Widespread contamination of wildflower and bee-collected pollen with complex mixtures of neonicotinoids and fungicides commonly applied to crops.

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Abstract

There is considerable and ongoing debate as to the harm inflicted on bees by exposure to agricultural pesticides. In part, the lack of consensus reflects a shortage of information on field-realistic levels of exposure. Here, we quantify concentrations of neonicotinoid insecticides and fungicides in the pollen of oilseed rape, and in pollen of wildflowers growing near arable fields. We then compare this to concentrations of these pesticides found in pollen collected by honey bees and in pollen and adult bees sampled from bumble bee colonies placed on arable farms. We also compared this with levels found in bumble bee colonies placed in urban areas. Pollen of oilseed rape was heavily contaminated with a broad range of pesticides, as was the pollen of wildflowers growing nearby. Consequently, pollen collected by both bee species also contained a wide range of pesticides, notably including the fungicides carbendazim, boscalid, flusilazole, metconazole, tebuconazole and trifloxystrobin and the neonicotinoids thiamethoxam, thiacloprid and imidacloprid. In bumble bees, fungicides carbendazim, boscalid, tebuconazole, flusilazole and metconazole were present at concentrations up to 73 nanogram/gram (ng/g). It is notable that pollen collected by bumble bees in rural areas contained high levels of the neonicotinoids thiamethoxam (mean 18 ng/g) and thiacloprid (mean 2.9 ng/g), along with a range of fungicides, some of which are known to act synergistically with neonicotinoids. Pesticide exposure of bumble bee colonies in urban areas was much lower than in rural areas. Understanding the effects of simultaneous exposure of bees to complex mixtures of pesticides remains a major challenge.

Keywords: neonicotinoids, fungicides, pollen, bumble bees, honey bees

Introduction

The extent, causes and consequences of bee declines have attracted much scientific and public attention in the last decade. It is clear that there is no single cause, but that several interacting factors including declines in floral abundance and diversity resulting from agricultural intensification, the spread of parasites and pathogens, and exposure to pesticides all contribute to these declines (Goulson et al., 2015). The impact of pesticides, in particular the class of insecticides known as neonicotinoids, on pollinator declines is the most controversial of these factors.

Neonicotinoids are neurotoxins which act as nicotinic acetylcholine receptor agonists in the central nervous system of insects and cause overstimulation, paralysis, and death (Goulson 2013). These pesticides are systemic and are widely applied as seed dressings to flowering crops, where they can be detected at the low ng/g level in the nectar and pollen (Fairbrother et al., 2014). Pollen is a major food source for growing bee larvae and nurse workers, and so is a likely source of exposure of bees to neonicotinoids (Sanchez-Bayo and Goka 2014).

A key part of the debate over the impacts of neonicotinoids has become focussed on the dose that bees are likely to be exposed to in the field. Laboratory and semi-field studies are often dismissed as using unrealistically high doses of pesticides. For example, Whitehorn et al. (2012) experimentally exposed bumble bee colonies to pollen containing 6 ng/g of the neonicotinoid imidacloprid, plus 0.70 ng/g in their nectar, and found an 85% drop in queen production compared to controls. However, it has since been argued that this dose was higher than bumble bees are likely to receive in the field because colonies will be feeding on a mix of contaminated crops and uncontaminated wildflowers (Carreck and Ratnieksi 2014). Thus, obtaining more information on what constitutes field realistic exposure to both bumble bee and honey bee colonies is vital to taking this debate forwards.

In addition to neonicotinoids, there is clear evidence that honey bees are routinely exposed to a complex mixture of many different agrochemicals (Johnson et al., 2012). An analysis of honey bees and their hive wax and pollen in the United States revealed that the majority of samples were contaminated with at least one pesticide, and a total of 121 different agrochemicals, including metabolites and miticides, were detected in samples (Mullin et al., 2010). Similarly, 37 insecticide and fungicide chemicals were detected in honey bees and hive products sampled in France (Lambert et al., 2013). In addition to the active ingredients, bees may also be exposed to additives used in pesticide formulations, and these have also been detected in pollen and honey with the potential to interact with pesticides and increase toxic effects (Mullin et al., 2015). Synergistic toxicity of some

68 combinations of insecticides and fungicides have been reported for honey bees or their larvae (Iwasa
69 et al., 2004; Schmuck et al., 2003; Thompson et al., 2014; Zhu et al., 2014). For example, the toxicity
70 of some neonicotinoids can be increased by as much as a factor of 1000 by simultaneous exposure
71 to demethylation inhibiting (DMI) fungicides (Iwasa et al., 2004; Schmuck et al., 2003). DMI
72 fungicides act by inhibiting Cytochrome P450 (CYP P450) mediated ergosterol biosynthesis in fungi
73 and are thought to inhibit CYP P450 enzymes in insects that are important for detoxification of
74 neonicotinoids and other insecticides (Schmuck et al., 2003).

75 Our study focusses on determining which mixtures of commonly used fungicides occur alongside
76 neonicotinoids in crop and wildflower pollen and in the pollen collected by honey bees and bumble
77 bees. Our aim is to investigate the potential for exposure of bees to mixtures of neonicotinoid and
78 fungicide pesticides that are present in crop and wildflower pollen. Pesticides were analysed in
79 pollen collected from oilseed rape (OSR) flowers, wildflowers growing in margins of OSR and winter
80 wheat (WW) crops, and from pollen collected by honey bee (*Apis mellifera*) and bumble bee
81 (*Bombus terrestris*) colonies placed in arable farmland. We also compare exposure of bumble bee
82 nests placed in urban versus rural areas, and quantify residues in the adult bumble bees. Mixtures of
83 a total of 20 agrochemicals were analysed comprising neonicotinoids and fungicides commonly used
84 in United Kingdom crops.

85

86 **2. Material and methods**

87 2.1 Sample collection

88 2.1.1 Pollen collected from plants

89 - *OSR pollen*

90 Pollen samples from OSR flowers were collected in 7 fields from three farms located in East Sussex
91 (United Kingdom) during the OSR blooming period (end of May – June 2013), and 1 to 3 sites per
92 OSR field were sampled (n=11 in total). The selected fields had varying cropping history following
93 normal farming practices in the region. The predominant crops were WW and OSR. Previous crops
94 were treated with a range of pesticides, including use of neonicotinoids and fungicides each year for
95 at least the three previous years (Table 1). In 2012, the seeds from the OSR fields were all treated
96 with Cruiser® seed dressing (active ingredients (a.i.): 280 g/L thiamethoxam, 8 g/L fludioxonil and

32.2 g/L metalaxyl-M) and the WW was treated with Redigo® Deter® (a.i.: 50 g/L prothioconazole and 250 g/L clothianidin).

To obtain pollen samples, OSR flowers were gathered, stored on ice in coolers in the field and then frozen immediately at -80°C until further handling. At processing, flower samples were gently defrosted and dried in an incubator at 37 °C for 24 hours to facilitate pollen release from the anthers. After drying, flowers were brushed over food strainers to separate pollen from anthers and sifted through multiple sieves of decreasing pore sizes (pore sizes from 250 to 45 µm).

