POLLINATORS IN PERIL

A systematic status review of North American and Hawaiian native bees

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EXECUTIVE SUMMARY

While the decline of European honeybees in the United States and beyond has been well publicized in recent years, the more than 4,000 species of native bees in North America and Hawaii have been much less documented. Although these native bees are not as well known as honeybees, they play a vital role in functioning ecosystems and also provide more than $3 billion dollars in fruit-pollination services each year just in the United States.

For this first-of-its-kind analysis, the Center for Biological Diversity conducted a systematic review of the status of all 4,337 North American and Hawaiian native bees. Our key findings:

- Among native bee species with sufficient data to assess (1,437), more than half (749) are declining.
- Nearly 1 in 4 (347 native bee species) is imperiled and at increasing risk of extinction.
- For many of the bee species lacking sufficient population data, it's likely they are also declining or at risk of extinction. Additional research is urgently needed to protect them.
- A primary driver of these declines is agricultural intensification, which includes habitat destruction and pesticide use. Other major threats are climate change and urbanization.

These troubling findings come as a growing body of research has revealed that more than 40 percent of insect pollinators globally are highly threatened, including many of the native bees critical to unprompted crop and wildflower pollination across the United States.

For this report we assembled a list of all valid native bee species and their current conservation status as established by state, federal or independent researchers. We then conducted a comprehensive review of all literature on those species as well as records documenting their occurrence. From that research we identified those bees with sufficient data to assess their status, including current and historical range, behavioral observations and studies, arriving at the first comprehensive analysis of the status of North American and Hawaiian native bees.

We also highlight five native solitary bee species that are seriously imperiled. These remarkable, underappreciated pollinators offer a snapshot of the threats driving the alarming declines in many native bee species — declines that must be reversed to save these irreplaceable native bees and the health of the ecosystems that depend on them.

INTRODUCTION

Bees are in serious trouble. Native bees indispensable to the health of the natural world are declining globally due to accelerating threats from agricultural expansion, habitat loss and climate change. [1][2] They are perilously underprotected.

Bees are the world's primary pollinators. With more than 20,000 species globally, they are an essential component of functioning ecosystems. [1][3] Without their pollination services, many wild plants and cultivated crops would be unable to thrive. [1][4][5] But bees are declining across the planet, [2][6][7][8] with more than 40 percent of insect pollinators — primarily native bees — highly threatened. [8]

For this report we undertook the first comprehensive review of the status of all 4,337 native bee species in North America and Hawaii. The report showcases the results of our overview and highlights five extraordinary native bees that are in need of immediate help to survive. Our analysis concludes that more than 50 percent of native bee species for which sufficient data is available are declining, while 24 percent are in serious peril.

The honeybees (*Apis mellifera*) most Americans associate as essential for food production are actually an introduced species from Europe. [9] The majority of native bees in North America are solitary, ground-nesting species that collect everything from pollen, nectar, leaves, petals and floral oils to be used as adult food sources, larval provisions or nest linings.

Almost 90 percent of wild plants are dependent on insect pollination, making bees indispensable pollinators in most ecosystems. [1][8] Pollination services provided by bees contribute to seed sets and plant diversity [1][2], as well as crop pollination that provides 35 percent of the global food supply or one of every three bites of food. [8] Native bees contribute to a significant portion to annual crop value [10], are critically important to their ecosystems and can be more effective pollinators than honeybees. [11] Native bees have profoundly shaped the world around us; they are a keystone to many habitats and have inspired our culture, from children's rhymes about bumblebees to the poetry of Emily Dickinson. Without these tiny, tireless creatures our world would be a less colorful and interesting place.

STATUS OF NORTH AMERICAN BEES

Bees are declining globally [6][7][8], including in North America. The most comprehensive global report thus far on the status of pollinators found that more than 40 percent of them, mostly bees, are facing extinction. [2] Europe is now tracking these declines, finding that 9.2 percent of European native bees are threatened with extinction and 37 percent are declining. [8][12] Their assessment likely greatly underestimates the magnitude of the threats because more than half the bee species native to Europe are too data-deficient for scientists to evaluate their status. [12]

Prior to our analysis, a similar comprehensive overview had never been

conducted for North American and Hawaiian bees. Status review provides critical new information that should spur more extensive study and protection of North American and Hawaiian native bees.

a. Methodology

Identification of Bees. We identified all bees recorded as native to Hawaii and North America, which we defined as Canada, the United States and Mexico, in the Discover Life database (www.discoverlife.org) [66], and checked them for taxonomic validity in the Integrated Taxonomic Information System database (www.itis.gov) and recent peer-reviewed journal articles, especially those published in ZooKeys. This resulted in a base list of 4,337 native bees to review for conservation status.

Conservation Status. We used Discover Life occurrence data, museum records, International Union for the Conservation of Nature (IUCN) and NatureServe species accounts, U.S. Department of Agriculture Farm Service Agency Cropland Conversion Datasets [37], U.S. Department of Agriculture State and County Profiles [63], U.S. Geological Survey National Synthesis Project for Pesticide Use Maps [64], and peer review and gray literature to determine whether the conservation status of each species was determinable and, if so, what the status was.

Each species was classified as *Data-Sufficient* (1,437) or *Data-Deficient* (2,900), indicating whether sufficient data were available to assign a conservation status with reasonable certainty.

Data-Sufficient species were classified as *Secure* or *Declining* based on changes in their population size or range between 2005 and 2015, or if data were lacking from that period, between 1985 and the last reported occurrence year. In keeping with IUCN methodology, we classified species as *Secure* if they declined by less than 30 percent between 2005 and 2015 and *Declining* if they declined by 30 percent or more during this period. Departing from the IUCN, species with no data after 2005 were classified as *Secure* if they declined by less than 40 percent between 1985 and the last reported occurrence, and *Declining* if they declined by 40 percent or more. Range change percent was calculated from presence/absence reports at the county level or a 30-mile radius of a latitude/longitude point.

We classified species *as Threatened* if they were categorized as *Threatened* (i.e. Vulnerable, Endangered, Critically Endangered) by the IUCN (Red List 3.1, Second Edition), *Vulnerable* or worse (G3, G2, G1, GH, GX) by NatureServe, *Vulnerable* or worse (Vulnerable, Imperiled, Critically Imperiled) by the Xerces Society, *Threatened* or *Endangered* by the Committee on the Status of Endangered Wildlife in Canada, or *Vulnerable* or worse (S3, S2, S1, SH, SX) by state natural heritage programs when species were absent from NatureServe, or *Critically Endangered* or *Vulnerable* by Griswold *et al.* [65] This resulted in our listing 184 species as *Threatened*.

We independently applied the IUCN and NatureServe ranking criteria to all species

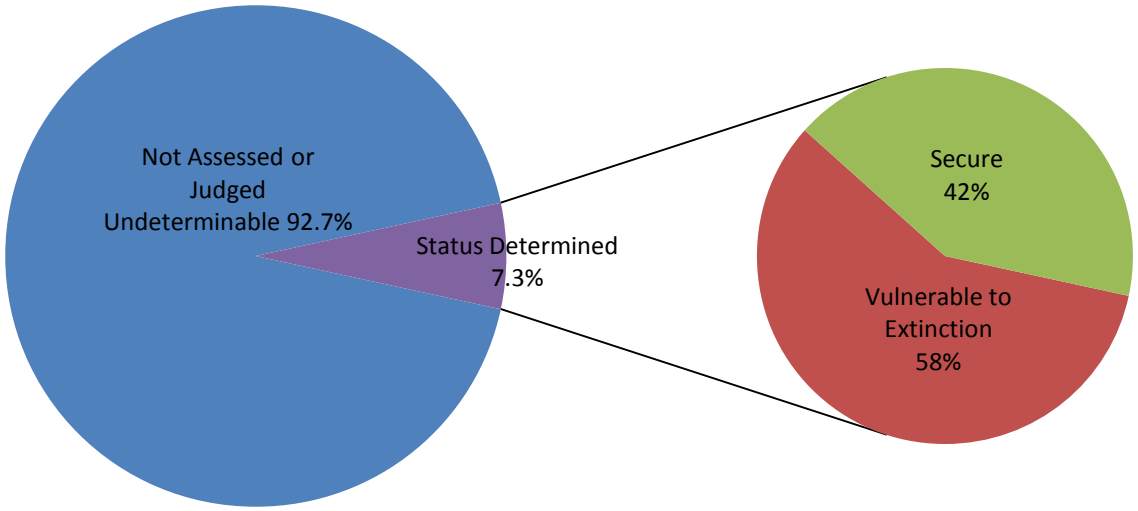
we judged to be Data-Sufficient but that were absent from, or unranked by, the above groups. We classified these species as *Threatened* if they met either the IUCN *Threatened* or the NatureServe *Vulnerable* or worse criteria. This resulted in another 163 species being classified as *Threatened*.

b. Relationship to Previous Studies

The status of various subsets of North American and Hawaiian bees has been assessed by individual researchers [e.g. 65], the IUCN's Bumblebee Specialist Group [15], NatureServe [14] and the Xerces' Society. [13] Taken together, they determined the conservation rank of 316 species: 7.3 percent of the region's 4,337 species (Figure 1). The vast majority of species remain unassessed or were determined to lack sufficient data to support a scientifically robust rank.

These prior studies found that 58 percent (184) of the 316 species with a determinable status were vulnerable to extinction.

Figure 1. Conservation Status of 4,337 North American and Hawaiian Native Bees as Reported by Prior Studies



Our study--which adds another 1,121 species with a known conservation status to the previous work--reached a similar result: 52 percent of species with a determinable status are declining and 24 percent are threatened with extinction.

c. Findings

We found that 24 percent of native bees (347) are imperiled, and population declines are occurring in 52 percent of native bees (749).

Many of these bees are endemic or have a highly restricted range, while others were once widespread but have been disappearing over the past several decades. All of these bees have something in common: Their habitat is shrinking, and so are their floral and nesting opportunities. A primary driver of their declines is agricultural intensification, which includes habitat destruction, widespread planting of monocultures and toxic pesticide use.

There is an urgent need for more research to better understand the bee species without current data. The number of imperiled and declining bee species would undoubtedly be clarified as higher if additional and current data were available.

However, we do know that many of these currently unrankable bees are often found in areas of great environmental degradation. Those include monocultures created by the escalating acreage planted only in crops such as pesticide intensive corn and soybeans. More research is urgently needed to better assess the threats to native bees so we can understand how to protect them. One study found that between 2008 and 2013, wild bee abundance declined across nearly a quarter of the United States, with California's Central Valley and the Midwest's Corn Belt ranking among the lowest in wild bee abundance. [16] This reduction in bee abundance was due to intense agricultural use of those areas. [16]

Clearly immediate action is needed if we are going to stop the widespread decline of native bees.

CASE STUDIES

Yellow carpet solitary bee (*Andrena blennospematis*)

Photo by Doug Wirtz

Though it lacks the familiar fuzziness and bright colors of many other bee species, a close look at the yellow carpet solitary bee reveals its dark, olive-green coloring and pale striped abdomen. [17] This beautiful bee's life is so intertwined with the life of the flower it depends on that they share the same name, yellow carpet (*Blennosperma nanum*). [17][18] The yellow carpet solitary bee depends solely on this plant genus for the pollen it needs to produce its offspring; [17][18] the bee's fate is completely tied to its specialized flower, and therefore the health and survival of the pockets of

California vernal pool ecosystems where they live. [5][18][19][20]

The yellow carpet solitary bee faces myriad threats, including severe reduction in habitat and other factors such as pesticide use, grazing and climate change. Habitat loss and modification is the primary threat facing the species because the vernal pool and upland habitats essential to its life cycle are being destroyed at alarming rates. [21][22][23] As much as 90 percent of the extant historic vernal pool habitat has been lost. [22] Three-quarters of it was lost by 1997, and by 2005 roughly 137,000 acres of vernal pool grassland had been lost in California's Central Valley. [24][25] An astounding additional 47,306 acres of vernal pool habitat was lost just between 2005 and 2012, despite conservation efforts put in place by the U.S. Fish and Wildlife Service's 2005 Vernal Pool Recovery Plan. [22][23] This loss is mainly due to agriculture [22][23][24], with increased pesticide use posing an escalating threat to the yellow carpet solitary bee. [26][27][28][29][30]

This loss of the yellow carpet solitary bee's habitat is reflected in the reduction of range, occurrence records and population size. [17][20][21] These bees are endemic to the vernal pool and upland habitat of Central California and the Bay Area [14][17], and went from occurring in 11 counties to being confirmed in only one county in the last decade. [20][21] The loss of the yellow carpet solitary bee is mirrored in the decline and possible loss of its specialized host (*Blennosperma* spp.), permanently changing the composition of the vernal pool ecosystem. [1][5][18][19][20]

Sunflower leafcutting bee (*Megachile fortis*)

Photo by Sam Droege / USGS Bee Inventory and Monitoring Lab

The sunflower leafcutting bee is the largest and most distinctive of all native North American leafcutting bees. [31] It is one of the few species within its genus to nest in the soil, instead of finding a hole in wood to rear its brood. [9][32] The bee uses its large mandibles or "bee teeth" to dig into hard packed soil, excavating a tunnel more than four times its length. [31]

The floral host for this grassland species is the sunflower (*Helianthus annuus*), which provides a pollen source for the brood. [14][31][32] This bee times its emergence and foraging with the bloom time of its bright-yellow host and could once be seen darting around sunflower patches from the Great Plains to Arizona. [33]

The sunflower leafcutting bee's grassland habitat is declining across its entire range, leaving it without forage and nesting habitat. [14] More than 90 percent of North America's natural grasslands have been converted to agricultural use, putting prairies among the rarest biomes in America [7], and replacing natural plant communities with monocultures of wheat and corn. [33] From 2006 to 2011, more than 1 million acres

(530,000 hectares) of U.S. grasslands were lost. [34] This conversion caused massive losses of nectar and pollen resources, reducing the range and abundance of the bee. [14][33][35] This important habitat has been declining since the 1950s, a decline that is expected to continue, with recent numbers revealing that states in sunflower leafcutting bee's range [31][36], including Nebraska, South Dakota and Texas, have the highest agricultural conversion rates in the United States. [37]

The sunflower leafcutting bee's floral host, the sunflower, is grown commercially in several states, including North Dakota and South Dakota. [38] However, sunflower monocultures can be detrimental to the bee, because they result in an overall loss of nesting sites. [39][40] In addition, the use of pesticides on the sunflower crop has been shown to harm and even kill solitary bees like the sunflower leafcutting bees. [1][14][27] Sublethal impacts caused by pesticides include decreased fitness, reduced brood rearing and reduced female production, all of which lead to smaller populations that can eventually cause local to large-scale extinctions. [27][29] Other threats to these bees are rangeland grasshopper spraying, grazing and climate change. [14] If current trends of land conversion and land-use practices continue, the already shrinking population of the sunflower leafcutting bee is projected to decline by more than 80 percent. [14] Soon this important creature may disappear from sunflower fields if steps are not taken to safeguard its future.

Wild sweet potato bee (*Cemolobus ipomoeae*)

Photo by Sam Droege / USGS Bee Inventory and Monitoring Lab

The wild sweet potato bee is the only known species in the world in its genus. [3] Its name, *Cemolobus,* means "lobed snout," referring to the three-lobed section on its face — the only bee to have this particular feature. [41] It is a floral specialist, foraging only on morning glory flowers (*Ipomoea*), especially wild sweet potato blooms (*Ipomoea pandurata*). [3][41][42][43] The bee emerges and is seen foraging in June and July, at the peak of flowering season for its hosts. [41][42]

Both the plant and the bee are found east of the Great Plains, from Missouri to Pennsylvania, in deciduous forest or at forest edges in the eastern United States. [41][42][43][44][45] The bee was once prevalent in forested areas, but due logging and land conversion has decreased in range and abundance. [46][47] It is also threatened by agricultural intensification and urban sprawl: As the bee's once-pristine habitat is paved or plowed over [45][46], its nesting and forging opportunities are greatly reduced, causing population declines. [4][48] Its floral host is not as fragile as some other native plants, and can survive in

a built environment, but occurrence records show that this unique bee does not adapt well to developed landscapes. [45][49]

The wild sweet potato bee was once most common in Illinois, yet has not been collected there since 2001 and before that had not been regularly collected in the state since the late 1970s. [45] Many of the counties in which it was once prevalent are now expanding towns or agricultural areas. [37][45][46][50] With its habitat continuing to be lost to development, this unique and once ubiquitous insect is now rarely seen.

Gulf Coast solitary bee (*Hesperapis oraria*)

Photo by John Bente

The Gulf Coast solitary bee is one of 34 bee species within the family Melittidae native to North America [3], and is the only bee within its genus to be found east of the Mississippi. [51] The species is also monolectic, meaning it forages on one plant and no others: the coastal plain honeycombhead (*Balduina angustifolia*), which provides for all its pollen and nectar needs. [51][52]

Endemic to a narrow band of barrier islands along the Gulf Coast, from eastern Mississippi to northwestern Florida, the bee nests in the deep sandy soil of dunes and forages on its specialized flower. [51] It emerges late in the season, exiting its ground nest from September to October — the peak bloom time of the coastal plain honeycombhead. [51][52] The honeycombhead is a self-incompatible plant, meaning it cannot reproduce without the help of this specialized bee, which transfers pollen from flower to flower. [51] Both flower and bee are thus heavily reliant on each other, and as one declines so does the other. Due to the bee's highly restricted host and range, the species has a high extinction risk.

The bee's entire range is estimated to be less than 38 square miles, and all known occurrences are in danger from development and hurricanes. [14] The Gulf Coast solitary bee only produces one generation a year, and any disturbance of this small population or its brood brings it closer to extinction. [51] Its distribution is becoming increasingly fragmented by urban growth, and remaining populations are becoming increasingly isolated. [51] The bee also has to contend with unrestricted recreation and aerial applications of broad-spectrum insecticides to control biting flies and mosquitoes. [51] The Gulf Coast solitary bee has never been found on the mainland despite its host flower's presence there, meaning that if its barrier islands habitat is further degraded, the bee will cease to exist.

The inevitable results of restricted range, isolated populations and habitat degradation are already playing out, as this bee is no longer found in one of the three counties where it was known to exist. [14] It is also

disappearing in other portions of its small range, including Choctawhatchee Bay, Pensacola Bay and Perdito Bay. [14] Without prompt action to conserve this species, it is likely to disappear.

Macropis cuckoo bee (*Epeoloides pilosula*)

Photo by The Packer Lab-Bee Tribes of the World

The macropis cuckoo bee is the only species of the cleptoparasitic tribe Osirini present in the United States and Canada, and is one of only two species of *Epeoloides* worldwide. [3][53] Cleptoparasitism is a form of feeding in which one bee's larvae feeds on food provided for a host larva. [3] The macropis cuckoo bee is an obligate cleptoparasitic of *Macropis* species. [54][55] Cleptoparasitic or cuckoo bees enter the nest of another bee (usually host specific) and lay their own egg in the cell. [3][56] Either the female cleptoparasite kills the host egg before leaving, or her larva destroys the host egg as it matures. [56][57] Hosts of the macropis cuckoo bee are bee species within *Macropis* (*M. nuda*, *M. ciliate*, *M. steironematis* and *M. patellata*), from which its name comes. [53]

The macropis cuckoo bee is a specialist, dependent upon nest aggregations of its *Macropis* hosts, and is often located in or near yellow or fringed loosestrife (*Lysimachia* spp.) habitat. [53][58] The loss or reduction of its host's nest is the main threat to the species. [55] Since *Macropis* species are dependent upon yellow or fringed loosestrife for pollen and floral oils, they are vulnerable to the loss or reduction of this plant. [55] Loosestrife plants are vulnerable to habitat loss and degradation as well as poor water quality since they're found in swamps and along streams and ponds edges. [55]

The macropis cuckoo bee was historically distributed in much of eastern and central North America and southern Canada. [53][54] A lack of records since 1942 led to the speculation that this species was extinct until the thrilling discovery of two males in Nova Scotia in 2004. [53][54] Its only known locality in the United States today is in New London, Conn., where it was discovered in June 2006 [14][59] — the first record of the bee in the United States since 1960. [59]

After the bee's rediscovery, some efforts have been made to protect it: It was listed as "endangered" in Connecticut in 2010 [60], and as "endangered" in Canada under the COSEWIC in May 2011. [14] The macropis cuckoo bee is considered "the most threatened and endangered bee species in New York (and the Northeast)." [61] Despite more attempts to locate the bee, unfortunately it has not been found in any of its previous range in the United States. [54][59] The story of the macropis cuckoo provides an important lesson that a species

should not have to decline to the point of being presumed extinct before receiving protection. Additional protections are still needed to ensure that this unique bee survives and recovers from the brink of extinction.

CONCLUSIONS

Native bees face myriad threats and are in desperate need of protection to safeguard their future. They contribute more than $3 billion in fruit-pollination services annually. [62] And these unique insects, and their pollination services, are vital to the survival of ecosystems. Our lives and culture would be significantly impoverished without these hardworking, underappreciated and declining animals.

The data compiled in this report offers a snapshot of magnitude of threats native bee species face and the extent of their decline. These findings are in line with those found globally and demonstrate the necessity of more research to fill the data gaps. But what we already know is troubling and should inspire us to act: 24 percent of data-sufficient native bees are imperiled, and 52 percent show population declines. We need to take aggressive steps to better understand and protect our precious bee species before it is too late.

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Chronic exposure to neonicotinoids increases neuronal vulnerability to mitochondrial dysfunction in the bumblebee (*Bombus terrestris*)

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ABSTRACT **The global decline in the abundance and diversity of insect pollinators could result from habitat loss, disease, and pesticide exposure. The contribution of the neonicotinoid insecticides (*e.g.*, clothianidin and imidacloprid) to this decline is controversial, and key to understanding their risk is whether the astonishingly low levels found in the nectar and pollen of plants is sufficient to deliver neuroactive levels to their site of action: the bee brain. Here we show that bumblebees (*Bombus terrestris audax*) fed field levels [10 nM, 2.1 ppb (w/w)] of neonicotinoid accumulate between 4 and 10 nM in their brains within 3 days. Acute (minutes) exposure of cultured neurons to 10 nM clothianidin, but not imidacloprid, causes a nicotinic acetylcholine receptor-dependent rapid mitochondrial depolarization. However, a chronic (2 days) exposure to 1 nM imidacloprid leads to a receptor-dependent increased sensitivity to a normally innocuous level of acetylcholine, which now also causes rapid mitochondrial depolarization in neurons. Finally, colonies exposed to this level of imidacloprid show deficits in colony growth and nest condition compared with untreated colonies. These findings provide a mechanistic explanation for the poor navigation and foraging observed in neonicotinoid treated bumblebee colonies.—Moffat, C., Pacheco, J. G., Sharp, S., Samson, A. J., Bollan, K. A., Huang, J., Buckland, S. T., Connolly, C. N. Chronic exposure to neonicotinoids increases neuronal vulnerability to mitochondrial dysfunction in the bumblebee (*Bombus terrestris*). *FASEB J.* 29, 2112–2119 (2015). www.fasebj.org**

Key Words: nicotinic acetylcholine receptors · neuronal culture

INSECTS POLLINATE >70% of our crops, contributing an estimated U.S.$215 billion to the global economy each year (1). In addition to their contribution to crop yield, insect pollinators can also improve the quality of the harvest (2). Beyond this, insect pollination provides ecosystem services that underpin biodiversity. Because of their clear importance in food security, global economics, and ecosystem stability, there is worldwide concern over the decline in insect pollinators, including wild and managed bees.

The known risks to insect pollinators include interacting pressures from parasites, disease, habitat loss, poor nutrition, and exposure to pesticides (1). A direct threat to insect pollinators is the use of insecticides that target the insect nervous system and are the principal means to control insect pests of crops, livestock, and people (3). The neonicotinoids are the most commonly used insecticide; it is widely accepted that very low levels exist in nectar (1.9 ppb) and pollen (6.1 ppb) (4); and they have been detected (3.8–13.3 ppb) in dead/dying but not healthy bees (5). Exposure to these chemicals extends beyond the period of crop flowering as relevant levels (tens of parts per billion) persist in the soil (6) and in nearby dandelions (*Taraxacum officinale*, 1–6 ppb) (5). Moreover, honeybees store food within their hives to maintain the colony's growth during poor weather and to sustain the colony over winter (7, 8).

Growing evidence indicates that sublethal levels of neonicotinoids may cause deficits in brain function (9), olfactory learning (10), navigation (11, 12), and colony development (13–15), therefore implicating their use in bee decline. However, others have failed to detect any deficits (16, 17). The target site of neonicotinoids is the nicotinic acetylcholine receptors (nAChRs) that, in insects, are found exclusively within the brain. However, despite our knowledge on exposure levels in the environment (4), we do not know if neonicotinoids reach the insect brain at a functional dose that is capable of perturbing neuronal function.

A second class of cholinergic insecticides is the cholinesterase inhibitors, the carbamates and organophosphates, which exert their effect by increasing acetylcholine to toxic levels. The organophosphate chlorpyrifos is used to treat a number of crops on which bumblebees forage, including

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; Ex/Em, excitation/emission; JC-1, 5,5′,6,6′ -tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbosyanine iodide; LC-MS/MS, liquid chromotography-mass spectrometry/mass spectrometry; nACh, nicotinic acetylcholine receptor

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grasslands, cranberries, top fruit, oilseed rape, and potatoes. In honeybee colonies, chlorpyrifos is detected commonly in wax (24.5 ppb), pollen (53.3 ppb), bees (3.4 ppb), and honey (46 ppb) (7, 8). Assuming a dietary exposure of 46 ppb (w/w) in honey, this equates to 30.8 ppb (w/v) (88 nM). Recently, additive toxicity between the neonicotinoids and organophosphates has been reported at the cellular (9) and whole bee (10) level in honeybees. This study tracks the dietary intake of neonicotinoid into the bumblebee brain and assesses its impact on neuronal function and colony performance, alone and in combination with raised levels of acetylcholine.

The field data, as the original Excel file, are available from the Environmental Information Data Centre Hub (*http://eidchub.ceh.ac.uk/metadata*).

MATERIALS AND METHODS

^{3}H-Imidacloprid feeding

Sugar syrup (Koppert Biologic Systems, Berkel en Rodenrijs, The Netherlands) was laced with 10 nM imidacloprid containing a ^{3}H-imdacloprid (specific activity = 40 Ci/mmol) radioactive tracer (American Radiolabeled Chemicals, St. Louis, MO, USA). The syrup was mixed by inversion overnight at room temperature in the dark. *Bombus terrestris* microcolonies of 20 intermediate sized bees (250–350 mg) sourced from 3 different colonies were fed syrup with or without imdacloprid tracer for 3 days. Microcolonies were maintained on a 12 hour light/dark cycle at room temperature. After 3 days, bee brains were removed by dissection and placed in scintillation cocktail, and each bee brain was counted individually.

Stable isotope dilution liquid chromotography-mass spectrometry/mass spectrometry analysis of imidacloprid in brains of bees

Bees were fed with sugar syrup containing 10 nM imidacloprid for 3 days. Bee brains were dissected and frozen at −80°C prior to analysis. A total of 63–100 bee brains were pooled together for analysis ($n = 3$). To each sample, 1 ml d_4-imidacloprid (10 ng/ml) in acetonitrile was added and dissociated on ice manually with a tissue homogenizer. The samples were then sonicated on ice (2 × 10 s) with an ultrasonicator probe. The homogenates were centrifuged at 13,000 rpm for 10 minutes, and the supernatant was dried in a vacuum dryer. The samples were then reconstituted in 50 μl acetonitrile followed by addition of 950 μl 0.1% formic acid in water. A solid phase extraction using Waters Sep-Pak C18 columns primed with 1 ml acetonitrile and preconditioned with 0.1% formic acid in 5% acetonitrile was used to enrich imidacloprid.

Liquid chromotography–mass spectrometry/mass spectrometry (LC-MS/MS) analysis was carried out using a Dionex 3000 LC system (Thermo Scientific, Hemel Hempstead, United Kingdom) linked to a Quantum Ultra Mass Spectrometer (Thermo Scientific) with an IonMax ESI interface. A C18 column (Pursuit, 3 μm, 50 × 1 mm; Thermo Fisher Scientific, Waltham, MA, USA) with a precolumn (Pursuit 3, MetaGuard; Thermo Fisher Scientific) was used to separate analytes. Five microliters of sample was injected, and each sample was analyzed in duplicate.

The LC was operated under gradient conditions with mobile phases of water/formic acid (99.9:0.1) (A) and acetonitrile/formic acid (99.9:0.1) (B) at a flow rate of 0.1 ml/min at 30°C. The initial mobile phase composition was 95% A, which was held for 1 minute, followed by a linear gradient over 5 minutes to 95% B, held at 95% B for 1 minute, and then returned to 95% A over 1 minute. The analytical column was then equilibrated at the initial conditions for 2 minutes for a total run time of 10 minutes.

Detection was in a multiple reaction mode, with transitions for imidacloprid being 256–209 and 256–175 and d_4-imidacloprid being 260.00–213.00. At the MS source, the voltage was set at 4500 V, sheath gas pressure at 50, ion sweep gas pressure at 5, auxiliary gas pressure at 0, and capillary temperature at 300°C. The tube length offset was set at 81, and collision energy at 18 V for both imidacloprid (256–209) and d_4-imidacloprid (260–213) and at 20 V for imidacloprid (256–175). The scan width was 0.05 (m/z), and the resolution for Q1 and Q3 was 0.7 (full width at half maximum). The argon pressure at Q2 was 1.5 mTorr. The optimized tuned condition was achieved by an infusion of imidacloprid (at 5 μl/min) to LC (0.1 ml/min, 80% B) using a T connector.

Data analysis was performed using XCalibur (version 2.0; Thermo Scientific) and LCQuan (version 2.5.6; Thermo Scientific). The extracted data were output to Microsoft Excel for further calculation.

B. terrestris primary neuronal culture

B. terrestris neuronal cultures were generated from the mushroom bodies of late-stage pupae. Mushroom bodies were dissected in cold supplemented Leibovitz's L-15 medium (22.2 mM glucose, 13.8 mM fructose, 128.5 mM sucrose, and 28.6 mM proline; Sigma-Aldrich, Paisley, United Kingdom) and pooled into ice-cold divalent cation-free Ringer solution (135 mM NaCl, 5 mM KCl, 180 mM sucrose, and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.2). Cells were trypsinized for 6 minutes and then incubated in 1 mg/ml trypsin inhibitor for 5 minutes. Cells were centrifuged for 1 min, 500 rpm, at room temperature. Supernatant was removed, and cells were resuspended and titurated in warm (28°C) supplemented L-15 medium. After being allowed to settle for 2 minutes, cells were plated onto poly-D-lysine (1 mg/ml)–coated glass coverslips. Cultures were maintained in the dark at 28°C in supplemented L-15 medium.

LIVE/DEAD viability/cytotoxicity assay

Viability assays on *B. terrestris* primary neuronal cultures were carried out using the LIVE/DEAD viability/cytotoxicity kit (Invitrogen, Carlsbad, CA, USA). Cells were pretreated with pesticides for 24 hours and then washed with phenol red free-supplemented L-15 medium. Cells were stained for 30 minutes in the dark at room temperature with a dye cocktail (4 μM EthD-1, 2 μM Calcein AM) made up in phenol red free-supplemented L-15 medium. Cells were washed for 5 minutes, imaged using an inverted wide-field imaging system, and analyzed using Volocity (PerkinElmer, Waltham, MA, USA) software. Excitation/emission (Ex/Em) wavelengths and bandwidth (in square brackets) used for the fluorescent dyes were Calcein AM (Ex/Em = 492[18]/535 [30]) and EthD-1 (Ex/Em = 572[23]/630[60]). Multiple fields of view were imaged from each coverslip.

JC-1 detection of mitochondrial membrane potential

B. terrestris primary neuronal cultures were washed with phenol red-free–supplemented L-15 medium and then incubated in the dark at 28°C for 15 minutes in 1 μg/ml JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbosyanine iodide; Invitrogen) made up in phenol red-free–supplemented L-15 medium. Cells were then washed with phenol red-free–supplemented L-15 medium for 15 minutes in the dark at 28°C. Cells were imaged live in phenol red-free–supplemented L-15 medium using an inverted wide-field imaging system and analyzed using Volocity (PerkinElmer) software, and chemical additions were added as 2× stock (300 μl). Images were obtained under ×400 magnification using

excitation/emission wavelengths and bandwidth (in square brackets) as follows: for polarized mitochondria (red; Ex/Em = 572 [23]/630[60]) and depolarized mitochondria (green; Ex/Em = 492 [18]/535[30]) with a 30 second capture rate.

$$\text{Relative mitochondrial membrane potential} = \frac{\text{ROI Em630} - \text{Bkgd Em630}}{\text{ROI Em535} - \text{Bkgd Em535}}$$

Acetylcholinesterase assay

Bumblebee brains were extracted by dissection and homogenized in PBS. Protein concentrations were determined by the Bradford assay, and acetylcholinesterase (AChE) activity was assayed at 14 mg/ml. AChE activity was determined using the Ellman assay. AChE inhibitors (appropriate concentrations) were incubated in bumblebee brain lysates for 20 minutes. Samples were then incubated at room temperature with a reaction mix containing the color indicator 50, 50 dithiobis (2-nitrobenzoic acid) (286 mM), and acetylcholine (ACh) iodide substrate (0.86 mM) for 30 minutes, and AChE activity was monitored by absorbance at 412 nm. AChE activity was normalized to control measurements. IC_{50} values were obtained from Hill equation fits of the data from 3 independent experiments.

Field experiment

Bumblebees (*B. terrestris audax*, the buff-tailed bumblebee) were housed 3 nests to a box, with entrances at the 2 ends and 1 in the middle. Two Tripols were assigned to each treatment group and placed in the field with ~1 m spacing between each Tripol. Therefore, any orientation mistakes (14) would be contained within a treatment group. The colonies were sited in a sheltered position within a wilderness/enriched grassland habitat in Wester Ross, The Highlands, Scotland. In this area, total pesticide (arable and grassland use) load is much reduced (~130-fold), as is the use of insecticides (~5000-fold) compared with intensively farmed arable areas such as East Fife (Scotland) (Supplemental Table S1, data provided by Science and Advice for Scottish Agriculture). No neonicotinoid or organophosphate use was encountered on farms sampled in the Highlands and Islands indicating that environmental contamination with these compounds is unlikely.

Treatment was provided in the form of pesticide addition to the supplemental sugar syrup feed provided with colonies. All colonies were provided with 1500 ml of sugar syrup containing the appropriate pesticide or were left untreated. Once spiked, colonies were closed and transported to the field site where they were opened within a day of exposure to treatment. At this point, bees were free flying throughout and were not forced to consume the sugar syrup provided. No pollen was provided and bees needed to forage for this. The order of the treatment boxes at the site was UT, single treatment, and double treatment to minimize any local effects. Experiments were performed on 2 separate occasions, with the first comparing untreated, chlorpyrifos (150 nM) and chlorpyrifos (150 nM)/imidacloprid (10 nM). The second experiment was placed on the same site and consisted of untreated, imidacloprid (10 nM)/chlorpyrifos (150 nM) plus imidacloprid (10 nM). Colonies were place in the field for 43 (second experiment; June 28–August 9, 2014) or 48 (first experiment; April 25–June 11, 2014) days (as access to the site permitted).

On the final day of the trial, entrance gates were set to permit bee entries only (no exits) and after ≥5 hours (the average foraging duration for bees exposed to imidacloprid is 42 minutes), the entrance gates were closed, and colonies returned to the laboratory for assessment. Colony assessment was determined by increase in colony mass, total live number of bees remaining, average bee mass, the number of healthy brood cells on the surface of the nest, and overall condition of the nest (Supplemental Fig. S2). Each individual nest mass was recorded at the beginning and end of the experiment (excluding the sugar syrup feed provided). Colonies were then anesthetized with CO_2, and live bees (identified by a combination of appearance and movement when handled) were removed, weighed, and euthanized quickly in ice-cold water containing detergent so that they didn't awaken.

Statistical analysis

We pool the data from the 2 field experiments. In our models, nests are nested within boxes, which allow us to incorporate any box effects and absorb any experiment effect into the box effects.

We used the following generalized linear mixed models:

1. Number of live bees/number of brood cells: A quasipoisson model with log link function was assumed. C and I were included as main effects (thus C = I = 0 corresponds to the control, C = 1 I = 0 to chlorpyrifos alone, C = 0 I = 1 to imidacloprid alone, and C = I = 1 to both chlorpyrifos and imidacloprid). Box was also included as a main effect, with nests nested within boxes.
2. Mean mass of live bees in nest/total bee mass in nest: A γ model with log link function was assumed. C and I were included as main effects. Box was also included as a main effect, with nests nested within boxes.
3. Final nest mass. Model same as for mean mass, except that log(initial nest mass) was included as a covariate, to adjust for any variation in initial nest size. For each model, we also tested for evidence of an interaction between C and I.

RESULTS

To determine the delivery of neonicotinoid to the brain following dietary intake of field relevant levels, adult bumblebees (*B. terrestris audax*) were fed sugar syrup containing imidacloprid [10 nM, 2.1 ppb (w/w)]. For rapid and sensitive detection, we tracked the accumulation of ^{3}H-imidacloprid. To exclude external contamination of the head and proboscis, we excised the brains for analysis and determined the concentration on the basis of the average size of a bumblebee brain (1.16 μl) (18). We find that imidacloprid (or its metabolites) does not reach significant levels within 42 minutes (an average foraging flight for bees exposed to imidacloprid) (14) but does accumulate to 9.7 ± 0.8 nM after 3 d (**Fig. 1*A***). The presence of intact (nonmetabolized) imidacloprid (at 3 days) was confirmed by using stable isotope dilution LC-MS (Fig. 1*B*) to be between 4.2 ± 1.7 (transition 256–209) and 5.2 ± 1.7 nM (256–175) (Supplemental Fig. S1). Imidacloprid is not lethal to brain neurons in culture (1 μM, 24 hours; Fig. 1*C*) or fed caged bees (10 nM, 5 days; data not shown). Therefore, any toxicity to adult bees is likely limited to neuronal dysfunction rather than acute brain damage.

As neurons are energetically demanding cells that require mitochondrial ATP production to maintain ion homeostasis (19), a constant mitochondrial membrane potential is critical for normal neuronal function (20). In mammalian neurons, excessive excitatory stimulation (by glutamate or its synthetic agonists) causes mitochondrial dysfunction (19) and long-term neural deficits (20). Therefore, we investigated whether the insect excitatory neurotransmitter, ACh, or its synthetic neonicotinoid agonists could influence mitochondrial function in bumblebee neurons. We find that exposure to high levels of ACh (1 mM, but not 100 μM), induces acute mitochondrial

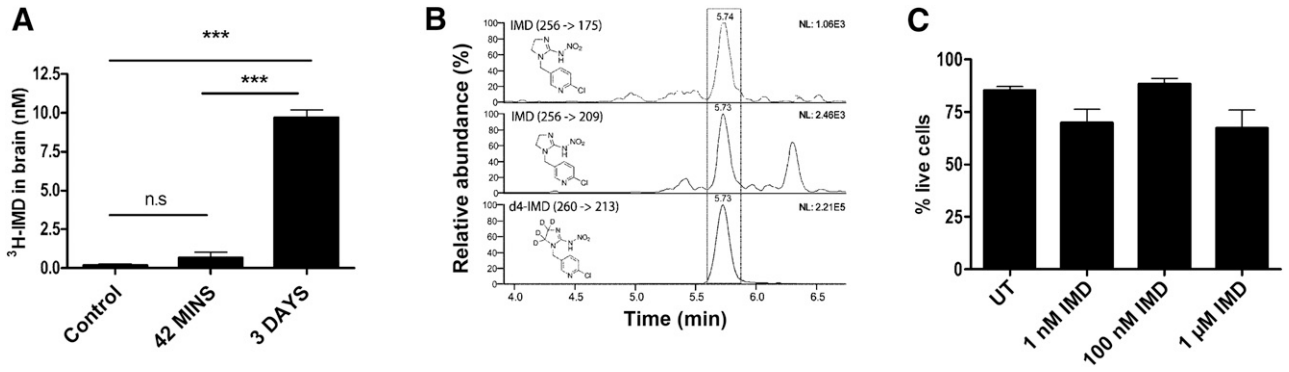

Figure 1. Imidacloprid accumulation in the brain does not affect neuronal viability. *A*) Bumblebees were fed radioactive imidacloprid (^{3}H-IMD) for times indicated, and brains (10 bees, $n = 3$) were isolated and counted by scintillation to determine imidacloprid concentration. ***$P < 0.001$ (1-way ANOVA with Bonferroni's multiple comparison test). *B*) Bumblebees were fed imidacloprid for 3 days, and the brain was excised and analyzed by stable isotope dilution LC/MS to determine the concentration of active ingredient [imidacloprid (transition 256→175 and 256→209)] with an internal standard [d_4-imidaclorid (260→213, 500 pg on-column)]. Examples of ion chromatograms from a bumblebee brain extract are shown. *C*) Bumblebee brain neurons (DIV 3–10) were exposed to imidacloprid (1 nM to 1 μM) for 24 hours, and cell viability was determined using calcein AM/EthD-1 staining (~8–11 fields, ~50 neurons per field, $n = 3$).

depolarization (**Fig. 2*A***). In contrast, both clothianidin (Fig. 2*B*) and imidacloprid (Fig. 2*C*) can induce acute mitochondrial depolarization at much lower levels (10 nM and 1 μM, respectively). As in mammals (19), this effect is receptor dependent and is blocked by the nAChR antagonist tubocurarine (500 μM; Fig. 2*D*). Therefore, on the basis of the accumulation of imidacloprid in bumblebee brains, an exclusive dietary exposure (over days) to clothianidin is sufficient to cause acute brain mitochondrial dysfunction in bumblebees. In contrast, for imidacloprid, the dose reached (5–10 nM) is insufficient to induce mitochondrial depolarization when presented acutely (30 minutes). However, the risk to bumblebees results from chronic exposure over many weeks during crop flowering and perhaps even longer due to its persistence in the soil (6) and re-emergence in wildflowers (5).

Even if the length of exposure may be extended, in a real landscape, alternative forage may be available, and therefore the actual exposure level may be reduced. Therefore, we probed further for potential deficits at even lower concentrations and over a longer duration. Neurons exposed chronically to ACh (100 μM, 48 hours) do not become sensitized to ACh, and they are resistant to a subsequent acute exposure to ACh (100 μM; **Fig. 3*A***). In contrast, although low-level imidacloprid (10 nM) does not induce mitochondrial depolarization acutely (Fig. 2*C*), when neurons are exposed chronically (48 hours) to just 1 nM imidacloprid, vulnerability to the normally innocuous ACh (100 μM) exposure occurs (Fig. 3*B*). Under these conditions, mitochondrial responses to imidacloprid can be divided into 3 cell groups; nonresponders (49.6 ± 21.2%; data not shown) and neurons undergoing mitochondrial depolarization either rapidly (37.4 ± 31.0%) or slowly (13.0 ± 11.4%). To confirm that the development of vulnerability to mitochondrial depolarization is receptor dependent, as seen for the acute effects of clothianidin (Fig. 2*D*), tubocurarine (500 μM) was included during the chronic exposure period (48 hours) to 1 nM imidacloprid.

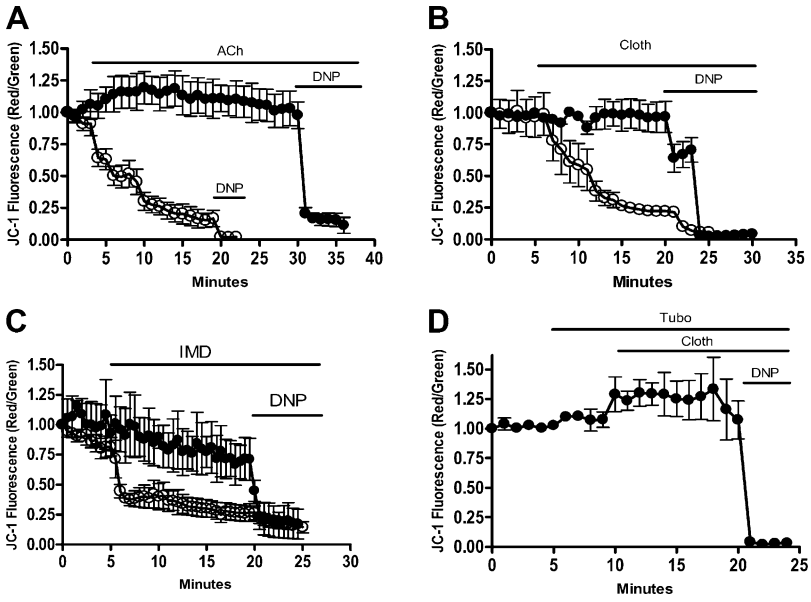

Figure 2. Bumblebee brain neurons undergo mitochondrial depolarization when nAChR are hyperstimulated. *A*) Bumblebee neurons in culture undergo mitochondrial depolarization in the presence of high levels (1 mM; open circles) but not low levels (100 μM; filled circles) of acetylcholine. The neonicotinoid, clothianidin (*B*), induces mitochondrial depolarization at 10 nM (open circles) but not at 1 nM (filled circles), and imidacloprid (*C*) induces mitochondrial depolarization at 1 μM (open circles) but not at 10 nM (filled circles). (D) Neurons pre-exposed to the nAChR antagonist *d*-tubucurarine (500 μM) do not undergo mitochondrial depolarization in the presence of clothianidin (100 nM), demonstrating an nAChR-dependent process. In all cases, mitochondrial depolarization was monitored using ratiometric (red/green) JC-1 imaging, and the experiment was terminated by full mitochondrial depolarization using 2,4-dinitophenol (1 mM). In all cases, 15–20 regions of interest were monitored ($n = 3$).

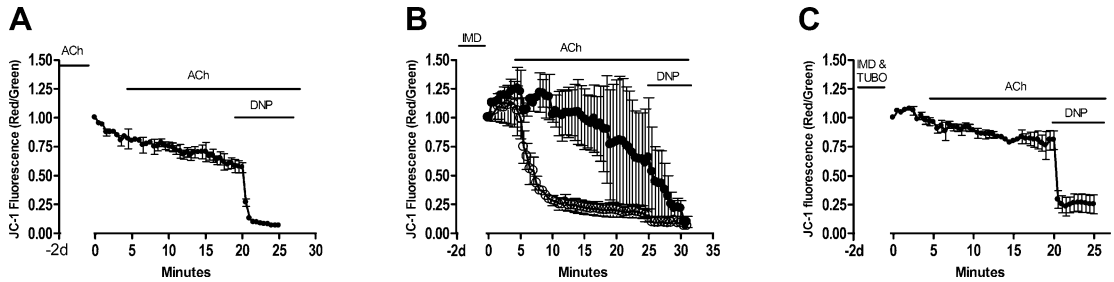

Figure 3. Chronic exposure to low levels of imidacloprid increases mitochondrial vulnerability. Bumblebee neurons (3–10 DIV) exposed chronically for 2 days (−2 days to 0 minutes) to (*A*) ACh (100 μM) do not induce vulnerability to mitochondrial depolarization by a subsequent exposure to subeffect ACh (100 μM). *B*) Low level imidacloprid (1 nM) induces vulnerability to a subsequent exposure to subeffect ACh (100 μM), revealing fast responders (open circles) and slow responders (filled circles). Nonresponders not shown. *C*) Low level imdacloprid (1 nM) and tubocurarine (500 μM) coexposure prevents development of mitochondrial vulnerability to subeffect ACh (100 μM). In all cases, mitochondrial depolarization was monitored using JC-1 and the experiment was terminated by full mitochondrial depolarization by 2,4-dinitophenol (1 mM).

Under these conditions, no increased vulnerability to ACh (100 μM) occurs (Fig. 3*C*), confirming that sustained nAChR activation is required to establish mitochondrial vulnerability to ACh.

