model was compared to all possible reduced models with the same random effect (i.e. none for thiacloprid and field identity for imidacloprid). If soil type was retained in the model and $P<0.05$, then Tukey post-hoc tests were used to determine whether neonicotinoid residues differed between individual soil types. Explained variability was determined by coefficients of determination or their equivalent for models fit on (restricted) maximum likelihood. These pseudo R^2 values were determined using Nagelkerke's method.

All analyses were done in *R* version 3.5.0. LMMs were fit using '*lmer'* of the '*lme4*' package. GLMMs for model selection were fit using '*glmmTMB'* of the '*glmmTMB'*. However, for illustrations the selected model was re-fit using '*glmer'* of the '*lme4*' package because '*glmmTMB'* does currently not allow to set random effects to zero. Pseudo coefficients of determination were obtained using the '*PseudoR2'* function of the '*DescTools*' package for the GLM on thiacloprid prevalence, while the '*r.squaredGLMM'* function of the *'lmtest'* package was used for models on imidacloprid prevalence or concentration. For (G)LMMs on imidacloprid, both marginal and conditional coefficients of determination were reported to show the theoretical variance explained by fixed factors only (R^2_m) and by the entire model (R^2_c), respectively. Tukey post-hoc analyses were conducted using the *'glht'* function of the *'multcomp'* package.

Risk assessment for foragers

We simulated acute and chronic mortality for honeybees, bumblebees and solitary bees foraging for 10 days on oilseed rape nectar to determine whether neonicotinoid exposure through oilseed rape can shorten their life span. The risk assessment included all four neonicotinoids that were detected as long as median lethal doses were available for the regarded bee type or scenario (acute/chronic). Honeybees forage about 10 days in their

lives (Schippers et al., 2006), while bumblebees and solitary bees can forage over even longer periods (Evans, Smith, & Raine, 2017; Michener, 2007). Honeybees, bumblebees and solitary bees forage intensively on oilseed rape during its bloom (Rollin et al., 2013; Stanley, Gunning, & Stout, 2013; Stanley & Stout, 2014; Van Reeth, Caro, Bockstaller, & Michel, 2018) and particularly honeybees but also some wild bees show high floral constancy (Amaya-Márquez, 2009; Gruter, Moore, Firmin, Helantera, & Ratnieks, 2011; Stout, Allen, & Goulson, 1998). Therefore, we assumed that bees would forage throughout the regarded timeframe on the same oilseed rape field. We adapted the risk assessment used by the European Food Safety Authority (EFSA) to obtain Exposure Toxicity Ratios (ETRs) for acute and chronic toxicity for 1000 hypothetical bees of each type per field (EFSA, 2013, 2014). ETRs constitute the quotient of an expected environmental dose (i.e. the neonicotinoid residue intake) and a median lethal dose. We related ETRs to a probability of death, which we then used to simulate death of individual foragers.

In a first step, neonicotinoid concentrations per volume were converted to mass fractions in ppb (i.e. µg kg^{-1}), by dividing them by the density of the nectar sample, ρ_{nectar} (in kg L^{-1}), which was estimated by the sugar content ω_{sugar} (in kg kg^{-1}), and assumed densities of water (1 kg L^{-1}) and sugar (1.6 kg L^{-1}) as follows:

$$\rho_{\mathrm{nectar}} = \left(1 + 0.6\,\omega_{sugar}\right) \quad \text{(equation 1).}$$

For each bee type, a theoretical normal distribution of daily sugar consumption amounts was derived from reported ranges of daily sugar consumption (in mg; Supplementary Fig. 3). The normal distributions were centered on the mean of the reported minimum and maximum sugar consumption amounts and standard deviations were estimated by a quantile function (qnorm) with alpha being set to 0.01, so that 99% of the estimated daily sugar consumption amounts were within the reported ranges (EFSA, 2014):

$$s.d. = \frac{mean - min}{qnorm(1 - alpha)} \quad \text{(equation 2).}$$

For 1000 bees per field and bee type, we randomly selected daily sugar consumption amounts from these probabilistic distributions and neonicotinoid mass factions with corresponding sugar content values from the available measures (if a field was more than once sampled) to calculate daily residue intake (µg bee^{-1}) as follows (EFSA, 2013):

$$daily\ residue\ intake = \frac{neonicotinoid\ mass\ fraction}{sugar\ content} \times \frac{daily\ sugar\ consumption}{\frac{10^6\ mg}{kg}}$$ (equation 3).

For acute mortality, ETRs for each bee type, field and neonicotinoid were then obtained by dividing residue intake over two days by twice the acute median lethal dose (48 h-LD_{50}, in µg bee^{-1}), as the LD_{50} is determined over two days but expressed as per day. Analogously, for chronic mortality, residue intake accumulated over ten days was divided by the tenfold chronic dietary median lethal dose (10 d-LDD_{50}, in µg bee^{-1} d^{-1}, Supplementary Table 2, EFSA 2018a; b; c). Bee mortality was subsequently simulated based on an assumed relationship between probability of death and ETR. For ETR≥0.1, probability of death was assumed to follow a logistic regression with ETR=0.1, corresponding to 10% mortality (Sanchez-Bayo & Goka, 2014) and ETR=0.5 corresponding to 50% mortality, while for ETR<0.1 no mortality was assumed (Supplementary Fig. 4). Mortality was simulated in five 2-day periods for acute mortality and in one 10-day period for chronic mortality. Finally, we calculated the proportion of bees that would die a premature death due to neonicotinoid-induced toxicity per field and determined then the proportion of fields that had mortality rates higher than threshold values between 0-100% in 1%-intervals.

Results

Prevalence and concentration of neonicotinoids in oilseed rape nectar

All neonicotinoids screened for, except acetamiprid, have been detected at least once within the sampling period of five years. Overall, 43% of samples were positive for exactly one neonicotinoid, 3.9% for two neonicotinoids and one sample contained three neonicotinoids. Despite never having been authorized for use on oilseed rape in France, imidacloprid was by far the most prevalent neonicotinoid, although with strong inter-annual variation in prevalence. In 2014 and 2016, imidacloprid was detected in over 60% of the samples (Fig. 2a). Due to repeated measures, the proportion of imidacloprid-positive fields was even higher, exceeding 90% in 2016. In contrast, in 2015, imidacloprid was detected in only 5.4% of the samples/fields. Thiacloprid, which has been authorized for spray applications on oilseed rape in France throughout the study period, was detected in all years except 2018 with the maximum prevalence being 13% in 2015. We also found that neonicotinoid concentrations spanned a wide range. Overall, a small portion of the samples exceeded 1 µg L^{-1} of imidacloprid (3.4%) or thiacloprid (2.4%), but some showed extremely high concentrations with maxima being 70 µg L^{-1} for imidacloprid and 541 µg L^{-1} for thiacloprid (Fig. 2b, Supplementary Fig. 5).

Imidacloprid was frequently enough detected to allow for the analysis of repeatability in the three years (2016, 2017 & 2018) in which most fields were multiple times sampled, while for thiacloprid this could only be done in 2016. Repeatability in prevalence was very high for thiacloprid in 2016 and for imidacloprid in 2017 (ICC~0.8, P<0.001; Supplementary Table 6). We found also in 2016 repeatability of imidacloprid prevalence, but at a considerably lower level (ICC=0.13, P=0.019). No repeatability of imidacloprid prevalence was found in 2018 when generally only two measures per field were available (Supplementary Fig. 1). Repeatability was lower for concentration than for prevalence and statistically non-significant in 2016 and 2018 for imidacloprid. However, $\log_{10}$ concentrations of thiacloprid in 2016 (ICC=0.38, P=0.002) and of imidacloprid in 2017 (ICC=0.57, P<0.001) were nonetheless moderately repeatable.

Relation between neonicotinoid residues and weather, time, soil or biological variables

Inter-annual differences in the prevalence of the two most frequently detected neonicotinoids (imidacloprid and thiacloprid) were not clearly linked to weather conditions (growing degree days and precipitation) in the oilseed rape growing season or the preceding wheat growing season (Supplementary Fig. 7). To determine which factors influenced imidacloprid and thiacloprid prevalence and concentration we conducted model selection analyses. These indicated that imidacloprid prevalence and concentration were related to year, Julian day number and precipitation (Table 1). In addition, imidacloprid prevalence was higher if cereals were cultivated in the preceding year on the same field (Fig. 3). Imidacloprid concentration was related to soil type with higher concentrations on red soil than on calcareous soil - a tendency that was also observed for imidacloprid prevalence (Table 1, Fig. 3). However, the difference between imidacloprid concentrations in red soil and shallow calcareous (P=0.066) or deep calcareous (P=0.064) soil was not statistically significant if a Tukey post-hoc correction was applied. Imidacloprid concentration decreased moderately with sugar content. Precipitation prior to sampling increased both prevalence and concentration of imidacloprid, although the regarded period of rainfall was considerably longer for prevalence than for concentration (Table 1).

The prevalence and concentrations of thiacloprid were not explained by the same factors. Thiacloprid prevalence varied between years, increased with time of day and decreased with sugar content, temperature and the number of cereal cultivations (Table 1, Supplementary Fig. 8). Thiacloprid concentration increased with precipitation and the number of years that oilseed rape has not been cultivated on the same field (Table 1, Supplementary Fig. 9). Just as imidacloprid concentration, thiacloprid concentration was higher on red soil than on calcareous soil. This difference remained statistically significant for the difference to both shallow (P=0.0279) and deep (P=0.026) calcareous soil when P-values were post-hoc adjusted.

Although pseudo coefficients of determination for models fit by (restricted) maximum likelihood are only too some extent comparable to coefficients of determination for ordinary least squares regressions, the selected models seemed to explain the prevalence of imidacloprid (R^2_m=0.45, R^2_c=0.64) better than its concentration (R^2_m=0.22, R^2_c=0.22). For thiacloprid the reverse was observed (prevalence: R^2=0.28; concentration: R^2=0.54).

Risk to foragers

Imidacloprid was the neonicotinoid that posed the highest risk. For honeybees, risk peaked in 2014 and 2016 (Fig. 4), with an estimated 30% of nectar foragers likely to die due to imidacloprid in 5% of fields in 2014 and 8% of fields in 2016. At 7% of fields in 2016, over 85% loss within 10 days was estimated. For wild bees an even higher risk was estimated with imidacloprid-induced mortality in all years (Fig. 4). For bumblebees, we determined in all years except 2015 fields where more than 30% of foragers would die and in 2014, 2016 and 2018 fields where more than 50% of foragers would die within 10 days. At around 11% of fields in 2014 and 2016, 85% of bumblebee foragers had a reduced life expectancy. For solitary bees a very similar risk pattern was found. However, in the year with the highest risk, 2016, a moderate risk was found at slightly fewer fields but at a high risk at more fields (Fig. 4). The proportion of fields with 85% solitary bee mortality was 19% in 2016 (11% for bumblebee foragers); however, in 2014 no difference was found in this proportion between solitary bees and bumblebees.

The most striking difference between risk estimates for solitary and bumblebee foragers was the proportion of fatalities due to acute toxicity. Estimated bumblebee losses were driven by chronic mortality (Fig. 4), while acute toxicity was negligible. In contrast, both acute and chronic mortality were relevant for solitary bees and honeybees (Fig. 4). Chronic mortality in bumblebees is based on a lethal dose that has been extrapolated from honeybees by EFSA (Supplementary Table 2, (EFSA, 2018b), and both acute and chronic median lethal doses for solitary bees were extrapolated from lethal doses for honeybees. For honeybees and solitary bees, chronic mortality was estimated to cause some mortality at a larger proportion of fields, while acute mortality was typically responsible for the extremely high mortality estimates at some fields (Fig. 4).

No risk for honeybees from neonicotinoids other than imidacloprid was determined. However, in one field in 2014, chronic clothianidin exposure was estimated to cause 12% mortality in solitary bees and 17% in bumblebee foragers. For thiamethoxam, 40% acute mortality of solitary bees was determined in 2017 at one field. Even though, thiacloprid was found in the highest concentrations, no risk of acute mortality for honeybee foragers was determined due to the lower toxicity of cyano-substituted neonicotinoids compared to nitro-substituted neonicotinoids (Iwasa, Motoyama, Ambrose, & Roe, 2004).

