Research Article

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Monitoring the effects of thiamethoxam applied as a seed treatment to winter oilseed rape on the development of bumblebee (*Bombus terrestris*) colonies

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Abstract

BACKGROUND: The development of bumblebee (*Bombus terrestris audax*) colonies that had foraged for 5 weeks on flowering winter oilseed rape grown from seed treated with thiamethoxam (as Cruiser OSR) was assessed (two control, one treated field). Colony development was evaluated by monitoring the colony mass, forager activity was assessed, both at the hive and within the crop, and the contribution of oilseed rape to the pollen stored within the colony was analysed.

RESULTS: Pollen collected from the treated crop contained residues of 1.0 μg thiamethoxam kg^{-1} and 3.0 μg CGA322704 (metabolite likely equivalent to clothiandin) kg^{-1}, and nectar contained residues of 1.8 μg thiamethoxam kg^{-1} and no metabolite. No residues of thiamethoxam or CGA322704 were detected in samples from the control fields. Up to 93% of bumblebee collected pollen sampled from within the colonies originated from oilseed rape, and *B. terrestris* were observed actively foraging on all the fields. Colonies on all three fields showed similar rates of mass gain during the exposure phase and comparable production of gynes and drones.

CONCLUSIONS: *B. terrestris* colonies placed adjacent to a field of flowering oilseed rape grown from thiamethoxam-treated seed developed at a comparable rate with colonies placed adjacent to oilseed rape grown from untreated seed.

Supporting information may be found in the online version of this article.

Keywords: bumblebees; thiamethoxam; field monitoring; oilseed rape

1 INTRODUCTION

Oilseed rape provides excellent resources for bumblebees (*Bombus terrestris*), resulting in rapidly developing colonies.[1] Systemic neonicotinoids, which are widely used as seed treatments globally, give protection to the growing plant but may also result in detectable residues in pollen and nectar.[2–4] There have been concerns that neonicotinoid seed treatments used on oilseed rape have the potential to cause adverse effects on bumblebee colonies, based on results from feeding studies.[5,6] When bumblebee colonies/microcolonies were exposed under laboratory conditions, the neonicotionid imidacloprid was reported to cause significant reductions in the number of queens and drones produced[5,7,8] and to have other adverse effects on individual workers, including reduced foraging efficiency[6,8–10] and effects on survival.[11] However, information for other neonicotinoids is limited. For thiamethoxam, results from queenless *B. terrestris* microcolonies have shown lowered brood (drones) when fed 10 μg thiamethoxam kg^{-1} or more in nectar and pollen.[9,12,13]

Laboratory studies of the effects of pesticide on bumblebee colonies and microcolonies can be readily conducted by providing dosed sucrose and/or pollen, and those published to date provide an indication of the potential for effects under conditions of continuous exposure to often relatively high concentrations when compared with residues in crops in the field.[14] A major gap in our knowledge has been the effects on bumblebee colonies from foraging on pesticide-treated crops under field conditions. Reductions in development have been reported in bumblebee colonies located adjacent to clothiandin-treated spring-sown oilseed rape, with relatively high residues in pollen and nectar.[15] The objective of this study was to compare bumblebee colony development and gyne production (unmated queens) between colonies placed adjacent to a field of flowering winter oilseed rape grown from thiamethoxam-treated seed (Cruiser OSR) and colonies placed adjacent to fields grown from untreated seed.

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2 MATERIALS AND METHODS

2.1 Field sites

The trial was conducted as an isolated field monitoring (open field) trial in Lincolnshire, United Kingdom. The area was selected because the predominant agriculture is intensive field vegetables, not a major cropping area for oilseed rape (there was no other oilseed rape within approximately 5 km), and there was negligible use of neonicotinoid pesticides in the vicinity (data supplied by the farmer). The exposure phase was carried out using one treated field and two fungicide only treated control fields drilled specifically for the purpose of the study, and each of approximately 2 ha (2.013–2.033 ha) and at least 5 km apart (see the supporting information). There were no applications of neonicotinoid compounds on the test fields for the two cropping seasons prior to drilling of the test crops.