Given the impact of neonicotinoids shown here, bee brain neurons would be unable to generate the energy required for homeostatic control and neuronal function. The accumulated loss of adult bee performance and/or developmental consequences to the brood, as seen previously at higher doses (20, 21), could impact colony growth (13, 14). Therefore, we replicated our feeding regime on whole bumblebee colonies to relate our cellular responses to colony performance. Bees were allowed to forage freely throughout the experiment in a predominantly wilderness environment, where few pesticides and no neonicotinoids or organophosphates, are used (Supplemental Table S1). As neonicotinoids increase the vulnerability of bee neurons to ACh, we were determined to increase ACh levels by coexposure to a cholinesterase inhibitor. We determined the IC_{50} (4.47 ± 0.16 nM) for the chlorpyrifos oxon active metabolite (of chlorpyrifos) in bumblebee brains to be well below the likely environmental dose (~88 nM) (7, 8).

Therefore, bumblebee colonies were provided with field relevant levels of imidacloprid (10 nM) and/or chlorpyrifos (150 nM) in sugar syrup and left at a single site to forage freely for 43–48 days. Three nests (all treated identically) were housed in each box. The individual values of all nests are indicated for colony growth, number of live bees and viable brood, and the individual bee masses plotted.

As expected for a natural environment, colony performance was variable, even in untreated colonies. Colony growth was significantly impaired in colonies exposed to imidacloprid (imidacloprid, 24.0 ± 1.0% or imidacloprid + chlorpyrifos, 14.4 ± 3.6%) compared with untreated colonies (38.0 ± 15.3%), or chlorpyrifos alone (51.5 ± 29%) (**Fig. 4*A***, individual nest values indicated). Similarly, the number of surviving bees was reduced significantly in the presence of imidacloprid (imidacloprid alone, 97.2 ± 11.1; imidacloprid/chlorpyrifos, 53.3 ± 14.1) compared with untreated (138.7 ± 24.7) or chlorpyrifos-treated (193.5 ± 127.5) colonies (Fig. 4*B*, individual nest values indicated). Finally, to indicate future colony potential, viable brood cell number on the exterior face of the nest was determined. Again, compared with untreated (32.7 ± 6.7) and chlorpyrifos-treated colonies (57.7 ± 41.5), this was reduced significantly by imidacloprid (imidacloprid alone, 15.8 ± 5.0; imidacloprid/chlorpyrifos, 12.0 ± 9.6) (Fig. 4*C*, individual nest values indicated). In all cases, there was no significant impact of chlorpyrifos on the deficits caused by imidacloprid. We observed no significant difference in the average bee mass for any treatment group (Fig. 4*D*). Finally, nest condition in the presence of imidacloprid was severely compromised by fungal contamination (Fig. 4*E*, see Supplemental Fig. S2 for images of all nests), and some weakened colonies were overrun by wasps (*Vespula vulgaris*).

Differences in colony performance were assessed statistically using generalized linear mixed models (**Tables 1 and 2**). The interaction between chlorpyrifos and imidacloprid was not significant at the 5% level for any of the analyses, and therefore we report results of fitting the models without interaction. In 2 cases, number of live bees ($P = 0.057$) and final nest mass ($P = 0.085$), the interaction was significant at the 10% level, providing weak evidence that the effect of imidacloprid was greater in the presence of chlorpyrifos. In Table 1, we show *P* values for testing the null hypotheses of no treatment effect on each response variable. These results show no indication of an effect of chlorpyrifos, whereas there was evidence of an effect of imidacloprid on all response variables except the mean mass of live bees (Table 1). For the other 4 response variables, the 95% confidence intervals for the coefficient of imidacloprid, together with the corresponding interval for percent reduction in the response variable in the presence of imdicaloprid, are shown (Table 2).

DISCUSSION

In terms of risk from neonicotinoids to bees, a prerequisite is that neonicotinoids reach a pharmacologically relevant level at their site of action: the insect brain. In this study, we demonstrate the delivery of neuroactive levels of imidacloprid to the brains of bumblebees fed at a field realistic level for 3 days. Brain levels were determined by both the use of a radioactive tracer and LC-MS to confirm, beyond doubt, the existence of active parental compound in the brain. This is likely an underestimate of exposure to active ingredient as the imidacloprid metabolite, olefin, is neuroactive in bees (9) and toxic to

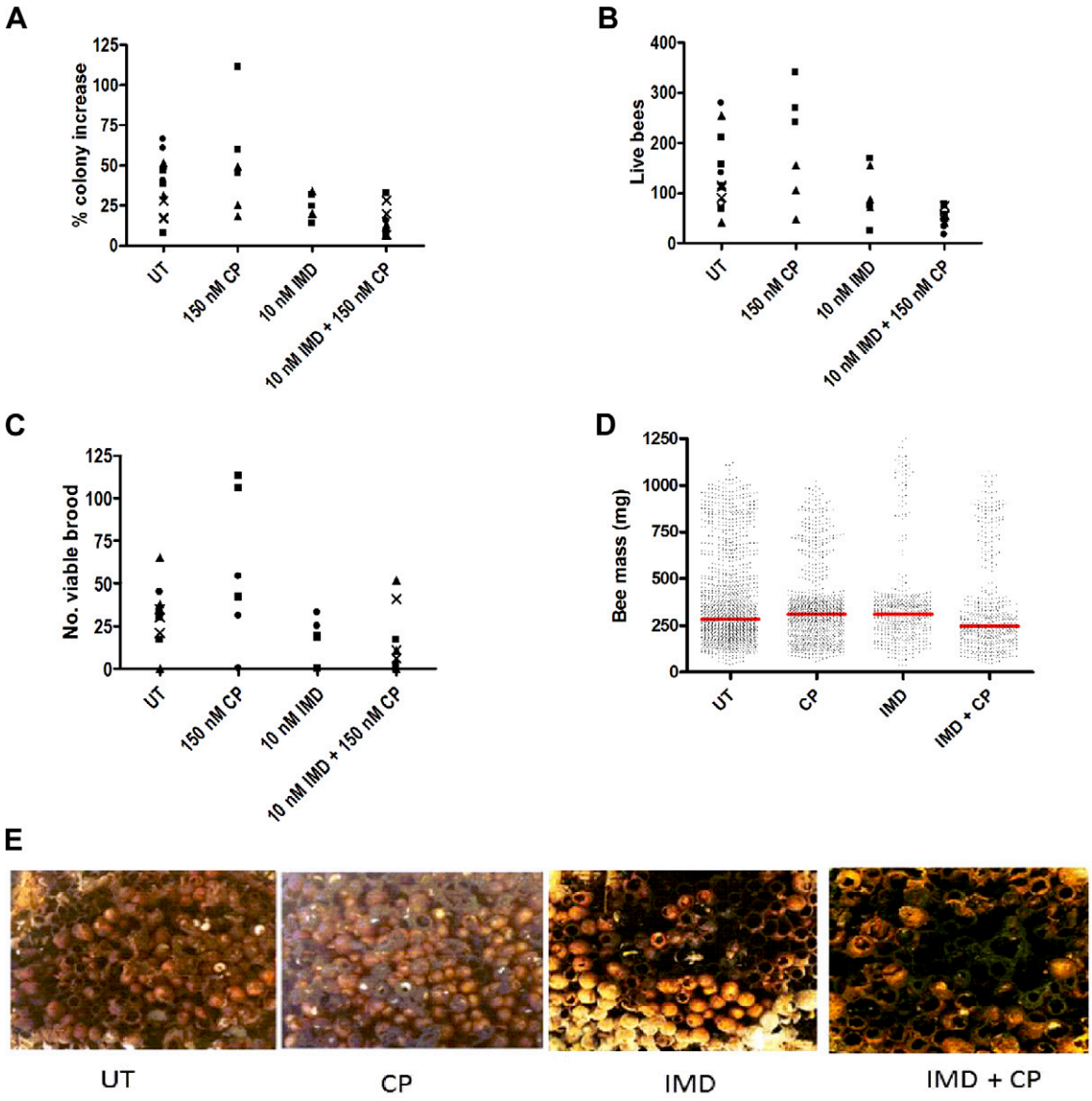

Figure 4. Exposure of bumblebee colonies to field-relevant levels of imidacloprid decreases colony performance. Bumblebee colonies of similar mass were provided with sugar syrup (UT, $n = 12$), containing chlorpyrifos (CP, 150 nM, $n = 6$), imidacloprid (IMD, 10 nM, $n = 6$), or both (IMD + CP, $n = 12$). No pollen was provided, and bees were free to forage in a wilderness/grassland area in the west of Scotland. After 43–48 d in the field, colony performance indicators were monitored. Nests within each box are depicted with the same symbol. *A*) Percentage colony mass increase for each nest. *B*) Total number of live bees remaining in each nest. *C*) Number of viable brood cells on the outer face of each nest. *D*) Size distribution scatter of individual bee masses within each treatment group. Median values are shown by red lines. *E*) To report on the condition of the nests, a representative image of each nest was collected. A representative example from each treatment group is illustrated (all images are available in Supplemental Fig. S2).

pests (22). Within the duration of a typical foraging bout (42 minutes when exposed to neonicotinoids) (14), no imidacloprid is detected. Therefore, no immediate impairment on bee function, such as homing ability, would be expected after initial exposure to normal levels of neonicotinoids. However, after approximately 3 days, imidacloprid accumulates to low nanomolar levels. Interestingly, even high levels (1 mM) of imidacloprid do not kill bumblebee neurons over 24 hours. Therefore, the consequences of normal neonicotinoid exposure would be expected to be subtle. Indeed, caged bees fed this level of imidacloprid over several days did not die (data not shown).

In terms of neuronal function, we observe that a low level of clothianidin (10 nM) activation of nAChRs does cause acute mitochondrial depolarization, making it 100,000-fold more potent, in this respect, than acetylcholine. Exposure to imidacloprid at this level (as realized after 3 days dietary exposure) did not cause acute (<25 minutes) mitochondrial depolarization. However, under the more realistic conditions identified in this study (present at <10 nM for days), as little as 1 nM imidacloprid increases neuronal sensitivity to acetylcholine, where a normally innocuous level (100 μM) is now capable of inducing mitochondrial depolarization in the majority of neurons.

TABLE 1. *Tests of the null hypothesis of no treatment effect for chlorpyrifos (C) and imidacloprid (I)*

Response	C	I
Number of live bees	0.822	0.009
Number of healthy brood cells	0.314	0.006
Mean mass of live bees	0.426	0.978
Total bee mass in nest	0.395	0.028
Final mass of nest	0.906	0.012

Tabulated values are *P* values.

One possible mechanism of increased sensitivity to acetylcholine is a pharmacological chaperone type effect by neonicotinoids, leading to an up-regulation in nAChR expression. In support of such a hypothesis, changes in nAChR expression have been observed in mammals exposed chronically to nicotine (23, 24) and even neonicotinoids (25). Although high levels (19–70 μM) of neonicotinoids were required to up-regulate mammalian receptors, this most likely reflects their low affinity for the mammalian receptors, and a similar up-regulation of insect nAChRs may occur at much lower, field-realistic, levels.

Mitochondrial dysfunction compromises ATP production and therefore disrupts neuronal homeostasis, plasticity, learning, and behavior in mammals (19, 26). Importantly, the neurons investigated here are Kenyon cells that constitute >40% of cells in the bee brain (27). They are the major neuronal component of the mushroom bodies, a higher-order insect brain structure that mediates multisensory integration, learning, and memory (28, 29). Therefore, mitochondrial dysfunction in Kenyon cells provides a reasonable explanation for the memory deficits (10) and poor navigation (11, 12) observed in honeybees and the reduced foraging efficiency in bumblebees (14, 30) exposed to neonicotinoids. Under more chronic conditions, a contribution from endogenous acetylcholine during intense synaptic activity, when bees are learning to forage on new flowers or in new areas, is likely. Therefore, the impact on colonies may be greater in challenging landscapes or weather conditions (31).

Previous colony studies used different conditions, with higher levels of imidacloprid (6), or included the exposure in both sugar and pollen (5), and colonies were laboratory based throughout (6) or during the period of exposure to imidacloprid (5). Therefore, to directly relate to our cellular studies, we performed a field trial on bumblebee colonies in a wilderness environment using the feeding regime that we used to track imidacloprid into the brain and assess its consequences. To mimic enhanced exposure to acetylcholine, we included a field-relevant level of the organophosphate chlorpyrifos in the sugar solution provided. Importantly, bees had to forage for their own pollen if they were to be successful at raising brood. Therefore, colonies suffering a deficit in their foraging ability (*e.g.,* olfactory learning or navigation) should fail to grow as strongly as control colonies. Chlorpyrifos, when present alone, exerted no significant effect on colony performance. In contrast, in colonies exposed to imidacloprid, few colonies exhibited strong nest growth, and they had fewer bees and brood cells. In the honeybee, very high doses (50–75 μM) of imidacloprid directly act on mitochondrial function (32), whereas at very low doses in the diet (0.15 pM), mitochondrial structure is normal in the midgut after 8 d of feeding (33).

The failure of chlorpyrifos to enhance the effect of imidacloprid may reflect that the negative impact of imidacloprid is already maximal. Accumulating evidence suggests that neonicotinoids (at field-relevant levels) exert their toxicity by a chronic deficit in neuronal function (9), leading to deficits in learning and memory (10) and poor colony foraging capacity (14, 30). Therefore, the effect of neonicotinoids on insect colonies may depend on how challenging the environment is in terms of food availability and weather (foraging opportunities). In our field trial, the area is typically wet and windy, and there was little garden or commercial forage available, suggesting that small deficits in foraging efficiency, compounded over time, may have had a high impact on our colonies.

The consequences of neonicotinoid exposure may be exacerbated by the coexistence of other environmental threats such as disease (34), other pesticides (7), or exposure to other sources of neonicotinoids from nearby wildflowers (5) or treated lawns (35), as synergistic interactions between neonicotinoids have been reported (patent no. U.S. 7,745,375 B2; 2010). Our study indicates that the consequences of neonicotinoid exposure would be subtle, affecting higher cognitive function. This is consistent with previous studies identifying deficits in learning (10), navigation (11, 12), foraging (14, 30), and colony growth (13, 14). Importantly, such deficits would be delayed while the impact of decreased foraging performance accumulates within a colony, and this has been reported (13, 14, 36). On the basis of imidacloprid accumulation, this study indicates that an acutely effective dose of clothianidin or a chronically effective dose of imidacloprid reaches the bumblebee brain within 3 days of dietary exposure to neonicotinoids. Future field trials will need to consider whether bees are challenged sufficiently (in terms of pesticide exposure time, forage availability, weather, and disease) if cognitive deficits resulting from pesticide exposure are to be revealed.

TABLE 2. *Estimates and 95% confidence intervals for the coefficient of imidacloprid*

Response	Estimated coefficient	95% confidence interval	Estimated % reduction	95% confidence interval
No. live bees	−0.81	(−1.36, −0.26)	55%	(23%, 74%)
No. healthy brood cells	−1.22	(−2.00, −0.45)	71%	(36%, 86%)
Total bee mass in nest	−0.85	(−1.58, −0.12)	57%	(11%, 79%)
Final mass of nest	−0.19	(−0.32, −0.05)	17%	(5%, 27%)

The coefficient would be zero in the absence of an effect; negative values indicate a negative impact of imidacloprid. Also shown are the corresponding estimates and 95% confidence intervals for the percent reduction of the response variable in the presence of imidacloprid.

Indeed, the improvement of forage availability for all insect pollinators may help to mitigate the negative impact of insecticides. FJ

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Chronic exposure to neonicotinoids increases neuronal vulnerability to mitochondrial dysfunction in the bumblebee (*Bombus terrestris*)

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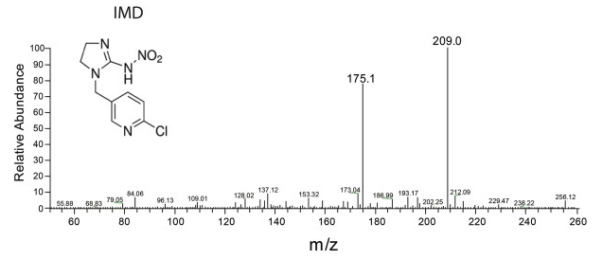

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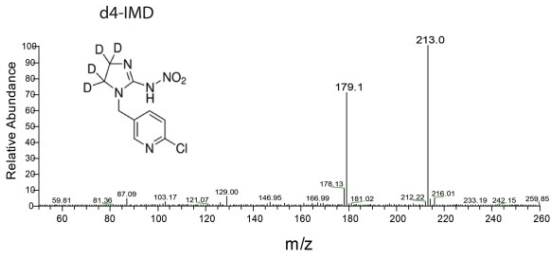

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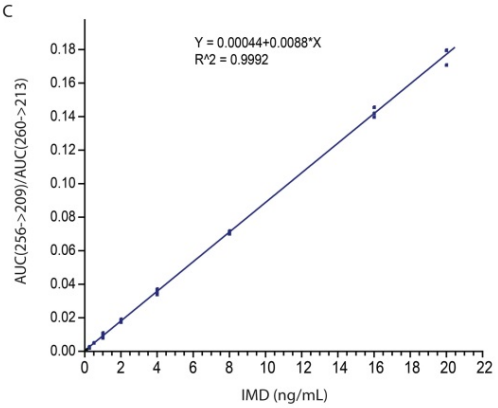
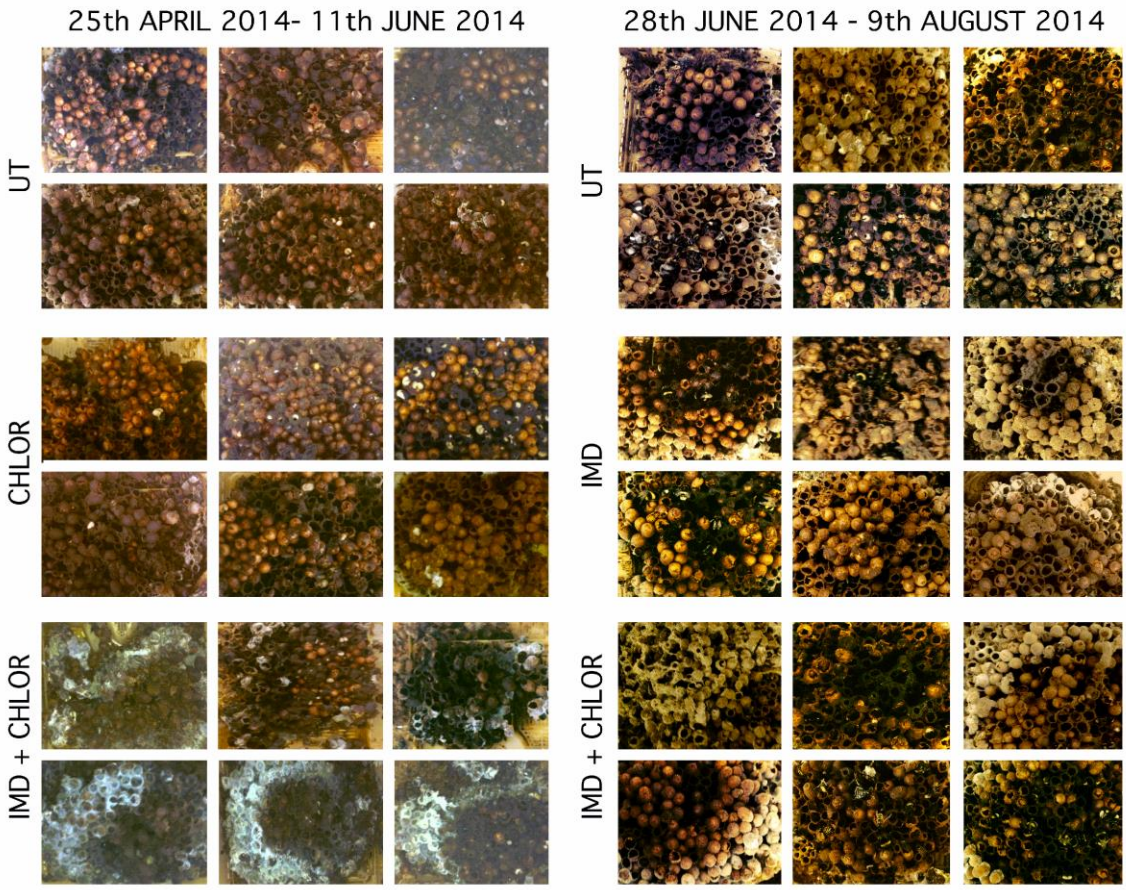


D

	N	[IMD] in bee brain (assuming brain volume= 1.16uL)(nM)
IMD (256->209)	3	4.2 ± 1.7
IMD (256->175)	3	5.2 ± 1.7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Sublethal neonicotinoid insecticide exposure reduces solitary bee reproductive success

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Abstract

1 Pollinating insects provide crucial and economically important ecosystem services to crops and wild plants, but pollinators, particularly bees, are globally declining as a result of various driving factors, including the prevalent use of pesticides for crop protection. Sublethal pesticide exposure negatively impacts numerous pollinator life-history traits, but its influence on reproductive success remains largely unknown. Such information is pivotal, however, to our understanding of the long-term effects on population dynamics.

2 We investigated the influence of field-realistic trace residues of the routinely used neonicotinoid insecticides thiamethoxam and clothianidin in nectar substitutes on the entire life-time fitness performance of the red mason bee *Osmia bicornis*.

3 We show that chronic, dietary neonicotinoid exposure has severe detrimental effects on solitary bee reproductive output. Neonicotinoids did not affect adult bee mortality; however, monitoring of fully controlled experimental populations revealed that sublethal exposure resulted in almost 50% reduced total offspring production and a significantly male-biased offspring sex ratio.

4 Our data add to the accumulating evidence indicating that sublethal neonicotinoid effects on non-*Apis* pollinators are expressed most strongly in a rather complex, fitness-related context. Consequently, to fully mitigate long-term impacts on pollinator population dynamics, present pesticide risk assessments need to be expanded to include whole life-cycle fitness estimates, as demonstrated in the present study using *O. bicornis* as a model.

Keywords Clothianidin, fitness, neonicotinoid, *Osmia*, pesticide risk assessment, pollinator, population dynamics, sublethal effect, thiamethoxam.

Introduction

Pollinating insects contribute significantly to agricultural productivity (Klein *et al.*, 2007; Garibaldi *et al.*, 2011a), revenue (Gallai *et al.*, 2009) and ecosystem stability (Bascompte *et al.*, 2006; Fontaine *et al.*, 2006), and are thus important components for the maintenance of biodiversity and food security. Recent reports on global pollinator declines (Biesmeijer *et al.*, 2006; Potts *et al.*, 2010; Cameron *et al.*, 2011) are alarming, especially with respect to the currently high and continuously increasing demands for pollination services (Klein *et al.*, 2007; Aizen *et al.*, 2008).

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Various managed and wild pollinators have been documented to exhibit similar population declines (Biesmeijer *et al.*, 2006; Goulson *et al.*, 2008; Potts *et al.*, 2010), despite having different life histories and habitat requirements. This suggests the involvement of common primary drivers, such as emerging parasites and pathogens (Cameron *et al.*, 2011; Nazzi *et al.*, 2012), or habitat degradation (Potts *et al.*, 2010; Garibaldi *et al.*, 2011b). In addition, the prevalent use of pesticides in crop protection is also suspected to represent a conspicuous threat throughout agricultural landscapes (Desneux *et al.*, 2007; Vanbergen & The Insect Pollinator Initiative, 2013), particularly including the application of systemic neonicotinoid insecticides, which has increased strongly on a global scale over the last decade (Elbert *et al.*, 2008; Mullin *et al.*,

2010; Jeschke *et al.*, 2011). Neonicotinoids act as agonists of acetylcholine receptors in insects and other invertebrates, thereby disrupting neuromuscular signalling pathways, leading to abnormal behaviour, immobility and death of target insect pests (Matsuda *et al.*, 2001; Elbert *et al.*, 2008). Because neonicotinoids are systemic, nontarget pollinating insects can also be directly exposed to these compounds in flowering crops through the translocation of residue trace levels from vegetative plant parts into nectar and pollen, eventually causing detrimental sublethal effects (Desneux *et al.*, 2007; Cresswell, 2011; Blacquière *et al.*, 2012; Cresswell *et al.*, 2012). For example, in honeybees, it was demonstrated that sublethal dietary exposure to neonicotinoids negatively affects learning abilities and memory, as well as foraging and homing behaviours (Decourtye *et al.*, 2004; Decourtye & Devillers, 2010; Belzunces *et al.*, 2012; Henry *et al.*, 2012; Williamson & Wright, 2013). Moreover, there is increasing evidence of detrimental synergism when honeybees are exposed to a combination of neonicotinoids and the prevalent gut parasite *Nosema* sp. (Alaux *et al.*, 2010; Vidau *et al.*, 2011; Pettis *et al.*, 2012) or viral pathogens (Di Prisco *et al.*, 2013). To date, there is neither consensus on how to target and incorporate chronic and sublethal effect bioassays in pesticide risk assessment guidelines for the standard model organism, the honeybee (OEPP/EPPO, 2010a,b; Cresswell, 2011; Blacquière *et al.*, 2012), nor any clear understanding of whether and how honeybee responses can be extrapolated to other pollinators exhibiting different life histories and foraging strategies (Desneux *et al.*, 2007; Mommaerts *et al.*, 2010; Biddinger *et al.*, 2013). However, irrespective of the not yet fully understood causal mechanisms underlying specific aberrances after sublethal neonicotinoid exposure, it could be assumed that even subtle side-effects collectively come at a cost, which is likely to be translated into reduced fitness performance. Therefore, it is surprising that life-time reproductive success, the ultimate fitness endpoint, has received little attention in pesticide hazard evaluations (Desneux *et al.*, 2007). Such information would not only be helpful for discovering yet unknown sublethal effects, but also is pivotal for understanding the long-term impact of insecticides on pollinator populations.

With regard to pesticide hazard evaluations, however, the honeybee might be a poor surrogate for pollinators in general because of its complex perennial life cycle, which leads to difficulties in quantifying reproductive success. Studies of pollinator species with annual and less complex life cycles could provide less ambiguous fitness quantifications. For example, in bumblebees (social bees with annual life cycles), it has been shown that chronic dietary exposure to field-realistic trace residues of imidacloprid strongly reduced colony performance and fitness (Mommaerts *et al.*, 2010; Gill *et al.*, 2012; Laycock *et al.*, 2012; Whitehorn *et al.*, 2012). In one semi-field study, Whitehorn *et al.* (2012) reported that 2 weeks of neonicotinoid exposure significantly decreased subsequent colony growth and vastly reduced daughter queen production; using a comparable experimental approach, very similar impacts on bumblebee colony fitness have recently been shown for clothianidin, another member of the neonicotinoids (Larson *et al.*, 2013). The mechanistic factor contributing most to apparent decreases in fitness in chronically exposed bumblebee colonies under field conditions is considered to be a strongly impaired foraging performance and efficiency, as found similarly in laboratory and semi-field studies (Mommaerts *et al.*, 2010; Gill *et al.*, 2012). This evidence clearly points to dietary neonicotinoid exposure having covert side-effects that are most likely expressed in relatively costly bumblebee life-history performances, such as reproductive investment, and that become most evident only under more realistic test conditions, which allow for the assessment of such informative endpoints (Cresswell *et al.*, 2012). A similar indication of the complexity of the impact of neonicotinoids comes from the detrimental synergism observed in honeybees that were immune challenged with gut parasites (Alaux *et al.*, 2010; Vidau *et al.*, 2011; Pettis *et al.*, 2012). In contrast to lethal poisoning incidences that result in readily visible and quantifiable mortality, sublethal effects of chronic insecticide exposure may rarely be recognized or masked by other factors in the field. If, however, sublethal effects of neonicotinoids can still trigger severely decreased output of daughter queens in bumblebees (Gill *et al.*, 2012; Whitehorn *et al.*, 2012; Larson *et al.*, 2013), the consequences on local populations are probably comparable to the immediate lethal intoxication of large proportions of workers. This is because in bumblebees only young (mated) queens are capable of surviving winter and establishing new colonies in the next season. Hence, the question arises as to whether sublethal exposure to routinely applied insecticides could be contributing to general pollinator population declines by negatively impacting on reproductive fitness in a manner similar to that observed in experimental studies on bumblebees using neonicotinoids (Bryden *et al.*, 2013).

Solitary bees represent an important species-rich group of wild pollinators providing crucial pollination services in both wild and crop plants (Klein *et al.*, 2007; Winfree *et al.*, 2007; Garibaldi *et al.*, 2013). Surprisingly, solitary bees are virtually ignored in pesticide regulations (Blacquière *et al.*, 2012). However, solitary bees represent much more convenient models for fitness quantification compared to social bees (honeybees and even bumblebees) because of the direct link between one individual female's performance and its reproductive success. Furthermore, detailed investigations of the impact of pesticides on solitary bees that differ in life-history traits (e.g. flight season, nesting habit, habitat and feeding specialization) would provide stronger and urgently needed insights into the wider environmental impacts of plant protection products.

The present study reports a novel methodological approach for quantifying the fitness effects of sublethal pesticide exposure on solitary bees under fully controlled experimental conditions. We assessed the impact of field-realistic dietary exposure to the neonicotinoids thiamethoxam and clothianidin on the life-time fitness performance of the red mason bee *Osmia bicornis* (syn. *O. rufa*, L. 1758; Hymenoptera, Megachilidae). We selected these particular neonicotinoids for two reasons. First, thiamethoxam is partly metabolized into clothianidin within plants (Maienfisch *et al.*, 2001; Nauen *et al.*, 2003), such that both neonicotinoids generally co-occurr in nectar and pollen of treated crops (Dively & Kamel, 2012; Pohorecka *et al.*, 2012). Second, in terms of sales, thiamethoxam is

the second most important neonicotinoid after imidacloprid, and several commercially available formulations are routinely applied via foliar spraying or seed treatment for systemic protection in 115 crops from at least 65 countries worldwide (Elbert *et al.*, 2008; Jeschke *et al.*, 2011). The neonicotinoid concentrations that we used in the present study (2.87 µg/kg for thiamethoxam and 0.45 µg/kg for clothianidin) were selected because they correspond to the range of field-realistic nectar residue-levels across several commonly treated crops in general (Blacquière *et al.*, 2012). More particularly, they reflect residue levels after the systemic treatment of oilseed rape with thiamethoxam (Pohorecka *et al.*, 2012), although considerably higher levels may be reached (e.g. in cucurbits after drip irrigation) (Dively & Kamel, 2012; Stoner & Eitzer, 2012).

The present study demonstrates that chronic sublethal neonicotinoid exposure of the solitary bee *O. bicornis* results in both strongly reduced reproductive success and male-biased offspring sex ratios. The bioassay has widespread value in that it can be readily adapted to laboratory, semi-field and field conditions, as well as be applied to other solitary bee species and, based on our findings, we urge that its principles be adopted in future pesticide risk assessment guidelines.

Materials and methods

Study organism

Osmia bicornis is a common above ground cavity-nesting megachilid species, native to Europe. It is univoltine, with a reproductive period from April to June, and uses a broad spectrum of floral resources. Although both males and females feed on flowers, it is exclusively the females who invest in brood care. Reproductive success depends on the female's ability to construct and provision brood cells with pollen, and to a lower extent also nectar, for progeny development. Females build nests in pre-made holes in wood or other structures, and sequentially construct linearly ordered brood cells separated by mud partitions. For the specific purpose of the present study, we used a whole population assessment, which is a natural situation because *O. bicornis* tends to be gregarious, with large nesting aggregations commonly observed in the field (Seidelmann, 2006; Seidelmann *et al.*, 2010). Similar to many other solitary bees, *O. bicornis* exhibits a pronounced sexual dimorphism in body size. Males are smaller than females, and thus are less costly to produce because they need less food for larval development (Seidelmann, 2006; Seidelmann *et al.*, 2010). Typically for hymenopterans, females have full control over offspring sex determination by regulating sperm release from the spermatheca for fertilization: fertilized eggs give rise to diploid daughters and unfertilized eggs result in haploid sons (Heimpel & de Boer, 2008). Reproductive performance and sex allocation is influenced by the female's physiological conditions, such as body size and health. Larger females are able to produce more daughters as a result of their higher provisioning performance, which is demonstrated by their ability to forage more efficiently: they collect the same amount of pollen and nectar in a shorter time compared to smaller females (Seidelmann *et al.*, 2010). Conversely, smaller females tend to shift their limited investment towards higher proportions of sons. In a similar manner, poor quality environments (e.g. weather, food plant abundance) that result in low provisioning efficiency, as well as the progressive senescence of reproducing females, lead to a reduced larval provisioning and a more pronounced investment in male offspring (Seidelmann, 2006; Seidelmann *et al.*, 2010).

Experimental set-up

Rearing cages. The present study was conducted on two bee populations kept in two identical climate controlled rooms, equipped with a sunlight simulation system (maximum of 1000 µmol photons/m^2/s) and air conditioning (York International, Germany). Light, temperature and humidity were controlled in both rooms in parallel, simulating a natural climate, and this was kept constant throughout the experiment (Fig. 1). Each room contained a flight cage (4.3 × 2.4 × 1.8 m) built from white nylon mesh (1.33 mm mesh size; Wondermesh, U.K.). Both flight cages were designed to allow access of the experimenter but to prevent the escape of bees, and were equipped identically: nectar substitutes, pollen powder, nest tubes and nest substrates were provided and arranged in the same way and position for each cage, with each food and nesting material being obtained from the same supply. None of the material used in this experiment, except the pollen (see below), had been in contact with bees prior to the study.

Bee populations. To establish two breeding populations of *O. bicornis*, cocoons were purchased from WAB Mauerbienenzucht (Germany). All cocoons originated from the last nesting season, and from the same nesting site of a single source population; upon collection in the field during the previous autumn, cocoons were kept at 4 °C, and then individually transferred to aerated containers at 22 °C in early May to allow the bees to emerge. Upon hatching from cocoons, adult bees were sexed and assigned randomly to one of the two study populations until each contained 125 females and 75 males. All bees used in the experiment hatched within 24 h and were released simultaneously into each climate room (males first and females last). Prior to this release, all females were chilled after defecation for 5 min at 4 °C and weighed to the nearest mg on a Mettler AE260 (Mettler Toledo, Inc., Columbus, Ohio) and then individually marked with numbered honeybee tags. Mating activity occurred immediately after releasing the bees, and bees were able to forage and reproduce freely.

Bee care and maintenance.

1 Food: In each flight cage, nectar substitutes (50% sugar content, containing equal amounts of glucose, fructose and sucrose; Hostettler's, Zurich, Switzerland) were provided *ad libitum* in twelve artificial flowers. Each flower was built from 10-mL laboratory plastic tubes (with closable lids) that were fixed upright to a small board hanging from the roof of the flight cage. At the bottom of each tube, a small piece of cardboard was fixed, serving as a landing place to

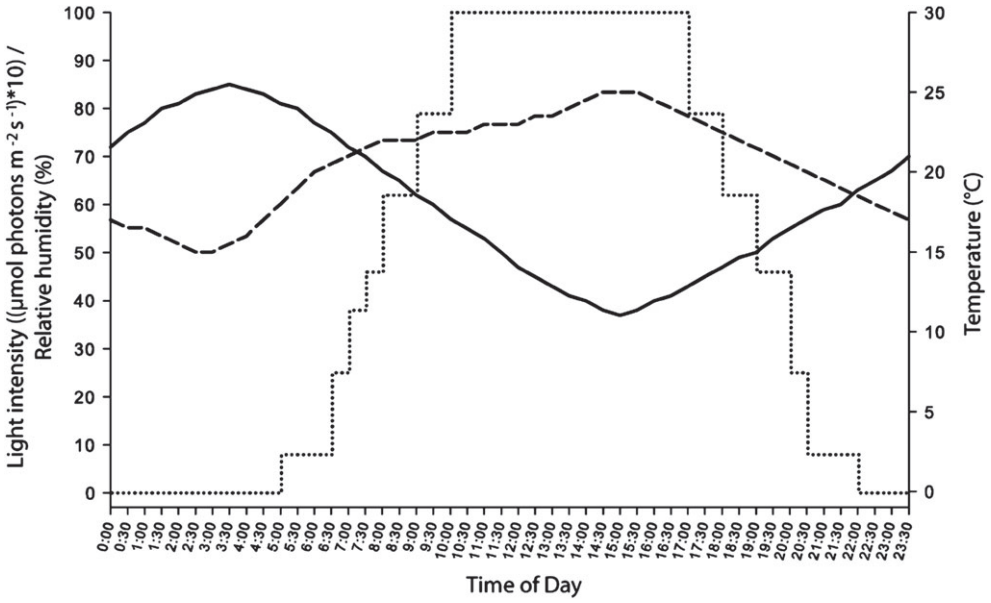

Figure 1 Climate simulation for the two study populations of *Osmia bicornis*. A computerized climate programme was run in both controlled environment rooms containing the bees and kept constant throughout the experiment. Light intensity (dotted line, left-hand *y*-axis) was adjusted stepwise, humidity (solid line, left-hand *y*-axis) and temperature (dashed line, right-hand *y*-axis) were adjusted gradually at 30-min intervals and are indicated for a 24-h period. Parameters were chosen to simulate a central European early summer day.

access nectar substitutes through two opposed holes (hand-made, using a hot needle), just above the vessel bottom and sufficiently large to allow the tongue of the bees to enter (diameter 1 mm). Attraction of bees to these artificial flowers was enhanced by simulating ultraviolet-reflective patterns using strips of commercial photographic paper, which were attached to the cardboard (landing place), as well as around the holes in the black lacquered tubes (see below). Artificial flowers were filled completely (10 mL) with freshly prepared nectar substitutes and were replaced every 3 days by new ones.

Bees in both flight cages were also provided with pollen, in the form of pulverized honeybee pollen pellets (one stock; Sonnentracht Imkerei, Germany). Microscopic examination of the pellets indicated that they contained pollen from at least 18 different floral resources. Pollen was gamma ray irradiated (Leoni Studer Hard AG, Däniken, Switzerland) to exclude honeybee pathogen spill-over effects. Four dishes containing pollen powder were placed in each flight cage, and their contents were replaced three times per day at intervals of 3–4 h.

2 Nesting materials: Notched wooden boards that, when piled on top of each other, formed successive holes (diameter 8 mm, length 16 cm) (WAB Mauerbienenzucht) were used to build nesting blocks; each flight cage was provided with a total of 696 nest holes. The backsides of the blocks were sealed, and the fronts were painted with identical colour patterns. A mixture of commercial potter's clay and silica sand, offered in tilted plastic trays (volume of 6 L), was provided as nesting substrate. Each tray contained a 250-mL water tank from which two cloth wicks extended into the substrate to create a moisture gradient along which the bees could choose their preferred degree of wetted substrate. The tanks were covered with a mesh to prevent bees from drowning and were refilled each morning.

Treatments and neonicotinoid residue analysis

To simulate chronic insecticide exposure, the experimentally treated bee population differed from the control in that the nectar-substitute contained the neonicotinoids thiamethoxam and clothianidin.

Because neonicotinoids are very sensitive to direct (ultraviolet) light exposure (Maienfisch *et al.*, 2001), artificial flowers were black-lacquered and covered with tinfoil to prevent nectar substitutes from direct exposure to the sunlight simulation system, as well as neonicotinoids from degradation. Pollen was not spiked with neonicotinoids because it could not be protected from light. Pure compounds (analytical standard; Fluka, Switzerland) were purchased from Sigma-Aldrich (Germany), dissolved in water, and stored at room temperature. Original sugar syrup and pollen stocks did not contain any of these chemicals (based on six random samples each). Sugar water was spiked using aliquots of neonicotinoid stock solutions to achieve concentrations of 2.87 µg/kg of thiamethoxam and 0.45 µg/kg of clothianidin, respectively; these concentrations were confirmed through residue analyses of random samples from each re-supply of nectar substitutes fed over the course of the experiment.

To determine neonicotinoid residue levels in current year's larval food provisions and offspring bees emerging in the subsequent year, from each flight cage (treatment and control populations), six samples of leftover larval provisions of ten nest cells each (from cells in which offspring failed to

develop; see below) and six samples of ten newly-emerged, randomly picked adult offspring each were subjected to residue analyses. All analyses were performed by the United States Department of Agriculture, Agricultural Marketing Service National Standards Laboratory in Gastonia, North Carolina, using established methods. Individual samples were analyzed using gas chromatography (GC)-mass spectroscopy (MS) and applicable standards for both compounds with a limit of detection of 0.1 p.p.b. Identification of the parent compounds was based on co-chromatography with known standards using GC/MS and/or liquid chromatography/MS-MS.

Data collection on bee fitness

During the bee's flight activity season, we documented female mortality by inspecting flight cages daily for dead adult females. We similarly tracked the cumulative numbers of completed nests (i.e. nest entrances sealed by a nest plug).

Four weeks after all adult bees had died, climate programmes for the rooms were stopped and nest blocks were kept in darkness at 22 °C for 4 months. Then, the nest blocks were separated to open the nest holes and to determine the number and conditions of nest contents. Both fully developed cocoons and undeveloped offspring were counted across all brood cells for each nest in each population. Leftover larval food provisions from undeveloped offspring were frozen for residue analysis (see above) and the cocoons were set aside and kept at 12 °C for 4 weeks and, subsequently, at 4 °C for 5 months. Furthermore, cocoons were then transferred to 22 °C to estimate hatching success and sex ratios (per nest tube and overall). Finally, after emergence, body weights were documented for 101 randomly picked male and female offspring from each population.

Statistical analysis

Analyses were performed using R (R Core Development Team, 2011). To explicitly test for inequality in body weights of parental females in control and treatment populations based on the lowest detectable difference of 1 mg, we sorted body weights of females within groups and used a Wilcoxon signed rank test with continuity correction based on paired differences of body weight between bees assigned to the two different experimental populations. A generalized linear model (GLM) with gamma probability distribution was applied to test for a difference in mortality between populations. We tested for differences in the number of hatched offspring per nest between populations using a GLM with Poisson probability distribution. Proportional data of overall offspring mortality and daughters produced were compared using binomial tests. Offspring sex ratios inferred from individual nest tubes were analyzed with a GLM with binomial probability distribution. We tested for inequality in the central tendency, as well as for differences in distribution of offspring body weight between populations. A Wilcoxon signed rank test with continuity correction was used to assess whether the location shift in body weights between the groups of offspring deriving from different populations was different from zero. We also performed a Fligner-Killeen test for offspring body weight variance homogeneity between the populations. All analyses were conducted separately for females and males.

Results

Neonicotinoid residue analysis

None of the samples from either leftover larval food provisions or bees collected from the control and treatment populations contained detectable levels of thiamethoxam and clothianidin.

Bee fitness

Body weight distributions in founder females did not differ between the control and treatment population ($V = 984$, $P < 0.001$).

Chronic exposure to field-realistic concentrations of thiamethoxam and clothianidin through nectar substitute had differing effects on the various fitness parameters.

There was no effect on adult females' longevity (Fig. 2 and Table 1): average life-spans reached in the treatment and control populations were 24.5 ± 7.2 and 23.8 ± 6.6 days, respectively. However, fewer nests were completed in the neonicotinoid-treated population: the control population completed 194 nests over the course of the breeding period, whereas the number in the treatment population was 22% lower (i.e. 151 nests) (Fig. 2). Further differences were evident with respect to brood cell number and larval mortality rate. Completed nests in the treatment population contained 43.7% fewer total brood cells than the control (i.e. 497 compared to 883). In addition, relative offspring mortality was almost two-fold higher in the treatment population; the proportion of offspring that completed larval development and/or were able to hatch after hibernation was thus lower compared to the control (i.e. 423 in the treatment population compared to 808 in the control) ($\chi^2 = 12.85$, d.f. $= 1$, $P < 0.001$), corresponding to 15% and 8.5% mortality, respectively. Overall, chronic exposure to neonicotinoids had a significant negative effect on the number of offspring that emerged per nest (Fig. 3 and Table 1).

Table 1 Summary of the results of the generalized linear models (GLM) performed to evaluate the effect of chronic exposure to neonicotinoids (thiamethoxam and clothianidin) on adult mortality, the number of cocoons per nest tube and offspring sex ratios of the red mason bee *Osmia bicornis*

GLM	Parameter	Estimate	SE	*P*-value
Mortality	Intercept	0.042	0.001	< 0.001
Gamma probability distribution	Neonicotinoids	−0.001	0.001	0.481
Number of cocoons per tube	Intercept	1.427	0.035	< 0.001
Poisson probability distribution	Neonicotinoids	−0.397	0.06	< 0.001
Offspring sex ratio	Intercept	0.224	0.071	0.002
Binomial probability distribution	Neonicotinoids	−0.342	0.12	0.004

Parameter estimates and SEs are given on the relative, canonical link scale. For details, see Materials and methods.

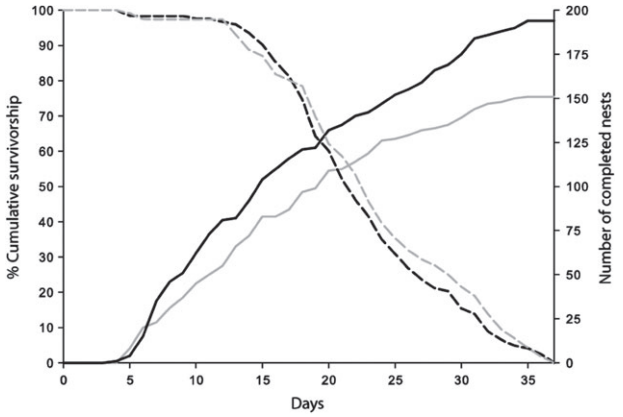

Figure 2 Cumulative survivorship and numbers of completed nest tubes. Dashed lines represent the percentage of cumulative survivorship of individually marked *Osmia bicornis* females (left-hand *y*-axis) plotted against time for the control bees (black) and treatment bees chronically exposed to neonicotinoids (grey). Mortality rates (measured as time-to-death) did not differ between the two bee groups ($P = 0.481$) (Table 1). Solid lines represent the cumulative numbers of completed nest tubes (right-hand *y*-axis) for the control bees (black) and treatment bees (grey). The treatment population completed 151 nests in total (i.e. 22% less than the 194 nests in the control population).

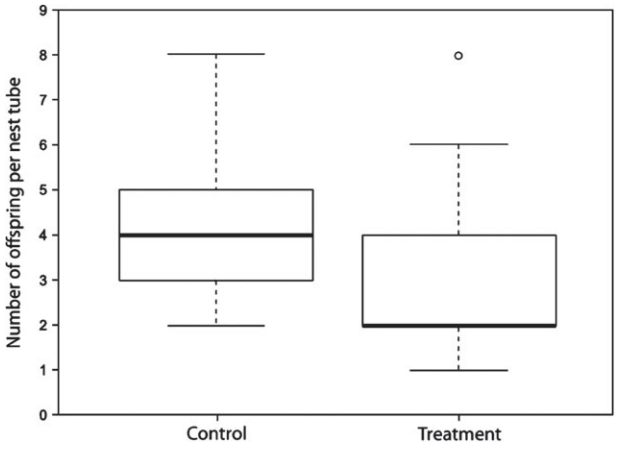

Figure 3 Number of offspring per nest tube. The numbers of offspring that hatched per nest tube are shown for the control and treatment populations of *Osmia bicornis*. Thick horizontal lines represent medians, boxes indicate 25–75th percentile range, and whiskers show the total range of the data (outliers are given as circles). Mean ± SD numbers of hatched offspring per nest were 4.17 ± 1.58 for the control population and 2.80 ± 1.46 for the treatment population. Chronic exposure to neonicotinoids (thiamethoxam and clothianidin) had a significant negative effect on the total number of hatched offspring per nest tube ($P < 0.001$) (Table 1) compared to the control bees.

Significantly male-biased offspring sex ratios were detected across nests within the treatment population (Table 1), resulting in a significantly lower proportion of daughters overall ($\chi^2 = 7.75$, d.f. = 1, $P < 0.003$) compared to the control population. On average, 47.1% of emerged bees in the treatment population and 55.6% of bees in the control population were females.

