The neonicotinoid insecticide thiacloprid impacts upon bumblebee colony development under field conditions

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Abstract

The impacts of pesticides, and in particular of neonicotinoids, on bee health remain much debated. Many studies describing negative effects have been criticised as the experimental protocol did not perfectly simulate real-life field scenarios. Here, we placed free-flying bumblebee colonies next to raspberry crops that were either untreated or treated with the neonicotinoid thiacloprid as part of normal farming practice. Colonies were exposed to the raspberry crops for a two week period before being relocated to either a flower-rich or flower-poor site. Overall, exposed colonies were more likely to die prematurely, and those that survived reached a lower final weight and produced 46% fewer reproductives than colonies placed at control farms. The impact was more marked at the flower-rich site (all colonies performed poorly at the flower poor site). Analysis of nectar and pollen stores from bumblebee colonies placed at the same raspberry farms revealed thiacloprid residues of up to 771ppb in pollen and up to 561ppb in nectar. The image of thiacloprid as a relatively benign neonicotinoid should now be questioned.

Introduction

Concerns have been growing about declines in bumblebee diversity and range in both Europe and North America, and the potential consequences for natural ecosystems and for food security[1,2]. While the causes of declines are likely to be multifactorial, recent studies describing the negative impacts of a group of systemic pesticides, the neonicotinoids, on foraging in honeybees and bumblebees, and on fecundity and colony success in bumblebees, have garnered widespread interest (e.g.[3-9]). These studies informed the European Union decision in 2013 to suspend use of the three most widely used neonicotinoids (imidacloprid, thiamethoxam and clothianidin) on flowering crops attractive to bees for 2 years, a suspension which has since been extended.

The studies that led to these restrictions have attracted criticism in some quarters because they were partly conducted in a laboratory setting, because bees were forced to consume treated food, and/or because bees were exposed to unrealistic concentrations of neonicotinoids[10]. Here, we describe a field study of the impacts of a neonicotinoid on bumblebee colonies in which bees were free-flying throughout, so that they were free to choose where to forage, and in which the pesticide applications followed normal farming practice. After exposure to the treated or untreated crop for two weeks, colonies were moved to either a flower-poor or flower-rich site, to examine how proximity to good forage mediated any impacts of pesticide exposure. The experiment is intended to be realistic of the scenario in which a wild bumblebee nest is situated near to a treated crop.

We focus here on the impacts of a less-studied neonicotinoid, thiacloprid, which has considerably lower toxicity to honeybees than the neonicotinoids that are the subject of the EU moratorium[11]. It is often described as "bee-safe" and hence suitable for use on flowering crops, in horticulture, and for garden use[12]. However, it has been found to cause elevated

mortality in honeybees, especially when combined with other stressors such as pathogens[13-14], and also to impair navigation[15-16]. There have been no previous attempts to evaluate the impact of this chemical on whole colonies of bees under field-realistic exposure.

Methods

Colony placement and monitoring

Fifty-four commercially reared colonies of *Bombus terrestris audax* (Biobest N.V., Belgium) were obtained on 15 June 2012 and randomly assigned to treatments in a full factorial design (controls or exposed to the neonicotinoid thiacloprid, flower-poor or flower-rich habitats). There was no difference in weight between the colonies at the beginning of the experiment (T-test, $t_{(33)}=1.16$, $p=0.255$). Colonies were initially kept in the grounds of the University of Stirling campus in an area comprising woodland, amenity grasslands, improved pasture, and ornamental gardens (for 0-21 days, see below).

A network of nine raspberry farmers in Perthshire and Angus (central Scotland) took part in the study. All raspberries were grown in polythene tunnels (polytunnels), all of which were open-ended, some were open-sided while others had closed sides. Pollination of raspberries in this region is delivered by a mixture of wild bumblebees of a range of species, honeybees and flies, supplemented on some farms with commercial colonies of *Bombus terrestris* (Lye *et al.* 2011; Ellis *et al*. *in press*).

Farmers informed us when they were about to spray a flowering raspberry crop with thiacloprid. No other insecticides were used on the farms in the year of our study. At each farm using thiacloprid, six colonies were placed at the ends of the rows of raspberries, within 1m of the flowering crop, as soon as possible after spraying (between 0 and 4 days, table S1). On the same day another six colonies were placed within 1 m of flowering raspberries on a

control farm that was not spraying within the next two weeks and had not previously applied an insecticide in 2012. Control farms were matched by size of soft fruit operation and where possible, geographical area (table S1). However, it is important to note that treatments were not randomized; we could not randomly allocate farms to treatments and dictate whether and when thiacloprid would be sprayed. Between 15th June and 5th July, five batches each of six colonies were deployed on five treated farms (30 colonies in total), and four batches of six colonies simultaneously placed adjacent to unsprayed raspberries on four control farms (24 colonies in total). The numbers of control and treatment are uneven as equal numbers of suitable control farms could not always be found to match the same time periods as treated farms, within the required geographical area, and of a similar farm size and management style. All farmers applied thiacloprid at the recommended manufacturer spray rate (up to 250mL/ha of Calypso 480 g/l thiacloprid). Bees in colonies were allowed to forage at the farms for two weeks. After the two week exposure period, colonies were removed from farms and randomly assigned to either the University campus or a site on flowering heather moorland approximately 5 km from the University. Colonies from different farms were placed at least 30m apart to minimise drifting between the colonies[17]. The University campus is probably reasonably typical of lowland UK, having relatively few floral resources in July and August, while the moorland site provided extensive dense patches of flowering *Calluna vulgaris* and *Erica* spp..

