Effects of neonicotinoid seed treatments on bumble bee colonies under field conditions

March 2013




Authors: Helen Thompson, Paul Harrington, Selwyn Wilkins, Stephane Pietravalle, Dinah Sweet and Ainsley Jones

Food and Environment Research Agency, Sand Hutton, York YO41 1LZ

1 Executive Summary

1. The current study tested the hypothesis that exposure of bumble bee colonies placed in the vicinity of crops treated with neonicotinoids had no major effect on the health of the colonies.
2. The study compared the development (mass and composition) of bumble bee Bombus terrestris colonies placed in three landscapes (A-C) near oilseed rape (OSR) crops which had been grown from (A) untreated seeds or from seeds treated with the neonicotinoid insecticides (B) clothianidin or (C) imidacloprid). Twenty bumble bee colonies were established at 3 sites.
3. Systematic differences in the pesticide residues were found across the three sites, but these were not related to the treatment applied to the adjacent crop suggesting bumble bees forage over large distances.
4. Differences existed in flowering phenology across the three sites necessitating colonies to be placed in the field at differing times. As a consequence of this there were systematic between-site differences in the size and possibly other aspects of the colonies. These differences particularly affected site C. In analysing the data attempts were made to control statistically for these baseline differences.
5. Commercially sourced bumble bee colonies of known size which contained a mean of 16-24 worker bees were placed in each site for the duration of the flowering period (6-7 weeks) of the OSR. At peak flowering times, samples of pollen and nectar were collected from each colony for residue analysis (limit of detection 0.025 µg/Kg in nectar and 0.5 µg/Kg in pollen) and palynology (pollen). Analysis was undertaken for the neonicotioinds and the two major honeybee-toxic metabolites of imidacloprid (there is no published data on the chronic toxicity of these neonicotinoids and metabolites to bumble bees).
6. All colonies grew and survived to the end of the experiment. At sites A and B colonies grew to a significantly greater terminal mass than at site C but all colonies grew to a greater mass than control colonies in published laboratory experiments.
7. The number of gynes (new queens) produced in the colonies was not significantly different across the three sites.
8. Despite the local abundance of flowering rape, pollen analysis showed bees foraged on a wide-variety of flowers with the former contributing on average 35-37% of pollen collected. Possibly because of the delay in placing colonies in the field, foraging patterns at site C differed somewhat from the other two sites.
9. At site A, colonies contained residues of thiamethoxam in all 19 available nectar samples available (mean 0.885 µg/Kg) and in 9 of the 20 pollen samples (mean 0.730 µg/Kg). Residues of clothianidin in nectar were detectable (but below the limit of quantitation;

LOQ) in 17 nectar and 20 pollen samples. There were no detectable residues of imidacloprid, nor its major (toxic) metabolites in pollen and nectar.

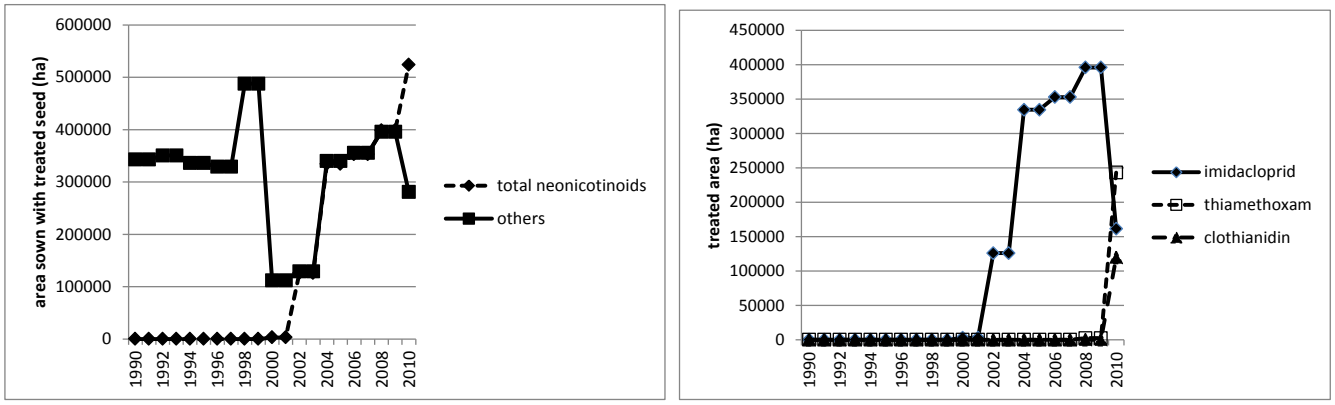
10. At site B, colonies also contained residues of thiamethoxam with a mean of 2.397 µg/Kg nectar and 0.718 µg/Kg pollen. Residues of clothianidin, a metabolite of thiamethoxam, were above LOQ in 14 nectar samples (mean 0.205 µg/Kg) and detectable, but below LOQ, in 6 nectar samples. There were no detectable residues of clothianidin in pollen. Neither imidacloprid, nor its major toxic metabolites, were detected in pollen and nectar.
11. At site C, of the 15 nectar samples, residues of imidacloprid were detected below LOQ in 6 samples, with both imidacloprid and clothiandin jointly detected (at or below LOQ) in a further 2 samples. In contrast, thiamethoxam was not determined in any of the nectar sampled. Residues of thiamethoxam, clothianidin or imidacloprid (or metabolites), were not detected in any of the 20 pollen samples taken.
12. Using the observed variation in neonicotinoid residues across colonies within and between sites, possible correlations with colony mass and the number of new queens produced were explored. No clear consistent relationships were observed.
13. This study was not a formal statistical test of the hypothesis that neonicotinoid insecticides reduce the health of bumble bee colonies. Nevertheless, were neonicotinoids in pollen and nectar from treated oilseed rape to be a major source of field mortality and morbidity to bumblebee colonies, we would have expected to find a greater contribution of insecticide residues from nearby treated crops and for there to have been a clear relationship between observed neonicotinoid levels and measures of colony success. The absence of these effects is reassuring but not definitive. The study underlines the importance of taking care in extrapolating laboratory toxicology studies to the field, as well as the great need of further studies under natural conditions.

Contents

2 Introduction

Neonicotinoid insecticides have been widely used in the UK as seed treatments on oilseed rape since the mid-1990s (Figure 1). Westphal *et al* (2009) considered that oilseed rape provides excellent resources for bumble bee colonies resulting in rapidly expanding colonies. However, the absence of alternative forage when flowering of the oilseed rape ceases was considered to have the potential to result in such positive effects being negated.

Figure 1 Usage of neonicotinoid seed treatments on oilseed rape in the UK (Pesticide Usage Surveys occur every 2 years)



There are limited data published for the toxicity of neonicotinoid insecticides to bumble bees with the acute toxicity of imidacloprid similar (on a per weight basis) to that in honeybees for which the no observed effect concentration (NOEC) for adult mortality is 24 µg/kg (EFSA 2013a). The clothianidin NOEC for mortality in adult honeybees following chronic dosing is 10 µg/L (EFSA 2013b). There is no chronic toxicity data for thiamethoxam in adult bees but a honeybee colony feeding study suggested that the NOEC for brood mortality is 12.5 µg/kg (EFSA 2013c).

Environmentally relevant concentrations of imidacloprid may cause adverse effects on *Bombus terrestris* colonies through significant reductions in the number of queens produced per colony (Whitehorn *et al* 2012). The Whitehorn *et al* study exposed commercially reared bumble bee colonies at an early stage of development to treated sucrose (0.7 µg imidacloprid/Kg) and pollen (6 µg imidacloprid/Kg) for 2 weeks in the laboratory before the colonies were moved outside to forage for a further 6 weeks from mid-July. Previous laboratory studies have reported reduced drone production in *B terrestris* at 1 µg imidacloprid/Kg (Laycock *et al* 2012), longer foraging times (Mommaerts *et al* 2010) and reduced foraging efficiency (Gill et al 2012) at 10 µg imidacloprid/Kg and at 16 µg imidacloprid/Kg resulted in both lower brood production rate and worker survival (Tasei *et al* 2000). Mommaerts *et al* (2010) showed no effects on offspring production at 2 µg imidacloprid/Kg but

adverse effects at 10 and 20 µg imidacloprid/Kg with cessation of brood production in the laboratory.

Nevertheless, there are limitations in our ability to extrapolate such results from laboratory studies to field conditions. This is because of the possibility that other factors could intervene including the availability of alternative forage, the foraging strategies of colonies, the effects of weather and the phenology of the crop cycle. The current study tested the hypothesis that exposure of bumble bee colonies placed in the vicinity of crops treated with neonicotinoids had no major effect on the health of the colonies.This study was established in a short-timescale to address the concerns raised by the publication in March 2012 of Whitehorn *et al*, by extending it to the field. It should therefore not be considered as a definitive field study but an assessment of whether major effects are observed in bumble bee colonies under real-life field conditions.

The most recent data about usages of neonicotinoids was the UK Pesticide Usage Survey 2010 (https://secure.fera.defra.gov.uk/pusstats/). The areas planted with imidacloprid, clothianidin and thiamethoxam seed treatments on oilseed rape were 161K, 119K and 243K ha respectively. The usage of imidacloprid on oilseed rape declined from 395K ha in 2008 and has been declining in the UK as seed treated with clothianidin and thiamethoxam has increased (Figure 1). Clothianidin is also a metabolite of thiamethoxam.

Bumble bee colonies were placed at the edges of flowering oilseed rape crops grown from untreated seed, seed treated with imidacloprid (Chinook™) or clothianidin (Modesto™) within landscapes that contained varying densities of other oilseed rape fields grown from untreated, thiamethoxam (Cruiser™) or clothianidin (Modesto™) treated seed. The present study was not designed to be an isolated worst-case field study, located away from other flowering crops (EPPO 2010). It was not possible to establish a statistically robust, replicated field study at isolated sites in the timescale required. The objective was to examine the effects on bumble bee colonies in conditions as close as possible to real-life field situations. *B. terrestris* have a usual foraging range in arable environments of 1.5-2 Km (Osborne et al 2008) although some foragers may exploit resources at greater distances (Benton 2006) and therefore crops beyond those immediately adjacent to that on which the colonies were located may be attractive forage sources. Even differences between varieties of oilseed rape may result in differences in the attractiveness as forage (Waddington 1979, Cook et al 2003).

The same parameters were assessed as those reported by Whitehorn *et al* (2012) together with additional parameters including residue analysis, foraging activity and analysis of the source of pollen (palynology) being returned to the colony.