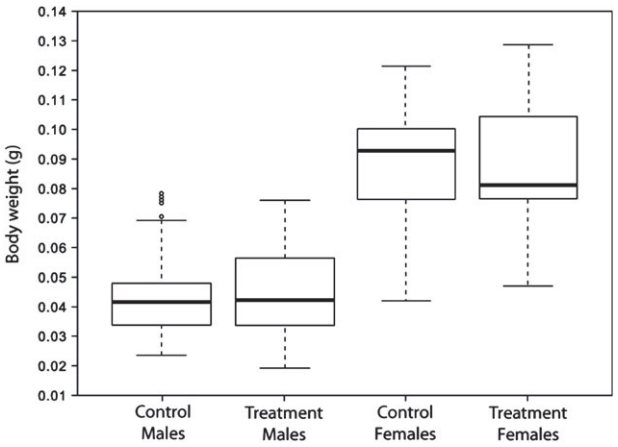

Figure 4 Offspring body weights. Body weight distributions of adult offspring are shown for the control and treatment populations of *Osmia bicornis*, based on 101 randomly picked individuals of each sex per population. Thick horizontal lines represent medians, boxes indicate 25–75th percentile range, and whiskers show the total range of the data (outliers are given as circles). Mean ± SD values for males: 42.7 ± 12.5 mg (control) and 45.2 ± 12.8 mg (treatment), and for females: 88.6 ± 18.8 mg (control) and 87.6 ± 18.8 mg (treatment). For each sex, offspring body weight distributions neither differed, nor varied significantly between the experimental populations.

Taken together, the treatment population produced 47.7% fewer offspring, including a 8.5% lower proportion of daughters, compared to the control population. The total numbers of female offspring in the treatment and control populations, with 199 versus 449 daughters, respectively, reveal a 2.3-fold reduced population growth upon chronic neonicotinoid exposure.

Emerged offspring from the two different populations, however, did not differ with respect to mean body weight (females: $W = 5496.5$, $P = 0.341$; males: $W = 4694.5$, $P = 0.329$) (Fig. 4) or body weight variance (females: $\chi^2 = 97.8$, d.f. = 90, $P = 0.269$; males: $\chi^2 = 100$, d.f. = 95, $P = 0.434$).

Discussion

The present study aimed to develop a feasible experimental procedure for quantifying the side-effects of chronic pesticide exposure on the life-time reproductive success of a representative of an important group of pollinators, solitary bees, which so far remain unconsidered in risk assessment guidelines. The results obtained reveal some major impacts on the red mason bee *O. bicornis* from low-level exposure to prevalent neonicotinoids. More specifically, we contribute data that strengthen our knowledge base regarding the ongoing debate on how widespread neonicotinoid applications in systemic crop protection could be influencing recent pollinator declines, and that point to the inclusion of solitary bees in future assessments.

Our findings show that while dietary exposure to field-realistic trace residues of two neonicotinoids, thiamethoxam and clothianidin, had no impact on the mortality of the actively foraging adult bees (Fig. 2), with mean longevities similar to those observed in the field (Seidelmann, 2006),

it significantly impacted the F_1 generation. Indeed, chronic sublethal exposure to less than 3.5 µg/kg neonicotinoids in nectar substitutes had marked negative consequences on the fitness performance of bees: while the nest building rate of non-exposed females approximated that reported in the field (Seidelmann *et al.*, 2010), exposed female bees both completed fewer nests and constructed fewer brood cells per nest (Fig. 3 and Table 1), thus exhibiting a reduction in offspring production of almost 50%. Moreover, while the offspring sex ratios of non-exposed females with more than 50% daughters indicate good environmental conditions, neonicotinoid exposure resulted in significantly male-biased offspring sex ratios (Table 1), thus further compounding the overall loss of reproductive potential. Our findings concur with recent studies on bumblebees, where dietary exposure to environmentally relevant residues of the neonicotinoids imidacloprid and clothianidin caused decelerated colony growth and a decrease of 85–100% in daughter queen production (Gill *et al.*, 2012; Whitehorn *et al.*, 2012; Larson *et al.*, 2013). These results reveal that neonicotinoid exposure has covert side effects that can negatively affect reproductive success and long-term population dynamics, and also that these effects can occur across different genera of wild bees.

Given that systemic neonicotinoids are broadly used to combat pests on various pollinator-attractive crops, red mason bees and related species found in agricultural landscapes could be chronically exposed to these chemicals during most of their breeding period, or even whole life cycles, as simulated in the present study. Members of the genus *Osmia* are generalist in their flower visitation and feed on a variety of floral resources, and common mass-flowering crops are readily incorporated into their diet (Westphal *et al.*, 2003; Jauker *et al.*, 2012; Holzschuh *et al.*, 2013). Although foraging in the field likely includes alternative resources, generalist bees such as *O. bicornis* commonly exploit mainly high-reward mass-flowering plants to maximize foraging efficiency (Westphal *et al.*, 2003; Winfree *et al.*, 2007; Teper & Biliński, 2009; Radmacher & Strohm, 2010). Of special concern is that the breeding season of *O. bicornis* includes the flowering period of oilseed rape, which can contribute considerable proportions of this species' nutrition, even if alternatives are available (Teper & Biliński, 2009; Holzschuh *et al.*, 2013), and which is most often treated with neonicotinoids. There could be additional exposure routes to neonicotinoids for noncrop plants, for example, via dust drift during drilling of coated seeds and its deposition on flowering weeds in the surroundings of agricultural areas (Krupke *et al.*, 2012).

Although our reported findings on sublethal neonicotinoid effects provide evidence of potentially severe impacts on solitary bee populations, the present study was conducted in a laboratory setting, and our conclusions need to be replicated in the field. However, our results are biologically important and carry strong weight for four reasons. First, the chosen set-up and procedures minimized the chance that there could have been undetected factors of either biological or mechanical nature influencing both populations differently: it can be assumed that the control and the treatment population did not differ with respect to their genetic background, nor with respect to the *a priori* conditions of the founder females, which could have influenced reproductive performance and offspring sex allocation, and that climate simulations, as well as the equipment in both flight cages, were identical.

Second, the fact that mortality rates did not differ between the control and treated populations (Fig. 2) makes it very unlikely that the effects from individual females interacting in a non-independent manner could have differed between populations, which furthermore had approximately equal numbers of adult females. Thus, the populations differed only with respect to the presence or absence of neonicotinoids. Third, equal mortality rates and longevities are indicative of the absence of repellence and anti-feeding effects of the applied environmentally relevant concentrations of neonicotinoids, as previously reported for honeybees and bumblebees (Faucon *et al.*, 2005; Gill *et al.*, 2012). Finally, the presence of any undetected factors, such as microbial infections, can be assumed to have been similar in both populations because all randomly allocated bees originated from the same source.

Considering the general mode of neurotoxic action of neonicotinoids in insects (Matsuda *et al.*, 2001; Desneux *et al.*, 2007), toxicological effects resulting in behavioural, cognitive or locomotive abnormalities could result in the devastating fitness response reported in the present study in solitary bees and previously in bumblebees (Whitehorn *et al.*, 2012). Physiological functions are tightly integrated with the nervous system of a bee (Belzunces *et al.*, 2012). Therefore, even subtle impairments of manifold fundamental mechanisms could collectively limit basic tasks, such as foraging performance (Cresswell *et al.*, 2012; Gill *et al.*, 2012; Henry *et al.*, 2012), and these have been suggested to explain the decelerated colony growth and lower reproductive success in bumblebees (Mommaerts *et al.*, 2010; Whitehorn *et al.*, 2012). Compared to honeybee colonies, which are perennial and consist of several thousands of workers exhibiting a complex division of labour, with a high degree of adaptive plasticity and generally high turn-over rates, bumblebees, which are also social bees but with small-sized colonies and annual life-cycles, appear to express sublethal effects more strongly (Gill *et al.*, 2012; Whitehorn *et al.*, 2012). The present study provides the first evidence suggesting that the same applies to solitary bees, as exemplified by the red mason bee *O. bicornis*.

In this species, reduced provisioning efficiency has been shown to result in decreased numbers of brood cells per nest, as well as male-biased offspring sex ratios (Seidelmann *et al.*, 2010), with both effects being observed in the present study in bees exposed to neonicotinoids. These shifts in breeding and offspring sex allocation strategies have mainly been attributed to evolutionary adaptations in mitigating brood parasitism (Seidelmann, 2006). Decreased foraging efficiency, as commonly induced by poor environments, high travelling costs or as an effect of female senescence, translates into an increased provisioning time per brood cell, which is positively correlated with a higher risk of brood parasitism. Accordingly, reduced numbers of offspring per nest and an apparent shift in offspring sex allocation towards less costly sons represent reliable general responses in reproductive investment to maximize fitness under adverse conditions in this and other solitary bee species (Seidelmann *et al.*, 2010). The fact that our findings on *O. bicornis* exposed to sublethal neonicotinoid dosages reflect

these patterns exactly (Figs 2 and 3 and Table 1) leads us to interpret them as most likely resulting from an overall impaired foraging performance and provisioning efficiency, as also demonstrated in several studies on bumblebees (Mommaerts *et al.*, 2010; Gill *et al.*, 2012; Whitehorn *et al.*, 2012) and honeybees (Cresswell, 2011; Belzunces *et al.*, 2012; Henry *et al.*, 2012; Schneider *et al.*, 2012).

With respect to the male-biased offspring sex ratios that we observed in *O. bicornis*, neonicotinoids may also have additional negative consequences in hymenopterans. In this order of insects, egg fertilization depends on the female voluntarily releasing stored sperm from her spermatheca (i.e. from where they are stored) (van Wilgenburg *et al.*, 2006) and neonicotinoids might directly impact this active process. Indeed, male-biased offspring sex ratios were reported for parasitoid wasps exposed to neurotoxic insecticides (Desneux *et al.*, 2007), which suggests that this may occur in other solitary hymenopterans. The putative effects of sublethal neonicotinoid exposure on egg fertilization in haplodiploid nontarget insects thus deserve further research.

An important point raised by our data is indicated by the negative effects that we observed on offspring survival in the neonicotinoid-exposed population. Although mortality rates in both study populations can be considered to be in the range of what might be expected in the field (when referring to mortality that is not caused by parasitizing arthropods, as was the case in the present study) (Seidelmann *et al.*, 2010), the difference between them was significant. Given that offspring body weight is strongly correlated with the amount of larval provisioning, which decreases with reduced foraging efficiency (Seidelmann, 2006; Seidelmann *et al.*, 2010), partially insufficient food provisioning by the mothers could have caused the lower offspring survivorship in our neonicotinoid-exposed population, and also could have contributed to biased offspring sex ratios if female offspring, requiring more food, were more likely to fail during development compared to males. However, offspring body weight means and variances of both sexes did not differ between the treatment and control populations (Fig. 4), which suggests that there were comparable amounts of food provision in the two populations. Therefore, when directly comparing mortality rates of the populations, it is possible that the restricted offspring development evident in our treatment population was a direct consequence of the neonicotinoids in the nectar substitutes. If exposure to neonicotinoids did impact bee development, this would suggest that the larval stages of *O. bicornis* are more sensitive than adults because we spiked only the nectar; in adults, nectar is a major food but it constitutes only a very small proportion of the larval food provisions of *O. bicornis* (Strohm *et al.*, 2002), which explains why we did not find any traceable amounts of either neonicotinoid in the larval provision samples subjected to residue analyses. In a field setting, the impact of neonicotinoids could be much higher than we observed because broods of solitary bees would be exposed to higher levels of neonicotinoids as a result of their feeding on pollen, which, in crops treated systemically with neonicotinoids, generally contains higher concentrations than nectar (Blacquière *et al.*, 2012; Dively & Kamel, 2012). Thus, we can expect solitary bee larvae in the field to commonly ingest greater quantities of neonicotinoids than did our laboratory-reared offspring.

It is very noteworthy that our findings regarding the impact of neonicotinoids in solitary bees are similar to those obtained in a separate investigation on bumblebees by Whitehorn *et al.* (2012), even though the two studies differed markedly in experimental set-up: in both, there was a reduction in numbers of female offspring upon field-realistic neonicotinoid exposure, implicating long-term impacts on effective population size. This impact is critical to population stability because small populations are more vulnerable to stochastic environmental processes and inbreeding depression (Goulson *et al.*, 2008). Of special relevance to bees, inbreeding depression can result in detrimental diploid male production instead of female offspring as a result of their complementary sex determination system (Zayed & Packer, 2005; Heimpel & de Boer, 2008; Whitehorn *et al.*, 2009; Darvill *et al.*, 2012). Assuming that the outcomes of both our study and that of Whitehorn *et al.* (2012), where reproductive output (offspring numbers) barely replaced parental females, can be extrapolated to field settings, it becomes clear that sublethal chronic neonicotinoid exposure could have a major impact on pollinator populations.

Dispersal and gene flow among populations of wild pollinators could counterbalance locally occurring detrimental effects as a result of neonicotinoid exposure. Yet, regarding the present dimensions of systemic neonicotinoid applications (Blacquière *et al.*, 2012), at some point, recurrent fitness losses in agricultural landscapes may fail to be compensated by functional metapopulation networks facing additional adverse conditions such as habitat degradation, emerging parasites or climate change (Goulson *et al.*, 2008; Potts *et al.*, 2010). Wild pollinator population declines are not only of concern for biodiversity, but also for food security (Garibaldi *et al.*, 2013). In the light of continuing honeybee losses (Winfree *et al.*, 2007; Aizen *et al.*, 2008; Aizen & Harder, 2009; Potts *et al.*, 2010; Pettis *et al.*, 2012), red mason bees and several related species are increasingly being established for managed pollination services (Bosch & Kemp, 2002; Teper & Biliński, 2009; Jauker *et al.*, 2012) to counteract suspected declines in crop pollination services. The sustainability of their use depends on the minimization of bee exposure to long-term sublethal environmental stressors, such as neonicotinoids.

In conclusion, our study of the solitary bee *O. bicornis* and several studies of bumblebees (Gill *et al.*, 2012; Whitehorn *et al.*, 2012; Larson *et al.*, 2013) concordantly demonstrate a link between chronic sublethal neonicotinoid exposure and reduced life-time reproductive success. Of crucial importance, these findings indicate that the current mandatory guidelines for pesticide risk assessment (i.e. the tiered approach of testing for side-effects of plant protection products on honeybees only) (OECD, 1998a, b; OEPP/EPPO, 2010a, b), is insufficient to ensure ecological sustainability for pollinators in a broad sense. Referring to the recently published guidance document by the European Food Safety Authority (http://www.efsa.europa.eu/en/efsajournal/doc/3295.pdf), the hazard evaluation schemes for pesticide regulations should urgently implement more stringent testing of sublethal and chronic effects on pollinators in general, and critically consider entire life-cycle fitness assessments of selected non-*Apis*

pollinators, such as the assay reported in the present study using the solitary bee *O. bicornis*.

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Neonicotinoid insecticides can serve as inadvertent insect contraceptives

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There is clear evidence for sublethal effects of neonicotinoid insecticides on non-target ecosystem service-providing insects. However, their possible impact on male insect reproduction is currently unknown, despite the key role of sex. Here, we show that two neonicotinoids (4.5 ppb thiamethoxam and 1.5 ppb clothianidin) significantly reduce the reproductive capacity of male honeybees (drones), *Apis mellifera*. Drones were obtained from colonies exposed to the neonicotinoid insecticides or controls, and subsequently maintained in laboratory cages until they reached sexual maturity. While no significant effects were observed for male teneral (newly emerged adult) body mass and sperm quantity, the data clearly showed reduced drone lifespan, as well as reduced sperm viability (percentage living versus dead) and living sperm quantity by 39%. Our results demonstrate for the first time that neonicotinoid insecticides can negatively affect male insect reproductive capacity, and provide a possible mechanistic explanation for managed honeybee queen failure and wild insect pollinator decline. The widespread prophylactic use of neonicotinoids may have previously overlooked inadvertent contraceptive effects on non-target insects, thereby limiting conservation efforts.

1. Introduction

Factors affecting reproductive success have a profound influence not only on a single individual's fitness, but on the dynamics of entire populations [1,2]. This principle provides a framework for pest control strategies that target reproduction. For example, modern-day agricultural practices frequently demand intensive insect pest management to ensure high-quality crops [3,4]. Strategies such as sterile insect techniques and insect growth regulator insecticides are designed for their sublethal effects on adult insect reproduction [5–7], whereas others may kill the pest insect outright [8,9].

Advances in agrochemical research highlight a lack of knowledge of the sublethal effects of insecticides on their target insect pests [10], as well as on sympatric beneficial insects such as bees that provide vital ecosystem services [11–13]. Frequently applied neonicotinoid insecticides can affect the nervous system of insects by acting as agonists of postsynaptic nicotinic acetylcholine receptors [14–16]. Recently, they have been shown to elicit sublethal effects on several bee genera, such as impairing bumblebee queen (primary reproductive females) production

and diminishing honeybee queen reproduction [17,18]. However, to date no data exist on how neonicotinoid insecticides may affect male insect reproduction.

Historically, the honeybee (*Apis mellifera*) has served as a model insect to investigate the effects of various anthropogenic and environmental stressors [9] because it can be easily maintained and is relatively well studied. Furthermore, honeybees contribute essential pollination services to agriculture [19] and wild plants [20]. Queens perform mating flights soon after emergence to collect and store sufficient quantities of sperm from multiple drones (male sexuals) to last their lifetime [21]. This highly polyandrous strategy [22] conveys several benefits, including increased colony functioning and resistance to disease [23–25].

Within the last decade, honeybees have experienced severe annual mortalities in the Northern Hemisphere [26], probably because of a diverse array of stressors acting in concert [20,27]. These events have paralleled declines of wild bees [28,29]. It is believed that poor queen health (i.e. premature queen replacement, frequent unfertilized egg-laying) is a major contributor to honeybee colony mortality [30,31], yet factors affecting honeybee reproductive success remain largely unexplored. Recent studies have demonstrated, however, that miticides can affect the production and storage of honeybee sperm in males [32–34] and stored sperm by mated females [35], respectively. Because queen survival and productivity are intimately connected to successful mating, any influence on sperm quality may have profound consequences for the fitness of the queen, as well as the entire colony [36–39].

Here, we tested for the first time the effects of neonicotinoid insecticides on male insect reproduction. We employed honeybee drones as models that were exposed during development to chronic field-realistic concentrations of the neonicotinoids thiamethoxam and clothianidin. We hypothesized that drones reared in colonies exposed to neonicotinoids would experience significant lethal (reduced longevity) and sublethal (sperm quality) effects compared with drones from control colonies based on previous studies demonstrating strong sublethal effects of neonicotinoids on female insect reproduction [17,18,30,40] and longevity [41–43], and because insecticide-induced reactive oxidative stress has been shown to reduce sperm quality [44–47].

2. Material and methods

The study was performed in Bern, Switzerland, between April and September 2015 using 20 *A. mellifera* L. honeybee colonies that were established at the beginning of the experimental period using the shook swarm method [48] to source drones and workers (primarily non-reproductive females). Each colony initially consisted of one laying sister queen, 1.8 kg workers, as well as five Dadant frames (each 435 mm by 298 mm) containing organic worker cell wax foundation that was tested for a broad array of agricultural chemical residues by the University of Hohenheim; an additional frame containing organic drone cell wax foundation was added approximately three weeks later to promote drone production [49].

(a) Insecticide exposure

In early May 2015, colonies were randomly assigned to one of two treatments (insecticide or control). Each colony was provided daily with 100 g pollen paste (60% fresh honeybee corbicular pollen, 10% organic honey, and 30% powder sugar) according to Sandrock *et al.* [50] and Williams *et al.* [18]. Pollen paste for insecticide colonies additionally contained 4.5 ppb thiamethoxam and 1.5 ppb clothianidin (both Sigma-Aldrich), which represents field-realistic concentrations found in plant pollen [51]; applied concentrations were confirmed (4.9 ppb thiamethoxam and 2.1 ppb clothianidin in insecticide patties; below the limit of quantification for thiamethoxam (less than 0.02 ppb) and clothianidin (less than 0.08 ppb) in control patties) by the French National Centre for Scientific Research using ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). Pollen paste feeding occurred over a period of 50 days to ensure colonies would be exposed to at least two complete brood cycles. Recent evidence suggests that foraging honeybees may be exposed to insecticide residues for a similar period due to contamination of non-agricultural foraging areas by surface run-off or drainage from nearby treated crops [52,53]. During the entire period, each colony was equipped with an entrance pollen trap to partially restrict forager-collected corbicular pollen entering the hive in order to promote pollen paste feeding [50].

(b) Source of drones and workers

Thirty-eight days post-initial pollen paste feeding, queens of each colony were first caged for approximately 48 h to a drone brood frame, and then 1 day later to a worker brood frame for an additional approximately 48 h to obtain sufficient numbers of drones and workers of the same known age cohort. Both experimental brood frames remained within their corresponding colonies until approximately 24 h prior to simultaneous drone and worker emergence; frames were then transferred to a laboratory incubator maintained in complete darkness at 34.5°C and 60% relative humidity [54].

(c) Teneral body mass and cage mortality

Upon emergence, each experimental drone and worker was visually examined to assess for physical abnormalities and the presence of the parasitic mite *Varroa destructor*. For each colony, the first 30 drones to emerge, which were free of *V. destructor* infestation and abnormalities, were weighed to the nearest 0.1 mg using an analytic scale (Mettler Toledo AT400). These drones, plus the next 30 of similar status (no *V. destructor* or abnormalities) to emerge per colony, were then placed in standard hoarding cages (250 cm^3) [54] corresponding to their source colony (and, therefore, respective treatment groups, i.e. insecticide or control). In total, each colony provided six hoarding cages of bees that each contained 10 drones and 20 workers from the same colony. The presence of workers in each cage was necessary because drones depend on worker attendance within the first few days of emergence [55–57]. Cages were subsequently maintained in complete darkness at 30°C and 60% relative humidity [54], and given 50% (w/v) sucrose solution and pollen paste (60% fresh honeybee corbicular pollen and 40% sugar powder) *ad libitum* to provide a carbohydrate energy source and ample proteins for organ and tissue development [58,59], respectively. Food was replaced every 72 h, whereas cage mortality was recorded every 24 h; dead individuals were removed using a forceps. After 8 days, all cages were exposed to indirect natural light for 1 h to promote and imitate an initial orientation flight [21]. The assay was terminated immediately after all experimental drones died.

(d) Sperm assessment

Three cages per colony were randomly selected to assess drone sperm quantity and viability at 14 days post-cage assay initiation, the typical age drones reach sexual maturity [60,61]. Drones in these cages were carefully removed using a forceps; to prevent sperm from migrating into the penis bulb, the drones were dissected alive by pinning them onto a wax plate [62]. Following Carreck *et al.* [63] the testes, mucus glands, and seminal vesicles were removed from each drone, placed in a 1.5 ml Eppendorf® tube containing 500 µl $Kiev^+$ buffer, and crushed to form a diluted stock sperm solution.

Immediately, a 50 µl aliquot of the stock sperm solution was set aside in a separate 1.5 ml Eppendorf® tube for analyses of sperm viability (proportion of sperm alive [64]). Sperm viability was quantified using the method previously described by Collins and Donoghue [65] and Stürup *et al*. [66]. In brief, each sample was diluted with 50 µl of Kiev^{+} buffer before 2 µl of propidium iodide (PI) solution (1 mg ml^{-1}) and 1 µl of Hoechst 33342 (0.5 mg ml^{-1}) [67] (both Sigma-Aldrich) were added to the suspension. Samples were then incubated for approximately 20 min in complete darkness and then gently vortexed. Ten microlitres were viewed at 400× magnification using a fluorescent microscope (Olympus BX41, Switzerland) equipped with filter cubes for UV excitation [67]. Ten visual fields were selected for each sample so that the quantity of living and dead sperm could be counted; an average value was then calculated from these fields [67].

In addition, 20 µl of each stock sperm solution were diluted with 80 µl Kiev^{+} buffer (1 : 5 dilution) in a 1.5 ml Eppendorf® tube to perform sperm counts. Sperm densities were measured using a Neubauer counting chamber under light microscopy (Thermo Fischer Scientific, USA). The final density of sperm was quantified using the following calculation [68]: total sperm quantity (500 µl) = average number of sperm counted in two Neubauer counting chambers × dilution factor (1 : 5) × sperm volume used for Neubauer counting chamber (10 µl) × stock solution volume (500 µl). Once both total sperm quantity and sperm viability were assessed, the total living sperm quantity was obtained by multiplying the two together.

(e) Statistical analyses

Three-level generalized regression mixed models with random intercepts were fitted using STATA14 [69], wherein individual drones were considered independent units, treatment (insecticide versus control) was included as the fixed term (or explanatory variable) and colonies and cages as random effects because of the clustering of individuals [70]. All statistical figures were created using NCSS v. 9.0.15 [71].

Drone teneral body mass was normally distributed (Shapiro–Wilk's test for normality, $p = 0.44$), so a general linear model was fitted using the meglm function. Total sperm quantity and the total living sperm quantity are count data, and were not normally distributed (Shapiro–Wilk's test for normality, $p < 0.001$) so were therefore fitted to negative binomial models using the menbreg function. Sperm viability is a score ranging from 0 to 100% and was also not normally distributed (Shapiro–Wilk's test for normality, $p < 0.001$) so an ordered logistic model was employed [72]. We used an XY scatter plot and Spearman's correlation coefficient to assess a possible correlation between sperm quantity and sperm viability. Lastly, survival times of drones and workers for both treatments were fitted using the mestreg function for multilevel survival models [70]. Median longevity was calculated as the 50th percentile of the survival time [73]. Drones sampled on day 14 for sperm assessments, as well as their accompanying caged workers, were censored. Whenever possible, every three-level model was compared with its single-level model counterpart using a likelihood ratio (LR) test [69]. LR tests, which do not rely on the assumption of asymptotic normal sampling distributions, can be used to demonstrate which model best fit the data.

Median differences and their 95% CI were calculated using the STATA14 package somersd. The function cendif calculates confidence intervals for Hodges–Lehmann median differences (or other percentile differences) between two groups [74].

3. Results

(a) Teneral body mass and cage mortality

No significant difference was observed between treatments for drone teneral body mass ($p = 0.80$; figure 1), which was

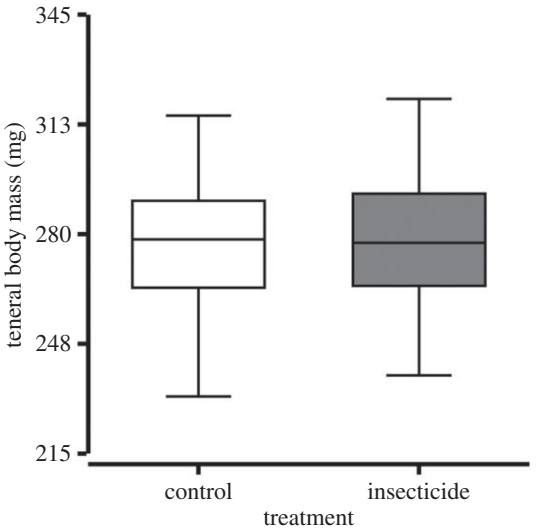

Figure 1. Drone (male) honeybee teneral body mass. Comparison of drone honeybee (*Apis mellifera*) teneral body mass (mg) showed no significant difference between controls ($N = 200$) and neonicotinoid insecticides ($N = 120$) ($p = 0.80$). The boxplots show the inter-quartile range (box), the median (black line within box), data range (horizontal black lines from box), and outliers (black dots).

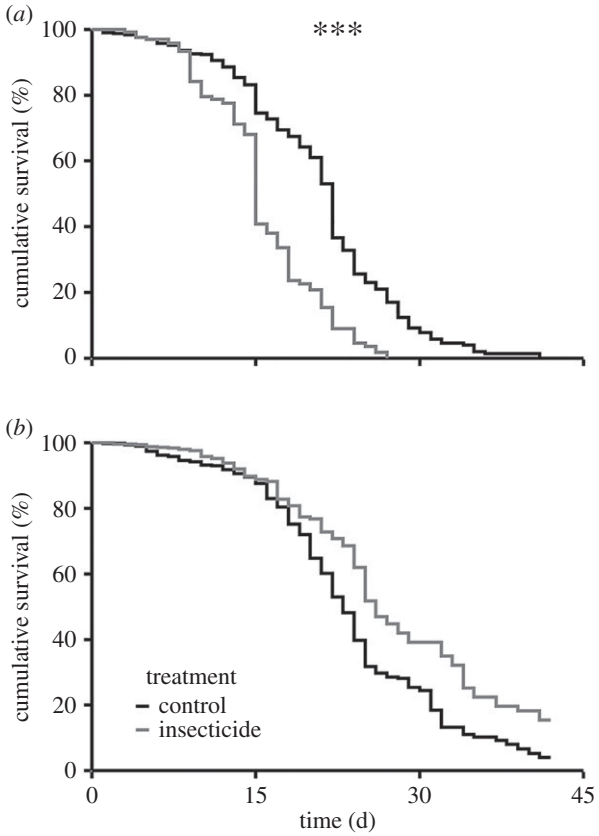

Figure 2. Honeybee drone (male) and worker (female) cage mortality. Survival curves (Kaplan–Meier) indicate the cumulative survival (%) of honeybee (*Apis mellifera*) drones ($N = 567$) (*a*) and workers ($N = 1120$) (*b*) under neonicotinoid insecticide exposure compared with controls. A significant difference was only observed for the mortality of drones ($p < 0.001$). A significant difference between treatment groups is indicated by ***$p < 0.001$.

277.06 ± 17.06 mg and 278.27 ± 18.16 mg for the controls and insecticides, respectively (mean ± standard error (s.e.)). However, median longevity of insecticide drones (15 ± 15–15 days) was significantly lower than controls (22 ± 21–22 days) ($p < 0.001$; median ± 95% CI; figure 2*a*). Furthermore,

insecticide drone survival was significantly reduced compared with controls for up to 14 days (point of sexual maturity); mortality was 16.82 ± 0.02% and 32.08 ± 0.03% for controls and insecticides, respectively, which represents an approximately 50% difference ($p < 0.001$; cumulative hazard% ± s.e.; figure 2*a*). By contrast, no significant difference in worker median longevity was observed between controls (23 ± 22–24 days) and insecticides (26 ± 25–29 days) ($p = 0.27$; median ± 95% CI; figure 2*b*).

(b) Sperm assessment

No evidence of treatment effect was found between control (2.19 ± 1.93–2.55 million) and insecticide (1.55 ± 1.33–2.05 million) drone sperm quantity 14 days post-cage assay initiation ($p = 0.14$; median ± 95% CI; figure 3*a*). By contrast, sperm viability was significantly different between the two treatment groups, with insecticide drones having 8 ± 4.6–11.3% (median difference ± 95% CI) lower sperm viability than controls ($p = 0.03$; figure 3*b*). Sperm viability was 92 ± 90–94% and 83.5 ± 80–86% in the controls and insecticides, respectively (median ± 95% CI). No correlation was observed between sperm quantity and sperm viability (Spearman's $|r| = 0.05$, $p = 0.44$). In addition, a significant difference was observed between control (1.98 ± 1.72–2.18 million) and insecticide (1.2 ± 0.20–1.6 million) treatments for total living sperm quantity ($p < 0.05$; median ± 95% CI; figure 3*c*), which represents on average approximately 39% less living sperm in insecticides compared with controls. The median difference and its 95% CI was 0.61 ± 0.32–0.90 million less living sperm in insecticides compared with controls.

4. Discussion

Factors governing reproductive success have a profound influence on shaping populations by affecting fitness [1,75]. Bountiful examples in nature include predation and parasitism [76,77]; however, anthropogenic influences such as industrial pollution and landscape fragmentation may also be important drivers [78–80]. Neonicotinoid insecticides represent a class of neurotoxins widely employed in agriculture for insect pest control [15]. Our study clearly demonstrates that neonicotinoid insecticides can have significant lethal (lifespan) and sublethal (sperm viability and living sperm quantity) effects on honeybee drones. Using the honeybee as a model, we hereby provide the first evidence that field-relevant concentrations of these chemicals can elicit effects on male insect reproductive capacity.

For eusocial insects such as honeybees, polyandry conveys several fitness benefits, such as reducing parasitism [81,82], buffering colony performance against environmental change [83], and improving task efficiency [84–86]; it also ensures sufficient sperm to maintain long-living queens and large colonies [85,87]. Therefore, evidence to suggest that neonicotinoids can impair reproduction provides one possible explanation for recent observations of increased annual mortality of managed honeybees [17,29,30,88], as well as the general decline of wild insect pollinators [29,89], throughout the Northern Hemisphere. Although drones (male honeybees) do not directly contribute to colony survival [90], their role via mating is vital for colony fitness [91]. Furthermore, queen survival and productivity are intimately connected to proper mating as the depletion of sperm results

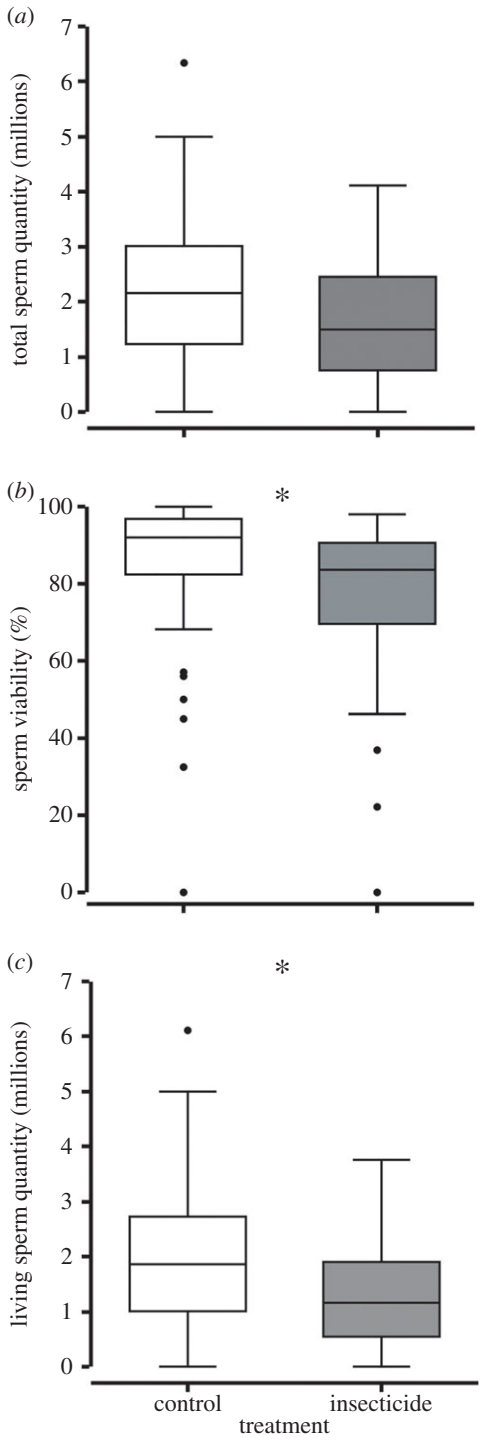

Figure 3. Honeybee sperm assessment. Assessment of various sperm traits in male (drone) honeybees (*Apis mellifera*) under neonicotinoid insecticide ($N = 90$) exposure compared with controls ($N = 145$). (*a*) Comparison of sperm quantity showed no significant differences ($p = 0.1375$). (*b*) Percentage of viable sperm in honeybee drones showed significant differences ($p = 0.03$). (*c*) Total quantity of living sperm in honeybee drones showed a significant difference ($p < 0.05$). All boxplots show the inter-quartile range (box), the median (black line within box), data range (horizontal black lines from box), and outliers (black dots). A significant difference between treatment groups is indicated by $*p < 0.05$.

in costly replacement of the queen by the colony, which can only successfully occur during specific periods of the year [92]. Recent data linking poor queen health to colony mortality [30], possibly because of low quality stored sperm from stressors such as miticides or insect growth regulator insecticides [33,93,94], highlight the urgent need for

investigating possible factors that may affect male reproductive success among non-target, beneficial insects.

Honeybee teneral body mass immediately succeeding pupation is often used as an index for an individual's overall condition [95,96]; both pathogens and insecticides reduce teneral body mass [43,97,98]. Our data revealed the teneral body mass of drones was not influenced by neonicotinoids, despite a previous investigation demonstrating reduced mass of neonicotinoid-exposed teneral workers [43]. Reasons for this disparity could be due to differences in neonicotinoid chemistries (the neonicotinoids, thiamethoxam and clothianidin versus imidacloprid), and routes of exposure (pollen versus sugar water). Nonetheless, our results demonstrated that neonicotinoid exposure strongly reduces the longevity of drones. Considering that sexual maturity is typically reached 9–14 days post-emergence, approximately 30% of neonicotinoid-exposed drones in our study would likely not be afforded the opportunity to mate with virgin queens. This could have severe consequences for colony fitness [99,100], as well as reduce the overall genetic variation within honeybee populations [101]. Conversely, female workers exposed to neonicotinoids did not experience a reduction in longevity, despite contrary evidence from previous studies [42,102]. This again could be due to differences among experimental treatments (the neonicotinoids, thiamethoxam and clothianidin versus thiacloprid), cage assay conditions (e.g. sugar and pollen feeding versus only sugar), or treatment exposure (colony versus individual level). This may, furthermore, be explained by the haploid–diploid susceptibility hypothesis, which proposes that hemizygous haploid individuals such as honeybee drones may experience increased susceptibility to environmental stressors due to decreased genetic variability [98,103]. Recent studies revealed that agrochemicals are capable of impairing immune function [104–107]; therefore, it is possible that neonicotinoid-exposed drones possess reduced detoxification abilities that subsequently affected lifespan.

The successful transfer of male sperm is the primary goal of copulation [23]. Therefore, honeybee mating success is highly dependent upon drones producing large quantities of sperm that must remain in excellent condition for an extended period within the queen's sperm storage organ (spermatheca). Although storage conditions afforded by the queen are important to ensuring long-term sperm survival [47], sperm received from the drone must nonetheless be of high quality. Even though neonicotinoids did not appear to influence the quantity of total sperm produced by males, we did observe a significant negative effect on sperm viability, which in turn resulted in a significant reduction in the number of living sperm produced by neonicotinoid drones. It is possible that this observation could be caused by reactive oxidative stress affecting sperm [44,46,47]; this possible mechanism should be studied in the future. The mean sperm quantity observed in this study was lower than found in previous cage and field studies [36,61,108,109]. The lower values could have resulted from laboratory cage conditions [36], as well as conditions of the drones during development [110].

Although only a small proportion of transferred sperm is stored by the queen [111], any decrease in sperm quality could have negative consequences [112]. Aided by muscular contractions in the female reproductive tract, transferred sperm actively swim from the oviducts to the female spermatheca, a process that can take up to approximately 40 h [60,111]. Considering that the majority of queen mating flights occur within 2–4 days [21,22,113], poor-quality sperm received during mating could result in a reduced quantity of stored sperm, or in extended, risky mating flight periods to ensure sufficient sperm is obtained [50,60,114,115].

As the primary egg layer and an important source of colony cohesion, the queen is intimately connected to colony performance [30]. Increased reports of queen failure have recently been reported in North America and Europe [30,31,116]; however, no studies have so far investigated the role of neonicotinoids and male health to explain this phenomenon. For the first time, we have demonstrated that frequently employed neonicotinoid insecticides in agro-ecosystems can elicit important lethal (reduced longevity) and sublethal (reduced sperm viability and living sperm quantity) effects on non-target, beneficial male insects; this may have broad population-level implications [17,29,117]. Furthermore, the observed effects of neonicotinoid insecticides on a highly polyandrous bee species are particularly worrying for monandrous insects that rely on a single successful mating event to provide fertilized eggs [118].

By demonstrating the effects of neonicotinoid insecticides on male insect reproduction, our study provides a possible mechanism, in addition to introduced parasites and other land-use practices, for honeybee queen failure [30,31] and a general decline of non-target beneficial insects throughout the Northern Hemisphere. Considering that neonicotinoid insecticides can affect non-target male vertebrate reproduction [119–122], our complementary findings for invertebrates are not surprising. Our research further highlights the urgent need for thorough investigations of possible unintended effects of agricultural insecticides on male insect reproductive traits, particularly among sympatric beneficial non-targets. For instance, it is not known if the insecticides had a direct effect on the male's reproductive traits via contaminated pollen, or an indirect effect because of poor nursing quality and reduced hypopharyngeal gland activity of young, exposed workers [123,124]. Furthermore, future research should be directed towards understanding how our results relate to broader implications for honeybee reproduction in the natural environment. Although recent improvements to regulatory requirements for evaluating the environmental impacts of insecticides have been adopted, none so far directly address the reproduction of beneficial insects [9].

Data accessibility. The complete raw data can be found at the Dryad repository. See doi:10.5061/dryad.bs515.

Authors' contributions. L.S., P.N, G.R.W. designed the experiment and wrote the manuscript; L.S., L.V.-B., S.B., A.T., G.R., L.G., K.K., G.R.W. collected field and laboratory data; P.C., L.G., P.N., G.R.W. provided materials, reagents; B.V. designed the statistical analysis and contributed to writing the statistical methods and results; L.S., G.R.W. analysed the data. All authors edited and approved the manuscript.

Competing interests. We have no competing interests.

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The Environmental Risks of neonicotinoid pesticides: a review of the evidence post-2013

EXECUTIVE SUMMARY

Neonicotinoid pesticides were first introduced in the mid-1990s and since then their use has grown rapidly so that they have become the most widely used class of insecticides in the world, with the majority being used as seed coatings. Neonicotinoids are water-soluble, and so a small quantity applied to a seed will dissolve when in contact with water in the soil and be taken up by the roots of the developing plant. Once inside the plant it becomes systemic and is found in vascular tissues and foliage, providing protection against herbivorous insects. This prophylactic use of neonicotinoids has become extremely widespread on a wide range of arable crops across much of the developed world.

However, only approximately 5% of the neonicotinoid active ingredient is taken up by crop plants and most instead disperses into the wider environment. Since the mid-2000s numerous studies have raised concerns that neonicotinoids may be having a negative effect on non-target organisms. In particular, neonicotinoids were associated with mass poisoning events of honeybees and were shown to have serious negative effects on honeybee and bumblebee fitness when consumed. In response to this growing body of evidence, the European Food Safety Authority (EFSA) was commissioned to produce risk assessments for the use of clothianidin, imidacloprid and thiamethoxam and their impact on bees. These risk assessments, published in January 2013, conclude that the use of these compounds on certain flowering crops poses a high risk to bees. On the basis of these findings, the European Union adopted a partial ban on these substances in May 2013 which came into force on 1st December 2013.

The purpose of this review is to collate and summarise scientific evidence published since 2013 that investigates the impact of neonicotinoids on non-target organisms and to bring it into one place to aid informed decision making. Due to international concern over the unintended impacts of neonicotinoids on wildlife, this topic has received a great deal of scientific attention in this three year period. As the restrictions were put in place because of the risk neonicotinoids pose to bees, much of the recent research work has naturally focussed on this group.

Risks to bees

Broadly, the EFSA risk assessments addressed risks of exposure to bees from neonicotinoids through various routes and the direct lethal and sublethal impact of neonicotinoid exposure. New scientific evidence is available in all of these areas, and it is possible to comment on the change in the scientific evidence since 2013 compared to the EFSA reports. This process is not meant to be a formal assessment of the risk posed by neonicotinoids in the manner of that conducted by EFSA. Instead it aims to summarise how the new evidence has changed our understanding of the likely risks to bees; is it lower, similar or greater than the risk perceived in 2013. With reference to the EFSA 2013 risk assessments baseline, advances in each considered area and their impact on the original assessment can be summarised thus:

- *Risk of exposure from pollen and nectar of treated flowering crops.* The EFSA reports calculated typical exposure from flowering crops treated with neonicotinoids as seed dressings. Considerably more data are now available in this area, with new studies broadly

supporting the calculated exposure values. For bees, flowering crops pose a **Risk Unchanged** to that reported by EFSA 2013.

- *Risk from non-flowering crops and cropping stages prior to flowering*. Non-flowering crops were considered to pose no risk to bees. No new studies have demonstrated that these non-flowering crops pose a direct risk to bees. They remain a **Risk Unchanged.**
- *Risk of exposure from the drilling of treated seed and subsequent dust drift*. Despite modification in seed drilling technology, available studies suggest that dust drift continues to occur, and that dust drift still represents a source of acute exposure and so is best considered a **Risk Unchanged.**
- *Risk of exposure from guttation fluid*. Based on available evidence this was considered a low-risk exposure path by EFSA 2013. New data have not changed this position and so it remains a **Risk Unchanged.**
- *Risk of exposure from and uptake of neonicotinoids in non-crop plants*. Uptake of neonicotinoids by non-target plants was considered likely to be negligible, though a data gap was identified. Many studies have since been published demonstrating extensive uptake of neonicotinoids and their presence in the pollen, nectar and foliage of wild plants. Bees collecting pollen from neonicotinoid-treated crops can generally be expected to be exposed to the highest neonicotinoid concentrations, but non-trivial quantities of neonicotinoids are also present in pollen and nectar collected from wild plants, and this source of exposure may be much more prolonged than the flowering period of the crop. Exposure from non-target plants clearly represents a **Greater Risk.**
- *Risk of exposure from succeeding crops*. A data gap was identified for this issue. Few studies have explicitly investigated this, but this area does represent some level of risk as neonicotinoids are now known to have the potential to persist for years in soil, and can be detected in crops multiple years after the last known application. However, as few data exist this is currently considered a **Risk Unchanged.**
- *Direct lethality of neonicotinoids to adult bees.* Additional studies on toxicity to honeybees have supported the values calculated by EFSA. More data have been produced on neonicotinoid toxicity for wild bee species and meta-analyses suggest a broadly similar response. Reference to individual species is important but neonicotinoid lethality should be broadly considered a **Risk Unchanged.**
- *Sublethal effects of neonicotinoids on wild bees.* Consideration of sublethal effects by EFSA was limited as there is no agreed testing methodology for the assessment of such effects. A data gap was identified. Exposure to neonicotinoid-treated flowering crops has been shown to have significant negative effects on free flying wild bees under field conditions and some laboratory studies continue to demonstrate negative effects on bee foraging ability and fitness using field-realistic neonicotinoid concentrations. **Greater Risk.**

Within this context, research produced since 2013 suggest that neonicotinoids pose a similar to greater risk to wild and managed bees, compared to the state of play in 2013. Given that the initial 2013 risk assessment was sufficient to impose a partial ban on the use of neonicotinoids on flowering crops, and given that new evidence either confirms or enhances evidence of risk to bees, it is logical to conclude that the current scientific evidence supports the extension of the moratorium, and that the extension of the partial ban to other uses of neonicotinoids should be considered.

Broader risks to environmental health

In addition to work on bees, our scientific understanding has also been improved in the following areas which were not previously considered by EFSA:

- Non-flowering crops treated with neonicotinoids can pose a risk to non-target organisms through increasing mortality in beneficial predator populations.
- Neonicotinoids can persist in agricultural soils for several years, leading to chronic contamination and, in some instances, accumulation over time.
- Neonicotinoids continue to be found in a wide range of different waterways including ditches, puddles, ponds, mountain streams, rivers, temporary wetlands, snowmelt, groundwater and in outflow from water processing plants.
- Reviews of the sensitivity of aquatic organisms to neonicotinoids show that many aquatic insect species are several orders of magnitude more sensitive to these compounds than the traditional model organisms used in regulatory assessments for pesticide use.
- Neonicotinoids have been shown to be present in the pollen, nectar and foliage of non-crop plants adjacent to agricultural fields. This ranges from herbaceous annual weeds to perennial woody vegetation. We would thus expect non-target herbivorous insects and non-bee pollinators inhabiting field margins and hedgerows to be exposed to neonicotinoids. Of particular concern, this includes some plants sown adjacent to agricultural fields specifically for the purposes of pollinator conservation.
- Correlational studies have suggested a negative link between neonicotinoid usage in agricultural areas and population metrics for butterflies, bees and insectivorous birds in three different countries.