All fields were drilled with 4.25 kg winter oilseed rape seed (var. Cabernet) ha^{-1} in October 2012 (actual rates were 3.29% below this for the control sites and 2.35% below this for the treated field, based on the mass of seed remaining in the drill subtracted from the mass put into the drill prior to sowing and the actual area drilled). The seed in the treated field contained the same treatment as commercially treated seed used in the United Kingdom, 420 g thiamethoxam nominal 100 kg^{-1} seed as Cruiser OSR (seed weight 6.3 mg; equivalent to 0.029 mg thiamethoxam $seed^{-1}$). The control seed was treated only with the fungicides Metalaxyl-M and fludioxinil. The control fields were drilled prior to the treated field using the same drill (an Accord DA Combi Drill UKW/5143/M) on the same day.

2.2 Experimental colonies

Seventy-five queen-right bumblebee colonies (*Bombus terrestris audax*, the native subspecies present in the United Kingdom) of the required size (10–20 workers) were obtained from the commercial supplier Biobest N.V. (Westerlo, Belgium) and were held for less than 24 h in cool conditions in the dark with access to feed before placement in the test fields.

Immediately prior to placement in the field, all sources of food supplied with the colonies were removed/sealed, the number of worker bees per colony was counted (none were added or removed) and the total nest was weighed (including the plastic nestbox, which is an integral part of the nest structure, but excluding the outer cardboard box). Twenty-five colonies were allocated to each of the three fields by stratified randomisation based on the number of worker bees. This resulted in a mean initial number of workers per colony in each field ranging from 16.3 ± 1.0 (SE) to 16.5 ± 1.0, with a mean initial mass (including the plastic nest box) ranging from 451 ± 3 g to 455 ± 4 g. Each colony was protected from rain by being placed inside a plastic box located on the field edge, with a spacing of at least 5 m between colonies (to minimise drift of foragers between colonies), and opened immediately. The nestbox entrances were restricted in size to prevent any newly emerged queens from leaving the colonies while allowing free passage of foraging workers.

To ensure that foraging on the crop was maximised and the duration in the field was identical, all colonies were placed on the test fields when the crop on the least developed field (the treated field) was 20% in flower. At the end of flowering (a total of 38 days from the start of the exposure phase), the colonies were moved to a monitoring site on a horticultural research farm at Cawood, North Yorkshire, which provided suitable non-crop forage, e.g *Phacelia*; the colonies were placed randomly on the site to avoid any bias. Colony weights started to decline after 4 weeks on the monitoring site, an indication that the colonies had switched from producing workers to producing drones and gynes, and a number of nest boxes were destroyed by badgers. Therefore, the decision was made to remove all the colonies from the monitoring site (68 days after the start of the study). All the colonies were returned to the laboratory, killed by freezing and then dissected.

2.3 Observations

Colony development was assessed by monitoring the colony weight every 5–8 days throughout the exposure and monitoring phase (on one occasion there was a 9 day interval owing to poor weather delaying assessment). Forager activity at the colony entrance was also monitored during the exposure phase every 5–8 days (on two occasions there was an 8–9 day interval owing to poor weather delaying assessment) by recording the number of bees entering and exiting from each individual colony over a 10 min period during daily peak activity periods (08:00–12:00 and 16:00–20:00). In order to avoid monitoring activity of all colonies from the different treatment groups on separate days, under possibly different climatic conditions, observers undertook colony activity assessments for the three sites spread over 2 days. One-third of the colonies (8 or 9) were monitored at each site in each peak activity period, i.e. nine individual colony observations at each site in the morning, the next eight in the late afternoon and the final eight in the morning of the next day. The order of site visits was rotated to reduce any bias.

Throughout the exposure phase, daily assessments were made (except on 2 days when there was persistent rain) of bee activity by counting the bees present in a 100 m by 1 m transect walked over a 10 min period through a flowering section within the crop. A different transect in each field was walked each day, and each transect was situated well within the crop area, i.e. not along the field edge. Numbers and species of bees observed within the crop were recorded. *B. terrestris* and *B. lucorum* cannot be visually distinguished and therefore in these assessments are reported as *B. terrestris/lucorum*.