Colonies were all weighed at the beginning of the experiment, and weekly throughout the experiment, apart from during the exposure period at the farms when they were not disturbed for two weeks. Weighing was conducted at night to ease handling, minimise disturbance and to ensure that most bees were present in the colony. The colonies were also checked for signs of poor health; 19 colonies died before the end of the experiment and hence

were not available for analysis of nest performance. Thirteen of these deaths were due to heavy infestation with wax moths (*Aphomia sociella*).

Dissections

At termination of the experiment, the surviving colonies were dissected and the following recorded: numbers of adult bees of each caste; numbers of pupae identifiable as future queens, males or workers; other pupae; empty pupal cells; numbers of dead bees. Bees that were dead before freezing are readily distinguished as they have matted fur, are often partly decayed, and are invariably located away from the comb around the periphery of the nest box, whereas live bees cluster together in the centre of the nest as the temperature drops. Reproductive output was calculated as the sum of queens and queen pupae plus 0.5 times the number of males and male pupae (since males are haploid).

Quantifying exposure to thiacloprid

We did not have funds or facilities for testing pesticide residues in 2012, and thus we did not collect samples. In 2013 we acquired access to suitable analytical facilities, and so we placed bumblebee nests on six of the nine farms used for the 2012 experiment, selecting only farms that were intending to spray thiacloprid. As before, nests were placed at the ends of the rows of raspberries, within 1m of the flowering crop, on 7 May 2013. Spraying with thiacloprid followed normal farming practice and commenced in mid June (approximately 6 weeks after the nests were placed in the field). When sufficient food stores were present in the nest, >100mg samples of nectar and pollen were collected 4, 8 and 10 weeks after nests were placed in the field. These were analysed for thiacloprid using methods slightly modified from Botias *et al.*[18] (see Supplementary Appendix 2). It should be noted that in our 2012 experiment colonies were placed on farms immediately after spraying, whereas in 2013

colonies were in place before spraying (a more field-realistic scenario). We might thus expect residues to be higher in 2013 than those that were experienced by experimental nests in 2012.

Statistical analysis

All statistics analyses were conducted in IBM SPSS 21. To assess the impact of treatment on measures of colony success, generalised linear mixed models (GLMMs) were fitted to the data with farm as a random factor. Explanatory factors within the model were final colony weight, treatment, location during the post-exposure period ("flower-rich" versus "flower-poor") and the interaction between these. Response variables were number of workers remaining in the colony, number of males produced (adults plus pupae), number of queens produced (adults plus pupae), and reproductive success (as described above). The model for colony weight was fitted using normal errors, while the remainder of analyses used gamma errors and a log link, with error structure chosen to minimise Akaike values. We also conducted a more conservative GLM analysis, identical to that described above but instead of treating nests as replicated and including farm as a random factor, we used the average value for each response variable across all nests placed at a particular farm / subsequent location (flower rich/ flower poor) combination.

Differences in colony failure rates between exposed and control colonies were examined using a χ^2 test of association.

Results

We found a number of significant interactions between the effects of pesticide exposure and the subsequent location of colonies (flower-rich or flower-poor sites) on colony performance. Broadly, colonies that were not exposed to thiacloprid and were then placed at the flower-rich site performed better than those in any other treatment combination (Figure 1, Table 1).

Colonies placed at the flower-poor site performed poorly regardless of pesticide treatment. For example, there was a significant treatment x site interaction on final colony weight; at the flower rich site the control colonies were 10% heavier than the exposed colonies (mean ± se of 780g ± 27.0 versus 709g ± 14.7), whereas at the flower poor site colony weights were low in both exposed and control colonies (overall mean of 701 g ± 16.6; Figure 1a). Similarly, there was a significant treatment x site interaction for the reproductive output of the colonies (measured as the number of new adult queens and queen pupae plus half the number of males and male pupae; Table 1, Figure 1b). Overall, reproductive output was 46% lower in treated colonies compared to controls (mean ± s.e. 23.9 ± 4.6 versus 13.0 ± 3.3, respectively), but the difference was more marked at the flower-rich site (Figure 1b). When analysed separately, the same pattern was observed for male production (Figure 1c), but not for queens; queen production was very low in all treatments (overall mean ± s.e.; new queens = 1.66 ± 0.47, queen pupae = 3.48 ± 0.59, Figure 1d). There were no treatment or site effects on the numbers of workers remaining in the colonies at the end of the experiment (Table 1). When response variables were subjected to a more conservative analysis in which farm (rather than nest) was treated as the unit of replication, patterns were broadly similar; there was a significant negative effect of treatment on reproductive output of colonies, and a strong interaction between treatment and subsequent nest location (flower rich or poor) (Table S2). However, using this approach the negative effect of treatment on colony growth was not significant (Table S2).

Marginally more of the colonies exposed to thiacloprid failed (14/30) before the end of the experiment compared to controls (5/24) ($\chi^2_1 = 3.89$, $p<0.05$).

Of the nine nests placed out in 2013, we were able to obtain sufficient samples of food stores for chemical analysis of one pollen and six nectar samples at four weeks, three nectar and five pollen samples at eight weeks, and five pollen samples at 10 weeks. No

thiacloprid was detected in nectar and very little in pollen at 4 weeks (4/6/13), which is as we would expect because this is before thiacloprid spraying commences. At eight and ten weeks (approximately 2 and 4 weeks after spraying with thiacloprid) residues of thiacloprid were detected in most pollen and nectar samples (up to 771 ppb in pollen and up to 561 ppb in nectar, Table 2).