Overall, this recent work on neonicotinoids continues to improve our understanding of how these compounds move through and persist in the wider environment. These water soluble compounds are not restricted to agricultural crops, instead permeating most parts of the agricultural environments in which they are used and in some cases reaching further afield via waterways and runoff water. Field-realistic laboratory experiments and field trials continue to demonstrate that traces of residual neonicotinoids can have a mixture of lethal and sublethal effects on a wide range of taxa. Susceptibility varies tremendously between different taxa across many orders of magnitude, with some showing a negative response at parts per billion with others show no such effects at many thousands of parts per billion. Relative to the risk assessments produced in 2013 for clothianidin, imidacloprid and thiamethoxam which focussed on their effects on bees, new research strengthens arguments for the imposition of a moratorium, in particular because it has become evident that they pose significant risks to many non-target organisms, not just bees. Given the improvement in scientific knowledge of how neonicotinoids move into the wider environment from all crop types, a discussion of the risks posed by their use on non-flowering crops and in non-agricultural areas is urgently needed.

1. INTRODUCTION AND STATE OF PLAY

Neonicotinoid pesticides were first introduced in the 1990s and since then their use has grown rapidly to become the most widely used class of insecticide in the world. This increase in popularity has largely occurred from the early 2000s onwards (Figure 1). This use has largely been driven by the adoption of seed treatments. Neonicotinoids are water-soluble, and so a small quantity applied to a seed will dissolve when in contact with water and be taken up by the roots of the developing plant. Once inside the plant it becomes systemic and is found in vascular tissues and foliage, providing protection against herbivorous insects. This prophylactic use of neonicotinoids has become extremely widespread – for example, between 79-100% of maize hectares in the United States in 2011 were treated with a neonicotinoid seed dressing (Douglas and Tooker 2015).

However, only approximately 5% of the neonicotinoid active ingredient is taken up by crop plants and most instead disperses into the wider environment. In recent years numerous authors have raised concerns about the impact neonicotinoids may have on non-target organisms. Neonicotinoids released in dust abraded by seed drilling machinery were implicated in mass poisonings of honeybees in Germany and Italy (Pistorius et al. 2009; Bortolotti et al. 2009), neonicotinoids were found in agricultural soils (Bonmatin *et al*. 2005) and also in the pollen and nectar of treated crops (Bonmatin *et al*. 2007). In 2012, two high profile studies were published that showed exposure to neonicotinoids in pollen and nectar could have serious effects on honeybee navigation and mortality (Henry *et al*. 2012) and bumblebee colony development and queen production (Whitehorn *et al*. 2012). In response to the growing body of work the European Food Safety Authority (EFSA), the body with regulatory oversight for agricultural chemicals, was commissioned to produce a risk assessment on the three most widely used agricultural neonicotinoids (clothianidin, imidacloprid and thiamethoxam) and the risk that they posed to bees (EFSA 2013a; 2013b; 2013c). On the basis of the available evidence EFSA recommended a moratorium on the use of neonicotinoids on treated crops which was accepted and implemented by the European Commission at the end of 2013.

This moratorium is due to conclude shortly. One of the specified objectives was to allow further research on the impact of neonicotinoids on bees in order to inform subsequent regulatory decisions. Since 2013, a great number of studies have been published that consider the impact of neonicotinoids on bees and also a wide range of other non-target taxa. Many large reviews of neonicotinoids impacts on non-target organisms have also been published, for example Nuyttens et al. (2013) on neonicotinoid contaminated dust, Godfray *et al*. (2014; 2015) on the risks neonicotinoids pose to pollinators, Bonmatin *et al*. (2015) on environmental fate of and exposure to neonicotinoids, Pisa *et al*. (2015) and Gibbons *et al*. (2015) on the impacts of neonicotinoids on non-target terrestris organisms and Morrissey *et al*. (2015) on contamination of aquatic ecosystems with neonicotinoids and their impact on aquatic organisms, to name a few.

The purpose of this review is to consider the scientific evidence published since 2013 that covers the impact of neonicotinoids on wild non-target organisms (therefore excluding the domesticated honeybee) and to bring it together into one place to aid informed decision making. It is not a formal risk assessment, though comparisons will be made with the knowledge base used in the EFSA risk assessments specifically and that which was known in 2013 more generally. The findings will be of interest to those considering the wider impact of neonicotinoid pesticide use when assessing their future use in agricultural environments.

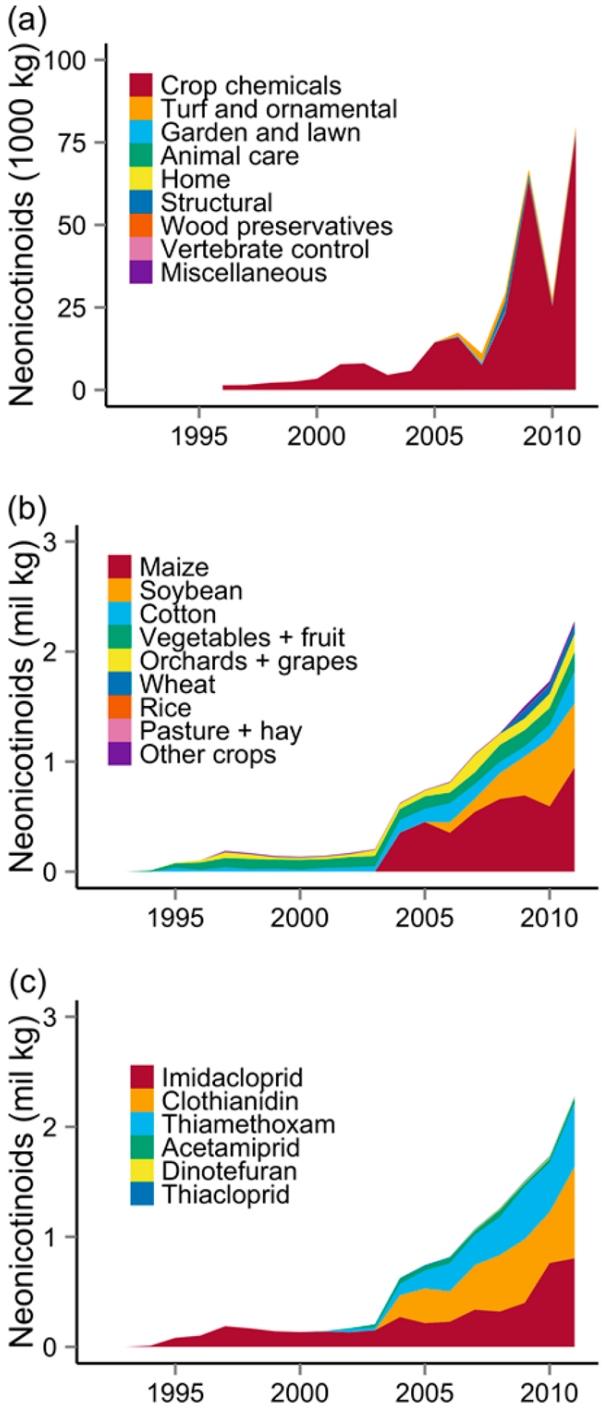

Figure 1. Neonicotinoid sales by (a) product type, (b) use by crop and (c) active ingredient, from 1992 to 2011. Data on use (a) is based on sales data from Minnesota. Data on crops and active ingredients are for the entire U.S., from United States Geological Survey. y-axes represent mass of neonicotinoid active ingredient in thousands or millions of kg. Reproduced from Douglas and Tooker (2015)

2. EVIDENCE FOR EXPOSURE TO NEONICOTINOID PESTICIDES

2.1 Risk of exposure for non-target organisms from neonicotinoids applied directly to crops

Due to their systemic nature, neonicotinoids applied to crops by any application method (e.g. seed dressing, foliar spray, soil drench) will be taken up by crop tissues and can subsequently be found in all parts of the treated plant (Simon-Delso *et al*. 2015). The EFSA (2103a; 2013b; 2013c) reports identify and discuss a number of exposure pathways through which bees can be exposed to neonicotinoids, where the risk of exposure is dependent on application rate, application type and crop type. However, knowledge about the extent and significance of these pathways was poor. Since then, a large number of studies have been published further documenting neonicotinoid exposure from treated crops. Important reviews include Nuyttens et al. (2013), Godfray *et al*. (2014), Long and Krupke (2015) and Bonmatin *et al*. (2015).

2.1.1 Risk of exposure from pollen and nectar of treated flowering crops

Using data from 30 (clothianidin), 16 (thiamethoxam) and 29 (imidacloprid) outdoor studies and known authorised application rates, EFSA (2013a; 2013b; 2013c) calculated expected residue rates in pollen and nectar of the studied crops (Table 1). Levels are variable but all are within one order of magnitude. Levels in pollen are consistently higher than levels in nectar. Godfray *et al*. (2014) reviewed 20 published studies to calculate an arithmetic mean maximum level of 1.9 ppb for nectar and 6.1 ppb for pollen in treated crops, in line with the EFSA findings.

Table 1. Summary of expected residues in pollen and nectar of various neonicotinoid-treated flowering crops calculated by EFSA from the review of outdoor field trials. No nectar values are available for maize as this plant does not produce nectar. Blanks are where no minimum values were stated

Crop	Pesticide	Residues in pollen (ng/g)		Residues in nectar (ng/g)	
		Minimum	Maximum	Minimum	Maximum
Oilseed rape	Clothianidin	5.95	19.04	5	16
Sunflower	Clothianidin		3.29		0.324
Maize	Clothianidin	7.38	36.88	*n/a*	*n/a*
Oilseed rape	Imidacloprid	1.56	8.19	1.59	8.35
Sunflower	Imidacloprid		3.9		1.9
Maize	Imidacloprid	3.02	15.01	*n/a*	*n/a*
Cotton	Imidacloprid	3.45	4.6	3.45	4.6
Oilseed rape	Thiamethoxam	4.592	19.29	0.648	2.72
Sunflower	Thiamethoxam	2.378	3.02	0.59	0.75
Maize	Thiamethoxam	13.419	21.513	*n/a*	*n/a*

Since 2014 a number of studies have been published which report neonicotinoid concentrations in the pollen and nectar of neonicotinoid-treated flowering crops. These results have been approximately in line with the concentrations reported by EFSA and Godfray *et al*. In oilseed rape treated with thiamethoxam, Botías *et al*. (2015) found average concentrations of 3.26 ng/g of thiamethoxam, 2.27 ng/g of clothianidin and 1.68 ng/g of thiacloprid in the pollen. Oilseed rape

Table 2. Summary of studies published since 2013 that document neonicotinoid residues in pollen and nectar collected by free flying bees at sites adjacent to treated and untreated flowering crops. Results for samples collected at treated sites are highlighted in bold. SS = spring-sown, WS = winter-sown, US = unclear sowing date

Species	Sample type	Samples collected	Nest location	Mean total neonicotinoid concentration (ng/ml or ng/g)	Reference
Apis mellifera	Nectar	2005-2009 (dates unknown)	Adjacent to untreated US OSR fields	<1 (*limit of quantification*)	Pilling *et al*. (2013)
Apis mellifera	Nectar	2005-2009 (dates unknown)	Adjacent to treated US OSR fields	**0.7-2.4** (range of reported median values)	Pilling *et al*. (2013)
Apis mellifera	Nectar	6th May 2014	Adjacent to untreated WS OSR fields	<0.3 (*limit of detection*)	Rolke *et al*. (2016)
Apis mellifera	Nectar	6th May 2014	Adjacent to treated WS OSR fields	**0.68**	Rolke *et al*. (2016)
Apis mellifera	Nectar	10th-14th May 2014	Adjacent to untreated WS OSR fields	<0.3 (*limit of detection*)	Rolke *et al*. (2016)
Apis mellifera	Nectar	10th-14th May 2014	Adjacent to treated WS OSR fields	**0.77**	Rolke *et al*. (2016)
Apis mellifera	Nectar	June 2013 (peak OSR flowering)	Adjacent to untreated SS OSR fields	0.1	Rundlöf *et al*. (2015)
Apis mellifera	Nectar	June 2013 (peak OSR flowering)	Adjacent to treated SS OSR fields	**10.3**	Rundlöf *et al*. (2015)
Bombus terrestris	Nectar	June 2013 (peak OSR flowering)	Adjacent to untreated SS OSR fields	0	Rundlöf *et al*. (2015)
Bombus terrestris	Nectar	June 2013 (peak OSR flowering)	Adjacent to treated SS OSR fields	**5.4**	Rundlöf *et al*. (2015)
Apis mellifera	Pollen	2005-2009 (dates unknown)	Adjacent to untreated maize fields	<1 (*limit of quantification*)	Pilling *et al*. (2013)
Apis mellifera	Pollen	2005-2009 (dates unknown)	Adjacent to treated maize fields	**1-7** (range of reported median values)	Pilling *et al*. (2013)
Apis mellifera	Pollen	2005-2009 (dates unknown)	Adjacent to untreated US OSR fields	<1 (*limit of quantification*)	Pilling *et al*. (2013)
Apis mellifera	Pollen	2005-2009 (dates unknown)	Adjacent to treated US OSR fields	**<1-3.5** (range of reported median values)	Pilling *et al*. (2013)
Apis mellifera	Pollen	First two weeks of July 2012	Located in untreated SS OSR fields	0.24	Cutler *et al*. (2014)
Apis mellifera	Pollen	First two weeks of July 2012	Located in treated SS OSR fields	**0.84**	Cutler *et al*. (2014)
Apis mellifera	Pollen	June 2013 (peak OSR flowering)	Adjacent to untreated WS OSR fields	<0.5 (*limit of detection*)	Rundlöf *et al*. (2015)
Apis mellifera	Pollen	June 2013 (peak OSR flowering)	Adjacent to treated WS OSR fields	**13.9**	Rundlöf *et al*. (2015)
Apis mellifera	Pollen	May to September 2011	Non-agricultural area	0.047	Long and Krupke (2016)
Apis mellifera	Pollen	May to September 2011	Adjacent to untreated maize fields	0.078	Long and Krupke (2016)
Apis mellifera	Pollen	May to September 2011	Adjacent to treated maize fields	**0.176**	Long and Krupke (2016)
Apis mellifera	Pollen	6th May 2014	Adjacent to untreated WS OSR fields	<0.3 (*limit of detection*)	Rolke *et al*. (2016)
Apis mellifera	Pollen	6th May 2014	Adjacent to treated WS OSR fields	**0.50**	Rolke *et al*. (2016)
Apis mellifera	Pollen	10th-14th May 2014	Adjacent to untreated WS OSR fields	<0.3 (*limit of detection*)	Rolke *et al*. (2016)
Apis mellifera	Pollen	10th-14th May 2014	Adjacent to treated WS OSR fields	**0.97**	Rolke *et al*. (2016)
Bombus terrestris	Pollen	10th May 2014	Adjacent to untreated WS OSR fields	<0.3 (*limit of detection*)	Rolke *et al*. (2016)
Bombus terrestris	Pollen	10th May 2014	Adjacent to treated WS OSR fields	**0.88**	Rolke *et al*. (2016)
Bombus impatiens	Pollen	July to August 2013	Adjacent to untreated maize fields	<0.1 (*limit of detection*)	Cutler and Scott-Dupree (2014)
Bombus impatiens	Pollen	July to August 2013	Adjacent to treated maize fields	**0.4**	Cutler and Scott-Dupree (2014)
Osmia bicornis	Pollen	14th May 2014	Adjacent to untreated WS OSR fields	<0.3 (*limit of detection*)	Rolke *et al*. (2016)
Osmia bicornis	Pollen	14th May 2014	Adjacent to treated WS OSR fields	**0.88**	Rolke *et al*. (2016)

nectar contained similar average concentrations of 3.20 ng/g of thiamethoxam, 2.18 ng/g of clothianidin and 0.26 ng/g of thiacloprid. Xu *et al*. (2016) found average levels of clothianidin in oilseed rape of 0.6 ng/g. No pollen samples were taken. In maize pollen, Stewart *et al*. (2014) found average thiamethoxam and clothianidin levels between the limit of detection (LOD) of 1 ng/g to 5.9 ng/g across a range of seed treatments. Xu et al. (2016) found average clothianidin concentration of 1.8 ng/g in maize pollen. Additionally, Stewart et al. (2014) found no neonicotinoid residues in soybean flowers or cotton nectar.

Several studies published since 2013 have used free flying bees to experimentally demonstrate that proximity to treated flowering crops increases their exposure to neonicotinoids (Table 2). Using honeybees, neonicotinoid concentrations in pollen taken from foragers returning to nests placed next to untreated flowering crops ranged from 0-0.24 ng/g compared to pollen from nests next to treated flowering crops which ranged from 0.84-13.9 ng/g. There have been fewer studies of bumblebees and hence the sample size is much smaller, with concentrations of neonicotinoids in pollen from untreated areas ranging from <0.1-<0.3 ng/g compared to 0.4-0.88 ng/g for nests placed next to treated areas. The only available study looking at solitary bee collected pollen found *Osmia bicornis* collecting <0.3 ng/g in untreated areas and 0.88 ng/g in treated areas. Similar trends are found in the nectar results, though fewer studies are available. Rolke *et al*. (2016) found neonicotinoid concentrations of 0.68-0.77 ng/ml in honeybee collected nectar samples from apiaries adjacent to neonicotinoid-treated oilseed rape, compared to <0.3 ng/ml from apiaries adjacent to untreated oilseed rape. However, Rundlöf *et al*. (2015) found concentrations of 5.4 ng/ml in bumblebee collected nectar and 10.3 ng/ml in honeybee collected nectar taken from bees originating from nests placed adjacent to treated oilseed rape compared to 0-0.1 ng/ml from bees from nests adjacent to untreated oilseed rape.

This level of variation of up to one order of magnitude in neonicotinoid concentrations found in bee collected pollen and nectar in different studies is substantial. The detected levels in pollen and nectar presumably depend significantly on the dose and mode of treatment, the studied crop, the season, the location, the soil type, the weather, time of day samples are collected, and so on. Even different crop varieties can result in significant variation in the residue content of pollen and nectar (Bonmatin *et al*. 2015). Because pollen samples taken from a series of bees will be from a mixture of different plants, most of which will not be crop plants, the neonicotinoid residues in crop pollen will be diluted by untreated, non-crop pollen. However, for the reported studies, the higher neonicotinoid concentrations are within an order of magnitude of the 6.1 ng/g in pollen and 1.9 ng/ml in nectar values calculated by Godfray *et al*. (2014). Additionally, in all cases, the concentrations of neonicotinoids in pollen and nectar were higher at sites adjacent to neonicotinoid-treated flowering crops than at sites adjacent to untreated crops. The available evidence shows that proximity to treated flowering crops increases the exposure of bees to neonicotinoid pesticides. The recent evidence for concentrations found in flowering crops is approximately in line with the levels reported by EFSA (2013a; 2013b; 2013c).

2.1.2 Risk from non-flowering crops and cropping stages prior to flowering

The EFSA studies state that some of the crops on which clothianidin is authorised as a seed-dressing do not flower, are harvested before flowering, or do not produce nectar or pollen, and therefore these crops will not pose any risk to bees via this route of exposure. Whilst non-flowering crops are clearly not a source of exposure through produced pollen and nectar, they do represent a source of neonicotinoids that can dissipate into the wider environment (discussed in Section 2.2). Additionally,

treated crops of any type represent additional pathways of neonicotinoid exposure to other organisms.

Depending on crop species and consequent seed size, neonicotinoid-treated seeds contain between 0.2-1 mg of active ingredient per seed (Goulson 2013). For a granivorous grey partridge weighing 390 g Goulson calculated that it would need to consume around five maize seeds, six sugar beet seeds or 32 oilseed rape seeds to receive a nominal LD_{50}. Based on US Environmental Protection Agency estimates that around 1% of sown seed is accessible to foraging vertebrates at recommended sowing densities, Goulson calculated that sufficient accessible treated seed would be present to deliver a LD_{50} to ~100 partridges per hectare sown with maize or oilseed rape. Given that grey partridges typically consume around 25 g of seed a day there is the clear potential for ingestion of neonicotinoids by granivorous animals, specifically birds and mammals. However, whilst some experimental studies have been conducted to investigate mortality and sublethal effects of treated seeds on birds (see Section 3.5), no studies are available that demonstrate consumption of treated seed by farmland birds under field conditions or quantify relative consumption of treated versus untreated seed to better understand total exposure via this route.

In addition to insect herbivores, developing seedlings treated with neonicotinoids are predated by molluscan herbivores. Because neonicotinoids have relatively low efficacy against molluscs, Douglas *et al.* (2015) investigated neonicotinoid residues in the slug *Deroceras reticulatum*, a major agricultural pest, using neonicotinoid seed-treated soybean in both laboratory and field studies. Total neonicotinoid concentrations from samples of field collected slugs feeding on treated soybean were as high as 500 ng/g with average levels over 100 ng/g after 12 days of feeding. No neonicotinoids were detected in slugs feeding on untreated control plants. After 169 days, no neonicotinoids were detected in either control or treated slugs. In the laboratory, slugs consuming soybean seedlings incurred low mortality of between 6-15% depending on the strength of the seed treatment. In laboratory experiments, slugs were exposed to the ground beetle *Chlaenius tricolor* after feeding on soybean. *C. tricolor* is a typical predatory beetle found in agro-ecosystems and is known to be an important predator of slugs. For beetles that consumed slugs, 61.5% (n=16/26) of those from the neonicotinoid treatment subsequently showed signs of impairment compared to none of those in the control treatment (n=0/28). Of the 16 that showed impairment, seven subsequently died. This study is also discussed in Section 3.3. A similar result was found by Szczepaniec *et al*. (2011) who found that the application of imidacloprid to elm trees caused an outbreak of spider mites *Tetranychus schoenei*. This increase was as a result of a reduction in the density of their predators which incurred increased mortality after ingesting imidacloprid-containing prey items. Many beneficial predatory invertebrates feed on pests of crops known to be treated with neonicotinoids, but to date no other studies have assessed whether neonicotinoids are transmitted to these predators through direct consumption of crop pests in agro-ecosystems.

Additionally, flowering crops in a non-flowering stage can also pose a potential threat to natural enemy populations. The soybean aphid parasitoid wasp *Aphelinus certus* is an important parasite of the soybean aphid *Aphis glycines*. Frewin *et al*. (2014) gave *A. certus* access to laboratory populations of aphids feeding on control and neonicotinoid-treated soybean plants. *A. certus* parasitised a significantly smaller proportion of aphids on treated plants than on untreated plants. Frewin *et al*. hypothesise two potential reasons for this effect – firstly that exposure to neonicotinoid residues within aphid hosts may have increased mortality of the immature parasitoid or the parasitism combined with residues may have increased aphid mortality. Secondly, *A. certus* may avoid parasitising pesticide-poisoned aphids. *Aphelinus* species are known to use internal cues to determine host suitability, and it is possible that they may use stress- or immune-related aphid

hormones to judge host suitability. Given that a key part of biological control of insect pests using parasitic wasps is to increase the parasitoid abundance early in the season, the reduction in the parasitism rate caused by neonicotinoid seed-treatment could potentially impair the ability of *A. certus* to control soybean aphid.

Non-flowering neonicotinoid crops present possible exposure routes through direct consumption of treated seed or consumption of seedling plants that may result in the transmission of neonicotinoids to higher trophic levels, including beneficial insects that offer a level of pest control through predatory behaviour. As the EFSA reports did not consider the impact of neonicotinoids on non-bees, no comparison can be made here.

2.1.3 Risk of exposure from the drilling of treated seed and subsequent dust drift

Numerous studies (12 listed by Godfray *et al.* 2014) prior to 2013 identified that neonicotinoids present in seed dressings can be mechanically abraded during the drilling process and can subsequently be emitted as dust. This dust can contain very high levels of neonicotinoids, up to 240,000 ng/g under certain conditions (see the review by Nuyttens *et al*. 2013). Acute contact with this dust can in certain cases result in the mass poisoning of honeybees (e.g. Pistorius *et al*. 2009; Bortolotti *et al*. 2009). Concentrations of neonicotinoids in dust created during sowing and the total volume released into the air depend on application rate, seed type, seed treatment quality (including additions such as talcum powder), seed drilling technology and environmental conditions. Girolami *et al.* (2013) demonstrated that the dust cloud created by seed drills is an ellipsoidal shape approximately 20 m in diameter. Using cage experiments, a single pass of a drilling machine was sufficient to kill all honeybees present. The use of tubes designed to direct exhaust air towards the ground did not substantially increase bee survival rate. Neonicotinoid concentrations of up to 4000 ng/g were detected in honeybees with an average concentration of 300 ng/g. Similar concentrations were detected in bees exposed to both unmodified and modified drills.

On the basis of the available evidence, the EFSA reports (2013a; 2013b; 2013c) concluded that maize produces the highest dust drift deposition, while for sugar beet, oilseed rape and barley seeds the dust drift deposition was very limited. No information was available for other crops, and given that seed type is an important factor determining neonicotinoid release, extrapolation to other crops is highly uncertain. A high acute risk was not excluded for bees foraging or flying in adjacent crops during the sowing of maize, oilseed rape, and cereals. In practice, this assessment indicates that forager honeybees or other pollinators flying adjacent to the crop are at high risk (e.g. via direct contact to dust) and may be able to carry considerable residues back to the hive (for social bees). Bees present further away or foraging upwind during the sowing will be considerably less exposed. The reports conclude that the aforementioned assessments do not assess potential risk to honeybees from sublethal effects of dust exposure. No information on neonicotinoid residues in nectar in the adjacent vegetation following dust drift was available.

In recent years, various types of improved seed drills have been adopted that direct air from the drills towards the soil, reducing the dust drift effect by up to 95% (see Manzone *et al*. 2015). Air deflectors have become mandatory for certain products in the Netherlands, France, Belgium and Germany (Godfray *et al*. 2014). Bonmatin *et al*. (2015) and Long and Krupke (2015) reviewed existing literature on the exposure of pollinators and other non-target organisms to contaminated dust from seed drilling machines, predominantly covering pre-April 2013 literature. The authors conclude that despite attention by regulators they consider dust drift to be a likely cause of environmental neonicotinoid contamination, in particular when best practice is not followed.

Recent studies continue to detect neonicotinoids in the tissues of wildflowers surrounding agricultural fields immediately after planting. Stewart *et al*. (2014) detected average neonicotinoid concentrations of 9.6 ng/g in whole wildflowers collected from field margins adjacent to fields planted with maize (n=18), cotton (n=18) and soybean (n=13). The samples were collected a few days after sowing (typically within three days), with the highest concentration of 257 ng/g collected adjacent to a maize field sown the previous day with thiamethoxam-treated seed. Detailed data on concentrations adjacent to each crop type are not available. No samples were taken from vegetation adjacent to crops sown without a neonicotinoid seed dressing. Rundlöf *et al*. (2015) collected flowers and leaves from wild plants growing adjacent to treated and untreated oilseed rape fields two days after sowing. Adjacent to the treated fields neonicotinoid concentrations were lower than in the previous study at 1.2 ng/g, but this was higher than the control fields where no neonicotinoids were detected. This is in line with previous findings that suggest a lower contamination risk from dust originating from oilseed rape seeds than for maize seeds.

2.1.4 Risk of exposure from guttation fluid

Some plants secrete small volumes of liquid (xylem sap) at the tips of leaves or other marginal areas, often referred to as guttation droplets. Six published studies and an EFSA review found extremely high neonicotinoid concentrations in guttation droplets of up to 4-5 orders of magnitude greater than those found in nectar, particularly when plants are young (see Godfray *et al*. 2014). Using a clothianidin concentration of 717,000 ng/g and an acute oral toxicity of 3.8 ng/bee for clothianidin (see Section 3.1.1), EFSA (2013a) calculated that a honeybee would only need to consume 0.005 µl to receive an LD_{50}. Given that honeybee workers can carry between 1.4-2.7 ml of water a day, there is the clear potential for lethal exposure via this route. The risk assessments for thiamethoxam and imidacloprid were similar (EFSA 2013b; 2013c). However, on the basis of experimental trials, the EFSA reports conclude that whilst guttation droplets were frequently produced, honeybees were rarely seen collecting water from them and therefore the risk should be considered low.

Few studies have looked at neonicotinoid exposure via guttation droplets since 2013. In the one available study, Reetz *et al*. (2015) assessed thiamethoxam concentrations in oilseed rape guttation droplets and measured residues in individual honeybee honey-sacs. The authors note that targeted observations of water-foraging honeybees in the field are nearly impossible, and so returning honeybees from apiaries placed out adjacent to treated oilseed rape crops were instead collected in the autumns of 2010 and 2011 when seedling oilseed rape crops were producing guttation droplets. Oilseed rape produced guttation droplets containing between 70-130 ng/ml clothianidin at the cotyledon stage. Out of 436 honey-sacs, neonicotinoids were only detected in 62 samples at concentrations between 0.1-0.95 ng/ml. However, because there was no behavioural observation it is not possible to state the providence of this contamination with certainty; neonicotinoids are also present in waterbodies and the nectar of wild flowers (see Section 2.2). As such, there is still little evidence documenting the extent to which honeybees or other insects collect or are otherwise exposed to neonicotinoids through contact with guttation droplets.

2.2 Risk of exposure for non-target organisms from neonicotinoids persisting in the wider environment

In identifying routes of exposure for honeybees the EFSA reports discussed the possibility of neonicotinoid residues in flowering arable weeds growing in fields with treated crops. This route of exposure was considered to be negligible as weeds would not be present in the field when the crop is sown and considerable uptake via weed plant roots was considered to be unlikely as the substance is concentrated around the treated seed. However, the reports note that potential uptake into flowering weeds cannot be ruled out for granular neonicotinoid applications, highlighting a data gap for this issue.

The persistence of neonicotinoids in soil, water and in wild plants is of potentially serious concern. If these pesticides are able to move into habitats surrounding agricultural fields the range of organisms that they could affect is much greater than simply crop-visiting invertebrates. If these pesticides last for extended periods in the wider environment then neonicotinoid exposure may be chronic, rather than an acute exposure associated with the sowing of treated seeds.

Since April 2013 much empirical data has been produced documenting the fate of residual neonicotinoids in the wider environment after application. Key review publications are Goulson (2013), Bonmatin *et al*. (2015) and Morrissey *et al*. (2015).

2.2.1 Persistence of neonicotinoids in soil

Although neonicotinoids applied through a seed dressing are designed to be taken up into the target crop plant, only 1.6-20% of the active ingredient is absorbed, with the majority remaining in the soil. A small proportion is dispersed through dust created whilst drilling (see Section 2.1.2). Neonicotinoids can bind to soil with the strength of the binding dependent on various factors. Neonicotinoids are water soluble (see section 2.2.2) and may leach from soils if water is present. Leaching is lower and sorption is higher in soils with a high content of organic material (Selim *et al*. 2010). In a recent comparison of soil types, Mörtl *et al*. (2016, Figure 2) found that clothianidin and thiamethoxam leached readily from sandy soils. Clay soils showed higher retention of neonicotinoids but the greatest retention was seen for loam soils. Correspondingly, the highest residual neonicotinoid concentrations were found in loam soils.

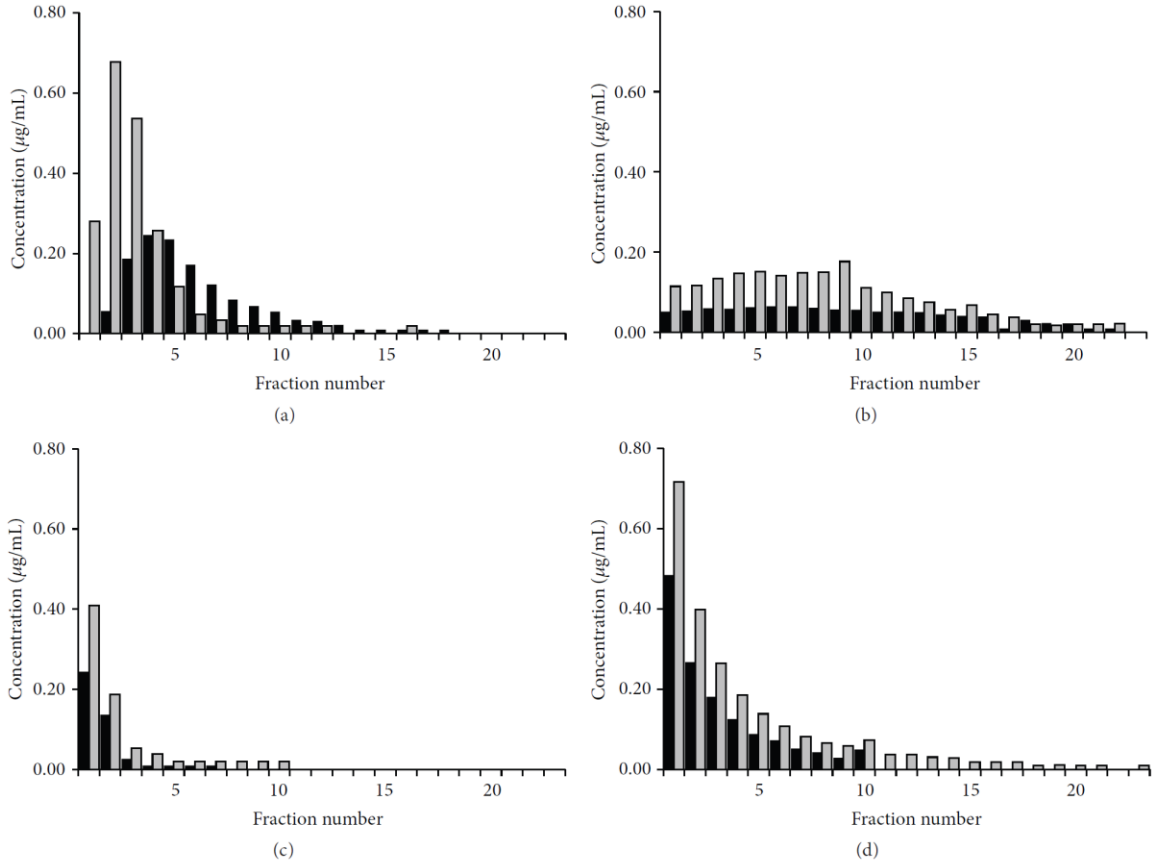

Figure 2. Elution profiles of clothianidin and thiamethoxam upon absorption on soils. Concentrations of clothianidin (black columns) and thiamethoxam (grey columns) measured in aqueous eluates from soil columns of (as) sand, (b) clay and (c) loam soils. Eluates from (d) pumice columns are shown as a control. Concentrations in 10 mL fractions of the eluate are shown in μg/mL, as a function of the fraction number. Reproduced from Mörtl *et al*. (2016)

Whilst several studies have assessed dissipation half-life times (DT_{50}) of neonicotinoids in soil, much of this work was conducted before the recent interest in the potentially deleterious effect of neonicotinoids on wider biodiversity. A review of available DT_{50} times from field and laboratory studies conducted between 1999 and 2013 were reviewed by Goulson (2013). Reported DT_{50}s are highly variable and typically range from 200 to in excess of 1000 days for imidacloprid, 7-353 days for thiamethoxam and 148-6931 days for clothianidin. DT_{50}s appear to be shorter for the nitro-substituted neonicotinoids, at 3-74 days for thiacloprid and 31-450 days for acetamiprid. DT_{50} values of over one year would suggest the likelihood of neonicotinoid bioaccumulation in the soil, assuming continuous input. However, these reported values are highly variable. At the time the EFSA reports were written only one field study was available that assessed neonicotinoid accumulation in the soil over multiple years with continued neonicotinoid input. Bonmatin *et al*. 2005 screened 74 samples of farmland soil from France for imidacloprid. Imidacloprid concentrations were higher in soils which had been treated in two consecutive years than those soils which had only received one treatment, suggesting the possibility of imidacloprid accumulation in the soil. However, as the study only looked at soils treated for a maximum of two years it is not clear whether residues would continue to increase. Two studies had been completed by 2013 but were not widely disseminated. These studies were carried out by Bayer and assessed levels of imidacloprid in soil over six years for seed-treated

barley in the UK (Placke 1998a) and spray application to orchard soils in Germany (Placke 1998b). Goulson (2013) reviewed this data and argued that the studies show accumulation of neonicotinoids in soils over time (Figure 3), with some indication that concentrations may begin to plateau after about five years. However, since the trials were terminated after six years it is not clear whether levels would have continued to increase.

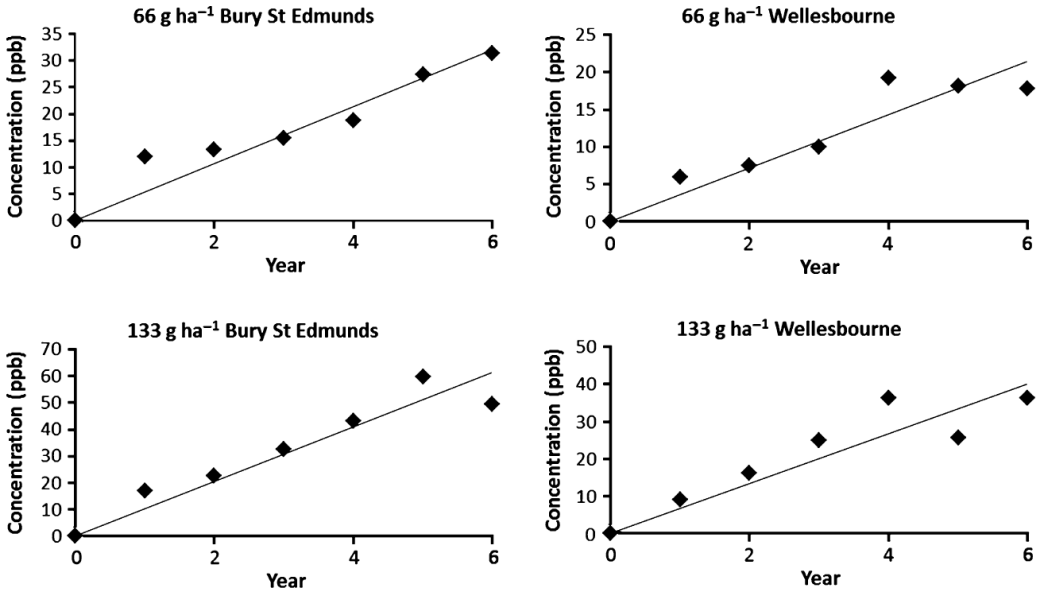

Figure 3. Levels of imidacloprid detected in soil into which treated winter wheat seeds were sown each autumn (1991–1996). Both study sites are in the east of England. Treatment rates were 66 and 133 g active ingredient ha^{-1} except in the first year, when it was 56 and 112 g, respectively. Data from Placke (1998a). Reproduced from Goulson (2013)

Since 2013 a number of studies have been published which have measured neonicotinoid levels in agricultural soils, have calculated DT_{50}s of neonicotinoids in real world soils and have measured accumulation in the soil using extensive field trials and field sampling. Data on field-realistic neonicotinoid samples are summarised in Table 3. Jones *et al*. (2014) measured neonicotinoid concentrations in centre and edge soil samples from 18 fields across 6 English counties. Samples were collected in the spring of 2013, prior to crop planting. Imidacloprid (range <0.09-10.7 ng/g), clothianidin (range <0.02-13.6 ng/g) and thiamethoxam (range <0.02-1.5 ng/g) were detected. Residues from the centre of the fields were higher than for the edge of the fields (average imidacloprid 1.62 against 0.76 ng/g, average clothianidin 4.89 against 0.84 ng/g and average thiamethoxam 0.40 against 0.05 ng/g). Neonicotinoids not previously applied in the previous three years (predominantly imidacloprid) were detected in 14 of the 18 fields. Limay-Rios *et al*. (2015) analysed soil samples collected in the springs of 2013 and 2014 from 25 agricultural fields in Ontario, Canada before crops were sown and found average concentrations of 3.45 ng/g of clothianidin and 0.91 ng/g thiamethoxam, with total average neonicotinoid concentration of 4.36 ng/g, similar to the findings of Jones *et al*. (2014).

Botías *et al*. (2015) analysed soil samples from seven winter-sown oilseed rape and five winter-sown wheat fields collected in summer 2013, 10 months after the crops were sown. Samples were collected from field centres (oilseed rape only) and field margins (oilseed rape and winter wheat).

Imidacloprid (range ≤0.07-7.90 ng/g), clothianidin (range 0.41-28.6 ng/g), thiamethoxam (range ≤0.04-9.75 ng/g) and thiacloprid (range ≤0.01-0.22 ng/g) were detected. Residues from the centre of the oilseed rape fields were higher than for the edge of the oilseed rape fields (average imidacloprid 3.03 against 1.92 ng/g, average clothianidin 13.28 against 6.57 ng/g, average thiamethoxam 3.46 against 0.72 ng/g and average thiacloprid 0.04 against ≤0.01 ng/g). Whilst these values are higher than those measured by Jones *et al*. (2014) and Limay-Rios *et al*. (2015) they are within an order of magnitude at their greatest difference.

Hilton *et al*. (2015) presented previously private data from 18 industry trials conducted between 1995 and 1998 for thiamethoxam applied to bare soils, grass and a range of crops (potatoes, peas, spring barley, winter barley, soybean, winter wheat and maize). Thiamethoxam DT_{50}s ranged between 7.1 and 92.3 days, with a geometric mean of 31.2 days (arithmetic mean 37.2 days). Across different application methods and environmental conditions, thiamethoxam declined to <10% of its initial concentration within one year. de Perre *et al*. (2015) measured soil clothianidin concentrations over 2011 to 2013, with clothianidin-treated maize sown in the springs of 2011 and 2013. Maize seeds were sown with seed dressings of 0.25 mg/seed and 0.50 mg/seed (Figure 4). At the lower concentration seed dressing, clothianidin residues in the soil ranged from approximately 2 ng/g before planting to 6 ng/g shortly after planting. At the higher seed dressing, clothianidin average residues ranged from 2 ng/g before planting to 11.2 ng/g shortly after planting. For the seed treatment of 0.5 mg/seed, de Perre *et al*. (2015) calculated a DT_{50} for clothianidin of 164 days. For the lower treatment of 0.25 mg/seed a DT_{50} of 955 days was calculated, though this model explained a much lower proportion of the data than the model for the 0.5 mg/seed data.

Table 3. Summary of studies published since 2013 that document neonicotinoid concentrations in agricultural soils.

Sample size (fields)	Country	Year(s) studied	Samples collected	Previously cropped with	Mean neonicotinoid concentration (ng/g)			Reference
					Imidacloprid	Clothianidin	Thiamethoxam	
28	USA	2012	Spring, pre-planting	Various	4.0	3.4	2.3	Stewart *et al.* (2014)
18	UK	2013	Spring	Various	1.62	4.89	0.4	Jones *et al.* (2014)
25	Canada	2013 and 2014	Spring, pre-planting	Maize		3.45	0.91	Limay-Rios *et al.* (2015)
7	UK	2013	Summer, with crop (10 months post planting)	Oilseed rape	3.03	13.28	3.46	Botías *et al.* (2015)
3	USA	2011 to 2013	Continuously	Maize and soybean		2.0-11.2		de Perre *et al.* (2015)
50	USA	2012 and 2013	Summer, with crop	Maize		7.0		Xu *et al.* (2016)
27	Canada	2012 to 2014	Summer, with crop	Oilseed rape		5.7		Xu *et al.* (2016)
35	Germany	2013	Autumn, pre-planting	Various		2.1		Heimbach *et al.* (2016)

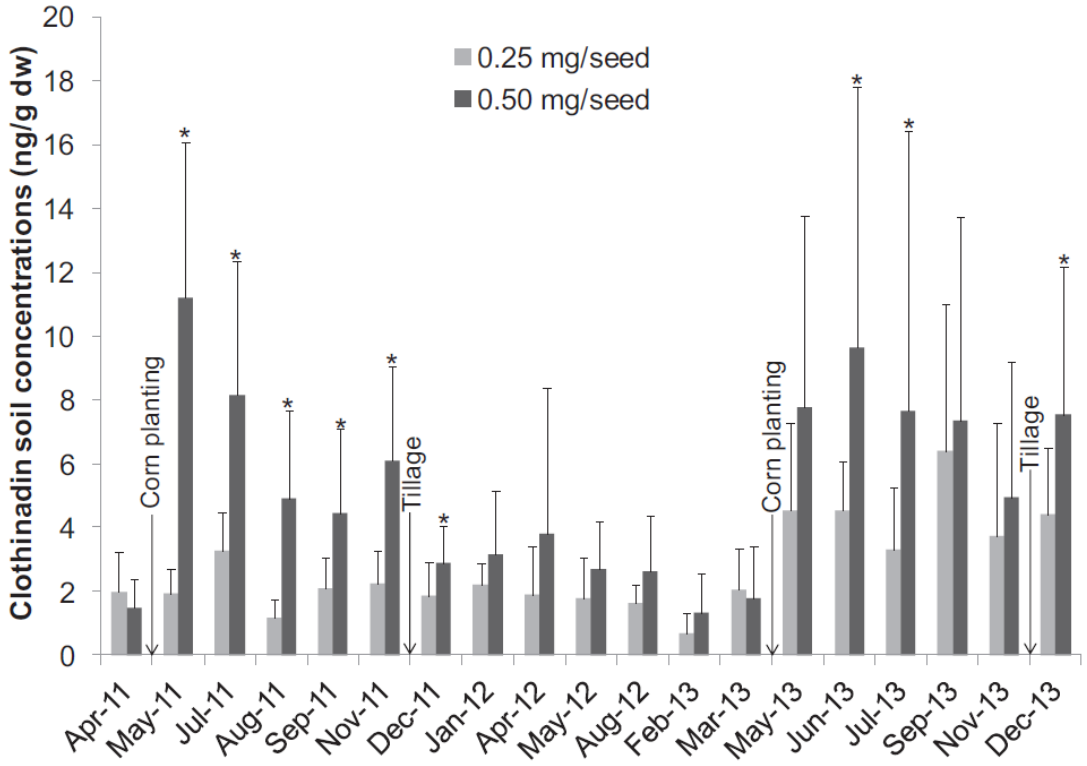

Figure 4. Mean clothianidin soil concentrations from 2011–2013 for each maize seed-coating rate (0.25 mg vs 0.50 mg of clothianidin/seed). Maize planting is presented because it represents the introduction of clothianidin in the field, and tillage events are also presented. Asterisks represent significantly different concentrations between seed-coating treatments for one sampling event (*t* test, $p \leq 0.05$, n=13 and n=17 for 0.25 mg/seed and 0.50 mg/seed, respectively, from April 2011 to March 2013; n=15 for both seed treatment rates since May 2013). Reproduced from de Perre *et al*. (2015). Note – untreated soybeans were sown in 2012

Schaafsma *et al*. (2016) calculated clothianidin DT_{50}s in maize fields in Ontario, Canada in 2013 and 2014, including data published in Schaafsma *et al*. (2015). Soil samples were collected from 18 fields in the spring before crop planting. Average neonicotinoid concentrations (clothianidin and thiamethoxam aggregated) were 4.0 ng/g in 2013 and 5.6 ng/g in 2014. Using the observed residues and the recharge rate applied at planting via treated maize seeds, fields studied in 2013 had an estimated DT_{50} of 0.64 years (234 days) and fields studied in 2014 had an estimated DT_{50} of 0.57 years (208 days). For fields studied in both years the DT_{50} was calculated at 0.41 years (150 days). Schaafsma *et al*. conclude that, at current rates of neonicotinoid application in Canadian maize cultivation, soil residues of neonicotinoids will plateau at under 6 ng/g.

Using the same method, Schaafsma *et al*. also calculated imidacloprid DT_{50} using the data from Placke (1998a; 1998b; Table 4), producing a very similar DT_{50} of 0.57 years (208 days). Schaafsma *et al*. argue the Placke studies show neonicotinoid concentrations plateauing after repeated use of neonicotinoid seed treatments. However, observed levels were high, so even if plateauing occurred after six years the average concentration of neonicotinoids in the soil would be around 30 ng/g (Table 4).