- *Wild plants in the field margins.*

Wildflower pollen samples were collected from 4 of the 7 OSR fields as well as in the margin of 4 WW fields present at the same 3 farms. Field boundaries in the region typically consist of a hedge of woody plants separated from the crop by a 0-2 m strip of herbaceous vegetation. The average sample distance from the crop edge was 1.5 m (range 1-2 m). Samples of pollen were collected from the wildflowers present in the field margins and hedge using the method described above for OSR plants. The species of wildflowers collected depended upon which species were available. Wildflowers were identified using a visual identification guide. In OSR field margins, pollen from 8 different wildflowers comprising 4 different species (*Ranunculus repens, Silene latifolia (x3), Matricaria recutita (x3), Cirsium vulgare*) were collected (the number in brackets after the species indicates the number of times different plants of the same species were sampled). In WW margins, pollen from 13 wildflowers comprising 6 different species (*Heracleum sphondylium (x5), Papaver rhoeas, Senecio jacobaea (x2), , Pimpinella saxifraga, Aethusa cynapium and Matricaria recutita (x3)*) were collected. Pollen samples were analysed separately from each species with the exception of low amounts (< 20 mg) of four wildflower pollen samples collected from plants growing at the same site of a WW margin, which were pooled and analysed as a single sample (see Table S5).

2.1.2 Pollen collected from bees.

- *Honey bees*

Five honey bee (*Apis mellifera*) colonies were placed in the vicinity of the OSR fields at the beginning of the OSR flowering period (May 2013) and stayed in the same sites until the end of August 2013. Distances between the hives and the nearest OSR fields ranged from 1 to 260 m (see Table S1). The hives were equipped with pollen traps during 4 consecutive days at the beginning of June 2013 (i.e., during the OSR blooming period), and for 4 days in mid-August 2013 (i.e., when no OSR was in flower) in order to collect pollen loads from the returning honey bee foragers. After 4 days, the traps

were removed from the hives and the pollen gathered and stored on ice in coolers in the field, and then at -80 °C until analysis. Trapped pollen samples from each hive were kept separately. Pollen loads within each sample were sorted and weighed by colour (Human et al., 2013; Kirk 2006). Pollen grains associated with plant species were identified under a microscope following standard methods and using reference specimens and published reference collections (Demske et al., 2013; Moore et al., 1991; Sawyer 1981).

- *Bumble bees*

Eight bumble bee nests (*Bombus terrestris audax*) were obtained from Agralan Ltd, Swindon, UK (originating from Biobest, Belgium). Five nests were placed in different farmland sites in South-East England (East and West Sussex) at the beginning of May 2013. Sites were at least 1 km apart and on average 590 m far from the nearest OSR crop (range 8-1116 m, see Table S1). Three other nests were located in gardens from urban areas of West Sussex, being separated more than 4 km apart, and with an average distance to the nearest OSR crop of 1577 m (range 240-2670 m). After 4 weeks of free foraging in the field (comprising most of the OSR blooming period), pollen samples (> 200 mg) were collected from the in-nest stores in every colony using stainless steel micro-spoons, and were stored in 1.5 ml micro-centrifuge tubes at -80° C. The stored pollen collected from each nest was individually analysed for pesticide presence. The pollen identification was done using the same method as for the honey bee pollen. Before the pesticide analysis, every pollen sample was manually homogenised using a micro-spatula. A subsample of approximately 2 mg was evenly spread in a microscope slide, using glycerine jelly as the mounting medium. Light microscopy was used to identify the source of the pollen grains within the samples, and the proportion of the different taxa present in the samples was estimated by identifying pollen grains in five microscope fields of view uniformly distributed across the slide coverslip until 200 pollen grains were counted. After ten weeks of free foraging in the field, three to eight workers per nest were also collected for pesticide analysis of individual bees.

2.2 Pesticide analysis

2.2.1 Chemicals and reagents

Choice of analytes: Details of test analytes used in the study are given in Table 1. The pesticides comprised eight classes of contaminants and included all five of the neonicotinoid chemicals that are registered for use in the UK. Fungicides were chosen based on the most used (by weight) in UK crops including oilseed rape, wheat, spring barley, field bean, strawberry and raspberry crops (https://secure.fera.defra.gov.uk/pusstats/surveys/2012surveys.cfm). In addition, levels of an

160 insecticide synergist piperonyl butoxide were also analysed as it is used in agrochemical formulations
161 and has been reported to synergise the activity of some neonicotinoids (Bingham et al., 2008; Khan
162 et al., 2015).

Table 1. The list of chemicals analysed in this work, their chemical classes and their last applications in the studied oilseed rape (OSR) or winter wheat (WW) fields.

Chemicals	Class	Last application				Application method	Comments
		OSR field		WW field			
		Month	Year	Month	Year		
Insecticides							
Thiamethoxam	Neonicotinoid	Aug	2012	Aug	2011	seed dressing	
Clothianidin	Neonicotinoid	March	2012	Oct	2012	seed dressing	
Imidacloprid	Neonicotinoid	Not used					used prior to 2011
Acetamiprid	Neonicotinoid	Not used					used for gardening
Thiacloprid	Neonicotinoid	Not used					used in neighbouring fields in 2011 and 2012 and in gardens
Fungicides							
Carbendazim	Methyl benzimidazole carbamates (MBC)	May	2013	April	2012	spray	
Carboxin	Succinate dehydrogenase inhibitors (SDI)	Not used					commonly used for barley crops [a]
Boscalid	Succinate dehydrogenase inhibitors	May	2013	May	2013	spray	
Spiroxamine	Amines ("Morpholines") (SBI: Class II)	April	2012	June	2013	spray	
Silthiofam	Thiophene	Not used					commonly used for WW [a]
Triticonazole	Demethylation inhibitors (DMI) (SBI: Class I)*			March	2011	spray	used for gardening
Epoxiconazole	Demethylation inhibitors (SBI: Class I)	April	2012	May	2013	spray	
Tebuconazole	Demethylation inhibitors (SBI: Class I)	June	2012	June	2013	spray	used for gardening
Flusilazole	Demethylation inhibitors (SBI: Class I)	Jan	2013	Nov	2011	spray	
Prochloraz	Demethylation inhibitors (SBI: Class I)			March	2011	spray	
Metconazole	Demethylation inhibitors (SBI: Class I)	May	2013	Jan	2012	spray	
Pyraclostrobin	Quinone outside inhibitors (QoI)	April	2012	May	2013	spray	
Fluoxastrobin	Quinone outside inhibitors	May	2011	May	2011	spray	
Trifloxystrobin	Quinone outside inhibitors			May	2011	spray	used for gardening
Synergist							
Piperonyl butoxide							used in the formulation of insecticides

[a] information from Defra report https://secure.fera.defra.gov.uk/pusstats/surveys/2012surveys.cfm.

* SBI = sterol biosynthesis inhibitor also known as Ergosterol biosynthesis inhibitor (EBI) - an inhibitor of sterol synthesis, which is essential for fungal growth. EBI fungicides include DMIs as well as the morpholines and piperidines.

Certified standards of carbendazim, thiamethoxam, thiamethoxam-d3, clothianidin, clothianidin-d3, imidacloprid, imidacloprid-d4, acetamiprid, thiacloprid, carboxin, boscalid, spiroxamine, silthiofam, triticonazole, epoxiconazole, tebuconazole, flusilazole, prochloraz, metconazole, pyraclostrobin, trifloxystrobin, fluoxastrobin, piperonyl butoxide and also formic acid, ammonium formate, magnesium sulphate, sodium acetate and Supel^{TM} QuE PSA/C18/GCB (ratio 1/1/1) were obtained from Sigma Aldrich UK. Certified standards of carbendazim-d3 and tebuconazole-d6 were purchased from LGC standards UK and prochloraz-d7 and carbamazepine-d10 from QMX Laboratories Limited UK. All pesticide standards were > 99% compound purity (except triticonazole: 98.8%, spiroxamine: 98.5% and piperonyl butoxide: 97.9%) and deuterated standards > 97% isotopic purity. HPLC grade acetonitrile, toluene, methanol and water were obtained from Rathburns UK. Individual standard pesticide (native and deuterated) stock solutions (1 mg/ml) were prepared in acetonitrile (ACN) as was an internal standard mixture of the seven deuterated pesticides at 100 ng/ml. Calibration points in H_20:ACN (70:30) were prepared weekly from the stock solutions. All solutions were stored at -20^{o}C in the dark.