2.4 Colony composition

At the end of the study, each colony was killed by freezing and then dissected. The nest wax material was broken up to identify eggs and young larvae, and the pupae were sorted into large (queen/gyne) and smaller (worker/drone). The numbers of adult workers, drones and queen/gyne bees, pupae (small – worker/drone; large – gyne), larvae and eggs were assessed. The remaining nest material, minus the bees and eggs/larvae/pupae, but including the stored pollen and nectar, was also weighed.

2.5 Pollen analysis

When the crop was in full flower (23–27 days from the start of flowering), pollen samples were collected from each of the nests. The samples from five nests were combined to give a total of five samples per field and stored frozen prior to analysis using microscopic palynology techniques. Owing to the differences in availability of pollen between nests, final sample weights were 0.66 ± 0.13 g from control site 1, 0.96 ± 0.32 g from control site 2 and 1.17 ± 0.23 g from the treated site. Each sample was homogenised with an Eppendorf pestle, and a subsample was suspended in water and vortex mixed. The resulting suspension (20 µL) was transferred onto a glass microscope slide and stained

using safranin stain in glycerol-gelatin. One hundred intact pollen grains per slide were examined at 400× magnification using a compound microscope with an eyepiece graticule. Where possible, each grain was identified to at least family level.

2.6 Residue analysis

Single samples of pollen (0.5–1.1 g) and nectar (0.5–1.1 g) were collected directly from the crop in each field (17–24 days after the start of the study). Pollen was collected by shaking the pollen from the stamens, and nectar was collected using microcapillary tubes. Samples were stored frozen at a maximum of −20 °C prior to analysis.

Pollen and nectar samples were analysed using the same methodology, which was fully validated according to SANCO/825/00. Each sample was extracted by vigorous shaking with methanol/0.2% formic acid in ultrapure water (50/50, v/v), centrifuged and diluted with ultrapure water. The sample was then transferred to an Oases HLB cartridge (Waters Corporation, En Yvelines Cedex, France), eluted with acetonitrile and evaporated at 45 °C to dryness. The extract was diluted with methanol/ultrapure water (10/90, v/v) before analysis using an API 5500 QTrap LC-MS/MS (Sciex Instruments, Les Ulis, France) with Analyst 1.5.1. A Develosil RP-aqueous column (3 µm, 150 mm × 3 mm) was used with a gradient of acetonitrile and acetic acid 0.2% v/v to ultrapure water and acetic acid 0.2% v/v with a flow rate of 0.6 mL min^{-1} and a 50 µL injection volume. Alternate injections of purified samples and external matrix-matched standards (extracts of pollen and nectar spiked with thiamethoxam 99.7% purity, CGA322704 98% purity) were used for analysis of both analytes in pollen and nectar samples. Quantification used transitions of 292.0–211.2 *m/z* for thiamethoxam and 249.9–169.0 *m/z* for CGA322704, and confirmatory transitions used were 292.0–181.1 *m/z* for thiamethoxam and 249.9–132.1 *m/z* for CGA322704 and were linear within the range used.

Recovery of thiamethoxam in nectar at the limit of quantification (LOQ) (0.5 µg kg^{-1}) was 90–111% with a mean recovery of 89% and 14% relative standard deviation (RSD), and at 5.0 µg kg^{-1} was 67–92% with a mean recovery of 82% and 13% RSD. Recovery of CGA322704 in nectar at the LOQ (1.0 µg kg^{-1}) was 82–99% with a mean recovery of 91% and 8% RSD, and at 10 µg kg^{-1} was 69–91% with a mean recovery of 80% and 12% RSD.

Recovery of thiamethoxam in pollen at the LOQ (1.0 µg kg^{-1}) was 78–96% with a mean recovery of 86% and 8% RSD, and at 10 µg kg^{-1} was 89–91% with a mean recovery of 90% and 1% RSD. The recovery values of CGA322704 in pollen at the LOQ (1.0 µg kg^{-1}) was 84–93% with a mean recovery of 90% and 4% RSD, and at 10 µg kg^{-1} was 87–92% with a mean recovery of 89% and 2% RSD.

