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Elizabeth Nicholls, Cristina Botias, Ellen L. Rotheray, Penelope Whitehorn, Arthur David, et al.. Monitoring Neonicotinoid Exposure for Bees in Rural and Peri-urban Areas of the UK during the Transition from Pre- to Post-moratorium. *Environmental Science and Technology*, 2018, 52 (16), pp.9391-9402. 10.1021/acs.est.7b06573 . hal-01901001

HAL Id: hal-01901001

<https://univ-rennes.hal.science/hal-01901001v1>

Submitted on 2 Jul 2019

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Environ. Sci. Technol., **Just Accepted Manuscript** • DOI: 10.1021/acs.est.7b06573 • Publication Date (Web): 28 Jun 2018

Downloaded from <http://pubs.acs.org> on July 30, 2018

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1 **Monitoring neonicotinoid exposure for bees in rural and peri-urban areas of the UK**
 2 **during the transition from pre- to post-moratorium.**

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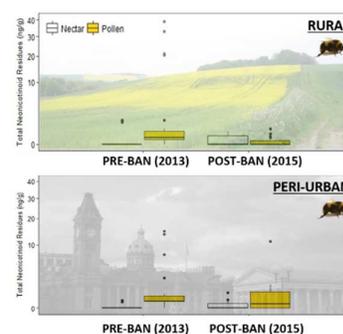
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12

13 **ABSTRACT:** Concerns regarding the impact of neonicotinoid
 14 exposure on bee populations recently led to an EU-wide
 15 moratorium on the use of certain neonicotinoids on flowering
 16 crops. Currently evidence regarding the impact, if any, the
 17 moratorium has had on bees' exposure is limited. We sampled
 18 pollen and nectar from bumblebee colonies in rural and peri-urban



19 habitats in three UK regions; Stirlingshire, Hertfordshire and Sussex. Colonies were sampled over
 20 three years; prior to the ban (2013), during the initial implementation when some seed-treated winter-
 21 sown oilseed rape was still grown (2014), and following the ban (2015). To compare species-level
 22 differences, in 2014 only, honeybee colonies in rural habitats were also sampled. Over half of all
 23 samples were found to be contaminated (n=408), with thiamethoxam being the compound detected at
 24 the highest concentrations in honeybee- (up to 2.29 ng/g in nectar in 2014, median ≤ 0.1 ng/g, n=79)
 25 and bumblebee-collected pollen and nectar (up to 38.77 ng/g in pollen in 2013, median ≤ 0.12 ng/g,
 26 n=76). Honeybees were exposed to higher concentrations of neonicotinoids than bumblebees in 2014.
 27 While neonicotinoid exposure for rural bumblebees declined post-ban (2015), suggesting a positive
 28 impact of the moratorium, the risk of neonicotinoid exposure for bumblebees in peri-urban habitats
 29 remained largely the same between 2013 and 2015.

30

31 INTRODUCTION

32 Neonicotinoids are the most commonly used insecticides worldwide¹. Their systemic nature
33 means that, following seed-application to crops such as oilseeds or cereals, neonicotinoids become
34 incorporated into the tissues of a plant as it grows, including pollen and nectar, the main source of
35 food for economically important pollinators, such as honeybees and bumblebees². Multiple studies
36 have raised concerns regarding the negative impacts of neonicotinoid exposure on bees³. Whitehorn *et*
37 *al.* (2012)⁴ found that exposure of bumblebees to pollen and nectar containing 6 ng/g and 0.7 ng/g of
38 imidacloprid respectively, resulted in slower colony growth and the production of fewer new queens,
39 relative to unexposed colonies. Other studies have observed detrimental impacts on foraging and
40 navigation^{5,6}, immunity⁷ and worker mortality⁸. Based on these findings, in 2013 the European
41 Commission instated a EU-wide moratorium on the use of three types of neonicotinoid,
42 thiamethoxam, clothianidin and imidacloprid on bee-attractive flowering crops such as oilseed rape⁹.
43 In 2018, this ban was subsequently expanded to include all field crops¹⁰⁻¹².

44 Criticism has been levied against studies cited in support of the moratorium, mainly for using
45 neonicotinoid concentrations purported to exceed those routinely experienced by foraging bees¹³,
46 sparking demand for further evidence as to what constitutes a ‘field-realistic’ dose. Several studies
47 have screened bee-collected pollen and nectar¹⁴⁻¹⁹ for neonicotinoid residues, quantifying the
48 ‘exposure landscape’ by incorporating multiple chemicals from several forage sources.
49 Concentrations have been shown to vary considerably across studies, depending on location, time of
50 year and species. Pollen sampled from rural bumblebee colonies in Sussex, England, prior to the
51 implementation of the moratorium in 2013, was found to contain 18 ng/g of thiamethoxam on
52 average, with pollen collected from nests in nearby peri-urban areas containing up to 20 ng/g
53 imidacloprid¹⁵ (mean=6.5 ng/g), well above the 6 ng/g used by Whitehorn *et al.*⁹. A large scale
54 Swedish field study found clothianidin concentrations averaging 5.4 ng/g in nectar sampled from
55 bumblebees foraging in fields of seed-treated oilseed rape (range 1.4-14 ng/g)¹⁶. In contrast, a study
56 conducted in Germany found considerably lower average concentrations (0.88 ng/g) in pollen
57 collected from bumblebee nests adjacent to neonicotinoids treated winter-sown oilseed rape²⁰, and a

58 more recent study conducted across the UK, Hungary and Germany reported that concentrations
59 detected in pollen and nectar collected by honeybees, bumblebees and the solitary bee *Osmia bicornis*
60 rarely exceeded 1.5 ng/g²¹. The wide ranging values reported by these studies highlights the need for
61 further data to determine the actual exposure risk, particularly for wild bees.

62 Here we monitored bees' risk of neonicotinoid exposure during the period from pre- to post-
63 moratorium, by screening pollen and nectar collected from bumblebee colonies located in several
64 regions; Sussex (2013-2015) and Hertfordshire (2014 only) in the south of England and Stirling,
65 Scotland (2013 only) in the north of the UK. Given the total weight of neonicotinoids applied in
66 Scotland is much lower compared to the south of England (FERA PUS STATS database²²), we
67 expected the exposure risk to be lowest for bees in this region. There is currently limited data on the
68 exposure risk for wild bees from foraging on ornamental plants grown using neonicotinoids^{15,23,24} and
69 the use of neonicotinoid-based garden sprays, therefore we monitored bumblebees in both rural and
70 peri-urban habitats (Sussex and Stirling only), the latter consisting of domestic gardens located on the
71 outskirts of urban areas. For bees in rural areas, we expected neonicotinoid concentrations in pollen
72 and nectar collected in 2015 to be lower than those collected in 2013, before the implementation of
73 the moratorium. In 2014, the impact of the ban may not have fully come into effect, as any winter-
74 sown oilseed crops would have been drilled prior to the implementation of the ban in December 2013
75 and therefore may still have been seed-treated with neonicotinoids. To compare species-level
76 differences in exposure risk during this transitional year (2014), we also screened pollen and nectar
77 from rural honeybee colonies located in Sussex and Hertfordshire.