Discussion

We found that bumblebee colonies exposed to thiacloprid are more likely to fail, and that those which survive reach a lower final weight and produced fewer reproductives than control colonies. These difference were more marked when colonies were placed in a flower-rich site in which control colonies thrived. Few previous experiments have studied the impacts of neonicotinoids on bee colony performance where the bees were exposed to pesticides while foraging on real crop-fields (rather than experimental plots), were free-flying throughout the experiment, and the pesticide application followed normal farming practice at working farms. Cutler and Scott-Dupree[20] conducted a similar experiment with colonies of the bumblebee *B. impatiens* placed next to clothianidin or thiamethoxam-treated or untreated corn and found few negative effects, although there were fewer workers in exposed colonies. However, bumblebees rarely forage on corn so none of the nests are likely to have received significant exposure. Rundlöf *et al.*[9] found that growth of bumblebee colonies and their reproductive output was significantly impaired when placed next to fields of oilseed rape treated with clothianidin; similar findings to ours. They also found strong negative impacts on solitary bees, but no significant impact on honeybee colonies. No similar experiment has previously been performed with thiacloprid. Like oilseed rape, bumblebees are highly attracted to raspberry flowers[21]. Our study replicates the common scenario of exposure when a wild bumblebee colony is situated close to a commercial raspberry crop, or when commercial colonies are placed next to such crops. The colonies were moved two weeks after

first exposure; normally, for wild and managed bumblebees residing in the farm landscape, colonies would be exposed to the treated crop for longer than two weeks, and might be subject to further pesticide applications. They would also be present when the crops were actually sprayed, rather than being placed next to crops after spraying. As our sites were working farms, we could not always anticipate when a farm would use thiacloprid and so colonies were first exposed between 0 and 4 days after the spray day (table S1), which again would reduce the expected exposure relative to naturally occurring colonies. In these respects our study likely underestimates exposure of bumblebee colonies to thiacloprid on working farms. However, it should also be noted that we were unable to randomly allocate farms to treatments. It is thus possible that farms using thiacloprid may have differed in other farming practices from control farms (although we attempted to match control farms as closely as possible), and if so this could conceivably confound results. In addition, wild bumblebee nests are unlikely to be as close to the crop as ours were, and in this respect our study might represent a worst-case scenario.

It is notable that all colonies produced few queens. A similar study using the same "flower-poor" site in 2011 recorded a mean of ~14 queens per control colony[6], but the weather in the summer of 2012 was the wettest in the UK for 100 years (Met Office, 2012), which may account for this difference. Our colonies were also subject to the dual disturbance of movement to and from the raspberry farms, which might have impaired their performance compared to those in Whitehorn *et al.*[6].

We did not investigate the mechanisms by which thiacloprid reduced colony performance in our study, but previous studies on other neonicotinoids may shed light on this. Exposure to thiamethoxam was found to impair navigation in honeybees[4] and reduce pollen collection in bumblebees[22] while exposure to imidacloprid has been found to reduce pollen collection[3,23,24] and reduce egg laying in bumblebees[5]. Honeybees fed thiacloprid at sublethal

doses were found to fly more slowly[15], and foraging behaviour, navigation performance and social communication were all impaired[16]. A study monitoring foraging honeybees exposed to thiacloprid in polythene tunnels found a drop in foraging activity after thiacloprid was sprayed, but this did not lead to hive level effects[25]. It has, however, been noted that the power to detect differences in this study was low due to a small number of replicates[26]. In addition, honeybee hives may be expected to be more resilient to short-term perturbations than bumblebee colonies, as honeybees colonies typically hold over 30,000 workers, compared to perhaps 50 to 200 in bumblebee colonies.

We found marked differences in colony performance between the 'flower-poor' and 'flower-rich' sites. These differences may have been due to any number of differences between sites (e.g. microclimate, local pathogen community), and we could only be sure that they were due to floral availability if we had many replicates of each habitat type. However, the direct effect of differences in food availability between sites would seem to be the most likely explanation. Despite very poor weather, control colonies at the 'flower-rich' site were presumably able to gather sufficient food and hence performed relatively well, while the treated colonies performed poorly perhaps because they were unable to efficiently harvest these resources. All colonies performed poorly in our flower-poor area, presumably because there was simply not enough food.

Our study builds on evidence of the impacts of neonicotinoids on bumblebees gained in laboratory and semi-field settings. By monitoring bees which were free to forage either on the crop or elsewhere, we can better infer the impacts of neonicotinoids on colonies in natural settings. It would have been valuable to quantify the exposure of nests in each treatment, for example by sampling and analysing food stores from the nests, but at the time the experiment was performed we did not have funding or facilities for such analysis, which is expensive. We cannot be sure that control colonies were not also exposed to additional neonicotinoids by

foragers travelling to nearby farms; although the average foraging distance of bees is modest in rewarding landscapes (~750m; [27]), foragers can travel considerable distances[28-30]. Soft-fruit farms can be considered "rewarding" landscapes particularly as raspberries are extremely attractive to bees, with high densities of wild bumblebees recorded on raspberries plants within the study region[21]. Therefore it is unlikely that bees would have had to travel far for forage. However, recent reviews have confirmed that neonicotinoids and other pesticides, particularly fungicides, are prevalent throughout farmed landscapes, so we cannot rule out the possibility that our bees were exposed to additional pesticides[18,31,32]. However, this would presumably have affected both treatment groups equally. Regardless of any such additional exposure, our experimental scenario accurately mimics the situation in which a bumblebee nest is situated close to a raspberry crop. The only difference between pesticide treatments groups was in whether the crop was sprayed with thiacloprid or not, and hence the marked difference in colony performance between treatment groups strongly indicates that applications of thiacloprid can have a negative impact on bumblebee colony performance under realistic field conditions.