Discussion

We found that neonicotinoid contamination of oilseed rape nectar is widespread despite EU-wide restrictions on the application of three neonicotinoids in bee-attractive crops. Imidacloprid, whose use has been banned in oilseed rape since December 2013, was found in all years and sometimes in a considerable fraction of fields. Generally, the prevalence of neonicotinoid contamination varied strongly among years, but no clear decline with the time passed since the introduction of the EU moratorium in 2013. We detected imidacloprid not only frequently but sometimes also in remarkably high concentrations. In 2016, imidacloprid concentrations were higher than what is typically found in the nectar of crops treated with imidacloprid (Bonmatin et al., 2015).

During the study period, imidacloprid use was restricted to winter-sown cereals in our study area. Indeed, we found that imidacloprid was more frequently detected in oilseed rape crops that succeeded winter cereal cultivation. The concentrations in positive samples were, however, not related to winter cereal use in previous years on the same field. The relatively weak link between imidacloprid occurrence in oilseed rape and its likely source, winter cereals, suggest that neonicotinoids contaminate the environment in a diffuse manner. Our analyses focused on the hypothesis that neonicotinoids are taken up by plants from residues remaining in the soil after cereal cultivation. Alternatively, imidacloprid use in cereals may contaminate oilseed rape through dust drift.

In fact, neonicotinoids can travel from treated crops to insect-pollinated plants at the moment of sowing through contaminated dust (Girolami et al., 2013; Greatti, Barbattini, Stravisi, Sabatini, & Rossi, 2006; Greatti, Sabatini, Barbattini, Rossi, & Stravisi, 2003; Krupke, Hunt, Eitzer, Andino, & Given, 2012; Pistorius, Bischoff, Heimbach, & Stähler, 2010; Pochi, Biocca, Fanigliulo, Pulcini, & Conte, 2012; Tapparo et al., 2012) or later on, through wind eroded soil (Limay-Rios et al., 2016; Schaafsma et al., 2015). To be systemic, neonicotinoids have to be fairly water-soluble (Bonmatin et al., 2015; Giorio et al., 2017), which makes them prone to transport by water in leachate or run-off (Huseth & Groves, 2014; Kurwadkar, Wheat, McGahan, & Mitchell, 2014).

Although moisture content and soil temperature can influence degradation and leaching in soils (Bonmatin et al., 2015), air temperature and precipitation in the beginning of the year or in previous year did not explain imidacloprid or thiacloprid prevalence. However, weather conditions in the days before sampling of oilseed rape nectar affected its neonicotinoid content. Imidacloprid prevalence and concentration and thiacloprid concentration increased with rainfall and tended to be higher on red than on calcareous soil. Neonicotinoids may be better retained on red soil in the region because of the finer texture, higher content of the clay mineral kaolinite and higher water holding capacity compared to the calcareous soil. Soil texture affects the leaching potential of neonicotinoids, which is highest in sandy soils and lowest in loams (Wood & Goulson, 2017) and neonicotinoids are much more persistent under aerobic than anoxic conditions (Giorio et al., 2017).

We found that the detected imidacloprid concentrations pose a substantial risk to bees foraging on oilseed rape nectar. At a large proportion of fields in several years, a reduction in forager longevity was estimated and, in a few fields, imidacloprid toxicity was estimated to kill the majority of foragers within 10 days. Overall, at 6 out of 291 examined fields 85% of honeybee foragers would die within 10 days of foraging on oilseed rape nectar. For bumblebees and solitary bees such a high risk was estimated at 24 and 28 fields, respectively.

We acknowledge a considerable uncertainty in the mortality estimates. We assumed that bees would forage exclusively on oilseed rape. This is likely an overestimation of oilseed rape use for many foragers, despite high floral constancy of many bee species (Amaya-Márquez, 2009; Gruter et al., 2011; Stout et al., 1998) and the attractiveness of mass-flowering oilseed rape (Rollin et al., 2013; Stanley et al., 2013; Stanley & Stout, 2014; Van Reeth et al., 2018). However, this does not imply an overestimation of neonicotinoid exposure risk. Neonicotinoids are frequently found in wild flowers (Botías et al., 2015; David et al., 2016; Tsvetkov et al., 2017) and the neonicotinoid that posed the largest risk to foragers in our study was imidacloprid, which was not applied to oilseed rape. Therefore, alternative forage does not necessarily contain less neonicotinoids. Besides, we restricted our analyses to nectar even though pollen contains often higher neonicotinoid concentrations (Bonmatin et al., 2015). The assumed foraging timespan of 10 days is a worst-case scenario for honeybees (Rortais, Arnold, Halm, & Touffet-Briens, 2005; Visscher & Dukas, 1997), but wild bees can forage over longer periods (Evans et al., 2017; Michener, 2007), which may imply even higher probabilities of premature death. To translate estimated exposure to mortality rates, we assumed a logistic dose-response curve with 10% mortality at ETR=0.1 and 50% mortality at ETR=1, which is typical for pesticides in many species (Sanchez-Bayo & Goka, 2014) and this seems to fit well imidacloprid toxicity for honeybees (Cresswell, 2011). Thereby we principally relied on the same lethal doses that EFSA uses for their risk assessments (EFSA, 2018b). However, there is some variability in dose responses and median lethal doses (Cresswell, 2011; Decourtye & Devillers, 2010). In addition, toxicity varies considerably between taxa and even between different subspecies of *Apis mellifera* (Suchail, Guez, & Belzunces, 2000).

Our risk assessment suggested a higher risk for individual wild bees than for individual honeybees. However, we used lethal doses for bumblebees and solitary bees that have been extrapolated by the European Food Safety Authority (EFSA) from values derived from honeybees (EFSA, 2018b). Therefore, the mortality risk is not necessarily higher for individual wild bees, but nonetheless implications for wild bee populations may be more severe than for honeybees. For solitary bees, elevated forager losses translate directly into

population declines. In contrast, social bees and particularly honeybees, which live in large colonies, can compensate for the loss of individual foragers (Henry et al., 2015) and may therefore be less susceptible to insecticide effects (Osterman et al., 2019; Rundlöf et al., 2015).

To estimate the risk for whole colonies accurately, a more advanced modelling approach would be needed that takes sublethal effects into account and determines mortality rates not only for foragers but also for in-hive bees. The approach would also need to take into account the reproductive capacity of colonies, its adaptability to individual bee losses and neonicotinoid effects on these.

However, the estimated proportion of honeybee foragers that would die a premature death due to imidacloprid exposure was so high that declines in colony functioning could be expected. Even in fields with moderate loss rates, long-term effects may occur. Elevated forager mortality may trigger for instance bees to forage at a younger age with negative consequences for brood care, foraging efficiency and worker longevity (Khoury, Myerscough, & Barron, 2011). All these aspects can also be affected by exposure to sublethal levels of neonicotinoids (Godfray et al., 2015; L. Pisa et al., 2017).

We found that neonicotinoid prevalence and concentrations vary with environmental conditions and particularly precipitation may substantially increase the risk of imidacloprid toxicity for foraging bees. Both the prevalence and concentration of imidacloprid increased with rainfall in the previous days. Bees, in particular honeybees, avoid foraging during rainy days (Javorek, Mackenzie, & Vander Kloet, 2002). Therefore, they may forage more intensively when rainfall ceases, which implies that intensive foraging and high prevalence and concentration would coincide. In bee species that do not store such large food reserves as honeybees, bees may additionally be starved and therefore more susceptible to pesticide effects.

We conclude that the EU moratorium restricting imidacloprid, thiamethoxam and clothianidin use in bee-attractive crops has not eliminated the risk for bees foraging on

oilseed rape nectar. This suggests that restricting bans on systemic insecticides to insect-pollinated crops does not eliminate the risk for bees. We therefore reaffirm the recent decision by the European Union to ban the outdoor use of these three neonicotinoids completely from December 2018 onwards. Neonicotinoids are, however, still used extensively outside the European Union as well as in permanent greenhouses within the European Union. Therefore, there remains a need to determine the factors that govern neonicotinoid spread in the environment and uptake by plants. The large variability in neonicotinoid prevalence and concentration that we determined suggests that pesticides should be extensively assessed under differing field conditions, before conclusions on their safety can be drawn.

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Tables

Table 1 | Model statistics of selected models on imidacloprid and thiacloprid prevalence and concentrations in positive samples.

Response	Predictor	Estimate	$\chi 2$	*DF* [a]	*P*
Imidacloprid prevalence	Sampling year	N/A	73.63	4	**<0.001**
	Julian day number	-0.044	7.52	1	**0.006**
	Sampling time	1.902	2.12	1	0.145
	Precipitation (10 d; 4 d lag [b])	0.401	11.49	1	**0.001**
	Temperature (4 d; 2 d lag [b])	-0.126	2.63	1	0.105
	Soil type	N/A	5.87	2	0.053
	Cereal cultivation in previous year	1.350	3.92	1	**0.048**
Imidacloprid concentration	Sampling year	N/A	13.95	4	**0.007**
	Julian day number	-0.010	8.80	1	**0.003**
	Sugar content	-0.002	5.61	1	**0.018**
	Precipitation (1 d; 3 d lag [b])	0.052	9.09	1	**0.003**
	Soil type	N/A	7.47	2	**0.024**
Thiacloprid prevalence	Sampling year	N/A	24.01	4	**<0.001**
	Sampling time	6.022	9.71	1	**0.002**
	Sugar content	-0.031	9.27	1	**0.002**
	Cereal cultivations in 3 years	-0.698	3.74	1	0.053
	Temperature (2 d)	-0.488	25.57	1	**<0.001**
Thiacloprid concentration	Precipitation (1 d; 2 d lag [b])	0.289	8.05	1	**0.005**
	Soil type	N/A	9.73	2	**0.008**
	Years without oilseed rape	0.096	12.75	1	**<0.001**

[a] *DF* = Degrees of freedom

[b] lag = number of days before sampling that the regarded period ended

Figures

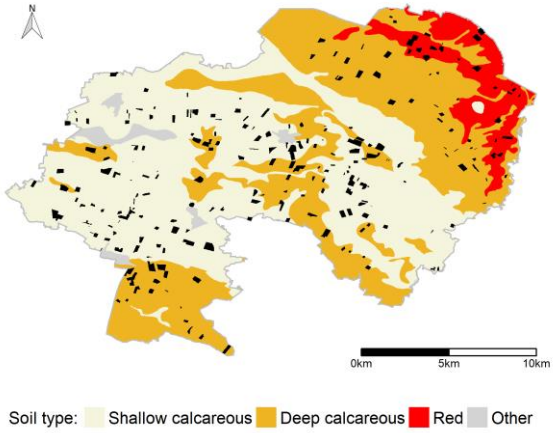


Figure 1 | Study site. Location of the oilseed rape fields (black) that nectar was collected from within the study period (2014-2018) and broad soil types within the Long-Term Socio-Ecological Research (LTSER) site *'Plaine & Val de Sèvre'*.

