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Quantifying exposure of wild bumblebees to mixtures of agrochemicals in agricultural and urban landscapes☆

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ABSTRACT

The increased use of pesticides has caused concern over the possible direct association of exposure to combinations of these compounds with bee health problems. There is growing proof that bees are regularly exposed to mixtures of agrochemicals, but most research has been focused on managed bees living in farmland, whereas little is known about exposure of wild bees, both in farmland and urban habitats. To determine exposure of wild bumblebees to pesticides in agricultural and urban environments through the season, specimens of five different species were collected from farms and ornamental urban gardens in three sampling periods. Five neonicotinoid insecticides, thirteen fungicides and a pesticide synergist were analysed in each of the specimens collected. In total, 61% of the 150 individuals tested had detectable levels of at least one of the compounds, with boscalid being the most frequently detected (35%), followed by tebuconazole (27%), spiroxamine (19%), carbendazim (11%), epoxiconazole (8%), imidacloprid (7%), metconazole (7%) and thiamethoxam (6%). Quantifiable concentrations ranged from 0.17 to 54.4 ng/g (bee body weight) for individual pesticides. From all the bees where pesticides were detected, the majority (71%) had more than one compound, with a maximum of seven pesticides detected in one specimen. Concentrations and detection frequencies were higher in bees collected from farmland compared to urban sites, and pesticide concentrations decreased through the season. Overall, our results show that wild bumblebees are exposed to multiple pesticides when foraging in agricultural and urban landscapes. Such mixtures are detected in bee tissues not just during the crop flowering period, but also later in the season. Therefore, contact with these combinations of active compounds might be more prolonged in time and widespread in the environment than previously assumed. These findings may help to direct future research and pesticide regulation strategies to promote the conservation of wild bee populations.

1. Introduction

Bees are exposed to environmental pollutants via contaminated food resources such as pollen, nectar or water (Bonmatin et al., 2015), and through external contact with aerosols during spraying and contaminated dust emitted during the sowing of dressed seeds as their hairy bodies trap particulate residues (Greig-Smith et al., 1994; Pistorius et al., 2015). Many studies have used honeybees as relevant organisms to monitor environmental pollution (Celli and Maccagnani, 2003; Porrini et al., 2003). Bumblebees also forage in a great diversity of places and strongly interact with the environment, mainly the flora, surrounding their nests in a range of maximum foraging distances of 363–1650 m depending on the species (Walther-Hellwig and Frankl, 2000; Wood et al., 2015), and are thus also suitable organisms for monitoring landscape-based ecological pollution.

While most pesticide research has been focused on managed bees, there has been less work on wild bee populations. For instance, the only European bumblebee that has been studied in relation to pesticide exposure and toxicology is *Bombus terrestris*, simply because this species is easy to rear in captivity and commercially reared colonies are readily available (Baron et al., 2014; Gill et al., 2012; Rundlöf et al., 2015; Whitehorn et al., 2012).

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There is increasing evidence that managed bees living in agricultural landscapes are routinely exposed to mixtures of agrochemicals (David et al., 2016; Lambert et al., 2013; Long and Krupke, 2016; Mullin et al., 2010; Pettis et al., 2013), but little is known about the exposure of wild bees in these environments (Hladik et al., 2016). Nevertheless, bees constitute a highly diverse group where different taxonomic groups differ widely in their vulnerability to pesticide exposure (Biddinger et al., 2013; Devillers et al., 2007; Piiroinen and Goulson, 2016; Thompson and Hunt, 1999). Furthermore, bee species exhibit pronounced differences in floral preferences and foraging habits, collecting pollen and nectar more frequently from particular plant species according to their morphological traits (e.g. tongue length, body shape and size) and nutritional needs (Goulson et al., 2008; Vanderplanck et al., 2014; Vaudo et al., 2016). Such foraging choices may profoundly influence the probability of bees to be more or less exposed to some active compounds (Woodcock et al., 2016). Treated crop plants growing in agricultural landscapes have often been regarded as the only source of exposure to agrochemicals for pollinators, but recent research revealed their presence in wild plants growing near crops (Botías et al., 2015; David et al., 2016; Long and Krupke, 2016; Mogren and Lundgren, 2016). We would expect bees that visit flowering arable crops to have higher exposure than those that do not, but also those that visit wild plant species may have varying exposure depending on the ecology, physiology and morphology of their preferred flowers (Botías et al., 2016). Therefore, it is essential to understand the possible differences in levels of exposure among bee species, since this could reveal which are the most likely exposed and the most frequent mixtures of agrochemicals that they are exposed to.

The widespread occurrence of mixtures of agrochemicals in bee tissues (Hladik et al., 2016) increases concerns regarding the possible detrimental effects of simultaneous exposure to a cocktail of compounds. In general, only the effects of single active substances are studied in toxicity studies both for research and pesticide registration protocols, and exposure to mixtures are only evaluated in risks assessments when they are part of the same formulation. However, the application of two or more plant protection products during the same cropping season is a common practice in conventional farming (Botías et al., 2015; Garthwaite et al., 2013), and hence complex mixtures of agrochemicals which are not co-formulants of a single product can be simultaneously detected in bee forage and bee tissues (David et al., 2016; Hladik et al., 2016; Long and Krupke, 2016). This issue is worrisome given that exposure to mixtures might pose higher risks for animal health than the single impact of a specific class of compounds (Cedergreen, 2014; Rizzati et al., 2016). For example, some combinations of insecticides (*e.g.* pyrethroids with neonicotinoids) and of insecticides with fungicides can lead to additive and synergistic toxicity for bees at the individual and the colony level (Gill et al., 2012; Iwasa et al., 2004; Schmuck et al., 2003; Sgolastra et al., 2016). The scarcity of information on the field-relevant mixtures of agrochemicals and levels of exposure for bees could lead us to overlook the possible additive or synergistic effects of pesticide mixtures when risk assessment studies are performed, some of which have been designed to evaluate the hazards of such combinations (Sánchez-Bayo and Goka, 2014).