By placing nests on nine farms using thiacloprid in 2013 and analysing their food stores we were able to confirm that bees in this environment are indeed exposed to pesticide residues; concentrations were variable, but sometimes were very high (up to 771 ppb in pollen). This is in the region of two orders of magnitude higher than concentrations of neonicotinoids in nectar and pollen of seed-treated crops[18]. Thiacloprid has considerably lower toxicity to honeybees than some other neonicotinoids; for example the LD_{50} by topical application is 14,600 ng/bee for thiacloprid compared to 18 ng/bee for imidacloprid[11]. As a result it has been described as "bee-safe" and hence suitable for use on flowering crops; it is widely used in horticulture and is also the predominant insecticide sold for garden use in Europe[12]. It is not covered by the EU moratorium, so some countries are moving towards

increasing the use of thiacloprid in response to the restrictions on other neonicotinoids. However spray application rates are much higher than those used in seed dressings and are less uniform[33], and our results demonstrate clearly that bee nests near a treated crop can be exposed to high concentrations of thiacloprid. High concentrations of thiacloprid have also been found in pollen in honeybee hives in Germany (up to 199 ppb)[34], and a mean concentration of 89.1 ppb of thiacloprid was found in apple pollen within honeybee hives in Poland[35]. Enhanced worker mortality has been found in laboratory studies when bumblebees were fed thiacloprid at the much lower concentration of 12 ppb[36], suggesting that foliar sprays of this chemical should be treated with the same caution as other neonicotinoids.

There is also evidence that thiacloprid is particularly potent when combined with other stressors such as fungicides, parasites and nutrient stress[11,37,38]. A laboratory study that exposed honeybees to thiacloprid and the commonly-used plant fungicide triflumizole found that this compound increased the potency of thiacloprid by 1,141 fold, decreasing the LD_{50} to 12.8 ng/bee[11]. Honeybees exposed to doses of thiacloprid of $1/100^{th}$ of the LD_{50} died more quickly when infected with the protozoan parasite *Nosema ceranae* than those with the parasite alone[38]. Honeybees fed thiacloprid when starved were more likely to die relative to controls, suggesting that nutrient deficiency could enhance lethal effects[37]. An environment with fungicides, parasites and occasional nutrient stress are likely to be the norm for free-flying bees; 97.3% of samples from wax, pollen, and bee bread from North American honeybees contained two or more pesticides[39], so the effective LD_{50} for thiacloprid in the field may be lower than expected.

The current study is the first study to find effects of thiacloprid on freely foraging bee colonies. It shows that types of neonicotinoids regarded as "bee safe" because of their relatively low toxicity are legally used at concentration that can harm bumblebee colonies.

308 The long-term impact of such use on wild bee populations and the pollination services they
309 provide in fruit-growing areas should be given due consideration.

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Table 1. Results of GLMMs to test whether response variables were influenced by pesticide treatment or subsequent location. Full outputs including parameter estimates are in Supplementary Appendix 1.

Response variable	Treatment	Location[a]	Treatment x Location[b]	Errors
Colony weight (final)	$F_{1,30}$ = 1.23, ns	**$F_{1,30}$ = 10.6, p = 0.003**	**$F_{1,30}$ = 6.62, p = 0.015**	Normal
Number of workers	$F_{1,31}$ = 0.0, ns	$F_{1,31}$ = 1.13, ns	$F_{1,31}$ = 0.67, ns	Gamma with log link
Reproductive output (inc pupae)	$F_{1,31}$ = 0.94, Ns	**$F_{1,31}$ = 5.37, p = 0.027**	**$F_{1,31}$ = 5.61, p = 0.024**	Gamma with log link
Number of males (inc pupae)	$F_{1,31}$ = 3.36, ns	$F_{1,31}$ = 2.16, ns	**$F_{1,31}$ = 4.28, p = 0.047**	Gamma with log link
Number of queens (inc pupae)	$F_{1,18}$ = 0.44 ns	$F_{1,18}$ = 4.35, ns	$F_{1,18}$ = 0.06, ns	Gamma with log link

ns = not significant.