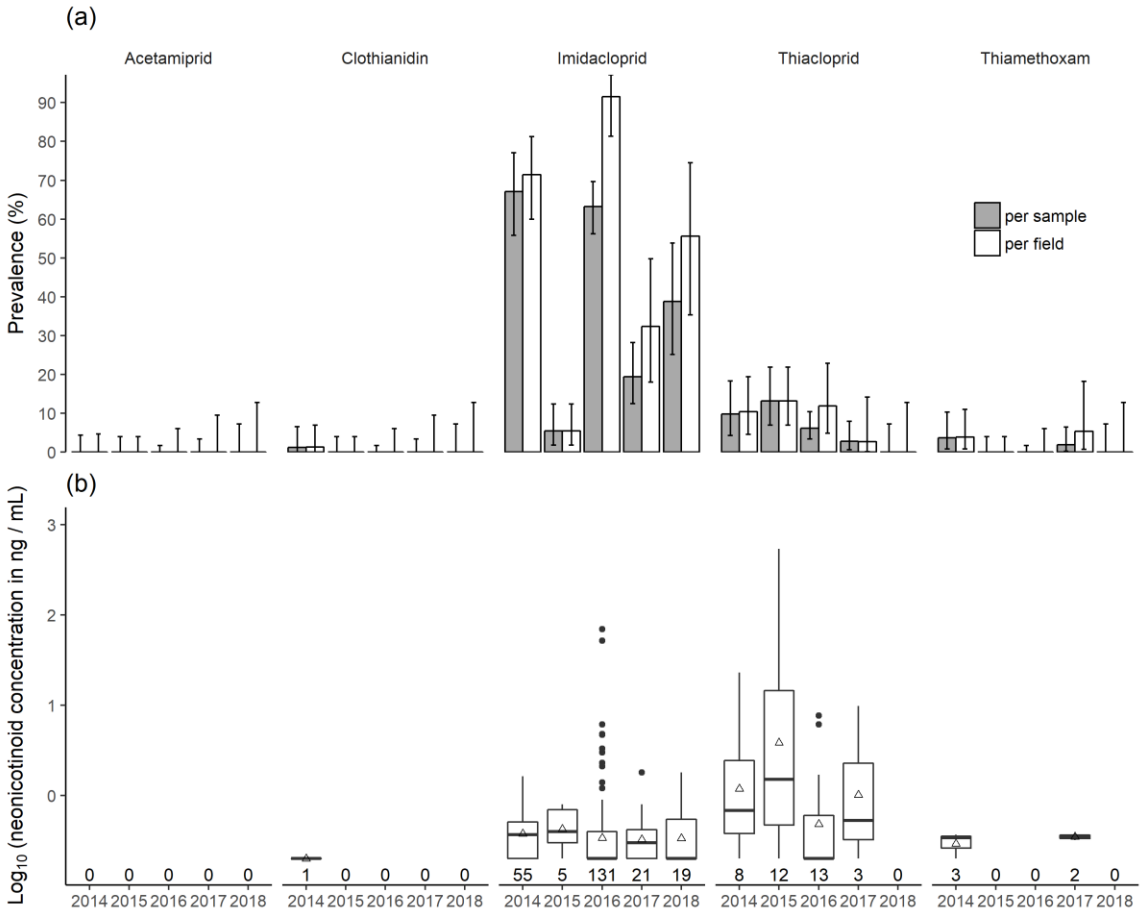


Figure 2 | Prevalences and concentrations of five common neonicotinoids in oilseed rape nectar by year. (a) prevalences are shown per sample (grey) and per field (white). Error bars denote 95% confidence intervals computed by binomial tests. (b) Tukey boxplots show $\log_{10}$ neonicotinoid concentrations in positive samples with horizontal lines denoting median values and triangles mean values. The bottom and the top of the boxed show the first and third quartiles, respectively. Whiskers illustrate minimum and maximum values within 1.5 interquartile ranges. The dots denote outliers. The number of positive samples is shown below the plots.

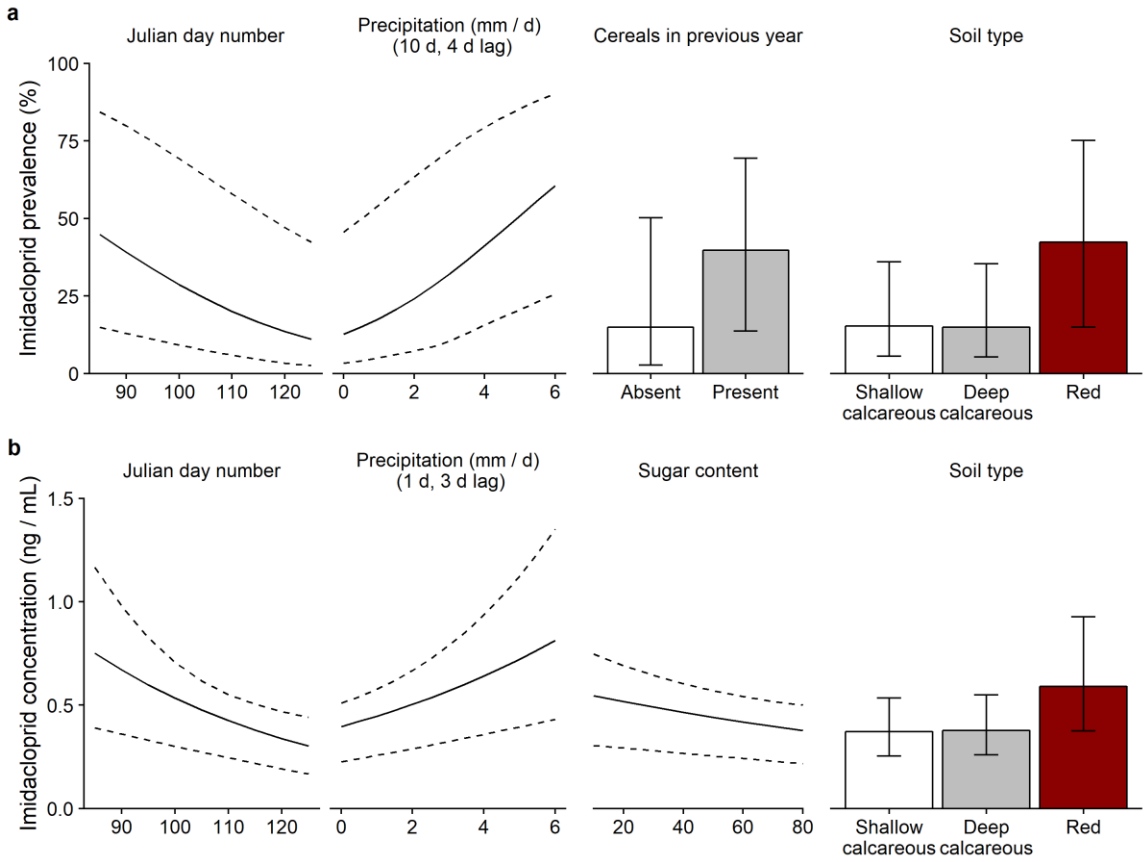


Figure 3 | Prevalence and concentration of imidacloprid in oilseed rape nectar in relation to selected predictors. Estimates and 95% confidence were obtained from (generalised) linear mixed effects models ((G)LMMs) containing field identity as random factor. For prevalence (a), GLMMs with a binomial error distribution and a log-link contained sampling Julian day number, precipitation in a 10-day period ending 4 days before sampling, presence of cereals on the same field in the preceding year, soil type, sampling time of day and mean air temperature in a 4-day period ending 3 days prior to nectar sampling as fixed factors. For concentration in samples in which imidacloprid was detected (b) LMMs (with a Gaussian error distribution) contained sampling Julian day number, precipitation in a 1-day period ending 3 days before sampling, sugar content of the nectar sample and soil type as fixed factors.

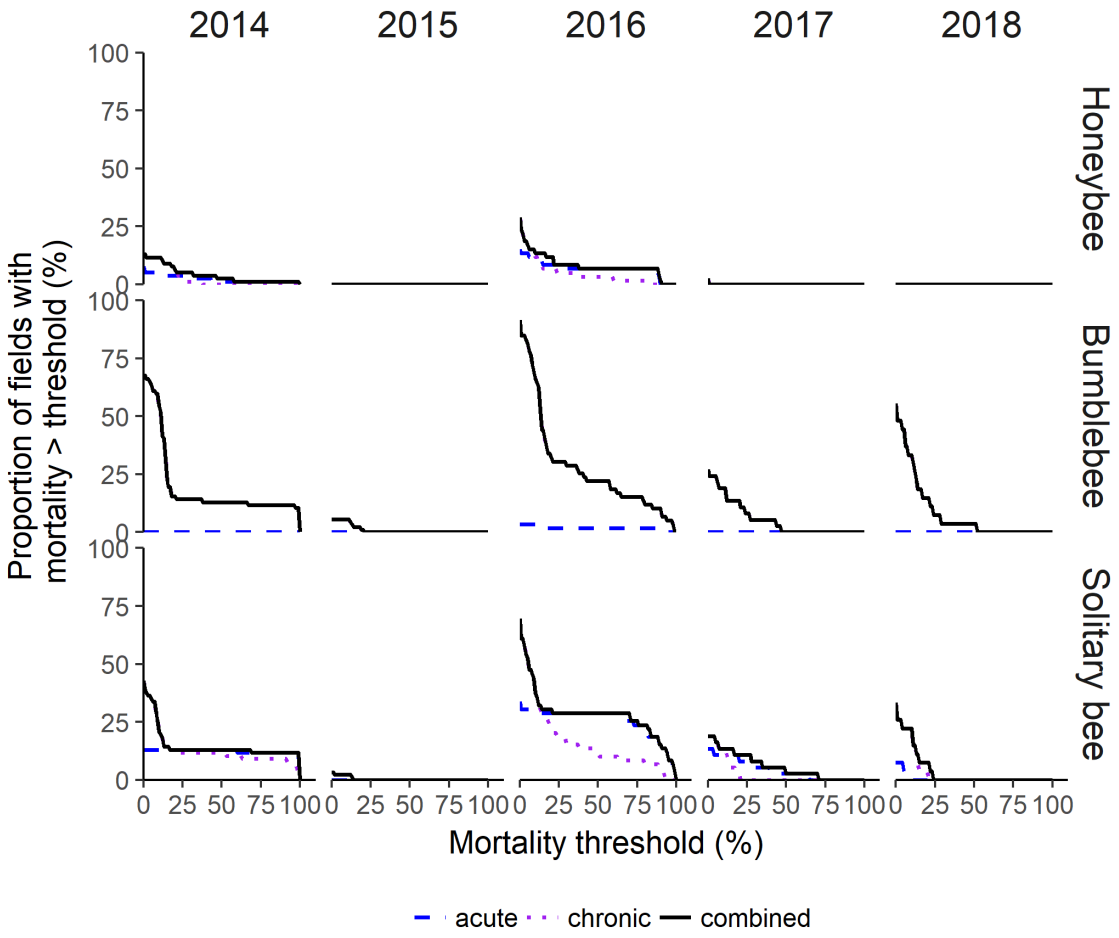


Figure 4 | Mortality risk for nectar foragers from imidacloprid exposure through oilseed rape over 10 days. Percentage of fields at which the estimated mortality of honeybees, bumblebees and solitary bees due to acute and chronic imidacloprid toxicity within 10 days of foraging exceeds a range of threshold values.

Supplementary Information

Supplementary Tables

Supplementary Table 1 | Sampling dates. Date of first and last sampling day as well as number of sampling days per year.

Year	First sampling	Last sampling	Sampling days
2014	15 April	24 April	5
2015	14 April	29 April	6
2016	23 March	10 May	12
2017	5 April	25 April	8
2018	13 April	9 May	5

Supplementary Table 2 | Median lethal doses. Acute oral median lethal dose (48 h-LD_{50}, in μg bee^{-1}) & chronic dietary median lethal dose (10 d-LDD_{50}, in μg bee^{-1} d^{-1})

Bee type	Scenario	Clothianidin [a]	Imidacloprid [b]	Thiacloprid [c]	Thiamethoxam [d]
Honeybee	Acute	0.0258	0.0037	12.6	0.005
	Chronic	0.00095	0.00282	N/A	N/A
Bumblebee	Acute	0.001911	0.038	N/A	0.005
	Chronic	0.000095	0.000282	N/A	N/A
Solitary bee	Acute	0.000379	0.00037	N/A	0.0005
	Chronic	0.000095	0.000282	N/A	N/A

References:

[a] (EFSA, 2018a); [b] (EFSA, 2018b); [c] (L. W. Pisa et al., 2015); [d] (EFSA, 2018b)