Another major gap in knowledge regarding exposure of bees to pesticides is the potential uptake and contact with these compounds in urban areas, where ornamental nursery plants can also be treated with pesticides (Brown et al., 2013; Fevery et al., 2016) and no information is available on their use in domestic gardens. The possible exposure of bees to harmful pesticides through forage collected in gardens is of high ecological concern, since these habitats are of great value for bees, providing nectar, pollen and nest sites, and sustaining a remarkably high pollinator species richness and abundance (Baldock et al., 2015; Kaluza et al., 2016; Samnegård et al., 2011), including bumblebees (Fetridge et al., 2008; Goulson et al., 2010). If foraging resources and nesting sites in urban habitats are contaminated with pesticide residues, it is likely that exposure to certain active compounds could be more widely spread in the landscape and more prolonged in time than previously assumed.

The aim of this study was to evaluate and compare exposure in different wild bumblebee species. To do this, we analysed the levels of five neonicotinoid insecticides, thirteen currently-used fungicides and a pesticide synergist in tissues of five bumblebee species (*B. hortorum*, *B. pascuorum*, *B. terrestris*, *B. lapidarius* and *B. pratorum*). These wild bumblebee samples were collected in agricultural and urban habitats to compare levels of exposure in both environments and to study distribution of agrochemicals in the landscape. The bees were gathered in three different periods (late spring, early summer and midsummer) in order to monitor the length of exposure to agrochemicals through the season.

Our results show evidence that wild bumblebees are frequently exposed to mixtures of agrochemicals when they forage in arable and urban habitats, with peak concentrations decreasing through the season.

2. Materials and methods

2.1. Sampling sites and field collection

Wild bumblebees were collected in five farms and five urban landscapes in East Sussex (South-East England, UK), all sites being at least 2 km apart from each other (Fig. 1) (Table S1). The sites selected to collect bees in agricultural land consisted of arable fields within mixed farms, where the predominant crops were oilseed rape, winter wheat and spring barley, and part of the land was pasture. The urban sampling sites consisted of ornamental public gardens and parks surrounded by houses that had private gardens in most cases. Foraging bumblebees were collected using insect nets and kept in individual labeled tubes and put on ice during transport back to the lab, and then kept at −80 °C until pesticide analysis was performed. Specimens of five bumblebee species with different ranges of tongue length were sampled (Brodie, 1996; Prys-Jones and Corbet, 2011): short-tongued bumblebees were *B. pratorum* (6.4–7.1 mm), *B. lapidarius* (6–8.1 mm), *B. terrestris* (5.8–8.2 mm); medium-tongued was *B. pascuorum* (7.6–8.6 mm); and long-tongued was *B. hortorum* (12–13.5 mm) (Table 1). The flowers where the bees were foraging at the time of capture were recorded (Tables S2a–S1c), since bumblebees exhibit a high degree of floral constancy (Wilson and Stine, 1996), and this may help predict exposure.

Bumblebee individuals were gathered during three sampling periods, spring (27/04/14–14/05/14), early summer (5/06/14–23/06/14) and midsummer (15/07/14–2/08/14), and 150 bee individuals were collected in total. Oilseed rape crops were in bloom during the first sampling period (late spring), and 18 out of the 25 individuals gathered in arable sites during that period were foraging in oilseed rape crops when collected (Table S2a). The pesticide usage information of the crops where bees were foraging was not provided by the farmers. The EU moratorium on the use of neonicotinoid insecticides started on the 1st December 2013, but the oilseed rape crops that were in bloom in the 2014 spring were sown at the end of August-beginning of September 2013, so these crops were still allowed to be seed-treated with neonicotinoids.

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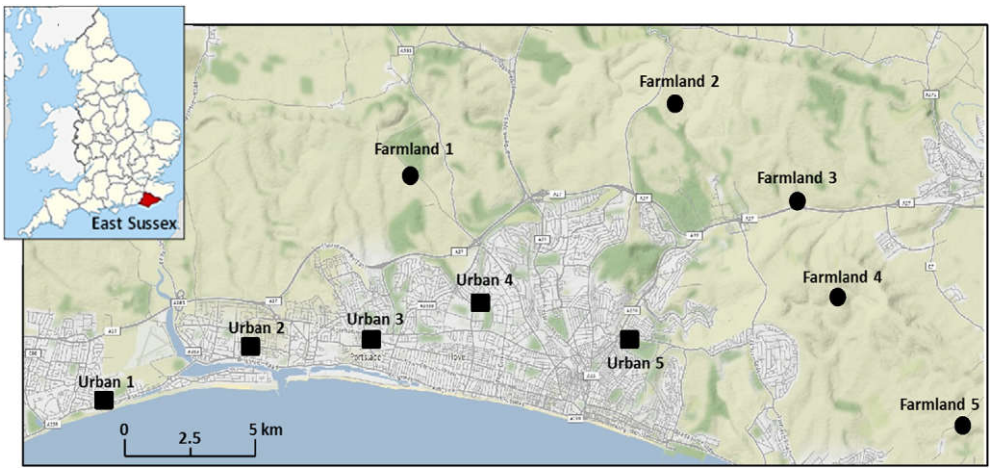

Fig. 1. Location of sites in East Sussex (UK), where wild bumblebees were collected from farmland sites (circles) and urban sites (squares). The sites selected to collect bees in agricultural land consisted of arable fields within mixed farms, where the predominant crops were oilseed rape, winter wheat and spring barley, and part of the land was pasture. The urban sampling sites consisted of ornamental public gardens and parks surrounded by houses that had private gardens in most cases.

Table 1
Mean (±standard deviation) and range of body mass values (mg), and tongue length range (mm) for the five bumblebee species analysed.