2.7 Statistical analysis

For this study, the only true replication comes from the two untreated fields, which provides just one degree of freedom for experimental error. Given that the experimental design incorporates multiple colonies per field, some observers may feel that there would be value in conducting a statistical analysis based on 'pseudoreplication', i.e. assessing the significance of the difference between treated and untreated fields using colony-to-colony variation as the error term. Such an approach is well known to be problematic.[16,17] The finding of spurious significant differences is a general concern whenever a statistical test is based on pseudoreplication, especially in cases where the number of pseudoreplicates is high, as is the case here. Therefore, with this major warning, statistical analysis was undertaken on the two key parameters – maximum colony mass and numbers of gynes produced. These data were analysed using the glm procedure within SAS v.9.4 (SAS Institute, Cary, NC). Prior to analysis, the gyne counts were subjected to a square root transformation in order better to satisfy the assumptions of normality and homogeneity of variance (both of which underlie the use of analysis of variance). The analysis of variance model contained a term for treatment (i.e. treated or control), and the error was partitioned into field-to-field variation (i.e. the variation between the two untreated fields) and within-field colony-to-colony variation.

3 RESULTS

3.1 Colony development

None of the colonies 'failed' (lost the queen and all worker bees) during the study period. Two colonies at one control site (control 2) were destroyed by a farm vehicle during week 4, and a further seven colonies (two each from the control groups and three from the treated group) were destroyed by badgers on the monitoring site in week 9. The activity and weight data for these latter colonies were complete and included within the analysis, giving data for 25 colonies for control 1 and for the treated site and 23 colonies for control 2. However, final colony composition information was available for 23 colonies for control 1, 21 colonies for control 2 and 22 colonies for the treated group.

The increase in mass for colonies in all treatment groups was similar during the exposure phase, with mean colony mass declining slightly during the first week after placing the bees on site, before increasing at a relatively steady rate until week 5 when the colonies were moved to the monitoring site (Fig. 1). At the end of the exposure phase, the mean increases in colony mass at the control sites were 174 ± 25 g (SE) and 187 ± 21 g, and at the treated site 215 ± 20 g. The control colony mass plateaued in week 6 and declined from week 7 onwards, but the treated group mass continued to increase for a further week before declining in week 8 (see the supporting information). The maximum mass gains for the colonies in the control groups were 251 ± 28 g and 220 ± 23 g, and in the treated group 332 ± 20 g. There was no indication of field-to-field variations over and above colony-to-colony variation ($F = 0.87$; df = 1, 72; $P = 0.353$). On this basis, it could be argued that the colony-to-colony variation is an appropriate error term for testing the treatment effect, and conducting the analysis in this way gave a *P*-value for the treatment effect of 0.001 ($F = 11.22$; df = 1, 72). While the treatment effect is highly significant, it is important to note that the average of the treated colonies was higher than that of the control colonies.

3.2 Colony activity

The total number of bees entering and exiting the hives at each site followed the same pattern on each field (Fig. 2). Activity increased over time as the colonies grew in size and more bees were available to forage, as the abundance of available forage increased with crop development and as mean daily temperatures increased throughout the exposure period. By the end of the exposure phase, the mean numbers of foragers leaving the colonies in a 10 min period were 3.9 ± 1.0 (SE) and 3.8 ± 0.8 in the controls, with 2.8 ± 0.5 in the treated group; the numbers returning in the same period were 5.5 ± 1.4 and 4.0 ± 0.8 in the controls, with 4.2 ± 0.7 in the treated group.

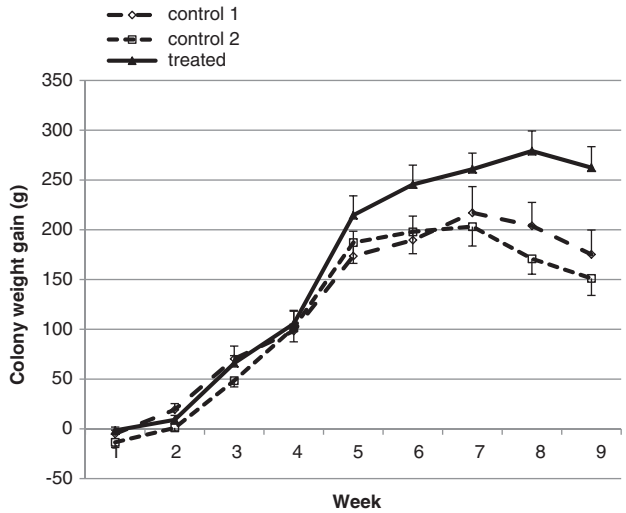

Figure 1. Mean colony weight gain throughout study (± SE). Colonies were moved to the monitoring site after the assessment in week 5.