78

79 **MATERIALS AND METHODS**

80 **Site Information** Bumblebee colonies (*B.terrestris audax*) were obtained from Agralan Ltd.,
81 Swindon, UK (originating from Biobest, Belgium) and in late spring (late May to early June, see
82 Table 1 for exact dates) were placed into the field:

83 i) to monitor exposure risk over the course of the implementation of the ban for both rural and
84 peri-urban habitats, bumblebee colonies were placed in rural (n=135, n=32-47/year) and peri-urban
85 (n=42, 12-15/year) locations across Sussex each year between 2013 and 2015. While the UK granted
86 a derogation to use neonicotinoids on oilseed rape in 2015, this was limited to a portion of East
87 England and did not affect the study area;

88 ii) to assess regional differences in neonicotinoid exposure between the north and south of the
89 UK, prior to the implementation of the ban (2013), bumblebee colonies were also placed in rural
90 (n=10) and peri-urban (n=20) locations in Stirling. In 2014 only, bumblebees were also placed in rural
91 locations across Hertfordshire (n=30) for comparison with Sussex colonies;

92 iii) to compare species-level differences in exposure risk, 15 rural bumblebee colonies were
93 each paired with a honeybee colony (located within 10m distance and placed into the field at the
94 beginning of April) in both Sussex and Hertfordshire in 2014 only. Queenright honeybee colonies
95 were obtained from experimental stocks at the University of Sussex and Rothamsted Research, which
96 at the beginning of the experiment consisted of a single brood box and a super containing frames of
97 fresh foundation wax, with additional space for bees to store pollen and nectar added as necessary.
98 We also mapped which crops were grown in ten, 5 km² surrounding the experimental colonies in
99 Sussex and Hertfordshire in 2014 (Fig. S4) and, where possible, asked farmers growing winter-sown
100 oilseed rape which seed treatments they had used (Table S4).

101 **Sampling** Pollen and nectar was collected from bumblebee colonies following four, eight and ten
102 weeks of foraging in the field. Pollen was scraped out of the colony using a stainless steel micro-
103 spoon, which was cleaned using methanol to avoid cross-contamination. From each colony, we aimed
104 to collect enough pollen to fill a 1.5 ml micro-centrifuge tube, to ensure enough material for chemical
105 analysis. Concurrently, 1.5 ml of nectar was obtained from nectar pots using disposable glass pipettes.
106 However, care was taken not to completely deplete bumblebee colony stores. Where stores were low,
107 no sample was collected (Table 2).

108 For honeybees, samples were collected once per month in April, May and June 2014, with the
109 last two sampling dates coinciding with sample collection from adjacent bumblebee colonies. Samples
110 were obtained from freshly drawn comb, where possible, to minimise contamination from previous
111 years. Enough pollen to fill a 1.5 ml micro-centrifuge tube was scraped out of ~10 cells using a
112 stainless steel micro-spoon as described above, and 1.5 ml of recently stored nectar was obtained from
113 uncapped and newly drawn comb using disposable glass pipettes. Freshness was determined by first
114 shaking the frame to ensure nectar dripped easily out of the comb. All pollen and nectar samples were
115 stored in individually labelled tubes and put on ice during transport back to the lab, and were then
116 frozen at -20°C until residue analysis was performed.

117 **Chemical analyses:** Pollen and nectar samples were extracted using the QuEChERS
118 method¹⁴ and screened for five neonicotinoids: thiamethoxam (TMX), clothianidin (CLO),
119 imidacloprid (IMC), acetamiprid (ACT) and thiacloprid (THC), using ultra high-performance liquid
120 chromatography tandem mass spectrometry (UHPLC-MS/MS). Pollen samples collected in Sussex in
121 2013 were not screened for acetamiprid.

122 **Sample preparation:** Pollen samples were extracted as described by Botias *et al.* (2015)¹⁴.
123 Briefly, 100 mg of pollen was weighed into an Eppendorf tube and 400 pg of deuterated pesticides in
124 ACN were added. The extraction was performed by the addition of 400 µl of water, 500 µl of ACN,
125 125 mg of magnesium sulphate: sodium acetate mix (4:1) and 125 mg of PSA/C18/ENVI-Carb for the
126 dispersive solid phase extraction (dSPE) step (QuEChERS method). After the first extraction, the
127 aqueous phase and re-suspended pellet were extracted again with 400 µl of ACN and the supernatants
128 combined. Extracts were mixed with PSA/C18/ENVI-Carb and centrifuged. The supernatant was
129 evaporated to dryness under vacuum, reconstituted with 120 µl ACN:H₂O (10:90) and spin filtered
130 (0.22 µm).

131 Nectar samples were centrifuged at 13,000 relative centrifugal force (RCF) for 10 min to
132 remove plant debris and the supernatant transferred into a clean eppendorf tube. Nectar samples were
133 very viscous and were therefore weighed for more accuracy (175 ± 50 mg depending on availability)
134 and the volume then increased to 400 µl with water. Four hundred pg of deuterated pesticide standard

135 mixture was added to the nectar and the samples were extracted using the same QuEChERS method
136 described for pollen.

137 **UHPLC-MS/MS analyses.** The ultra high-performance liquid chromatography tandem mass
138 spectrometry (UHPLC-MS/MS) method described by Botias *et al.* (2015)¹⁴ was used for the analysis
139 of samples. UHPLC-MS/MS analyses were carried out using a Waters Acquity UHPLC system
140 coupled to a Quattro Premier triple quadrupole mass spectrometer from Micromass (Waters,
141 Manchester, UK). Data were acquired using MassLynx 4.1 and the quantification was carried out by
142 calculating the response factor of neonicotinoid compounds to their respective internal standards.
143 Concentrations were determined using a least-square linear regression analysis of the peak area ratio
144 *versus* the concentration ratio (native to deuterated). Method detection and quantification limits (MDL
145 and MQL, respectively) as well as recoveries were determined as described by Botias *et al.* (2015)¹⁴
146 (Table S1-3).

147 **Quality control.** One blank workup sample (*i.e.* solvent without matrix) per batch of eleven
148 samples was included and injected on the UHPLC-MS/MS to ensure that no contamination occurred
149 during the sample preparation. Solvent samples were also injected between sample batches to ensure
150 that there was no carryover in the UHPLC system that might affect adjacent results in analytical runs.
151 Samples were analysed in a random order and quality control samples (*i.e.* standards) were injected
152 during runs every ten samples to check the sensitivity of the machine. Identities of detected
153 neonicotinoids were confirmed by comparing ratio of MRM transitions in samples and pure standards.