Bumblebee species	Body mass (mg)		Tongue length range (mm)
	MEAN ± S.D.	Range	(Brodie, 1996; Prys-Jones and Corbet, 2011)
B. hortorum	105 ± 45	40–223	12–13.5
B. pascuorum	97 ± 34	29–171	7.6–8.6
B. terrestris	142 ± 46	45–236	5.8–8.2
B. lapidarius	117 ± 49	40–226	6–8.1
B. pratorum	79 ± 32	21–161	6.4–7.1

2.2. Pesticide analysis

2.2.1. Chemicals and reagents

Eight classes of contaminants were chosen to be tested in the bumblebee samples, including the five neonicotinoid insecticides that are registered for use in the UK, based on the most used (by weight or area treated) in UK crops including oilseed rape, wheat, spring barley, field bean, strawberry and raspberry crops (https://secure.fera.defra.gov.uk/pusstats/surveys/2014surveys.cfm) (Table S3). Except for certified standards of carbendazim-d3 and tebuconazole-d6, which were purchased from LGC standards UK and carbamazepine-d10 and prochloraz-d7 from QMX Laboratories Limited UK, all the other certified standards as well as formic acid, magnesium sulphate, ammonium formate, sodium acetate and Supel™ QuE PSA/C18/GCB (ratio 1/1/1) were obtained from Sigma Aldrich UK. The compound purity of the pesticide standards was >99%, apart from spiroxamine (98.5%), triticonazole (98.8%), piperonyl butoxide (97.9%). Isotopic purity of deuterated standards was >97%. HPLC grade acetonitrile, toluene and water were obtained from Rathburns UK.

2.2.2. Preparation of samples and residue analysis

The extraction of pesticides from the bumblebee samples was performed as reported in David et al., 2015.

Briefly, individual whole bumblebee specimens (N = 150) were ground in liquid nitrogen and weighed (mean weight ± S.D = 108 ± 46 mg; range = 21–236 mg). Each sample was spiked with 10 μl acetonitrile containing the mixture of deuterated internal standards (IS) at 40 ng/ml (400 pg of each IS). Subsequently, the extraction was performed by the addition of 400 μl of water, 500 μl of acetonitrile, 250 mg of magnesium sulphate: sodium acetate mix (4:1) and 50 mg of SupelTM QuE PSA/C18/GCB for the dispersive solid phase extraction step (dSPE) (QuEChERS method). After the dSPE step, the sorbent was extracted with acetonitrile/toluene (3/1, 150 μl) and the supernatant was combined with that of the previous acetonitrile extract and spin filtered (0.22 μm). After evaporation, reconstitution was made with 120 μl acetonitrile:water (30:70) and the extract was centrifuged (20 min). The supernatant obtained was stored in the dark at −20 °C before analysis.

2.2.3. UHPLC-MS/MS analyses

Extracts of samples were analysed by ultra high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) as described in David et al. (2015), using a Waters Acquity UHPLC system coupled to a Quattro Premier triple quadrupole mass spectrometer from Micromass (Waters, Manchester, UK). Acquisition of data was performed with the software MassLynx 4.1 and the quantification was established by the calculation of the response factor of each pesticide to its internal standard. Least-square linear regression analysis of the native analyte to deuterated internal standard (concentration ratio) versus the peak area was used to determine analyte concentrations. Further methodological details are described in David et al. (2015).

Table S4 reports the method detection limits (MDL) and the method quantification limits (MQL) for all the compounds that were analysed in bumblebees.

2.3. Statistical analysis

Shapiro-Wilk test was used to assess whether data (i.e. concentration of pesticides detected in the bumblebees per species, habitat, sampling period and bumblebee body mass) met the assumptions of normality. Since pesticide concentrations did not

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meet the assumptions of normality, non-parametric Kruskal-Wallis tests were used to compare this variable among the 5 different bee species and 3 sampling periods. Pairwise comparisons between bee species, sampling periods and habitats were performed using Mann–Whitney U-tests. Bumblebee body mass and frequencies of pesticide detection in the 5 species were normally distributed, so one-way ANOVA analyses were carried out to compare weights of the 5 species, using Bonferroni post-hoc tests for pairwise comparisons, and to compare the frequencies of detection among them. For the statistical analysis, the MDL value was assigned to concentrations above the limits of detection (≥MDL) but below the limits of quantification (>MQL), whereas those below the MDL were considered to be zero. The software used to perform the statistical analysis was SPSS 21.

3. Results

3.1. Ranges, frequencies and average levels of neonicotinoid and fungicide residues detected in wild bumblebee samples

In total, 60.7% of the 150 individuals tested had detectable levels of at least one of the compounds analysed, with boscalid being the most frequently detected (in 35.3% of all the bees analysed), followed by tebuconazole (27.3%), spiroxamine (18.7%), carbendazim (10.7%), epoxiconazole (8%), imidacloprid (7.3%), metconazole (6.7%) and thiamethoxam (6%) (Table 2) (Tables S2a–2c). In general, from all the bees where pesticides were detected, the majority (71.4%) had more than one compound, and 43.4% of the bees contained two or more, with a maximum of 7 different pesticides being detected in one specimen (*B. lapidarius* collected from urban site 2 in June) (Table S2b).

3.2. Levels of pesticide exposure for the five bumblebee species studied

Although the combinations of pesticides detected varied notably among individuals of the same and the other species, some combinations occurred very frequently in farmland bumblebees. Boscalid with DMI-fungicides was the most common mixture detected in bumblebees (64%) foraging in farmland in spring (April–May), except for *B. pratorum* individuals where such combination was not detected (Table S2a). In early summer (June) spiroxamine with DMI-fungicides was detected in the great majority of farmland bumblebees (88%), regardless of the species (Table S2b) (Figs. 2 and 3). In general, the detection frequency of the 19 agrochemicals analysed did not significantly differ among the 5 species (ANOVA, $F(4, 25) = 0.78$, $P = 0.55$). However, when the concentrations of pesticides detected in the 5 bumblebee species were compared, we found that *B. pratorum*, the species with the lowest body mass and shortest tongue length range (Table 1), had significantly lower residue levels (mean ± SD = 1.7 ± 3.6 ng/g) than *B. hortorum* (4.7 ± 10.1 ng/g) (M-W test: $U(58) = 291$; $P = 0.013$), *B. terrestris* (6.8 ± 10.4 ng/g) ($U(58) = 275$; $P = 0.006$), *B. lapidarius* (7.2 ± 11.8) ($U(58) = 260$; $P = 0.003$) and also tended to have lower concentrations than *B. pascuorum* (2.8 ± 4.9) ($U(58) = 330$; $P = 0.056$).