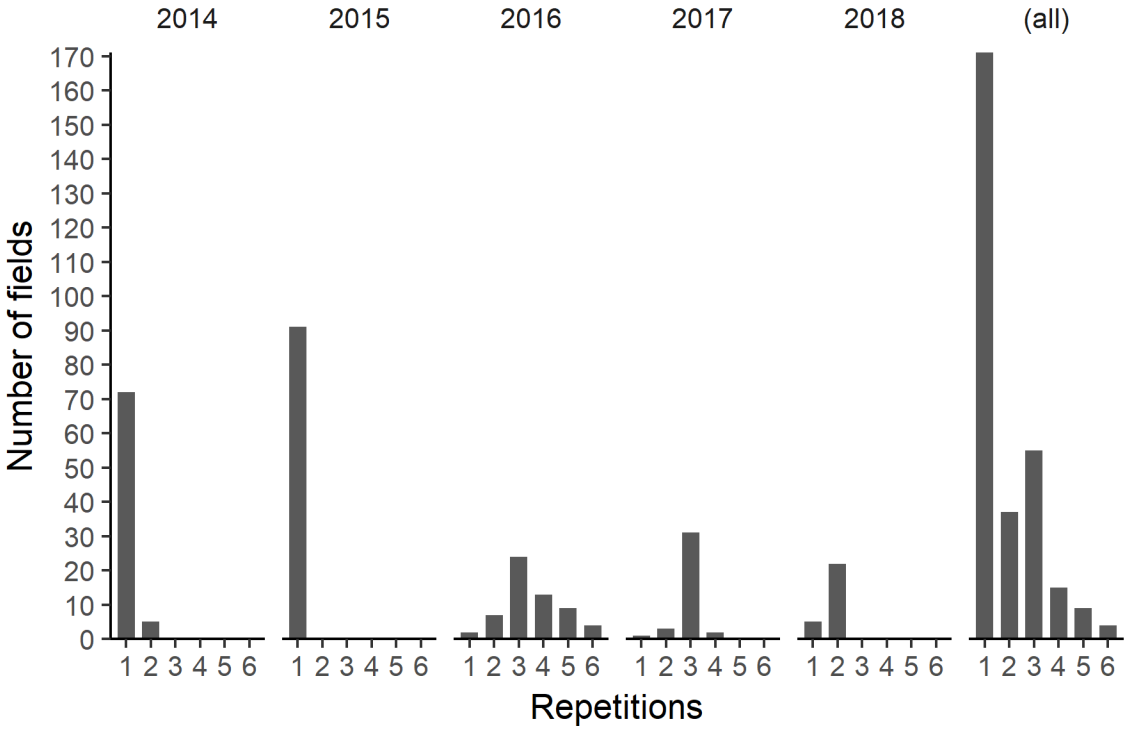
Supplementary Table 3 | Repeatability in prevalence and log_{10}

concentrations of imidacloprid and thiacloprid

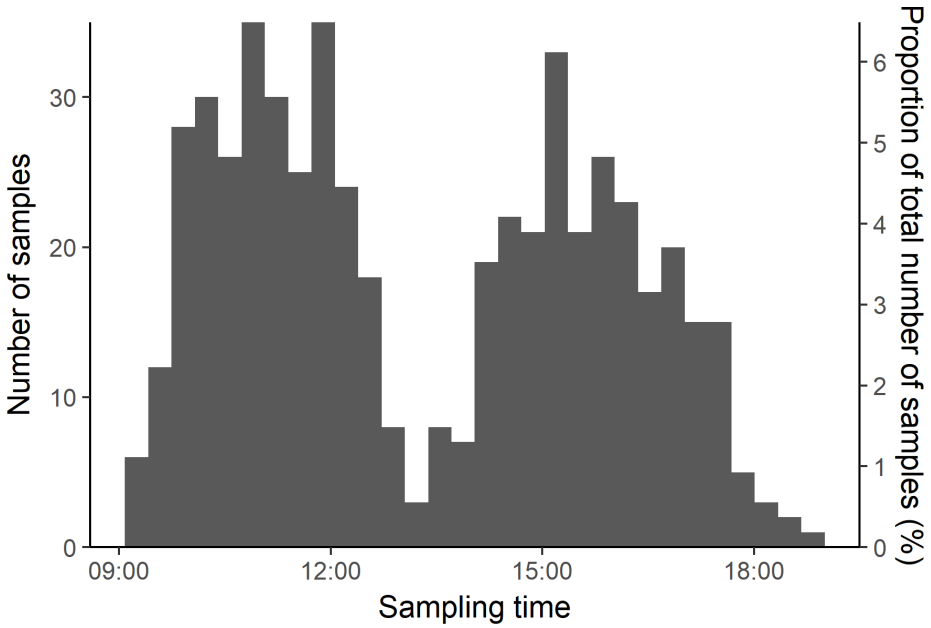
Insecticide	Year	Prevalence		Log_{10} concentration	
		ICC [a]	***P*** [a,b]	***ICC***	***P*** [b]
Imidacloprid	2016	0.13	**0.019**	0.09	0.066
	2017	0.78	**<0.001**	0.57	**<0.001**
	2018	0.08	0.253	0.15	0.237
Thiacloprid	2016	0.80	**<0.001**	0.38	**0.002**

[a] Estimation was done on the logit scale. [b] *P*-values were computed by permutation tests with 1500 simulations.

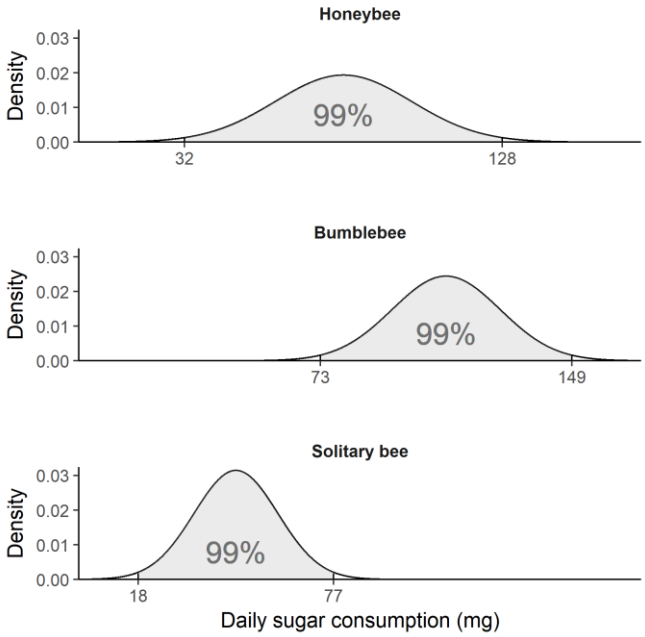
Supplementary Figures



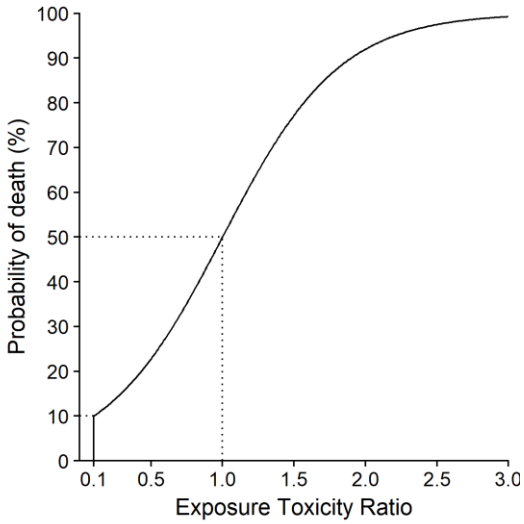
Supplementary Figure 1 | Sample size. Histograms of the number of sampling repetitions per field, separately and combined for all years.



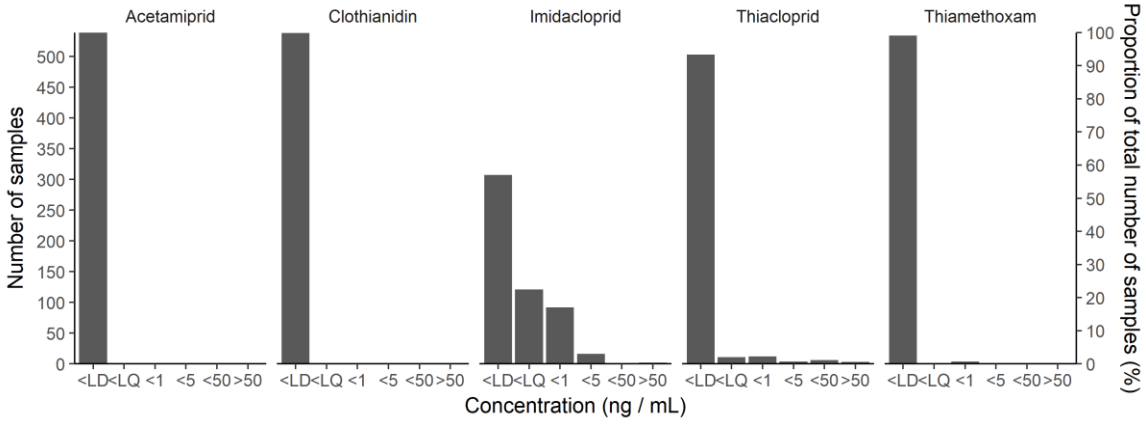
Supplementary Figure 2 | Sampling time. Histogram of times when samples were collected.



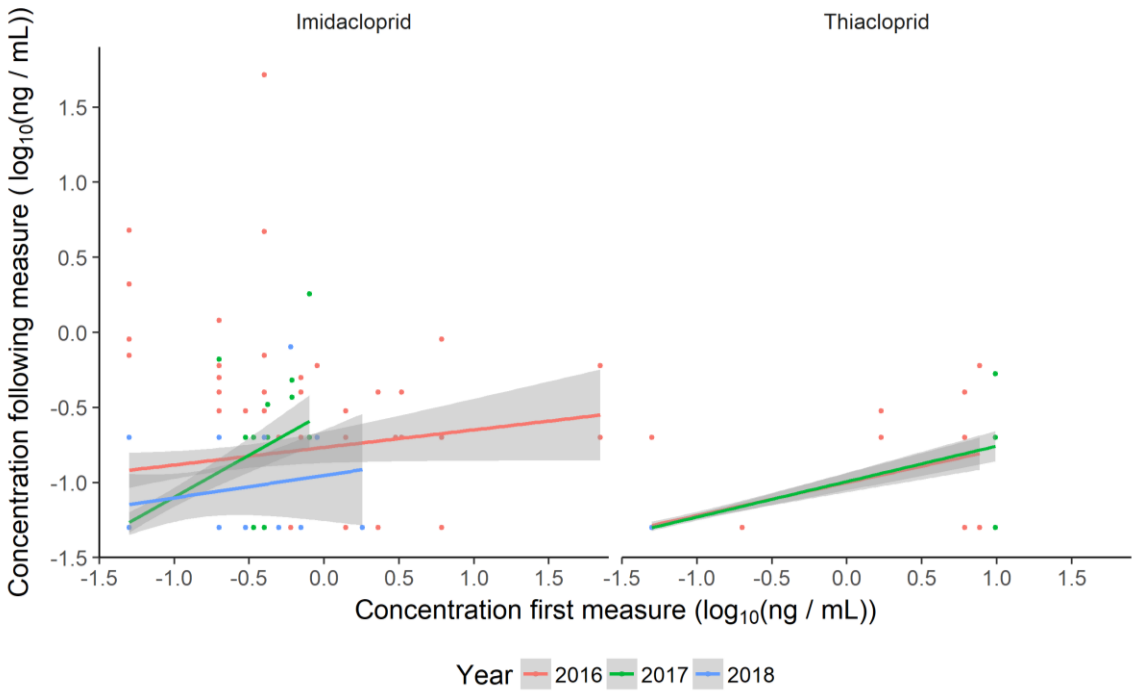
Supplementary Figure 3 | Daily sugar consumption. Normal distributions of daily sugar consumption amounts in which 99% are within reported ranges for honeybees (32-128 mg), bumblebees (73-149 mg) and solitary bees (18-77 mg).



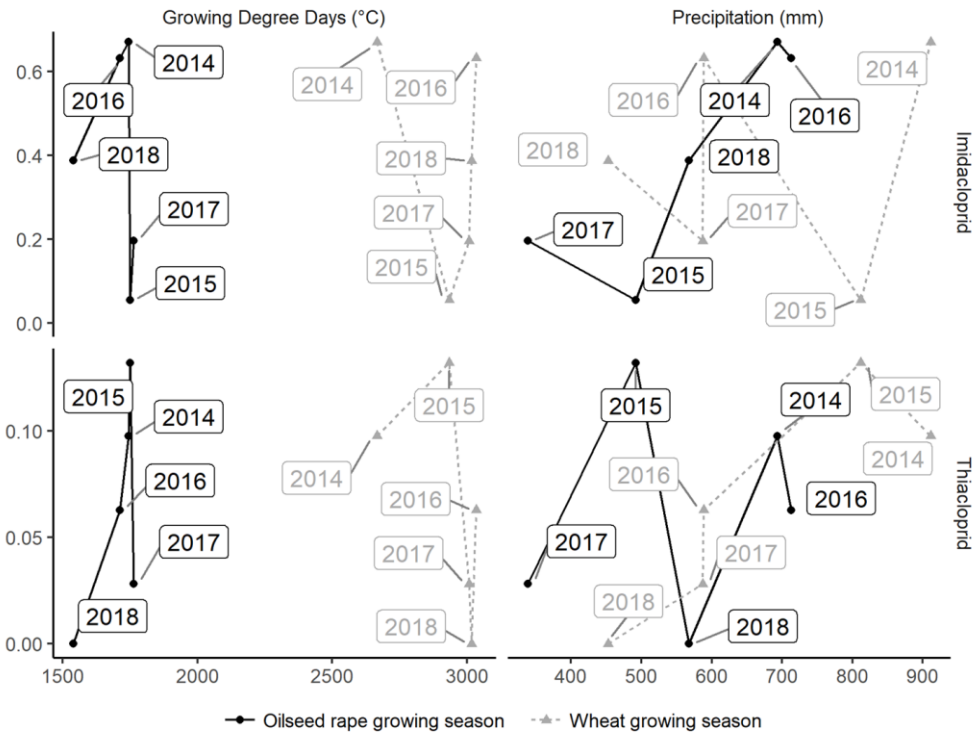
Supplementary Figure 4 | Probability of death. Assumed relationship between probability of death and Exposure toxicity ratio (ETR) per bee following a logistic regression for ETR ≥ 0.1 with probability of death being 10% at ETR=0.1 and 50% at ETR=1. No mortality is assumed for ETR < 0.1.