2.2.2 Sample preparation for neonicotinoid analyses

- *Pollen samples*

Pollen samples were extracted as described in David et al. (2015). Briefly, 100 mg (± 5 mg) of pollen sample was weighed, and 400 pg of the mix of deuterated internal standards in ACN were added to each sample, which was then extracted using a modified QuEChERS method. First, 400 µl of water was added and samples were then extracted by adding 500 µl of ACN and mixing on a multi-axis rotator for 10 min. Then, 250 mg of magnesium sulphate: sodium acetate mix (4:1) was added to each tube. After centrifugation (13,000 RCF for 5 min), the supernatant was removed into a clean Eppendorf tube containing 50 mg of Supel^{TM} QuE PSA/C18/GCB and vortexed (10 s). The extract was mixed on a multi-axis rotator (10 min) and then centrifuged (10 min). The supernatant was transferred into a glass tube. The PSA/C18/GCB phase was then extracted with ACN/toluene (3/1, 150 µl vortex 15 s). After centrifugation, the supernatant was combined with that of the previous ACN extract and spin filtered (0.22 µm). The extract was evaporated to dryness under vacuum, and finally reconstituted with 120 µl ACN:H_2O (30:70). Finally, the extract was centrifuged for 20 min and the supernatant stored at -20^{o}C in the dark until analysis.

- *Bumble bee samples*

Pollen baskets on bumble bee legs were first checked for adhering pollen residues in order to remove them before analysis. Individual whole bumble bee samples were ground in liquid nitrogen

with a pestle and mortar followed by manual homogenisation using a micro-spatula. Each bumble bee sample was then accurately weighed (average weight ± standard deviation was 123 ± 83 mg). Then, 400 µl of water was added, and the samples were homogenised for 20 s using a vortex. Samples were then extracted using the same modified QuEChERS method as above (i.e, 500 µl of ACN, 250 of magnesium sulphate: sodium acetate mix (4:1) and 50 mg of PSA/C18/GCB). Extracts were reconstituted, centrifuged and stored as above. A sample of bumble bee workers from Biobest nests was analysed for target pesticides prior to the experiment, and levels of all test analytes in bumble bee extracts were found to be below the method detection limits.

2.2.3 UHPLC-MS/MS analyses

The ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) method described in David et al. (2015) was used for the analysis of samples. Briefly, sample extracts were analysed using a Waters Acquity UHPLC system coupled to a Quattro Premier triple quadrupole mass spectrometer from Micromass (Waters, Manchester, UK). Pesticides in extracts were separated using a reverse phase Acquity UHPLC BEH C18 column (1.7 µm, 2.1 mm × 100 mm, Waters, Manchester, UK) fitted with a ACQUITY UHPLC BEH C18 VanGuard pre-column (130 Å, 1.7 µm, 2.1 mm X 5 mm, Waters, Manchester, UK) and maintained at 22 °C. Injection volume was 20 µL, and mobile phase solvents were 95% water, 5% ACN, 5 mM ammonium formate, 0.1% formic acid (A) and 95% ACN, 5% water, 5 mM ammonium formate, 0.1% formic acid (B). Methods were developed to separate all 20 test analytes within a 25 min run. The initial ratio (A:B) was 90:10 and separation was achieved at 22°C using a flow rate of 0.15 ml/min with the following gradient: 90:10 to 70:30 in 10 min; from 70:30 to 45:55 at 11 min, from 45:55 to 43:57 at 20 min, from 43:57 to 0:100 at 22 min and held for 8 min prior to return to initial conditions and equilibration for 5 min.

MS/MS was performed in the multiple reaction monitoring (MRM) using ESI in the positive mode, and two characteristic fragmentations of the protonated molecular ion $[M+H]^+$ were monitored for quantification and confirmation (David et al., 2015). Argon was used as collision gas (P collision cell: 3×10^{-3} mbar), and nitrogen was used as desolvation gas (600 L/h). Mass calibration of the spectrometer was performed with sodium iodide. Data were acquired using MassLynx 4.1, and the quantification was carried out by calculating the response factor of neonicotinoid and fungicide compounds to their respective internal standards. Analyte concentrations were determined using a least-square linear regression analysis of the peak area ratio versus the concentration ratio (native analyte to deuterated IS). A minimum of six point calibration curves (R^2> 0.99) were used to cover the range of concentrations observed in the different matrices for all compounds, within the linear

range of the instrument. Method detection limits (MDL) and method quantification limits (MQL) for pollen and bumble bee matrices are given in Table S2.

2.2.4 Quality control

One workup sample (i.e., using extraction methods without a pollen/bee sample) per batch was injected on the UHPLC-MS/MS at the beginning of the run to ensure that no contamination occurred during the sample preparation. Solvent samples (ACN:H_2O (30:70)) were also injected between sample batches to ensure that there was no carryover in the UHPLC system that might affect adjacent results in analytical runs. Identities of detected neonicotinoids and fungicides were confirmed by comparing ratios of MRM transitions in samples and pure standards. The standard calibration mixture was injected before and after all sample batches to monitor sensitivity changes, and quality control samples (QCs, i.e., standard solutions) were injected every 10 samples to monitor the sensitivity changes during the analysis of each batch.

2.3 Statistical analysis

All statistical analyses were carried out using GraphPad Prism 6 software. Pesticide concentrations in the different pollen matrices were tested for normality using the D'Agostino-Pearson test. As pesticide concentrations were not normally distributed for many pesticides in the different pollen types, non-parametric Mann-Whitney U-tests were used to compare the concentrations of neonicotinoids and fungicides in pollen collected from 1) OSR flowers vs OSR wildflower 2) OSR flowers vs WW wildflower 3) OSR flowers vs honey bee pollen in June 4) OSR wildflowers vs WW wildflowers 5) honey bee pollen in June vs August. To perform the statistical analyses, all concentrations that were over the limits of detection (≥MDL) but below the limits of quantification (<MQL) were assigned the value considered as the MDL in each case. Concentrations below the MDL were considered to be zero.

3. Results

3.1 Neonicotinoid and fungicide residues in pollen samples from oilseed rape, wildflowers from field margins and pollen collected by honey bees.

3.1.1 Frequencies, ranges and mean concentrations

Mixtures of neonicotinoids and fungicides were analysed in pollen samples from OSR flowers, wildflowers from OSR and WW margins and pollen collected by honey bees (during and after the OSR bloom) in order to estimate exposure of bees to these pesticides. All the different types of

pollen were collected in each of the 3 different farms. Frequencies of each pesticide (i.e., percentage of samples with detectable levels of pesticides) as well as the ranges, mean and median concentrations found in the different pollens are presented in Table 2 (for raw data see Table S3 to S7).

Table 2. The mean, median and range of concentrations (ng/g) and frequency of detection of neonicotinoid (highlighted in bold) and fungicide chemicals in pollen collected from oilseed rape flowers, wild flowers and by honey bees during and after the OSR bloom.