In order to evaluate the relationship between bee body size and levels of exposure, we compared the body mass of the five bumblebee species, and we found that *B. pratorum* was significantly lighter than the two species with the highest pesticide concentrations, *B. terrestris* (1-way ANOVA with Bonferroni's multiple comparison test: $P = 0.03$) and *B. lapidarius* ($P = 0.017$).

3.3. Levels of pesticide exposure in arable and urban habitats

In general, bees foraging in agricultural landscapes had significantly higher levels of agrochemicals (6.8 ± 9.5 ng/g) than those foraging in urban sites (2.5 ± 7.8 ng/g)(M-W test: $U(148) = 1635.5$; $Z = -4.6$; $P < 0.001$)(Fig. 2). However, the highest levels and frequencies of detection for neonicotinoids (10 ng/g of imidacloprid on a *B. terrestris* specimen) and the most frequently detected fungicide boscalid (54.5 ng/g in a *B. lapidarius* specimen) were recorded in urban bumblebees collected during the early summer (June) (Table S2b). Overall, neonicotinoids were found in more bees in urban sites than in farmland (9.3% versus 2.7%), with all five neonicotinoids registered for use in the UK found in at least one urban bee.

3.4. Changing levels of pesticide exposure through the season

The levels of exposure to agrochemicals for wild bumblebees were examined for the period of highest foraging activity in the studied area (East Sussex, England), and we found that the frequencies of detection decreased both in arable and urban habitats for the 5 species evaluated in midsummer (July) (Fig. 3), when only 28% of the bees collected had at least one agrochemical, compared to 76% in late spring (April–May) and 78% in early summer (June). Consequently, the average concentrations detected were lower in midsummer (July: 0.6 ± 2.3 ng/g) than in spring (April–May: 5.9 ± 7.6 ng/g) (M-W test: $U(98) = 474$; $Z = -5.67$; $P < 0.001$) and early summer (June: 7.5 ± 12.4 ng/g) (M-W test: $U(98) = 462.5$; $Z = -5.74$; $P < 0.001$) (Table 2).

4. Discussion

Our field study revealed that free-flying wild bumblebees are exposed to multiple pesticide residues, with different levels and frequencies of detection according to the species, sampling period and landscape context. Several studies have reported the presence of mixtures of agrochemicals in honeybee matrices (Lambert et al., 2013; Mullin et al., 2010; Pettis et al., 2013), where more than 170 compounds have been detected so far (Sánchez-Bayo and Goka, 2014), but little is known about the exposure of wild bees. Recent research detected up to 19 different chemicals in native bees collected from wheat fields and grassland in Colorado (USA) (Hladik et al., 2016), reporting levels as high as 310 ng/g for thiamethoxam, 87 ng/g for clothianidin and 82 ng/g for imidacloprid. The maximum concentrations detected in our bumblebee samples were much lower (i.e. 2.35 ng/g for thiamethoxam, 1.4 ng/g for clothianidin and 10 ng/g for imidacloprid), which could be explained by the differences between both experimental designs. While we collected free-flying bumblebees with nets and analysed them individually, Hladik et al. (2016) performed the analysis on composite samples containing approximately 10 individuals of different wild bee species that were collected using bee monitoring traps. The collection and analysis of composite samples containing individuals from different bee genera might conceivably increase the chances of including particular specimens that could have been exposed to very high concentrations of pesticides due to their foraging and feeding behaviour, metabolic rates and morphological traits. Although the bumblebee species analysed in our study can present differences in their foraging distances, they seem to use and benefit similarly from the resources available in farmland (Wood et al., 2015), while bees from other genera, specially solitary bees, may present more variation in their foraging choices (Wood et al., 2016), and thus, a wider range of levels of exposure to agrochemicals. On the other hand, the dissimilarities in pesticide use patterns between the UK and the USA could partly explain the

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Table 2
Frequencies of detection (%), maximum concentrations (Max), average (Avg) and median values (Mdn)(ng/g) of neonicotinoid insecticides and fungicides detected in wild bumblebees. The analytical methods do not allow us to differentiate what fraction of the pesticide was on the surface (contact toxicity) or inside the bumblebee (oral) since all specimens were individually processed as whole samples. Therefore, LD_{50} values of contact (C) and/or oral (O) toxicity for honeybees (hb) or bumblebees (bb), according to availability of data (Sánchez-Bayo and Goka, 2014), are reported for each of the pesticides that were detected in wild bumblebees.