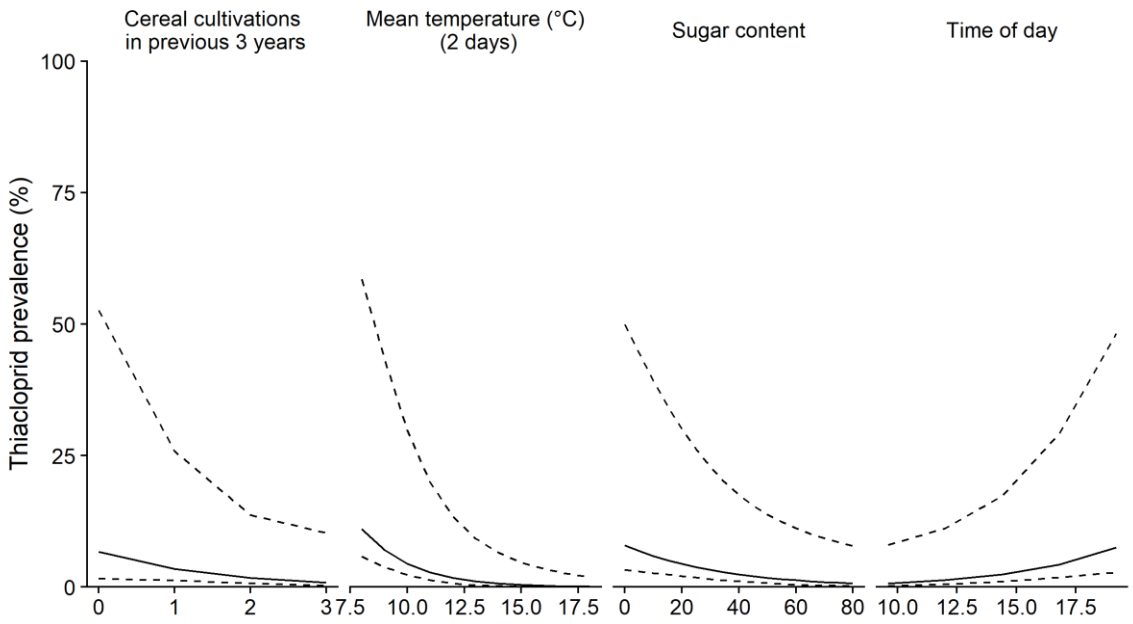
Supplementary Figure 5 | Frequency distribution of fields per neonicotinoid concentration class



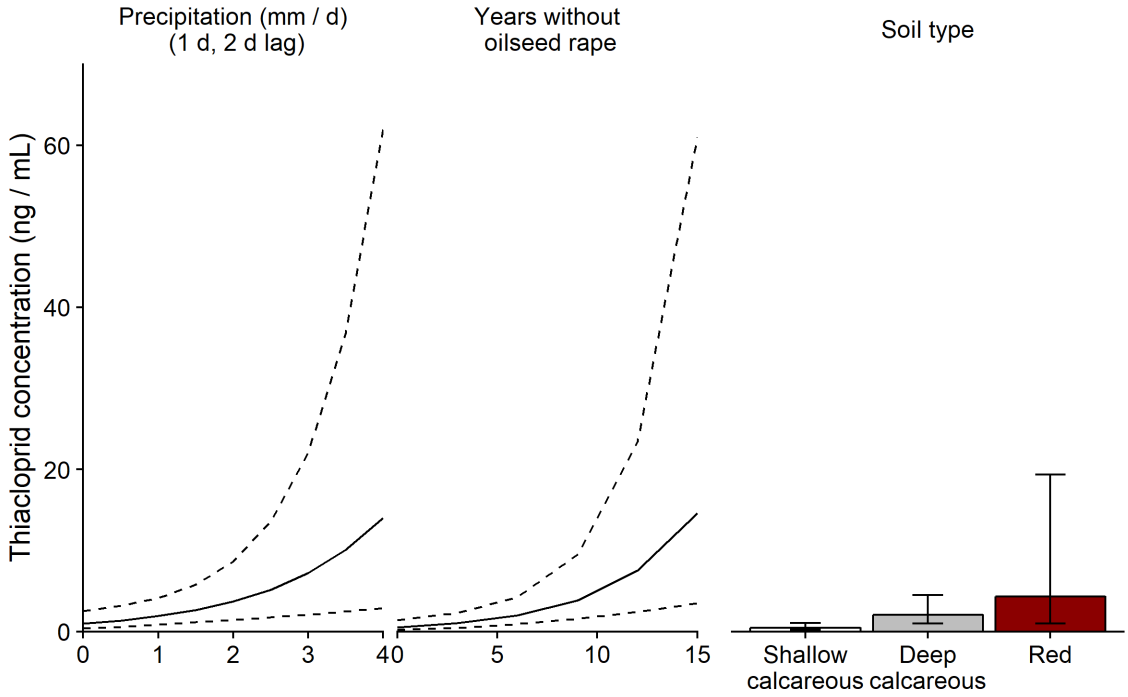
Supplementary Figure 6 | Imidacloprid and thiacloprid neonicotinoid concentrations in repeated measures in relation to the first measure per field.



Supplementary Figure 7 | Imidacloprid and thiacloprid prevalence in relation to growing degree days and precipitation. Growing degree days for winter wheat (base temperature = 0°C) and oilseed rape (base temperature = 5°C) as well as the amount of precipitation accumulated over the wheat and oilseed rape growing seasons in relation to sampling year. The regarded oilseed rape growing season ends with the sampling date, while the regarded wheat growing season was ended a month before oilseed rape sowing.



Supplementary Figure 8 | Thiacloprid prevalence in relation to abiotic and biotic variables. Thiacloprid prevalence was related to the number of cereal cultivations in the previous 3 years, mean air temperature in the 2 days before sampling, sugar content in the nectar sample, sampling time of day and sampling year (not shown). Dashed lines indicate 95% confidence intervals.



Supplementary Figure 9 | Thiacloprid concentration in relation to environmental variables. Thiacloprid concentration was related to the precipitation in a 1-day period in the 2 days before sampling, the number of years without oilseed rape and soil type using a linear model (LM) from which the shown estimates and 95% confidence intervals were obtained.

General discussion

In this doctoral thesis, we set out to study the effects of pesticides on bees in agricultural landscapes under real-world conditions. We address several knowledge gaps, such as the effect of organic farming and indirectly the general use of commonly used combinations of chemical pesticides on honeybees (Chapter I), the effect of field-level neonicotinoid exposure on colony performance and pathogen susceptibility of honeybees (Chapter II) and bumblebees (Chapter III). Lastly, we assess in a multi-season study the risk of neonicotinoid exposure for bees foraging on contaminated oilseed rape under the EU-wide moratorium on three neonicotinoids in bee-attractive crops (Chapter IV). In line with this, we study the factors that favour the spread of neonicotinoids in the environment.

We address these research questions in well-replicated landscape-scale studies to measure pesticide effects on the foraging scale of bees, while the effect of potential covariates is randomized. Thereby, we focus on the colony level for honeybees (Chapter I, II, IV) and bumblebees (Chapter III, IV) as colonies represent the reproductive units of social bees. For bumblebees, we examined, however, also neonicotinoid effects at the individual level (Chapter III) as these can give insight into trade-offs in resource and energy allocation in bumblebee colonies. Three out of four of our studies were conducted over two to six seasons to capture inter-annual variation (Chapter I, II, IV), to measure long-term clothianidin effects on honeybees (Chapter II) and to identify whether neonicotinoid contamination of oilseed rape is related to the time passed since the European Union has started to restrict neonicotinoid use in insect-pollinated crops (Chapter IV).

We show that organic farming and the associated reduction in the general use of various pesticides as compared to conventional farming can provide benefits to honeybees in agricultural landscapes (Chapter I). The ban of synthetic pesticides in organic farming benefits honeybees likely not only through a reduction of direct toxic effects but also through an increase of weed availability resulting from decreased herbicide use. We reveal that field exposure to clothianidin seed-treated oilseed rape can severely impact the

size and numbers of bumblebees, particularly of reproductives (Chapter III), but does generally not negatively affect honeybee colony performance (Chapter III). Under field conditions, neonicotinoid exposure had no major effect on pathogen susceptibility or the abundance of beneficial gut microbiota in honeybees and bumblebees (Chapter II, III). In line with this, we did not find an effect of exposure to clothianidin through seed-dressed oilseed rape on immune gene expression in honeybees (Chapter III). Lastly, we show that despite being banned in bee-attractive crops, imidacloprid poses a risk to honeybees, bumblebees and solitary bees foraging on oilseed rape nectar (Chapter IV). The environmental conditions that drive the spread of neonicotinoids in the environment and the uptake by oilseed rape crops remain rather elusive, but we showed that imidacloprid prevalence and concentrations are higher after rainy days and on red rather than calcareous soil.

Do landscape-scale studies confirm pesticide effects on bees identified in laboratory studies?

Pesticide use is considered a major driver of bee declines as numerous laboratory studies and artificial feeding experiments showed sublethal and lethal effects of insecticides on bees. In addition, fungicides were shown to enhance insecticide toxicity to bees. However, at the beginning of this thesis, we noted a lack of field- and landscape-scale studies assessing pesticide effects. Using a large-scale monitoring program, we determined predominantly positive effects of organic farming on honeybee colonies (Chapter I), which are suggestive of adverse effects of pesticides, since the hallmark of organic farming is the ban of synthetic agro-chemicals. In a field study with replicated landscapes, we also demonstrated and confirmed severe effects of exposure to the neonicotinoid clothianidin on bumblebee colonies, particularly in respect to the number and weight of reproductives (Chapter III). However, honeybee colony performance tended to be positively related to exposure to clothianidin through seed-dressed oilseed rape in two flowering periods. We found also no major effect of clothianidin on the pathosphere of honeybee and bumblebee colonies (Chapter II, III).

We identified two discrepancies between findings from field and laboratory studies: effects of neonicotinoids on pathogen susceptibility in honeybees and bumblebees and on honeybee performance were often observed under laboratory conditions but only rarely under field conditions. Laboratory-based research has shown that neonicotinoids can impair the innate and social immune system of bees (Brandt et al., 2016, 2017; Czerwinski & Sadd, 2017; Di Prisco et al., 2013; Fauser-Misslin et al., 2014). However, we found no major effect on pathogen susceptibility in bumblebees and honeybees and the few differences in microbial composition (Chapter III) or in the change in microbial composition during exposure (Chapter II) between clothianidin-exposed and control colonies suggested mostly a positive association between clothianidin exposure and bee health.

For honeybees, we also tested and failed to detect an effect of clothianidin on immune gene expression. In field studies there may be a time lag between exposure and sampling of bees, which can indicate that suppression of the immune system does not persist for a sufficiently long period to raise pathogen levels. However, a recent study (Chaimanee, Evans, Chen, Jackson, & Pettis, 2016) found that imidacloprid exposure can suppress immune gene expression for a whole week, but the administered concentration was relatively high (20 ppb) and unfortunately we have some doubts (Chapter II) about the primers used in their study and the original study by Di Prisco *et al.* 2013. Other studies with realistic exposure found contrasting findings on the effect of neonicotinoids on pathogen and parasite levels (Collison et al., 2016). For instance, the number of the *Varroa* mites increased in both years of a study with exposure to neonicotinoid-treated maize (M. Alburaki et al., 2016; Mohamed Alburaki et al., 2015), while another found no effect of clothianidin exposure on *Varroa* and virus levels (Rolke et al., 2016). *Varroa* and clothianidin did also not interact in a study with experimentally-induced exposure to both (Siede et al., 2018) and long-lasting in-hive feeding of thiacloprid had no effect on honeybee colonies or their levels of parasites, pathogens and expressed immune genes (Siede et al., 2017).