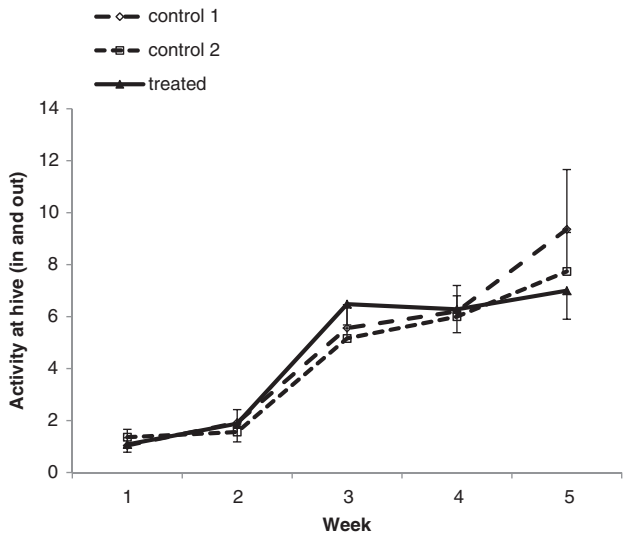

Figure 2. Mean total activity at colony entrance per 10 min observation period during the exposure phase (weeks 1 to 5) (bees in and out) (± SE).

3.3 In-crop activity

Throughout the course of the exposure phase, *B. terrestris/lucorum* were active within the crop at all three field sites and increased over time, with the greatest numbers foraging observed during weeks 4 and 5 when flowering was at its maximum and mean daily temperatures were highest (Fig. 3). The maximum number of individuals observed on a single day was 21 on control 1, 35 on control 2 and 31 on the treated field. Other bumblebee species (*B. pascuorum* and *B. lapidarius*) were occasionally observed actively foraging within the crop (a total of 3–4 observations on each field).

3.4 Colony dissection

Table 1 shows the mean numbers of emerged adult bees per colony (queens/gynes, drones and workers), together with the numbers of eggs, larvae and pupae and remaining nest mass.

The mean total numbers of adult queens/gynes per colony were similar but ranged widely between colonies from 1 to 60 in one control group ($n = 23$), from 1 to 67 in the other control

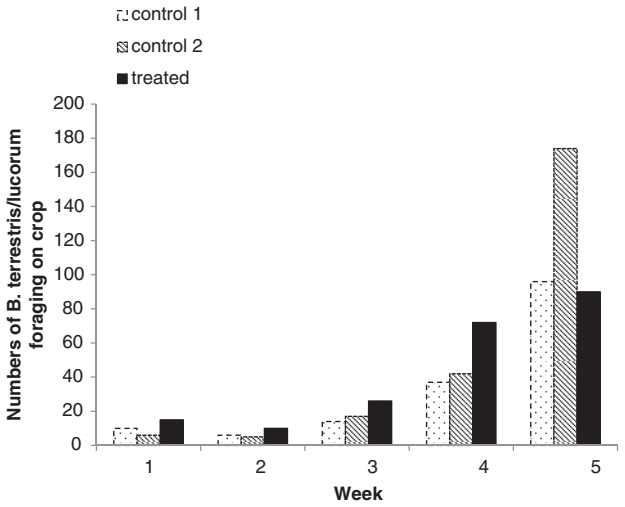

Figure 3. Total number of *B. terrestris/lucorum* observed foraging on the crop in each of the weeks during the exposure phase.

group ($n = 21$) and from 1 to 88 in the treated group ($n = 22$) (all figures include the foundress queen). One colony in control 1, two colonies in control 2 and one colony in the treated group failed to produce any gynes. There was no indication of field-to-field variations over and above colony-to-colony variation ($F = 0.25$; df = 1, 63; $P = 0.621$). On this basis, it could again be argued that the colony-to-colony variation is an appropriate error term for testing the treatment effect, and conducting the analysis in this way showed no significant effect of treatment ($F = 0.66$; df = 1, 63; $P = 0.420$). The mean numbers of emerged workers and drones, larvae and large (gyne) pupae produced by all colonies across the treatments were also similar.