3 Materials and Methods

Experimental colonies

Queen-right *Bombus terrestris* Audax colonies at an early stage of development and an expected similar stage to wild populations (first workers emerging early April (see Thompson and Hunt 1999)) were obtained from a commercial supplier (Biobest, Belgium).

At the start of the exposure phase (the bees were placed on each field site within one day of receipt) the colonies placed on site A contained 21 ± 2 bees and weighed 0.579 ± 0.003 kg; the colonies placed on site B contained 24 ± 2 bees and weighed 0.578 ± 0.003 kg, these colonies were randomly assigned to treatment. The colonies placed on the site C contained 16 ± 1 bees and weighed 0.546 ± 0.002 kg (as these were established 13 days later than the colonies at sites A and B they could not be randomly assigned to treatment). The number of workers per colony at site C were significantly lower ($p=0.04$) and this was included in all statistical analyses; correction for the number of workers also ensured differences in mass were accounted for in the analysis.

The supplied cardboard colony boxes were placed within an outer corrugated plastic box to protect them from direct rainfall (Figure A3 in the Appendix). The doors on the nest boxes were adapted to prevent queens leaving the colonies and the supplied proprietary liquid food was removed. Colonies were randomly allocated to locations on identified farms and placed 2-3m apart under hedges to limit direct sunlight and rainfall and to limit interference between the colonies. Due to the later flowering of the crop at site C these colonies were purchased and placed on the site 13 days later than on the other two sites. Despite this later placement at site C the duration of the exposure phase was the same on all sites and termination of the colonies was related to the development (decline in mass) of the colonies.

Field sites

Farms in northern England were located which had fields of flowering winter-sown oilseed rape identified by farmers.

Site A. (near Lincoln, Lat 53.489, Long -0.778) Twenty bumble bee colonies were placed at the edge of a 6.5 ha (300 x 200m) crop of flowering oilseed rape which was identified by the farmer as grown from untreated seed (home-saved, Variety: Catana (conventional)) sown at 3.5 Kg/ha. Information obtained from the agronomist and the farmer was that all oilseed rape on the farm was grown from untreated seed and no neonicotinoids were used on any field within 1 km of the control field in this or the previous year. Due to the ubiquity of oilseed rape, other fields in the locality, i.e.

beyond 1 km, may have been grown from seed treated with clothianidin or thiamethoxam. Other flowering plants at the field edge included hawthorn (*Crataegus monogyna)* and there were gardens nearby.

Site B.(near York (Lat 54.082, Long -1.045) Twenty bumble bee colonies were placed at the edge of a 10.7 ha (350m x 350m) flowering oilseed rape crop identified by the farmer as grown from seed (Variety: Excalibur (hybrid)) treated with clothianidin (Modesto™: beta-cyfluthrin/clothianidin 8/40) 0.0125 l/Kg seed) and sown at 3 Kg/ha giving a rate of 15g ai/ha (based on 4.5 mg seed weight this is 0.0225mg/seed). Adjoining and nearby fields contained winter wheat, pasture or flowering oilseed rape grown from seed which had also been treated with clothianidin. Other oilseed rape in the locality was identified by the neighbouring farmer as grown from seed (Variety: Catana (conventional)) treated with Cruiser™ (thiamethoxam) with the nearest fields 0.9-1 Km away. Other flowering plants at the field edge included hawthorn (*Crataegus monogyna),* blackthorn (*Prunus spinosa*) and crab apple (*Malus sylvestris).*

Site C. (near Scunthorpe, Lincs (Lat 53.137, Long -0.552) Twenty bumble bee colonies were placed at the edge of a 12.1 ha (200m x 500m) crop of flowering oilseed rape which was grown from seed (Variety: Catana (conventional)) treated with imidacloprid (Chinook™: beta-cyfluthrin/imidacloprid 10:10) 0.02 l/Kg seed) and sown at 5.41 Kg/ha giving a rate of 11g ai/ha, or based on 4.5 mg seed weight this is 0.009 mg/seed. Adjoining fields contained crops winter wheat, winter barley or flowering oilseed rape grown from seed (Variety: ExPower (hybrid)) which had been treated with clothianidin (200m away) and oilseed rape in the locality had been grown from seed (Varieties: Tactic (conventional) and ExPower (hybrid)) treated with Cruiser™ (thiamethoxam) (the nearest 600m away). Other flowering plants at the field edge included hawthorn (*Crataegus monogyna)*, horse chestnut (*Aesculus hippocastanum*) and hogweed (*Heracleum sphondylium)* although these were at lower density than at sites A and B.

No other neonicotinoid pesticide applications were made to the oilseed rape crops before or during flowering.

At the end of the flowering period of the oilseed rape (6-7 weeks compared with the expected 3-4 weeks) all the colonies were moved to sites providing appropriate forage on plants not treated with neonicotinoids. After a total of 8-9 weeks from placement in the field the colonies were in decline (decreased mass and thus at the same stage of development at all sites) and producing reproductive (drones and gynes (queens)). At this point all of the colonies were at the same developmental stage and were freeze-killed, i.e. the colonies from site C were killed 2 weeks after the colonies on sites A and B.

Observations

Colony mass (Kg) (inner colony box and nest) was measured weekly after the foraging assessments (between 1030 and 1800). The number of foragers moving in and out of the nest were counted for a 10-minute period. Foraging activity on the flowering oilseed rape was measured by counting the number of bees within a 100 metre strip transect (1 m wide) along the edge of the crop at a rate of movement of 10 m per minute. These counts took place between 1030 and 1800 when peak foraging was expected based on the environmental conditions at each site.

Temperature was recorded using TinyTag dataloggers at each site.

At the end of the study period all colonies were dissected and the number and mass of queens (gynes), drones, workers, larvae, pupae and numbers of eggs, nectar and pollen storage cells present were recorded as well as the mass of the remaining wax structure. The presence of spores of *Nosema bombi* or *Crythidia bombi* parasite was assessed in the queens present at the end of the study by microscopy. All the founding queens present were assessed and where 10 or less gynes were present these were all assessed, where more than 10 gynes were present 10 were randomly selected for assessment.

Pollen and nectar sample collection

Samples of nectar and pollen were collected from each of the colonies (treated and control) for analysis of the active ingredient and major toxic metabolites. These were taken 25 days at Site A, 26 days at site B and 34 days at site C after placement of the colonies at each site and during peak flowering of the oilseed rape. Samples of pollen were also collected from returning foragers (by removing pollen loads from individual foragers) for identification of the source of the pollen being returned to the colony (palynology). If a sample of pollen could not be collected from the foragers returning to a colony a subsample of the pollen collected from the store within the colony was used. Light microscopy was used to identify the source of the pollen grains within the sample, and approximate proportion of each within the sample, was undertaken (Sawyer 1981).

Samples of pollen and nectar were collected from the flowering crop for analysis of the presence and concentrations of pesticides and major toxic metabolites. These samples were collected by confining a small honeybee colony to the headland area of the crop using a mesh tent (3m x 3m x 2.5m) (Figure A2), although this proved not to be bee-proof in high winds, and collecting samples of nectar and pollen directly from the comb. All samples were stored at a maximum of -25^{o}C prior to analysis.

Residue analysis

Nectar samples were diluted with water and partitioned with ethyl acetate. The ethyl acetate solution was evaporated to dryness and re-dissolved in methanol:water (10:90; v/v) and the concentration of neonicotinoids in the sample were determined by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS)

Pollen samples were extracted with methanol (Schoning and Schmuck 2003). A portion of the extract was diluted with water and cleaned up on an Oasis HLB cartridge. The eluate was evaporated to dryness and re-dissolved in methanol:water prior to LC-MS/MS determination.

LC-MS/MS was performed using an Agilent 6490 Triple Quadrupole Mass Spectrometer operated in selective ion monitoring mode using electrospray ionisation in positive ion mode. Quantification was by means of matrix-matched external calibration standards. The neonicotinoids determined were imidacloprid (and its olefin and 5-OH metabolites), clothianidin and thiamethoxam. The method was validated for all compounds except thiamethoxam by fortifying control samples and determining recoveries. Thiamethoxam was not included in recovery experiments as it was not originally intended to include this compound in the analysis procedure. However recovery samples were included during analysis of the batches. Since control samples of nectar were not available, honey diluted 6:4 with water was used. The mean recoveries and % RSDs (CVs) obtained from validation experiments are listed below (Table 1). All residues were corrected for recovery. The limit of detection (LOD) for imidacloprid, clothianidin and thiamethoxam was 0.025 - 0.05 µg/Kg in nectar and 0.5 µg/Kg in pollen. The LOD for the toxic olefin and 5-hydroxy metabolites of imidacloprid were 0.1-0.2 µg/Kg in nectar and 0.5 µg/Kg in pollen.

Statistical analysis

Site based analysis

The numbers of each caste, developmental stage and pollen and nectar storage cells identified within the colonies at the end of the study were analyzed using a Generalized Linear Model (GLM), assuming a Poisson distribution and using a logarithm link function whilst accounting for any over-dispersion of the data. The number of worker bees present (developmental stage of the colony) at the start of the exposure period was taken into account as a covariate. When looking at mass of each parameter and of the remaining nest at the end of the study, a similar approach was followed, but assuming a normal distribution.

When looking at colony mass over time, the data were analyzed using a repeated measure analysis of variance to account for any possible autocorrelation between

observations and, once again, adjusting for differences in the number of worker bees present at the start of the exposure period through the use of a covariate. A similar approach was followed when looking at foraging activity over time (where foraging activity is defined as the ratio of the number of bees flying in over the total number of bees flying in and out).

Residue based analysis

As neonicotinoid residues were detected in colonies at all three sites an alternative approach was used to assess the effects of exposure to residues of thiamethoxam and clothianidin. Three explanatory variables (analysed separately): thiamethoxam in nectar, clothianidin in nectar and thiamethoxam in pollen and three response variables were used: the number of queens, the colony mass in the week that the residue samples were collected and the colony mass at the end of the study.

The number of queens (count data), were analysed using a Generalized Linear Model, assuming a Poisson distribution (and using a log link function). In order to account for differences in initial colony size the number of bees at start was used as an offset variable (note the number of bees at start was log-transformed so that the analyses look at the number of queens produced per bee present at start). "Site" was used as an additive term in the regression to first remove any potential effect of the site.

A straightforward regression model was used for colony mass at the time that the residue sample was taken. Again, in order to account for differences at the start of the season, the mass at the start of the season (untransformed) was used as an offset variable. "Site" was again used as an additive term in the model to first remove any potential effect of site.

In both analyses, the explanatory variables were therefore the "Site" and the neonicotinoid residue level in pollen or nectar.