	OSR pollen				Wildflower pollen								Honey bee pollen							
					OSR Margins				WW Margins				During OSR bloom				After OSR bloom			
	n = 11				n = 8				n = 10				n = 25				n = 19			
	Freq	Range	Mean	Median	Freq	Range	Mean	Median	Freq	Range	Mean	Median	Freq	Range	Mean	Median	Freq	Range	Mean	Median
	%	*ppb*	*ppb*	*ppb*	%	*ppb*	*ppb*	*ppb*	%	*ppb*	*ppb*	*ppb*	%	*ppb*	*ppb*	*ppb*	%	*ppb*	*ppb*	*ppb*
Thiamethoxam	100	2.4 - 11	5.7	3.9	50	<0.12 - 21	2.8	<0.36	30	<0.12 - 0.50	0.13	<0.12	64	<0.12 - 1.6	0.15	<0.36	21	<0.12 - <0.36		
Clothianidin	73	<0.72 - 11	3.6	3.8	0	<0.72			10	<0.72 -5.0	0.50	<0.72	8	<0.72 - <2.2			0	<0.72		
Imidacloprid	0	<0.36			13	<0.36 - <1.1			0	<0.36			12	<0.36 - 3.5	0.20	<0.36	5	<0.36 - <1.1		
Acetamiprid	0	<0.02			0	<0.02			0	<0.02			4	<0.02 - <0.07			0	<0.02		
Thiacloprid	100	<0.22 - 78	19	7.5	63	<0.07 - 4.0	0.60	<0.22	20	<0.07 - 2.9	0.30	<0.07	48	<0.07 - 10	0.90	<0.07	0	<0.07		
Carbendazim	100	0.60 - 163	39	13	100	1.3 - 6.8	3.5	3.5	0	<0.08			96	<0.08 - 120	12	2.5	74	<0.08 - 1.4	0.40	0.34
Carboxin	0	<0.12			0	<0.12			0	<0.12			0	<0.12			0	<0.12		
Boscalid	18	<0.12 - 25	3.2	<0.12	63	<0.12 - 38	5.8	0.53	60	<0.12 - 38	8.5	1.7	52	<0.12 - 21	5.2	<0.36	37	<0.12 - 17	2.5	<0.12
Spiroxamine	100	13 - 328	80	58	88	<0.02 - 151	47	7.3	70	<0.02 - 26	7.7	6.3	28	<0.02 - 74	3.4	<0.02	47	<0.02 - 1.1	0.20	<0.02
Silthiofam	0	<0.24			0	<0.24			0	<0.24			0	<0.24			0	<0.24		
Triticonazole	0	<0.24			0	<0.24			0	<0.24			0	<0.24			0	<0.24		
Epoxiconazole	64	<0.84 - 27	4.3	2.5	0	<0.84			0	<0.84			0	<0.84			5	<0.84 - 8.3	<0.84	<0.84
Tebuconazole	100	1.5 - 21	5.2	2.9	75	<0.24 - 8.5	3.3	3.2	90	<0.24 - 34	7.0	3.2	76	<0.24 - 19	1.4	<0.72	79	<0.24 - 6.4	1.2	0.85
Flusilazole	18	<0.24 - 16	1.6	<0.24	25	<0.24 - 5.0	0.80	<0.24	0	<0.24			12	<0.24 - 6.1	0.30	<0.24	0	<0.24		
Prochloraz	0	<0.36			0	<0.36			0	<0.36			0	<0.36			0	<0.36		
Metconazole	27	<0.30 - 19	2.5	<0.30	0	<0.30			0	<0.30			12	<0.30 - 12	1.0	<0.30	0	<0.30		
Pyraclostrobin	9	<0.24 - 5.4	0.50	<0.24	38	<0.24 - 4.3	1.0	<0.24	10	<0.24 - 2.8	0.30	<0.24	28	<0.24 - 9.8	0.90	<0.24	16	<0.24 -3.7	0.40	<0.24
Trifloxystrobin	45	<0.24 - 18	2.6	<0.24	63	<0.24 - 104	13	<0.72	20	<0.24 - 1.0	0.10	<0.24	40	<0.24 - 10	1.6	<0.24	16	<0.24 - 1.0	0.10	<0.24
Fluoxastrobin	18	<0.01 - <0.02			50	<0.01 - <0.02			30	<0.01 - <0.02			12	<0.01 - <0.02			11	<0.01 - 3.9	0.20	<0.01
Piperonyl butoxide	0	<0.72			0	<0.72			0	<0.72			0	<0.72			0	<0.72		

Pollen traps were used to collect pollen brought back to honey bee hives (5) both during the OSR blooming period and later in the summer. Pollen was separated into wildflower species and analysed separately (n=3, 4, 5, 5 and 8 for hives 1, 2, 3, 4 and 5, respectively during the OSR bloom and n=5, 4, 2, 5 and 3 for hives 1, 2, 3, 4 and 5, respectively after the OSR bloom). ppb = ng/g wet weight of sample.

- *OSR flowers*

As expected, the number of detected pesticides, their frequencies, their ranges as well as their mean concentrations were generally higher in pollen from OSR flowers than in wildflower pollen and pollen collected by honey bees (Table 2). All individual OSR pollen samples contained at least 6 neonicotinoid and fungicide residues, and most samples contained between 7 and 12 different pesticides. Thiamethoxam, thiacloprid, carbendazim, tebuconazole and spiroxamine were the most frequently detected compounds (all present in 100% of samples), followed by clothianidin (73%), epoxiconazole (64%) and trifloxystrobin (45%). The other fungicides (i.e., boscalid, flusilazole, metconazole, pyraclostrobin and fluoxastrobin) were detected in less than 30% of these samples from OSR flowers. Pesticides such as carbendazim and spiroxamine were present in some samples at concentrations > 100 ng/g. The range of concentrations for other fungicides were between < MDL – 27 ng/g, and neonicotinoid concentrations were detected at between < MDL – 78 ng/g. With the exception of thiacloprid, which was only applied to neighbouring fields, thiamethoxam, clothianidin, carbendazim, boscalid, spiroxamine, epoxiconazole, tebuconazole flusilazole, metconazole, pyraclostrobin and fluoxastrobin had been applied in the studied OSR fields in the year of the sampling or up to two years before the sampling (i.e., before the rotation to OSR crop). Trifloxystrobin had been applied to WW fields present in the same farms two years before the sampling period (Table 1).