3.5 Residue analysis of crop collected samples

No residues of either thiamethoxam or the metabolite CGA322704 were detected in any of the samples collected from the control fields. The sample of pollen collected from the oilseed rape in the treated plot contained 1.0 µg thiamethoxam kg^{-1} and 3.0 µg CGA322704 kg^{-1}. The sample of nectar collected from the oilseed rape in the treated plot contained 1.8 µg thiamethoxam kg^{-1} with no detectable residues of CGA322704.

3.6 Palynological analysis of pollen nest collected samples

Within all of the treatment groups, the highest proportion of pollen within the samples collected from the colonies was from Cruciferae and most likely originated from oilseed rape (*Brassica napus*), ranging from 68 ± 7% (SE) to 84 ± 4% in the controls and 70 ± 9% in the treated group. The remainder were predominantly from Aquifoliaceae, Rosaceae and Umbelliferae (see the supporting information). The presence of a high proportion of Cruciferae pollen in the absence of significant alternative sources suggests that the bumblebee colonies were actively foraging on the oilseed rape and that it was a major source of forage for these colonies.

4 DISCUSSION AND CONCLUSIONS

No adverse effects on the bumblebee colonies located in the oilseed rape grown from thiamethoxam-treated seed were detectable. Analysis using the field as the replicate (the only source of true replication) showed no significant effect on mass gain or

Table 1. Composition of colonies at the end of the monitoring period (week 9)

	Queens/gynes		Workers		Drones		Eggs	Larvae	Large pupae (gynes)	Small pupae (workers/drones)	
	Mean number (SE)	Mean weight (g) (SE)	Mean number (SE)	Mean weight (g) (SE)	Mean number (SE)	Mean weight (g) (SE)	Mean number (SE)	Mean number (SE)	Mean number (SE)	Mean number (SE)	Net nest weight[a] (g) (SE)
Control 1, $n = 23$	18.6 (3.5)	0.70 (0.03)	54.4 (10.0)	0.20 (0.01)	32.5 (5.3)	0.18 (0.01)	34.9 (7.9)	14.5 (4.2)	3.9 (1.8)	58.4 (10.3)	560.2 (19.5)
Control 2, $n = 21$	17.9 (4.0)	0.65 (0.04)	47.2 (7.4)	0.20 (0.02)	34.1 (6.6)	0.19 (0.02)	21.4 (6.4)	13.1 (5.0)	1.5 (0.9)	47.8 (7.0)	553.3 (13.1)
Treated, $n = 22$	21.3 (5.0)	0.68 (0.05)	57.7 (7.3)	0.21 (0.01)	32.3 (5.4)	0.22 (0.01)	66.1 (20.7)	18.5 (5.8)	5.4 (1.9)	74.7 (10.0)	628.3 (14.7)

[a] Excluding emerged adults and brood.

numbers of gynes produced. Analysis using colony-to-colony variation as the error term (pseudoreplication) showed that the colonies on the treated field had apparently highly significantly greater mass gain than the control colonies with no effect on production of gynes. That the treatment could be responsible for a greater increase in mass seems unlikely, and it is much more plausible that the difference observed is entirely a consequence of random field-to-field variation. This highlights the danger of conducting statistical analyses based on pseudoreplication.

There is a high likelihood that the *B. terrestris/lucorum* seen foraging within the crops on the study fields were from the study colonies. *B. terrestris/lucorum* bees were observed actively foraging on all three fields during the exposure period, and a significant level of oilseed rape pollen was identified within samples collected from the study colonies. *B. terrestris* have a foraging range of up to 1.5–2 km,[18] but based on the isolated location and separation between the field sites of at least 5 km, it is likely that the bees used within this study foraged on the adjacent oilseed rape field. Residues of thiamethoxam were identified within the nectar, and residues of thiamethoxam and the metabolite CGA322704 were detected in pollen collected from plants on the treated site, and therefore it can be assumed that bees foraging on the treated crop would be exposed.