154 **Statistical Analysis.** All analyses were performed using R-3.3.3. Residue concentrations that were
155 above the MDL but below the MQL were assigned the MDL (Tables 2-3, range 0.03-0.10 ng/g).
156 Concentrations below the MDL were assumed to be zero¹⁴. Shapiro-Wilk tests, combined with
157 inspection of *q-q* plots, confirmed that residue data were not normally distributed. Therefore we
158 compared the frequency of neonicotinoid contamination using contingency tables and either χ^2 or
159 Fisher's exact tests (where expected frequencies were <5). To compare total neonicotinoid
160 concentrations between regions (Sussex *vs.* Stirling; Sussex *vs.* Herts), habitats (Rural *vs.* Peri-Urban)
161 and years of the study (2013 *vs.* 2015) we used non-parametric Mann-Whitney tests. For honeybee

162 data, where frequencies of contamination and residue concentrations were compared between samples
163 from the same hive over several months, we used Cochran's Q test (with McNemar's test for post-hoc
164 comparisons) and the Wilcoxon Signed-Rank test, with Bonferroni corrections to account for multiple
165 comparisons. Given the relatively small number of pollen and nectar samples collected from each
166 bumblebee colony, for analyses involving bumblebees we pooled samples collected after four and
167 eight weeks in the field.

168 RESULTS

169 **Bumblebees:** In total, 233 pollen and nectar samples were collected from bumblebee colonies placed
170 in rural and peri-urban habitats in the regions of Stirling, Sussex and Hertfordshire between 2013 and
171 2015. Forty percent of all samples screened were found to be contaminated with neonicotinoids,
172 predominantly thiamethoxam (23%), thiacloprid (15%) and imidacloprid (10%). Pollen samples were
173 more often contaminated (62% samples) than nectar (25% samples) and the mean combined total
174 residues detected in pollen (Pollen N=132, 62% samples, mean± standard deviation (SD) =1.44±5.44
175 ng/g, median <MDL, max= 38.77 ng/g) were more than ten times higher (Nectar N=101, mean± SD=
176 0.12±0.44 ng/g, median <MDL, max=3.58 ng/g).

177 **Differences in exposure by habitat and year:** In 2013, the frequency of neonicotinoid
178 contamination was similar for pollen (Table 1, $\chi^2_1=0$, $p=1.000$, Rural =58%; Peri-urban= 59%) and
179 nectar ($\chi^2_1=0$, $p=1.000$, Rural=14%, Peri-urban =14%) sampled from peri-urban (PU) and rural (R)
180 bumblebee colonies across the regions of Sussex (SU) and Stirling (ST) (Table 1). Concentrations of
181 neonicotinoids were very similar in nectar (Mann-Whitney $U_{21, 21}=225$, $p=0.867$, mean_{PU}≤0.10,
182 median_{PU}≤0.10, mean_R±SD=0.22±0.55 ng/g, median_R <MDL), and though higher in pollen from rural
183 colonies, this difference was not significant ($U_{36, 32}=603.5$, $p=0.73$; mean_R=3.37±9.36 ng/g,
184 median_R≤0.12, mean_{PU}= 1.28±3.62 ng/g, median_{PU}≤0.12). While nectar from both habitats contained
185 only one type of neonicotinoid, predominantly thiamethoxam, over a quarter of pollen samples from
186 bumblebee colonies in rural (28%) and peri-urban (26%) habitats contained more than one residue.
187 Thiamethoxam (up to 38.77 ng/g, median <0.12, mean±SD= 2.08±7.47 ng/g) and clothianidin (up to
188 2.08 ng/g, mean ≤0.12 ng/g, median <0.12 ng/g) were present at the highest concentrations in rural

189 colonies. While thiamethoxam was also present in a high percentage of pollen samples collected from
190 peri-urban colonies in Sussex (79% samples), thiacloprid was found at the highest concentration in
191 these samples (up to 14.8 ng/g, mean ≤ 0.04 ng/g, median < 0.04 ng/g).

192 In 2014, less than 10% of pollen (n=13) and nectar (n=13) samples from rural bumblebee
193 colonies in Sussex contained neonicotinoids, all thiamethoxam and below the method quantification
194 limit, whereas a significantly higher proportion of both pollen (85%, $\chi^2_1=8.987$, $p=0.003$, n=7) and
195 nectar samples (80%, Nectar $\chi^2_1=6.152$, $p=0.013$, n=5) from peri-urban nests were contaminated
196 (N=12), frequently with multiple residues (40% nectar samples, 29% of pollen). Again, thiacloprid
197 (up to 9.32 ng/g in pollen, mean=1.34 \pm 3.52 ng/g, median ≤ 0.04 ng/g) and thiamethoxam (up to 3.48
198 ng/g in pollen, mean= 0.76 \pm 1.52, median=0.10 ng/g) and were detected at the highest concentrations.

199 In 2015, the frequency of neonicotinoid detection was similar for nectar collected from rural
200 and peri-urban bumblebee colonies in Sussex ($\chi^2_1=0.158$, $p=0.691$, Rural=47%, Peri-urban=33%) as
201 were the concentrations present (Mann-Whitney $U_{19, 12}=130.5$, $p=0.469$, mean_R=0.10 \pm 0.15 ng/g,
202 median_R $<$ MDL, mean_{PU}=0.08 \pm 0.17 ng/g, median_{PU} $<$ MDL). While the frequency of detection
203 (Rural=35%, Peri-urban=64%), proportion of samples with multiple residues (Rural=9% vs. Peri-
204 urban=18%) and mean concentration of neonicotinoids were higher in pollen from peri-urban nests,
205 the difference was not significant ($\chi^2_1=1.238$, $p=0.266$, $U_{22, 11}= 75.5$, $p=0.06$, mean_R=0.06 \pm 0.14 ng/g,
206 median_R $<$ MDL, mean_{PU}=1.29 \pm 3.30 ng/g, median_{PU} $<$ MDL). Both habitats were contaminated
207 predominantly with thiacloprid (up to 0.44 ng/g, mean \pm SD=0.04 \pm 0.11 ng/g, median $<$ MDL), and
208 imidacloprid (up to 11.16 ng/g in peri-urban nests, mean \pm SD=0.21 \pm 1.40 ng/g, median $<$ 0.14), though
209 a small proportion of peri-urban samples also contained acetamiprid (4% up to 1.4 ng/g, mean \leq 0.03
210 ng/g, median $<$ MDL).