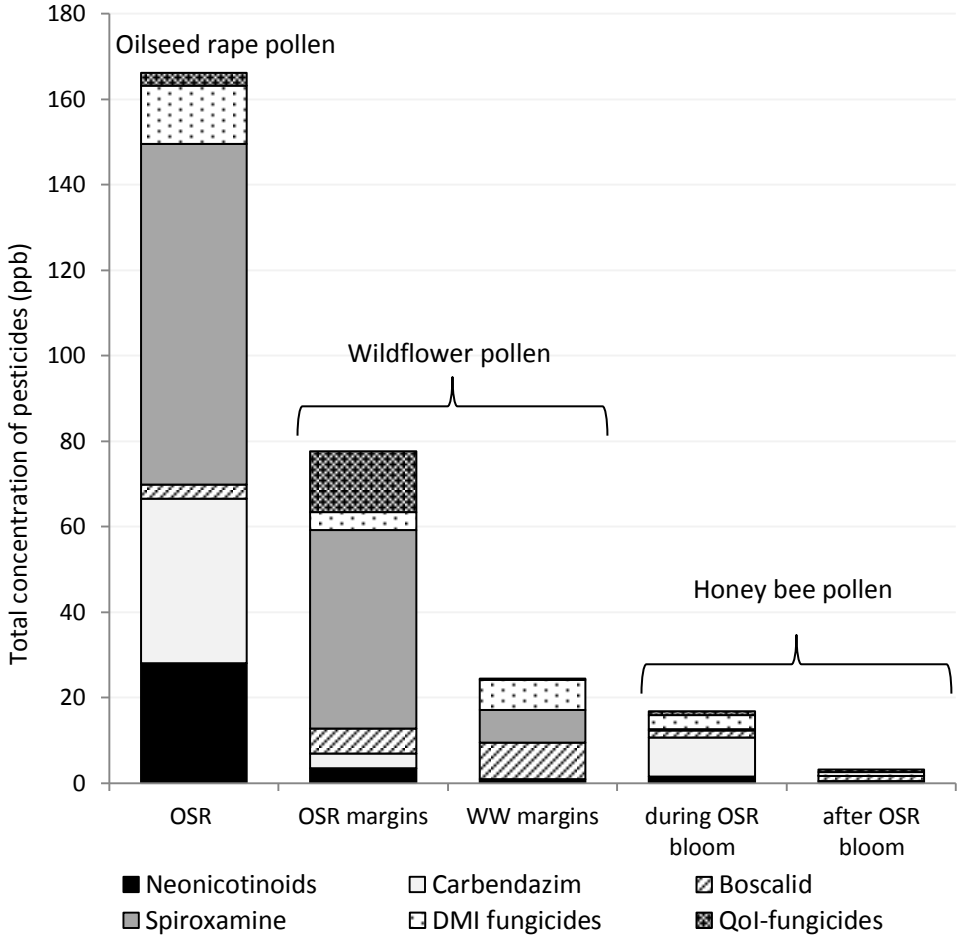
- *Wildflower pollen*

Pollen from four wildflower species was collected from 8 OSR field margins between June and August 2013. A similar mixture of pesticides as OSR pollen was detected in pollen from wildflowers growing in the OSR field margins; however, their frequencies of detection and concentration ranges were generally lower than for OSR pollen (Table 2, Figure 1). Concentrations of thiamethoxam (Mann-Whitney test, U=11, p=0.0045) and thiacloprid (Mann-Whitney test, U=6, p=0.0006) were significantly lower in wildflower pollen compared with OSR pollen. Nevertheless, it is worth noting that the highest concentration of thiamethoxam was measured in the pollen from a wildflower (21 ng/g detected in pollen from *Matricaria recutita* flowers growing in the margin from OSR field 2 in farm 2, Table S4). Pollen was collected from 13 wildflower samples comprising 6 different species growing in 9 margins of WW fields between July and August. Three neonicotinoids and six fungicides were also detected in wildflower pollen collected in WW field margins, and all the agrochemicals had been applied previously to WW or to nearby fields. Concentrations of most pesticides were the same in pollen samples collected from the wildflowers growing in WW and OSR field margins with the

exception of thiacloprid (Mann-Whitney test, U=3, p=0.002), which was lower in wildflower pollen from WW field margins.

- *Pollen collected by honey bees*

The weight of pollen collected from all hives ranged between 15-303 g during the OSR bloom and between 14-103 g after the OSR bloom (Table S6 and S7), suggesting that all hives were active but that collection of pollen was very variable among hives due to unknown factors that may have affected their foraging behaviour (Beekman et al., 2004; Dussaubat et al., 2013). Honey bee pollen loads were sorted by species in order to study the variability in exposure levels, and sub-samples that were > 100 mg were analysed separately. The pesticide concentrations for the composite samples brought to the hives were also calculated for later comparison with pollen samples collected from the bumble bee nests. During June 2013, the honey bee-collected pollen included ten wildflower species and OSR pollen, and twelve wildflower species in August. The total pollen analysed comprised >86% of the total honey bee-collected pollen in June and >75% of the total honey bee-collected pollen in August (Tables S6 and S7). In terms of weight, the majority of these pollen samples collected by honey bees during the OSR flowering was from wildflowers, with just 10% of pollen coming from OSR (Botías et al., 2015). All pollen samples collected by honey bees were contaminated with a mixture of neonicotinoids and fungicides; a total of 14 compounds in pollen collected during OSR blooming and 10 after the bloom period. The number of pesticides found in any one pollen sample during OSR blooming ranged between 2 to 8 compounds. A similar mixture of neonicotinoids and fungicides were detected in honey bee-collected pollen in June as that present in wildflowers and OSR pollen: however, these compounds were at lower concentrations in honey bee corbicular pollen. The concentrations of pesticides in honey bee pollen were lower in August compared with June and significantly reduced for carbendazim (Mann-Whitney test, U=54, $p<0.0001$), thiamethoxam (Mann-Whitney test, U=131.5, p=0.0047) and trifloxystrobin (Mann-Whitney test, U=170.5, p=0.0459). In addition, clothianidin, thiacloprid, flusilazole and metconazole were no longer detected in honey bee collected pollen at this time.



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334 **Figure 1. The sum of the mean concentrations of neonicotinoids and fungicides in pollen samples from oilseed rape (OSR) flowers (n=11), wildflowers**
335 **from OSR margins (n=8) and WW margins (n=10), and collected by honey bees during OSR bloom (n=5) and after OSR bloom (n=5). OSR and wildflower**
336 **pollens were collected in 3 farms, honey bee pollen samples were collected from hives sited on the vicinity of these farms.** For the honey bee collected
337 pollen, concentrations of the whole composite samples brought to the hives were used for the calculation of the means (i.e. .one sample per hive was
338 analysed). ppb = ng/g wet weight of sample.

Overall these results reveal that pollen collected by honey bees are contaminated by similar mixtures of pesticides as those present in wildflower pollen collected from OSR or WW field margins. The most frequently detected pesticides both in honey bee collected pollen and wildflower pollen were thiamethoxam, thiacloprid, carbendazim, boscalid, spiroxamine, tebuconazole, pyraclostrobin and trifloxystrobin. Carbendazim and spiroxamine were detected at concentrations up to several hundreds of ng/g in some pollen samples. The totals for the mean measured concentrations of pesticides in pollen were 167 ng/g from OSR, and for wildflowers sampled from OSR and WW margins were 78 and 25 ng/g respectively. For honey bee pollen sampled during and after the OSR blooming period, concentrations were 16 and 3 ng/g, respectively (concentrations of the whole composite pollen samples brought to the hives were used for the calculation of the means) (Figure 1).

3.2 Neonicotinoid and fungicide levels in stored pollen and bumble bee individuals from nests placed in rural and urban areas

The presence of neonicotinoids and fungicide mixtures in pollen and individual bumble bees sampled from nests placed either in rural farmland or urban environments was determined. The weight of bumble bee nests at the time of collection ranged between 501- 705 g in rural areas and between 549-707 g in urban areas (Table S8), suggesting that all colonies were viable and actively foraging. The range, mean and median of the pesticide levels found are presented in Table 3.

Pollen samples collected from the stores of individual nests placed in rural areas (n=5) contained between 3 to 10 pesticide compounds (Table S8). The most frequently detected compounds (40-100%) included thiamethoxam, thiacloprid, carbendazim, boscalid, tebuconazole, flusilazole, metconazole and trifloxystrobin and at concentrations up to 68 ng/g for carbendazim and 84 ng/g for flusilazole. Imidacloprid, prochloraz and pyraclostrobin were also detected in 20% of the samples. Spiroxamine, although frequently detected at high concentrations in OSR and wildflower margin pollen, was below the MDL in bumble bee-collected pollen. The pollen from every nest was analysed as a whole, but the analysis of identity and proportion of pollen types under light microscopy revealed that it comprised a number of wildflower taxa with Rosaceae (*Crataegus monogyna/Malus* type) representing 42% on average of the visited plants, and 32% on average coming from OSR flowers (Table S9). In bumble bee individuals (Tables S10-S11), the neonicotinoids thiamethoxam, acetamiprid and thiacloprid were detected at concentrations below their MQLs. Carbendazim (up to 73 ng/g), boscalid (up to 10 ng/g), tebuconazole (up to 5 ng/g), flusilazole and metconazole were detected above the MQLs in several individuals. Carbendazim, boscalid, tebuconazole, flusilazole and thiacloprid were the most frequently detected in 14-64% of individual

372 bees. A comparison of the total pesticide concentrations in bumble bee and pollen samples revealed
373 large differences in pesticide contamination and exposure between each nest (Figure 2).