	Contact (C) and/or oral (O) LD50s reported for bumblebees (bb) or honeybees (hb)	Concentrations (ng/g)																							
		Late spring (27/4/14 - 14/5/14)								Early summer (5/6/14–23/6/14)								Midsummer (15/7/14–2/8/14)							
		Arable (N = 25)				Urban (N = 25)				Arable (N = 25)				Urban (n = 25)				Arable (n = 25)				Urban (n = 25)			
		Freq. %	Max	Avg	Mdn	Freq. %	Max	Avg	Mdn	Freq. %	Max	Avg	Mdn	Freq. %	Max	Avg	Mdn	Freq. %	Max	Avg	Mdn	Freq. %	Max	Avg	Mdn
Neonicotinoid insecticides																									
Thiamethoxam	C = 25 ng/hb; O = 5 ng/hb	8%	<0.90			12%	<0.90	<0.30	<0.30	0%				16%	2.35	<0.30	<0.30	0%				0%			
Clothianidin	C = 16 ng/bb	4%	<1.4			0%				0%				4%	1.4	<0.48	<0.48	0%				0%			
Imidacloprid	C = 20 ng/bb; O = 27 ng/bb	0%				16%	<2.2	<0.72	<0.72	0%				24%	10	<0.72	<0.72	4%	<2.2	<0.72	<0.72	0%			
Thiacloprid	C = 36,000 ng/hb; O = 17,000 ng/bb	0%				0%				0%				12%	1.17	<0.07	<0.02	0%				0%			
Acetamiprid	C = 100,000 ng/hb; O = 22,000 ng/bb	0%				0%				0%				4%	0.17	<0.01	<0.01	0%				0%			
MBC-Fungicide																									
Carbendazim	C = > 50,000 ng/hb	8%	1.2	<0.14	<0.05	24%	28.8	1.49	<0.05	8%	1.2	<0.14	<0.05	12%	4.37	0.23	<0.05	8%	<0.14	<0.05	<0.05	8%	0.92	<0.14	<0.05
SDHI-Fungicides																									
Carboxin		0%				0%				0%				0%				0%				0%			
Boscalid	C = > 200,000 ng/hb; O = 166,000 ng/hb	76%	31.7	7.03	6.2	44%	13.4	1.35	<0.24	44%	5.94	1.39	<0.05	36%	54.4	2.65	<0.05	12%	8.51	0.50	<0.05	0%			
Amine fungicide																									
Spiroxamine	C = 4200 ng/hb; O = 92,000 ng/hb	4%	3.8	0.15	<0.05	0%				96%	37.7	6.51	2.91	0%				12%	2.19	0.20	<0.05	0%			
DMI fungicides																									
Triticonazole		0%				0%				0%				0%				0%				0%			
Epoxiconazole	C = > 100,000 ng/hb	48%	6.07	1.03	<0.96	0%				4%	<2.9	<0.96	<0.96	0%				0%				0%			
Tebuconazole	C = > 200,000 ng/hb; O = 83,000 ng/hb	12%	1.7	<0.12	<0.12	8%	<0.36	<0.12	<0.12	88%	11.7	2.91	1.59	28%	1.5	<0.36	<0.12	32%	4.95	<0.36	<0.12	0%			
Flusilazole		0%				0%				0%				0%				0%				0%			
Prochloraz	C = > 50,000 ng/hb; O = 60,000 ng/hb	0%				0%				4%	<0.90	<0.30	<0.30	4%	<0.90	<0.30	<0.30	0%				0%			
Metconazole	C = > 100,000 ng/hb; O = 80,000 ng/hb	20%	<0.72	<0.24	<0.24	8%	4	<0.24	<0.24	0%				4%	2.58	<0.24	<0.24	0%				8%	<0.72	<0.24	<0.24
QoI-fungicides																									
Pyraclostrobin	C = > 100,000 ng/hb; O = 73,000 ng/hb	4%	<0.72	<0.24	<0.24	0%				0%				0%				0%				0%			
Trifloxystrobin	C = > 200,000 ng/hb; O = 200,000 ng/hb	4%	2.74	0.11	<0.01	4%	2.74	0.11	<0.01	12%	<0.04	<0.01	<0.01	0%				0%				0%			
Fluoxastrobin	C = > 200,000 ng/hb; O = 843,000 ng/hb	12%	<0.72	<0.24	<0.24	4%	<0.72	<0.24	<0.24	4%	<0.72	<0.24	<0.24	0%				0%				0%			
Synergist																									
Piperonyl butoxide		0%				0%				0%				0%				0%				0%			

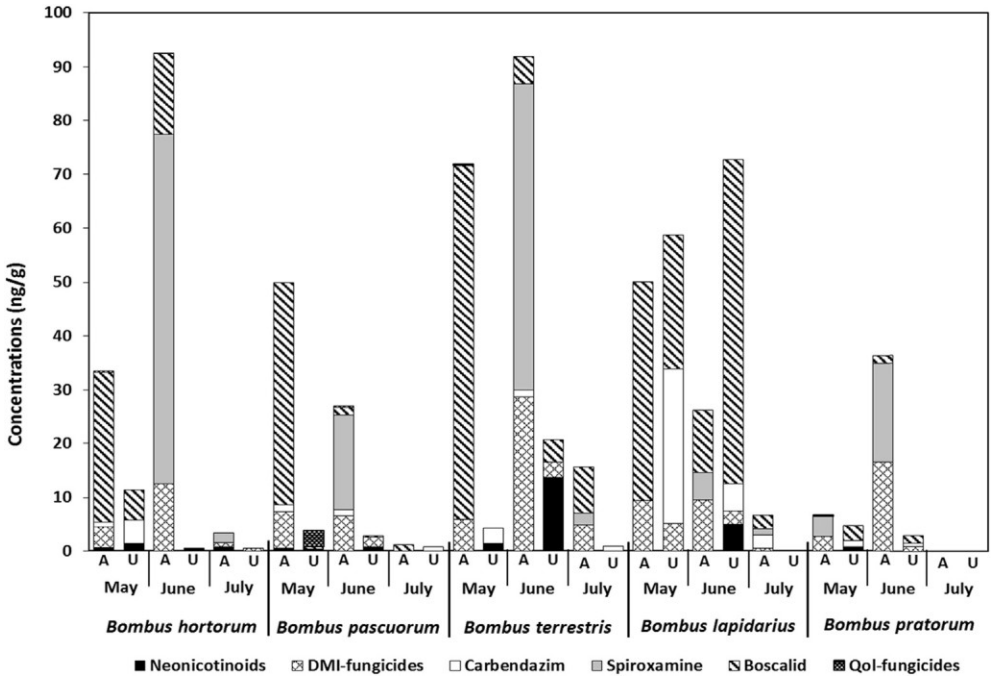

Fig. 2. Sum of total concentrations of neonicotinoid insecticides and fungicides detected in 5 different species of wild bumblebees collected in arable (A) and urban (U) sites in three sampling periods (May, June and July). Concentrations reported refer to the amount of active substance (ng) detected per bumblebee body weight (g).

differences found, as for instance, the maximum application rate for thiamethoxam in oil seed crops in the UK is 33.6 g a.i./ha (European Food Safety Authority, 2013), and 157 g a.i./ha in the USA (USEPA, 2011). Studying the link between pesticide application rates and the levels of exposure for bees, and how different bee species can be more or less susceptible to exposure is essential for a full understanding of the risk posed by pesticides.