211 To compare the changing risk of exposure to peri-urban and rural bees over the transitional period
212 from pre- to post- moratorium, we compared residue concentrations in 2013 and 2015 for Sussex
213 bumblebee colonies only. For pollen collected from rural colonies there was a significant decrease in
214 overall combined residue concentrations between years (Mann-Whitney $U_{23, 22}=385$, $p=0.002$,
215 mean₂₀₁₃= 5.10 \pm 11.40 ng/g, median ≤ 0.12 ng/g, mean₂₀₁₅=0.06 \pm 0.14 ng/g, median $<$ MDL), but not for

nectar ($U_{14, 19}=98$, $p=0.134$; $\text{mean}_{2013}=0.20\pm 0.51$ ng/g, median <MDL, $\text{mean}_{2015}=0.10\pm 0.15$ ng/g, median <MDL). When considering just those neonicotinoids affected by the moratorium (thiamethoxam, clothianidin and imidacloprid), the same effect is observed, with a significant decrease in residue concentrations in pollen ($U_{23, 22} = 389$, $p < 0.001$, $\text{mean}_{2013}=5.02\pm 11.32$ ng/g, median ≤ 0.12 ng/g, $\text{mean}_{2015}=0.05\pm 0.14$ ng/g, median <MDL) but not nectar between 2013 and 2015 ($U_{14, 19}=140$, $p=0.676$; $\text{mean}_{2013}=0.20\pm 0.51$ ng/g, median <MDL, $\text{mean}_{2015}<\text{MDL}$, median <MDL). In contrast, concentrations of thiacloprid, which was unaffected by the ban, increased significantly in nectar between 2013 and 2015 ($U_{14, 19}=84$, $p=0.013$, $\text{mean}_{2013}<\text{MDL}$, median <MDL, $\text{mean}_{2015}=0.09\pm 0.15$ ng/g, median <MDL). Concentrations of thiacloprid in pollen remained unchanged over this period ($U_{23, 22}=267$, $p=0.627$, $\text{mean}_{2013}=0.08\pm 0.31$ ng/g, median <MDL, $\text{mean}_{2015}<\text{MDL}$, median <MDL).

For peri-urban nests, there was no significant difference in overall residue concentrations in either pollen ($U_{19, 11}=124$, $p=0.408$, $\text{mean}_{2013}=2.11\pm 4.56$ ng/g, median=0.12 ng/g, $\text{mean}_{2015}=1.29\pm 0.14$ ng/g, median ≤ 0.04 ng/g) or nectar ($U_{13, 12}=62.5$, $p=0.276$, $\text{mean}_{2013}=0.02\pm 0.05$ ng/g, median <MDL, $\text{mean}_{2015}=0.08\pm 0.17$ ng/g, median <MDL), samples collected between 2013 and 2015. When considering either the banned neonicotinoids only (Pollen, $U_{19, 11}=134.5$, $p=0.188$; $\text{mean}_{2013}=0.63\pm 1.64$ ng/g, median ≤ 0.12 , $\text{mean}_{2015}=1.14\pm 3.33$ ng/g, median <MDL; Nectar $U_{13, 12}=76$, $p=0.898$, $\text{mean}_{2013}<\text{MDL}$, median <MDL, $\text{mean}_{2015}<\text{MDL}$, median <MDL) or thiacloprid, which was unaffected by the ban (Pollen $U_{19, 11}=104$, $p=1$, $\text{mean}_{2013}=1.47\pm 4.41$ ng/g, median <MDL, $\text{mean}_{2015}<\text{MDL}$, median <MDL, Nectar $U_{13, 12}=58.5$, $p=0.067$, $\text{mean}_{2013}<\text{MDL}$, median <MDL, $\text{mean}_{2015}=0.05\pm 0.13$ ng/g, median <MDL), again there was no difference in the concentrations detected in pollen and nectar collected from peri-urban nests between 2013 and 2015.

Regional differences in exposure In 2013, pollen collected from bumblebee colonies in Sussex (SU) was more frequently contaminated ($\chi^2_1=15.62$, $p<0.001$, Sussex=79%; Stirling=27%), with significantly higher concentrations of neonicotinoids than pollen collected from colonies in Stirling (ST) (Mann-Whitney $U_{42,26}=276$, $p<0.001$; $\text{mean}_{\text{SU}}\pm\text{SD}=3.74\pm 9.01$ ng/g, $\text{median}_{\text{SU}}\leq 0.12$ ng/g, $\text{mean}_{\text{ST}}\pm\text{SD}=0.20\pm 0.49$ ng/g, $\text{median}_{\text{ST}}<\text{MDL}$). Nectar was contaminated at similar frequencies

243 (Fisher's Exact Test $p=1.00$, Sussex=14%; Stirling 12.5%) and concentrations ($U_{27,15}=200$, $p=0.931$;
244 $\text{mean}_{\text{SU}}=0.11\pm 0.37$ ng/g, $\text{median}_{\text{SU}} < \text{MDL}$, $\text{mean}_{\text{ST}}=0.13\pm 0.47$ ng/g, $\text{median}_{\text{ST}} < \text{MDL}$).

245 Pollen sampled from Sussex colonies was more frequently contaminated with multiple
246 residues (Peri-urban=37%, Rural=35%) compared to Stirling samples (Peri-urban=8%, Rural=15%),
247 and the concentrations of thiamethoxam detected in pollen were considerably higher
248 ($\text{mean}_{\text{SU}}=0.58\pm 1.64$ ng/g, $\text{median}=0.12$ ng/g vs. $\text{mean}_{\text{ST}}\leq 0.12$ ng/g, $\text{median} < 0.12$ ng/g). Sussex peri-
249 urban colonies in particular also contained higher concentrations of thiacloprid compared to Stirling
250 ($\text{mean}_{\text{SU}} = 1.47\pm 4.41$ ng/g $\text{median} < 0.03$ ng/g vs. $\text{mean}_{\text{ST}} = 0.07\pm 0.22$ ng/g, $\text{median} < 0.03$ ng/g).
251 Imidacloprid was also frequently detected in pollen from Sussex nests in 2013, but was not detected in
252 any samples from Stirling. Clothianidin was not detected in any Sussex nests, but accounted for the
253 highest residue concentrations detected in nests in Stirling ($\text{mean}_{\text{ST}} = 0.16\pm 0.58$ g/g, $\text{median} < \text{MDL}$,
254 $\text{max}_{\text{ST}} = 2.08$ ng/g).

255 In 2014, residues detected in pollen and nectar samples collected from bumblebee colonies
256 placed in rural habitats in Hertfordshire (H) and Sussex (SU) were all below the limits of
257 quantification (< 0.04 - 0.1 ng/g). Though there was a higher frequency of contamination of both pollen
258 (H=36%, SU=7%) and nectar (H=20%, SU= 8%) from Hertfordshire colonies, this difference was not
259 significant (Nectar: Fisher's Exact Test $p=0.560$; $N_{\text{SU}}=13$, $N_{\text{H}}=10$; Pollen $p=0.142$, $N_{\text{SU}}=13$, $N_{\text{H}}=11$).
260 A small proportion of pollen from Sussex (10%), and nectar from both regions was contaminated with
261 thiamethoxam (SU=10%; H=20%). Pollen from Hertfordshire colonies also contained acetamiprid
262 (10%) and, more frequently, thiacloprid (40%).