Plenty of laboratory-based research determined severe sublethal and (chronically) lethal effects of neonicotinoids on honeybee performance (Blacquière et al., 2012; C. J.

Godfray et al., 2015; H. C. J. Godfray et al., 2014; Goulson et al., 2015; L. Pisa et al., 2017; L. W. Pisa et al., 2015). In contrast, we found only a temporary positive association of clothianidin exposure and the amount of brood and no effect on the number of adult bees even though our study design was robust enough to determine with over 80% power an effect size of <7%, which is the threshold set by EFSA for field studies attempting to assess the safety of plant protection products. Several other field studies found also no deleterious effects on honeybee colonies (M. Alburaki et al., 2016; Mohamed Alburaki et al., 2015; G. C. Cutler & Scott-Dupree, 2007; G. C. Cutler et al., 2014; Pilling et al., 2013; Rundlöf et al., 2015). Interestingly, a well-replicated study conducted in the United Kingdom, Germany and Hungary found country-specific differences in neonicotinoid effects on honeybees, including both negative and positive effects (B. A. Woodcock et al., 2017). However, the negative effects were long-lived, while the positive ones disappeared over the winter. The authors suggested that differences between countries may be due to regional differences in parasite levels or in the extent that bees forage on the treated crop. The study detected also the banned neonicotinoid imidacloprid in hive matrices. Widespread environmental pesticide contamination is another source of variation that may confound findings from field studies. Widespread environmental pesticide contamination (David et al., 2016; Noa Simon-Delso, Martin, Bruneau, Delcourt, & Hautier, 2017; Tsvetkov et al., 2017; B. A. Woodcock et al., 2017; Ben A. Woodcock et al., 2018) is another source of variation that may confound findings from field studies and therefore explain not only differences between field and laboratory studies but also differences among field studies. This is particularly the case for studies with low (M. Alburaki et al., 2016; Mohamed Alburaki et al., 2015; Rolke et al., 2016) or intermediate (G. C. Cutler & Scott-Dupree, 2007; G. C. Cutler et al., 2014; Pilling et al., 2013) levels of site replication, which makes them prone to the influence of uncontrolled covariates.

To disentangle pesticide effects from confounding factors, Tsvetkov et al., 2017 analysed pesticide residues in bee samples and identified neonicotinoids as the potentially most harmful pesticides based on their toxicity and the detected concentrations. The authors exposed honeybees subsequently to clothianidin levels found in pollen samples and determined adverse effects on foraging behaviour, worker longevity, hygienic behaviour

and the presence of laying queens. They also identified synergistic interactions between neonicotinoids (clothianidin and thiamethoxam) and the fungicide boscalid, which was frequently found in combination with these neonicotinoids.

Differential exposure to neonicotinoid treated sites may also partly explain differences in the susceptibility of different bee species to the impact of neonicotinoids. Under field conditions, bees can forage on a wide range of flowers and it is conceivable that neonicotinoid-impaired honeybees may have a limited impact on their colonies' flower choice. Honeybees communicate sites with suitable forage via an elaborated dance language (Beekman, Gilchrist, Duncan, & Sumpter, 2007; T. Seeley, 1995; T. D. Seeley, 1986). Foragers that fail to return to their hives due to neonicotinoid-induced morality or homing failure (Fischer et al., 2014; Henry et al., 2012) cannot advertise the sites they have been feeding on. The neurotoxic pesticides may potentially also alter the willingness or effectiveness of honeybee foragers that return to their hives to advertise sites with available forage to their nest-mates. This may therefore limit the colonies' overall foraging activities on heavily contaminated sites. However, neonicotinoid residues are often found in (honey)bees and the pollen and nectar they collect (Botías et al., 2015; David et al., 2016; Giorio et al., 2017; Tsvetkov et al., 2017). Residue levels varied, however, strongly between studies and may therefore partly explain differences between different field studies. Low neonicotinoid residue levels in several studies may be the reason for the failure to detect effects. However, in our study (Chapter II), honeybees foraged intensively on oilseed rape and a large differential in clothianidin residue levels could be detected between sites with clothianidin treated and untreated oilseed rape. This suggests that honeybee colonies are relatively robust to the effect of clothianidin under field conditions.

A study attempting to bridge the gap between laboratory studies and field studies indicated that honeybee colonies experience neonicotinoid-induced forager losses also under field conditions, but the colonies managed to compensate for these by increasing the production of workers at the expense of energetically more costly males (Henry et al., 2015). Bees that do not live in large colonies may, therefore, be more susceptible to the impacts of neonicotinoids. Indeed, several large-scale studies showed neonicotinoid

effects on free-foraging wild bees (Rundlöf et al., 2015; B. A. Woodcock et al., 2017; Ben A. Woodcock et al., 2016), although some field studies with lacking site replication or low levels of exposure did not find a major effect on mason bees (Peters et al., 2016) or bumblebees (C. G. Cutler & Scott-Dupree, 2014; Sterk et al., 2016). Considerably lower neonicotinoid residue levels in treated oilseed rape in some fields studies as compared to our study, may be because they used winter-sown rather than spring-sown oilseed rape. The timeframe between sowing and flowering is much longer for winter-sown than for spring-sown oilseed rape, which means there is more time for neonicotinoids to leach out, resulting in lower residue uptake by the crop (Wood & Goulson, 2017). Differential detoxification efficiency may also explain differences between species (Cresswell et al., 2014).

What are the mechanisms by which agricultural pesticide use affects bee populations?

We showed that honeybee colonies in landscapes rich in organic farmland performed overall better than colonies in conventionally farmed landscapes (Chapter I). This may suggest that honeybees benefit from the ban of synthetic pesticides in organic farmland. The restrictions on pesticide use may benefit bees not only through a reduction of direct toxic effects, but also through an increase in floral availability. Extensive herbicide use simplifies and reduces the weed cover in conventional cropland. Although organic farmers also attempt to limit weed proliferation, organic farmland harbours typically less abundant and less diverse weeds (Bengtsson, Ahnström, & Weibull, 2005; Ekroos, Hyvönen, Tiainen, & Tiira, 2010; Gabriel & Tscharntke, 2007; Ponce, Bravo, de León, Magaña, & Alonso, 2011). Weeds are valuable resources for bees (Bretagnolle & Gaba, 2015), which may compensate for the lack of flowers in the absence of mass-flowering crops (Requier et al., 2016, 2015). Indeed, we found most consistent positive effects of organic farming on the amount of worker brood during the floral dearth between the blooms of oilseed rape and sunflower (Chapter I). These translated later into benefits for the number of adult bees and honey production. Organic farming differs, however, also in aspects other than pesticide use from conventional agriculture. Organic land is covered to

a greater extent by grassland (Bengtsson et al., 2005), and semi-natural elements (Gibson, Pearce, Morris, Symondson, & Memmott, 2007) and less by oilseed rape and mineral fertilizer use is banned in organic farming. All of these may alter the floral availability for bees. However, the relation between organic farming and colony performance was generally unaffected by the proportions of oilseed rape, grassland and sunflower in the surroundings of the hives. Mineral fertilizers simplify weed communities, while perennial or legume cover crops, which organic farmers use for nitrogen fixation (Decourtye, Mader, & Desneux, 2010), increase floral availability for bees. Although our results suggest that bees benefit from a more continuous supply of floral resources in organic farmland, it remains speculative to what extent differential crop choice, semi-natural elements or herbicide and mineral fertilizer use impact honeybee colonies. Sporadically, we also observed positive effects of organic farming on the number of adult bees independently of increases in worker brood production. These indicate increased survival, potentially due to a reduction in direct toxic effects from pesticide exposure.

We studied the toxic effects of the neonicotinoid insecticide clothianidin in a field study with replicated landscapes (Rundlöf et al., 2015). We found only after two seasons of exposure a moderate positive association with brood production of honeybees (Chapter II). The reproduction of bumblebees was, however, severely affected by exposure to clothianidin seed-treated oilseed rape (Chapter III). Clothianidin exposure had only little impact on the pathogen susceptibility of both honeybees and bumblebees, as inferred from data on pathogen prevalence and abundance or immune gene expression or the relationship between parasite abundance and colony performance. This suggests that clothianidin impairs bee colonies under field conditions not majorly through a suppression of the immune system. Bumblebees may rather be affected through a decrease in foraging efficiency. Neonicotinoids impact particularly pollen foraging ability, as pollen is more difficult to handle than nectar. Pollen is the principal food for brood and reproductives (drones and queens) require more feed than workers. Therefore, reduced pollen foraging ability may explain the adverse impacts on brood production, the reduced worker size and the more severe effects on drone pupal weight as well as queen and drone numbers in bumblebees.

Does neonicotinoid contamination of non-target plants pose a risk to bees?

Neonicotinoids are commonly found in wild flowers (Botías et al., 2015; David et al., 2016; Tsvetkov et al., 2017) and untreated crops (Henry et al., 2015; Thompson H et al., 2013; B. A. Woodcock et al., 2017). In addition, neonicotinoids that have been banned in bee-attractive crops in the EU are still frequently detected in hive matrices (Calatayud-Vernich et al., 2016; Daniele et al., 2017; Mitchell et al., 2017; Tosi et al., 2018; B. A. Woodcock et al., 2017; Ben A. Woodcock et al., 2018). Therefore, we questioned the effectiveness of the current EU moratorium on the use of three neonicotinoids in bee-attractive crops. We found, even after the moratorium came into effect, imidacloprid and thiamethoxam in honeybees and the pollen and nectar they had collected in oilseed rape fields (Chapter II) as well as in nectar directly collected from oilseed rape flowers (Chapter IV). In a French study site, we found imidacloprid in oilseed rape nectar in all years, in which the moratorium has been in effect. Imidacloprid prevalence varied strongly between years, but there was no consistent decline. In fact, highest prevalence and concentrations were detected in 2016, even though imidacloprid was only detected in a few samples in 2015. We demonstrated that the detected imidacloprid concentrations can be harmful to honeybees and wild bees to an extent that may reduce colony survival and population sizes. Imidacloprid prevalence in oilseed rape tended to be higher in fields which were in the preceding year cultivated with cereals. However, imidacloprid concentrations in positive samples could not be linked to number of winter cereal cultivations in previous years, even though imidacloprid use in the study site was restricted to winter cereals. We showed that imidacloprid prevalence and concentration were higher after rainy days and on red soil as compared to calcareous soil. This suggests that neonicotinoids may be very mobile in the environment and travel through water and be retained in soil with relatively high water retention capacity. Rainfall constrains bee foraging (Javorek et al., 2002); therefore bees may forager more intensively after several days of rainfall, when high imidacloprid prevalence and concentration coincide.

Therefore, our findings re-affirm the appropriateness of the recent decisions to ban neonicotinoid use completely in France (Daniele et al., 2017) and imidacloprid, thiamethoxam and clothianidin for all outdoor uses within the European Union (European Commission, 2018). The latter was triggered by a risk assessment by the European Food Safety Authority (EFSA). EFSA collected data mostly through a systematic review of published literature on these three neonicotinoids and determined, in line with our results, a considerable risk for bees of most uses including applications in crops that are not attractive to bees.

Methodological limitations and comparison of study designs

We set out to determine pesticide effects under real-world conditions. We did this in large-scale studies in real agricultural landscapes. Field and landscape studies are always subject to potential confounding effects from covariates. To limit this, we principally relied on an exceptionally high degree of site replication. In addition, we accounted for differences in land use between sites in different ways. In the studies conducted in Southern Sweden (Chapter II, III), we paired sites based on land use and geographical proximity and randomly selected one site per pair to be treated with the insecticide clothianidin, while the other served as a control. In contrast, in the study on the effect of organic farming on honeybee colonies in a French study site (Chapter I), we did not have true controls or an experimental treatment. The colonies were randomly placed in the landscape, which resulted in gradients of the variable of interest (the proportion of organic farmland) and potential land use covariates that we included in models to account for differences between sites. Our study on the effect of environmental factors such as the frequency of cereal cultivations in previous years or weather conditions before sampling on the neonicotinoid contamination of oilseed rape nectar (Chapter IV) is also correlational.