The comparison of pesticide concentrations among the five bumblebee species that we studied showed that *B. pratorum*, the species with the smallest body mass and tongue length range, had lower residue levels than the other four species. Different explanations are plausible; for example smaller bees may consume lower amounts of food, and hence they would be less exposed to these active compounds present in pollen and nectar. Smaller body size may lead to greater mass-specific metabolic rates (Heinrich, 1993), and so pesticides might be metabolised faster in smaller bees such as *B. pratorum*, whose body mass was significantly lower than that of the two species with the highest pesticide concentrations, *B. terrestris* and *B. lapidarius*.

Neonicotinoid insecticides can be metabolised relatively fast, with metabolites being the main residues detected in bees a few minutes after ingestion of the parent compound (Brunet et al., 2005; Suchail et al., 2004). Also the metabolites of some fungicides have been detected in bees and bee-collected pollen (Jabot et al., 2016; Mullin et al., 2010; Stoner and Eitzer, 2013), so the analysis of neonicotinoid and fungicide metabolites could have revealed the presence of other potentially toxic compounds that bees might have been exposed to. Some neonicotinoid metabolites have been proven to be highly toxic for bees (Simon-Delso et al., 2015; Suchail et al., 2004, 2001), while the possible effects of both parent fungicides and their metabolites has been scarcely studied in bees, with some reports showing detrimental effects (Bernauer et al., 2015; Pettis et al., 2013; Syromyatnikov et al., 2016; van Engelsdorp et al., 2009). It is possible that the action of fungicides on bees may not be directly toxic, as is the case with insecticides, but may alter the beneficial microbiome present in the pollen and nectar (Bartlewicz et al., 2016; van Engelsdorp et al., 2009; Yoder et al., 2013) and as a consequence, in the bee gut, which could have important implications for bee nutrition and health (Engel et al., 2016; Mattila et al., 2012; Pettis et al., 2013).

The tongue length of the different bumblebee species could be hypothesized as a possible predictor of residue exposure, since this trait determines whether or not, and how fast, a bee can manipulate a particular flower to extract nectar, and thus it is crucial for the division of resources between different species (Brian, 2016; Cariveau et al., 2016; Goulson et al., 2008; Harder, 2013). Long-tongued bees generally forage from long-corolla flowers, and short-tongued bees from short corolla flowers (Hobbs, 1962). In the case of *B. pratorum*, as a short-tongued bee, the most commonly visited flowers should be those with short corolla (*e.g.* many Rosaceae and Asteraceae flowers), which have both nectaries and stamens more exposed to environmental conditions and wind-blown aerosols. Oilseed rape flowers are shallow and more frequently visited by short-tongued bees, even though the long-tongued bumblebee *B. hortorum* often collects pollen from this plant (Stanley et al., 2013), and all our *B. hortorum* specimens sampled in farmland in late spring were foraging in these crop flowers when collected (Table S2a). Some of the pesticides analysed in this study have been reported to degrade after exposure to sun light and/or high temperatures (*i.e.* some neonicotinoids, carbendazim, carboxin, epoxiconazole and prochloraz) (Bonmatin et al., 2015; Burrows et al., 2002; Mazellier et al., 2002), so it is possible that the flowers with pollen and nectar more exposed to the environmental conditions might have lower concentrations of the parent active compounds. Therefore, the bees feeding on these shallow flowers would be less exposed to them. However, the tongue length range of *B. pratorum* is not very different to that of the short-tongued bumblebees *B. terrestris* and *B. lapidarius* (Table 1). Moreover, the range of flowers visited by the three species did not differ remarkably, since more than half (53%) of the plant species visited by *B. terrestris* and *B. lapidarius* were also visited by *B. pratorum* in May and June (*i.e.* when concentrations