263 **Honeybees:** In total, 175 pollen and nectar samples were collected from honeybee hives in Sussex
264 and Hertfordshire between April and June May 2014, with over two thirds (68%) found to be
265 contaminated with neonicotinoids. Total residue concentrations in nectar ($N= 85$, $\text{mean}\pm \text{SD} = 0.64 \pm$
266 0.84 ng/g, $\text{median}=0.20$ ng/g, $\text{max}= 4.23$ ng/g) were approximately three times the concentrations
267 detected in pollen ($N= 90$, $\text{mean}\pm \text{SD} = 0.20 \pm 0.32$ ng/g, $\text{median}\leq 0.12$ ng/g, $\text{max}=1.74$ ng/g), with
268 40% of nectar samples containing more than one residue, compared to just 9% of pollen samples.
269 Alongside thiamethoxam, which was highly prevalent in both pollen (61% of samples) and nectar

270 (69%), clothianidin was also frequently detected in nectar collected from honeybee hives (40%), but
271 only once in pollen (Table 2). Imidacloprid and thiacloprid were detected in a very small percentage
272 of samples (4-5%) and acetamiprid was not detected.

273 **Seasonal differences:** Frequency of neonicotinoid detection in pollen (Cochran's $Q=24.67$,
274 $df=2$, $p<0.001$) and nectar ($Q=20.38$, $df=2$, $p<0.001$) sampled from honeybee colonies in 2014
275 changed significantly across the season. The highest frequency and concentration of neonicotinoid
276 residues were detected in April (Fig. 3), when nearly all nectar samples collected from hives in
277 Hertfordshire (H) and Sussex (SU) were contaminated with neonicotinoids (H=100%, $\text{mean}_H \pm \text{SD}$
278 $=1.46 \pm 0.66$ ng/g; median=1.17 ng/g; SU=93%, $\text{mean}_{SU}=0.95 \pm 1.13$ ng/g, median ≤ 0.12 ng/g).
279 Likewise, almost all pollen samples contained neonicotinoid residues (H=80%, $\text{mean}_H=0.41 \pm 0.47$
280 ng/g, median ≤ 0.12 ng/g; SU=100%, $\text{mean}_{SU}=0.23 \pm 0.19$ ng/g, median ≤ 0.12 ng/g) in April.

281 Between April and May, there was a similar frequency of neonicotinoid detection in both
282 pollen (April= 90%, May=73%, McNemar test, $p=0.287$) and nectar (April=81%, May=80%
283 $p=0.760$). While the concentration of neonicotinoid residues in pollen remained the same as the
284 previous month (Wilcoxon signed-rank test, $Z_{30}=0.28$, $p=0.120$, $\text{mean}_{\text{April}}=0.32 \pm 0.37$ ng/g, median
285 ≤ 0.12 ng/g $\text{mean}_{\text{May}}=0.22 \pm 0.33$, median ≤ 0.12 ng/g), neonicotinoid concentrations in nectar,
286 previously high in comparison to pollen, declined significantly between April and May ($Z_{26}=0.75$,
287 $p<0.001$; $\text{mean}_{\text{April}}=1.20 \pm 0.95$ ng/g, median= 1.06 ng/g, $\text{mean}_{\text{May}}=0.65 \pm 0.72$, median=0.27 ng/g).

288 At the final sampling point in June, neonicotinoid concentrations detected in samples from
289 both regions were below the limit of quantification, and were significantly lower than in May (Pollen
290 $Z_{30}=0.55$, $p=0.003$; Nectar $Z_{27}=0.73$, $p<0.001$). The frequency of neonicotinoid detection in both
291 pollen (30% samples, McNemar test, $p=0.002$) and nectar (34% samples, $p=0.002$) was also
292 significantly lower than the previous month (Table 2)

293 **Regional differences:** While overall neonicotinoid concentrations in pollen contamination
294 did not differ between Hertfordshire and Sussex (Mann-Whitney $U_{45, 45}=1014$, $p=0.100$,
295 $\text{mean}_H=0.23 \pm 0.36$, median ≤ 0.12 ng/g, $\text{mean}_{SU}=0.17 \pm 0.27$, median ≤ 0.12 ng/g), concentrations in

nectar were significantly higher in Hertfordshire hives ($U_{44, 42}=1301$, $p\leq 0.001$, $\text{mean}_H=0.88\pm 0.81$,
median=0.75 ng/g, $\text{mean}_{SU}=0.40\pm 0.80$ ng/g, median ≤ 0.10 ng/g). Crop mapping of the five 5 km²
study areas in each region in 2014, showed that arable crops accounted for 55% of land cover in
Hertfordshire (9% oilseed rape), and 32% in Sussex (5% oilseed rape, Figure S4).

Species-specific differences: A comparison of residue concentrations in pollen and nectar
collected from adjacent honeybee (HB) and bumblebee (BB) nests located in rural habitats in
Hertfordshire and Sussex revealed significantly higher concentrations of neonicotinoid exposure for
honeybees compared to bumblebees (Table 1, 2, $U_{18, 18}= 112$, $p=0.04$; $\text{mean}_{HB}=0.17\pm 0.39$ ng/g,
median <MDL, max=1.38 ng/g; $\text{mean}_{BB}\leq 0.12$ ng/g, median <MDL, max ≤ 0.12 ng/g).

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306 DISCUSSION

In December 2013, an EU-wide moratorium on the use of certain neonicotinoids on bee-attractive
flowering crops was implemented by the European Commission, which in early 2018 was
subsequently expanded to include all field crops. To monitor bees' exposure to neonicotinoids during
the initial transitional period from pre- to post-ban, between 2013 and 2015 we collected more than
400 pollen and nectar samples from bumblebee and honeybee colonies located in rural and peri-urban
habitats in three regions across the UK, finding just over half of all samples to be contaminated with
neonicotinoids. While combined total concentrations of neonicotinoids in pollen collected by rural
bumblebees declined post-ban from an average of 5.1 ng/g in 2013, to 0.06 ng/g in 2015, suggesting a
positive impact of the moratorium, neonicotinoid concentrations detected in samples collected from
peri-urban bumblebee colonies remained largely unchanged between 2013 and 2015, indicating that
the risk of exposure for peri-urban bees was not altered during the transitional period, and that more
could be done to mitigate the risk for bees foraging in such habitats.