Table 4. Observed concentrations of imidacloprid and estimated dissipation rates (half-life) in orchard soil in Germany and in winter barley fields in the United Kingdom. Data taken from Placke (1998a; 1998b). Half-life calculated iteratively by varying the half-life incrementally until the predicted and measured values are equal. Reproduced from Schaafsma *et al*. (2016)

Field	Observed imidacloprid concentration (ng/g)	Half-life (years)
Barley_66_1	31.4	0.74
Barley_133_1	49.4	0.63
Barley_66_2	17.8	0.53
Barley_133_2	36.3	0.54
Orchard_1	23.3	0.48
Orchard_2	34.5	0.59
Orchard_3	23.1	0.47
Mean ± Standard Error	30.8	0.57 ± 0.04

Xu *et al*. (2016) analysed soil samples from 50 maize producing sites in the Midwestern USA across 2012 and 2013 and soil samples from 27 oilseed rape producing sites in western Canada across 2012, 2013 and 2014. Samples were collected after planting, but it is not clear exactly how long after. Average clothianidin soil concentration at Midwestern maize producing sites with a range of 2-11 years of planting clothianidin-treated seeds was 7.0 ng/g with a 90th percentile concentration of 13.5 ng/g. Xu *et al*. argue that this average is similar to the theoretical soil concentrations (6.3 ng/g) expected from a single application of 0.25 mg clothianidin-treated maize seed. Clothianidin levels in soil appear to plateau after 4 years (Figure 5a), but the sample size for sites with a history of more than four years is much smaller than the number of sites with a history of under four years of use. At the oilseed rape producing sites, average clothianidin concentrations were 5.7 ng/g with the 90th percentile concentration of 10.2 ng/g. This is also similar to the theoretical soil concentration (6.7 ng/g) from a single application of oilseed rape seed treated at 4 g clothianidin per kg of seed (Figure 5b). The oilseed rape sites do not have the same history of clothianidin use but levels appear to be fairly stable over the four years of applications. For reference, 10 g clothianidin per kg of oilseed rape seed is the most common dosage rate in recent field trials (the Elado seed dressing, Section 3.1.2.1).

The current body of evidence shows that detectable levels of neonicotinoids are found in agricultural soils over a year after treated seeds were planted, clearly demonstrating a level of neonicotinoid persistence greater than the annual agricultural cycle. Moreover, neonicotinoids known not to have been recently used can still be present in soils several years after the last application date. The available data suggest that, whilst a proportion of the total neonicotinoids applied can and do persist in the soil from year to year, there appears to be sufficient degradation that means they do not continue to accumulate indefinitely (bioaccumulation) but instead plateau after 2-6 years of repeated application. However, these studies also show that overall, the annual sowing of neonicotinoid-treated seed results in chronic levels of neonicotinoid soil contamination in the range of 3.5-13.3 ng/g for clothianidin and 0.4-4.0 ng/g for thiamethoxam which will act as a constant source of exposure for soil dwelling organisms, and for neonicotinoid transport into the wider environment.

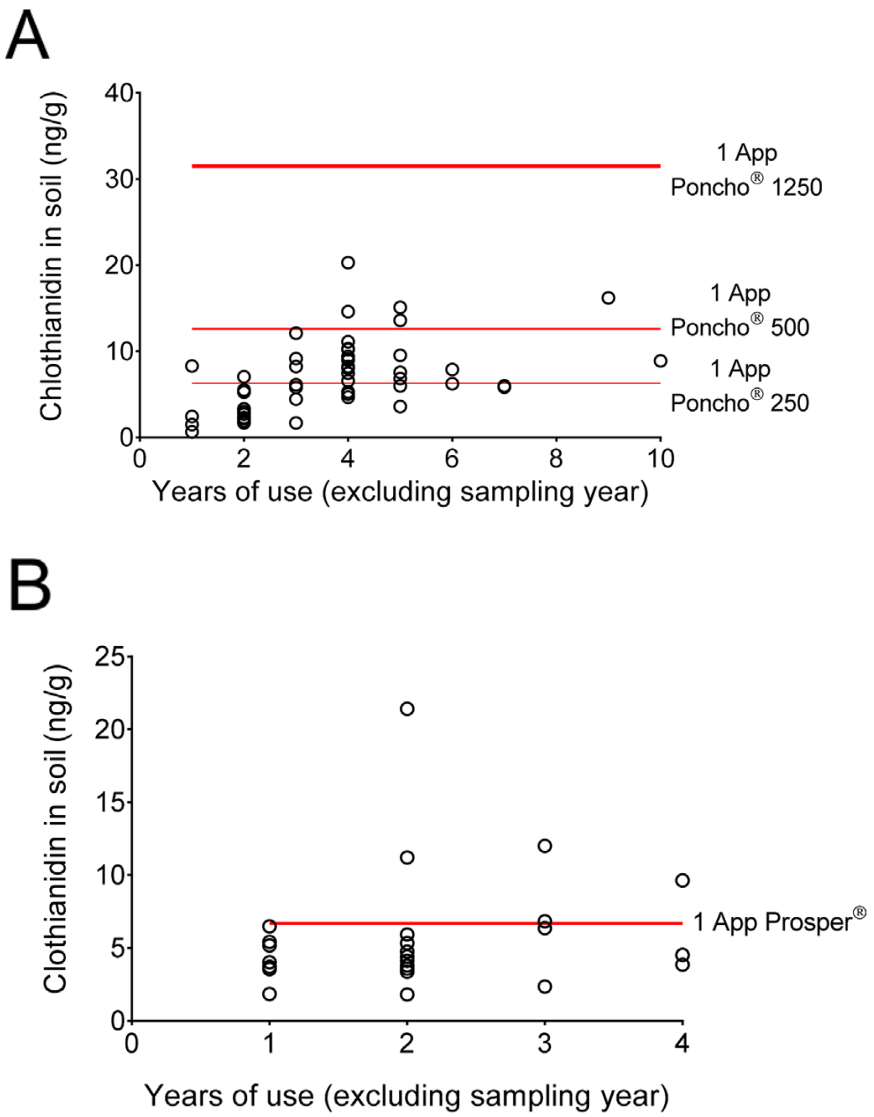

Figure 5. (a) Comparison of clothianidin concentrations in soil with years of clothianidin use for maize producing sites. Red lines indicate theoretical concentrations from a single application of clothianidin-treated seeds for three formulations. (b) Comparison of clothianidin concentrations in soil with years of clothianidin use for oilseed rape producing sites. Red lines indicate theoretical concentrations from a single application of clothianidin-treated seeds. Reproduced from Xu *et al*. (2016)

2.2.2 Persistence of neonicotinoids in water and transport mechanisms for contamination of aquatic systems

Neonicotinoids are soluble in water, a property that is necessary for them to function effectively as systemic pesticides which can be taken up by crops. The solubility of neonicotinoids depends on local conditions such as ambient temperature, water pH and the form that the neonicotinoids are applied in, such as granules, as a seed dressing or as dust drift from seed drilling (Bonmatin *et al*. 2015). Under standard conditions (20°C, pH 7), neonicotinoid solubility varies between 184 (moderate) to 590,000 (high) mg/L for thiacloprid and nitenpyram respectively (PPDB 2012). The values for clothianidin, imidacloprid and thiamethoxam are 340 (moderate), 610 (high) and 4,100 (high) mg/L respectively. In contrast, Fipronil has a solubility 2-3 orders of magnitude lower at 3.78 mg/L under the same conditions.

Because of the high solubility of neonicotinoids in water, concerns were raised that neonicotinoids might be passing into water bodies in the wider environment and that this may pose a risk for aquatic organisms. Available evidence to 2015 was reviewed by Bonmatin *et al*. 2015 and Morrissey *et al*. 2015. In general, under simulated environmental conditions, neonicotinoids readily leach into water (Gupta *et al*. 2008; Tisler *et al*. 2009). Neonicotinoids have been identified passing into waterways through several different routes. These include direct leaching into ground water and subsequent discharge into surface water, decay of treated plant material in waterways and direct contact from dust from the drilling of treated seed, treated seeds or spray drift into water bodies (Krupke *et al*. 2012; Nuyttens *et al*. 2013). The majority of this contamination is thought to occur from run-off after acute rainfall (Hladik *et al*. 2014; Sánchez-Bayo and Hyne 2014; Main *et al*. 2016). Run-off will be particularly severe where soil organic content is low and on steep slopes (Goulson 2013).

Whilst rainfall during or shortly after the planting season appears to be the main mechanism for neonicotinoid transport into waterbodies, detectable levels of neonicotinoids can be found in prairie wetlands in Canada during early spring before the planting season (Main *et al*. 2014). Main *et al*. (2016) analysed snow, spring meltwater, particulate matter and wetland water from 16 wetland sites adjacent to agricultural fields that had been used to grow either oilseed rape (canola, treated with neonicotinoids) or oats (not treated). They found that all meltwater samples were contaminated with clothianidin and thiamethoxam in the range of 0.014-0.633 µg/L (1 µg/l = 1 ppb). Levels of contamination in meltwater were higher adjacent to fields planted with neonicotinoid-treated oilseed rape in the previous year (mean 0.267 µg/L). However, fields planted with non-neonicotinoid-treated oats in the previous year still showed similar levels of contamination (mean 0.181 µg/L). Treated oilseed rape and untreated oats are frequently rotated from year to year (Main *et al*. 2014), and the small difference in neonicotinoid concentration in meltwater from fields previously planted with treated and untreated crops suggests the persistence of neonicotinoids in the soil over multiple years (see Section 2.2.2). The findings of this study suggest that neonicotinoid active ingredients previously bound to soil particles are eroded during spring freeze-thaw cycles. The demonstration of this route of transport in addition to general rainfall suggests a more chronic transport of neonicotinoids into water bodies outside the main period of crop planting.

The effect of neonicotinoids on aquatic habitats will depend on their persistence therein. Field and laboratory studies investigating the breakdown of imidacloprid, thiamethoxam and clothianidin in water report half-lives of minutes to several weeks depending on the conditions, several of which are not field-realistic (see Anderson *et al*. 2015; Lu *et al*. 2015). There has been no formal review of the degradation of neonicotinoids in water and existing literature consists of published peer review studies and grey literature government studies, all using different methodologies. However, a

number of studies have attempted to measure neonicotinoid degradation under field-realistic conditions. Peña *et al*. (2011) measured degradation of thiamethoxam in wastewaters and sewage in Spain finding maximum absorption at 250-255 nm, suggesting high susceptibility to direct photolysis from natural light. In control waters thiamethoxam half-life was found to be 18.7 hours (Peña *et al*. 2011). Under natural light in rice paddies in Japan, imidacloprid had a half-life of 24.2 hours (Thuyet *et al*. 2011). Under natural light in Switzerland von Gunten *et al*. (2012) reported a half-life of 2 hours for imidacloprid and 254 hours for acetamiprid. Under laboratory conditions, Lu *et al*. (2015) measured half-lives for five neonicotinoids under differing conditions to mimic the seasonal change found in Canada (Table 5). They found 7-8-fold variation in the rate of neonicotinoid photolysis due to the variation in light levels across the season. The results are broadly similar to previously published studies with nitro-substituted neonicotinoid half-lives in the region of <1-3 days depending on light levels.

Table 5. Estimated photolysis and half-lives ($t_{1/2E}$) (days) for neonicotinoid pesticides in surface water at 50°N latitude for spring, summer, autumn and winter by sunlight on clear days. Reproduced from Lu *et al*. (2015)

Compound	Spring	Summer	Autumn	Winter
Thiamethoxam	0.32	0.20	0.63	1.49
Clothianidin	0.53	0.35	1.23	3.31
Imidacloprid	0.36	0.24	0.83	2.22
Acetamiprid	16.5	9.67	29.7	67.9
Thiacloprid	14.3	8.75	26.6	60.3

In addition to these peer reviewed studies, Lu *et al*. drew comparison with European Commission regulatory studies on neonicotinoid compounds (EC 2004a; EC 2004b; EC 2005; EC 2006). The European Commission studies found half-lives in water of 3.3 hours for clothianidin, 2.3-3.1 days for thiamethoxam, 34 days for acetamiprid and 80 days for thiacloprid. The exact methodology used in these studies is unclear and inconsistent (see Lu *et al*. 2015 discussion). Nevertheless, the overall trend is consistent with the cyano-substituted neonicotinoids (acetamiprid and thiacloprid) taking 1-2 orders of magnitude longer to degrade than the nitro-substituted neonicotinoids (thiamethoxam, clothianidin and imidacloprid). The short half-lives of these three, most widely used neonicotinoids suggests that, under field conditions, free neonicotinoids in surface waters should be broken down by natural light in a matter of hours or days. However, local environmental conditions can affect this, with increasing turbidity increasing neonicotinoid persistence. Moreover, in mesocosm experiments, photolysis of thiamethoxam was found to be negligible at depths of greater than 8 cm (Lu *et al*. 2015). This significant light attenuation through the water column suggests that neonicotinoids may be shielded from photolysis even in shallow waterbodies. In waterbodies such as groundwater that are not exposed to light there will be no photolysis. In these circumstances clothianidin is persistent and has the potential to accumulate over time (Anderson *et al*. 2015), though empirical data demonstrating this is lacking.

2.2.3 Levels of neonicotinoid contamination found in waterbodies

The most comprehensive review of levels of neonicotinoid contamination in global surface waters was conducted by Morrissey *et al*. (2015), though see also Anderson *et al*. (2015). Morrissey reviewed reported average and peak levels of neonicotinoid contamination from 29 studies from 9

countries between 1998 and 2013. The water bodies studied included streams, rivers, drainage, ditches, groundwater, wetlands, ponds, lakes, puddled surface waters and runoff waters. Study systems were adjacent to or receiving run-off water from agricultural land. From this dataset (Figure 6), the geometric mean for average surface water neonicotinoid concentration was 0.13 µg/L (=0.13 ppb, n=19 studies) and the geometric mean for peak surface water concentration was 0.63 µg/L (=0.63 ppb, n=27 studies). Because most monitoring schemes use spot sampling, they are likely to underreport the true maximum concentrations that occur immediately after maximum periods of neonicotinoid influx (Xing *et al*. 2013). As peak concentrations are often found after acute events such as heavy rainfall, this limits our understanding of the true average and maximum concentrations that are found in waterbodies.

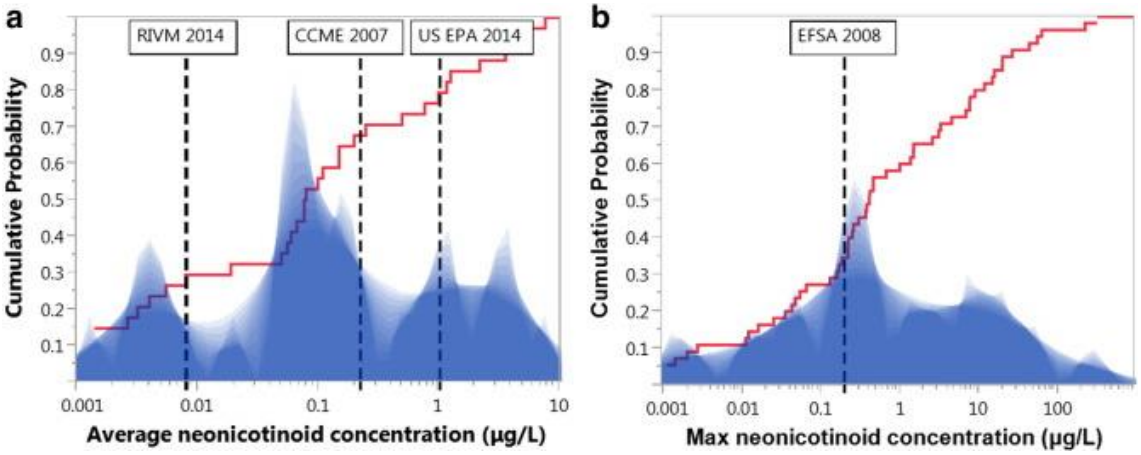

Figure 6. Shadow histogram of a) average and b) maximum individual neonicotinoid concentrations (log scale, µg/L) reported from water monitoring studies. Overlaid is the cumulative distribution probability (red ascending line) using all available surface water monitoring data showing proportion of data below any given neonicotinoid concentration. Vertical dashed lines illustrate multiple ecological quality reference values set for average imidacloprid water concentrations (RIVM 2014: 0.0083 µg/L, CCME 2007: 0.23 µg/L and US EPA 2014: 1.05 µg/L) or for maximum imidacloprid water concentrations (EFSA, 2008: 0.2 µg/L). Reproduced from Morrissey *et al*. 2015

Since Morrissey *et al*. (2015) was published, a number of studies have become available documenting broadly similar neonicotinoid contamination levels in a wide range of aquatic environments. At a small scale in agricultural regions, Schaafsma *et al*. (2015) measured concentrations in surface water (puddles and ditches) in and around 18 maize fields in Ontario, Canada. They found arithmetic mean residues of 0.002 µg/L of clothianidin (maximum = 0.043 µg/L) and 0.001 µg/L of thiamethoxam (maximum = 0.017 µg/L). In Iowa, USA, Smalling *et al*. (2015) assessed six wetlands surrounded by agricultural land and found arithmetic mean neonicotinoid concentrations of 0.007 µg/L (maximum 0.070 µg/L). Away from agricultural land, Benton *et al*. (2016) measured concentrations in mountain streams in the southern Appalachians, USA, where eastern hemlock forests are treated with imidacloprid to control pests. Average concentrations of 0.067 µg/L of imidacloprid (maximum = 0.379 µg/L) were found in seven of the 10 streams investigated. de Perre *et al*. (2015) measured concentrations of clothianidin in groundwater below fields of treated maize. Data on average concentrations are not available but concentrations peaked at 0.060 µg/L shortly after crop planting.

At a wider scale, Qi *et al*. (2015) and Sadaria *et al*. (2016) measured concentrations in wastewater treatment plants. Qi *et al*. (2015) recorded imidacloprid at concentrations between 0.045-0.100 µg/L in influent and 0.045-0.106 µg/L in effluent at five waste water treatment plants in Beijing, China with no data available on arithmetic mean concentrations. Sadaria *et al*. (2016) assessed influent and effluent wastewater at 13 conventional waste water treatment plants around the USA. For influent, imidacloprid was found at arithmetic mean concentrations of 0.061 µg/L, acetamiprid at 0.003 µg/L and clothianidin at 0.149 µg/L. For effluent, imidacloprid was found at concentrations of 0.059 µg/L, acetamiprid at 0.002 µg/L and clothianidin at 0.070 µg/L.

Two nationwide surveys for neonicotinoids were also published. Hladik and Kolpin (2016) measured neonicotinoid concentrations in 38 streams from 24 US states plus Puerto Rico. Five neonicotinoids (acetamiprid, clothianidin, dinotefuran, imidacloprid, thiamethoxam) were recorded with at least one compound found in 53% of sampled streams, with an arithmetic mean contamination of 0.030 µg/L and median contamination of 0.031 µg/L. Thiacloprid was not recorded. Székács et al. (2015) conducted a nationwide survey of Hungarian watercourses, finding clothianidin at concentrations of 0.017-0.040 µg/L and thiamethoxam at concentrations of 0.004-0.030 µg/L.

Across all studies, the highest levels of neonicotinoid contamination were found in agricultural areas. In the most comprehensive nationwide survey of streams across the USA conducted between 2012 and 2014, levels of clothianidin and thiamethoxam contamination (the now dominant agricultural neonicotinoids) were significantly positively correlated with the proportion of the surrounding landscape used for crop cultivation (Hladik and Kolpin 2016). The most acute levels of neonicotinoid contamination in agricultural areas are reported from surface water in the immediate vicinity of cultivated crops. Puddles adjacent to fields planted with neonicotinoid-treated maize seeds were found to contain maximum concentrations of 55.7 µg/L clothianidin and 63.4 µg/L thiamethoxam in Quebec, Canada (Samson-Robert *et al*. 2014). Surface water in the Netherlands had imidacloprid concentrations up to 320 µg/L (van Dijk *et al*. 2013) and transient wetlands found in intensively farmed areas of Texas had thiamethoxam and acetamiprid concentrations of up to 225 µg/L (Anderson *et al*. 2013). In Hungary, the highest neonicotinoid concentrations of 10-41 µg/L were found in temporary shallow waterbodies after rain events in early summer (Székács et al. 2015). More generally, watercourses draining agricultural fields had high levels of neonicotinoids after rainfall in Canada, the USA and Australia (Hladik *et al*. 2014, Sánchez-Bayo and Hyne 2014). Where repeated sampling of the same site has been carried out, the highest neonicotinoid concentrations have been found in early summer and are associated with rainfall during the planting season (Main *et al*. 2014; Hladik *et al*. 2014). Hladik and Kolpin (2016) measured neonicotinoid concentrations in three agriculturally affected streams in Maryland and Pennsylvania and found peak levels after rain events during the crop planting season in May, though this could not be formally statistically analysed due to low sample size (Figure 7).

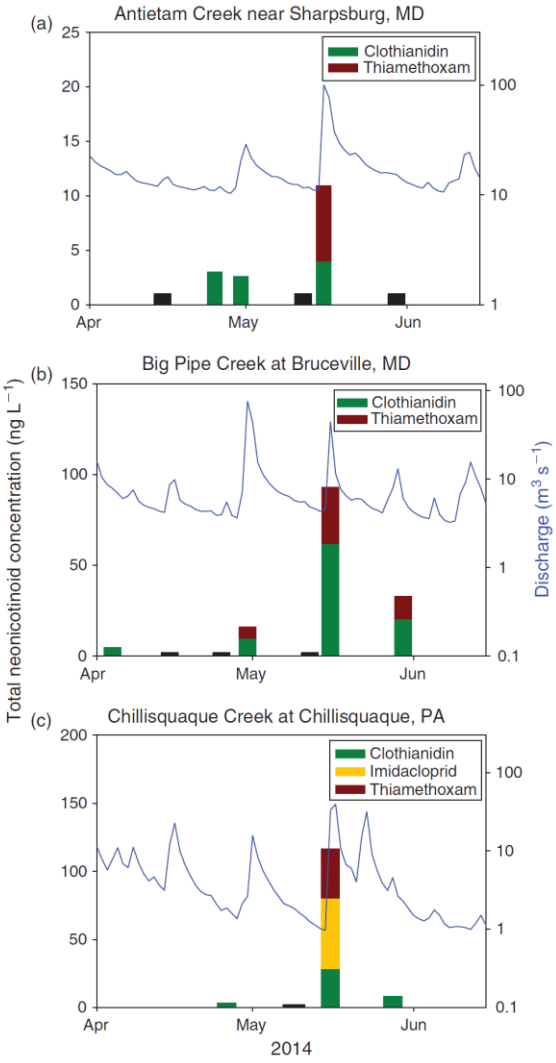

Figure 7. Concentrations of clothianidin, imidacloprid and thiamethoxam and the corresponding stream discharge at three sites in the Chesapeake Bay area sampled in 2014. Black bars represent samples where no neonicotinoids were detected. Reproduced from Hladik and Kolpin (2016)

In addition to agricultural run-off, urban areas also contribute towards neonicotinoid contamination of waterbodies. Whilst the use of imidacloprid as an agricultural pesticide has declined it is still found in a wide range of domestic products and veterinary treatments for pets (Goulson *et al.* 2013). Hladik and Kolpin (2016) continuously monitored neonicotinoid levels in Slope Creek, a stream surrounded by a largely urban catchment (39% urban) and the Chattahoochee river which includes the drainage of Slope Creek and overall has a lower proportion of urbanisation (9%). Imidacloprid was the dominant neonicotinoid found, present in 87% of the 67 collected samples (Figure 8). Dinotefuran and acetamiprid were less frequently encountered. Unlike in the studied watercourses draining agricultural land, no significant relationship was seen with stream flow in either Slope Creek or the Chattahoochee river. Hladik and Kolpin suggest that this may be because, unlike for the planting period of arable crops, there is no distinct period of use for domestic imidacloprid in an urbanised catchment. No clothianidin or thiamethoxam were detected, probably because neither catchment contained cultivated crops.

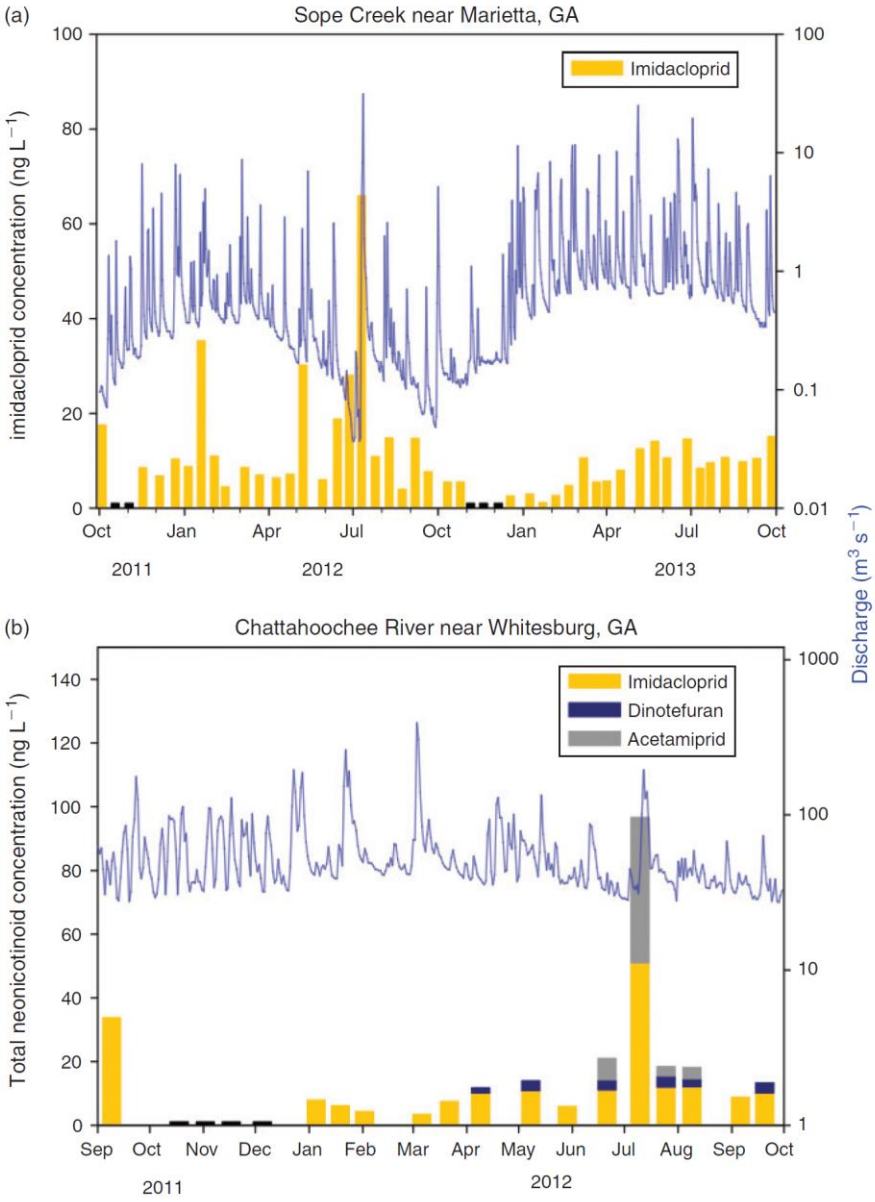

Figure 8. (a) Concentrations of imidacloprid and the corresponding stream discharge from October 2011 to October 2013 for Sope Creek (a largely urban catchment) and (b) Concentrations of imidacloprid, dinotefuran and acetamiprid along with the corresponding stream discharge from September 2011 to September 2012 for Chattahoochee River. Black bars represent samples where no neonicotinoids were detected. Reproduced from Hladik and Kolpin (2016)

2.2.4 Risk of exposure from and uptake of neonicotinoids in non-crop plants

Since neonicotinoids are water soluble and can persist in soils and waterbodies there is the possibility that they may be taken up by any wild plants present nearby. In April 2013 little empirical data was available documenting neonicotinoid contamination of wild plants. The EFSA reports considered that uptake of neonicotinoids by wild weed plants and subsequent exposure would be negligible, as weeds will not be present in the field when the crop is sown and considerable uptake via the roots would be unlikely as the substance is concentrated around the treated seed. No comment was made on the potential uptake of neonicotinoids by other wild plants in the agricultural environments. In the single study available in 2013, Krupke *et al*. (2012) found that dandelions *Taraxacum agg.* growing near to fields planted with neonicotinoid-treated maize contained between 1.1 to 9.4 ng/g clothianidin and <1.0 (LOD) to 2.9 ng/g thiamethoxam. They did not assess whether the pesticides were found in the pollen or nectar. It was not clear whether the contamination came from neonicotinoid dust settling on the external surface of the plants or if the neonicotinoids had been directly taken up through the roots, in which case we would expect them to be present inside all plant tissues, pollen and nectar. Since April 2013, a number of studies have been published which demonstrate that neonicotinoids are frequently taken up in wild plants surrounding agricultural fields (Table 6).

Botías *et al*. (2015) collected pollen and nectar from wildflowers growing in field margins adjacent to agricultural fields planted with neonicotinoid-treated oilseed rape and wheat. Pollen samples from 54 wild flower species were collected. Thiamethoxam, imidacloprid and thiacloprid were all detected. Thiamethoxam was the most frequently encountered neonicotinoid and levels were highly variable with the highest concentrations found in *Heracleum sphondylium* at 86 ng/g and *Papaver rhoeas* at 64 ng/g. There was substantial variation in the levels of contamination in the same wildflower species found in different field margins. Average levels of total neonicotinoid contamination in wildflower pollen were significantly higher in margins adjacent to treated oilseed rape (c. 15 ng/g) than for margins adjacent to treated wheat (c. 0.3 ng/g). Levels of neonicotinoids were much lower in wild plant nectar. Only thiamethoxam was detected at average levels of 0.1 ng/g in wild flowers adjacent to oilseed rape fields and <0.1 ng/g adjacent to wheat fields.

Botías *et al*. (2015) is the only available study which has specifically measured neonicotinoid concentrations in pollen and nectar directly taken from wild plants growing in close proximity to neonicotinoid-treated crops. Mogren and Lundgren (2016) assessed neonicotinoid concentrations in the nectar of five wild flower species sown as part of pollinator conservation measures which were located adjacent to neonicotinoid-treated maize. This was achieved by collecting honeybees seen to visit these flowers for nectar and extracting the contents of their crop for neonicotinoid residue analysis. Honeybees generally have a very high fidelity to visiting the same flower species on a single forage flight so the authors assumed that the nectar was representative of that particular species. Average clothianidin concentrations found in this nectar ranged between 0.2 and 1.5 ng/g, with significant differences found between wild plant species. Mogren and Lundgren (2016) also tested the foliage of seven wildflower species for neonicotinoid residues directly. There was high variability in clothianidin uptake between and within plant species (Figure 9). Sunflowers *Helianthus annuus* accumulated the highest levels with concentrations of 0-81 ng/g, with buckwheat *Fagopyrum esculentum* and phacelia *Phacelia tanacetifolia* accumulating lower levels at 0-52 ng/g and 0-33 ng/g respectively. Similarly high levels of variation were found by Botías *et al*. (2016) who sampled the foliage of 45 species of wild plant in field margins adjacent to treated oilseed rape crops. Average total neonicotinoid contamination was 10 ng/g, with the highest levels seen in creeping thistle *Cirsium arvense* of 106 ng/g of thiamethoxam. Pecenka and Lundgren (2015) looked specifically at

Table 6. Summary of studies published since 2013 that document mean neonicotinoid residues in wild plant tissues, pollen and nectar in plants growing close to neonicotinoid-treated agricultural crops. The results of Krupke *et al*. (2012) are included for reference

Sample size	Vegetation adjacent to	Samples collected	Sample type	Mean neonicotinoid concentration (ng/g)				Reference
				Thiamethoxam	Clothianidin	Imidacloprid	Thiacloprid	
43	Oilseed rape	May-June 2013	Pollen	14.81		0.56	<0.04	Botías *et al*. (2015)
55	Wheat	May-June 2013	Pollen	0.14		<0.16	<0.04	Botías *et al*. (2015)
24	Oilseed rape	May-June 2013	Nectar	0.10				Botías *et al*. (2015)
8	Wheat	May-June 2013	Nectar	<0.10				Botías *et al*. (2015)
33	Maize	Summer 2014 and 2015	Nectar *		0.2-1.5			Mogren and Lundgren (2016)
40	Maize	June 2014	Foliage		0.4			Pecenka and Lundgren (2015)
50	Maize	July 2014 (1 month after planting)	Foliage		0.69			Pecenka and Lundgren (2015)
100	Oilseed rape	May-June 2013	Foliage	8.71	0.51	1.19		Botías *et al*. (2016)
375	Maize	Summer 2014 and 2015	Foliage		0.5-13.5**			Mogren and Lundgren (2016)
6	Maize	Summer 2011	Complete flower	1.15	3.75			Krupke *et al*. (2012)
78	Various	Summer 2012	Complete flower	7.2	1.4	1.1		Stewart *et al*. (2014)
7	Oilseed rape	April-May 2013 (2 days after sowing)	Complete flowers and foliage		1.2			Rundlöf *et al*. (2015)
8	Oilseed rape	April-June 2013 (2 weeks after sowing)	Complete flowers and foliage		1.0			Rundlöf *et al*. (2015)

* Mogren and Lundgren (2016) sampled honeybees foraging on wild plants and directly extracted nectar from their crop. See main body of text for further discussion

** Range of concentrations, data on mean concentrations not available

clothianidin concentrations in milkweed *Asclepias syriaca* in field margins adjacent to clothianidin-treated maize. Levels were lower than the previous two studies, with mean levels of 0.58 ng/g with a maximum concentration of 4.02 ng/g.

Whilst not looking at specific concentrations in pollen, nectar or foliage, Stewart *et al*. (2014) and Rundlöf *et al*. (2015) found total mean neonicotinoid concentrations of 10 ng/g and 1ng/g respectively in whole wild flower samples collected around neonicotinoid-treated fields. As discussed in Section 2.1.3, these levels may have been a direct result of neonicotinoid-contaminated dust drift onto surrounding vegetation and do not in and of themselves demonstrate uptake of neonicotinoids from contaminated soil and/or water.

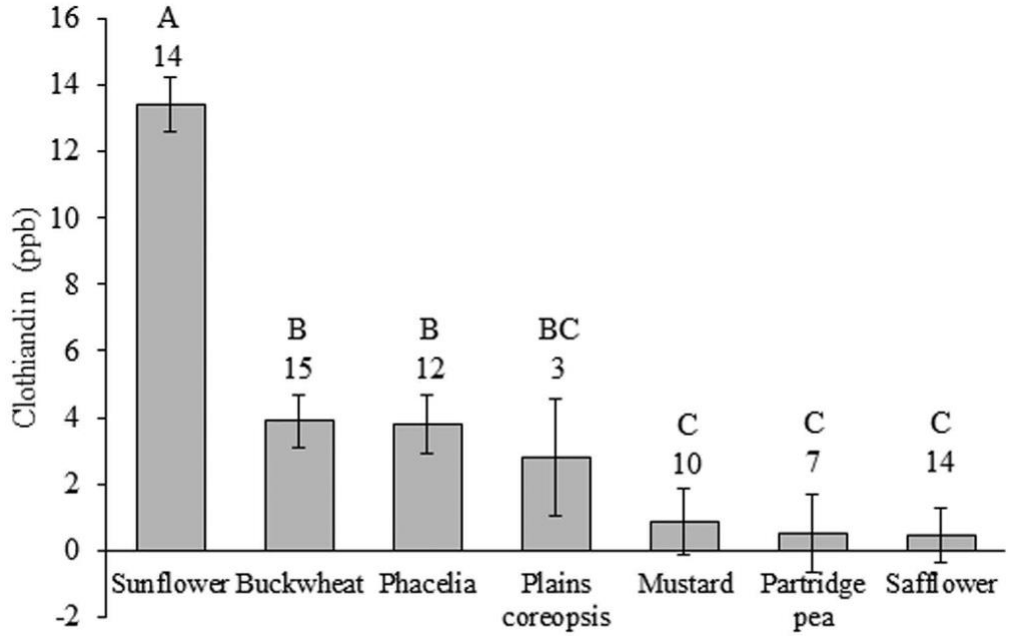

Figure 9. Concentrations of clothianidin in leaf tissues (mean±SE). Letters above bars show significant differences between plant species and numbers represent the number of site-years in which a particular species was analysed. Reproduced from Mogren and Lundgren (2016).

Across all studies published since 2013, average levels of neonicotinoids in wild plants range from 1.0-7.2 ng/g in whole flower samples, 0.4-13.5 ng/g in foliage samples, <0.1-1.5 ng/g in nectar samples and <0.04 to 14.8 ng/g in pollen samples. Due to the limited number of studies available, it is difficult to make a comparison with levels in directly treated crop plants. However, they are broadly comparable to the levels found in the treated crop itself (see Section 2.1.1)

In 2013 it was known that honeybees collected neonicotinoid contaminated pollen from crop plants, but the extent to which this was diluted by uncontaminated pollen from wild plants was unknown. Krupke *et* al. (2012) found levels of clothianidin and thiamethoxam in honeybee-collected pollen that ranged between 0 and 88 ng/g, with the proportion of pollen collected from maize (the main treated crop in their study area) also varying substantially between 2.6 and 82.7%. There was no correlation between the proportion of maize pollen collected and the total neonicotinoid concentration. Given the uncertainty over the contamination of wild plants it was not clear what long term chronic neonicotinoid exposure was from pollen or nectar over a whole season. A number

of studies have attempted to quantify the levels of neonicotinoids in bee-collected pollen and, through microscopic identification of the constituent pollen grains, to identify the major source of neonicotinoid contamination throughout the season. Most of these studies have used honeybee-collected pollen as the model, as pollen traps are easy to fit to apiaries that can be moved into targeted locations.

Studies are summarised in Table 7. Most of these studies used honeybees, placing apiaries out next to neonicotinoid-treated and untreated crops. As summarised in Section 2.1.1, bees placed near to treated crops collected pollen with higher concentrations of neonicotinoids (Cutler *et al*. 2014; Rundlöf *et al*. 2015; Long and Krupke 2016; Rolke *et al*. 2016). The highest levels of acute contamination are found when a large proportion of crop pollen is collected. Pohorecka *et al*. (2013) found average clothianidin concentrations of 27.0 ng/g in pollen samples (73.7% wildflower pollen) collected from apiaries adjacent to treated maize fields. Rundlöf *et al*. (2015) found average clothianidin concentrations of 13.9 ng/g in pollen samples (37.9% wildflower pollen) collected from apiaries adjacent to treated oilseed rape fields. Apiaries adjacent to untreated oilseed rape fields collected pollen consisting of 47.4% wildflower pollen with no detectable levels of neonicotinoids (<0.5 ng/g).

Where bees collect a greater proportion of wildflower pollen, neonicotinoid concentrations are lower. Botías *et al*. (2015) measured neonicotinoid concentrations in pollen during the peak flowering period of oilseed rape and two months after this period. During peak flowering, honeybees collected 91.1% of their pollen from wildflowers and 8.9% from oilseed rape, with a total neonicotinoid concentration of 3.09 ng/g. In the later period, 100% of their pollen was collected from wildflowers, with a total neonicotinoid concentration of 0.20 ng/g. Cutler *et al*. (2014) also sampled honeybee pollen from apiaries adjacent to treated and untreated oilseed rape for a two week period in July during peak flowering. Honeybees collected low levels of crop pollen and higher levels of neonicotinoid contamination were found adjacent to treated fields (9.0% wildflower pollen week 1 to 45.2% week 2, 0.84 ng/g) than untreated fields (15.1% wildflower pollen week 1 to 62.5% week 2, 0.24 ng/g). Long and Krupke (2016) collected data over a longer period of time, from May to September, covering the flowering period of maize, the flowering crop at their study sites. At all sites a high proportion of pollen was collected from wildflowers. Average neonicotinoid concentrations were lowest at non-agricultural sites (93.9% wildflower pollen, 0.047 ng/g), higher at untreated agricultural sites (95.8% wildflower pollen, 0.078 ng/g) and highest at treated agricultural sites (95.3% wildflower pollen, 0.176 ng/g). Alburaki *et al*. (2015 and 2016) found low levels of neonicotinoids when honeybees collected predominantly wildflower pollen, with none detected in loads of 99% wildflower pollen and average neonicotinoid concentrations of 0.04 ng/g in loads of 93.5% wildflower pollen.

Only two studies are available which measured neonicotinoid concentrations in bumblebee collected pollen and quantified the proportion of pollen collected from wildflowers. Cutler and Scott-Dupree (2014) placed out *Bombus impatiens* nests next to neonicotinoid-treated and untreated maize fields. Bumblebees collected a very low proportion of their pollen from maize, less than 1%, in contrast to honeybees which can collect large quantities of maize pollen during its flowering period (Krupke *et al*. 2012; Pohorecka *et al*. 2013, though see Alburaki *et al*. 2015; 2016; Long and Krupke 2016). Levels of neonicotinoid residues were low, at <0.1 ng/g by untreated fields and 0.4 ng/g by treated fields. In contrast, David *et al*. (2016) placed out five *B. terrestris* nests adjacent to treated oilseed rape fields, a crop with pollen attractive to bumblebees. Pollen was sampled from nest stores at the end of June. Bumblebees collected an average of 68.1% wildflower pollen and 31.9% oilseed rape pollen.

Table 7. Summary of studies published since 2013 that document mean neonicotinoid residues in pollen collected by free-flying bees. The results of Krupke *et al*. (2012) and studies described in Section 2.1.1 are included for reference. SS = spring-sown, WS = winter-sown, US = unclear sowing date

Species	Sample type	Samples collected	Nest location	Proportion of pollen collected from wildflowers	Mean total neonicotinoid concentration (ng/g)	Reference
Apis mellifera	Pollen	Summer 2011	Adjacent to treated maize fields	55.5	9.71	Krupke *et al*. (2012)
Apis mellifera	Pollen	July to August 2011 and July 2012	Adjacent to treated maize fields	73.7	27.0	Pohorecka *et al*. (2013)
Apis mellifera	Pollen	April to May and June to September 2012	Adjacent to treated fields (various crops, 180 m mean distance)	*Data not collected*	<1.0 (*limit of detection*)	Stewart *et al*. (2014)
Apis mellifera	Pollen	First two weeks of July 2012	Located in untreated SS OSR fields	15.1 (week 1) to 62.5 (week 2)	0.24	Cutler *et al*. (2014)
Apis mellifera	Pollen	First two weeks of July 2012	Located in treated SS OSR fields	9.0 (week 1) to 45.2 (week 2)	0.84	Cutler *et al*. (2014)
Apis mellifera	Pollen	August to early September 2012	Adjacent to treated and untreated maize fields	c.99	*None detected*	Alburaki *et al*. (2015)
Apis mellifera	Pollen	June 2013 (peak OSR flowering)	Adjacent to treated WS OSR fields	91.1	3.09	Botías *et al*. (2015)
Apis mellifera	Pollen	August 2013	Adjacent to treated WS OSR fields	100.0	0.20	Botías *et al*. (2015)
Apis mellifera	Pollen	June 2013 (peak OSR flowering)	Adjacent to untreated SS OSR fields	47.4	<0.5 (*limit of detection*)	Rundlöf *et al*. (2015)
Apis mellifera	Pollen	June 2013 (peak OSR flowering)	Adjacent to treated SS OSR fields	37.9	13.9	Rundlöf *et al*. (2015)
Apis mellifera	Pollen	Late July to September 2013	Adjacent to treated and untreated maize fields	93.5	0.04	Alburaki *et al*. (2016)
Apis mellifera	Pollen	May to September 2011	Non-agricultural area	93.9	0.047	Long and Krupke (2016)
Apis mellifera	Pollen	May to September 2011	Adjacent to untreated maize fields	95.8	0.078	Long and Krupke (2016)
Apis mellifera	Pollen	May to September 2011	Adjacent to treated maize fields	95.3	0.176	Long and Krupke (2016)
Apis mellifera	Pollen	2005-2009 (dates unknown)	Adjacent to untreated maize fields	*Data not collected*	<1 (*limit of quantification*)	Pilling *et al*. (2013)
Apis mellifera	Pollen	2005-2009 (dates unknown)	Adjacent to treated maize fields	*Data not collected*	1-7 (range of reported median values)	Pilling *et al*. (2013)
Apis mellifera	Pollen	2005-2009 (dates unknown)	Adjacent to untreated US OSR fields	*Data not collected*	<1 (*limit of quantification*)	Pilling *et al*. (2013)
Apis mellifera	Pollen	2005-2009 (dates unknown)	Adjacent to treated US OSR fields	*Data not collected*	<1-3.5 (range of reported median values)	Pilling *et al*. (2013)
Apis mellifera	Pollen	6th May 2014	Adjacent to untreated WS OSR fields	*Data not collected*	<0.3 (*limit of detection*)	Rolke *et al*. (2016)
Apis mellifera	Pollen	6th May 2014	Adjacent to treated WS OSR fields	*Data not collected*	0.50	Rolke *et al*. (2016)
Apis mellifera	Pollen	10th-14th May 2014	Adjacent to untreated WS OSR fields	*Data not collected*	<0.3 (*limit of detection*)	Rolke *et al*. (2016)
Apis mellifera	Pollen	10th-14th May 2014	Adjacent to treated WS OSR fields	*Data not collected*	0.97	Rolke *et al*. (2016)
Bombus terrestris	Pollen	June 2013 (peak OSR flowering)	In urban areas (average 1600 m from treated WS OSR)	*Data not collected*	6.5	David *et al*. (2016)
Bombus terrestris	Pollen	June 2013 (peak OSR flowering)	In farmland (average 590 m from treated WS OSR)	68.1	21.2	David *et al*. (2016)
Bombus impatiens	Pollen	July-August 2013	Adjacent to untreated maize fields	99.35	<0.1 (*limit of detection*)	Cutler and Scott-Dupree (2014)
Bombus impatiens	Pollen	July-August 2013	Adjacent to treated maize fields	99.35	0.4	Cutler and Scott-Dupree (2014)
Bombus terrestris	Pollen	10th May 2014	Adjacent to untreated WS OSR fields	*Data not collected*	<0.3 (*limit of detection*)	Rolke *et al*. (2016)
Bombus terrestris	Pollen	10th May 2014	Adjacent to treated WS OSR fields	*Data not collected*	0.88	Rolke *et al*. (2016)
Osmia bicornis	Pollen	14th May 2014	Adjacent to untreated WS OSR fields	*Data not collected*	<0.3 (*limit of detection*)	Rolke *et al*. (2016)
Osmia bicornis	Pollen	14th May 2014	Adjacent to treated WS OSR fields	*Data not collected*	0.88	Rolke *et al*. (2016)

Thiamethoxam was found in this pollen at an average concentration of 18 ng/g and thiacloprid at an average concentration of 2.9 ng/g. These levels are much higher than the levels found in honeybee collected pollen from the same study area in the same year of 3.09 ng/g total neonicotinoids, though a much higher proportion (91.9%) of pollen was collected from wildflowers (Botías *et al*. 2015). Comparisons are difficult because few other studies have assessed neonicotinoid concentrations in bumblebee collected pollen with reference to pollen origin. Rolke *et al*. (2016) placed *B. terrestris* colonies out next to treated oilseed rape fields and found much lower concentrations of 0.88 ng/g of clothianidin in pollen taken directly from returning bumblebees, but the origin of this pollen is unknown. The concentrations found by David *et al*. are however lower than the levels reported by Pohorecka *et al*. (2013) and within a factor of two of the levels reported by Rundlöf *et al*. (2015) who found neonicotinoid concentrations of 27.0 ng/g and 13.9 ng/g in honeybee-collected pollen respectively, samples which also contained a high proportion of crop pollen.