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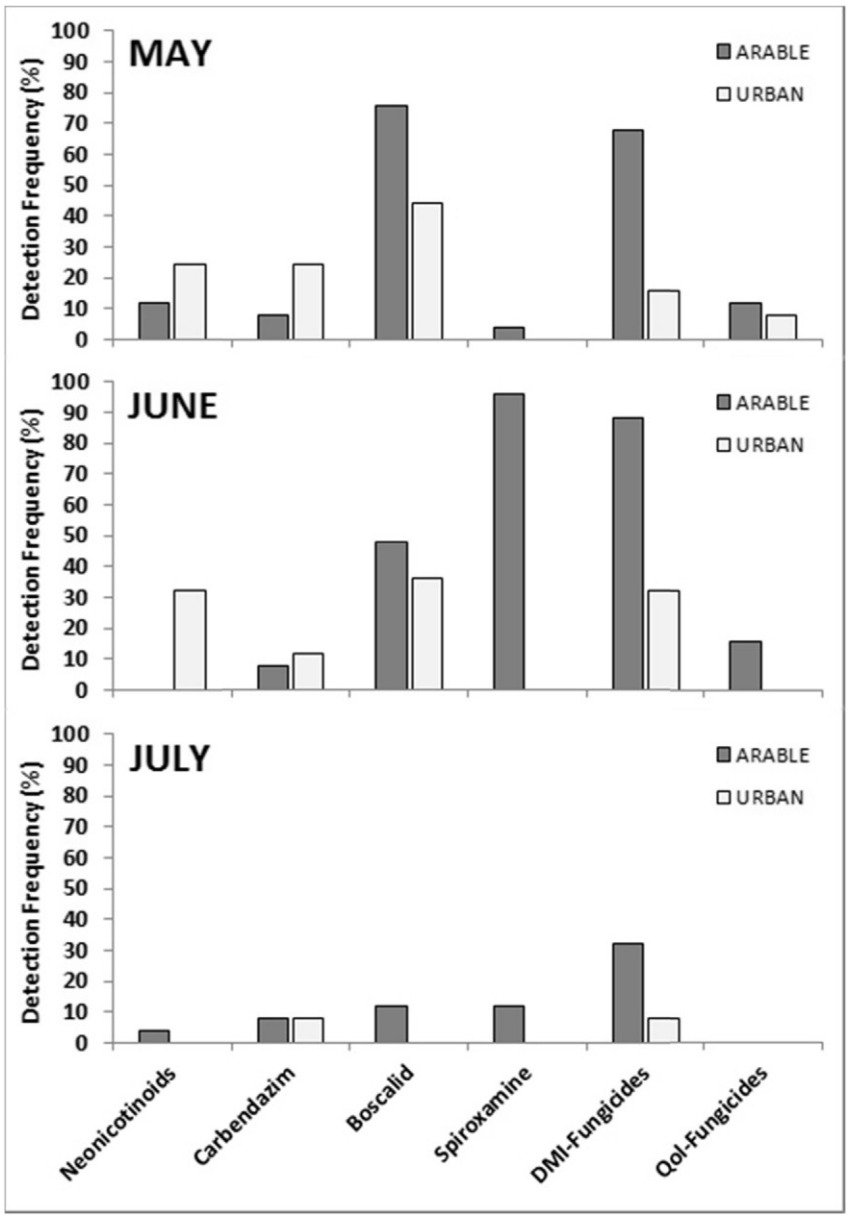

Fig. 3. Frequencies of detection (%) of pesticide residues in wild bumblebees collected in arable and urban habitats in May, June and July.

detected were higher) (Tables S2a and S2b). Nevertheless, as mentioned above, *B. terrestris* and *B. lapidarius* showed significantly higher levels of pesticide concentrations than *B. pratorum*, so the tongue length doesn't seem to be a suitable predictor of residue exposure for the group of bumblebees species studied here. Otherwise, a bigger sample size might be needed to test this hypothesis.

Regarding the toxicity of the pesticides detected, it is worth noting that the bumblebee specimens collected in the present study were individually processed as whole samples to include residues on external as well as internal parts of the bees, so it is not possible to differentiate if the pesticides detected were on the cuticle (contact toxicity) or inside the organism (oral). Thus, both routes of exposure should be considered when the levels detected in the samples are compared to lethal doses reported for the compounds analysed. Moreover, as the bee gut was not removed before processing the samples, there is a chance that some of the residues detected were present in the nectar and pollen contained in the digestive tract, although we consider that the bulk of the bee weights were formed by bee tissues. None of the residues detected in bumblebees were found to overlap with contact or oral acute LD_{50} values tested on bumblebees or honeybees (Table 2), which is to be expected since the bees screened for pesticides in this study were performing foraging tasks and appeared to be healthy at the time of collection; it would be very unlikely to catch bees alive had they been exposed to lethal doses. Additionally, we cannot determine what doses the bees had been exposed to since pesticides are metabolised at varying rates (and we do not know the time of exposure), so that the residues we detected represent an unknown proportion of the dose received and actual exposures may have probably been higher. It should also be mentioned that bees are subjected to chronic exposure when foraging on contaminated flowers, and acute LD_{50}s are frequently higher than chronic LD_{50}s, particularly for neonicotinoids (Alkassab and Kirchner, 2016; Rondeau et al., 2014). Thus, the potential risk of chronic exposure to the levels of pesticides detected in the bumblebees cannot be

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ruled out. Studies where pesticide mixtures are analysed in bees showing health problems or mortality in the field would be of high interest (Kasiotis et al., 2014), since this could provide key information about the hazard posed by specific mixtures and the threshold levels of risk. Nevertheless, such studies are highly challenging to perform in the field due to sampling difficulties, and because the detection of accurate levels at the time of death are problematic since bee samples need to be fresh to avoid degradation of pesticides following exposure to environmental factors and microbial activity (Katagi, 2004; Liu et al., 2011).

Our results revealed that 43.3% of bees contained two or more pesticides, suggesting that simultaneous exposure is likely to be encountered regularly in a field realistic scenario. Although contamination with mixtures of pesticides has been detected in flower pollen, bee collected pollen and bees (David et al., 2016; Hladik et al., 2016; Long and Krupke, 2016; Mullin et al., 2010), evaluating the impact of the exposure to such combinations of agrochemicals poses a major challenge and warrants more research. A few laboratory and field studies have explored the impact of simultaneous exposure to different chemicals on bees, some of them reporting detrimental effects of certain combinations (Gill et al., 2012; Iwasa et al., 2004; Johnson et al., 2013; Schmuck et al., 2003; Sgolastra et al., 2016; Thompson and Wilkins, 2003). Given the mixtures detected in bees in the present study and in previous research (Hladik et al., 2016; Lambert et al., 2013), the number of possible combinations of pesticides is very high and variable. Therefore, focussing on the most frequent ones when performing assays of bee exposure might be the most sensible approach. Moreover, these scenarios are especially important to consider in cases when two or more pesticides that exhibit synergy are detected simultaneously in bees. For instance, the toxicity of certain insecticides (*e.g.* neonicotinoids and pyrethroids) can be enhanced in the presence of demethylation-inhibiting (DMI) fungicides (e.g. epoxiconazole, tebuconazole). In our study, 55.6% of the bumblebees where neonicotinoid insecticides were detected also contained DMI-fungicides, so exposure to these combinations seems to be likely in the field although it is not known if these concentrations were high enough to induce biological effects. These DMI-fungicides can act as synergists by inhibiting the detoxification system in bees (Iwasa et al., 2004; Johnson et al., 2013; Pilling et al., 1995), and thus the insecticide residues are metabolised or eliminated more slowly. It is also important to remark that this is a limited list of pesticides; due to analytical constrains, insecticides such as pyrethroids, usually detected using gas chromatographic methods and which are known to interact with neonicotinoids and DMI-fungicides, could also be present in these bees.