Experimental treatment is generally to be preferred over a correlational approach when cause and effect relationships are to be established. Correlational approaches are useful to determine relationships for prediction but are prone to unintentional co-variation between

the predictor variable of interest and other potential predictors. For instance, it is conceivable that in landscapes with more abundant and more diverse flowers, farmers are more likely to convert to organic farming. Honeybees benefit from a high abundance and diversity of floral resources. Therefore, a positive relationship between the proportion of organic farmland in the landscape and honeybee colony performance may not only be due to direct effects of applying organic farming practices but also due to pre-existing differences that influence the decision of farmers to convert to organic farming.

Similarly, controlling physically rather than statistically for unintended covariation, such as land use, is generally to be preferred, as the inclusion of covariates in models can only to some extent account for differences in the presence of the covariate between sites. This is further complicated by the fact that the covariate may not only directly affect the response variable, but also reduce or increase the effect that the predictor variable of interest has on the response variable. However, even in our studies with matched landscapes, differences in land use in the surroundings existed. We abstained to control statistically for those, as the inclusion of additional predictor terms reduces statistical power.

A study design based on landscape gradients (such as organic farming) has the advantage over a treatment-control design that an exposure-response relationship can be established. In addition, analyses with continuous predictors have higher statistical power than analyses with a binary predictor (Altman & Royston, 2006).

Landscape gradients can be obtained at different spatial scales and therefore be used to explore the variability of effects over space. We determined the effects of clothianidin-seed treatment by placing honeybee or bumblebee colonies next to single oilseed rape fields that were either sown from seeds that were treated or not treated with clothianidin. The foraging scale of bumblebees and particularly honeybees exceed, however, the distance covered by the field. Therefore, neonicotinoid exposure of bees foraging in the wider landscape cannot be excluded. However, our residue analyses of bee tissue and bee collected pollen and nectar showed that the experimental colonies foraged intensively on

oilseed rape and that those colonies placed by treated fields were exposed to high concentrations of clothianidin, while those at control fields contained no or only trace amounts of clothianidin. The residue analyses suggest that clothianidin was the only neonicotinoid with differential exposure between treatments and therefore validated our study design. In contrast, in our study on the effect of organic farming on honeybee colonies, it was not feasible to determine the extent by which experimental honeybees foraged on organically farmed land.

In each of our studies that involved bees (Chapter I-III), experimental colonies were used that were managed by the same beekeepers to reduce variation due to differences in beekeeping practices. Management of bees can affect the impact environmental stressors have on them. For instance, mass-breeding of bumblebees may increase their robustness to diseases, as they had to grow up and stay healthy under conditions that are conducive to many pathogens (Goulson et al., 2015; Graystock et al., 2013). We managed honeybee colonies in alignment with local beekeeping practices, to determine pesticide effects under real-world conditions. Honeybees are a predominantly managed species and beekeepers typically treat against the *Varroa* mite, supplement sugar solutions and collect honey. In comparison to feral honeybees, managed honeybees may for instance be exposed during a shorter period of time to pesticides as the honey containing pesticides is removed. Therefore, pesticide effect sizes may differ between managed and feral honeybee populations.

Our studies involving honeybee colonies differed also in the way colony performance parameters were assessed. In Chapter I, we used objective measures to assess the size of brood areas and number of adult bees, i.e. the dimensions of brood areas were measured and approximated to an eclipse and number of bees was determined by weight. In contrast, in Chapter II, we used the subjective Liebefeld method to visually assess number of adult bees and brood areas (Imdorf, Buehlmann, Gerig, Kilchenmann, & Wille, 1987). The objective measures are generally more reliable but also more invasive and disruptive (Delaplane et al., 2013). Particularly, the estimation of number of adult bees by weight causes disturbance of colonies as bees are shaken off frames. In Chapter I, we estimated the total area covered by open (eggs and larvae) and sealed brood, whereas

in Chapter II, only sealed brood was determined. The estimation of the whole brood area is more holistic and adapted to identify more quickly effects on the egg-laying capacity of queens but also less reliable as estimates of open brood are typically less accurate (Imdorf et al., 1987).

Another key difference between these two studies (Chapter I & II) was the extent by which colonies were standardised. In Chapter II, colonies were equalised with respect to the number of brood frames, honey frames and adult bees. This requires disturbance of the colonies, which takes, however, place before the main period of interest – the oilseed rape flowering season. In Chapter I, colonies were not standardised but approximate equal strength of apiaries was obtained by ensuring that weak and strong colonies are equally distributed between apiaries.

The study in Chapter I had with measurements every two weeks during six beekeeping seasons an outstanding temporal resolution and duration that allows for testing relationships between honeybee colony performance and agricultural practices in different periods of the year. The study in Chapter II, was designed as a repeated (i.e. in two years) before-after-control-impact (BACI) study with a measurement before and one after exposure of honeybee colonies to oilseed rape that was either treated or not treated with clothianidin. This study had an exceptionally high statistical power and met the EFSA requirement to detect an effect size on colony size of < 7% with a power of 80%, which was previously deemed unfeasible (Ben A Woodcock et al., 2016). This study provides therefore also a stronger evidence base than the parallel study on bumblebees (Chapter III), which was done over a single season without measurements before exposure. Nonetheless, clothianidin effects on bumblebees were validly determined with large effect sizes.

Knowledge gaps

Our results suggest a positive effect of organic farming on honeybee colony performance (Chapter I), which remains however to be confirmed by additional studies that may be

designed to examine effects of organic farming on honeybee colonies. Colonies may intentionally be placed along a gradient of organic farming to obtain a stronger contrast as well as an equal sample size per amount of exposure. Study sites may be chosen based on the surrounding landscape to reduce collinearity with other variables of interest such as the acreage of oilseed rape, grassland or semi-natural elements. We suggested several differences between conventional and organic farming that may explain the observed relationship between the availability of organic farmland and honeybee colony performance, such as reduced intoxication risk and a more continuous supply of floral resources due to the abstention from synthetic herbicides and mineral fertilizers. To confirm the impact of these factors they may be studied in isolation or in fully crossed experiments. It remains also unclear whether and to what extent the impact of organic farming depends on the overall pesticide use intensity in the landscape. Organic farming may represent a particularly valuable haven for honeybees in landscapes with intensive pesticide use in conventional cropland (Tuck et al., 2014).

Both laboratory and field studies have demonstrated negative effects of neonicotinoids. Impacts varied, however, between bee species and geographical region (L. Pisa et al., 2017; Rundlöf et al., 2015; B. A. Woodcock et al., 2017). The reasons for this variation are not fully understood. Future studies may investigate under which conditions honeybee colonies are able to cope with pesticide-induced stress and for instance what role landscape composition plays in the pesticide tolerance of bee populations. For neonicotinoids, there is a distinct lack of studies at the population level (Lundin et al., 2015), even though imidacloprid use in oilseed rape in the United Kingdom could be linked to long-term declines of wild bees (Ben A. Woodcock et al., 2016) and honeybee colony losses (Budge et al., 2015).

We showed that despite the EU moratorium, imidacloprid was highly prevalent in oilseed rape and in concentrations that pose a risk to foraging bees (Chapter IV). This risk varied, however, strongly between years and fields. We identified that certain soil and weather conditions were associated with elevated neonicotinoid prevalence and concentration, but the factors that govern the spread and uptake of neonicotinoids remain largely elusive. Therefore, it is also unclear whether the ban of imidacloprid, thiamethoxam and

clothianidin for all outdoor uses will eliminate their risk for bees. Neonicotinoids are highly persistent in soil and fairly water-soluble. It is conceivable that they may be leached from greenhouses due to their high mobility. We (Chapter II, IV) and several other studies (Bonmatin et al., 2015; Botías et al., 2015; Giorio et al., 2017; Krupke et al., 2012) have shown that neonicotinoids have the potential to spread in the environment. Therefore, it remains to be studied whether neonicotinoids continue to pose a risk to bees in European landscapes and whether the stricter regulation in France effectively eliminates this potential risk.

It is also unclear how the ban of clothianidin, imidacloprid and thiamethoxam in outdoor crops will affect the use of alternative insecticides and the extent to which this affects bee populations. The initial restrictions of these neonicotinoids have already prompted the use alternative insecticides such as unrestricted neonicotinoids (acetamiprid & thiacloprid) or pyrethroids (Kathage, Castañera, Alonso-Prados, Gómez-Barbero, & Rodríguez-Cerezo, 2018). Other potential replacements include sulfoximines (Mark J.F. Brown et al., 2016), organophosphates, carbamates, pyridin-azomethines (e.g. pymetrozine), phenylpyrazoles (e.g. fipronil) and oxadiacines (e.g. indoxacarb) (Klatt et al., 2016). In general, the impact of these alternative insecticides is less well studied, but many were already shown to be harmful to honeybees, bumblebees and solitary bees (Klatt et al., 2016; Siviter, Brown, & Leadbeater, 2018). It is therefore important to monitor real-world pesticide use and study its effect on bees under agronomically realistic conditions.

The ban of the outdoor use of neonicotinoids may also cause an overall reduction in pesticide use e.g. through triggering a more stringent application of integrated pest management (IPM) practices such as diversifying and modifying crop rotations, using crops that are relatively robust to regionally prevailing pests or altering the timing of sowing, tillage and irrigation (Furlan & Kreutzweiser, 2015). In addition, the neonicotinoid ban may contribute to an ongoing stable growth in organic farming (EC, 2018). This raises the question how altering food resources in agricultural landscapes affect bee populations. For instance, reduced cultivation of oilseed rape may temporarily reduce honeybee colony performance (Requier et al., 2015; Rollin et al., 2013), but increased floral diversity may also benefit populations of both specialist and generalist

foragers throughout the year and particularly in periods of generally low resource availability (Requier et al., 2016).

Implications for the pesticide authorization process

The European Union regulatory framework for plant protection products is vaunted as one of the strictest in the world (European Commission - Group of Chief Scientific Advisors -Scientific Opinion 5/2018, 2018; Handford, Elliott, & Campbell, 2015). Indeed, neonicotinoids are only restricted in the European Union, the Philippian province Marinduque (Gordon, Calatayud, Le Gall, & Garnery, 2019) and to some extent in Ontario, Canada (Goulson, 2018), where farmers have to conduct a pest assessment to justify the need for certain pesticides in specific crops (Ontario Government, 2017).

The recent decision to ban three neonicotinoids in open land illustrates, however, also the need for a more proactive regulatory approach to effectively avoid severe environmental repercussions from pesticides. In fact, post-authorization bans after the discovery of unexpected and unacceptable risks to the environment are commonplace in the European Union (Storck, Karpouzas, & Martin-Laurent, 2017). These discoveries are often made many years after the pesticide has been placed on the market (European Commission - Group of Chief Scientific Advisors -Scientific Opinion 5/2018, 2018). This implies that hazardous substances are released into the environment over long timeframes with potentially severe implications for wildlife and human health and raises questions about the scrutiny of the pre-authorization risk assessment process and compliance with the precautionary principle.

The European Union follows a stepwise authorization process that involves national and international authorities (Fig. 2). The process is initiated by the submission of a dossier by a pesticide company that request authorization to sell a plant protection product. In the first step, an EU-designated Rapporteur Member State creates an initial draft assessment report by modifying, amending and concluding on the risk assessment provided by the pesticide company. The active substance is evaluated based on its efficacy, toxicology,

residue levels, metabolites, environmental fate and ecotoxicological impact on non-target organisms. Other Member States are subsequently invited to peer-review the initial draft report. The European Food Safety Authority publishes then a conclusion, which facilitates the decision on approval of the active substance in the European Union by the European Commission. Each Member State can then decide whether or not to authorize the use of the active substance and determine in what pesticide formulations it can be sold (European Commission - Group of Chief Scientific Advisors -Scientific Opinion 5/2018, 2018).

The initial draft report includes a tiered ecological risk assessment, as it has been implemented in principle by most regulatory authorities in the 36 member states of the Organisation for Economic Cooperation and Development (OECD) (Sánchez-Bayo & Tennekes, 2015). The first-tier assessment uses simple ratios (HQ, ETR) of expected environmental concentrations to toxic doses (mostly LD50, LC50) in order to identify and exclude highly hazardous substances. In the following tiers risk is assessed under increasingly realistic conditions.