Overall, these studies show that the highest acute exposure (0.84-27.0 ng/g) comes during the flowering period of insect-attractive neonicotinoid-treated flowering crops in situations where over a quarter of total pollen intake comes from crop plants. Reported values vary by up to two orders of magnitude depending on crop type, date of sample collection, initial strength of neonicotinoid seed coating and the proportion of wildflower pollen collected. Because only one study has explicitly measured neonicotinoid concentrations in wildflower pollen it is difficult to judge whether wildflower pollen consistently contains higher or lower concentrations of neonicotinoids than crop pollen. However, when looking at honeybee pollen diets in neonicotinoid-treated agricultural areas outside of the main flowering period of attractive crops, or where flowering crops are unattractive to a specific bee species, neonicotinoid concentrations are generally low, in the region of 0.04-0.40 ng/g from pollen diets comprised of 95.3-100% wildflower pollen (Cutler and Scott-Dupree 2014; Botías *et al*. 2015; Long and Krupke 2016; Alburaki et al. 2016). Whilst the highest levels of acute exposure come from pollen diets containing a proportion of crop pollen, because honeybees collect pollen over the whole season, total exposure to neonicotinoids may primarily be determined by concentrations in wildflowers. Botías *et al*. (2015) calculated, based on pollen collected in June and August, that 97% of the total neonicotinoids present in pollen were of wildflower origin. Non-crop plants surrounding agricultural areas represent an additional and chronic source of neonicotinoid exposure.

2.2.5 Risk of exposure from succeeding crops

The risk of neonicotinoid exposure from succeeding crops was identified as a key knowledge gap by the EFSA reports. The available studies suggested that residues in succeeding crops are below LOQ, but the data set was limited. Since 2013, few studies have explicitly looked at neonicotinoid levels in untreated crops grown in soil that had previously been used to grow neonicotinoid-treated crops, as most crops will be sown with a new dose of neonicotinoids each year. However, where specific neonicotinoid formulations are changed this analysis is possible. Botías *et al*. (2015; 2106) analysed neonicotinoid concentrations in oilseed rape treated with thiamethoxam. The fields had been used to grow clothianidin treated cereals over at least the previous two years. Imidacloprid had not been used for the previous three years. Oilseed rape pollen and foliage was found to contain 3.15 ng/g and 1.04 ng/g of thiamethoxam, 1.90 ng/g and 2.91 ng/g of clothianidin and 0 ng/g and 0.23 ng/g of imidacloprid, respectively. As clothianidin can be produced as a metabolite of thiamethoxam it is not possible to comment on the origin of these detected residues. Imidacloprid was absent from the pollen samples, reflecting the time since the last known agricultural use. Given that these compounds can persist in soil for multiple years, the level of exposure from succeeding crops will

broadly depend on the date since the last application, as well as the other factors determining neonicotinoid persistence in soil (Section 2.2.1). However, as demonstrated by the presence of imidacloprid in foliage samples, succeeding crops can take up residues of neonicotinoids remaining from applications made at least two years previously. Given the presence of neonicotinoids in annual, perennial and woody vegetation surrounding agricultural land (Section 2.2.4), and the medium-term persistence of neonicotinoids in soil and water (Sections 2.2.2 and 2.2.3), the risk of exposure from succeeding crops is likely to be in line with levels reported from general vegetation in agricultural environments. However, more explicit investigation in this area is required.

3. EVIDENCE FOR IMPACT OF NEONICOTINOIDS ON ANIMAL HEALTH

3.1 Sensitivity of bumblebees and solitary bees to neonicotinoids

3.1.1 Direct lethality of neonicotinoids to adult wild bees

Almost all of the studies conducted on the toxicity of neonicotinoids to bees have been conducted on honeybees, *Apis mellifera*. Fourteen studies conducted up to 2010 were reviewed in a meta-analysis by Cresswell (2011) who concluded that for acute oral toxicity imidacloprid has a 48-h LD_{50}=4.5 ng/bee. The EFSA studies (2013a; 2013b; 2013c) reviewed existing studies for acute oral toxicity up to 2013, including both peer reviewed studies and also private studies that are not in the public domain (summarised in Godfray *et al*. 2014). These analyses produced LD_{50}s of 3.7 ng/bee for imidacloprid, 3.8 ng/bee for clothianidin and 5.0 ng/bee for thiamethoxam. Equivalent LD_{50}s for acute contact have also been calculated by EFSA (2013a; 2013b; 2013c) for honeybees to be 81 ng/bee for imidacloprid, 44 ng/bee for clothianidin and 24 ng/bee for thiamethoxam.

However, the EFSA reports highlighted a knowledge gap for the effects of neonicotinoids on bees other than honeybees. Arena and Sgolastra (2014) conducted a meta-analysis comparing the sensitivity of bees to pesticides relative to the sensitivity of honeybees. This analysis combined data from 47 studies covering 53 pesticides from six chemical families with a total of 150 case studies covering 18 bee species (plus *A. mellifera*). Arena and Sgolastra calculated a sensitivity ratio R between the lethal dose for species *a* (*A. mellifera*) and for species *s* (other than *A. mellifera*), R = $LD_{50}a/LD_{50}s$. A ratio of over 1 indicates that the other bee species is more sensitive to the selected pesticides than *A. mellifera* and vice versa. There was high variability in relative sensitivity ranging from 0.001 to 2085.7, but across all pesticides a median sensitivity of 0.57 was calculated, suggesting that *A. mellifera* was generally more sensitive to pesticides than other bee species. In the vast majority of cases (95%) the sensitivity ratio was below 10.

Combining data for all neonicotinoids (acetamiprid, imidacloprid, thiacloprid and thiamethoxam) and for both acute contact and acute oral toxicity, nine studies covering nine bee species (plus *A. mellifera*) were found. These studies showed a median sensitivity ratio of 1.045 which is the highest median value of all the analysed pesticide chemical families. The most relatively toxic neonicotinoids to other bees were the cyano-substituted neonicotinoids acetamiprid and thiacloprid as these exhibit lower toxicity to honeybees than the nitro-substituted neonicotinoids imidacloprid and thiamethoxam.

Selecting pesticides covered by the moratorium (excluding acetamiprid and thiacloprid and including fipronil) and including both acute contact and acute oral toxicity, 12 studies covering 10 bee species (plus *A. mellifera*) were found. These studies showed a median sensitivity ratio of 0.957 which is close to the calculated sensitivity ratio for all neonicotinoids. The greatest discrepancy between honeybees and other bees was found for stingless bees (Apidae: Meliponini). The effect of acute contact of fipronil on *Scaptotrigona postica* (24-fold greater), of acute contact of fipronil on *Melipona scutellaris* (14-fold greater) and of acute contact of Thiacloprid on *Nannotrigona perilampoides* (2086-fold) were the only three cases with a sensitivity ratio of over 10. Stingless bees are predominantly equatorial with the greatest diversity found in the neotropics. No species are found in Europe (Nieto *et al*. 2014). In contrast, studies on *B. terrestris* consistently report a lower sensitivity ratio between 0.005 and 0.914, median 0.264. *B. terrestris* is widespread in Europe and is the most commonly used non-*Apis* model system for assessing the effects of neonicotinoids on wild bees (see Section 3.1.2). Differences in bee body weight have been proposed to explain these differences, with sensitivity to pesticides inversely correlated with body size (Devilliers *et al*. 2003).

However, this has not been consistently demonstrated and other mechanisms have been suggested such as species level adaptation to feeding on alkaloid-rich nectar (Cresswell *et al*. 2012) and differential abilities to clear neonicotinoid residues from their bodies (Cresswell *et al*. 2014). With the limited data available Arena and Sgolastra could not comment on the strength of these claims.

Spurgeon *et al*. (2016) calculated various toxicity measures of clothianidin on honeybees, the bumblebee species *B. terrestris* and the solitary bee species *O. bicornis*. Acute oral toxicity 48-h, 96-h and 240-h LD_{50}s for honeybees were 14.6 ng/bee, 15.4 ng/bee and 11.7 ng/bee respectively. For *B. terrestris*, the corresponding values were 26.6 ng/bee, 35 ng/bee and 57.4 ng/bee respectively. For *O. bicornis*, the corresponding values were 8.4 ng/bee, 12.4 ng/bee and 28.0 ng/bee respectively. These findings are generally in line with the findings of Arena and Sgolastra, with *B. terrestris* less sensitive than *A. mellifera* at all time points and *O. bicornis* less sensitive at 240-h.

Sgolastra *et al*. (2016) calculated relative sensitivity to clothianidin to these same three species over a range of time periods from 24-96 hours. The highest LD_{50} values were obtained after 24 hours for *A. mellifera* and *B. terrestris* and after 72 hours for *O. bicornis*. At these time points, *O. bicornis* was the most sensitive of the three species, with LD_{50} measurements of 1.17 ng/bee and 9.47 ng/g, compared to 1.68 ng/bee and 19.08 ng/g for *A. mellifera* and 3.12 ng/bee and 11.90 ng/g for *B. terrestris*. These results are in line with the values calculated by Spurgeon *et al*. (except for the 240 hour values), with decreasing sensitivity in the order of *O. bicornis* > *A. mellifera* > *B. terrestris*. Together, these studies support the position that small bodied species show greater sensitivity to neonicotinoids.

Around 2000 bee species are known from Europe. The biology, behaviour and ecology of each of these species differ from those of honeybees. Consequently, extrapolating from the limited toxicological data available for 19 bee species to the effects of neonicotinoids on the wider European fauna is fraught with difficulties given the wide variation in relative sensitivity. Current data suggests that wild bees are equally to slightly less sensitive to neonicotinoids compared to honeybees when considering direct mortality. However, care must be taken when considering individual bee species, genera and families, as different taxonomic groups may show consistently different individual level sensitivity. Most European wild bees are smaller than honeybees and there is the potential for them to be more sensitive on a ng/bee basis. In general, continuing to use honeybee neonicotinoid sensitivity metrics is likely to be a reasonable proxy measure for the direct sensitivity of the wild bee community to neonicotinoids (Arena and Sgolastra 2014), but further work is needed in this area to cover the wide range of bee species present in agricultural environments.

3.1.2 Sublethal effects of neonicotinoids on wild bees

In 2013 a number of studies looking at sublethal effects of neonicotinoids were available, predominantly using honeybees as a model organism in laboratory conditions. Blacquière *et al*. (2012) reviewed studies on neonicotinoid side effects on bees published between 1995 and 2011 with a specific focus on sublethal effects. The authors found that whilst many laboratory studies described lethal and sublethal effects of neonicotinoids on the foraging behaviour and learning and memory abilities of bees, no effects were observed in field studies at field-realistic dosages. Two major studies that substantially contributed towards the initiation and subsequent implementation of the European Union neonicotinoid moratorium were published after this review in 2012.

Henry *et al*. (2012) gave honeybee workers an acute dose of 1.34 ng of thiamethoxam in a 20 µl sucrose solution, equivalent to 27% of the LD_{50} (see Section 3.1.1) then released them 1 km away

from their nests and measured their return rate. Dosed bees were significantly less likely to return to the nest than control bees. Whitehorn *et al*. (2012) exposed *B. terrestris* colonies to two levels of neonicotinoid-treated pollen (6 and 12 ng/g plus control) and nectar (0.7 and 1.4 ng/g plus control) in the laboratory for two weeks before moving them outdoors to forage independently for six weeks, aiming to mimic a pulse exposure that would be expected for bees foraging on neonicotinoid-treated oilseed rape. Bees in the two neonicotinoid treatments grew significantly more slowly and had an 85% reduction in the number of new queens produced when compared to control colonies.

Both of these studies have been criticised for using neonicotinoid concentrations greater than those wild bees are likely to be exposed to in the field (see Godfray *et al*. 2014, Carreck and Ratnieks 2014). The 1.34 ng of thiamethoxam in a 20 µl sucrose solution used by Henry *et al*. is a concentration of 67 ng/g. Taking maximum estimated concentrations of thiamethoxam in oilseed rape nectar of 2.72 ng/g (see Section 2.1.1), a honeybee would have to consume 0.49 g of nectar to receive this dose. Honeybees typically carry 25-40 mg of nectar per foraging trip, equivalent to 0.025-0.040 g, some 10% of the volume necessary to receive a dose as high as the one used by Henry *et al*. Moreover, as honeybee workers regurgitate this nectar at the hive, the total dose consumed is likely to be a fraction of the total amount carried. Consequently, it is extremely unlikely that the findings of Henry *et al*. are representative of a real world situation.

The pollen and nectar concentrations used by Whitehorn *et al*. are much closer to field-realistic levels with the lower treatment within maximum estimated concentrations of imidacloprid in oilseed rape pollen and nectar (see Section 2.1.1). However, the experimental set up, where bees had no choice but to consume treated pollen and nectar has been criticised as unrealistic, as in the real world alternative, uncontaminated forage sources would be available. Studies that have measured residues in both crop and wildflower pollen and have assessed the origin of bee-collected pollen (see Section 2.2.4) have recorded neonicotinoid concentrations of between 0.84-27.0 ng/g in wild bee-collected pollen where a substantial proportion of this pollen is collected from crop plants during their period of peak flowering. Pollen extracted from bumblebee nests contained neonicotinoid concentrations of 6.5 ng/g in urban areas and 21.2 ng/g in rural areas during the peak flowering period of oilseed rape, though the number of nests sampled (three and five) were low. However, other studies measuring levels in pollen taken directly from bumblebees found concentrations of <1 ng/g, so there is still a lack of clarity surrounding true levels of neonicotinoid exposure for wild bumblebees. On the basis of these described concentrations, the results of Whitehorn *et al*. are likely to be closer to real world conditions than the findings of Henry *et al*.

Post-April 2013, much work on sublethal effects of neonicotinoids on bees has been carried out on individual honeybees and honeybee colony fitness metrics, such as colony growth, overwintering success and the production of sexuals. This work is beyond the scope of this review, but important recent publications include Pilling *et al.* (2013), Cutler *et al*. (2014a), Rundlöf *et al*. (2015) and Dively *et al*. (2015) who all found limited to negligible impacts of neonicotinoids at the colony level. See also Cresswell (2011) for a meta-analysis of 13 laboratory and semi-field studies conducted before 2011. Various authors note that interpreting the findings of studies on honeybees to wild bees is fraught with difficulty, given the differing size of individual bees and the social behaviour of honeybees that gives rise to colonies containing many thousands of workers.

3.1.2.1 Impact on colony growth and reproductive success

Several authors have investigated the effects of neonicotinoids on bumblebees using micro-colonies. These are small groups of worker bumblebees that are taken from a queenright colony and isolated in a new nest box. These workers, lacking a queen, will begin to rear their own male offspring. As such, micro-colonies are useful for generating a large sample size for investigating pesticide impacts on bee mortality and larval rearing behaviour and reproductive success.

Elston *et al*. (2013) fed micro-colonies of three *B. terrestris* workers a 'field-realistic' dose of 1 ng/g thiamethoxam and a 'field-maximum' dose of 10 ng/g in both pollen paste and sugar solution for a 28-day period. Micro-colonies from both thiamethoxam treatments consumed significantly less sugar solution than control colonies. There was no impact on worker mortality, but colonies fed 10 ng/g thiamethoxam had reduced nest-building activity and produced significantly fewer eggs and larvae, with the 10 ng/g thiamethoxam treatment the only one to produce no larvae over the 28-day experimental period.

Laycock *et al*. (2014) fed micro-colonies of four *B. terrestris* workers thiamethoxam-treated sugar solution at a range of concentrations up to 98 ng/g. Pollen was not treated with thiamethoxam. Sugar solution consumption was significantly reduced at the 39 and 98 ng/g treatments. Worker mortality was only increased at the highest dose of 98 ng/g. Worker oviposition failure was only significantly higher at the 39 and 98 ng/g treatments, with no significant differences seen between the lower concentration treatments between 0 and 16 ng/g.

The findings of these two studies are generally in line with pre-2013 knowledge. Mommaerts *et al*. (2010) exposed *B. terrestris* micro-colonies to sugar solution treated with thiamethoxam concentrations of up to 100 ng/g. Whilst the 100 ng/g level reduced brood production, the 10 ng/g treatment had no detectable effect. The difference between the findings of Elston *et al*. and Laycock *et al.* may partially be explained by the fact that Elston *et al*. treated pollen with thiamethoxam as well as sugar solution. Laycock *et al*. confirm that concentrations of 98 ng/g increase worker mortality, but as such concentrations are not usually encountered in the field this is of limited relevance.

Scholer and Krischik (2014) exposed greenhouse queenright colonies of *B. impatiens* to imidacloprid- and clothianidin-treated sugar syrup at concentrations of 0, 10, 20, 50 and 100 ng/g for 11 weeks. Queen mortality was significantly increased at six weeks for the 50 and 100 ng/g treatments, and at 11 weeks for the 20 ng/g treatment for both clothianidin and imidacloprid. Surprisingly, no significant impact was found on numbers of workers or new queens produced, though this was in part because very low numbers of new queens were produced across all treatments (average of four per colony). Colonies in treatments above 10 ng/g imidacloprid and 20 ng/g of clothianidin gained significantly less weight over the course of the study. Neonicotinoid concentrations of 20 ng/g and above are very high and are unlikely to be consistently encountered by bees for prolonged periods of times under real world conditions. As a result, queen mortality in the real world is unlikely to be significantly affected by currently observed neonicotinoid concentrations.

Several field studies have also been published since 2013 that investigate the impact of neonicotinoid-treated mass flowering crops on wild bee colony growth and reproductive success. Cutler and Scott-Dupree (2014) placed *B. impatiens* colonies adjacent to maize fields during pollen shed in Ontario, Canada. Four neonicotinoid-treated conventional and four untreated organic fields were used. Colonies were placed out adjacent to each field on the first day of major pollen shed. Colonies were left for 5-6 days and then transported to an area of semi-natural habitat for 30-35 days, after which they were frozen. Colonies placed next to treated maize produced significantly

fewer workers than those placed next to organic farms. All other metrics (colony weight, honey and pollen pots, brood cells, worker weight, male and queen numbers and weights) were not significantly different. Bumblebees collected less than 1% of their pollen from maize (Section 2.2.4) and neonicotinoid residues in collected pollen were low, at 0.4 ng/g from bees foraging adjacent to treated fields and below the LOD for bees adjacent to organic fields. Given that it is well known that bumblebees collect very low volumes of maize pollen, the relevance of this study is unclear.

Rundlöf *et al*. (2015) conducted an extensive field trial of the effects of clothianidin-treated oilseed rape on wild bees. Sixteen oilseed rape fields separated by at least 4 km were selected across southern Sweden and were paired on the basis of similar landscape composition. In each pair, one of the fields was randomly selected to be sown with oilseed rape treated with 10 g clothianidin/kg of seed and the other field was sown without a neonicotinoid seed treatment. Twenty-seven cocoons of the solitary bee *O. bicornis* (15 male, 12 female) were placed out alongside each field a week before the oilseed rape began to flower, and six colonies of *B. terrestris* were placed alongside each field on the day the oilseed rape began to flower. The *O. bicornis* placed adjacent to treated oilseed rape showed no nesting behaviour and did not initiate brood cell construction. *O. bicornis* adjacent to untreated fields showed nesting behaviour in six of the eight fields studied. The reasons for these differences in nest initiation are unclear and it is difficult to draw firm conclusions with a small sample size. Bumblebees placed next to treated oilseed rape showed reduced colony growth and reproductive output. Bumblebee colonies were collected and frozen when new queens began to emerge, with this happening between the 7th of July and 5th of August depending on each colony. The number of queen and worker/male cocoons present was counted. At the point of freezing, colonies placed next to treated oilseed rape fields had significantly fewer queen and worker/male cocoons present.

Sterk *et al*. (2016) performed a similar field experiment to Rundlöf *et al*. Two 65 km^2 areas in northern Germany were selected in which the only flowering crops comprised winter-sown oilseed rape. In one area the oilseed rape was treated with the same seed coating used by Rundlöf *et al.* of 10 g clothianidin/kg seed. The other area was an untreated control. In each area, ten *B. terrestris* colonies were placed at each of six localities. Colonies were left adjacent to oilseed rape between April and June, covering its main flowering period. After this the colonies were moved to a nature reserve. No differences were found in colony weight growth, number of workers produced or reproductive output as measured by the production of new queens.

That these two field studies using the same neonicotinoid seed dressing found markedly different results is interesting. The major difference is that whilst Rundlöf *et al*. used spring-sown oilseed rape, Sterk *et al*. used winter-sown oilseed rape. The length of time between sowing and peak flowering is much greater for winter-sown oilseed rape (mid-August to May) than for spring-sown oilseed rape (April/May to mid-June). As such, there is more time for neonicotinoids to leach into soil and water for winter-sown oilseed rape, reducing the amount of active ingredient available to be taken up by the crop. This may explain some of the order of magnitude differences in neonicotinoid concentrations in pollen collected from the two crops (Section 2.2.4) and the difference in reported colony growth and number of reproductives produced. An additional difference is that in the Sterk *et al*. study, colonies were moved to a nature reserve consisting of forests, lakes and heaths after the flowering period of oilseed rape ended. The quality of available forage at this nature reserve is likely to have been of both a higher quality and quantity than what was available in a conventional agricultural landscape and is not typical of the experience of a bumblebee colony located in such a landscape that will have to continue foraging there after crops such as oilseed rape cease flowering. In addition, a major problem with the experimental design of Sterk *et al*. is that only one treated and

one control area were used, so there is no true site level replication, as opposed to Rundlöf *et al*. who used eight treated and eight control fields. These differences in experimental design should be taken into account when considering why the studies produced such different results.

One of the studies conducted in response to the results of Henry *et al*. (2012) and Whitehorn *et al*. (2013) was produced by FERA (2013). It consisted of a field trial with bumblebee colonies placed out adjacent to oilseed rape treated with either clothianidin, imidacloprid or an untreated control. Colonies were allowed to forage freely for 6-7 weeks whilst the oilseed rape flowered and then were moved to a non-agricultural area to continue developing. The initial aim was to measure colony growth and development across these three treatments and compare this with neonicotinoid concentrations collected from food stores within the nests, but the study was criticised for a number of methodological problems such as variable placement date and initial colony size, lack of site level replication and contamination of control colonies with neonicotinoid residues during the experiment. The study was ultimately not published in a peer reviewed journal but it came to the conclusion that there was no clear relationship between bumblebee colony success and neonicotinoid concentrations. Goulson (2015) reanalysed the FERA data using linear models and retaining two colonies excluded in the original study as outliers, but which do not meet the statistical definition of this term. This reanalysis found that the concentration of clothianidin in nectar and the concentration of thiamethoxam in pollen significantly negatively predicted both colony weight gain and production of new queens.

Only one study is available that looked at the impact of neonicotinoids on the reproductive success of a solitary bee in controlled conditions. Sandrock *et al*. (2014) established laboratory populations of *O. bicornis*, a solitary stem nesting bee. Bees were fed on sugar solution treated with 2.87 ng/g thiamethoxam and 0.45 ng/g clothianidin along with untreated pollen. There was no impact of neonicotinoids on adult female longevity or body weight. However, treated bees completed 22% fewer nests over the course of the experiment. Nests completed by treated bees contained 43.7% fewer total cells and relative offspring mortality was significantly higher, with mortality rates of 15% and 8.5% in the treated and untreated groups, respectively. Overall, chronic neonicotinoid exposure resulted in a significant reduction in offspring emergence per nest, with treated bees producing 47.7% fewer offspring. These results suggest that exposure to these low level, field-realistic doses of neonicotinoids (<3.5 ng/g) did not increase adult mortality but did have sublethal impacts on their ability to successfully build nests and provision offspring.

Overall, the studies produced since 2013 are generally in line with existing knowledge at this point but have advanced our knowledge in several key areas. Laboratory studies have continued to demonstrate negative effects of neonicotinoids on bumblebee reproductive output at generally high concentrations, with the lowest sublethal effects on reproductive output detected at 10 ng/g. Field studies using bumblebees demonstrate that exposure to neonicotinoid-treated flowering crops can have significant impacts on colony growth and reproductive output depending on the levels exposed to, with crop flowering date relative to sowing and availability of uncontaminated forage plants likely to explain variation in the detected residues between the available studies. Our understanding of the impact on solitary bees is much improved with the findings of Sandrock *et al*. (2014) suggesting substantial impacts on solitary bee reproductive output at field-realistic concentrations of 3.5 ng/g. Field studies demonstrating this under real-world conditions are limited with the work of Rundlöf *et al*. (2015) suffering from no nest-building activity at the neonicotinoid treatment sites.

3.1.2.2 Impact on foraging efficiency

In 2013 a limited amount was known about how neonicotinoids affected the foraging behaviour of individual bees, and whether this affected colony level fitness. Gill *et al*. (2012) exposed *B. terrestris* colonies to 10 ng/g imidacloprid in sugar solution in the nest for a period of four weeks. Colonies were housed indoors but access tubes allowed them to forage freely outdoors. Imidacloprid exposed colonies grew more slowly but there were substantial effects on worker foraging behaviour. Compared to controls, imidacloprid treated colonies had more workers initiating foraging trips, workers brought back smaller volumes of pollen on each successful trip and successful pollen foraging trips were of a significantly longer duration. Treated workers also collected pollen less frequently, with 59% of foraging bouts collecting pollen versus 82% for control workers, a decline of 28%. The authors conclude that exposure to imidacloprid at these concentrations significantly reduced the ability of bumblebee workers to collect pollen in the field. The reduced ability to collect pollen resulted in imidacloprid treated colonies collecting less pollen than control colonies, subsequently resulting in reduced growth through pollen limitation. Since the publication of this paper, several new studies assessing neonicotinoid impacts on the foraging behaviour of bumblebees have been published.

Feltham *et al*. (2014) exposed *B. terrestris* colonies to sugar solution treated with 0.7 ng/g and pollen treated with 6 ng/g of imidacloprid for two weeks. These sugar solution concentrations were an order of magnitude lower than the 10 ng/g used by Gill *et al*. (2012). Colonies were then placed out in an urban area in Scotland. The foraging workers from each nest were then monitored for a further four weeks. There was no difference in the length of time spent collecting nectar or the volume of nectar collected between workers from treated and control colonies. However, treated workers collected significantly less pollen, bringing back 31% less pollen per time unit to their colonies. Treated workers also collected pollen less frequently, with 41% of foraging bouts collecting pollen versus 65% for control workers, a decline of 23%.

Gill and Raine (2014) performed a similar experiment to Gill *et al*. (2012) where *B. terrestris* colonies were exposed to sugar solution treated with 10 ng/g of imidacloprid whilst also having access to forage freely outside. Colonies and individual worker bumblebees were studied over a four week period. In common with their previous findings (Gill *et al*. 2012), imidacloprid treated workers initiated significantly more foraging trips across all four weeks of the experiment. The authors note that this is likely driven by an acute individual-level response in the first weeks (neonicotinoids acting as a neural partial agonist, increasing desire to forage) and by a chronic colony-level response in the latter part of the experiment, with treated colonies allocating a higher proportion of workers to pollen collection. Pollen foraging efficiency of treated workers decreased as the experiment progressed with the smallest collected pollen loads recorded in week four, suggesting a chronic effect of imidacloprid on pollen foraging ability. It is not clear whether this is as a result of individual performance deteriorating, or new emerging workers having been exposed for a greater period of time.

Stanley *et al*. (2015) exposed *B. terrestris* colonies to 2.4 or 10 ng/g thiamethoxam treated sugar solution for 13 days. Colonies were then moved to pollinator exclusion cages where they were allowed to forage freely on two varieties of apple blossom. Bees from colonies exposed to 10 ng/g spent longer foraging, visited fewer flowers and brought back pollen on a lower proportion of foraging trips compared to bees from control colonies. Stanley and Raine (2016) also exposed *B. terrestris* colonies to 10 ng/g thiamethoxam sugar solution for a nine to ten day period. At this point, colonies were moved to a flight arena provisioned with two common bird's-foot trefoil *Lotus corniculatus* and one white clover *Trifolium repens* plants. Worker bees were individually released

and their interaction with the flowers was recorded. Significantly more treated workers displayed pollen-foraging behaviour compared to control workers. However, control workers learnt to handle flowers efficiently after fewer learning visits.

Arce *et al*. (2016) placed *B. terrestris* nests out in an area of parkland for a five week period whilst also supplying them with sugar solution treated with 5 ng/g of clothianidin. The volume of sugar solution provided was estimated to be half that which colonies typically consume over the course of the experiment. No pollen was provided, so workers had to forage for this and to make up the shortfall in nectar resources. In contrast to the previous papers, only subtle changes to patterns of foraging activity and pollen collection were detected. There was no clear difference in colony weight gain between treatments or number of brood individuals. However, by the end of the experiment, treated colonies contained fewer workers, drones and gynes when compared with control colonies.

Switzer and Combes (2016) studied the impact of acute imidacloprid ingestion on sonicating behaviour of *B. impatiens*. Sonicating is a behaviour whereby a bumblebee lands on a flower and vibrates loudly to shake pollen loose from anthers. Bumblebee workers were fed a dose of 0, 0.0515, 0.515 or 5.15 ng of imidacloprid in 10 µL of sugar solution. These are equivalent to concentrations of 0, 5.15, 51.5 and 515 ng/g, with the highest volume consumed equivalent to 139% of the honeybee LD_{50}, a moderate proxy for bumblebees, as bumblebees are generally less sensitive than honeybees (Section 3.1.1). Bees were then allowed to forage from tomato *Solanum lysopersicum* plants and sonicating behaviour was observed. At the lowest dose of 0.0515 ng of imidacloprid, no impact was found on wingbeat frequency, sonication frequency or sonication length. No analysis could be made for higher doses, as bees in these treatments rarely resumed foraging behaviour after ingesting imidacloprid. Given the neonicotinoid concentrations used in this study and sample size problems it is difficult to draw many conclusions other than that high levels of exposure impair bumblebee pollen foraging behaviour.

Overall, these studies suggest that exposure to neonicotinoids in nectar at concentrations of between 0.7-10 ng/g can have sublethal effects on the ability of bumblebees to collect pollen at both the individual and colony level. This shortfall in pollen and subsequent resource stress is a plausible mechanism to explain diminished colony growth and production of sexuals in the absence of increased direct worker mortality. Given that concentrations as high as 10 ng/g are at, but within, the upper limit of what bumblebees are likely to experience in the field (Section 2.1.1 and Section 2.2.4), it is likely that wild bumblebees exposed to neonicotinoids in contemporary agricultural environments suffer from a reduced ability to collect pollen, with a subsequent impact on their reproductive output.

3.1.2.3 Impact on bee immune systems

Bee diseases (including both parasites and pathogens) have been implicated as the major factor affecting managed honeybee colony survival in recent years (vanEngelsdorp *et al*. 2010). Whilst most evidence for the negative effects of diseases comes from studies of honeybees, most diseases can affect a wide range of bee species. For example, the microsporidian parasite *Nosema ceranae* originates in Asia and has been spread around the world by the trade in honeybees. *N. ceranae* has now been detected in four different genera of wild bees (*Bombus*, *Osmia*, *Andrena*, *Heriades*) across Europe and the Americas (see Goulson *et al*. 2015). The spread of diseases between wild and managed bees can occur at shared flowering plants (Graystock *et al*. 2015).

Sánchez-Bayo *et al.* (2016) reviewed evidence that linked the use of neonicotinoids to the incidence and severity of bee diseases. Prior to 2013, several studies demonstrated a link between neonicotinoid exposure and increased susceptibility to diseases in honeybees (Vidau *et al*. 2011; Pettis *et al*. 2012). Exposure of honeybees infected with *N. ceranae* to imidacloprid reduced their ability to sterilise the brood, increasing the spread of *N. ceranae* within the colonies (Alaux *et al*. 2010). In addition, exposure to sublethal doses of imidacloprid or fipronil increased honeybee worker mortality due to a suppression of immunity-related genes (Aufauvre *et al*. 2014). Di Prisco *et al*. (2013) found that sublethal doses of clothianidin adversely affected honeybee antiviral defences. By enhancing the transcription of the gene encoding a protein that inhibits immune signalling activation, the neonicotinoid pesticides reduce immune defences and promote the replication of deformed wing virus in honeybees bearing covert viral infections. At the field level, a positive correlation is found between neonicotinoid treatment and Varroa mite infestation and viral load of honeybee colonies (Divley *et al*. 2015; Alburaki *et al*. 2015). No studies are available that measure the impact of neonicotinoids on the immune systems of wild bees or on the incidence of diseases in wild bees in conjunction with neonicotinoid usage. However, given that wild bees share a very similar nervous and immune system it is highly likely that neonicotinoids will have similar effects, increasing wild bee susceptibility to parasites and pathogens.

3.1.3 Population level effects of neonicotinoids on wild bees

Nothing was known about the population level effects of neonicotinoids on wild bees in 2013. As a managed domesticated species, population trends are available for honeybees, but no such data are available for wild bees. One study has attempted to investigate the impact of neonicotinoids on wild bee population trends. Woodcock *et al*. (2016) used an incidence dataset of wild bee presence in 10 x 10 km grid squares across the United Kingdom. The dataset is comprised of bee sightings by amateur and professional entomologists and is probably the most complete national bee distribution database currently in existence. Sixty-two wild bee species were selected and their geographic distance and persistence over an 18 year period between 1994 and 2011 was calculated. Neonicotinoid seed-treated oilseed rape was first used in the UK in 2002, and so the authors calculated spatially and temporally explicit information describing the cover of oilseed rape and the area of this crop treated with neonicotinoids. The 62 species were split into two groups – species that foraged on oilseed rape (n=34) and species that did not (n=28). Species persistence across this time period was then compared with expected neonicotinoid exposure. Over the 18 year period, wild bee species persistence was significantly negatively correlated with neonicotinoid exposure for both the foraging and non-foraging group, with the effect size three times larger for the oilseed rape foraging group.

The characterisation of bees as foragers or non-foragers has one major problem. Many species of bees are obligately parasitic on other bees and do not forage for their own pollen. Some parasitic bees were included in the oilseed rape forager category (n=2), and some in the non-forager category (n=12) based on observed nectar visits from a previous study. Some of the parasitic bees in the non-forager group are parasitic on bees included in the forager group (n=10/28). Given that these species are highly dependent on their host's abundance this classification does not make ecological sense. A decline due to a decline in their host or because of increased direct mortality cannot be separated, introducing an additional confounding issue into the analysis. In addition, given the presence of neonicotinoids in wild plants adjacent to agricultural areas (Section 2.2.4), the amount applied to oilseed rape is not necessarily a true measure of actual neonicotinoid exposure for wild bees.

Overall, the study suggests that bee species were more likely to disappear from areas with a high exposure to neonicotinoids as measured by the amounts applied as seed dressings to oilseed rape, and that this trend was more pronounced for species known to forage on oilseed rape. Whilst more work is needed, this is a major correlational study that suggests a link between levels of neonicotinoid exposure and bee community persistence at a national scale.

3.2 Sensitivity of butterflies and moths to neonicotinoids

Pisa *et al*. (2015) reviewed the existing literature on the impact of neonicotinoids on butterflies and moths (Lepidoptera). In contrast to bees, very few comparative toxicity tests have been conducted for butterflies. Most existing studies have compared butterfly abundance and diversity on organic versus conventional farms. Organic farms host a greater diversity of species, but the specific reasons for this cannot be isolated. For example, the relative importance of herbicide use that reduces the abundance of larval food and adult nectar plants versus direct mortality or sublethal stress from pesticides is unknown.

Most available toxicological studies looking at the sensitivity of Lepidoptera to neonicotinoids and fipronil have been conducted on 32 species of moths from nine families that are pests of crops (Pisa *et al*. 2015). There is considerable variation in reported sensitivities between species, with the susceptibility to acetamiprid of two cotton pests differing almost 3-fold (LC_{50}=11,049 and 3,798 ppm). There is also variation between different stages of larval development, with first instar caterpillars more than 100 times as sensitive as fifth instar caterpillars with a LC_{50}/LC_{90} of 0.84/1.83 and 114.78/462.11 ppm, respectively. Botías *et al*. (2016) listed LC_{50} values for three moth species that are agricultural crop pests, with 24 h LC_{50} values between 2400 and 186,000 ppb clothianidin. These levels are generally very high and there are multiple examples of neonicotinoid resistance in wild populations (see Pisa *et al*. 2015). Because many of the studied moths species are pests of major crops they have been exposed to multiple pesticides over many generations in recent decades, and their sensitivity to neonicotinoids many not necessarily be representative of non-pest wild Lepidoptera species.

Since 2013, few studies looking at the sensitivity of wild Lepidoptera to neonicotinoids are available. Pecenka and Lundgren (2015) assessed the lethality of clothianidin to caterpillars of monarch butterflies *Danaus plexippus*. First instar caterpillars were fed treated leaves for a 36 hour period. A LC_{50} of 15.63 ng/g was calculated. In addition, sublethal effects on growth were measured at 0.5 ng/g with first instar larvae taking longer to develop, having reduced body length and lower weight. These differences did not extend into the second instar. Yu *et al*. (2015) fed second instar silkworm *Bombyx mori* caterpillars leaves treated with imidacloprid and thiamethoxam for a 96 hour period. They calculated LC_{50} values of 1270 ng/g for imidacloprid and 2380 ng/g for thiamethoxam. This wide range of reported tolerances for a limited number of ecologically different species means that thorough assessment of butterfly and moth sensitivity to neonicotinoids is difficult. Much more research is required in this area.

Whilst there is a paucity of toxicological data on wild butterflies and moths, two recent studies have used long term butterfly population datasets to assess the relative impact of neonicotinoid usage in agricultural areas. Gilburn *et al*. (2015) used data from the UK butterfly monitoring scheme. The data consists of butterfly counts from a wide variety of habitats and the period studied was 1984-2012, a more extensive time period that than used for UK wild bees by Woodcock *et al*. (2016, Section 3.1.3) in order to have a ten year period before the introduction of neonicotinoids onto British farmland. Seventeen UK butterfly species were selected that are predominantly generalists and are found in a wide range of habitats including agricultural habitats. The area of the UK treated with neonicotinoids and a range of temperature and weather variables were included in the model, as local climatic conditions are a very important factor impacting butterfly populations. In line with expectations, summer temperature was significantly positively and spring rainfall significantly negatively correlated with the butterfly population indexes. Neonicotinoid usage was also significantly negatively associated with butterfly population indices after controlling for the effects of weather. The pattern of association varied between butterfly species, but most (14 out of 17) had a negative

association. In the most recent time period between 2000-2009 when neonicotinoid usage was at its highest, 15 of the 17 studied species showed a negative population trend.

Forister *et al*. (2016) conducted a similar analysis on Californian lowland butterfly populations. Butterflies have been monitored continuously with biweekly walks at four sites in a region of northern California since 1972, 1975 and 1988 depending on the individual site. These sites are situated across a land gradient that includes arable, semi-natural and urban habitats. The data were used to examine the impact of annual neonicotinoid input and other factors such as summer temperature and land-use change.

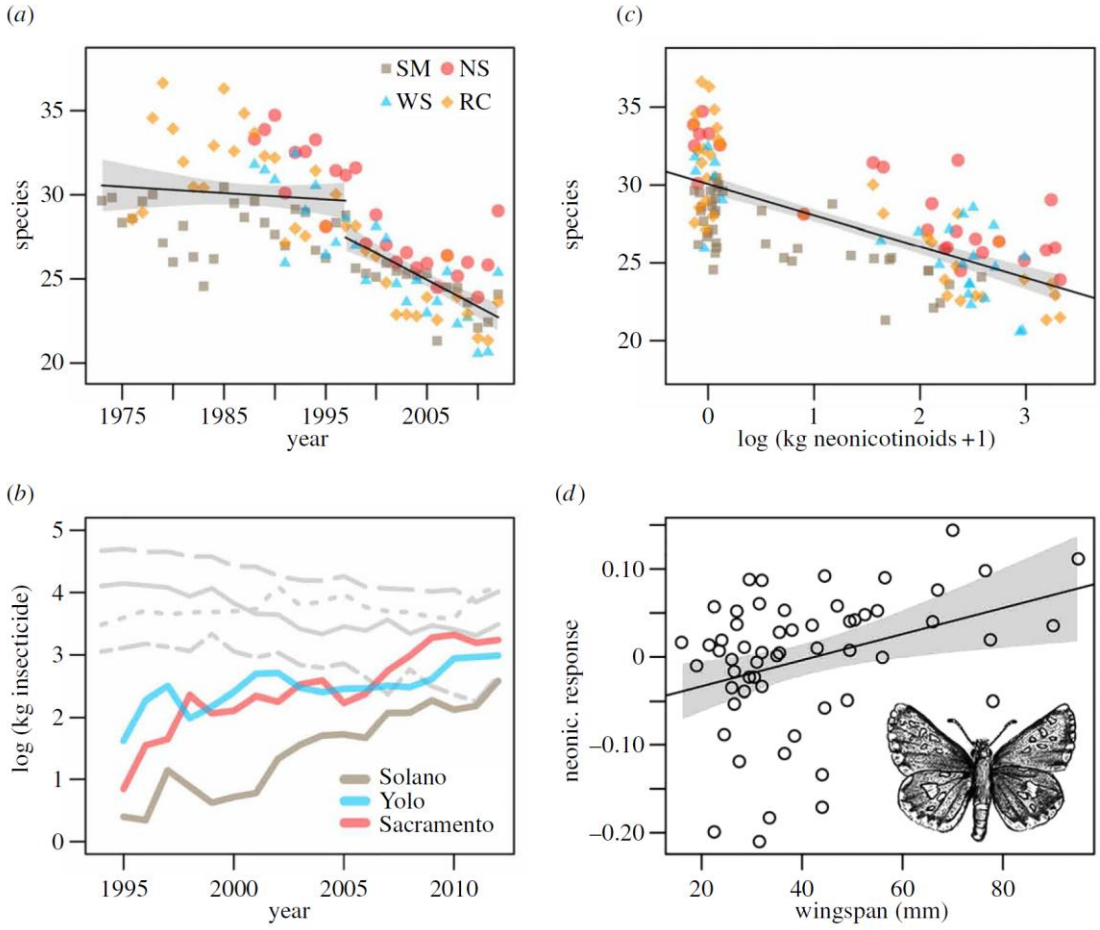

Figure 10. (a) The number of observed butterfly species at four sites. The response variable (in (a) and (c)) is the exponential of Shannon diversity, i.e. the effective number of species; the spline knot in (a) is 1997 (95% confidence interval: 1990–2001). (b) Pesticide application for neonicotinoids in focal counties (coloured lines), and for the four most commonly applied non-neonicotinoid classes (grey lines). The non-neonicotinoids are, in decreasing order of line elevation in 1995; organophosphates, carbamates, pyrethroids and organochlorines (lines are county averages). Note the different range of years in the first two panels, as (b) starts in the year in which neonicotinoids are first reported. (c) Relationship between number of butterfly species and neonicotinoids (values of the latter at zero jittered for visualization). (d) Response of individual species to neonicotinoids as predicted by wingspan; more negative values on the y-axis indicate species with more negative associations with neonicotinoids. Grey polygons in panels (a), (c), and (d) are 95% confidence intervals. Reproduced from Forister *et al*. 2016

A substantial decline in butterfly species richness was seen from 1997 onwards (Figure 10a, 1997 being the breakpoint identified by the statistical models). Neonicotinoid usage in the region began in 1995 and has increased since that point (Figure 10b). Neonicotinoid use was significantly negatively correlated with butterfly species richness (Figure 10c) and smaller bodied butterflies had the strongest negative response to neonicotinoids (Figure 10d).

Both of these analyses are strictly correlational and neonicotinoid usage may simply be a proxy measurement for some other factor that is driving declines. Gilburn *et al*. note that if habitat deterioration and loss of food plants is the main cause of butterfly declines, and agricultural intensification is playing a key role in this habitat deterioration, then levels of neonicotinoid usage might be acting as a proxy for agricultural intensification and therefore habitat deterioration. Thus, neonicotinoid usage could be responsible for driving butterfly declines or alternatively it could provide the first useful quantifiable measure of agricultural intensification that strongly correlates with butterfly population trends. As most of the UK butterfly monitoring scheme survey areas are not directly on agricultural land, Gilburn *et al*. suspect that it is the transport of neonicotinoids into the wider environment (Section 2.2.4) and farmed areas acting as population sinks that is driving the declines of butterflies, rather than neonicotinoid use acting as a proxy for agricultural intensification. No data is available to assess this hypothesis.

Overall, recent studies have demonstrated that Lepidoptera show a wide range of tolerances to ingested neonicotinoids in their larval stages. No data is available on sensitivity to neonicotinoids ingested during the adult stage, for example from crop plant nectar. Two correlational studies using long term datasets show a strong association between neonicotinoid use and declines in butterfly abundance and species-richness, though more laboratory and field studies are required to establish the exact mechanism causing this decline.

3.3 Sensitivity of other terrestrial invertebrates to neonicotinoids

Most available studies that have assessed neonicotinoid sensitivity for insect species have focussed on pest species of economically important crops. Pisa *et al*. (2015) reviewed existing literature on the impacts of neonicotinoids on other terrestrial invertebrates and Botías *et al*. (2016) presented a summary on reported LC_{50}s for 24 species of insects across four orders (Hymenoptera, Lepidoptera, Hemiptera and Coleoptera) from studies conducted between 1996 and 2015. Pisa *et al's*. (2015) review found no post-2013 research on the effects of neonicotinoids on Neuroptera, Hemiptera and Syrphidae (hoverflies).

3.3.1 Sensitivity of natural enemies of pest insects

Douglas *et al*. (2015) investigated the impact of thiamethoxam seed-treated soybean on the agricultural pest slug *Deroceras reticulatum* and one of their natural predators, the carabid beetle *Chlaenius tricolor*, using both laboratory assays and field studies. Slugs collected from the field that had been allowed to feed freely on developing soybean seedlings contained total neonicotinoid concentration as high as 500 ng/g with average levels over 100 ng/g after 12 days of feeding. In the laboratory, slugs consuming soybean seedlings incurred low mortality of between 6-15% depending on the strength of the seed treatment. Under laboratory conditions, 61.5% (n=16/26) of *C. tricolor* beetles that consumed slugs from the neonicotinoid treatment subsequently showed signs of impairment compared to none of those in the control treatment (n=0/28). Of the 16 that showed impairment, seven subsequently died. In the field, seed-treated soybean reduced potential slug predator activity-density by 31% and reduced predation by 33%, resulting in increased slug activity-density by 67%.

Douglas *et al*. argue that the introduction of neonicotinoids into soybean results in a trophic cascade, whereby the predators of slugs are more significantly affected than the slugs themselves, resulting in an increase in the slug population as predation pressure is relaxed. This trophic cascade argument may also explain the results of Szczepaniec et al. (2011) who found that the application of imidacloprid to elm trees caused an outbreak of spider mites *Tetranychus schoenei*. This increase was as a result of a reduction in the density of their predators which incurred increased mortality after ingesting imidacloprid-containing prey items. Many beneficial predatory invertebrates feed on pests of crops known to be treated with neonicotinoids, but to date no other studies have assessed whether neonicotinoids are transmitted to these predators through direct consumption of crop pests in agro-ecosystems.

Frewin *et al*. (2014) studied the impact of imidacloprid and thiamethoxam seed-treated soybean on the soybean aphid parasitoid wasp *Aphelinus certus*. Mated females were placed in petri dishes containing soybean leaves with soybean aphid *Aphis glycines* populations for 24 hours. Petri dishes were then monitored for eight days with the numbers of alive, dead and juvenile aphids recorded. The effects of pesticide treatment was significant on the proportion of aphids parasitised, with no difference between the two different neonicotinoid seed treatments (Figure 11). Frewin *et al*. hypothesise two potential reasons for this effect – firstly that exposure to neonicotinoid residues within aphid hosts may have increased mortality of the immature parasitoid or the parasitism combined with residues may have increased aphid mortality. Secondly, *A. certus* may avoid parasitising pesticide-poisoned aphids. *Aphelinus* species are known to use internal cues to determine host suitability, and it is possible that they may use stress- or immune-related aphid hormones to judge host suitability. Given that a key part of biological control of insect pests using parasitic wasps is to increase the parasitoid abundance early in the season, the reduction in the

parasitism rate caused by neonicotinoid seed-treatment could potentially impair the ability of *A. certus* to control soybean aphid. It is not known if *A. certus* emerging from contaminated hosts will incur lethal or sublethal effects which may further impair this ability.