As for the differences in levels of exposure for bees living in farmland or urban habitats, we found that concentrations of pesticides in wild bumblebees foraging in agricultural land were higher than in urban land, as reported for commercial bumblebee colonies in a previous study (David et al., 2016). However, the maximum values for neonicotinoids were recorded in bumblebees collected in urban gardens (i.e. 10 ng/g of imidacloprid, 2.35 ng/g of thiamethoxam and 1.4 ng/g of clothianidin), even though these maximum values were high in comparison to the levels detected for these compounds in the rest of the bee specimens collected, so it is possible that these particular bees had been visiting freshly sprayed plants just before they were sampled. The use of imidacloprid, clothianidin and thiamethoxam has been banned since December 2013 on ornamental plants flowering in the year of treatment (as well as on flowering crops) (European Commission, 2013), and so the high levels of imidacloprid in particular are hard to explain. Nonetheless, the result is corroborated by David et al. (2016) who detected high levels (20 ng/g) of imidacloprid in pollen collected by commercial bumblebee colonies placed in urban areas. The imidacloprid may be persisting in urban environments from applications before the ban, or it might still be available in some stores and presumably may be used in gardens for months or even years after the ban. Alternatively, this exposure of bees could originate from other uses of imidacloprid; it is widely used in baits to kill ants and for flea control in dogs and cats. Orally applied imidacloprid in dogs and other mammals is completely eliminated in the urine (70–80%) and faeces (20–30%) in 48 h as the main metabolites 6-chloronicotinic acid and its glycine conjugate together with significant amounts of the parent compound, whereas topical application spreads over the skin for 24 h and the compound is stored in the oil glands of the skin and slowly shed with hair and sebum (European Food Safety Authority, 2006; Hovda and Hooser, 2002). The environmental impacts of the use of pesticides in ornamental gardens and on pets has been scarcely studied (Brown et al., 2013; Fevery et al., 2016). There is no policing of homeowner use of garden pesticides to ensure that they follow label instructions, and amounts used in gardens are not known. Similarly, we can find no information on the number of dogs and cats treated with imidacloprid as a prophylactic flea treatment, and the environmental fate of such chemicals has not been studied. Urban gardens are an important food source and refuge for bees and other wildlife in cities and towns because they represent the only green space in these large environments, and they can host a great diversity of pollinators and high density of bumblebee nests (Baldock et al., 2015; Fetridge et al., 2008; Goulson et al., 2010), resulting in enhanced pollination services for both urban and nearby agricultural landscapes (Kaluza et al., 2016; Samnegård et al., 2011; Theodorou et al., 2016). If bees are sometimes exposed to high doses of harmful pesticides through forage collected in ornamental gardens, this might be a matter of high ecological concern, meaning that more attention should be paid to this route of contamination for bees.

The frequencies of detection and concentrations of agrochemicals in bee tissues decreased significantly in midsummer (July), agreeing with findings reported before (Botías et al., 2015; David et al., 2016) where residue levels decreased in the pollen collected by bees as the season progressed. Midsummer is usually the period for crop harvesting in the studied area, and fewer pesticides are normally applied during this period to crops, so this may partly explain this finding. Concurrently, the decrease in residue levels may be due to a reduction in plant tissue concentrations, and thus on pollen and nectar collected by bees through summer because of plant/soil metabolism, photolysis and increasing temperatures (Bonmatin et al., 2015; Lassalle et al., 2014; Mazellier et al., 2002).

5. Conclusions and perspectives

The extensive incidence of multiple pesticide residues and the scarcity of scientific literature on the biological consequences of exposure to such combinations on wild bees calls for more attention on regulatory strategies concerning monitoring procedures and registration of agrochemicals as they relate to pollinator protection, since the combined toxicity and synergism of all these chemicals may pose a real threat to the health and survival of managed and wild bees. Thus, investigations on the toxicological effects of field-realistic levels and mixtures are crucial to prevent potential exposure of bees to damaging combinations of agrochemicals. Also, the possible impact of metabolites, and of agrochemicals other than insecticides on bees should not be overlooked, as they could have direct or indirect detrimental effects.

Understanding the factors involved in the degree of exposure, such as the type of flowers that tend to incorporate more residues

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or where certain pesticides are more persistent, as well as the morphological and physiological traits of pollinators that makes them more or less susceptible to the exposure, is also crucial to mitigate the damaging effects for the most vulnerable species.

Finally, the widespread detection of pesticides in bumblebees foraging in urban areas indicates a pressing need for further research on the prevalence and doses present in ornamental gardens, and on the environmental fate of pesticides upon domestic uses in order to better inform homeowners and garden centers of the potential risk the use of these products poses. Furthermore, surveillance programs on domestic uses would improve the current lack of safety and control in the application of agrochemicals in non-commercial agricultural situations.

Our results show evidence that wild bumblebees are frequently exposed to mixtures of agrochemicals when they forage in arable and urban habitats, with peak concentrations decreasing through the season. The effects of exposure to pesticide mixtures in wild bees remains to be determined, but studying the temporal distribution of such combinations in habitats favored by bees is crucial to identify timing and routes of pesticide exposure, which may help us to properly direct our conservation efforts regarding pesticide regulation and bee health protection.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2017.01.001.

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