Mean gyne production was 17–21 per colony and weight gain was 220–330 g, both of which are comparable with those figures reported for controls in other studies where commercial *B. terrestris audax* were held in the laboratory for 2 weeks prior to placement in the field,[5] and for commercially sourced colonies of closely related subspecies in the field (*B. terrestris terrestris*[19] and *B. terrestris dalmatinus*[20]). The number of queens (gynes) produced in each *B. terrestris* colony was highly variable, and this is consistent with other studies.[5,19,20] Only a small number of colonies (4–10%) failed to produce any gynes at each site. This is similar to the 10–15% that failed to produce gynes in a previous unpublished field study (Fera 2013), but far lower than the 44% (11 of 25 controls) identified in Whitehorne *et al.*[5] when bees were fed in the laboratory before placement in the field (data accessed at http://datadryad.org/resource/doi:10.5061/dryad.1805c973/1). The numbers of gynes produced by colonies have been linked to the amount of forage available to the colony, the presence within the colony of sufficient workers to forage and the nectar foraging rate (related to forager size), as well as being controlled by the founding queen.[20,21] In this study, the mass of the colonies, the numbers of workers present at the start of the study and the forage availability (same area and variety of oilseed rape) were similar across the sites. This is also reflected in the increased mass of the colony, which can be used as an indicator of both food availability and worker numbers/foraging activity.[1,19] It appears that commercially sourced *B. terrestris audax* colonies are more similar to other commercially sourced subspecies of *B. terrestris* than to their native collected counterparts.[20] Queen production in *Bombus* species in the field is highly variable,[19–21] and it is unclear how it relates to population maintenance and growth, particularly in extrapolating between species with widely varying colony size. Population dynamics are obviously also dependent on the presence of suitable forage to enable the queen to overwinter successfully, as well as suitable hibernation and nest sites.

Owing to the variability between colonies in the production of drones and gynes, another measure of colony fitness has been proposed, but has the same inherent weaknesses as queen production in that the acceptable value to sustain the population is not known. The index is based on the sum of the number of drones and twice the number of queens (as the mass of a queen is approximately twice that of a drone).[1] Using this measure, the fitness indices of the colonies (control 1, 67.7 ± 8.3; control 2, 67.8 ± 10.1; treated, 72.9 ± 9.8) were comparable with those reported by Goulson *et al.*[19] for *B. terrestris terrestris* placed on conventional farms (mean fitness index 67.4) and lower than those placed in gardens (mean fitness index 124) and farms with conservation measures (mean fitness index 99.8).

The results in this study are in contrast to the conclusions of Goulson,[22] which relied on a correlation with residues in pollen, but not nectar, and were based on a dataset from a study that did not have isolated fields, and thus, owing to the foraging of the bees on other fields, there were no true control data for comparison. The lack of effect on mass gain in this study is also in clear contrast to recent data on the development of bumblebee colonies exposed to spring-grown oilseed rape grown from clothiandin-treated seed in Sweden.[15] Far higher residues in pollen and nectar were reported by Rundlof *et al.*[15] than in this study (or other studies with winter oilseed rape, e.g. Pilling *et al.*[3]) and were closer to those used in laboratory feeding studies with bumblebees showing an effect with imidacloprid.[5,6] Although residues in spring-drilled crops would be expected to be higher than in overwintered crops,[23] previously reported residues in pollen and nectar from spring oilseed rape have been far lower.[23–25]

In summary, these data show no adverse effects on gyne production or maximum mass gain in *B. terrestris* colonies placed adjacent to, and identified from surveys and palynology as most likely foraging on, a field of flowering winter oilseed rape grown from thiamethoxam-treated seed. These results provide additional information to the expanding evidence base on bees and neonicotinoids, which increasingly highlights the importance of realistic

exposure under field conditions and the ability of studies at the colony level to address sublethal effects.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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