To use as much information as possible from the data (i.e. use the fact that some of the residue levels were below the limit of detection (LOD)), the data were simulated (bootstrapped) below the LOD to produced actual values (uniformly distributed between zero and the LOD). The regressions were fitted as described above and the process repeated 1000 times to allow for different sets of simulated data below the LOD (the data below the LOD are simulated separately for each of the 1,000 runs). For each simulation, whether the effect of the dose was significant or not (at the 5% significance level) was assessed and the proportion of those 1,000 runs where the effect of dose was found to be significant determined.

In addition, a non-parametric analysis was undertaken with simulation for residues below the LOD undertaken as described above. Instead of fitting a model, a Spearman correlation between the variable (number of queens, colony mass at 3-4 weeks or colony mass at end) and the dose was assessed and the proportion of times (out of the 1,000 runs) that the correlation coefficient was significant was

determined. Note that this analysis does not use any information about differences between sites nor any information about the colony mass at the start of the season or the number of bees at the start of the season.

Table 1 Validation data for clothianidin, imidacloprid and the toxic metabolites of imidacloprid (imidacloprid- olefin and 5 hydroxy –imidacloprid) in pollen and nectar

Nectar			
		% Recovery	
	Fortification level (µg/kg)	Mean	RSD
Imidacloprid	0.16	75.8	3.0
	4.0	81.5	2.7
imidacloprid olefin	0.16	66.6	32.6
	4.0	76.6	3.4
imidacloprid 5-OH	0.16	73.7	10.2
	4.0	74.3	3.0
Clothianidin	0.16	70.7	20.5
	4.0	79.6	1.0
Pollen			
		% Recovery	
	Fortification level (µg/kg)	Mean	RSD
Imidacloprid	0.5	87.6	9.6
	25	84.6	3.7
imidacloprid olefin	0.5	86.2	15.7
	25	85.9	4.8
imidacloprid 5-OH	0.5	97.3	14.1
	25	91.6	4.5
Clothianidin	0.5	90.5	10.1
	25	92.1	3.3

4 Results

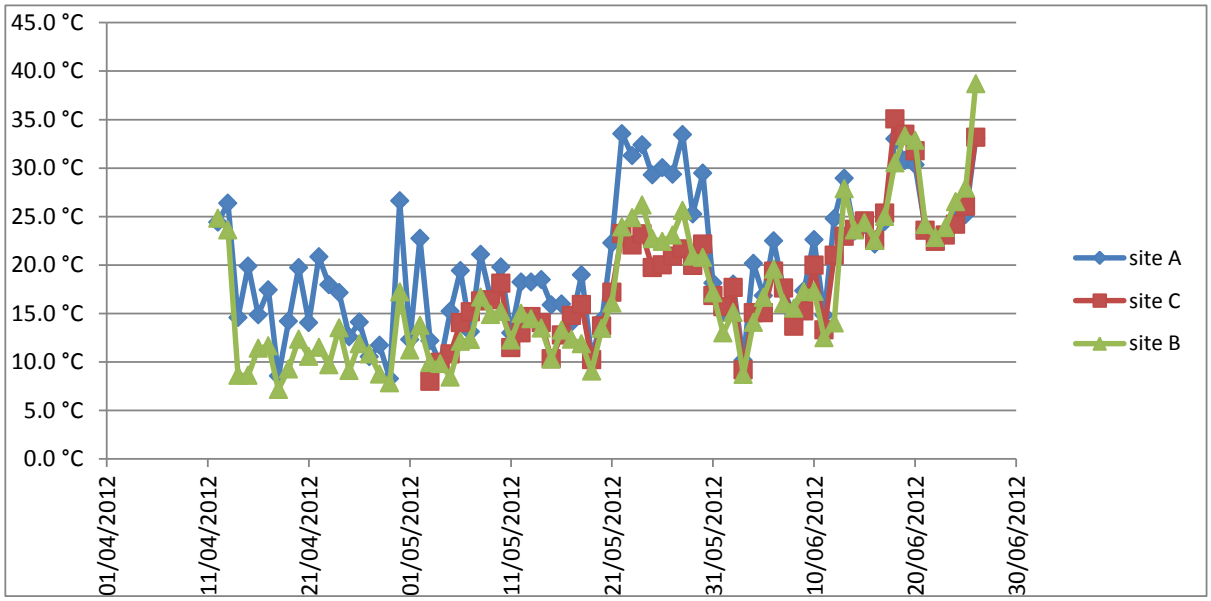
The oilseed rape started flowering in early-mid April and flowered for a total of 6-7 weeks before the colonies were moved to alternative forage. This was well in excess of the expected 3-4 weeks typical of the UK and probably related to the cool weather experienced in late spring/ early summer 2012. The mean temperatures at each site were similar but with cooler temperatures initially at site B (Table 2 and Figure 2). Rainfall data for the region shows that this was an exceptionally wet spring/summer and rainfall was not a limiting factor in nectar production. The total time in the field was 63 days for the colonies at site C compared with 60 and 61 days total at the other 2 sites, i.e. in excess of the 8 weeks required to complete colony development at all sites.

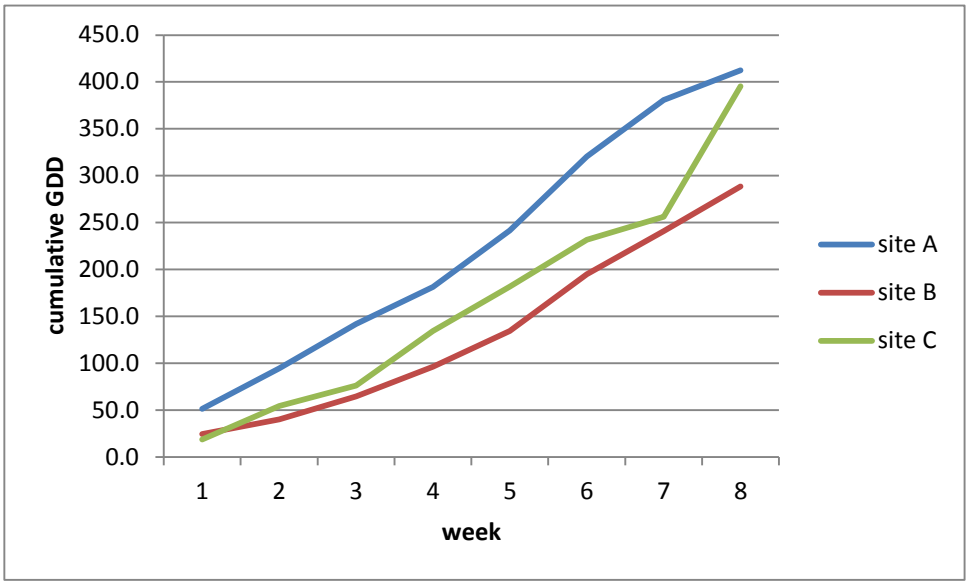
Table 2 Mean minimum and maximum daily temperatures (^{o}C) recorded on each site during the study

Month	Site A	Site B	Site C	Daily rainfall York (mm)
Flowering period	13 April -2 June	13 April – 2 June	26 April – 11 June	
April (mean min-mean max ^{o}C)	3.0-16.5	3.0-12.0	-	0-30
May (mean min-mean max ^{o}C)	5.8-20.5	5.5-15.9	6.3-16.6	0-7
June (mean min-mean max ^{o}C)	13.6-22.5	12.9-21.7	13.5-21.7	0-15
Days in field	60	61	63	-
Cumulative growing degree days[a]	412	289	395	-

[a] GDD = $\sum((Tmin+Tmax)/2) -6)_n$ where 6^{o}C is the minimum temperature for bumble bee flight (Corbet *et al* 1993) and n = days in field; where lower temperature is less than 6^{o}C this is set to 6^{o}C for the purpose of the calculation.

Figure 2 Mean maximum temperature and calculated cumulative growing degree days (GDD) at each site





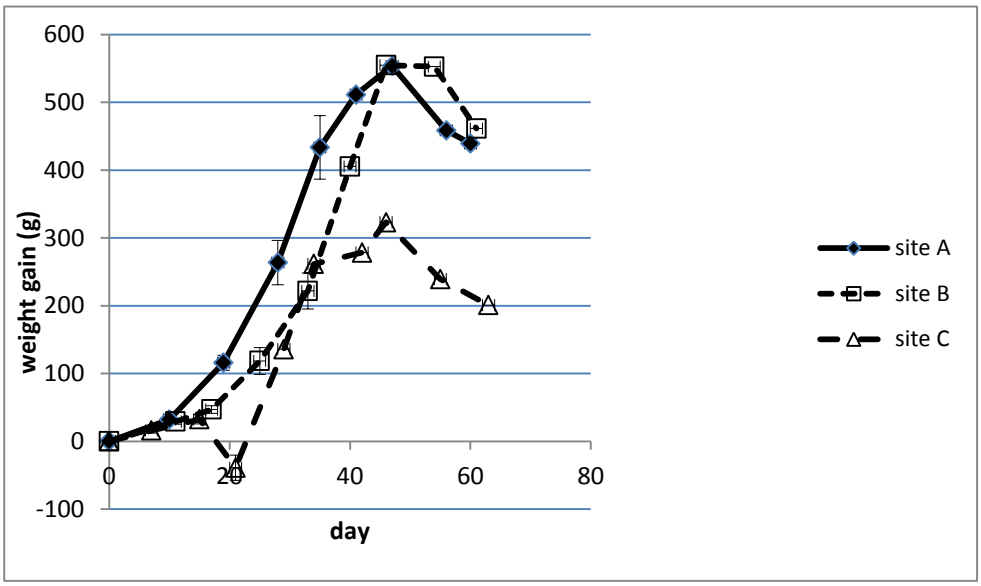
Site based analysis

Although it was not possible to establish a replicated study in isolated field plots, as required for a statistically robust study design, the foraging activity, mass growth and composition of the colonies could be compared between the three sites. This provides an indication of any major differences between the development of the colonies at the three sites which could be ascribed to the varying exposure to neonicotinoid insecticides. Details of the statistical analysis are shown in the Appendix.

Colony mass over time

When corrected for the number of workers per colony there were significant differences in changes in colony mass both between sites and between sites over time ($F<0.001$). The change in colony mass over time after placement in the field are shown in Figure 3 and a significant difference ($p<0.05$) was detected at Site C compared with Sites A and B from week 3 onwards. After correcting for the initial number of workers in the colonies (which also corrected for any differences in initial colony mass) there were no significant differences in the maximum mass of the colonies at sites A and B (mean peak mass 1.130kg and 1.119 kg respectively when adjusted for initial size), whereas the maximum mass of the colonies at site C (mean peak mass 0.885 kg when adjusted for initial size) was significantly lower ($p<0.05$) than those at both site A and site B.