Table 3. The range, mean and median concentrations (ng/g) and frequency of detection of neonicotinoid and fungicide levels detected in stored pollen and in individual bumble bees sampled from nests sited in rural and urban landscapes.

	Rural area								Urban area							
	Bumblebee pollen 5 nests				Bumblebee n= 28 / 5 nests				Bumblebee pollen n= 13 / 3 nests				Bumblebee n= 15 / 3 nests			
	Freq %	Range *ppb*	Mean *ppb*	Median *ppb*	Freq %	Range *ppb*	Mean *ppb*	Median *ppb*	Freq %	Range *ppb*	Mean *ppb*	Median *ppb*	Freq %	Range *ppb*	Mean *ppb*	Median *ppb*
Thiamethoxam	100	1.7 - 35	18	21	7	<0.3 - <0.9			0	<0.12			7	<0.3 - <0.9		
Clothianidin	0	<0.72			0	<0.48			0	<0.72			0	<0.48		
Imidacloprid	20	<0.36 - <1.1			0	<0.72			33	<0.36 - 20	6.5	<0.36	0	<0.72		
Acetamiprid	0	<0.02			7	<0.01 - <0.04			0	<0.02			0	<0.01		
Thiacloprid	60	<0.07 - 13	2.9	0.45	18	<0.02 - <0.07			0	<0.07			40	<0.02 - 0.17	0.02	<0.02
Carbendazim	100	1.8- 68	25	12	64	<0.05 - 73	4.6	0.25	67	<0.08 - 0.80	0.40	0.36	0	<0.05		
Carboxin	0	<0.12			0	<0.24			0	<0.12			0	<0.24		
Boscalid	80	<0.12 - 13	5.4	4.6	36	<0.24 - 9.8	0.60	<0.24	0	<0.12			0	<0.24		
Spiroxamine	0	<0.02			0	<0.05			0	<0.02			0	<0.05		
Silthiofam	0	<0.24			0	<0.24			0	<0.24			0	<0.24		
Triticonazole	0	<0.24			0	<0.48			0	<0.24			0	<0.48		
Epoxiconazole	0	<0.84			0	<0.96			33	<0.84 - 2.8	0.90	<0.84	0	<0.96		
Tebuconazole	80	<0.24 - 15	4.6	2.8	18	<0.12 - 5.2	0.20	<0.12	67	<0.24 - 1.1	0.40	0.20	7	<0.12 - <0.36		
Flusilazole	40	<0.24 - 84	17	<0.24	14	<0.12 - 1.9	0.15	<0.12	0	<0.24			0	<0.12		
Prochloraz	20	<0.36 - 11	2.2	<0.36	0	<0.30			0	<0.36			0	<0.30		
Metconazole	40	<0.30 - 19	4.3	<0.30	4	<0.24 - 1.1	<0.24	<0.24	0	<0.30			0	<0.24		
Pyraclostrobin	20	<0.24 - 2.4	0.50	<0.24	0	<0.24			33	<0.24 - 1.0	0.30	<0.24	0	<0.24		
Trifloxystrobin	40	<0.24 - 4.4	1.7	<0.24	0	<0.01			0	<0.24			0	<0.01		
Fluoxastrobin	20	<0.01 - 0.1	0.02	<0.01	0	<0.24			0	<0.01			0	<0.24		
Piperonyl butoxide	0	<0.72			0	<0.24			0	<0.72			0	<0.24		

Pollen and bumble bees were collected from the same nests. Between 5 and 8 individuals per nest were analysed (except for one nest where only 3 workers were available). For the calculations of means and medians, all concentrations that were over the limits of detection (≥MDL) but below the limits of quantification (<MQL) were assigned the MDL value, whilst concentrations below the MDL were considered to be zero. ppb = ng/g wet weight of sample. Compounds highlighted in bold correspond to pesticides that were commonly found in pollen from both rural and urban areas.

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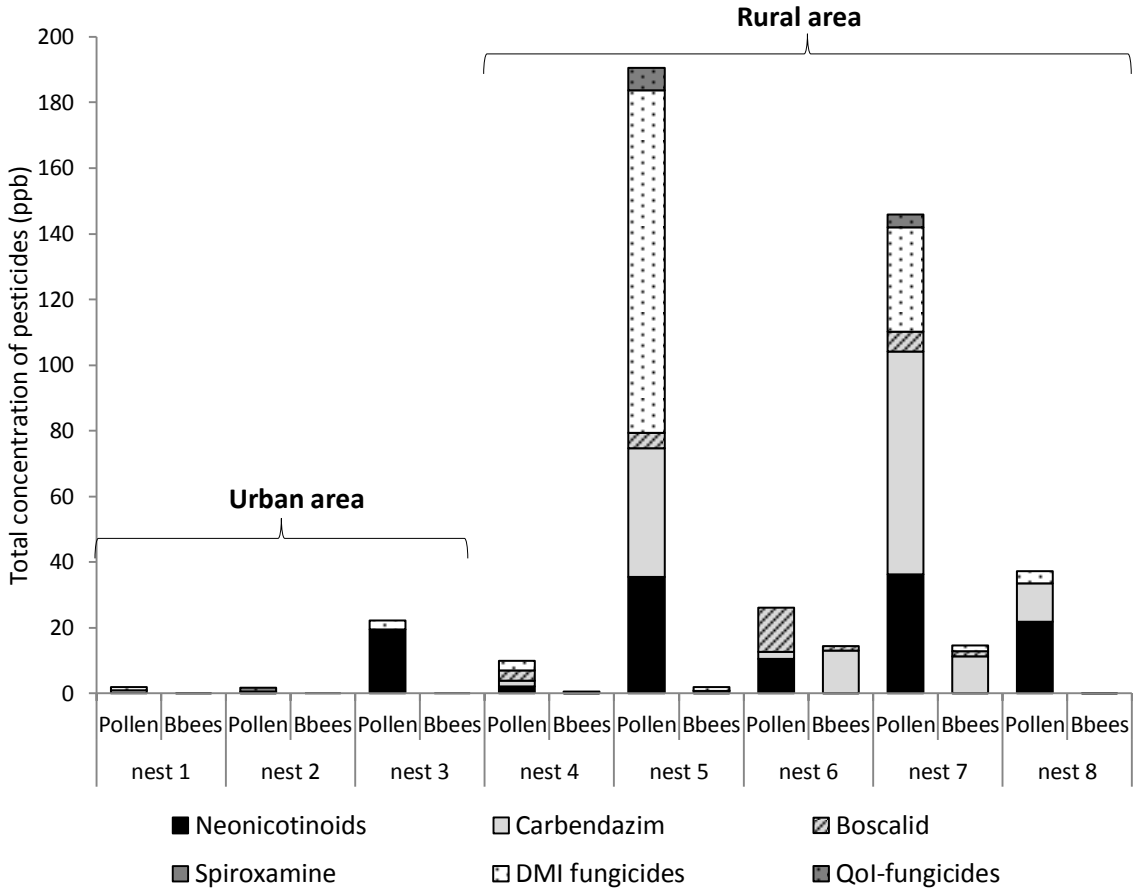

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383 **Figure 2. The sum of the mean concentrations of neonicotinoids and fungicides in individual bumble bees (bbees) and collected pollen in nests sited in**
384 **urban and rural areas.** ppb = ng/g wet weight of sample.

Concentrations of pesticides in pollen and bees sampled in urban areas (n=3) were much lower compared with rural areas (Figure 2). In nests placed in urban areas, five pesticides were detected in pollen collected by bumble bees; imidacloprid, carbendazim, epoxiconazole, tebuconazole and pyraclostrobin. Imidacloprid was detected in pollen at up to 20 ng/g. Thiamethoxam, thiacloprid and tebuconazole were detected in bumble bee individuals at concentrations < 1 ng/g. Imidacloprid, carbendazim, tebuconazole and pyraclostrobin are the pesticides that were commonly found in pollen from both rural and urban areas.