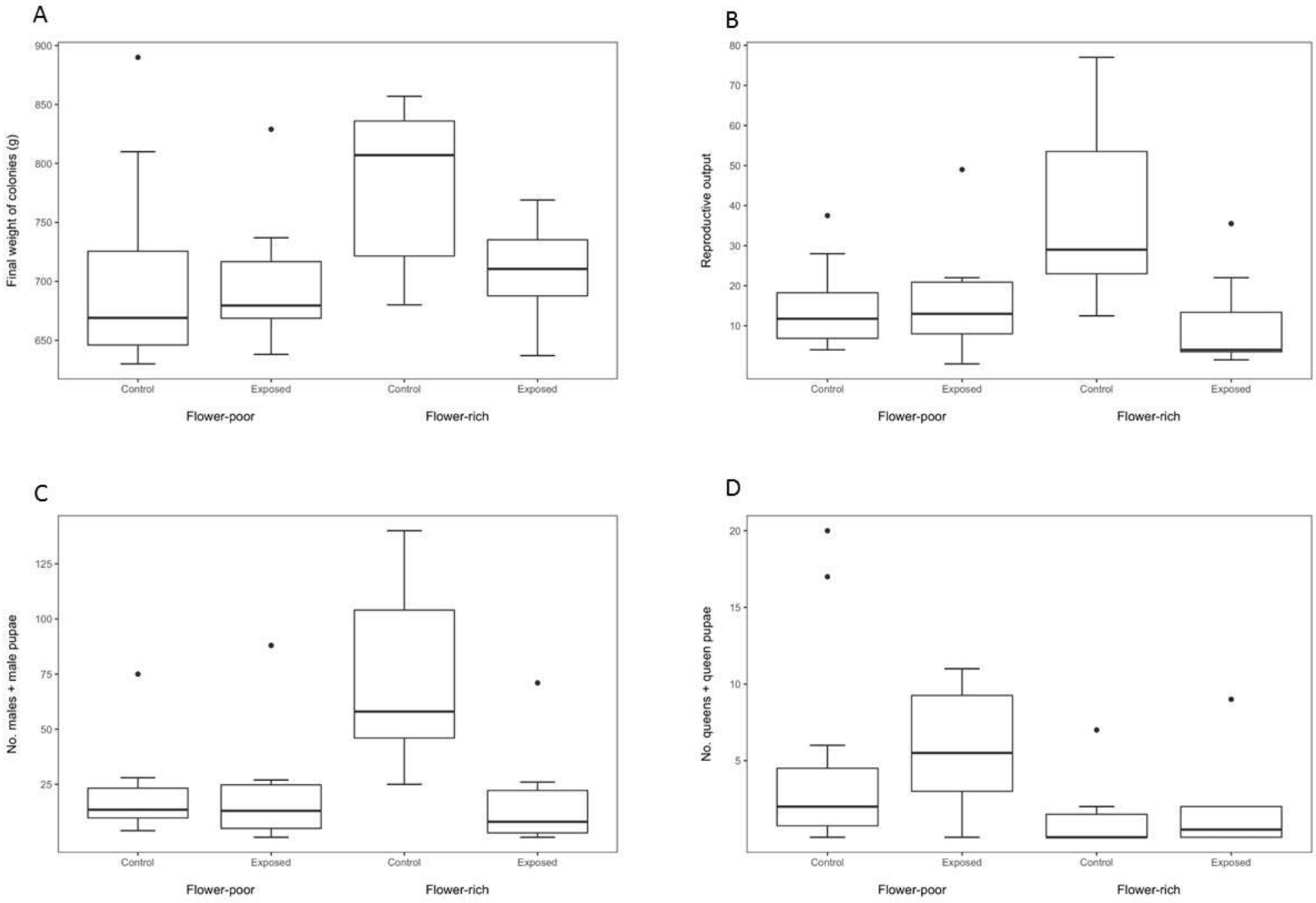
Table 2. Thiacloprid residues detected in food stores collected by bumblebee nests placed on raspberry farms in 2013. Values are in parts per billion. <MDL = less than the detection limit; <MQL = less than the quantification limit.

- = no sample could be collected

Nest number	Matrix	Week 4	Week 8	Week 10
1	Pollen	-	0.34	<MDL
1	Nectar	<MDL	-	-
2	Pollen	-	-	0.33
2	Nectar	-	-	-
3	Pollen	-	-	771
3	Nectar	<MDL	12	-
4	Pollen	-	656	320
4	Nectar	<MDL	-	-
5	Pollen	0.56	135	70
5	Nectar	<MDL	561	-
6	Pollen	-	-	-
6	Nectar	-	-	-
7	Pollen	-	0.96	-
7	Nectar	<MDL	-	-
8	Pollen	-	<MDL	-
8	Nectar	<MDL	-	-
9	Pollen	-	-	-
9	Nectar	-	<MDL	-

Figure Legends

Figure 1. Effects of exposure to thiacloprid on measures of bumblebee colony performance (median and interquartile range). After exposure for two weeks to treated or control crops, nests were split equally between flower-rich or flower-poor habitats. a) Final weight of colonies; b) Reproductive output, measured as the number of queens plus half the number of males; c) The number of workers remaining in colonies at the end of the experiment; d) The proportion of dead bees within nests at the end of the experiment.


A
Final weight of colonies (g)
900
850
800
750
700
650
Control
Exposed
Control
Exposed
Flower-poor
Flower-rich
B
Reproductive output
80
70
60
50
40
30
20
10
Control
Exposed
Control
Exposed
Flower-poor
Flower-rich
C
No. males + male pupae
125
100
75
50
25
Control
Exposed
Control
Exposed
Flower-poor
Flower-rich
D
No. queens + queen pupae
20
15
10
5
Control
Exposed
Control
Exposed
Flower-poor
Flower-rich

453

454 **SUPPLEMENTARY MATERIALS**

455 **Table S1:** Location of farm sites, flower-rich and flower-poor sites, and site details

Latitude	Longitude	Area soft-fruit (ha)	Treatment	Spray Date	Placement Date	Map code (Fig S1)
56.615509	-3.2462661	80	Thiacloprid	11th June	15th June	A.1
56.5914	-3.3329856	85	Thiacloprid	11th June	15th June	A.2
56.601626	-3.289783	85	Thiacloprid	13th June	15th June	A.3
56.564543	-3.4141517	40	Control		15th June	A.4
56.608748	-3.1902087	80	Control		15th June	A.5
56.739685	-2.4548419	7	Control		3rd July	B.1
56.32925	-3.6076717	9	Thiacloprid	2nd July	3rd July	B.2
56.521725	-2.6811709	65	Thiacloprid	6th July	6th July	C.1
56.899158	-2.3951671	65	Control		6th July	C.2
56.1499	-3.9095986		Flower-poor site			X
56.185824	-3.8974535		Flower-rich site			Y