Figure 3 Mass gain over time of bumble bee colonies exposed on sites A, B and C



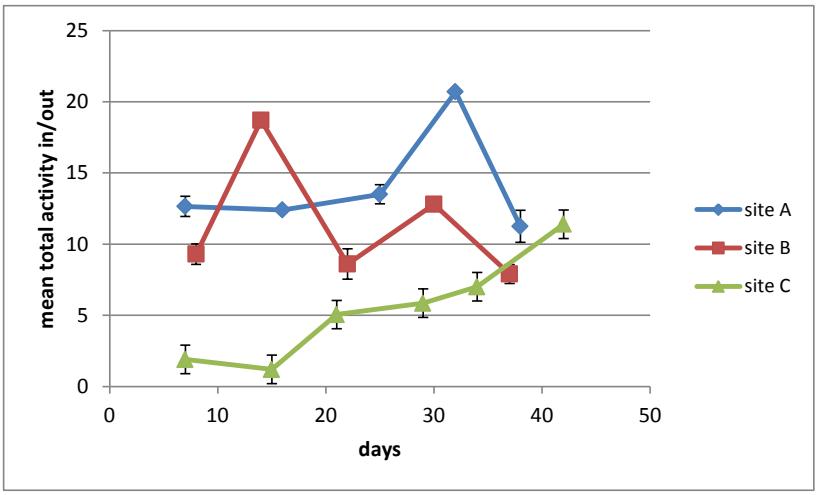
Foraging activity over time

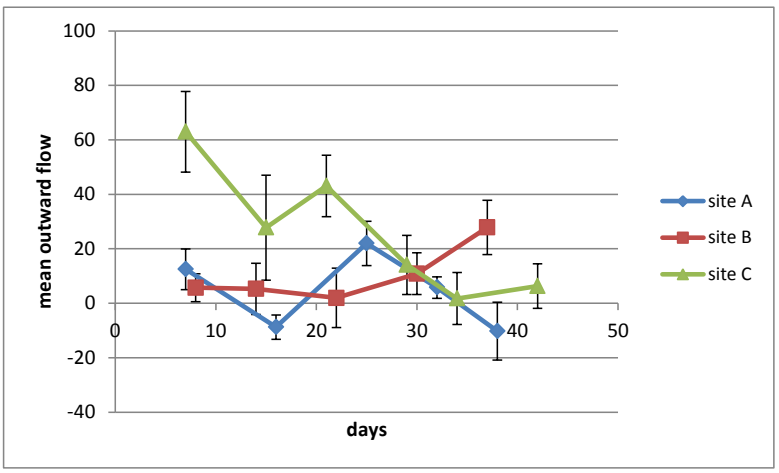
No more than 2 bees were ever observed foraging on the 100m flowering oilseed rape strip assessed in any field and these were only recorded on 1 occasion each on the control and clothianidin treated fields over the 7 week period. Although foraging activity (Figure 4) is likely to be highly dependent on the environmental conditions at the time (Table 3) more detailed analysis of the foraging data showed that there was a significantly different pattern of foraging activity between sites ($F=0.002$) and between sites over time ($F=0.003$) with significant differences ($p<0.05$) between colonies at site C and those at the other two sites in weeks 1-3 after placement on the field. The local climatic conditions during the foraging assessment at each site may in part account for these differences but bumble bees forage at temperatures above 6^{o}C and foraging is reduced at higher temperatures (Corbet et al 1993); no temperatures were close 6^{o}C (Table 3).

Table 3 Timing and conditions during two hours of foraging assessments

	date	Start time	date	Start time	date	Start time	date	Start time	date	Start time
Site A	23/04/12	16:02	02/05/12	12:15	11/05/12	11:00	18/05/12	12:15	24/05/12	13:05
Max/min temp	10.5/12.3		22.7/17.0		11.1/8.8		19.0/12.8		31.5/28.8	
Site B	24/04/12	10:35	30/04/12	11:30	08/05/12	11:00	16/05/12	11:30	23/05/12	10:50
Max/min temp	11.1/9.1		17.2/15.6		15.3/11.4		13.3/11.6		24.9/21.7	
Site C	03/05/12	13:15	11/05/12	15:45	17/05/12	12:55	25/05/12	13:20	30/05/12	14:00
Max/min temp	9.2/9.0		10.2/7.8		14.8/12.7		18.1/17.2		20.6/19.1	

Figure 4 Foraging activity at the colonies over time after placement in the field, total number entering (in) and leaving (out) the colony (in + out) and proportion of total bees observed leaving the colony (number out-number in/total number in + out)



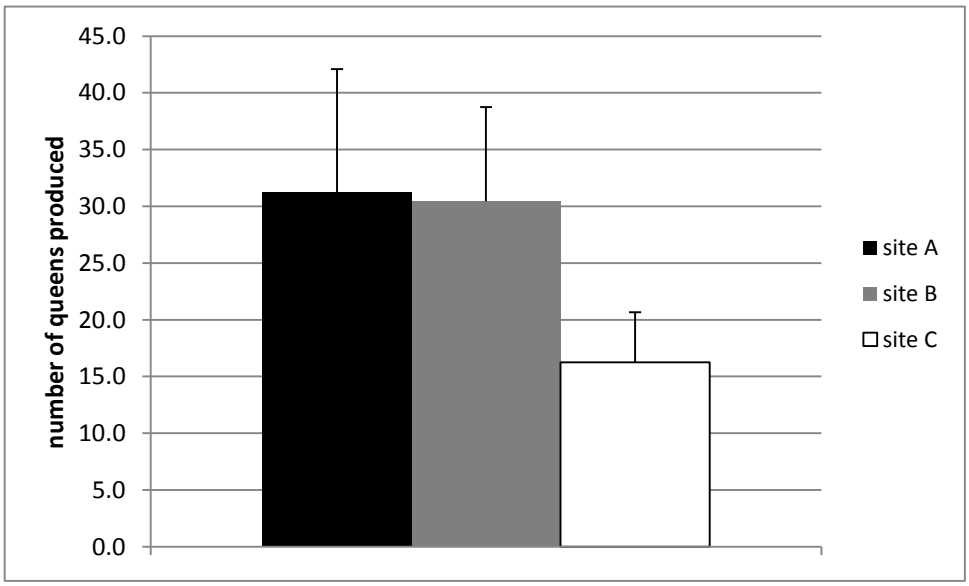


Colony structure

Dissection of the colonies confirmed the lower level of development of the colonies from site C in terms of the numbers of each life stage present (there were no significant effects on the mass of the life stages). These are summarised in Table 4 and the detailed statistical analysis is shown in the Appendix.

The number of queens which had emerged and those present as queen pupae in the colonies are shown in Table 4 and Figure 5. The founding queen was present at the end of the study in 14 of the colonies on site A, 14 of the colonies on site B and 17 of the colonies on site C. In the colonies where the founding queen was absent between 1 and 52 gynes were produced in colonies at site A, between 0 (2 colonies) and 58 queens were produced in colonies at site B and between 0 (1 colony) and 2 queens were produced at site C. The presence of gynes within a colony at the end of the study confirmed the presence of a viable queen within the previous 4 weeks, i.e. the queens may have died in 2 colonies before the start of gyne production at site B and in 1 colony at site C. The differences in the number of gynes produced were not statistically significant even after controlling for initial size using the initial number of workers in each colony. Adjusting for the initial number of workers present in each of the colonies the adjusted total queen numbers were 30.6 (95% confidence limits 18.5-50.7) at site A, 28.7 (95% confidence limits 16.8-48.8) at site B and 17.3 (95% confidence limits 8.5-35.1) at site C. The adjusted total queen pupae were 23.4 (95% confidence limits 13.2-41.4) at site A, 14.6 (95% confidence limits 7.0-30.4) at site B and 5.3 (95% confidence limits 1.5-18.1) at site C.

Figure 5 Numbers of queens produced in bumble bee colonies exposed to flowering oilseed rape grown at sites A, B and C



The number of workers per colony at dissection was significantly lower ($p<0.05$) at site B than at site A. There were no significant differences in the number of workers

between site C and sites A and B. The number of adult drones per colony at dissection was higher in the colonies from site B than in those from sites A and C ($p<0.05$) but colonies from the latter two sites did not differ significantly from each other. Adjusting for the initial number of workers present in each of the colonies the adjusted total drone numbers were 36.5 (95% confidence limits 24.4-54.8) at site A, 64.0 (95% confidence limits 46.9-87.3) at site B and 31.2 (95% confidence limits 19.2-50.5) at site C.The number of drones varied widely between colonies but both drones and workers could exit from the colonies without restrictions (they are of similar size and therefore the doors could not be adapted to prevent drones leaving the colonies) which may have influenced the overall numbers. Adjusting for the initial number of workers present in each of the colonies the adjusted total worker numbers were 58.5 (95% confidence limits 43.0-79.7) at site A, 24.4 (95% confidence limits 14.9-39.9) at site B and 35.1 (95% confidence limits 23.2-53.1) at site C.

The numbers of larvae within the colonies were significantly lower ($p<0.05$) in the colonies from site C than in the colonies from sites A and B; this was true for both younger larvae in multi-occupancy cells and older single larvae. The number of single larvae in colonies from site C was also significantly lower ($p<0.05$) than the number in colonies from site B but there was no significant difference in the numbers of younger larvae in multi-occupancy cells. There was no significant difference between the numbers of larvae (both single and multi-occupancy) in the colonies from site B and site A. Adjusting for the initial number of workers present in each of the colonies the adjusted total single occupancy cell larvae numbers were 39.5 (95% confidence limits 28.2-55.3) at site A, 40.7 (95% confidence limits 28.9-57.2) at site B and 20.7 (95% confidence limits 12.8-33.6) at site C. The adjusted total multi-occupancy cell larvae numbers were 61.5 (95% confidence limits 42.7-88.5) at site A, 36.6 (95% confidence limits 22.3-60.3) at site B and 12.8 (95% confidence limits 5.9-27.7) at site C.

Similarly the number of drone/ worker pupae (at this stage of colony development it is probable most were drones) was significantly lower ($p<0.05$) in the colonies from site C, than those in colonies from sites A and B; there was no significant difference between the colonies at site A and site B. The adjusted total small pupae (drone) numbers were 190.8 (95% confidence limits 150.2-242.3) at site A, 232.9 (95% confidence limits 185.7-292.1) at site B and 68.0 (95% confidence limits 45.1-102.5) at site C.