Across all samples, the highest neonicotinoid residue concentrations were detected in 2013, in
pollen samples collected from rural bumblebee colonies in Sussex. Concentrations of up to 38.77 ng/g
of thiamethoxam were detected, with the average total neonicotinoid concentrations of 5.1 ng/g

322 similar to that detected by previous studies conducted prior to the moratorium^{25,15,26}, and within the
323 range demonstrated to have negative impacts on bumblebee physiology^{27,28}, foraging efficiency²⁹ and
324 colony growth²⁸. Pre-ban (2013), the frequency of neonicotinoid contamination was extremely high
325 for pollen sampled from bumblebee colonies in both rural and peri-urban habitats in Sussex (74% and
326 84% of pollen samples respectively, mean=3.74 ng/g). As predicted, pollen samples collected from
327 nests near Stirling in 2013 were contaminated to a lesser degree (23-30% of pollen samples), and with
328 lower concentrations (mean=0.20 ng/g). This likely reflects the fact that across Scotland,
329 neonicotinoid use in 2013/2014 was approximately four times lower than in South East England (4,
330 186 kg, over 78, 345 ha vs. 16, 820 kg, over 197,507 ha²²), though differences in the growth season
331 and therefore timing of neonicotinoid application between regions may also have played a role.

332 Pollen and nectar samples collected from honeybee colonies in 2014, post-implementation of
333 the ban, but when any winter-sown oilseed rape may still have been seed-treated with neonicotinoids,
334 also had a high prevalence of neonicotinoid contamination (68% samples). Contamination was highest
335 in April when oilseed rape was flowering (93% samples), and declined throughout the season, a
336 phenomenon observed in several earlier studies^{14,15,23,30}, and hypothesised to arise from temperature
337 increases and photo-degradation of neonicotinoid residues in plant tissues as the season progresses³¹.
338 During this early part of the year, concentrations detected in honeybee-collected nectar averaged 1.2
339 ng/g, close to the average maximum concentration detected in seed-treated crop nectar, as reported by
340 Godfray *et al.*³² (1.9 ng/g, averaged from 20 published studies). Concentrations in pollen were
341 considerably lower (0.32 ng/g, average maximum concentration in seed-treated crop pollen=6.1
342 ng/g³²), likely reflecting honeybees' preference for collecting nectar from oilseed rape. For both
343 bumblebees and honeybees, early spring is a period when the colony might be expected to be
344 particularly vulnerable^{33,34}, and levels detected in pollen were within the range known to impair
345 honeybee foraging performance³⁵, immune function⁷ and alter gene expression pathways³⁶.
346 Furthermore, as observed in several previous studies^{15,17,18}, many of the samples we screened were
347 found to contain more than one neonicotinoid residue, which gives rise to the potential for additive or
348 synergistic effects. Tosi *et al.*¹⁷ found when screening honeybee pollen collected from multiple

349 apiaries across Italy for 66 different pesticides, that the frequency of detection actually peaked in
350 summer months. Though here we did not screen for the presence of other chemical classes such as
351 fungicides, there is evidence to suggest that exposure to certain fungicides can make bees more
352 susceptible to the adverse effects of neonicotinoids³⁷.

353 Although the concentration of neonicotinoids in pollen and nectar sampled from rural
354 bumblebee colonies declined between 2013 and 2015, bumblebees from both rural and peri-urban
355 habitats were nevertheless still exposed to neonicotinoids following the implementation of the ban.
356 Indeed 47% of nectar and 36% of pollen samples collected from rural colonies in 2015 contained
357 neonicotinoid residues, a similar frequency as observed for peri-urban nests (33% nectar, 64%
358 pollen), albeit at lower concentrations (mean concentration detected in pollen from rural nests = 0.06
359 ng/g vs. 1.29 ng/g detected in peri-urban pollen in 2015). This echoes the findings of Woodcock *et*
360 *al.*³⁰ who screened honey samples submitted by beekeepers across the UK, and found that while
361 samples harvested in 2014 were more likely to be contaminated (52% samples), 22.9% of samples
362 harvested post-ban in 2015 also contained neonicotinoids. Similarly, a worldwide study of honey
363 contamination spanning five years between 2012 and 2016, found 75% of 198 samples to contain
364 neonicotinoids, with the highest prevalence in honey from North America, Asia and Europe³⁸.

365 Not only did exposure to neonicotinoids change for rural bees between 2013 and 2015, so did
366 the chemical type. Across all samples, thiamethoxam was the most frequently detected, which is
367 unsurprising given that, prior to the moratorium, it was the active ingredient in the mostly commonly
368 used seed dressing on oilseed rape across Great Britain. Indeed, of fifteen farmers growing winter-
369 sown oilseed rape within a 5 km radius of our experimental bee colonies that we interviewed in 2014,
370 twelve had used seeds dressed with a thiamethoxam-based formulation (Cruiser®). Clothianidin, a
371 metabolite of thiamethoxam and still in use as a seed-dressing on non-flowering cereal crops, was also
372 frequently detected in honeybee nectar (69% samples), but only once in pollen, and was rarely
373 detected in any samples collected from bumblebee colonies. Post-ban, acetamiprid and thiacloprid,
374 the use of which is unaffected by the moratorium, were detected more often and at higher levels than
375 thiamethoxam. For nectar samples collected from rural bumblebee colonies, thiacloprid

376 concentrations actually significantly increased between 2013 and 2015. Thiacloprid is an active
377 ingredient in many bug sprays sold in garden centres, and a recent study in which ornamental ‘bee-
378 friendly’ plants were screened for multiple pesticide and fungicide residues found more than 70% of
379 plants contained neonicotinoids, with thiacloprid present in almost half²⁴.

380 Imidacloprid was detected in a moderate proportion (10%) of samples collected from
381 bumblebee nests throughout the duration of the study. Considering that use of imidacloprid in arable
382 farming has dramatically declined in the UK (50% and 90% decline in weight of imidacloprid applied
383 to cereals and oilseeds respectively between 2012 and 2014, PUS Stats database, Table S6), having
384 been replaced by thiamethoxam and clothianidin, it is somewhat concerning that it was detected to
385 such an extent. Woodcock et al.³⁰ also noted that imidacloprid was present in honey samples
386 harvested in 2014 at a rate ‘disproportional to its use’ and Tosi et al.¹⁷ detected imidacloprid in 9.1%
387 of honeybee-collected pollen sampled from multiple apiaries across Italy in 2014 at mean
388 concentrations of 2 ng/g, raising concerns about the persistence of this chemical in agro-
389 environments. As previously observed when screening pollen from bumblebee colonies¹⁵ and wild
390 bumblebees collected in peri-urban areas²³, the highest concentrations of imidacloprid were detected
391 in peri-urban colonies, at levels up to 11.16 ng/g in 2015 (mean=1.13 ng/g). Again, this may originate
392 from use by the horticulture industry, since screening of ornamental plants detected imidacloprid in
393 38% of samples²⁴. An alternative, yet untested source, is the use of imidacloprid for flea control in
394 domestic pets and as ant poison.

395 Honeybees in Hertfordshire were exposed to significantly higher neonicotinoid concentrations
396 in nectar compared to Sussex honeybees, which is most likely explained by the fact that, in 2014,
397 there was almost double the percentage cover of treated oilseed crops (9% land cover in Hertfordshire
398 vs. 5% in Sussex), and generally a higher percentage of arable land cover (55%) compared to Sussex
399 (32%).