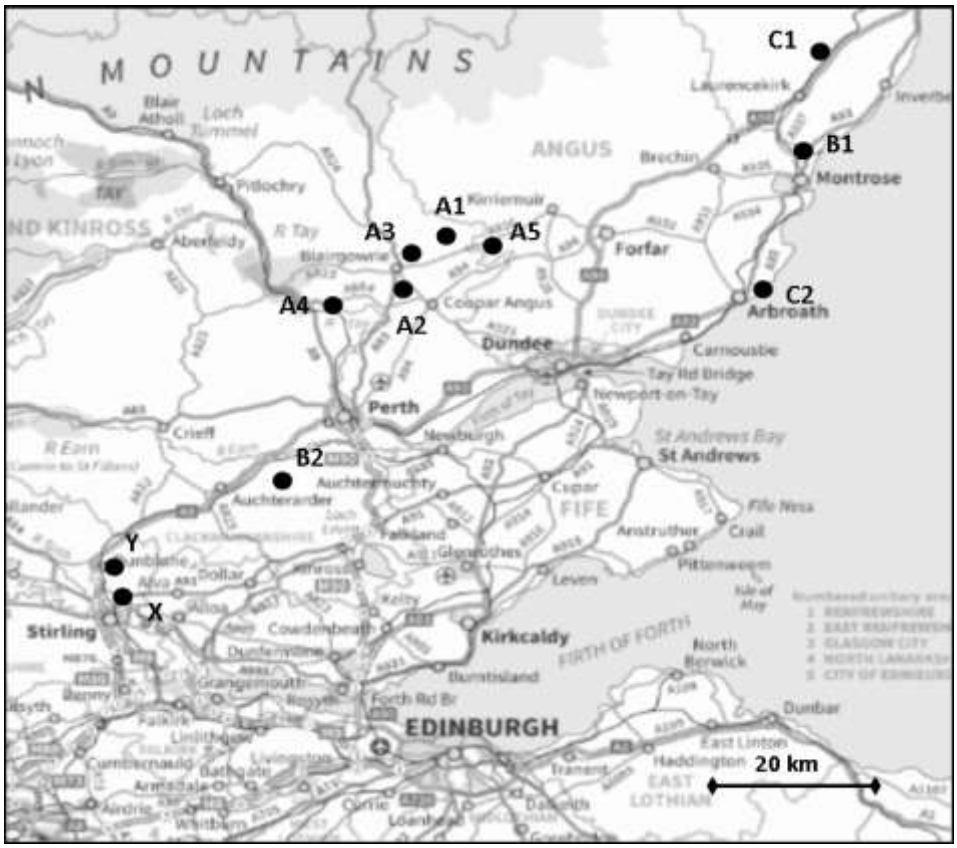
456

457

Table S2. Results of a more conservative analysis of the effects of treatment and subsequent location (flower rich/flower poor) using GLMs and averaging values for all nests at each farm/location combination.

Response variable	Treatment	Location	Treatment x Location	Errors
Colony weight (final)	$F_{1,11}$ = 0.45 ns	$F_{1,11}$ = 0.12 ns	$F_{1,11}$ = 1.53 ns	Normal
Number of workers	χ_1 < 0.00, ns	χ_1 = 0.05 ns	χ_1 = 0.04 ns	Gamma with log link
Reproductive output (inc pupae)	**χ_1 = 4.47 p = 0.035**	χ_1 = 0.72 ns	**χ_1 = 6.63 p = 0.010**	Gamma with log link
Number of males (inc pupae)	χ_1 = 3.17 ns	χ_1 = 2.41 ns	**χ_1 = 5.35 p = 0.021**	Gamma with log link
Number of queens (inc pupae)	χ_1 = 0.11 ns	**χ_1 = 5.71 p = 0.017**	χ_1 = 0.07 ns	Gamma with log link

Figure S1: Map of farm sites. Letters refer to placement dates, see table S1. Letters A to C are farm sites, with letters corresponding to the dates of placement (A = 15 June, B = 3 July, C = 6 July). Sites A4, A5, B1 and C2 are controls, A1, A2, A3, B2 and C1 received thiacloprid. X and Y are the flower-poor and flower-rich post exposure locations, respectively.



Supplementary Appendix 1. Output from Generalized Linear Mixed Models conducted in SPSS 21. Treatment (pesticide / no pesticide) and location (flower rich / flower poor were included as fixed factor, plus the interaction between them. Farm was included as a random factor

Response variable: Final nest weight. Error structure: linear

Fixed Effects[a]

Source	F	df1	df2	Sig.
Corrected Model	6.047	3	30	.002
Treat	1.227	1	30	.277
Loc	10.597	1	30	.003
Treat * Loc	6.623	1	30	.015

Probability distribution: Normal

Link function: Identity

a. Target: Final nest weight

Fixed Coefficients[a]

Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	713.401	30.7403	23.207	.000	650.621	776.181
Treat=Co	90.662	45.6197	1.987	.056	-2.506	183.830
Treat=Tr	0[b]	.	.	.	.	.
Loc=FP	-12.040	25.2585	-.477	.637	-63.625	39.545
Loc=FR	0[b]	.	.	.	.	.
[Treat=Co]*[Loc=FP]	-90.887	35.3175	-2.573	.015	-163.015	-18.759
[Treat=Co]*[Loc=FR]	0[b]	.	.	.	.	.
[Treat=Tr]*[Loc=FP]	0[b]	.	.	.	.	.
[Treat=Tr]*[Loc=FR]	0[b]	.	.	.	.	.