The number of eggs was significantly greater ($p<0.05$) in the colonies from site B than those at site C but there were no differences between site A and the other two sites. The adjusted total egg numbers were 32.5 (95% confidence limits 18.8-56.2) at site A, 55.1 (95% confidence limits 35.1-86.6) at site B and 12.7 (95% confidence limits 5.5-29.4) at site C.

The number of nectar cups in the colonies from site C and site B were significantly lower than those present in colonies from site A ($p<0.05$) but not significantly different from each other. The adjusted total nectar cup numbers were 31.7 (95%

confidence limits 22.1-45.5) at site A, 4.5 (95% confidence limits 1.7-12.0) at site B and 2.8 (95% confidence limits 0.9-9.3) at site C.

There were no significant differences between the sites in the numbers of pollen cells per colony. Differences in the development of these colonies were reflected in the mass of the colonies after removal of all adults, pupae, larvae and eggs, i.e. wax, nectar and pollen cells only, which was significantly lower ($p<0.05$) in the colonies from site C than those from the other two sites. The adjusted total pollen cell numbers were 3.8 (95% confidence limits 1.8-8.1) at site A, 6.9 (95% confidence limits 3.9-12.4) at site B and 2.5 (95% confidence limits 1.0-6.6) at site C.

Mass of the nest structure after removal of all life stages was significantly lower in colonies from site C than at the other two sites ($p<0.5$). This reflects the differences in mass during development of the colonies. The adjusted mass of the colonies after removal of the bees and brood were 0.793 kg (95% confidence limits 0.738-0.848) at site A, 0.804 kg (95% confidence limits 0.749-0.859) at site B and 0.673 kg (95% confidence limits 0.615-0.731) at site C.

Table 4. Summary data for the colonies placed at each site

Mean number or mass ± SE	Site A	Site B	Site C
Queens (incl. pupae)	31.2 ± 11.1	30.4 ± 8.3	16.2 ± 4.4
Queen pupae	23.7 ± 9.7	15.2 ± 7.5	5.1 ± 1.8
Drones	40.8 ± 13.1[a,b]	77.3 ± 10.7[b]	26.8 ± 5.8[a]
Workers	58.4 ± 11.8[b]	23.9 ± 4.7[a]	36.1 ± 6.8[a,b]
Eggs	34.6 ± 7.7	48.7 ± 19.3	16.8 ± 5.8
Multi-occupancy larvae	61.9 ± 10.8[b]	34.0 ± 9.7[a,b]	14.4 ± 6.4[a]
Single occupancy larvae	40.1 ± 6.9[b]	42.8 ± 6.9[b]	19.7 ± 6.3[a]
Drone/worker pupae	191.2 ± 22.5[b]	235.3 ± 29.1[b]	67.2 ± 10.9[a]
Nectar cells	31.6 ± 9.2[b]	4.3 ± 1.4[a]	3.0 ± 1.0[a]
Pollen cells	3.9 ± 1.0	7.2± 3.2	2.4 ± 1.1
Maximum brood mass increase (kg)	0.574 ± 0.051[b]	0.587 ± 0.062[a]	0.345 ± 0.042[b]
Brood nest mass at colony dissection (kg)	0.797 ± 0.032[b]	0.818 ± 0.029[b]	0.655 ± 0.019[a]
Fitness[1]	103 ± 24	138 ± 21	59 ± 12

[1] fitness = number of drones + 2* (number of queens) (Westphal *et al* 2009)
Treatments with different letters are significantly different (P<0.05) and all analysis was undertaken after adjusting for initial colony size

Palynology

The results of palynological analysis of the pollen sampled from returning foragers or from the colony nest showed that the overall mean contribution of oilseed rape to all pollen returned to the colonies was 26 ± 5.6% at site A, 20 ± 6.8% at site B and 13 ± 5.6% at site C (Fig. 6). Oilseed rape pollen was present in 75%, 55% and 35% of the pollen samples, both in those collected from the colony and those collected directly from bees, at Sites A, B and C respectively (Table 5). Of those samples containing oilseed rape pollen the mean contribution of this source to the total was 35-37% at all sites even though the colonies were sited at the edge of the flowering oilseed rape (Table 5). Figure 6 shows the wide diversity of pollen sources in samples from the site C when compared with the other two sites and this is shown in more detail in Table A4 (Appendix).

Figure 6. Composition of pollen loads or in-colony pollen stores (where corbicular loads were not available) from colonies on site A (4 nest samples 16 corbicular samples), site B (1 nest sample 19 corbicular samples) and site C (11 nest samples 6 corbicular samples)

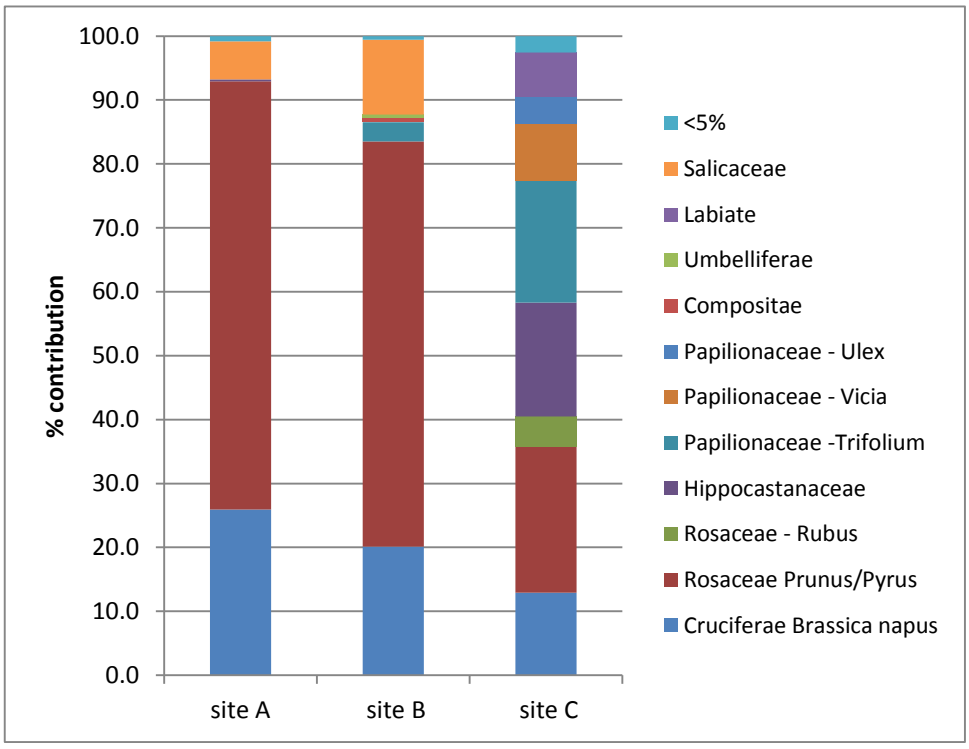


Table 5 Contribution of oilseed rape pollen to samples collected from and stored by bumble bees

	Site A		Site B		Site C	
	no. samples	%	no. samples	%	no. samples	%
Total containing osr pollen	20	75.0	20	55.0	17	35.3
Mean % contribution of osr in those containing osr	34.6 ± 5.9		36.5 ± 9.9		36.7 ± 9.0	
Forager collected containing osr pollen	17	70.6	19	57.9	6	33.3
Forager collected mean % contribution of osr in those containing osr	30.8 ± 5.8		36.5 ± 9.9		20 ± 0	
Stored in-colony containing osr pollen	3	100	1	0.0	11	36.4
Stored in-colony mean % contribution of osr in those containing osr	49.7 ± 17.9		-		45 ± 14.2	

Residue analysis

The results of analysis of the pollen and nectar collected from each colony are shown in Tables A1-A3 (Appendix) and summarized in Table 6, together with the field collected samples (from the confined honeybee colonies). The concentrations of clothianidin detected were close to the limit of detection and lower than reported in pollen and nectar from a clothianidin treated crop (Table 6). However, analysis of thiamethoxam, of which clothianidin is a metabolite, showed levels above the limit of quantitation (LOQ) (Table 6). The residue data from the honeybee collected pollen and nectar was of limited value as there appeared to be carry-over of residues from pollen and nectar due to bees foraging on oilseed rape during the process of

preparing the colonies for the study but provide some information on clothianidin residues in nectar where thiamethoxam resdiues were absent as well as residues of imidacloprid (Table 6).

Residue analysis showed that the bumble bees were collecting pollen and nectar from oilseed rape crops other than those immediately adjacent to the field on which they were placed.

The bumble bee colonies at site A contained mean residues in nectar of 0.885 ± 0.102 µg thiamethoxam/Kg and residues of clothianidin were below the limit of detection (< LOD) (0.025 µg /Kg) – below the limit of quantitation (<LOQ) (0.16 µg /Kg). The residue of clothianidin in pollen was below the LOD (0.5 µg /Kg) but residues of <0.5- 0.730 ± 0.0675 µg thiamethoxam /Kg pollen were detected. There were no detectable residues of imidacloprid in pollen or nectar.

At site B residues of thiamethoxam were detected at 2.397 ± 0.160 µg thiamethoxam /Kg nectar and 0.718 ± 0.058 µg thiamethoxam /Kg pollen. The concentration of clothianidin in nectar from colonies at Site B were from <LOQ- 0.205 ± 0.008 µg/Kg. There were no detectable residues of clothianidin in pollen from Site B. There were no detectable residues of imidacloprid in pollen or nectar.

In the 15 nectar samples available from the bumble bee colonies at site C, 8 contained low but detectable residues of imidacloprid and only 2 contained low but detectable residues of clothianidin). No thiamethoxam was detected in the 5 nectar samples which provided sufficient matrix to be analysed specifically for this component. There were no detectable neonicotinoid residues in any of the 15 pollen samples available.

Residue-based analysis

The summary of the statistical analysis in comparisons of the residue data and the colony mass at the time the samples were taken, the colony mass at the end of the season and the number of queens produced by each colony are shown in Table 7. The data are shown (excluding effects of starting mass and colony size at the start of the exposure phase) in Figures 7A-7C. There is a correlation between the residues of thiamethoxam and clothianidin in nectar (Figure 8) supporting the suggestion that the clothiandin resdieus were primarily present as a metabolite of thiamethoxam.