A comparison of pollen collected by honey bees and bumble bees during the OSR bloom in rural landscapes revealed that many of the neonicotinoid and fungicide compounds that were present at concentrations > 1 ng/g were common to pollen collected by both bee species (Figure 3).

The insecticide synergist piperonyl butoxide was not detected in any of the pollen samples in this study.

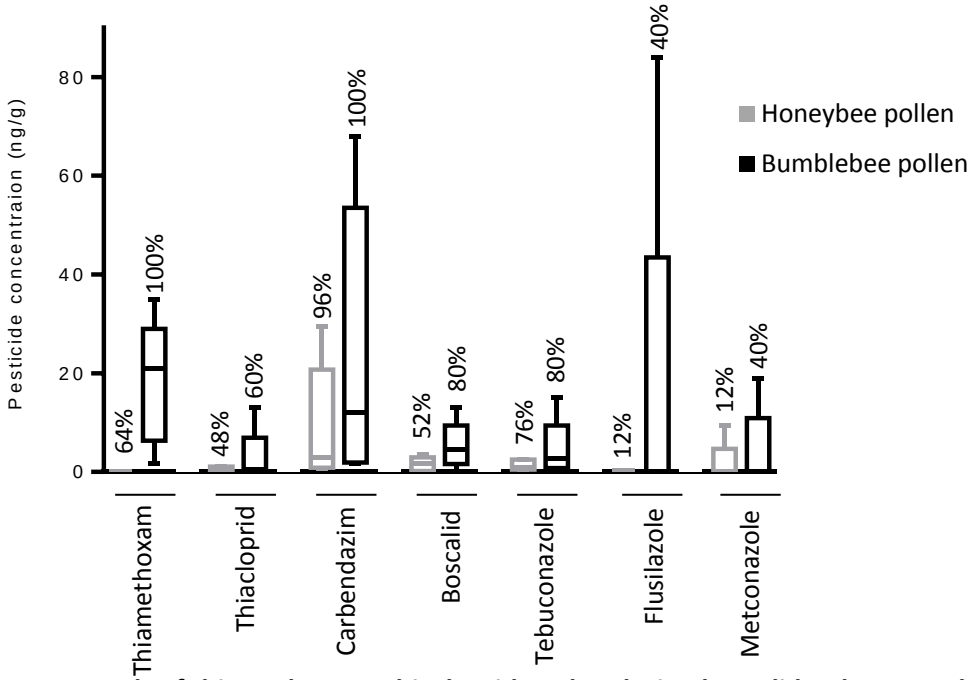

Figure 3. Levels of thiamethoxam, thiacloprid, carbendazim, boscalid, tebuconazole, flusilazole and metaconazole in pollen samples collected by honey bee (n=5 beehives) and bumble bees (n=5 nests). Honey bee hives were placed in farms near OSR fields and the pollen was collected during the OSR bloom for 4 days using pollen traps. Concentrations of the whole composite samples brought to the hives were used for the calculation of the means. Bumble bee nests were placed in rural areas in arable landscapes, and the pollen was collected after 4 weeks of free foraging in the field. The frequency of detection of neonicotinoid and fungicide are indicated above each box-and-whiskers- plot. The length of each box corresponds to the interquartile range, the upper and lower boundary of the box representing 75th and 25th percentiles, respectively. The upper and lower

whiskers represent the maximum and the minimum values, respectively. The line in the box indicates the median value.

4. Discussion

Debates over the impacts of pesticides on bees have tended to focus on the effects of specific compounds or groups of compounds, with much attention in recent years on neonicotinoid insecticides. However, it has recently become clear that honey bees are chronically exposed to complex mixtures of pesticides (Johnson et al., 2012). Here, we show that both flowering crops and nearby wildflowers are contaminated with a broad range of pesticides, and that this translates into exposure of both honey bees and bumble bees to similar complex mixtures, with marked differences in concentrations of pesticides in pollen collected by the two bee species. However, these differences in concentrations between honey bee and bumble bee pollen must be tempered by the fact that the bumble bee nests and the honey bee hives were placed in different rural areas and also that honey bee pollen was gathered for 4 days using traps, whereas bumble bees foraged for 4 weeks before the pollen was collected in the nests. Nevertheless, it is likely that the pollen sample collected by bumble bees was gathered in the previous two-three days as they keep low storage levels to avoid theft of honey and pollen by mammals (Heinrich 2004).

Our data show that the pollen of oilseed rape crops is contaminated with a broad range of pesticides, notably spiroxamine, carbendazim, the neonicotinoids thiamethoxam and clothianidin, a range of DMI fungicides and trifloxystrobin. Other fungicides, i.e. boscalid, pyraclostrobin and fluoxastrobin were also present, but less frequently detected. Broadly similar cocktails, at generally slightly lower concentrations, were found in hand-collected pollen from wildflowers in arable field margins. It should be noted that this is not an exhaustive list of the pesticides present; in particular we did not screen for pyrethroids that were used on the farms we studied because these require an entirely different analytical approach.

Some of the neonicotinoids and fungicides that we have detected in honey bee collected pollen had already been detected in similar pollen samples in other studies, although this is the first study providing data in bee pollen for this mixture of pesticides in UK. It should be noted, however, that these other studies used composite pollen samples (as opposed to pollen from individual species here) and therefore, provide less information on the variability of exposure levels. In pollen samples from honey bee colonies in western France, carbendazim and flusilazole were detected at concentrations up to 2595 ng/g and 52 ng/g, respectively (as opposed to 120 and 6.1 ng/g

441 respectively in our study) (Lambert et al., 2013). Higher concentrations of thiacloprid, imidacloprid,
442 carbendazim, trifloxystrobin, boscalid, tebuconazole, pyraclostrobin and trifloxystrobin were also
443 observed in honey bee pollen collected in hives from North America (up to 962 ng/g for boscalid)
444 (Mullin et al., 2010), but their frequencies were generally much lower than those detected in this
445 study. However, differences between studies may also be due to various factors such as the timing
446 of pesticide spray, residual duration and the timing of pollen collection. Overall, our results and
447 these studies indicate that these mixtures of insecticides and fungicides appear ubiquitous in pollen
448 samples and that even higher concentrations than the ones observed in our study can be
449 encountered.

450 Honey bees and the bumble bee *Bombus terrestris* are both highly polylectic in their flower visits.
451 Both taxa are regular visitors to OSR flowers (Cresswell and Osborne 2004), but both also visit a
452 broad range of wildflowers present in field margins and hedgerows, gardens, and uncropped areas,
453 although the two species exhibit different floral preferences (Wood et al., 2015). We would, thus,
454 expect both species to be exposed to the chemicals we found in pollen of the crop and wildflowers,
455 and indeed this was the case. For both species, pollen from hawthorn represents a major part of the
456 collected pollen (up to 87%) and that the pollen from hawthorn collected by honey bees was often
457 contaminated by several pesticides (up to 6) and notably at concentrations up to 29 ng/g for
458 carbendazim.

459 For pollen collected by honey bees, the major pesticide contaminants were (in declining order of
460 mean concentration) carbendazim, boscalid, spiroxamine, trifloxystrobin and tebuconazole, with
461 small amounts of the neonicotinoids thiacloprid, imidacloprid and thiamethoxam. Overall, the
462 concentrations tend to be lower than in the crop or adjacent wildflowers, likely to be because the
463 bees are also collecting pollen from uncontaminated wildflowers distant from arable fields, diluting
464 the overall concentration returning to the hive. There was a reduction in the concentrations of
465 neonicotinoids and fungicides detected in honey bee pollen collected after OSR blooming,
466 presumably because the bees are no longer feeding on treated crops, but also perhaps because of
467 ongoing biodegradation and photolysis of pesticide residues in the environment as summer
468 progresses (Bonmatin et al., 2015; Gupta et al., 2008).