Probability distribution: Normal

Link function: Identity

a. Target: Final nest weight

b. This coefficient is set to zero because it is redundant.

Response Variable: Number of workers. Error: Gamma with log link.

Fixed Effects[a]

Source	F	df1	df2	Sig.
Corrected Model	.578	3	31	.634
Treat	.000	1	31	.983
Loc	1.130	1	31	.296
Treat * Loc	.673	1	31	.418

Probability distribution: Gamma

Link function: Log

a. Target: No. workers

478

Fixed Coefficients[a]

Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	3.454	.2672	12.926	.000	2.909	3.999
Treat=Co	.189	.3951	.478	.636	-.617	.995
Treat=Tr	0[b]	.	.	.	.	.
Loc=FP	.418	.3205	1.304	.202	-.236	1.072
Loc=FR	0[b]	.	.	.	.	.
[Treat=Co]*[Loc=FP]	-.364	.4438	-.821	.418	-1.269	.541
[Treat=Co]*[Loc=FR]	0[b]	.	.	.	.	.
[Treat=Tr]*[Loc=FP]	0[b]	.	.	.	.	.
[Treat=Tr]*[Loc=FR]	0[b]	.	.	.	.	.

Probability distribution: Gamma

Link function: Log

a. Target: No. workers

b. This coefficient is set to zero because it is redundant.

479

480 **Response Variable: Reproductive Output. Error: Gamma with log link.**

481

Fixed Effects[a]

Source	F	df1	df2	Sig.
Corrected Model	3.880	3	31	.018
Treat	.942	1	31	.339
Loc	5.365	1	31	.027
Treat * Loc	5.612	1	31	.024

Probability distribution: Gamma

Link function: Log

a. Target: Reproductive output

482

Fixed Coefficients[a]

Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	2.031	.3388	5.996	.000	1.340	2.722
Treat=Co	1.086	.4985	2.178	.037	.069	2.102
Treat=Tr	0[b]	.	.	.	.	.
Loc=FP	.017	.4553	.036	.971	-.912	.945
Loc=FR	0[b]	.	.	.	.	.
[Treat=Co]*[Loc=FP]	-1.492	.6298	-2.369	.024	-2.776	-.207

[Treat=Co]*[Loc=FR]	0[b]	.	.	.	.	.
[Treat=Tr]*[Loc=FP]	0[b]	.	.	.	.	.
[Treat=Tr]*[Loc=FR]	0[b]	.	.	.	.	.

Probability distribution: Gamma

Link function: Log

a. Target: Reproductive output

b. This coefficient is set to zero because it is redundant.

483

484 **Response Variable: Number of males (including pupae). Error: Gamma with log link.**

485

Fixed Effects[a]

Source	F	df1	df2	Sig.
Corrected Model	2.900	3	31	.051
Treat	3.364	1	31	.076
Loc	2.161	1	31	.152
Treat * Loc	4.281	1	31	.047

Probability distribution: Gamma

Link function: Log

a. Target: Number of males

486

Fixed Coefficients[a]

Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	2.869	.3792	7.567	.000	2.096	3.643
Treat=Co	1.444	.5551	2.602	.014	.312	2.576
Treat=Tr	0[b]	.	.	.	.	.
Loc=FP	.222	.5363	.413	.682	-.872	1.315
Loc=FR	0[b]	.	.	.	.	.
[Treat=Co]*[Loc=FP]	-1.531	.7401	-2.069	.047	-3.041	-.022
[Treat=Co]*[Loc=FR]	0[b]	.	.	.	.	.
[Treat=Tr]*[Loc=FP]	0[b]	.	.	.	.	.
[Treat=Tr]*[Loc=FR]	0[b]	.	.	.	.	.

Probability distribution: Gamma

Link function: Log

a. Target: Number of males

b. This coefficient is set to zero because it is redundant.

487

488 **Response Variable: Number of queens (including pupae). Error: Gamma with log link.**

489

Fixed Effects[a]

Source	F	df1	df2	Sig.
Corrected Model	1.559	3	18	.234

Treat	.436	1	18	.517
Loc	4.349	1	18	.052
Treat * Loc	.056	1	18	.815

Probability distribution: Gamma

Link function: Log

a. Target: queenspup

490

Fixed Coefficients[a]

Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	1.037	.4844	2.141	.046	.020	2.055
Treat=Control	-.290	.7293	-.398	.695	-1.822	1.242
Treat=Exposed	0[b]	.	.	.	.	.
Loc=Flower-poor	.808	.4808	1.680	.110	-.202	1.818
Loc=Flower-rich	0[b]	.	.	.	.	.
[Treat=Control]*[Loc=Flower-poor]	-.165	.6956	-.238	.815	-1.627	1.296
[Treat=Control]*[Loc=Flower-rich]	0[b]	.	.	.	.	.
[Treat=Exposed]*[Loc=Flower-poor]	0[b]	.	.	.	.	.
[Treat=Exposed]*[Loc=Flower-rich]	0[b]	.	.	.	.	.

Probability distribution: Gamma

Link function: Log

a. Target: queenspup

b. This coefficient is set to zero because it is redundant.

491

Supplementary Appendix 2: information on chemical analyses

Chemicals and reagents

Certified standards of thiacloprid (> 99% compound purity) and imidacloprid-d4 (> 97% isotopic purity), and formic acid, ammonium formate, magnesium sulphate, sodium acetate and Supel^{TM}QuE PSA/C18/ENVI-Carb were obtained from Sigma Aldrich UK. HPLC grade acetonitrile and water were obtained from Rathburns UK. Individual standard pesticide (native and deuterated) stock solutions (1 mg/ml) were prepared in acetonitrile (ACN). Calibration points in H_20:ACN (90:10) were prepared weekly from the stock solutions. All stocks were stored at -20^{o}C in the dark.