The non-parametric approach, which makes no assumptions about the distribution of the data, however, these analyses do not incorporate the effects of “Site” and any effect “detected” may be related to the differences between the sites, the initial colony mass or the number of bees at the start of the season (site B showed lower colony mass at weeks 3-4 and higher thiamethoxam levels than the other two sites). The use of parametric approaches are more robust in taking into account both “Site” and initial colony size (mass and number of bees) but may be considered to have a

disadvantage in assuming both the distribution of the data (normal or Poisson) and that a given relationship exists between the dose and the response variable (as well as with the offset variable). Therefore, in assessment of the evidence for relationships the results of both parametric and non-parametric approaches were considered; although the parametric approach is considered more robust. Neither the non-parametric nor the parametric approaches showed evidence of a relationship between queen production and residues of thiamethoxam or clothianidin in nectar or thiamethoxam in pollen.

Table 7 Results of parametric (including and excluding datapoints with high leverage) and non-parametric analysis of correlation of residues with queen production and colony mass

Residue/matrix	**Parametric percentage significant all data (excluding points with high leverage)**	**Non-parametric percentage significant***
Thiamethoxam pollen	20.5 (2.3)	6.6
Thiamethoxam nectar	0	0
Clothianidin nectar	35.9	0
Thiamethoxam pollen	90 (36.3)	100
Thiamethoxam nectar	36.5	100
Clothianidin nectar	0.3	100
Thiamethoxam pollen	74.8 (0)	0.2
Thiamethoxam nectar	0	0
Clothianidin nectar	100	0

*Does not take into account site, colony mass at start of exposure or number of bees at start of exposure

Thiamethoxam in pollen

Based on the non-parametric approach significant relationships were identified between residues in pollen and colony mass at the time of sampling but not at the

end of the study. Using a parametric approach there was evidence of a (negative) relationship between residues of thiamethoxam in pollen and colony mass (both at the time of the sampling of the residues and at the end of the study): there was a significant relationship in 90% and 75% of the 1,000 simulations run respectively. However, further assessment showed that the identified relationships were due to two observations with high leverage on the regression (colony C4 at Site A and colony M7 at site B). This supported by the fact that there was no evidence of any relationship between residues of thiamethoxam in pollen and colony mass (only found significant in a third of cases at weeks 3-4 and no cases at the end of the study) when these datapoints were removed..

Thiamethoxam in nectar

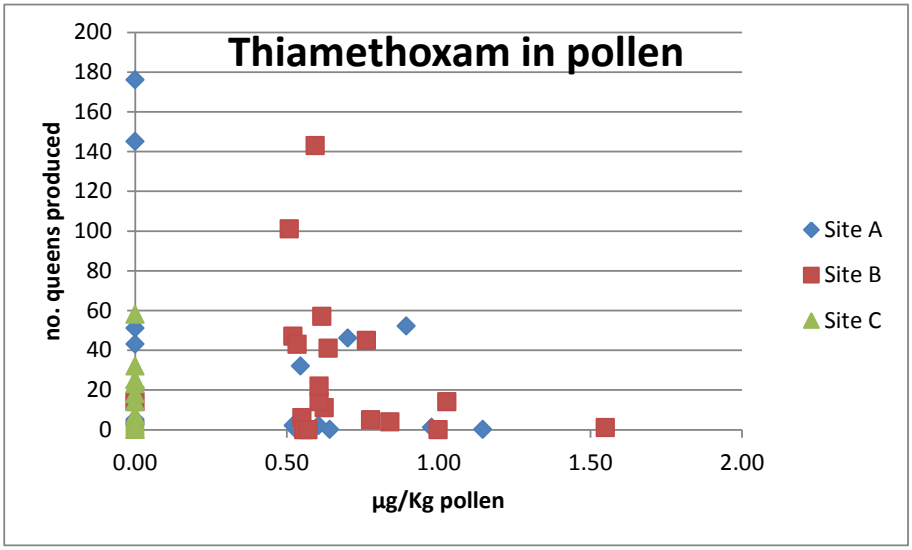
Based on the non-parametric approach a significant relationship was identified between residues in nectar and colony mass at the time of sampling but not at the end of the study. Using a parametric approach there was no strong evidence of any relationship with thiamethoxam residues in nectar and colony mass at the time of sampling (a significant relationship was found in less than 40% of cases) suggesting the relationship identified was site- or starting size-related. The relationship was driven by, again, one point with high leverage on the regression and, in this case, the point with high leverage did not have an exact residues level observed (below the LOD).

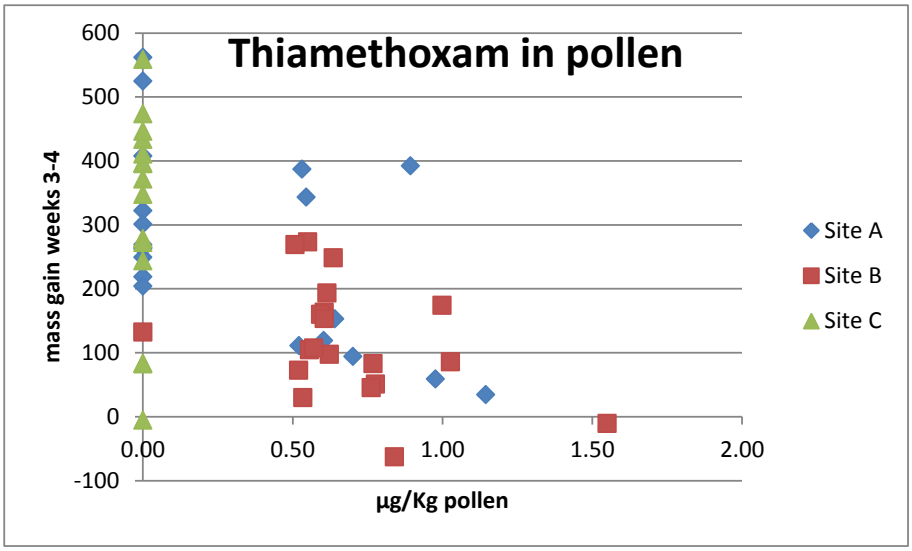
Clothianidin in nectar

Based on the non-parametric approach there was evidence of a relationship between residues in nectar and colony mass at the time of sampling. However, using the parametric approach there was no evidence of any relationship with clothiandin residues in nectar and colony mass at the time of sampling (a significant relationship was found in less than 1% of cases) suggesting the relationship was site- or starting size-related. When the colony mass at the end of the study was assessed there was some evidence of a relationship using the parametric approach but not using the non-parametric approach. This relationship should be treated with caution due to the required assumptions made about the data using parametric approaches, the clear site variation in residues detected and, although there were no observations with high leverage, assessment of the fitted regressions showed that the signal was weak as highlighted in Figure 7C.

.

Figure 7A Comparison of residues of thiamethoxam in pollen with the total number of queens produced per colony and the mass gain of the colony at the time the sample was collected (weeks 3-4) and at the end of the study (weeks 8-9) (values shown as 0 µg/kg are <0.5 µg/kg)





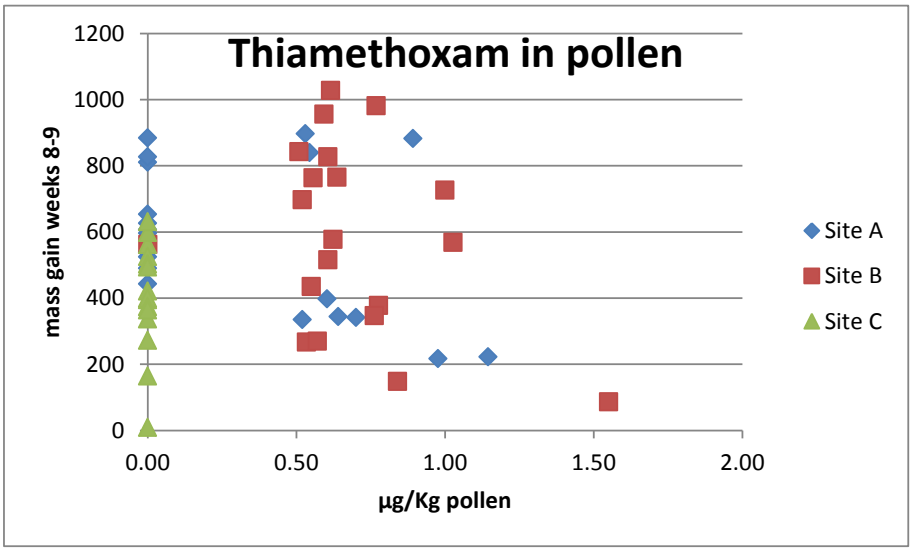


Figure 7B Comparison of residues of thiamethoxam in nectar with the total number of queens produced per colony and the mass gain of the colony at the time the sample was collected (weeks 3-4) and at the end of the study (weeks 8-9) (values shown as 0 µg/kg are <0.025 µg/kg)