400 Overall, honeybee samples had higher concentrations of neonicotinoids compared to
401 bumblebees. This contrasts with findings from an earlier study conducted in 2013 where the reverse
402 was found to be true¹⁵. However in the previous study, colonies of each species were not placed in

403 identical locations, therefore in addition to differences in foraging range and flower preferences^{39,40},
404 colonies may simply have been in proximity to a different range of plant species. Clearly more paired
405 sampling of both species is required to establish whether there are consistent differences in exposure.

406 On the basis of evidence published post-2013, the European Food Standards Agency recently
407 concluded that neonicotinoids do indeed pose a risk to bees⁴¹, and in 2017 the EU commission
408 proposed extending the moratorium to include all field crops (barring permanent greenhouse crops),
409 which was passed by the European Union in early 2018¹⁰⁻¹². Here we have shown for the first time
410 how exposure to neonicotinoids has changed for bees foraging in rural and peri-urban areas across the
411 UK, since the initial implementation of the moratorium on their usage in December 2013. The
412 exposure of rural bumblebees appears to have declined post-ban, suggesting that continued limitation
413 of their use on flowering crops could have a positive impact on the risk for bees and other pollinators
414 in rural areas. However, exposure for peri-urban bees remains largely unaffected, presumably as a
415 result of contaminated ornamental plants sold in garden centres and ongoing domestic usage of
416 neonicotinoid-based bug sprays. This is concerning given the growing interest in encouraging
417 pollinators in urban areas; more research is needed to understand the sources of exposure and find
418 ways to reduce it.

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433 **FIGURES**

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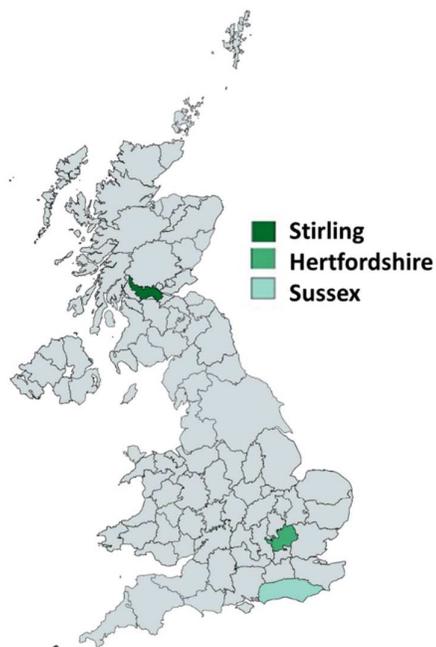
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448 Figure 1 Map of the UK showing the regions in which honeybee (Hertfordshire and Sussex, 2014) and
449 bumblebee (Stirling, 2013; Hertfordshire, 2014; Sussex 2013-2015) colonies were placed in rural
450 (honeybees and bumblebees) and peri-urban (bumblebees only) habitats (see Fig. S1-3 for exact
451 locations).

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Moratorium Status	Year	Region	Bee Species	Habitat	N Colonies	Sampling Dates
Pre-ban	2013	Stirling	Bumblebee	Rural	10	12 th June; 11 th July; 18 th July
				Peri-urban	20	6 th June; 4 th July; 17 th July
		Sussex	Bumblebee	Rural	32	30 th May; 9 th June; 23 rd June
				Peri-urban	12	30 th May; 9 th June; 23 rd June
During ban (Winter-sown crops still seed-treated)	2014	Sussex	Bumblebee	Rural	47	28 th May; 25 th June; 9 th July
				Peri-urban	15	28 th May; 25 th June; 9 th July
		Herts	Honeybee	Rural	15	16 th April; 28 th May; 25 th June
			Bumblebee	Rural	30	28 th May; 25 th June; 9 th July
During ban	2015	Sussex	Bumblebee	Rural	45	15 th June; 13 th July; 27 th July
				Peri-urban	15	15 th June; 13 th July; 27 th July

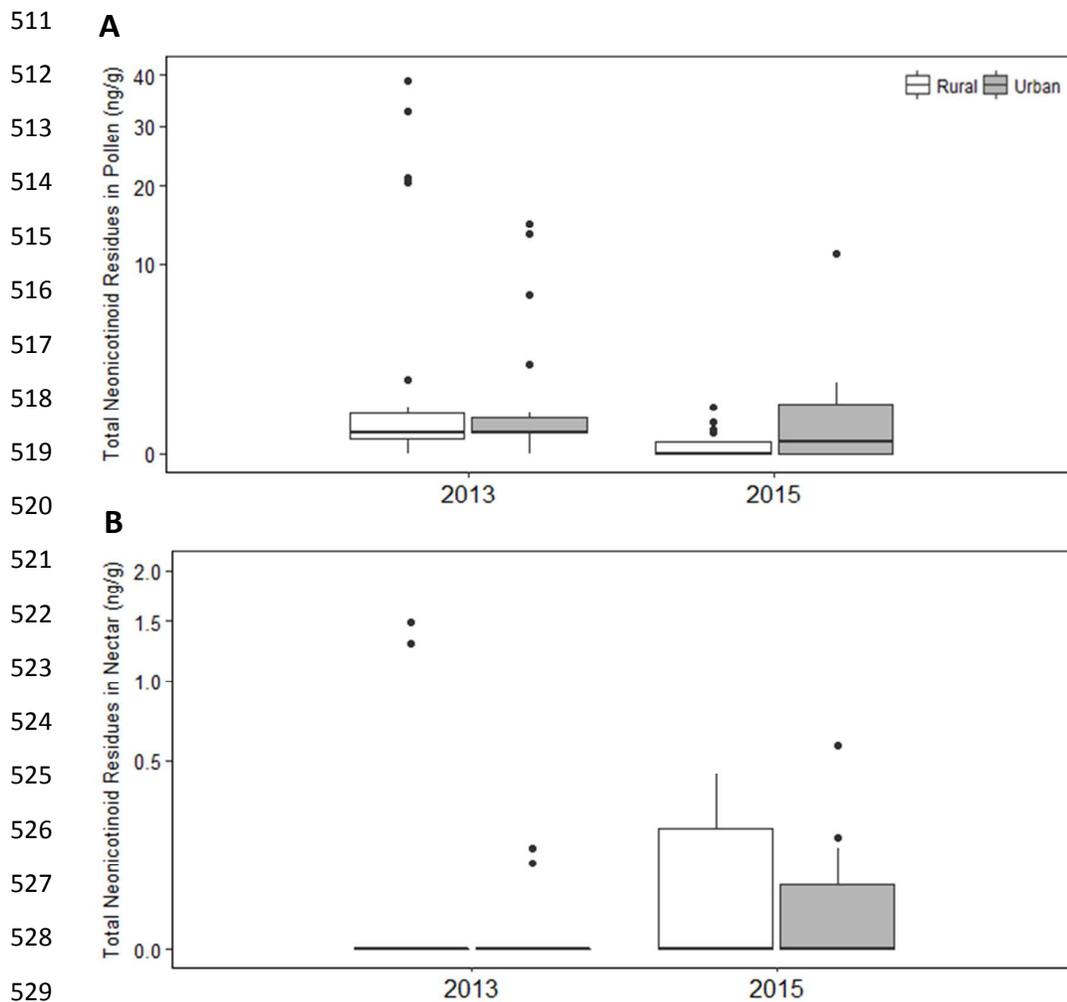
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Table 1 Number of honeybee and bumblebee colonies placed in each habitat type (Peri-urban vs. Rural), in each region (Sussex, Stirling, Hertfordshire (Herts)) across the three years of the study (2013-2015). The specific dates colonies were sampled for pollen and nectar are listed.