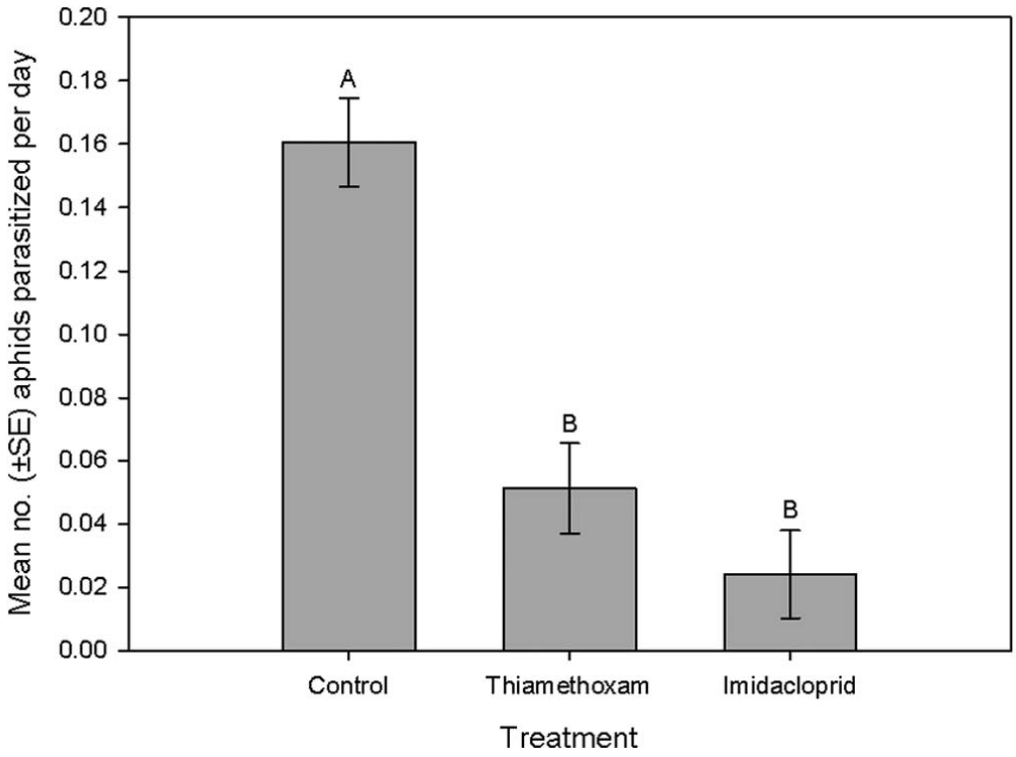

Figure 11. Parasitism rates (±SE) of *Aphelinus certus* on *Aphis glycines* feeding on soybean plants grown from seed not treated (control) with insecticidal seed treatment compared with those feeding on plants grown from seed treated with imidacloprid or thiamethoxam. Bars with the same letter are not significantly different (Tukey's honestly significant difference, $\alpha = 0.05$), n=35 for each treatment. Reproduced from Frewin *et al*. 2014.

Overall, where predatory species have a greater sensitivity to neonicotinoids than their prey species, such as insect predators of non-insect groups like molluscs and arachnids which have differing neuroreceptors that renders them less sensitive to neonicotinoids, there is the possibility of unintended negative effects on populations of beneficial natural enemies.

3.3.1 Sensitivity of ants to neonicotinoids

Four studies are available that have looked at the impact of neonicotinoids on ants. Galvanho *et al*. (2013) treated *Acromyrmex subterraneus* leafcutter ants with imidacloprid to investigate impacts on grooming, an important behaviour for limiting the spread of fungal pathogens. Workers were treated with 10, 20 or 40 ng/insect imidacloprid. Only workers with a head capsule of 1.6-2.0 mm in width were selected. This is a large size relative to most species of ants in the world. At this size, individual ants would weigh around 10-20 mg, giving a concentration of 10-40 ng active ingredient per 0.015 g of ant, or 666.7-2666.7 ng/g. The lowest dose was sufficient to significantly decrease

grooming behaviour. Mortality was not measured, but a previous study found that another species of leaf-cutter ant, *Atta sexdens*, had significantly increased mortality when exposed to a fungal pathogen and imidacloprid at the same concentration 10 ng/insect concentration compared to ants exposed only to the fungal pathogen (Santos *et al*. 2007).

Barbieri *et al*. (2013) exposed colonies of the Southern ant *Monomorium antarcticum* (native to New Zealand where the study was conducted) and the invasive Argentine ant *Linepithema humile* to imidacloprid in sugar water at a concentration of 1.0 µg/ml, equivalent to 1000 ng/g. Relative aggression was affected by neonicotinoid exposure, with native ants lowering their aggression to invasive ants, and conversely exposed invasive ants increasing their aggression, resulting in a lower survival probability. Brood production was not affected in the Southern ant, but exposure to neonicotinoids reduced Argentine ant brood production by 50% relative to non-exposed colonies. No effect of neonicotinoid exposure on foraging ability was detected.

Wang *et al*. (2015a) fed colonies of fire ants *Solenopsis invicta* sugar water at concentrations of 0.01, 0.05, 0.25, 0.50 and 1.00 µg/ml, equivalent to 10-1000 ng/g. The impact on feeding, digging and foraging were quantified. Ants exposed to the 10 ng/g concentration consumed significantly more sugar water and increased digging activity. Concentrations greater than or equal to 250 ng/g significantly supressed sugar water consumption, digging and foraging behaviour.

Wang *et al*. (2015b) fed *Solenopsis invicta* newly mated queens water containing imidacloprid concentrations of 10 or 250 ng/g. Neither concentration increased queen mortality but they did both significantly reduce queen's brood tending ability and the length of time taken to respond to light, an indication of disturbance and colony threat. In *Solenopsis* species, eggs are groomed and coated with an adhesive substance that maintains moisture levels and allows for rapid transport of egg clumps. At the 250 ng/g concentration, the number of egg clumps was significantly increased (indicating low egg care and an increase in the effort needed to transport brood), suggesting that the queens had a reduced ability to groom eggs. Untended eggs become mouldy, reducing colony growth. Colonies exposed to 10 ng/g showed no difference in egg clump numbers compared to controls.

Across these ant studies, the neonicotinoid concentrations used are generally very high, in most cases far higher than expected exposure rates under field-realistic conditions (Section 2.1 and 2.2). Few sublethal effects were detected at 10 ng/g, the levels that might be reasonably expected to be encountered under field conditions. More laboratory and field work is required using lower concentrations to better understand the likely effects of neonicotinoids on ants.

3.3.2 Sensitivity of earthworms to neonicotinoids

Pisa *et al*. (2015) reviewed existing literature on the impact of neonicotinoids on earthworms. Earthworms have similar neural pathways to insects, and earthworms are highly likely to be exposed to neonicotinoids through direct contact with soil, ingestion of organic material bound to neonicotinoids and consumption of contaminated plant material (Wang *et al*. 2012, Section 2.2.1) Reported neonicotinoid LC_{50}s for earthworms from 13 studies range from 1,500 to 25,500 ppb, with a mean of 5,800 ppb and a median of 3,700 ppb (see Pisa *et al*. 2015). Fewer studies are available that measured sublethal effects on reproduction. Negative impacts on cocoon production were measured at between 300-7,000 ppb depending on earthworm species and neonicotinoid type.

Very little data is available for realistic neonicotinoid exposure to earthworms under field conditions. Neonicotinoid concentrations in soils can range from 2-50 ng/g depending on organic matter composition, application rate and other factors, although they may be much higher in immediate proximity to dressed seeds (Section 2.2.1). Douglas *et al*. (2015) detected neonicotinoids in earthworms present in thiamethoxam-treated soybean fields. Two earthworms were casually collected during soil sample collection. The two samples were found to contain total neonicotinoid concentrations of 54 and 279 ppb corresponding to ~16 and ~126 ng per worm. In addition to thiamethoxam and its degradates, the two earthworm samples contained imidacloprid at 25 and 23 ppb. The fields from which they were taken had not been treated with imidacloprid for at least one year previously, adding further to the evidence that neonicotinoids can persist in soils for over one year (Section 2.2.1). Because only live earthworms were collected and the small sample size, it is not clear if these are representative of typical concentrations or are an underestimate. For example, if earthworms are exposed to higher levels that cause mortality, they cannot be subsequently sampled for residue analysis. More work is needed in this area.

Overall, these studies continue to increase our understanding of the negative effects of neonicotinoids on non-target organisms. In contrast to bees, most studied groups had lower sensitivity to neonicotinoids, in some cases by several orders of magnitude. The trophic level of the study organism may be important, with low trophic level insects better able to detoxify neonicotinoids due to their obligately herbivorous lifestyle that results in frequent contact with harmful plant metabolites. The most pronounced reported effects have been on predatory insects.

3.4 Sensitivity of aquatic invertebrates to neonicotinoids

The most comprehensive review of the acute and chronic effects of neonicotinoids on aquatic invertebrates was conducted by Morrissey *et al*. (2015). This followed on from and updated the reviews of Goulson (2013), Mineau and Palmer (2013) and Vijver and van den Brink (2014). Morrissey's analysis covered 214 toxicity tests for acute and chronic exposure to imidacloprid, acetamiprid, clothianidin, dinotefuran, thiacloprid and thiamethoxam for 48 species of aquatic invertebrate species from 12 orders (Crustacea: Amphipoda (11.7% of tests), Cladocera (21.0%), Decapoda (1.9%), Isopoda (4.2%), Mysida (7.9%), Podocopida (12.6%), Insecta: Diptera (22.9%), Ephemeroptera (6.5%), Hemiptera (3.7%), Megaloptera (1.9%), Odonata (1.9%), Trichoptera (3.3%)) from peer reviewed and government studies. Both LC_{50} and ED_{50} values were included. Acute and chronic toxicity of neonicotinoids vary greatly across aquatic invertebrates with differences of six orders of magnitude observed (Figure 12). In general, insects were more sensitive than crustaceans, in particular the Ephemeroptera (mayflies), Trichoptera (caddisflies) and Diptera (flies, most specifically the midges, Chironomidae) were highly sensitive.

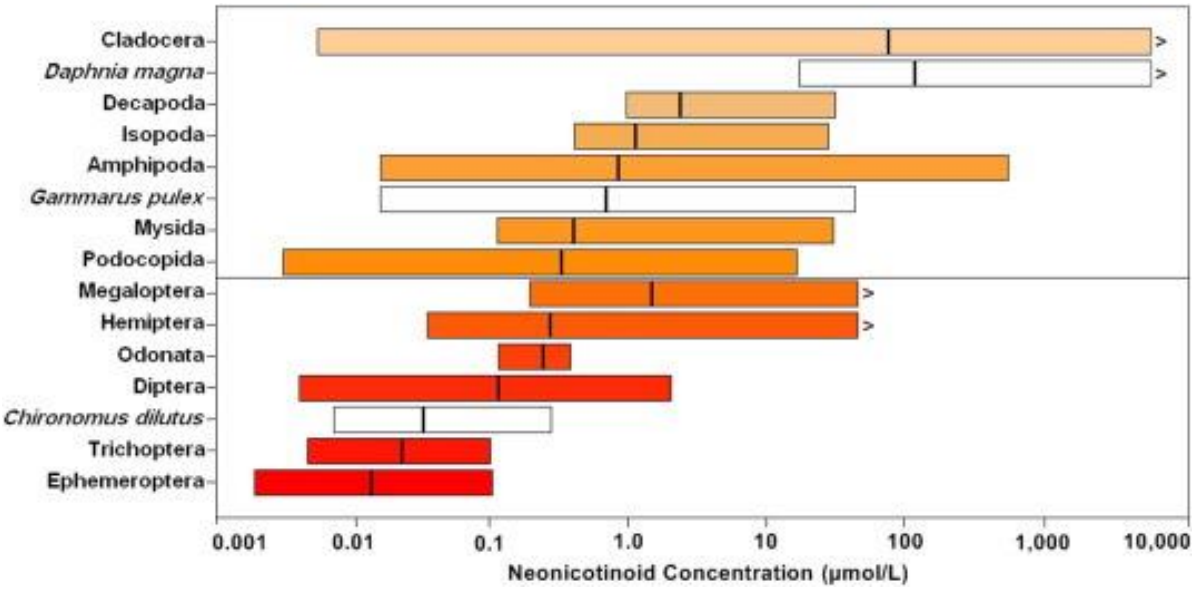

Figure 12. Range of neonicotinoid toxicity (L[E]C_{50}: 24–96 h in µmol/L, both lethal and sublethal values included) among all tested aquatic invertebrate orders. For context, three of the most common test species (white bars) for the orders Cladocera (*Daphnia magna*), Amphipoda (*Gammarus pulex*) and Diptera (*Chironomus dilutus*) are shown to illustrate differences in sensitivity by species. Vertical lines within bars represent geometric means of test values. Concentrations are given as molar equivalents µmol/L to standardise for the variable molecular weights of the different neonicotinoids. Back conversions to concentrations in µg/L (ppb) can be obtained by multiplying the molar concentration by the molar weight of the neonicotinoid compound. Reproduced from Morrissey *et al*. 2015

The Cladoceran water flea *D. magna* was the most commonly used model organism, represented in 34 of the 214 toxicity tests (16%). Its widespread use is because of its position as a global industry standard for the majority (82%) of commercial chemicals tested (Sánchez-Bayo 2006). It shows a wide variation in sensitivity to neonicotinoids but the mean short term L[E]C_{50} is at least two to three

orders of magnitude greater than for all other tested invertebrate groups (Figure 12). This has been highlighted by several authors (e.g. Beketov and Liess 2008) who argue that given the low sensitivity of *D. magna* to neonicotinoids, a different model organism such as a Dipteran should be selected when conducting tests on this class of pesticide. This is illustrated by the most recent study to calculate LC_{50}s for a range of aquatic invertebrates that was not included in Morrissey's review. de Perre *et al*. (2015) found no sublethal or lethal effects of clothianidin on *D. magna* at concentrations of over 500 µg/L. In contrast, *C. dilutus* showed EC_{50} effects at 1.85 µg/L and LC_{50} effects at 2.32 µg/L, in line with previous findings (Figure 12).

Kunce *et al*. (2015) also investigated the impacts of neonicotinoids on the similar *C. riparius*. First instar midge larvae were exposed to thiacloprid and imidacloprid at 50% of the 96-h LC_{50}s reported in the literature, corresponding to 2.3 µg/L for thiacloprid and 2.7 µg/L for imidacloprid. Three day old larvae were pulse exposed to these concentrations for 1 hour then transferred to clean water and allowed to develop normally. The one hour exposure to thiacloprid significantly decreased the proportion of larvae surviving to adulthood from 94% in the control to 68%. However, imidacloprid alone and thiacloprid and imidacloprid combined had no observable effect. No difference on adult egg production levels was detected.

These recent studies in conjunction with the review of Morrissey *et al*. strongly support the position that insect larvae are most sensitive to neonicotinoids in aquatic environments. Morrissey *et al*. conclude that chronic neonicotinoid concentrations of over 0.035 µg/L or acute concentrations of over 0.200 µg/L can affect the most sensitive aquatic invertebrate species. This finding is consistent with the value suggested by Vijver and van der Brink (2014) of 0.013-0.067 µg/L for imidacloprid. A number of water quality reference values have been published by governmental regulatory bodies and independent researchers in Europe and North America (Table 8). Most of these studies are based on assessments for imidacloprid only. Values for acceptable long term concentrations vary by three orders of magnitude from 0.0083 µg/L in the Netherlands (RIVM 2014; Smit *et al*. 2014) to 1.05 µg/L in the USA. There is considerable difference in the methodologies used to calculate these reference values, with the US EPA value likely to have been strongly based on results from *D. magna*, a species known to have relatively low sensitivity to neonicotinoids (Morrissey *et al*. 2015).

Current levels of neonicotinoids in aquatic habitats regularly exceed this threshold. Morrissey *et al*. reviewed 29 studies from nine countries and found geometric mean surface water concentrations of 0.130 µg/L (73.6%, 14/19 studies over 0.035 µg/L threshold) with geometric mean peak surface water concentration of 0.630 µg/L (81.4% 22/27 studies over 0.200 µg/L). Studies published since 2015 that are not included in Morrissey's review have also reported average neonicotinoid levels exceeding this threshold (see Section 2.2.2). Qi et al. (2015) and Sadaria *et al*. (2016) found levels of neonicotinoids above the threshold in influent and effluent wastewater at processing plants in the China and the USA. Benton *et al*. (2015) found average and peak imidacloprid levels above the thresholds in Appalachian streams in the USA. In contrast, low average levels of neonicotinoids were found in standing water and ditches on arable land in Ontario, Canada (Schaafsma *et al*. 2015) and in Iowan wetlands in the USA (Smalling *et al*. 2015). de Perre *et al*. (2015) found peak concentrations of 0.060 µg/L of clothianidin in groundwater below maize fields shortly after crop planting. In a nationwide study, Hladik and Kolpin (2016) found arithmetic mean neonicotinoid concentrations in streams across the USA to be just below the chronic threshold at 0.030 µg/L. However, peak concentration was 0.425 µg/L. Székács *et al*. (2015) also conducted a nationwide survey of Hungarian watercourses, finding clothianidin at concentrations of 0.017-0.040 µg/L and thiamethoxam at concentrations of 0.004-0.030 µg/L. The highest concentrations, of 10-41 µg/L, were only found in temporary shallow waterbodies after rain events in early summer.

Table 8. Summary of published ecological quality reference values for neonicotinoids (imidacloprid except this review) in freshwater environments against which average (chronic or long-term) or maximum (acute or peak) exposure concentrations are to be compared. Reference values are placed in descending order. Reproduced from Morrissey *et al*. (2015)

Source	Average concentration (µg/L)	Maximum concentration (µg/L)	Justification
EPA (2014) USA	1.05	35.0	Aquatic life benchmark – methodology uncertain
CCME (2007) Canada	0.23		EC_{15} for the most sensitive of two freshwater species tested with assessment factor of 10 applied
EFSA (2008) European Union		0.2	No Observable Effect Concentration (NOEC) (0.6 µg/L) from a 21 d German microcosm study to which an assessment factor of 1–3 has been applied based on expert deliberations
RIVM (2008) Netherlands	0.067		Maximum permissible concentration (MPC) for long term exposure derived from the lowest NOEC value for chronic toxicity studies with assessment factor of 10 applied
Morrissey *et al*. (2015)	0.035	0.2	Lower confidence interval of HC_5 from SSDs generated using 137 acute (LC_{50}) and 36 chronic ($L[E]C_{50}$) toxicity tests considering all neonicotinoid compounds weighted and standardized to imidacloprid and all available test species
RIVM (2014) Netherlands (see Smit *et al*. 2014)	0.0083		Updated MPC for long-term exposure derived from chronic studies using species sensitivity distribution (SSD) approach and Hazard Concentration (HC_5) applied to NOEC/LC_{10}/EC_{10} values with assessment factor of 3 applied
Mineau and Palmer (2013)	0.0086 or 0.029		The higher of two empirically-determined acute–chronic ratios applied to the most sensitive of 8 aquatic species tested to date; or HC_5 from SSD applied using NOECs from chronic studies of 7 single species and 1 species assemblage

Combining these recent studies with those included in Morrissey's 2015 review a total of 65.3% of studies (17/26) report average neonicotinoid concentrations of over the 0.035 µg/L chronic threshold and 73.5% of studies (25/34) report peak concentrations over the 0.200 µg/L acute threshold. The number of countries that have been studied and their widespread distribution (Australia, Brazil, Canada, China, Hungary, Japan, the Netherlands, Sweden, Switzerland, the United States and Vietnam) indicates the widespread contamination of watercourses of all kinds with levels of neonicotinoids known to be harmful to sensitive aquatic invertebrates. This is now a chronic global problem, likely to be impacting significantly on aquatic insect abundance and on food availability for their predators, including fish, birds and amphibians.

3.5 Sensitivity of birds and bats to neonicotinoids

Gibbons *et al*. (2015) reviewed the direct and indirect effects of neonicotinoids and fipronil on vertebrate wildlife including mammals, fish, birds, amphibians and reptiles. LD_{50} values for imidacloprid, clothianidin and fipronil are available for 11 species of bird (Table 9). There is considerable variation in the lethality of these compounds to birds, both between bird species and pesticide type. Using US EPA (2012) classifications for toxicity (see legend for Table 9), imidacloprid ranged from moderately toxic to highly toxic, clothianidin from practically non-toxic to moderately toxic and fipronil from practically non-toxic to highly toxic.

Table 9. Single (acute) dose LD_{50} for bird species (mg/kg, equivalent to ppm) for imidacloprid, clothianidin and fipronil. Toxicity classification follows US EPA (2012): *PNT* practically non-toxic, *ST* slightly toxic, *MT* moderately toxic, *HT* highly toxic, *VHT* very highly toxic. For birds: *PNT* >2,000, *ST* 501–2,000, *MT* 51–500, *HT* 10–50, *VHT* <10. Reproduced from Gibbons *et al*. (2015)

Species	Pesticide	LD_{50}	Reference
Mallard, *Anas platyrhynchos*	Imidacloprid	283 (MT)	Fossen (2006)
Grey partridge, *Perdix perdix*	Imidacloprid	13.9 (HT)	Anon (2012)
Northern bobwhite quail, *Colinus virginianus*	Imidacloprid	152 (MT)	SERA (2005)
Japanese quail, *Coturnix japonica*	Imidacloprid	31 (HT)	SERA (2005)
Feral pigeon, *Columba livia*	Imidacloprid	25-50 (HT)	SERA (2005)
House sparrow, *Passer domesticus*	Imidacloprid	41 (HT)	SERA (2005)
Canary, *Serinus canaria*	Imidacloprid	25-50 (HT)	SERA (2005)
Mallard, *Anas platyrhynchos*	Clothianidin	>752 (ST)	European Commission (2005)
Northern bobwhite quail, *Colinus virginianus*	Clothianidin	>2,000 (PNT)	Mineau and Palmer (2013)
Japanese quail, *Coturnix japonica*	Clothianidin	423 (MT)	Mineau and Palmer (2013)
Mallard, *Anas platyrhynchos*	Fipronil	2,150 (PNT)	Tingle *et al*. (2003)
Ring-necked pheasant, *Phasianus colchicus*	Fipronil	31 (HT)	Tingle *et al*. (2003)
Red-legged partridge, *Alectoris rufa*	Fipronil	34 (HT)	Tingle *et al*. (2003)
Northern bobwhite quail, *Colinus virginianus*	Fipronil	11.3 (HT)	Tingle *et al*. (2003)
Feral pigeon, *Columba livia*	Fipronil	>2,000 (PNT)	Tingle *et al*. (2003)
Field sparrow, *Spizella pusilla*	Fipronil	1,120 (ST)	Tingle *et al*. (2003)
Zebra finch, *Taeniopygia guttata*	Fipronil	310 (MT)	Kitulagodage *et al.* (2008)

Many of these studied species are granivorous and can be expected to feed on sown seeds shortly after the sowing period. Depending on crop species and consequent seed size, neonicotinoid-treated seeds can contain between 0.2-1 mg of active ingredient per seed. Goulson (2013) calculated that a granivorous grey partridge weighing 390 g would need to consume around five maize seeds, six sugar beet seeds or 32 oilseed rape seeds to receive a nominal LD_{50}. Based on US Environmental Protection Agency estimates that around 1% of sown seed is accessible to foraging vertebrates at recommended sowing densities, Goulson calculated that sufficient accessible treated seed would be present to deliver a LD_{50} to ~100 partridges per hectare sown with maize or oilseed rape. Given that grey partridges typically consume around 25 g of seed a day there is the clear potential for ingestion of neonicotinoids by granivorous birds. However, no studies are available that demonstrate consumption of treated seed by farmland birds under field conditions or quantify relative consumption of treated versus untreated seed. More work is needed in this area to better understand total neonicotinoid exposure via this route.

In addition to lethal effects, several studies have identified sublethal effects of neonicotinoid ingestion on birds (Table 10). House sparrows can become uncoordinated and unable to fly, and

studies of Japanese quail and red-legged partridges have reported DNA breakages and a reduced immune response, respectively. Many of these sublethal effects occur at lower concentrations than the lethal dose. A single oral dose of 41 mg/kg of imidacloprid will cause mortality in house sparrows, a substantially lower dose (6 mg/kg) can induce uncoordinated behaviour and an inability to fly (Cox 2001). While imidacloprid is highly toxic to Japanese quail, with an LD_{50} of 31 mg/kg, chronic daily doses of only 1 mg/kg/day can lead to testicular anomalies, DNA damage in males, and reductions in embryo size when those males are mated with control females (Tokumoto *et al*. 2013).

Table 10. Other studies of the direct effects of imidacloprid, clothianidin and fipronil on birds. Exposure could either be acute or chronic, the latter shown as /day (per day). All studies demonstrated deleterious effects at the given dosage, except those marked NE (no effect). Reproduced from Gibbons *et al*. (2015)

Species	Effect on:	Imidacloprid	Clothianidin	Fipronil	Source and detailed effect
Mallard, *Anas platyrhynchos*	Reproduction	16 mg/kg/day	>35 mg/kg/day (NE)		Adapted from figures in Mineau and Palmer (2013); various effects on reproduction
Chicken, *Gallus gallus domesticus*	Growth and development			37.5 mg/kg	Kitulagodage *et al.* (2011a); reduced feeding and body mass, and developmental abnormalities of chicks
Chicken, *Gallus gallus domesticus*	Neurobehavioural			37.5 mg/kg	Kitulagodage *et al.* (2011a); behavioural abnormalities of chicks
Red-legged partridge, *Alectoris rufa*	Survival	31.9-53.4 mg/kg/day			Lopez-Antia *et al.* (2013); reduced chick survival at low dose, and reduced adult survival at high dose
Red-legged partridge, *Alectoris rufa*	Reproduction	31.9 mg/kg/day			Lopez-Antia *et al.* (2013); reduced fertilisation rate and chick survival
Red-legged partridge, *Alectoris rufa*	Immunotoxic	53.4 mg/kg/day			Lopez-Antia *et al.* (2013); reduced immune response
Northern bobwhite quail, *Colinus virginianus*	Reproduction		>52 mg/kg/day		Adapted from figures in Mineau and Palmer (2013); various effects on reproduction
Northern bobwhite quail, *Colinus virginianus*	Growth and development	24 mg/kg/day[a]		11 mg/kg[b]	[a]Adapted from figures in Mineau and Palmer (2013); various effects on weight [b]Kitulagodage *et al*. (2011b); birds stopped feeding so lost weight
Japanese quail, *Coturnix japonica*	Reproduction	1 mg/kg/day			Tokumoto *et al.* (2013); testicular anomalies; reductions in embryo length when those males mated with un-dosed females
Japanese quail, *Coturnix japonica*	Genotoxic	1 mg/kg/day			Tokumoto *et al*. (2013); increased breakage of DNA in males
House sparrow, *Passer domesticus*	Neurobehavioural	6 mg/kg			Cox (2001); in-coordination, inability to fly
Zebra finch, *Taeniopygia guttata*	Reproduction			>1 mg/kg	Kitulagodage *et al.* (2011a); reduced hatching success

In addition to the studies reviewed by Gibbons *et al*., one additional study is available that assessed the impact of neonicotinoid ingestion on birds. Lopez-Anita *et al*. (2015) fed red-legged partridge *Alectoris rufa* imidacloprid-treated wheat seeds for a period of 25 days in the autumn and an additional period of 10 days in the spring, matching the pattern of cereal cropping in Spain. One treatment contained seeds treated at the recommended dosage rate and the second at 20% of the recommended rate, to mimic a diet comprised 20% of treated seeds. Treated seeds contained concentrations of imidacloprid of 0.14-0.7 mg/g at the two dose rates. As the 400 g partridges used in this study consume around 25 g of seeds a day, a daily ingestion of 8.8 and 44 mg/kg/day was expected, above the LD_{50} for Japanese quail (Table 9, SERA 2005).

Imidacloprid at the highest dose killed all adult partridges in 21 days, with first deaths occurring on day three. Mortality in the low dose and control groups was significantly lower at 18.7% and 15.6% respectively. As all partridges in the high dose died, effects on reproductive output were only measured in the low dose treatment. Compared to controls, low dose females laid significantly smaller clutches, and the time to first egg laying was also significantly increased. There was no difference in egg size, shell thickness, fertile egg rate and hatching rate. There was no detectable impact on chick survival, chick growth or sex ratio between these two groups. These results are in line with previous findings for lethal (Table 9) and sublethal (Table 10) effects of neonicotinoid consumption by birds. Whilst LD_{50}s vary across two orders of magnitude from 11.3->2,000 mg/kg, sublethal effects are seen across a more consistent range of doses over one order of magnitude between 1-53 mg/kg. The greatest outstanding issue is that no data exist that quantify the actual exposure rate to granivorous birds from neonicotinoid-treated seeds. As such, it is difficult to judge whether these clearly demonstrated lethal and sublethal effects are manifested in wild bird populations in the field.

In addition to sublethal and lethal effects potentially caused by the ingestion of neonicotinoids from treated seeds, bird populations may also be affected by a reduction in invertebrate prey. Hallmann *et al*. (2014) used bird population data from the Dutch Common Breeding Bird Monitoring Scheme, a standardised recording scheme that has been running in the Netherlands since 1984. Surface water quality measurements are also regularly collected across the Netherlands, including data on imidacloprid levels. Hallmann *et al*. compared surface water imidacloprid levels between 2003-2009 with bird population trends for 15 farmland bird species that are insectivorous at least during the breeding season to assess the hypothesis that neonicotinoids may cause bird population declines through a reduction in invertebrate food availability. The average intrinsic rate of increase in local farmland bird populations was significantly negatively affected by the concentration of imidacloprid. At the individual level, 14 of the 15 bird species showed a negative response to imidacloprid concentrations, with 6 out of 15 showing a significant negative response. As previously discussed in Section 3.2, it is difficult to disentangle the effects of neonicotinoids from the effects of general agricultural intensification. Hallmann *et al*. attempt to control for proxy measures of intensification including changes in land use area, areas of cropped land and fertiliser input, but imidacloprid levels remained a significant negative predictor.

The only available study that has quantified changes in invertebrate prey availability after neonicotinoid treatment and concurrent changes in the bird community was conducted in the USA. Falcone and DeWald (2010) measured invertebrates in eastern hemlock *Tsuga canadensis* forests in Tennessee after trees has been treated with imidacloprid to control hemlock woolly adelgid *Adelges tsugae*. The imidacloprid treatment had a significantly negative effect on non-target Hemiptera and larval Lepidoptera. However, there was no corresponding decline in insectivorous bird density between treatments. Direct comparison between this study and the findings of Hallmann *et al*. 2014 are difficult due to the very different ecological conditions. It is likely sufficient untreated areas existed in hemlock forests for insectivorous birds to find sufficient forage. In the Netherlands, one of the most agriculturally intensified regions in the world, unaffected semi-natural habitat is scarce and a reduction in prey availability caused by neonicotinoid application would have a more severe impact.

No studies are available that measure the effect of neonicotinoids on bats and bat populations. A link between neonicotinoid use and declining farmland butterfly populations has been suggested (Gilburn *et al*. 2015; Forister *et al*. 2016) and given the ecological similarity between butterflies and moths a similar trend may be ongoing, though this has not yet been investigated. Many bat species

feed on moths, so a reduction in the moth population is likely to impact bat populations through a reduction in food availability. Mason *et al*. (2014) link neonicotinoid use with an increase in the frequency of bat diseases such as White Nose Syndrome (caused by the fungus *Geomyces destructans*) in both the US and Europe. They hypothesise that consumption of neonicotinoid residues in insect prey weakens the immune system of bats. However, no evidence is presented demonstrating the presence of neonicotinoid residues in moths or bats, passage across these trophic levels or that exposure to neonicotinoids weaken the immune system of bats, resulting in increased rates of fungal infection. The position of Mason *et al*. must currently be considered unsupported.

3.6 Synergistic effects of additional pesticides with neonicotinoids

The EFSA (2013a; 2013b; 2013c) risk assessments for clothianidin, imidacloprid and thiamethoxam considered these pesticides and their impacts on honeybees individually. In the field, multiple neonicotinoids, other insecticides and other pesticides such as herbicides and fungicides are commonly applied to a single crop. Bees are frequently exposed to complex mixtures of pesticides, with 19 detected in trap caught bees from an agricultural region of Colorado (Hladik *et al*. 2016). It is possible that combinations of neonicotinoids and other pesticides may have antagonistic (become less effective), additive (equivalent to adding together existing effectiveness) or synergistic (multiplicative) effects. Morrissey *et al*. (2015) briefly listed known examples of synergistic effects between neonicotinoids and other pesticides. Several examples have been demonstrated by pesticide companies themselves. For example, Bayer demonstrated that the combination of clothianidin and the fungicide trifloxystrobin resulted in a 150-fold increase in kill rate to *Phaedon* leaf beetle larvae over clothianidin alone (Wachendorff-Neumann *et al.* 2012). Bayer scientists also demonstrated that treatments of 8,000 ppb of thiacloprid and 8,000 ppb of clothianidin resulted in aphid population kill rates of 25% and 0% after 6 days. Combining the two increased the kill rate to 98% (Andersch *et al.* 2010). Specifically for honeybees, Iwasa *et al*. (2004) demonstrated that the combination of thiacloprid with the fungicide propiconazole increased the toxicity of the mixture several hundred fold. Whilst synergies have been demonstrated, few environmental risk assessments have been made for neonicotinoids in combination with other pesticides.

Since 2013, a number of studies have investigated possible synergistic effects in neonicotinoids. Several have focussed on the interaction between neonicotinoids and ergosterol biosynthesis inhibitor (EBI) fungicides (which include propiconazole) and their impact on bees. Biddinger *et al*. (2013) studied the interaction between the contact toxicity of acetamiprid, imidacloprid and the fungicide fenbuconazole, a substance virtually non-toxic to bees (except at extremely high concentrations), using *A. mellifera* and Japanese orchard bees *Osmia cornifrons*. These pesticides are commonly found together in formulated products used in orchards. The doses ranged from 1.38-60 µg/bee 1:1 acetamiprid plus fenbuconazole mixture and 0.86-983 µg/bee 2:1 imidacloprid plus fenbuconazole mixture. At the LD_{50}, the acetamiprid and fenbuconazole mixture was ~5 times more toxic than acetamiprid alone for *A. mellifera* and ~2 times more toxic than acetamiprid for *O. cornifrons*. However, these doses are exceptionally high, for example the 0.86 µg/bee imidacloprid:fenbuconazole mixture is equivalent to 567.6 ng/bee, with the *A. mellifera* contact toxicity to imidacloprid LD_{50} calculated as 81 ng/bee (Section 3.1). Unsurprisingly, this dose killed 85% of honeybee in this treatment. At unrealistically high concentrations it is not clear how informative these results are.

Thompson *et al*. (2014) investigated synergies between several EBI fungicides (flusilazole, propiconazole, myclobutanil and tebuconazole) and a range of neonicotinoids (clothianidin, thiacloprid, imidacloprid and thiamethoxam) on *A. mellifera*. Individual pesticides and mixtures of one neonicotinoid and one fungicide were administered through both contact and ingestion at a range of concentrations sufficient to increase mortality and bees were observed for a 96 hour period. LD_{50}s were calculated after 48 hours as mortality did not significantly increase after this point. Single neonicotinoid and fungicide doses showed similar toxicity to previous published results, with no individual fungicide causing toxic effects even at concentrations of 22.4 µg/bee.

For neonicotinoid/fungicide mixtures, neonicotinoids were applied at calculated LD_{50}s, in the region of 0.035-0.124 µg/bee for clothianidin, imidacloprid and thiamethoxam and 122.4 µg/bee for thiacloprid (cyano-substituted neonicotinoids having lower toxicity to bees, Section 3.1.1). Fungicides were applied at doses of between 0.161 and 0.447 µg/bee depending on the particular

compound. These values of were calculated as realistic worst-case exposures based on approved application rates for UK crops. For these mixtures, a synergy ratio was calculated where the LD_{50} of the neonicotinoid was divided by the LD_{50} of the neonicotinoid plus fungicide mixture. Consequently, a value of over one indicates the mixture was more toxic and a value under one indicates the mixture was less toxic. Combinations of fungicides with thiacloprid and clothianidin showed negligible synergy for contact toxicity, with an average synergism ratio of 0.30 and 1.07 respectively. Imidacloprid and thiamethoxam were higher at 1.53 and 2.02. For oral toxicity, thiacloprid and imidacloprid showed low synergy at 0.60 and 0.48 whereas clothianidin and thiamethoxam were higher at 1.52 and 1.31 respectively. Only two combinations showed significant synergy, for a contact dose of tebuconazole and thiamethoxam with a synergy of 2.59 and for an oral dose of clothianidin and tebuconazole at a synergy of 1.90.

Sgolastra *et al*. (2016) investigated the interaction between clothianidin and the fungicide propiconazole in three bee species, *A. mellifera*, *B. terrestris* and *O. bicornis*. Each species was administered a LD_{10} dose of clothianidin (0.86, 1.87 and 0.66 ng/bee respectively, see Section 3.1.1 for more detail), a non-lethal dose of propiconazole (7 µg/bee) and a combination of the two treatments. Bees were then observed for a 96 hour period and mortality quantified. Some synergistic effects were seen. In *A. mellifera*, mortality was significantly higher for the combined dose in the first two time periods (4 and 24 hours). Mortality in *B. terrestris* for the combined dose was only significantly higher in the first time period, after 4 hours. However, in *O. bicornis*, exposure to the combination of clothianidin and propiconazole resulted in significantly higher mortality at all time points (Figure 13).

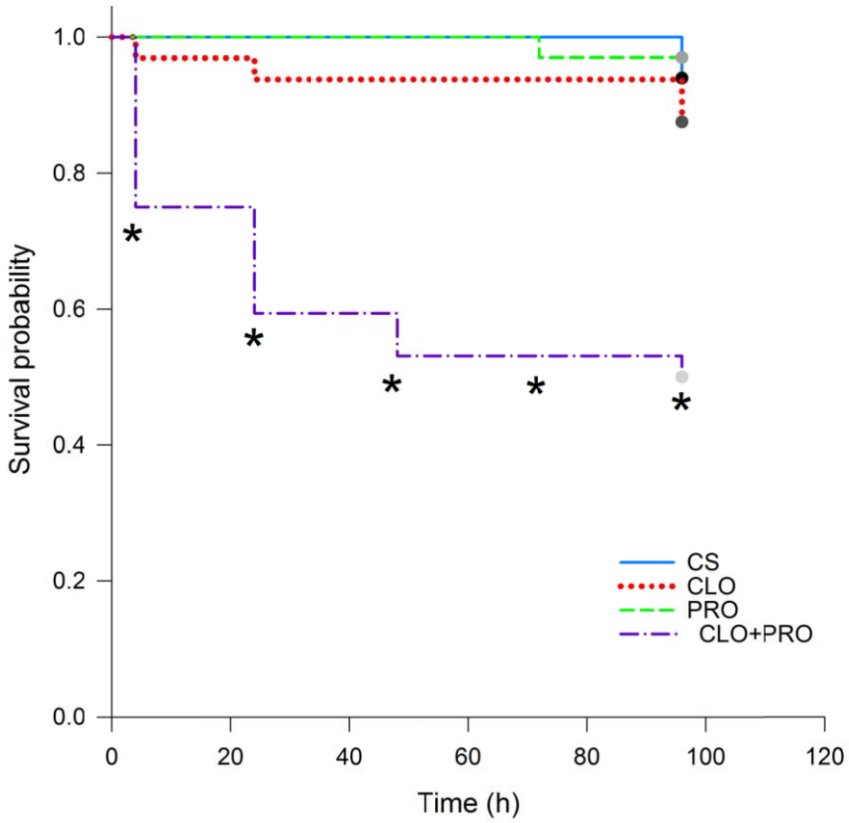

Figure 13. Cumulative proportion of surviving *Osmia bicornis* females exposed to a control solution (CS – sugar water solution with 3% acetone), clothianidin (CLO – 0.63 ng/bee) propiconazole (PRO – 7 µg/bee), and clothianidin plus propiconazole (CLO+PRO – 0.63 ng/bee plus 7 µg/bee). Statistically

significant synergistic effects at the various assessment times (4, 24, 48, 72, 96 h) are marked with an asterisk

Spurgeon *et al.* (2016) conducted similar experiments to Sgolastra *et al*., investigating the effect of a combination of clothianidin and propiconazole on *A. mellifera*, *B. terrestris* and *O. bicornis*. In order to calculate an LD_{50}, clothianidin concentrations were varied and propiconazole concentrations were held at zero, a low dose and a high dose. The low dose was taken from the EFSA Panel on Plant Protection Products (2012) reported environmental concentrations, and the high dose was 10 times the low dose to represent a plausible worst case scenario, but it is not clear what these values actually are. Mortality was quantified over 48, 96 and 240 hours. For *A. mellifera*, clothianidin LC_{50}s with and without propiconazole were always within a factor of 2, with no clear negative trend at higher propiconazole concentrations. For *B. terrestris*, clothianidin LC_{50}s with propiconazole were between 1.5 to 2 fold lower. For *O. bicornis*, clothianidin LC_{50}s with propiconazole was up to 2 fold lower with a negative trend as propiconazole concentrations increased. Spurgeon *et al.* concluded that the clothianidin and propiconazole combination had no to slight synergy for *A. mellifera* and slight to moderate synergy for *B. terrestris* and *O. bicornis*.

In an additional trial, Thompson *et al*. (2014) demonstrated that the dose of fungicide applied is a key factor determining neonicotinoid toxicity using propiconazole and thiamethoxam mixtures (Table 11). The authors argue that their low rates of significant synergies between neonicotinoids and fungicides was because of their lower, more field-realistic fungicide doses of 0.161-0.447 µg/bee compared to 10 µg/bee used by Iwasa *et al.* (2004), an early study demonstrating this interaction. The values of 0.161-0.447 µg/bee were calculated as realistic worst-case exposures based on approved application rates for UK crops. However, data are lacking demonstrating true field-realistic exposure rates to fungicides for free flying bees. Whilst studies such as Sgolastra *et al*. (2016) show a clear synergistic effect between fungicides and neonicotinoids on *O. bicornis*, the dose of fungicide used is more than an order of magnitude greater than that used by Thompson *et al*. Bees are consistently exposed to fungicides with 40 types found in honeybee pollen, wax and nectar (Sánchez-Bayo and Goka 2014). Pollen collected by bumblebees and stored in their nests has also been found to contain fungicides at average concentrations between 0.15-25 ppb (EBI fungicides 0.15-17 ppb, David *et al*. 2016). However, almost nothing is known about how concentrations present in bee-collected material translate into acute or chronic exposure to bees. It is currently not possible to comment on what fungicide doses represent a realistic situation that bees are likely to encounter in the wild.

Table 11. Comparison of the ratio of propiconazole to the doses of thiamethoxam and the resultant LD_{50} in the contact and oral studies. Synergy ratios marked with an * were significantly different. Reproduced from Thompson *et al*. (2014).

Contact dose propiconazole µg/bee	Ratio fungicide: thiamethoxam contact LD_{50}	Contact LD_{50} thiamethoxam µg/bee	Synergy ratio	Ratio fungicide: thiamethoxam oral LD_{50}	Oral LD_{50} thiamethoxam µg/bee	Synergy ratio
0	-	0.0373	-	-	0.0641	-
0.0224	0.6	0.0288	1.3	0.349	0.0268	2.4
0.224	6	0.0247	1.5	3.49	0.0277	2.3
2.24	60	0.0134	2.8*	34.9	0.0265	2.4
22.4	600	0.0104	3.6*	349	0.00776	8.3*

In addition to work on bees, Kunce *et al*. (2015) investigated the impact of one hour pulse exposure of imidacloprid and thiamethoxam and two pyrethroids, deltamethrin and esfenvalerate in single, pairwise and combined doses on the development of the aquatic midge *C. riparius* (see Section 3.4 for more methodological and concentration details). Most pesticide treatments reduced the survival of the larvae, but the deleterious effects did not appear to be synergistically amplified by a combination of pesticides. Kunce *et al*. conclude that at the low doses and period of exposure used, the risk of synergistic or additive effects is very low. Much more work on the potential synergistic effects of pesticides in aquatic ecosystems is required.

Overall, these studies support the position that neonicotinoids can act synergistically with fungicides, increasing their lethality to bees. However, the dose rate of both neonicotinoids and fungicides, time of exposure, neonicotinoid and fungicide chemical class and length of time after exposure are all important explanatory factors affecting this relationship. The concentration of fungicide used in laboratory studies appears to be the most important factor determining synergistic lethality. Fungicides are regularly sprayed during the period when flowering crops are in bloom under the assumption that these compounds are safe for bees. Further work is needed in this area to establish realistic levels of fungicide exposure for free flying bees in order to assess the likely impact of neonicotinoid/fungicide synergies on bee populations.

Studies to date have only examined pairwise interactions between pesticides. It is clear that bees and other non-target organisms inhabiting farmland are routinely exposed to far more complex cocktails of pesticides than any experimental protocol has yet attempted to examine. For example, honeybee and bumblebee food stores commonly contain 10 or more pesticides (e.g. David *et al*. 2016). A major challenge for scientists and regulators is to attempt to understand how chronic exposure to complex mixtures of neonicotinoids and other chemicals affects wildlife.

4. CONCLUDING REMARKS

4.1 Advances in scientific understanding and comparison with the 2013 knowledge base

The EFSA reports into clothianidin, imidacloprid and thiamethoxam are naturally narrow in scope, focusing specifically on the risks that these neonicotinoids pose to bees, with almost all data consisting of and referring to the honeybee *Apis mellifera*. Because the scope of this review is much wider, focusing on neonicotinoid persistence in the wider environment and possible impacts on many non-target organisms, a simple comparison with the EFSA reports is not possible as there is no well-defined baseline of existing knowledge prior to 2013 for most topic areas. However, it is possible to comment on the change in the scientific evidence since 2013 compared to the EFSA reports. This process is not meant to be a formal assessment of the risk posed by neonicotinoids in the manner of that conducted by EFSA. Instead it aims to summarise how the new evidence has changed our understanding of the likely risks to bees; is it lower, similar or greater than the risk perceived in 2013. With reference to the EFSA risk assessments baseline, advances in each considered area and their impact on the original assessment can be briefly summarised thus:

- *Risk of exposure from pollen and nectar of treated flowering crops.* The EFSA reports calculated typical exposure from flowering crops treated with neonicotinoids as seed dressings. Considerably more data are now available in this area, with new studies broadly supporting the calculated exposure values. For bees, flowering crops pose a **Risk Unchanged** to that reported by EFSA 2013.
- *Risk from non-flowering crops and cropping stages prior to flowering*. Non-flowering crops were considered to pose no risk to bees. No new studies have demonstrated that these non-flowering crops pose a direct risk to bees. They remain a **Risk Unchanged.**
- *Risk of exposure from the drilling of treated seed and subsequent dust drift*. Despite modification in seed drilling technology, available studies suggest that dust drift continues to occur, and that dust drift still represents a source of acute exposure and so is best considered a **Risk Unchanged.**
- *Risk of exposure from guttation fluid*. Based on available evidence this was considered a low-risk exposure path by EFSA 2013. New data have not changed this position and so it remains a **Risk Unchanged.**
- *Risk of exposure from and uptake of neonicotinoids in non-crop plants*. Uptake of neonicotinoids by non-target plants was considered likely to be negligible, though a data gap was identified. Many studies have since been published demonstrating extensive uptake of neonicotinoids and their presence in the pollen, nectar and foliage of wild plants, and this source of exposure may be much more prolonged than the flowering period of the crop. Bees collecting pollen from neonicotinoid-treated crops can generally be expected to be exposed to the highest neonicotinoid concentrations, but non-trivial quantities of neonicotinoids are also present in pollen and nectar collected from wild plants. Exposure from non-target plants clearly represents a **Greater Risk.**
- *Risk of exposure from succeeding crops*. A data gap was identified for this issue. Few studies have explicitly investigated this, but this area does represent some level of risk as neonicotinoids and now known to have the potential to persist for years in the soil, and can be detected in crops multiple years after the last known application. However, as few data exist this is currently considered a **Risk Unchanged.**
- *Direct lethality of neonicotinoids to adult bees.* Additional studies on toxicity to honeybees have supported the values calculated by EFSA. More data have been produced on neonicotinoid toxicity for wild bee species and meta-analyses suggest a broadly similar

response. Reference to individual species is important but neonicotinoid lethality should be broadly considered a **Risk Unchanged.**

- *Sublethal effects of neonicotinoids on wild bees.* Consideration of sublethal effects by EFSA was limited as there is no agreed testing methodology for the assessment of such effects. A data gap was identified. Exposure to neonicotinoid-treated flowering crops has been shown to have significant negative effects on free flying wild bees under field conditions and some laboratory studies continue to demonstrate negative effects on bee foraging ability and fitness using field-realistic neonicotinoid concentrations. **Greater Risk.**

Within this context, research produced since 2013 suggest that neonicotinoids pose a similar to greater risk to wild and managed bees, compared to the state of play in 2013. Given that the initial 2013 risk assessment was sufficient to impose a moratorium on the use of neonicotinoids on flowering crops, and given that new evidence either confirms or enhances evidence of risk to bees, it is logical to conclude that the current scientific evidence supports the extension of the moratorium.