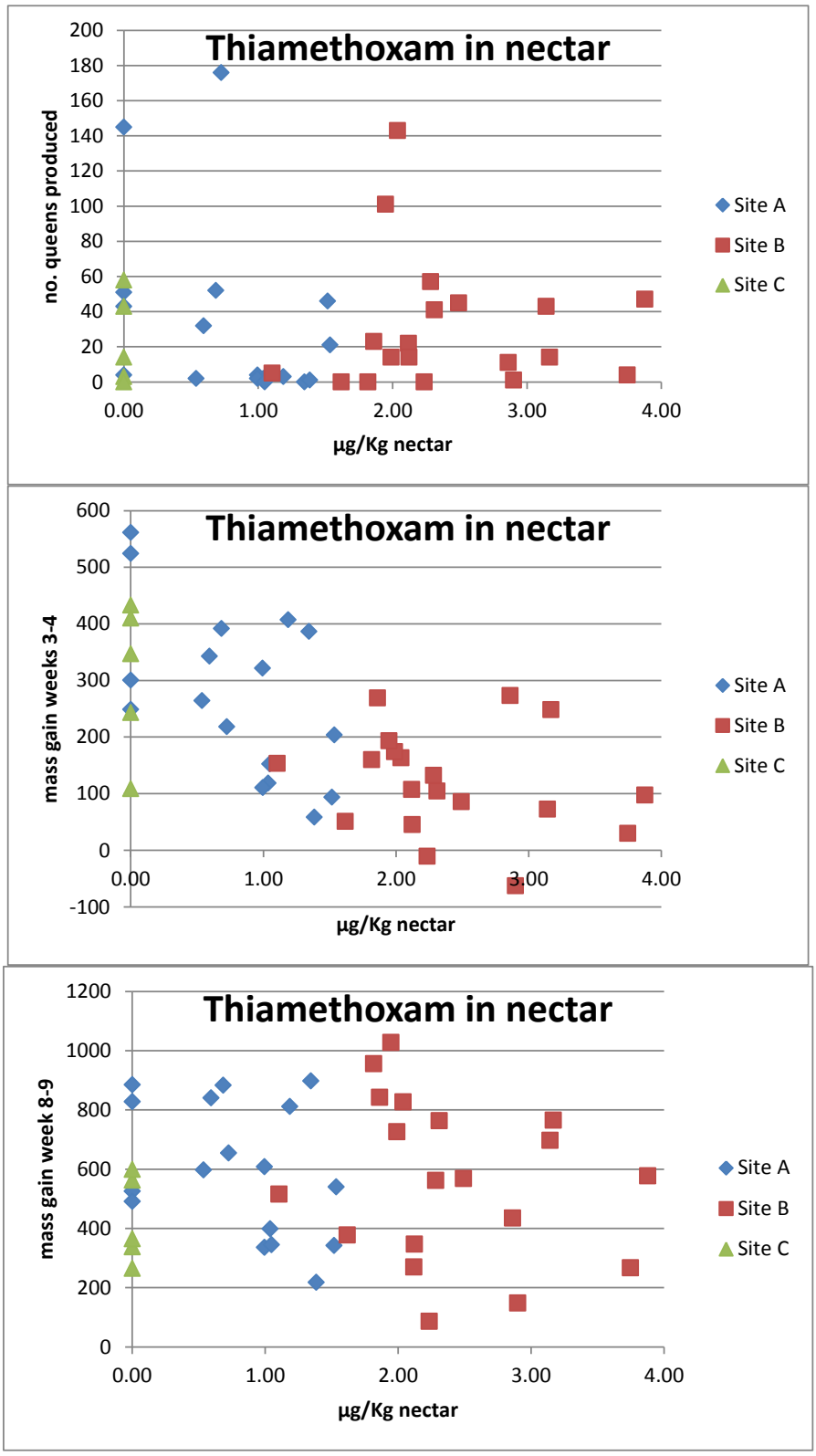


Figure 7C Comparison of residues of clothianidin in nectar with the total number of queens produced per colony and the mass gain of the colony at the time the sample was collected (weeks 3-4) and at the end of the study (weeks 8-9) (values shown as 0 µg/kg are <0.025 µg/kg)

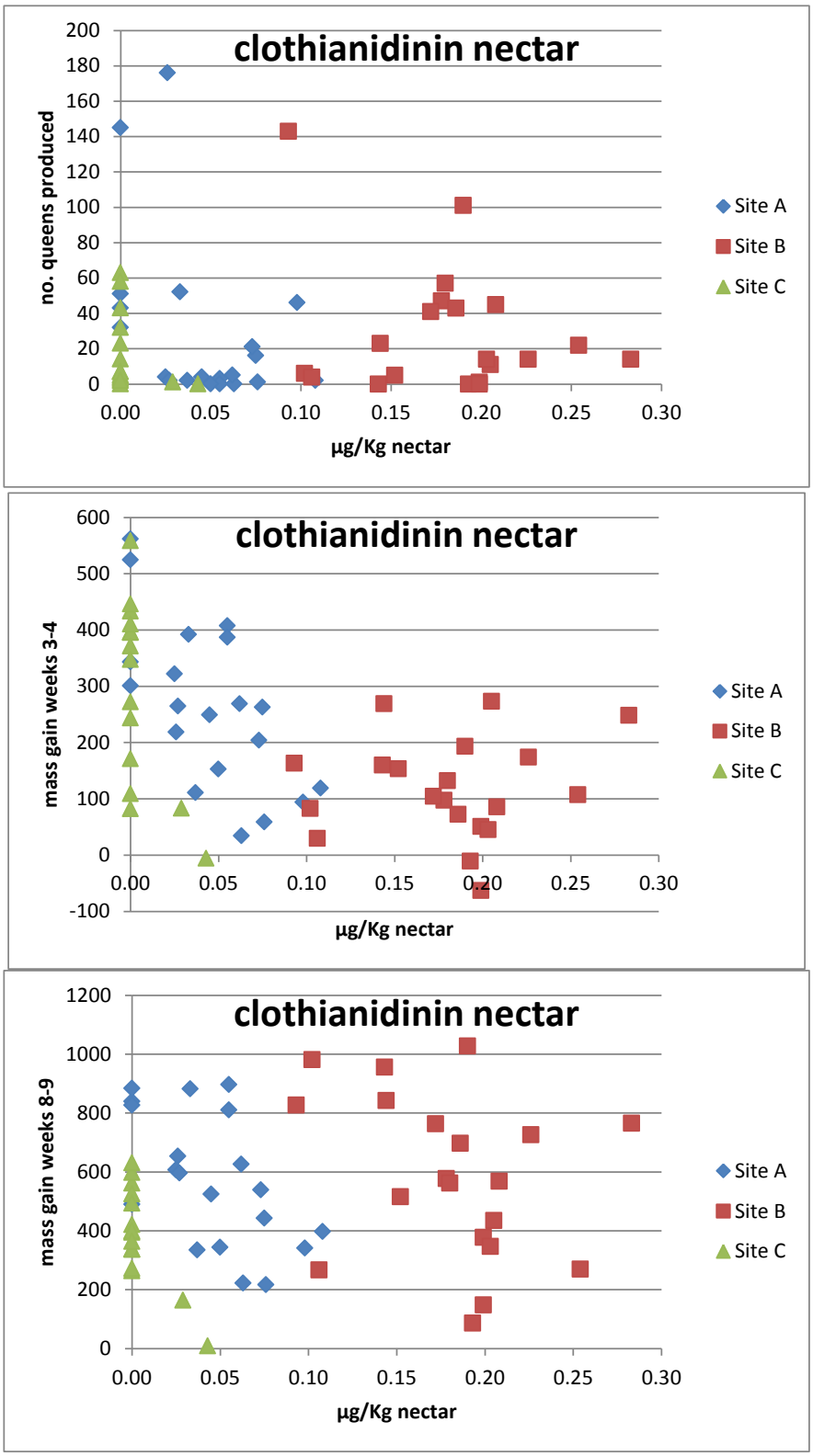


Figure 8. Relationship between clothiandin and thiamethoxam residues in nectar in samples collected from colonies.

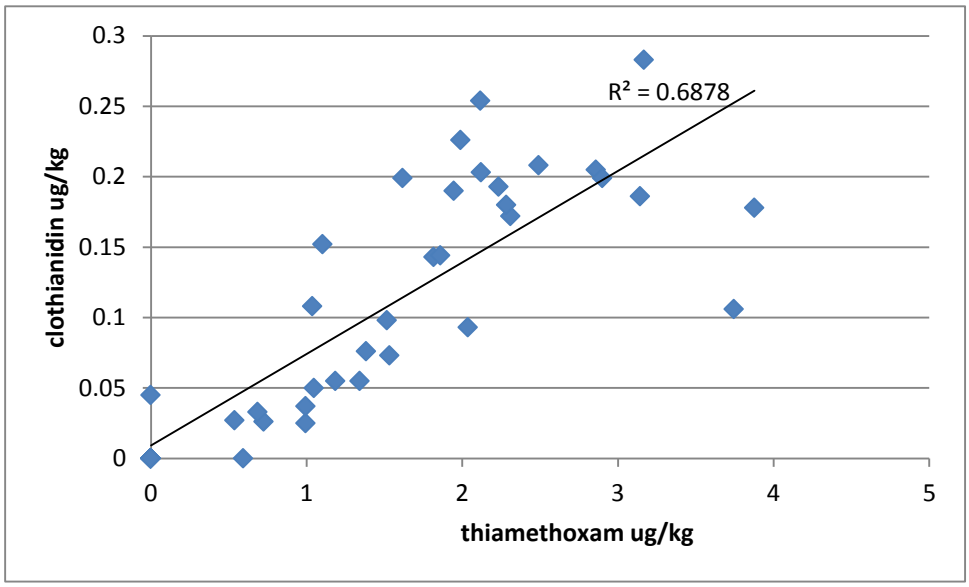


Table 6. Neonicotinoid residues detected in samples from colonies and field samples (from honeybee colonies confined on the crop) and published data (EFSA 2012). LOD was 0.5 µg/Kg in pollen and 0.025-0.05 µg/Kg in nectar. Residues of 5-hydroxy or olefin metabolites of imidacloprid were below the LOD (0.05-0.1 µg/Kg).

Mean ± SE (µg/Kg)		Site A colonies	Site A field honeybee collected samples	Site B colonies	Site B field honeybee collected samples	Site C colonies	Site C field honeybee collected samples	Range reported EFSA (2012)
Thiamethoxam in nectar	>LOQ	0.885 ± 0.102 (n=17)	-	2.397 ± 0.160 (n=19)	<LOD	-	<LOD	1.4-4.6
	>LOD, <LOQ	-		-		-		
	<LOD	-		-		(n=5)		
Thiamethoxam in pollen	>LOQ	0.730 ± 0.075 (n=9)	2.301	0.718±0.058 (n=19)	2.723	-	<LOD	4-8
	>LOD, <LOQ	-		-		-		
	<LOD	n=11		n=1		n=20		
Clothianidin in nectar	>LOQ	-	-	0.205 ± 0.008 (n=14)	0.053	-	0.131	1-8.6 (<1-1 as metabolite of thiamethoxam)
	>LOD, <LOQ	0.057 ± 0.006 (n=17)		0.124 ± 0.010 (n=6)		0.036 ± 0.007 (n=2)		
	<LOD	n=3		-		n=13		
Clothianidin in pollen	>LOQ	-	<LOD	-	0.718	-	<LOD	1-4 (<1-3 as metabolite of thiamethoxam)
	>LOD. <LOQ	-		-		-		
	<LOD	n=20		n=20		n=20		
Imidacloprid in nectar	>LOQ	-	-	-	0.450	-	0.133	<LOQ (5)
	>LOD. <LOQ	-		-		0.061 ±0.009 (n=8)		
	<LOD	n=15		n=15		n=7		

Mean ± SE (µg/Kg)		Site A colonies	Site A field honeybee collected samples	Site B colonies	Site B field honeybee collected samples	Site C colonies	Site C field honeybee collected samples	Range reported EFSA (2012)
Imidacloprid in pollen	>LOQ	-	<LOD	-	<LOD	-	<LOD	2-<LOQ (10)
	>LOD. <LOQ	-		-		-		
	<LOD	n=20		n=20		n=20		

5 Discussion

The study tested the hypothesis that exposure of bumble bee colonies placed in the vicinity of crops treated with neonicotinoids had no major effect on the health of the colonies. As stated in the introduction the study was limited by the lack of replication and the variation between sites, e.g. temperature, available alternative forage and timing of flowering, as well as the exposure to neonicotinoids on all three sites due to the wide foraging range of the colonies. However, the study has shown that bumble bee colonies remained viable and productive in the presence of the neonicotinoid pesticides under these field conditions. Given these underlying problems with conducting tightly controlled experiments in field conditions in which the crops are not isolated, the quantitative results suggest that there could be a reduced performance when the colonies were sited next to a field of flowering oilseed rape grown from imidacloprid treated seed (site C) in an area with a high density of treated oilseed rape and with limited non-oilseed rape forage available. However, this was difficult to detect conclusively because of the effects of various uncontrolled co-variates, e.g. the delay in the timing of the initiation of the study at site C due to late flowering of the crop, the availability of alternative forage, and the level of exposure to imidacloprid and clothiandin.