489	NECTAR										POLLEN										
	Method Quantification Limit (ng/g)										0.3	0.3	0.4	0.08	0.08	0.36	0.36	0.48	0.12	0.12	
490	Method Detection Limit (ng/g)										0.1	0.1	0.14	0.03	0.03	0.12	0.12	0.16	0.04	0.04	
491	Month	Region	N	TMX	CLO	IMC	ACT	THC	TOTAL	% Multi-residue	N	TMX	CLO	IMC	ACT	THC	TOTAL	% Multi-residue			
491	APRIL	HERTS	15	Frequency of detection %	100%	73.3%	6.7%			100%	80.0%	80%		6.6%		13.3%	80%	20.0%			
492				Mean ± SD (ng/g)	0.83 ± 0.48	0.63 ± 0.51	≤0.14				1.46±0.66		15	0.26±0.28		≤0.16		0.14±0.42	0.41±0.47		
493				Median (ng/g)	0.77	0.66	≤0.14				1.17			0.12		≤0.16		≤0.04	0.12		
494				Max (ng/g)	1.83	1.38	≤0.14				1.83			0.94		≤0.16		1.62	1.62		
495		SUSSEX	15	Frequency of detection %	93%	47%	7%		7%	93.3%	60.0%	100%						100%	0%		
496				Mean ± SD (ng/g)	0.56± 0.14	0.37±0.18	≤0.14		≤0.03	0.95 ±1.13		15	0.23±0.19						0.23±0.19		
497				Median (ng/g)	0	≤0.1	≤0.14		≤0.03	0.58				0.12					0.12		
498				Max (ng/g)	1.76	2.47	≤0.03		≤0.03	2.47				0.6					0.60		
499		MAY	HERTS	15	Frequency of detection %	86.6%	73.3%			93.3%	66.7%	80%						80%	0%		
500					Mean ± SD (ng/g)	0.60±0.16	0.38±0.11					1.04±0.74		15	0.19±0.24					0.19±0.24	
501					Median (ng/g)	0.45	0.10					1.08			0.12					0.12	
502					Max (ng/g)	2.29	1.26					2.29			0.92					0.92	
503	JUNE	SUSSEX	12	Frequency of detection %	66.7%	16.7%		16.70%	66.7%	25.0%	53.3%	6.7%	6.7%		20%	66.7%	20%				
504				Mean ± SD (ng/g)	0.12±0.05	≤0.10		≤0.03	0.19±0.34		15	≤0.12	≤0.12	≤0.16		0.16±0.4	0.24±0.4				
505				Median (ng/g)	0.10	≤0.10		≤0.03	0.10					≤0.12	≤0.12	≤0.16		≤0.04	0.1		
506				Max (ng/g)	0.53	0.68		≤0.03	0.68					≤0.12	≤0.12	≤0.16		1.19	1.2		
507	HERTS	14	Frequency of detection %	50%	21.4%	7.1%			66.3%	21.4%	26.7%		6.7%				26.7%	8.9%			
508			Mean ± SD (ng/g)	≤0.10	≤0.10	≤0.14				0.08±0.08		15	≤0.12		≤0.16			0.09±0.26			
509			Median (ng/g)	≤0.10	≤0.10	≤0.14				0.10			≤0.12		≤0.16			≤0.12			
510			Max (ng/g)	≤0.10	≤0.10	≤0.14				≤0.14			≤0.12		0.88			0.88			
511	SUSSEX	15	Frequency of detection %	13.3%					13.3%	0%	26.7%		6.7%		6.7%		33.3%	6.7%			
512			Mean ± SD (ng/g)	≤0.10						≤0.10		15	≤0.12		≤0.16		≤0.04	0.05±0.07			
513			Median (ng/g)	≤0.10						≤0.10			≤0.12		≤0.16		≤0.04	≤0.12			
514			Max (ng/g)	≤0.10						≤0.10			≤0.12		≤0.16		≤0.04	≤0.16			

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506 Table 3 Frequency of detection (% samples), mean (± standard deviation (SD)), median and maximum concentrations of five neonicotinoids
507 (TMX=thiamethoxam, CLO= clothianidin, IMC= imidacloprid, ACT=acetamiprid, THC=thiacloprid) and the combined total concentration of neonicotinoids
508 detected in honeybee nectar and pollen sampled from colonies located in in Sussex (N=15) and Hertfordshire (Herts, N=15) between April and June. Multi-
509 residue samples are those where more than one type of neonicotinoid was detected. *MQL*= Method quantification limit, *MDL*=Method detection limit, *nt*= not
510 tested, ≤ less than or equal to.



531 Figure 2 Total neonicotinoid concentrations (Thiamethoxam, clothianidin, imidacloprid, acetamiprid
 532 and thiacloprid combined) detected in A) Pollen and B) Nectar samples collected from bumblebee
 533 colonies in Rural (White, N Pollen samples=45; Nectar=33) and Peri-urban (Grey, N Pollen samples=
 534 30; Nectar=25) habitats across the region of Sussex in the years 2013 and 2015. Concentrations are
 535 plotted on a square-root scale. Black horizontal bars show median values. Box limits denote the first
 536 and third quartiles, and boxplot whiskers extend to 1.5 times the interquartile range. Outliers are
 537 represented by solid black circles.

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541 **ASSOCIATED CONTENT**

542 **Supporting Information**

543 The following file is available free of charge.

544 Additional figures and tables as mentioned in the text (PDF)

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557 **Notes**

558 The authors declare no competing financial interest.

559 **ACKNOWLEDGEMENTS**

560 We are grateful to BBSRC (BB/K014498/1; BB/J014915/1) and Defra (Research Project PS2372) for
561 funding this work and for the farmers and land-owners who allowed us to work on their property and
562 shared their pesticide usage data. We thank Bill Hughes, Mark Crumpton, Steve Kennedy and
563 Luciano Scandian for beekeeping equipment, assistance and advice, and Pete Kennedy and Martyn
564 Stenning for useful discussions regarding experimental design and logistics. Thanks also to Julia

565 Horwood and Daniel Ingram for technical advice and assistance and to Grace Twiston-Davies for
566 digitising crop maps.

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