In addition to the use of neonicotinoids on flowering crops, research since 2013 has demonstrated neonicotinoid migration into and persistence in agricultural soils, waterways and constituent parts of non-crop vegetation. Where assessments have been made of concentrations likely to significantly negatively affect non-target organisms, levels have been demonstrated to be above these thresholds in numerous non-crop agricultural habitats.

The strongest evidence for this is found in waterbodies surrounding agricultural areas, both temporary and permanent. The impact of neonicotinoids on aquatic organisms appears to be the easiest to quantify, as field-realistic concentrations can be easily obtained through sample collection and once neonicotinoids are present in waterbodies, aquatic organisms cannot limit their exposure to them. In contrast, assessing the field-realistic exposure of bees to neonicotinoids is much harder, as it will depend on numerous factors including but not limited to: the type of flowering crop, its relative attractiveness compared to existing available forage, the crop type and levels of neonicotinoid loss into the wider environment through seed dust and leaching, soil type and organic content and consequent retention of neonicotinoid active ingredient, uptake of neonicotinoids by surrounding vegetation and relative collection of pollen and nectar from various wild plants containing variable levels of neonicotinoids at different parts of the year. In addition, wild and managed bees have traits such as flight period, floral choice preferences and social structure that vary radically between different bee species, as can be clearly seen in the three most commonly used bee model organisms *A. mellifera*, *B. terrestris* and *O. bicornis*. As such, it is much more difficult to gain a completely accurate and consistent measure of neonicotinoid exposure for taxa such as these.

However, whilst these aforementioned factors are all important, it is still possible to comment on likely outcomes based on average exposure levels across a range of studies. This is as true for other taxa as it is for bees. Given these caveats, it is clear that since 2013, new research has substantially advanced our understanding of the effect of neonicotinoids on non-target organisms in the following areas:

- Non-flowering crops treated with neonicotinoids can pose a risk to non-target organisms through increasing mortality in beneficial predator populations.
- Neonicotinoids can persist in agricultural soils for several years, leading to chronic contamination and, in some instances, accumulation over time.

- Neonicotinoids continue to be found in a wide range of different waterways including ditches, puddles, ponds, mountain streams, rivers, temporary wetlands, snowmelt, groundwater and in outflow from water processing plants.
- Reviews of the sensitivity of aquatic organisms to neonicotinoids show that many aquatic insect species are several orders of magnitude more sensitive to these compounds than the traditional model organisms used in regulatory assessments for pesticide use.
- Neonicotinoids have been shown to be present in the pollen, nectar and foliage of non-crop plants adjacent to agricultural fields. This ranges from herbaceous annual weeds to perennial woody vegetation. We would thus expect non-target herbivorous insects and non-bee pollinators inhabiting field margins and hedgerows to be exposed to neonicotinoids. Of particular concern, this includes some plants sown adjacent to agricultural fields specifically for the purposes of pollinator conservation.
- Correlational studies have suggested a link between neonicotinoid usage in agricultural areas and population metrics for butterflies, bees and insectivorous birds in three different countries.

4.2 Existing knowledge gaps and future research

Whilst much research has been conducted on neonicotinoid pesticides and their impact on non-target organisms since 2013, a number of key knowledge gaps exist. As stated by Godfray *et al*. (2015) in their update on the existing scientific literature concerning neonicotinoids and insect pollinators, it is important to remember that major gaps in our understanding occur and different policy conclusions can be drawn depending on the weight given to important (but not definitive) scientific findings and the economic and other interests of different stakeholders. This review is not intended as a risk assessment, simply as a review of advances in our scientific understanding of the environmental risks that neonicotinoids pose.

From the perspective of better understanding the impacts of neonicotinoids on non-target organisms, further research is needed in the following areas:

- Whilst the impact of neonicotinoids on bees have been relatively well studied, few data exist for most taxa. The sensitivity of non-pest herbivorous taxa and important natural enemies of crop pests to neonicotinoids are particularly poorly understood.
- Continue to improve our understanding of realistic neonicotinoid and other pesticide exposure in agricultural and non-agricultural areas for understudied taxa. The implications of laboratory studies assessing the lethal and sublethal impacts of neonicotinoids are unclear without a realistic baseline for comparison with real world conditions. Data are most lacking for herbivorous, soil dwelling, parasitic and predatory invertebrates and granivorous and insectivorous terrestrial vertebrates.
- In addition to sensitivity and exposure, the movement of neonicotinoids through trophic levels is poorly understood with the exception of a few field studies which demonstrate the principle. Some authors have linked direct neonicotinoid exposure with declines in higher trophic level organisms, but little to no data exist regarding these claims.
- Long-term datasets exist that have demonstrated recent population declines across various taxa, with the most pronounced declines correlating with neonicotinoid use. Whilst these studies are suggestive in their own right, the effects of general agricultural intensification relative to the effects of neonicotinoid pesticides must be teased apart if long term declines in taxa are to be better understood and reversed.

- Possible synergistic and additive effects of neonicotinoids with other pesticides are still poorly understood for bees, and almost nothing is known about their effects on other non-target taxa. This problem is compounded by a lack of understanding of field-realistic exposures to the various constituent active ingredients, with different taxa likely to be receiving different doses depending on their interaction with agricultural environments.

4.3 Closing statement

Recent work on neonicotinoids continues to improve our understanding of how these compounds move through and persist in the wider environment. These water soluble compounds are not restricted to agricultural crops, instead permeating most parts of the agricultural environments in which they are used and in some cases reaching further afield via waterways and runoff water. Field-realistic laboratory experiments and field trials continue to demonstrate that residual neonicotinoid traces can have a mixture of lethal and sublethal effects on a wide range of taxa. Relative to the risk assessments produced in 2013 for clothianidin, imidacloprid and thiamethoxam which focussed on their effects on bees, new research strengthens arguments for the imposition of a moratorium on their use, in particular because it has become evident that they pose significant risks to many non-target organisms, not just bees. Given the improvement in scientific knowledge of how neonicotinoids move into the wider environment from all crop types, a discussion on the risks posed by their use on non-flowering crops and in non-agricultural areas is urgently needed.

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Impacts of neonicotinoid use on long-term population changes in wild bees in England

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Wild bee declines have been ascribed in part to neonicotinoid insecticides. While short-term laboratory studies on commercially bred species (principally honeybees and bumblebees) have identified sub-lethal effects, there is no strong evidence linking these insecticides to losses of the majority of wild bee species. We relate 18 years of UK national wild bee distribution data for 62 species to amounts of neonicotinoid use in oilseed rape. Using a multi-species dynamic Bayesian occupancy analysis, we find evidence of increased population extinction rates in response to neonicotinoid seed treatment use on oilseed rape. Species foraging on oilseed rape benefit from the cover of this crop, but were on average three times more negatively affected by exposure to neonicotinoids than non-crop foragers. Our results suggest that sub-lethal effects of neonicotinoids could scale up to cause losses of bee biodiversity. Restrictions on neonicotinoid use may reduce population declines.

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Insect pollinators are estimated to support 9.5% of world food production[1] and wild bees have an important role in the delivery of this ecosystem service[2]. However, wild bees have undergone global declines that have been linked to habitat loss and fragmentation, pathogens, climate change and insecticides[3–7]. Recent debate about causal factors has focused on the role of neonicotinoid insecticides that are used worldwide as seed dressings to control pests of economically important crops[8–10]. The active compound of these insecticides is expressed systemically throughout plant tissues, leading to potential ingestion where honeybees[8] and wild bees[9–12] feed on the pollen and nectar of treated crops. The exposure risk to pollinators is large; in 2008 neonicotinoids comprised 80% of the worldwide insecticide seed treatment market (24% of the total insecticide market)[13].

The primary evidence for detrimental impacts of neonicotinoids is from small-scale or short-term exposure studies on bees that are commercially bred and thus suitable as model systems, principally honeybees (*Apis mellifera* L.), some bumblebees (notably *Bombus terrestris* L.) and solitary bees of the genus *Osmia* (for example, *O. bicornis* L.)[8–10,12]. Such studies have identified an 85% drop in queen production in *B. terrestris*[9] and a 50% reduction in the reproductive output of *O. bicornis*[14] following exposure to neonicotinoids. A recent large-scale field study conducted over a single year also identified negative impacts on the colony growth rate of *B. terrestris* and reductions in the densities of breeding *O. bicornis*[12]. In 2013, the European Union imposed a 2-year moratorium on the use of neonicotinoids to protect both domesticated and wild bees. This moratorium is scheduled to be formally reviewed in 2016, although exemptions to this ban have already been implemented in the UK.

While there is considerable experimental evidence for short-term sub-lethal effects of exposure to neonicotinoids for a few bee species, it remains unknown whether these findings can explain large-scale and long-term changes in wild bee distributions and community structure. The short-term nature of these experiments means that while they are appropriate for determining potential drivers of change and exploring underlying causal mechanisms, they cannot determine whether a particular driver is linked to bee declines over time scales relevant to population level processes[15]. However, long-term and spatially explicit distributional data exist in the UK and are suitable for addressing this question. These data have been collected mostly through volunteer surveys by skilled naturalists and collated by the Bees, Ants and Wasps Recording Society (http://www.bwars.com/). The data cover time scales relevant to population-level processes and are suited to understanding the impacts of historic changes in agricultural management.

This study tests whether commercial use of neonicotinoids on oilseed rape crops in England can be linked to bee declines in the wild at a national scale. Oilseed rape (*Brassica napus* L.) is one of the principal crops treated with neonicotinoids worldwide and is the main arable crop on which bees actively forage in the UK: the crop covers 8.2 million ha in Europe (34.1 million ha worldwide). We test the hypothesis that spatial and temporal variation in exposure to neonicotinoids applied to commercial oilseed rape crops was correlated with population extinctions of wild bees foraging on this crop. Our results provide the first evidence that sub-lethal impacts of neonicotinoid exposure can be linked to large-scale population extinctions of wild bee species, with these effects being strongest for species that are known to forage on oilseed rape crops. These results support the findings of previous studies on commercially bred pollinators that have identified the underlying mechanisms affecting mortality. This study extends existing evidence from a limited number of model species to the wider community of bees found in agricultural landscapes. These findings provide an important contribution to the evidence base underpinning the current moratorium on the use of this insecticide in the European Union.

Results

Multi-species dynamic Bayesian occupancy models. We constructed a multi-species dynamic Bayesian occupancy model[16–18] to assess change in the occurrence of 62 wild bee species in England over a 18 year period (1994–2011). We use this model to explore the relationship between population persistence and exposure to neonicotinoid-treated oilseed rape over this period. This time period was centered on the first wide-scale commercial use of neonicotinoid seed treatments on oilseed rape in 2002. This model included spatially and temporally explicit information describing the cover of oilseed rape[19], the area of the crop treated with neonicotinoids[20] and an index of the combined toxicity of all foliar-applied insecticides (referred to as the foliar insecticide impact (FII) index). Note that although the FII index includes a small number of neonicotinoid based foliar applied insecticides, their non-systemic mechanism of action makes their incorporation into this index appropriate. The model used in this analysis was hierarchical and incorporates an observation sub-model that accounts for bias associated with volunteer-collected data[21,22]. We restricted our analysis to 1 km^2 grid cells with surveys in at least two of the 18 years to produce a final data set that contains 31,818 surveys from 4,056 km^2, which were nested in 1,658 25 km^2 grid cells (Fig. 1). We excluded honeybees, since these are regularly moved across landscapes by beekeepers. Our analysis included wild bee species with records on at least 500 survey visits. Finally, we tested the prediction that bees known to forage on oilseed rape would be more likely to experience population extinctions due to higher neonicotinoid exposure than species not known to forage on this crop.

Responses to neonicotinoid seed treatments on oilseed rape. By grouping bees according to whether or not they forage on oilseed rape (foragers = 34 species; non-foragers = 28 species) we found

Figure 1 | The grid cells from which bee species distributional data were derived. These were used to assess the response of individual species to oilseed rape cover, neonicotinoid exposure and the FII index. All data were derived from the Bees, Ants and Wasps Recording Scheme. Scotland and Ireland were not included in the analysis.

substantial evidence for negative impacts of neonicotinoids on wild bees. Persistence was negatively correlated with neonicotinoid exposure for both oilseed rape foraging (mean = −1.37; 95% credible intervals (CI): −1.87, −0.89; >99.99% of the posterior distribution is below zero) and non-foraging species (mean = −0.46; 95% CI: −0.98, 0.09; 95.2% below zero) (Fig. 2a). The difference between the effect sizes of these two groups (0.91; 95% CI: 0.20, 1.64; 99.5% of the posterior is above zero) indicates that the negative effect of neonicotinoid exposure on persistence is three times greater for oilseed rape foragers than for non-foraging species. The difference in effect size is represented by the posterior distribution of effect sizes for oilseed rape foragers subtracted from the posterior distribution of effect sizes for non-foragers (Fig. 2a). When individual species occupancy from 1994 to 2010 was compared with occupancy predicted under the model where neonicotinoids were not used, it is clear that the detected loss of species occupancy was typically small (Figs 3 and 4). Therefore, while neonicotinoid seed treatments on oilseed rape are correlated with a reduction in population persistence for some wild bees this effect has not led to population extinction at a national scale. We estimate that neonicotinoid dose alone is responsible for a loss of greater than 20% of extant grid cells for *Halictus tumulorum*, *Lasioglossum fulvicorne*, *L. malachurum*, *L. pauxillum* and *Osmia spinulosa* since 2002 (>15% for 11 species, >10% for 24 species).

Responses to the cover of oilseed rape. Persistence was positively correlated with oilseed rape cover (OSR) for species that forage on the crop (mean = 1.06; 95% CI: 0.66, 1.49; >99.99% above zero), but not for other wild bee species (mean = −0.09; 95% CI: −0.52, 0.35; 67% below zero; Fig. 2b). This suggests that only oilseed rape foraging species benefit from the presence of oilseed rape in the landscape. Benefits of oilseed rape cover do not compensate for the negative impacts of neonicotinoid dose.

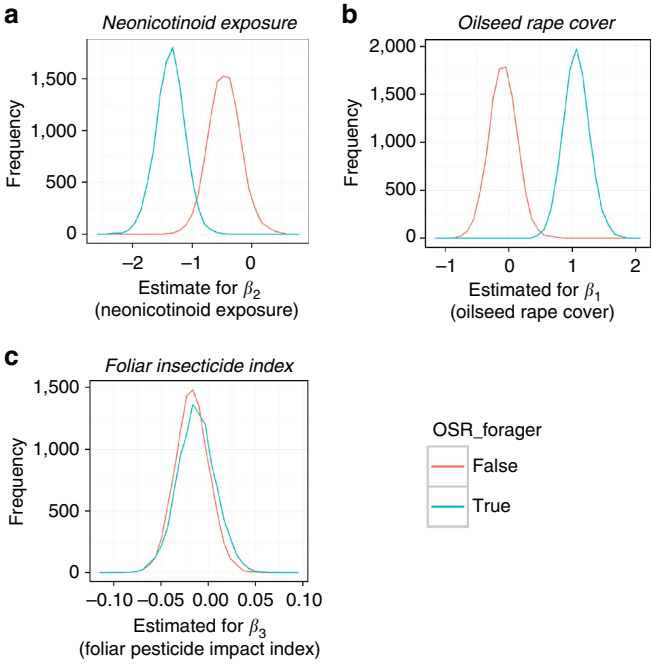

Figure 2 | Posterior distributions for the effect sizes describing wild bee population persistence in England. The posterior distributions show the probability of parameter estimates explaining wild bee population persistence for (**a**) neonicotinoid dose rate, (**b**) oilseed rape area and (**c**) the foliar insecticide index. Posterior distributions for oilseed rape foraging and non-foraging wild bee species are shown in blue and red respectively. Mean probabilities below zero suggest negative effects of these environmental factors. Supplementary Fig. 1 provides the precision for these parameter estimates.

Responses to foliar applied insecticides on oilseed rape. For groups of wild bee species we found weak negative correlations between their persistence and the application of foliar applied pesticides (foragers: mean = −0.017; 95% CI: −0.051 0.018; 83% below zero; non-foragers: mean = −0.013; 95% CI: −0.052, 0.027; 74% below zero) (Fig. 2c). The small difference in effect size between these two groups (mean = −0.004, 95% CI: −0.058, 0.049) suggests a common response to foliar applied pesticides.

Discussion. This study provides the first evidence for community level national scale impacts on the persistence of wild bee populations resulting from exposure to neonicotinoid treated oilseed rape crops. While correlational, the identification of reduced persistence rates suggest that sublethal impacts reported by previous studies do 'scale up' to cause population extinctions over long time scales[8,9,12,14]. Wild bee species that forage on oilseed rape were three times as negatively affected by exposure to neonicotinoids than non-foragers. This supports the hypothesis that the application of this pesticide to oilseed rape is a principle mechanism of exposure for wild bee communities[12,23]. Although not tested in the existing study this finding also suggests that other mass flowering crops (for example, sunflower) could similarly provide a route of exposure to neonicotinoids that could lead to the loss of population persistence for wild bees.

Negative correlations between population persistence and neonicotinoid exposure were also found for species not known to forage on oilseed rape. One interpretation for this is that 'non-foraging' species have been exposed to neonicotinoids expressed in non-crop plants growing in soils contaminated with neonicotinoids. This indirect mechanisms of exposure has increasingly being identified as a potential problem in arable farming systems for wild bees[24,25] and may pose a risk for species that are active outside of the flowering period of oilseed rape. An alternative, but not mutually exclusive explanation, it is some of these species may also forage on oilseed rape at a level high enough to experience reductions in population persistence, but low enough to have escaped identification as an oilseed rape forager[8]. Variation in the use of oilseed rape by different bee species is likely a common feature of wild bee communities in agricultural systems, from those that habitually forage on oilseed rape (for example, *B. terrestris*) to those that use the crop on a more opportunistic basis when other floral resources are absent[26]. Such differences in resource utilization would not only affect the risk of exposure to neonicotinoids for known foragers, but also the likelihood of identifying a species as a potential forager in the first place. However, classifying wild bees into foraging and non-foraging species based on existing observational data provided the only tractable approach for assessing exposure risk to neonicotinoids. Increased resolution in both inter-and intra-specific crop foraging preferences would improve the explanatory power of these models. It should be noted that there is also a lack of phylogenetic independence between species allocated to either oilseed rape foraging or non-foraging groups. Ultimately, the evidence from this study suggests that while there may be alternative mechanisms of exposure to neonicotinoids for wild bees (for example, soil contamination), foraging on treated oilseed rape for pollen and nectar represents the principal mechanism of exposure affecting population persistence[27].

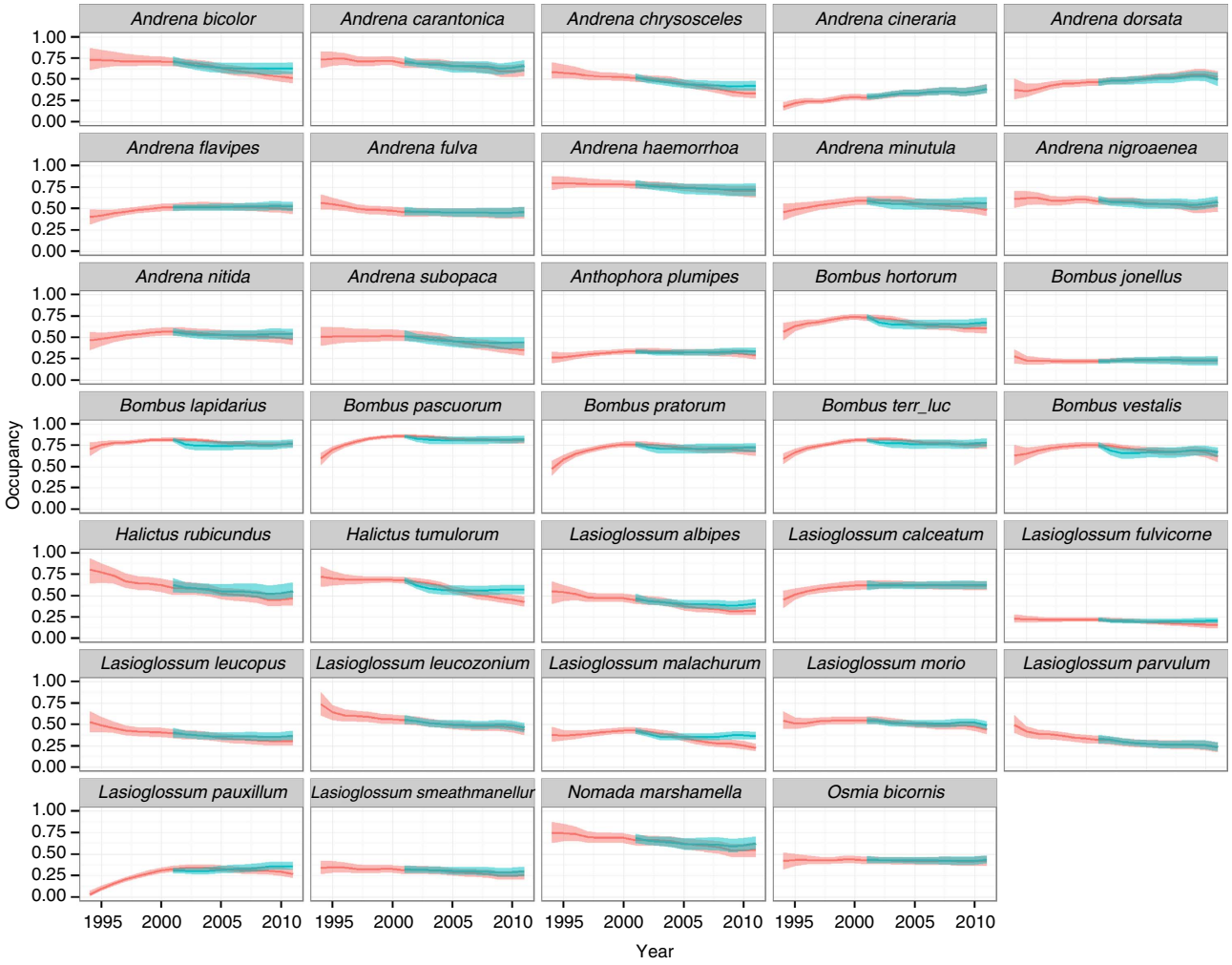

Figure 3 | Estimates of the net effect of neonicotinoid exposure on wild bee species that forage on oilseed rape. Species population persistence trajectories are based on fitted values from individual species models (red line) and are compared with an idealized model in which no neonicotinoids were applied following their first widespread use in 2002 (blue line). Shaded areas show 95% credible intervals.

The short duration of flowering for oilseed rape (typically 4–6 weeks in early summer) is thought to limit the importance of this crop for many species as it is unable to provide a continuous foraging resources over the entire breeding season[28–30]. For example, early season foraging resources (such as those provided by oilseed rape) are important for worker production in bumblebees[23,29], but current evidence suggests that queen production and subsequent population growth depends on foraging resource over the whole season[29]. However, mechanistic models demonstrate that for generalist solitary bees oilseed rape can have a positive effect on population growth[31]. Our results support these later findings with wild bee species known to forage on oilseed rape crops having increase population persistence in response to the cover of this crop. As the current analysis lacked data of sufficient resolution to assess whether the benefits of OSR were conditional on the availability of other flowering resources this finding does not dispute the importance of whole season foraging resources[28]. The spatially complex structure of English landscapes and the creation of flower rich habitats under the agri-environment schemes may mean that a sufficient continuity of foraging resources do exist that allow wild bees to benefit from the short flowering period of oilseed rape. Ultimately, the intensive nature of oilseed rape crop management, in particular its dependence on insecticides, means that its value as a foraging resource for wild bees may be outweighed by the management required to ensure crop yields[32]. However, it may be possible to cultivate oil-seed rape without extensive use of neonicotinoids: a recent UK based analysis demonstrated that on average neonicotinoid seed treatments do not boost farmer profits[32].

Interestingly, we found that the application of foliar applied insecticides had little or no negative consequences for population persistence of wild bees. Management operations are widely implemented in English farming systems to minimize the risk of exposure for domesticated and wild bees to foliar applied insecticides. For example, codes of practice limit application times to periods of low bee activity, particularly the evening or early morning[33]. The small effect sizes for foliar applied insecticides suggest that these codes of practice may have been effective in minimizing the exposure risk for wild bees, however, it is not possible to directly test this assertion within the current analysis. Such codes of practice were developed principally to protect honeybees that forage over a well-defined daily feeding period[34]. Other wild bee species, in particular bumblebees, may forage over a larger proportion of the day and so may be more likely to suffer mortality from foliar insecticides even where codes of practice to protect them are adhered to[34,35].

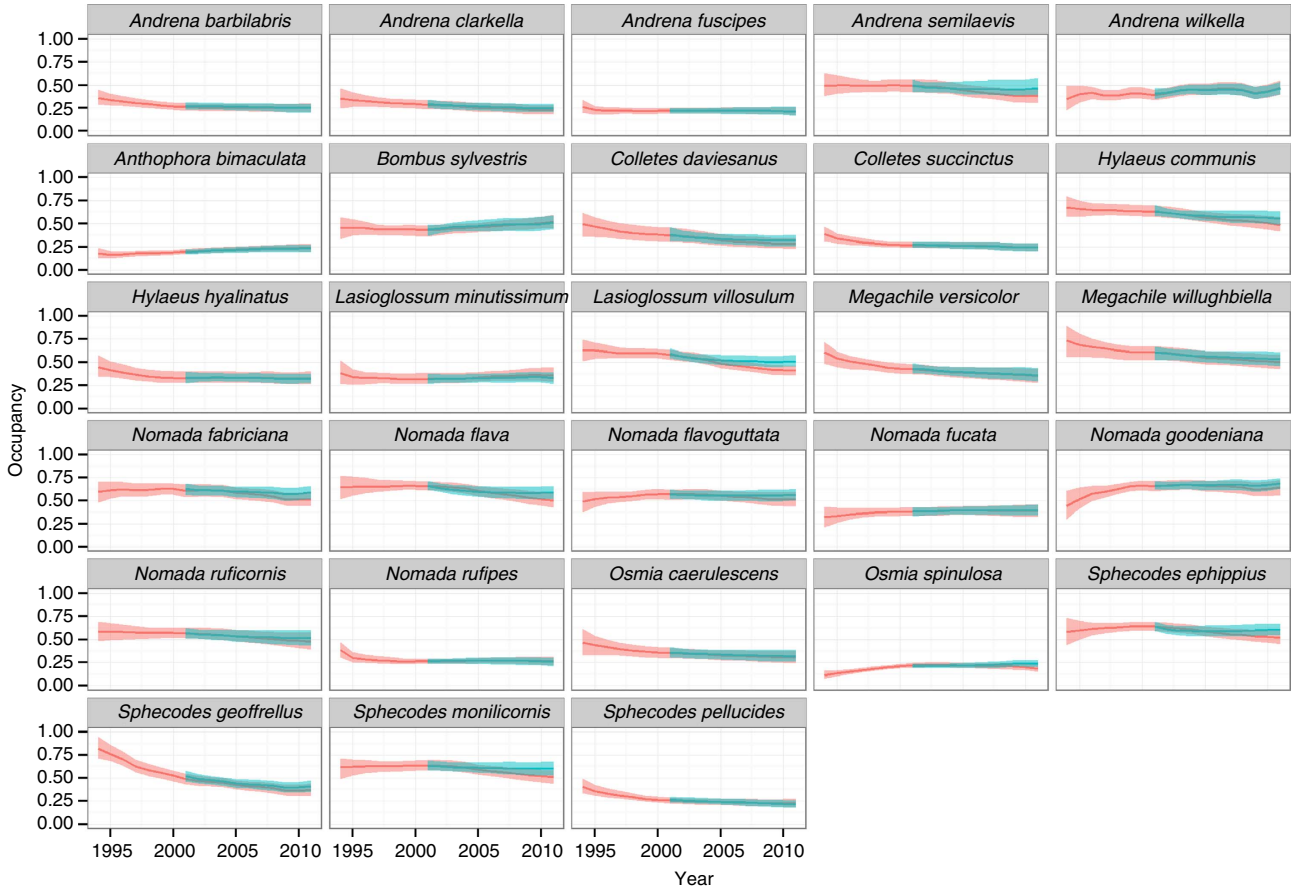

Figure 4 | Estimates of the net effect of neonicotinoid exposure on wild bee species that do not forage on oilseed rape. Species population persistence trajectories are based on fitted values from individual species models (red line) and are compared with an idealized model in which no neonicotinoids were applied following their first widespread use in 2002 (blue line). Shaded areas show 95% credible intervals.

In conclusions, the results presented here significantly expand upon previous short-term studies by demonstrating how exposure to neonicotinoids seed treatments have impacted upon the population persistence of wild bee communities foraging on oilseed rape. Although a relatively small number of bee species typically play the dominant role in crop pollination[36], the resilience of pollination services will often depend on the overall community[37,38]. Our results, therefore, have implications for the conservation of not only bee communities in intensively farmed landscapes, but the capacity of these systems to maintain stable crop pollination services in the face of changing environmental conditions[2]. These findings ultimately provide a crucial component of the evidence base needed to develop national scale management strategies that support wild bees over temporal and spatial scales that ensure population persistence over the long-term. While the evidence presented here shows that neonicotinoids were a contributing factor leading to reduced population persistence it is unlikely that its effects would act in isolation of other environmental pressures. A complex array of drivers, from land use to climatic change, may be interacting with neonicotinoid exposure in non-linear ways to affect wild bee population persistence[3–7]. Although not assessed in the current study, the capacity of species to recover from the impacts of neonicotinoid exposure would likely vary on an individual basis should the current moratorium on neonicotinoid use continue. However, in the absence of neonicotinoid use the benefits of oilseed rape as an early season foraging resource may mean that the recovery of at least some wild bee species may be relatively rapid.

Methods

Wild bee distributional data and foraging preferences. There are ~250 species of native bees (Order Hymenoptera) known to occur in England, comprising solitary bees (for example, some Apidae, Andrenidae, Megachilidae and Halictidae), 24 species of bumblebee (Apidae: *Bombus* spp.) and the domesticated honeybee (*A. mellifera*). Approximately 20% of these species are known to act as pollinators of oilseed rape and so occur within arable farmland[37,39,40]. To assess distributional changes in these wild bee species we analysed long-term occurrence records from 1994–2011. These records were collected and verified by the Bees, Ants and Wasps Recording Society (BWARS: http://www.bwars.com/) and represent a largely volunteer-collected, national-scale distributional database that is globally unique in coverage and detail. We used only bee distributional records from England to match available data on insecticide use and oilseed rape cropping patterns. We focused on the period 1994–2011 to quantify trends both before and after the date of first use of neonicotinoids on oilseed rape in England in 2002[41]. We excluded honeybees from the current study as their hives are both artificially managed and moved around landscapes and so are not comparable with wild species[42].

As citizen science data can be collected via wide participation projects using non-experts it has a reputation for being variable in quality. However, the UK recording schemes for invertebrates are typically more refined. Individual records are normally collected by local experts/entomologists rather than the general public (that is, who have no taxonomic experience). Under the auspices of the Bees, Wasps and Ants Recording Society, identifications are verified through photographic evidence and/or physical specimens in questionable cases. Records are also compiled centrally and subject to computer checks to identify potential outliers, such as those outside the previously known range, from atypical habitats or outside typical flight periods. In terms of the taxonomic rigour of individual records this data set is of high quality. As the data on bee distributions were collected by volunteers, not all areas are sampled with the same effort. As such while the data set is taxonomically robust there is no structured framework for how records are collected in terms of sites sampled or methods used[43]. It is for this reason that these data sets contain information only on occupancy of grid squares and not abundance. However, variation in recorder activity is a potentially significant issue in the analysis of these data sets. We use methods recommended by Isaac *et al.*[21] to account for the effect of variation in recorder activity on trend

estimation which are described in detail in the statistics section below. The data sets used here have been used as a basis for the identification of declines in pollinator species richness in the UK[4,44].

Wild bee distributional foraging preferences for oilseed rape. We classified wild bee species according to whether or not they have been observed foraging on oilseed rape in England. This was based upon published surveys from 30 English farms comprising 114 h of direct observations[7,8] to produce a list of 50 bee species (including honeybees) recorded as foraging on oilseed rape (Supplementary Table 1). This list was used to classify species of bee into two categories: oilseed rape foragers and non-foragers. Due to differences in methods used to collect the data from which this list was compiled it is solely qualitative, and makes no assessment of the relative use of the crop by different species. However, the list is derived from surveys undertaken in areas of high diversity of wild bees in the UK, in particular those associated with Salisbury Plain (the largest area of pristine chalk grassland in Europe). To our knowledge this is the most comprehensive UK list of bees that forage on oilseed rape, and comprises *c.* 20% of UK bee species. All of the 20 wild bee species identified as pollinators of oilseed rape by the Kleijn *et al.*[36] study of world crop pollinators were represented in this list, with an additional 29 wild bee species.

Criteria for species inclusions in analysis. We converted the occurrence records into a data frame suitable for analysis by first selecting all 1 km^2 grid cells in England with surveys in at least two-years during the period 1994–2011. We then identified all unique combinations of date and 1 km^2 grid cell, which we henceforth refer to as a survey. Surveys with coarser spatio-temporal resolution were excluded. Our final data set contained 31,818 surveys from 4,056 1 km^2 grid cells in England. Half the surveys were of just a single bee species, but the maximum number of species per survey was 45 species out of the total bee fauna of *c.* 250 (Fig. 1). We selected the 62 species that were recorded on at least 500 surveys from our final data set, representing 28 species of non-foraging and 34 species of oilseed rape foraging bees (Supplementary Table 2). This 500 survey threshold served two purposes. First, it excluded data poor species that could affect model convergence. Second, by including only relatively well-represented species we increased the reliability of our classification of bees as either oilseed rape foragers or non-foragers. Specifically species that may potentially have fed on oilseed rape, but due to their rarity would have been unlikely to be observed doing so, were excluded from the analysis using this threshold. In the analysis we treat *B. terrestris* and *B. lucorum* as an aggregate: workers of these species are extremely difficult to distinguishing from one another. Treating them as an aggregate avoids the possibility that our model could be biased by misidentifications, while minimizing the amount of discarded data (these are two of the commonest bees in the database).

OSR and neonicotinoid exposure rates. Oilseed rape represents an important forage resource for many wild bees, so we hypothesized that the cover of this crop had a positive effect on population persistence[27,31]. This contrasted with the potentially negative impacts of exposure to neonicotinoids expressed in the pollen and nectar of pesticide-treated crop[8–10,12]. To account for this, our analysis defined two separate variables that describe both the area of oilseed rape grown and neonicotinoid exposure resulting from the treatment of that crop with neonicotinoid seed treatments. The area of sown oilseed rape was derived from the Department for Environment, Food and Rural Affairs June Survey of Agriculture and Horticulture[19]. This quantified OSR in each 5 × 5 km grid square of England from 1994 to 2010. These data were collected every two-years, so we interpolated the data values for alternate years (for example, 2005 values were set as the mean of 2004 and 2006 values).

To define the temporal and spatial changes in the exposure of wild bees to neonicotinoids we defined the extent of neonicotinoid seed treatment use as recorded by the UK Pesticide Usage Survey[45]. Neonicotinoids were widely used in oilseed rape from 2002 following the first full commercial UK licensing of this insecticide for this crop (first, Imidacloprid in 2002, followed by Clothianidin and Thiamethoxam[41]). Note, our data set includes a small number of grid cells where regulatory trials were conducted before this (1999–2001). The use of neonicotinoid seed treatments rose rapidly from 37.4% (s.e. ± 8.0) in 2002, to 83.0% (s.e. ± 5.2) of the crop treated by 2011. Data on the use of neonicotinoids was collected as part of UK commitments to the EU Statistics Regulation (1185/2009/EC) by the Food and Environment Research Agency. These data are collected every two-years (and so concurrent with the crop cover data) but are derived at a considerably coarser scale of eight Department for Environment, Food and Rural Affairs regions in England (East Midlands, Eastern, London and South East, North East, North West, South West, West Midlands and Yorkshire and the Humber). The Pesticide Usage Survey is based on information provided from 1,200 surveyed farms, stratified by region and size. Surveys incorporate inbuilt anomaly checks, including verification that application rates on individual sites lie within manufacturer recommendations. For each of the eight regions standard errors for the extrapolated rates of application are derived using non-parametric bootstrapping techniques. Following regulatory requirement these standard errors must fall below 5% (ref. 46). As such this data are considered highly reliable both within and between years. Neonicotinoid exposure in each 25 km^2 grid square (5 × 5 km) for each year was estimated as the product of the area of oilseed rape and the proportion of that crop treated with neonicotinoids in the region within which the grid square was located. We provide a discussion of issues relating to potential collinearity problems between OSR and the proportion of the crop treated with neonicotinoids in Supplementary Note 1, and show that they do not affect the general reliability of our conclusions.

FII index. The extent of foliar insecticide use (that is, that applied as a spray as opposed to a systemically expressed seed treatment) was defined by an aggregate index describing both the application rates of foliar insecticides on oilseed rape, as well as information on their relative toxicity for bees. This FII index produces a composite estimate of the impact of foliar sprayed non-systemic insecticides (of which the most frequently used were pyrethroids) and was based on the bee component of the Environmental Impact Quotient (EIQ)[47]. The FII was defined as:

$$\mathrm{FII} = \sum_{i=1}^{\mathrm{ai}} (Z_{\mathrm{ai}} \times P_{\mathrm{ai}} \times 3) \times \left(\frac{M_{\mathrm{ai,R}}}{A_{\mathrm{R}}}\right) \quad (1)$$

where: Z_{ai} is a measure of the toxicity of the active ingredient (ai) to bees. The factor 3 in equation 1 represents a weighting for comparing the relative exposure of bees to other taxa and is an integral component of the full EIQ calculation (bees and birds are given a weighting of 3, beneficial arthropods are given a weighting of 5). While redundant in the current equation it has been retained to provide consistency with the original EIQ assessment[47]. To calculate this measure of toxicity for bees, each foliar insecticide was classified by its lethal dose score (LD_{50}) into high, medium or low toxicity compounds. Following established protocols[47], high toxicity compounds ($LD_{50} < 1$ ug per bee) were given a coefficient (Z_{ai}) of 5, medium toxicity compounds (100 ug per bee $> LD_{50} > 1$ ug per bee) a coefficient of 3 and low toxicity compounds ($LD_{50} > 100$ ug per bee) a coefficient of 1. This 5:3:1 ratio is a developed as part of the EIQ and has been widely applied in a variety of assessments of the impacts of pesticides, including studies on wild bees[48–50]. The variable P_{ai} is the plant surface half-life for active ingredient ai, which is estimated by dividing the soil deterioration half-life of the insecticide (DT_{50}) by four[51]. Lethal dose toxicity (LD_{50}) and soil degradation (DT_{50}) data for insecticide active ingredients were derived from the Pesticide Properties Data Base[20]. $M_{\mathrm{ai,\ R}}$ is the mass of active ingredient applied in a region R, and A_{R} is the area of crop sprayed in that same region. Treating the mass and area separately was avoided to limit the potential impact of correlations between the area of oilseed rape and the pesticide pressure score. Summary data on the mass of active ingredient applied and the area treated for each region of England was derived from the Pesticide Usage Survey undertaken on alternate years[45]. Regional FII scores were calculated for each year by interpolation (as above) and assigned to each 25 km^2 grid cell based on the region that the majority of the cell area fell within.

Landscape structure. While landscape structure has an important role in the population persistence of many bee species[3], its inclusion as a fixed effect in the current analysis was precluded by the absence over the 18 year period of spatially explicit data of an appropriate temporal resolution (for example, annual or biennial). However, evidence from the UK Countryside Survey undertaken in 1990, 2000 and 2007 (ref. 52) indicates there has been no significant change in the cover of Broad Habitats in England between these three time periods[52]. The main change in land use over the period has been in crop types, with the total area of cropped land and wheat cover remaining relatively constant[53] and the cover of oilseed rape increasing largely at the cost of barley. Neither wheat nor barley are used by wild bees. It is worth noting that this survey shows that plant species richness on arable and horticulture land increased by 30% between 2000 and 2007, partly due to an increase in sown wildflower field margins which are used by wild bees[52]. Arable land therefore improved rather than declined in its quality for many wild bees over the period we study. This conclusion is supported by Carvalheiro *et al.*[44], who show that the great loss of semi-natural habitat to agricultural intensification—which is linked to declines in wild bees—occurred before the 1990s in NW Europe.

Statistical analysis. We created a multi-species dynamic Bayesian occupancy-detection model[18,54] (BOD) to characterize distributional changes in wild bee species, implemented in the BUGS language (see Supplementary Note 2 for the BUGS code). A key feature of BOD models is that the occupancy of each grid cell (presence or absence) is separated statistically from the data collection process (detection versus non-detection): specifically, observations are conditional on the species being present. This makes BOD models well-suited to modelling change using opportunistic surveys collected by volunteers[22], and the resulting trends are robust to multiple sources of error and bias[21]. The model we employed is 'dynamic'[17], in that persistence and colonization of individual grid cells is modelled explicitly (equation 2), and 'multispecies'[16], in that we fitted a single model to the full data set with species-specific parameter estimates. We modelled occupancy at 25 km^2 resolution (that is, 5 × 5 km grid square) to match the spatial scale at which our covariates were calculated. In the model the expected value of $z_{i,j,t}$ (occupancy of species i in gird square j in year t) was modelled as a function of occupancy in the previous year, $z_{i,j,t-1}$. Unoccupied grid squares could be colonized with species-specific probability γ_i, while occupied grid squares could persist from one year to

the next with a probability $\varphi_{i,j,t}$.

$$\mathrm{E}[z_{i,j,t}] = z_{i,j,t-1} * \varphi_{i,j,t} + (1 - z_{i,j,t-1}) * \gamma_i \quad (2)$$

Population persistence, $\varphi_{i,j,t}$, was modelled as a linear function of OSR, the neonicotinoid exposure and the FII in the previous year ($t-1$). Specifically:

$$\mathrm{logit}(\varphi_{i,j,t}) = \beta_{0i} + \beta_{1i} * \mathrm{OSR}_{j,t-1} + \beta_{2i} * \mathrm{NNI}_{j,t-1} + \beta_{3i} * \mathrm{FII}_{j,t-1} \quad (3)$$

Parameter β_{2i} is an estimate of the annual change in the log odds ratio of the persistence from one year to the next for species i within the average occupied 25 km^2 grid square. This is assessed for each unit increase in the neonicotinoid exposure; parameters β_1 and β_3 estimate the effect sizes of oilseed rape area and FII. Our central hypothesis is that high doses of neonicotinoids cause a reduction in population persistence (that is, $\beta_2 < 0$).

Our detection sub-model states that the *kth* survey to a site occupied by species i will yield an observation with probability $p_{i,k}$. We modelled this probability as a function of the total number of species recorded on that survey, since this provides a convenient measure of sampling effort[55]. Specifically, $p_{i,k}$is a function of two binary variables indicating whether the survey produced a short (two or three species) or long (>3 species) list[22]:

$$\mathrm{logit}(p_{i,k}) = \alpha_t + \beta_{4i} + \beta_{5i} * \mathrm{Short}_k + \beta_{6i} * \mathrm{Long}_k \quad (4)$$

Parameter β_4 is the probability that a single-species list is a survey of the focal species in the average year; parameters β_5 and β_6 estimate how the detection probability changes with survey effort and α_t is a random effect for year. This formulation treats short lists, long lists and single species surveys as separate data sets with different statistical properties[22] and does not assume that all surveys record 'complete lists' of what was present[43]. An alternative would be to use the list length as a continuous covariate on detectability[55]. However, such a monotonic function is not appropriate for bee records in the UK where a large (but unknown) proportion of records derive from casual (or 'incidental') observations rather than formal surveys. Such incidental records disproportionately represent charismatic and easy to identify species, so that the probability of recording such a species on an incidental observation (that is, list length 1) could be higher than the probability of being recorded on a complete list derived from a short survey (list length 2–3).

Our species-specific parameter estimates treat species identity as a random effect. For parameters γ, β_0, β_4, β_5, and β_6 we assumed all species are drawn from a common distribution by estimating a single mean and variance for each parameter. For the covariates on population persistence (β_1, β_2 and β_3) we assumed that species foraging on oil seed rape were drawn from a different distribution from non-foragers, following Ruiz-Gutiérrez *et al.*[16]. Comparing the posterior distributions for these groups allowed us to test the hypothesis that foragers and non-foragers respond differently, whilst fully accounting for all forms of uncertainty in the model. The covariates (OSR, neonicotinoid exposure and FII) were centered on their mean values for analysis. We ran the model described above using uninformative priors in three Monte Carlo chains of 10,000 iterations each, following a burn-in of 5,000 iterations and a thinning rate of three. We confirmed that the parameter estimates had reached convergence through a combination of quantitative (Rhat statistics[56]) and qualitative assessments (for example, visual inspection of the posterior density). We implemented the BOD model using BUGS[57] and conducted all other analysis in the R statistical environment[58].

There were many sites for which there were several years between surveys. In these cases, the state variable (presence-absence) was imputed following standard practice in Bayesian statistics. This imputation is likely to have smoothed our estimates of persistence (and hence occupancy) across years. Note that parameter estimates (β_1, β_2, and so on) were estimated over all the entire state space (that is, a large number of permutations of which sites were occupied in different years), so the posterior distributions that we derived from the model were unbiased with respect to the sparseness.

Data availability. The wild bee distributional data that support the findings of this study are available at the BWARS data holdings accessible via the National Biodiversity Network's Gateway http://data.nbn.org.uk/ as are the Food and Environment Research Agency Pesticide Usage Survey Statistics https://secure.fera.defra.gov.uk/pusstats/myindex.cfm. The PPPB: Pesticide Properties Data Base that support the findings of this study available at http://sitem.herts.ac.uk/aeru/ppdb/en/. Finally, OSR data that support the findings of this study are available from the EDINA agcensus http://edina.ac.uk/agcensus/description.html.

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Author contributions

N.J.B.I. and B.A.W. had equal contributions to the production of this manuscript. B.A.W. wrote the manuscript with significant input from R.F.P., N.J.B.I. and J.M.B. Statistical analysis and modelling were performed by N.J.B.I. with support from J.M.B. and D.B.R. Insecticide use data and the derivation of the foliar insecticide impact index was by D.G.G. and A.C. The study was conceptualized by R.F.P.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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