469 Concentrations of pesticides in pollen collected by bumble bees markedly differed from those for
470 pollen collected by honey bees during the OSR bloom (Figure 3). The major contaminants were
471 carbendazim, thiamethoxam and tebuconazole. The high levels of thiamethoxam are particularly
472 noteworthy, for this is an insecticide of high toxicity to bees. Experimental studies such as Whitehorn
473 et al. (2012), which describe severe impacts of neonicotinoids on bumble bees, have been criticised

for using unrealistically high concentrations of pesticide (in this example 6 ng/g of imidacloprid) (Carreck and Ratnieksi 2014). Our data suggest that real-world exposure may often be much higher than this, for the mean concentration of thiamethoxam in our samples from 5 nests located in farmland was 18 ng/g, and one of the nests located in urban environment showed more than 19 ng/g for imidacloprid. It has also been demonstrated that there are synergies between neonicotinoids and DMI fungicides such as flusilazole (Iwasa et al., 2004; Schmuck et al., 2003), so the presence of both compounds at high concentrations in pollen stores of bumble bees is a cause for concern.

Recently, Ründlof et al. (2015) found that bumble bee colonies were adversely affected by proximity to fields of OSR treated with clothianidin (the major bioactive metabolite of thiamethoxam), but that honey bees showed no significant harm, at least within one season. Our results suggest an explanation for this disparity; bumble bees may simply be exposed to the pesticide more, perhaps because of a greater propensity to collect OSR pollen (i.e. proportion of OSR pollen was 10% on average for honey bees as opposed to 32% on average for bumble bees). It may also be because bumble bees tend to forage over shorter distances compared to honey bees (Knight et al., 2005), which may mean that there is less dilution of pesticide residues coming in to the nest when these are located in the vicinity of arable lands. However, it should be noted that our data set is small, and that honey bee hives and bumble bee colonies were not placed in exactly the same localities. They were also sampled in different ways; honey bee pollen was collected from returning bees using a pollen trap, whereas pollen traps are not effective for bumble bees for which in-nest pollen stores were sampled instead. Further research is clearly needed to confirm whether bumble bees really are more prone to collect pollen contaminated with pesticides, and if so, why.

Our sampling was conducted in the spring and summer of 2013. Since then, a moratorium on the use of neonicotinoids as seed dressings on flowering crops has come into effect in the EU (though some individual countries have granted derogations for continued use). It would be fascinating to repeat our work to examine whether contamination of wildflowers and bee pollen with neonicotinoids has dropped as a result.

In contrast to rural areas, there were generally few pesticide residues in pollen collected by bumble bee colonies in the 3 nests placed in urban areas. Imidacloprid was the biggest contaminant, and the only neonicotinoid detected. To our knowledge, these are the first data pertaining to exposure of bees to pesticides in urban environments, and a more extensive study is needed to determine whether pesticide exposures are much lower in these areas. While pesticide usage data in the UK is available for farmland, no data are publicly available on sales or usage of pesticides by gardeners and

local authorities, and very little information is available on likely levels of contamination of ornamental plants with pesticides, so we can only speculate as to the source of this exposure. Imidacloprid was widely sold in the UK as a garden insecticide in the past, but has been largely replaced by thiacloprid and acetamiprid in recent years (D.G. pers. obs.). It is unclear whether the imidacloprid found in our samples is due to persistent residues from past use, or due to ongoing environmental contamination from other sources – for example imidacloprid is the active ingredient in formulations widely used for ant control (e.g. "Maxforce Quantum", Bayer Crop Science) and for flea control on domestic animals (e.g. "Advantage", Bayer Crop Science).

It has previously been found that bumble bee populations in gardens are higher than those in farmland (Goulson et al., 2010; Osborne et al., 2008), and our results may in part explain why – because they could be exposed to fewer pesticides. However, they also probably have access to a greater abundance and diversity of floral resources in gardens, and without further experimental manipulations, we cannot determine which of these factors is most important.

Screening of whole bees for pesticides detected generally low concentrations, compared to pollen samples (Table 3), although a range of DMI fungicides were found at concentrations exceeding 1 ng/g in some samples, and carbendazim was found at a mean concentration of 4.6 ng/g in bumble bees from rural areas. There were also detectable traces of the neonicotinoids thiamethoxam, acetamiprid and thiacloprid in some bees. For practical reasons, bumble bee pollen and bumble bee individuals were collected at different times (individuals were collected 6 weeks after the pollen was collected, i.e. after the OSR bloom) and this could partially explain the lower concentrations observed for some pesticides in bumble bees. Despite this, it seems likely that pesticides are metabolised at varying rates once consumed by bees; for instance, it has been shown that bumble bees can clear imidacloprid from their body after 2 days of exposure (Cresswell et al., 2014) and a half-life of 5 hours has been recorded for honey bees (Suchail et al., 2004). A recent study has revealed that bee detoxification of the xenobiotic nicotine was associated with increased energetic investment and antioxidant and heat shock response (du Rand et al., 2015). The process of detoxifying an array of xenobiotics arising from exposure to agrochemicals and secondary plant products may result in metabolic stress and increased susceptibility of the bee to pathogens and disease (Goulson et al., 2015).

It is notable that the bulk of pesticides found in both honey bee pollen and bumble bee pollen were fungicides, particularly carbendazim, boscalid, tebuconazole, flusilazole, metconazole, pyraclostrobin and trifloxystrobin. Although fungicides have generally low toxicity to bees (Johnson 2015) it has been shown recently that spray applications of a commercial-formulation Pristine (a combination of

two fungicides-12.8% ai pyraclostrobin and 25.2% ai boscalid) at the highest recommended field rates (1.6 kg/ha) can disrupt the nest recognition abilities of females from two solitary bee species: *Osmia lignaria* and *Megachile rotundata* (Artz et al. 2015). Furthermore, little is understood about the impacts they may have on beneficial fungi commonly found in stored pollen (bee bread). Classes of fungicides commonly found in bee pollen in our study (boscalid, DMIs and quinone outside inhibitors, QoIs) have been reported to be fungicidal against 12 fungal species isolated from bee bread (Yoder et al., 2012). Bee bread is produced by fungal fermentation of stored pollen and is important food for honey bee larvae. Alterations in the diversity of fungi may affect food value and also allow pathogenic fungi such as the etiological agent of chalkbrood disease, *Ascosphaera apis*, to thrive in the hive, thus affecting colony performance (Yoder et al., 2013).

In summary, our study confirms that bees foraging in arable farmland are exposed to a complex cocktail of neonicotinoid insecticides and fungicides in the pollen they collect. While quantifying realistic levels of exposure via pollen as we have done here is an important step forwards, we did not examine exposure via nectar, which we intend to address in future work. A major challenge which has yet to be tackled is attempting to understand what effects simultaneous exposure to multiple pesticides has upon bees in the field.

Acknowledgments

We are grateful to the Soil Association (Bristol, UK) for part funding of this work, and to the farmers for allowing us to collect samples on their farms and for sharing their pesticide usage data.

Conflict of Interest

The authors declare that they have no conflict of interest.

Statement on animal ethical care

The work reported here conforms to the regulatory requirements for animal experimentation in the UK. No ethics approval was required for this study. Honey bee hives and bumble bee nests were housed on private land for which research permission was granted by the owners. This study did not involve endangered or protected species.

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