Sample preparation for neonicotinoid analyses

Pollen

Pollen samples were extracted as described in Botias et al. (2015). Briefly, one hundred milligrams of pollen sample was weighed into an Eppendorf tube, 400 pg of deuterated pesticide in ACN were added and the samples were extracted using the QuEChERS method. First, 400 µl of water was added to form an emulsion and samples were then extracted by adding 500 µl of ACN and mixing on a multi axis rotator for 10 minutes. Then, 125 mg of magnesium sulphate: sodium acetate mix (4:1) was added to each tube and after centrifugation; the supernatant was removed into a clean Eppendorf tube containing 125 mg of PSA/C18/ENVI-Carb. After the first extraction, the aqueous phase and resuspended pellet were extracted again with 400 µl of ACN and the supernatants combined. Extracts were mixed with PSA/C18/ENVI-Carb (10 min) and centrifuged (10 min). The supernatant was evaporated to dryness under vacuum, reconstituted with 120 µl ACN:H_2O (10:90) and spin filtered (0.22 µm).

Nectar

Nectar samples were centrifuged at 13,000 relative centrifugal force (RCF) for 10 min to remove pollen and plant debris and the supernatant transferred into a clean eppendorf tube. Nectar samples were very viscous and were therefore weighted for more accuracy (175 ± 50 mg depending on availability). Four hundred pg of deuterated pesticide standard mixture was added to the nectar and the samples were extracted using the same QuEChERS method than described previously for pollen.

UHPLC-MS/MS analyses

The Ultra high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) method described in Botias et al. (2015) was used for the analysis of samples. UHPLC-MS/MS analyses were carried out using a Waters Acquity UHPLC system coupled to a Quattro Premier triple quadrupole mass spectrometer from Micromass (Waters, Manchester, UK). Samples were separated using a reverse phase Acquity UHPLC BEH C18 column (1.7 µm, 2.1 mm × 100 mm, Waters, Manchester, UK) fitted with a ACQUITY UHPLC BEH C18 VanGuard pre-column (130Å, 1.7 µm, 2.1 mm X 5 mm, Waters, Manchester, UK). Injection volume was 20 µl and mobile phase solvents were 95% water, 5% ACN, 5 mM ammonium formate, 0.1% formic acid (A) and 95% ACN, 5% water, 5 mM ammonium formate, 0.1% formic acid (B). Initial ratio (A:B) was 90:10 and separation was achieved using a flow rate of 0.2 ml/min with the following gradient: 90:10 to 70:30 in 10 min; then from 70:30 to 0:100 in two minutes and held for 7 min, and return to initial condition and equilibration for 7 min.

MS/MS was performed in Multiple Reaction Mode (MRM) using ESI in the positive mode and two characteristic fragmentations of the protonated molecular ion $[M+H]^+$ were monitored.

Retention times, ionisation and fragmentation settings are reported in Table S3. Data were acquired using MassLynx 4.1 and the quantification was carried out by calculating the response factor of thiacloprid compounds to imidacloprid-d4. Concentrations were determined using a least-square linear regression analysis of the peak area ratio versus the concentration ratio (native to deuterated). At least five point calibration curves (R^2> 0.99) were used to cover the range of concentrations observed in the different matrices for all compounds, within the linear range of the instrument. The very high THC concentrations (i.e. >100 ppb) were calculated using an external calibration. Method detection and quantification limits (MDL and MQL, respectively) as well as recoveries were determined as described in Botias et al. (2015) and are given respectively in Table S4 and S5.

Quality control

One blank workup sample (*i.e.* solvent without matrix) per batch of twelve samples was included and injected on the UHPLC-MS/MS to ensure that no contamination occurred during the sample preparation. Solvent samples were also injected between sample batches to ensure that there was no carryover in the UHPLC system that might affect adjacent results in analytical runs. Samples were analysed in a random order and QC samples (i.e. standards) were injected during runs every 10 samples to check the sensitivity of the machine. Identities of thiacloprid was confirmed by comparing ratio of MRM transitions in samples and pure standards.

Table S3. Multiple reaction monitoring conditions used for UHPLC–MS/MS analysis of thiacloprid (ESI, positive mode) and its retention time. IMC-d4 = imidacloprid-d4, and THC = thiacloprid.

Pesticide	**Transition mass (m/z)[a]**	**Dwell-time**	**CV (V)**	**CE (eV)**	**Rt (min)**
IMC-d4	260.1>213.1	0.3	20	13	6.32
	253.0>132.0	0.3	22	14	
THC	253.0>126.0	0.3	30	18	9.46
	253.0>186.0	0.3	22	22	

Table S4. Method detection limits (MDLs) and method quantification (MQLs) limits of thiacoprid for nectar and pollen samples extracted using the QuEChERS method and analysed by UHPLC-MS/MS. THC = thiacloprid.

	Nectar		Pollen	
	MDL	MQL	MDL	MQL
	ng/g ww		*ng/g ww*	
THC	0.03	0.08	0.04	0.12

571 Table S5. Absolute recoveries (%) of four neonicotinoids from spiked nectar and pollen
572 extracted with the QuEChERS method. THC = thiacloprid.

	Nectar (n=4)		Pollen (n=4)	
	1 ppb dw		*1.2 ppb ww*	
	Av	SD	Av	SD
THC	80	11	93	8

573