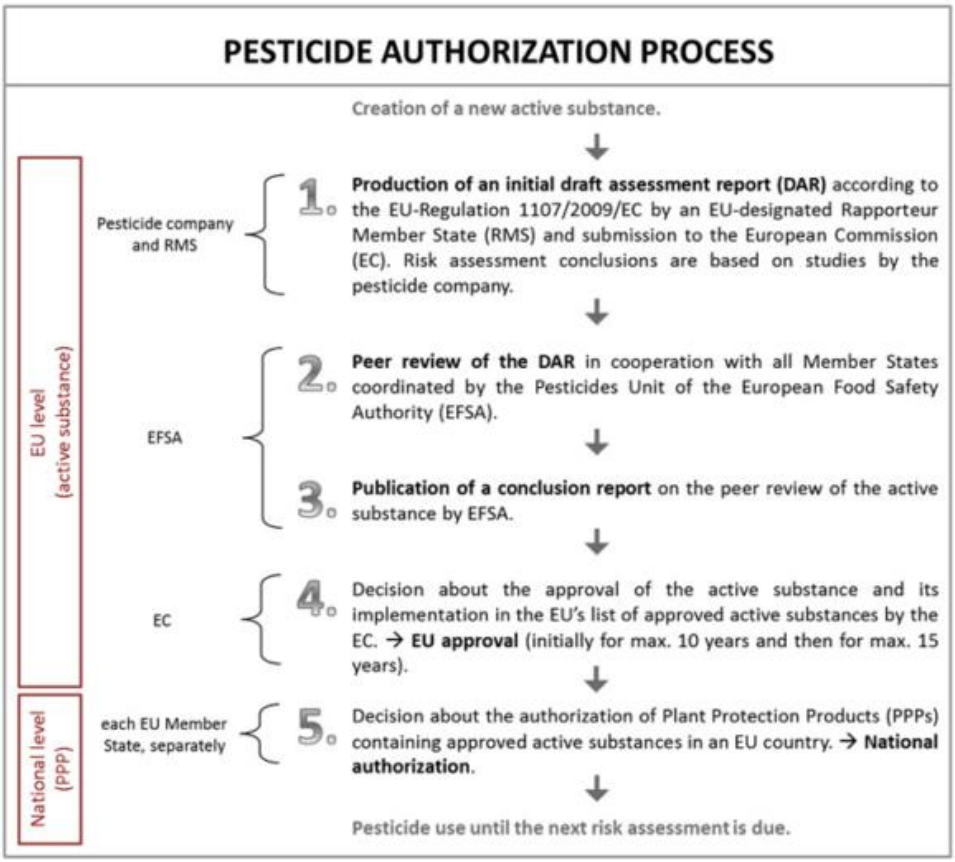


Figure 2 | Schematic presentation of the stepwise process of pesticide authorization in the EU (taken from Storck, Karpouzas & Martin-Laurent 2017).

OECD guidelines for compulsory ecological risk assessment schemes have been criticized for relying almost exclusively on acute toxicity tests – particularly in the first tier (Sánchez-Bayo & Tennekes, 2017). However, the European Union and the United States have recently adopted more comprehensive testing standards which include chronic tests for bees, as shown by current guidance documents for risk assessments on bees by the European Food Safety Authority (EFSA; (EFSA, 2013)) and the United States Environmental Protection Agency (USEPA; (US EPA, 2014)). However, there is a lack of chronic toxicity data for already registered pesticides. For instance, EFSA noted the absence of reliable data to determine a chronic lethal dose for thiamethoxam in honeybees, even though the pesticide's effect has been more carefully scrutinized than many other pesticides (EFSA, 2018a). Thiamethoxam has been assessed over years after

its authorization due to ongoing public concern and a following request by the European Commission for further evaluation, which recently led to its ban in outdoor crops.

Neonicotinoids are a prime example for pesticides that may have severe consequences at the population level even though most individuals are exposed to sublethal doses. They can exhibit delayed mortality after exposure over a long time to doses well below acute lethal levels (Sánchez-Bayo & Tennekes, 2015, 2017). The degree to which the pesticides exhibit such time-cumulative mortality depends on their mode of action. Time-cumulative mortality is common in neurotoxins, as they bind virtually irreversibly to specific receptors, cause their continuous excitation and ultimately cell death. For pesticides whose effect is strongly dependent on the time of exposure, time-to-event toxicity tests may be more conclusive than tests of acute toxicity. Time-to-event toxicity tests measure for example the time to cause 50% mortality in a population under realistic exposure scenarios (Sánchez-Bayo & Tennekes, 2015, 2017; Noa Simon-Delso, San Martin, Bruneau, & Hautier, 2018).

Risk assessments of pesticides should also include stringent tests on sub-lethal effects (Sánchez-Bayo & Tennekes, 2017). Impairments of the reproductive capacity can have similarly severe or even more severe consequences for population sizes than lethal effects on individuals (Bryden et al., 2013; Sánchez-Bayo & Tennekes, 2017). Neonicotinoids were shown to affect fecundity, drone sperm quality and ovary development in honeybees (Baron, Raine, & Brown, 2017; Ciereszko et al., 2017; L. Pisa et al., 2017; Straub et al., 2016). Similarly, before its ban, DDT was implicated in the decline of predatory bird populations, as it reduced hatching success through a thinning of eggshells, even though adult birds were alive and appeared to be healthy (Sánchez-Bayo & Tennekes, 2017).

Pesticide risk assessments require a determination of environmentally relevant doses. These should encompass mean/median and maximum doses under realistic conditions. Maximum concentrations are likely to determine main ecological effects. The initial draft report is done in accordance with the EU Regulation 1107/2009/EC on the placing of plant protection products on the European Union market. The regulation demands that

residue levels in food, feed and succeeding crops are reliably predictable before the approval of a pesticide. However, even years after approval of neonicotinoids, residue levels of only few nectar and pollen samples were publicly available (Carreck & Ratnieks, 2014). Residue levels can widely vary between geographic regions due to climatic conditions, soils and agricultural practices. We demonstrated that even within the same region, neonicotinoid residue levels in oilseed rape nectar and honeybee-collected nectar samples of colonies that foraged predominately on oilseed rape, can vary strongly between and within years (Chapter II, IV) for both crops that have been deliberately treated with the pesticide (Chapter II, IV) and those that have been accidently contaminated (Chapter IV). Residue levels varied strongly in honeybee-collected nectar that was sampled at the same day within the same treated field and among different fields of the same region (Chapter II). Clothianidin residues in oilseed rape nectar sampled directly from flowers varied for both treated and accidently contaminated oilseed rape of samples taken the same day in different fields or on different days in the same fields (Chapter IV).

The European Union first called in 2012 for an EFSA review of imidacloprid, thiamethoxam and clothianidin after beekeeper reports and scientific studies suggested implications in exceptional honeybee colony loss incidents. EU Regulation 1107/2009/EC specifically requires that only plant protections with negligible are to be approved. The regulation does not request a risk assessment for other pollinators. The EFSA guidance document for the risk assessment of ecological impacts on bees includes to some extent bumblebees and solitary bees, but in practice, this comprises mostly extrapolations of toxic doses from honeybees (EFSA, 2018a, 2018b, 2018c) .

Honeybees may, however, be a poor model organism for other bees and pollinators. Our (Chapter II, III) and several other studies (Cresswell et al., 2012; Rundlöf et al., 2015) suggest that honeybee colonies are less susceptible to pesticide effects than wild bee communities. Not only may individual honeybees differ from other bees in sensitivity to pesticides (Arena & Sgolastra, 2014; Cresswell et al., 2012), but the large colony size may act as a shield against environmental pressures (Henry et al., 2015). Honeybees have large colonies consisting of tens of thousands of individuals. The social structure and

reproductive capacity of honeybee colonies may help them to buffer losses and impairments of individual bees. In contrast, for solitary bees, mortality of individual bees translates directly into declines at the population level. Considerable differences in life strategies between taxa (Michener, 2007) may cause marked differences in exposure and vulnerability. Compulsory landscape experiments with pollinator taxa of varying degrees of sociality would better capture the risk for pollinator communities. Generally, a lack of landscape-scale studies, measuring 'out of field' impacts has been determined by scientific advisors of the European Commission (European Commission - Group of Chief Scientific Advisors -Scientific Opinion 5/2018, 2018).

They also recommend an improvement of the post-authorization risk assessment, including a systematic landscape-scale environmental monitoring of residues (European Commission - Group of Chief Scientific Advisors -Scientific Opinion 5/2018, 2018). To limit the release of substances with large ecological impact, high risk substances may also be authorized for only a limited timeframe, cultivation area and use. After intensive monitoring under real-world conditions, wide-scale use of the pesticide could be granted (European Commission - Group of Chief Scientific Advisors -Scientific Opinion 5/2018, 2018). Environmental risk assessments in the EU have to be done no more than ten years after authorization (Storck et al., 2017). This process could be shortened to identify risks of real-world pesticide use more timely (Storck et al., 2017). In addition, risk assessors at EU-level may monitor peer-reviewed literature on environmental impacts in appropriate intervals (European Commission - Group of Chief Scientific Advisors -Scientific Opinion 5/2018, 2018).

Increased transparency of the authorization and post-authorization risk assessments may further reduce the timespan to identify unexpected ecological risks. The disclosure of relevant information in real time starting from the beginning of the authorization process rather than from the launch on the market could initiate private and public investigations early on (Storck et al., 2017). The full and quick release of the manufacture's risk assessment and EFSA's post-authorization findings could streamline research and prevent unnecessary redundancies in studies. In addition, comprehensive reporting of

pesticide use by farmers may help identify the most relevant combinations of pesticides, which may facilitate for instance studies on potential cocktail effects.

Final conclusions

We have shown that the use of pesticides in agricultural landscapes have adverse effects on bees. Our results suggest that both insecticides and herbicides may negatively impact bees and that organic farming may provide a haven for honeybees in predominantly conventionally farmed landscapes. We generally confirm findings from laboratory studies, but also identify a discrepancy between laboratory and field studies in the degree of impact of neonicotinoids on honeybee colony performance and bee pathogen susceptibility. Our results indicate that bumblebees may be more susceptible to the neonicotinoid clothianidin than honeybees. The large size and reproductive capacity of honeybee colonies may act as a shield against environmental stressors. We conducted a risk assessment that suggests that foraging on imidacloprid contaminated oilseed rape can shorten the life span of a considerable proportion of bees. The risk varied, however, strongly between years and fields. Cereal cultivation in preceding years did not increase imidacloprid levels, even though imidacloprid is extensively used in winter cereals and banned in bee-attractive crops. This suggests a diffuse contamination of the environment, but the mechanisms that govern the spread and uptake of neonicotinoids remain poorly understood. Soil and weather conditions explained only partly the observed variation in neonicotinoid prevalence and concentration in oilseed rape nectar.

The case of neonicotinoids illustrates the need for a more stringent assessment of the ecological consequences of pesticide use prior to their authorisation. Severe impacts of neonicotinoids have been detected and verified long after their massive release into the environment had been approved. Even though in the European Union, the three most toxic neonicotinoids have recently been banned for all outdoor uses, they may continue to impact pollinators due to their persistence and mobility. Most regulatory authorities assess the risk for pollinators through laboratory tests of acute mortality of honeybees.

However, field-realistic concentrations of neonicotinoids are typically sublethal for honeybees, even though they can cause chronic mortality and impair the functioning of bee colonies. In addition, other bee species seem to be more susceptible.

Therefore, we acclaim that the European Union has recently adopted more comprehensive testing standards that involve the assessment of more bee species and of sublethal effects and chronic mortality. Nonetheless, we argue that the ecological repercussions of pesticides need to be assessed under more realistic conditions before their release at a national or international scale is authorised.

More stringent pesticide authorisation frameworks are, however, likely insufficient to stop current pollinator and biodiversity declines. Insecticides are designed to kill insects and unsurprisingly pollinating insects are not exempt from their negative effects. The use of insecticides and other pesticides has massively increased from the 1960s to 2007 (Carvalho, 2017; Zhang, 2018) and despite a decline in the applied amount in the last decade (Zhang, 2018), the toxic load for bees further increased (Goulson, Thompson, & Croombs, 2018). To reverse this trend, alternative pest control methods need to be developed and popularised. We believe that agricultural landscapes should not only be considered in the assessment of pesticide effects but also in their mitigation. Future research should identify farming practices and landscape elements that decrease pesticide exposure of pollinators or increase their resilience.

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