The detection of varying levels of neonicotinoid residues in colonies at all sites reflected the wide foraging areas of bumble bees (Osborne et al 2008) and allowed assessment of the relationship between the residues detected in the colonies and both colony mass (at the time of sampling and at the end of the study) and the number of queens produced. There was a significant relationship identified in relation to colony mass (thiamethoxam in pollen) and was due to a very small number of observations (2 colonies, 3.6%) with high leverage. These “outliers” suggest further data are required to determine the wider prevalence of these higher levels of colony exposure (>1μg thiamethoxam/kg pollen) and, if warranted, the consequent effects identified, e.g. in isolated field studies. Overall, there were no consistent relationships between neonicotinoid residues in pollen and nectar with colony mass at the time of sampling or at the end of the study or with the numbers of queens produced. Within this context, the study did not show conclusively that exposure to neonicotinoids used within a normal agricultural setting had major effects on bumble bee colonies.

Colony mass

The mean maximum colony mass gains shown in the colonies at sites A and B (>570g) were greater than those reported by Whitehorn *et al*. (2012) for control colonies after 2 weeks of laboratory dosing followed by a 6 week period of free-

foraging in July (<300g). There are limited data available for natural or commercial *B. terrestris* colony development in the field but the colonies at both sites A and B gained similar mass to commercial *B. terrestris terrestris* colonies placed in suburban gardens and on farms with and without conservation measures (Goulson *et al.* 2002).

The colonies at site C gained a maximum mass greater than that reported by Whitehorn *et al*. (2012) for control commercially-sourced colonies dosed in the laboratory but less than those reported by Goulson *et al.* (2002) for colonies sited on farms. This suggests that confinement of colonies for laboratory dosing prior to free foraging has an adverse impact on overall mass gain when compared with similar colonies in the field. It also suggests that the environment at site C limited the development of the colonies at this site.

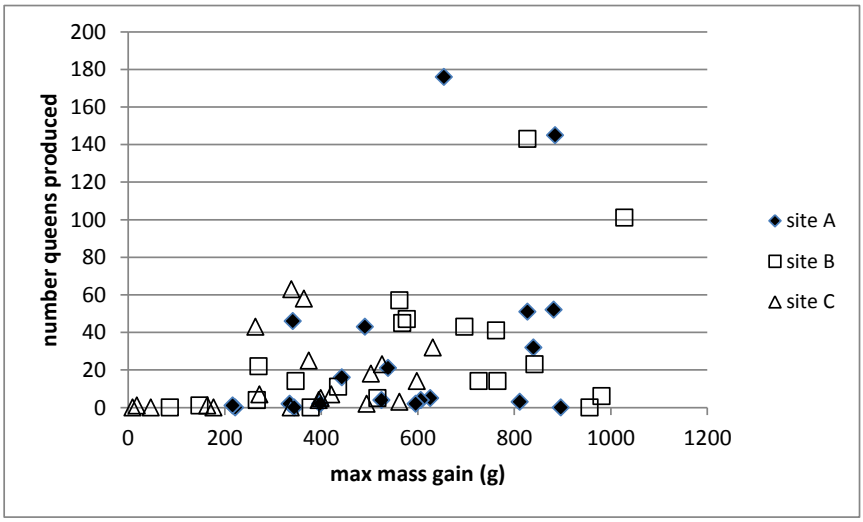
Colony composition

The variation between colonies in the number of queens (gynes) produced is consistent with other studies (Goulson *et al.* 2002; Whitehorn *et al.* 2012). The mean number of queens produced in the colonies on sites A and B was comparable with those previously reported for *B. terrestris terrestris* in suburban gardens and on farms with conservation measures (Goulson *et al* 2002).

The mean number of queens produced in the colonies at site C was not statistically different from those at other sites. The colonies had reached their full potential by the end of the field phase of the study: the colonies were declining as shown by their loss in mass and the number of larvae remaining within the colonies, and thus with the potential to develop into further queen pupae, were significantly lower than at the other two sites. Thus the delay in the start of the field phase at site C, with the colonies placed two weeks later than on sites A and B, did not affect the duration of colony development. Production of queens on site C (16.2 ± 4.4) was comparable to that reported for control colonies and significantly greater than following imidacloprid dosing in the laboratory (13.7 ± 5.7 and 2.0 ± 1.3 respectively) (Whitehorn *et al* 2012). The numbers of queens produced by colonies has been linked to both the amount of forage available to the colony and the presence within the colony of sufficient workers to forage (Shykoff and Mullar 1995). The availability of forage is important as queen production is predicated on the larvae receiving additional food at a key stage in their development and once the production of reproductives is initiated no further significant numbers of workers are produced. The mass of the colony can be used as an indicator of both food availability and worker foraging activity (Goulson *et al* 2002, Westphal *et al* 2009, Whitehorn *et al* 2012) but showed no clear relationship with queen production (Figure 9) highlighting the importance of other factors in colony decision making (Shykoff and Muller 1995, Benton 2006). Queen production in the field is highly variable (Shykoff and Muller 1995) and it is

unclear how it relates to population maintenance and growth particularly in extrapolating between species with varying colony size.

Figure 9 Relationship between colony mass and queen production, in bumble bee colonies and between thiamethoxam residues detected (total µg/Kg in pollen and nectar) and mass and queen production on sites A, B and C



Foraging behaviour

B. terrestris are reported to forage over a 1-2 Km radius from their colony in arable environments (Osborne *et al* 2008) although some foragers disperse over a wider area (Benton 2006). The palynology analysis supported the view that many of the bumble bees did not forage on the oilseed rape crops that were adjacent to the colonies and at site C foraged to a lesser extent on the neighbouring oilseed rape crops than at the other two sites. *B. terrestris* is known to extend foraging beyond the monocultures present close to the colony (Osborne *et al* 2008; Goulson *et al* 2002).

The wide foraging area is demonstrated at site A where no neonicotinoids had been used on any field within 1km in the current or previous crop and on site B where the bees foraged on thiamethoxam treated oilseed rape over 900m away rather than on the adjacent clothiandin treated fields. There is evidence that varieties of oilseed rape vary in nectar production and that the production of nectar by the crop varies with microclimate (Farkas and Zajarcz 2007) with production being greatest in cloudy, warm conditions. This may in part explain the use of oilseed rape crops beyond those immediately adjacent to the colonies.

Exposure to neonicotinoids in each treatment

The presence of residues of thiamethoxam in pollen and nectar sampled from the bumble bee colonies sited next to untreated crops at site A demonstrates that they were also foraging on thiamethoxam treated crops within their foraging range.

Clothianidin was probably present in nectar as a metabolite rather than collected from plants grown from clothiandin treated seed as it was present at only 6% of the thiamethoxam residue. Based on an overall percentage contribution of oilseed rape pollen to the total of 26% and a mean residue of 0.471 µg thiamethoxam /Kg pollen (taking all residues below the LOD as LOD/2) this suggests the residue at source was 1.8 µg thiamethoxam /Kg pollen. This is below the expected residue in pollen from a thiamethoxam treated crop (Table 6) and suggests residues were diluted by colonies foraging on both untreated and treated oilseed rape crops.These residues demonstrate the difficulties in identifying true reference sites for these types of studies if sites cannot be identified as truly isolated from other flowering crops. The presence of thiamethoxam treated seed in the close vicinity can be excluded as the farmer used only home-saved untreated seed on the farm and immediate neighbouring farms did not grow oilseed rape.

At site B, with all adjacent oilseed rape crops treated with clothianidin, the residue data identified that the colonies sited on this field were actually foraging on crops treated with thiamethoxam (the nearest was 900m-1Km away) with the residues in the colonies detected up to 3.9 µg thiamethoxam/Kg nectar and 1.6 µg thiamethoxam/Kg pollen. There were no detectable residues of clothianidin in pollen and residues of clothianidin in nectar suggest that it was as a metabolite of thiamethoxam (the mean residue was only 7% of that of thiamethoxam). The EFSA (2012) review identified residues from a clothianidin treated oilseed rape crop of 1-8.6 µg clothianidin/Kg nectar and 1-4 µg clothianidin/Kg pollen.

The residues detected in pollen and nectar collected from the colonies on site C, next to a crop grown from imidacloprid treated seed, suggested very limited foraging on the treated crop or on the neighbouring clothianidin treated oilseed rape crops. The pollen residue data are consistent with the low number of samples (35%) in which oilseed rape pollen was detected. Unfortunately there are very limited published data with which to compare expected imidacloprid residue levels, e.g. <5 - 10 µg imidacloprid/Kg nectar and 2- <10 µg imidacloprid/Kg pollen (EFSA 2012). The palynology and the lower maximum mass gain of the colonies support the observation that the oilseed rape was not a significant source of forage for these colonies and also suggests that they sought other sources of pollen to a greater extent than the colonies on the other two sites. It is well established that bumble bees use more diverse pollen sources than honeybees even when there is a large area of a single source available (Osborne *et al* 2008). Site C provided apparently less readily accessible alternative forage when compared to the other two sites, both of which had flowering blackthorn hedges immediately adjacent to the colonies. The suggestion that the reduced mass gain was an adverse effect of imidacloprid on foraging (Gill *et al* 2012) cannot be excluded but the palynology and residues identified in the colony suggested oilseed rape was not a primary forage source.

The importance of diverse forage sources to bumble bees has been raised by a number of authors particularly in relation to oilseed rape cropping (Westphal *et al* 2009) and the results presented here may suggest this extends to the effects of some chemicals and possibly varietal differences on the attractiveness of crops. The absence of significant imidacloprid and clothiandin residues suggests it is not a direct toxic effect on the foragers, as suggested by Gill et al (2012), but further work is required to demonstrate whether there is avoidance of imidacloprid and/or clothianidin treated oilseed rape by bumble bees; reduced intake in the absence of choice has been demonstrated for bumble bee microcolonies exposed to imidacloprid above 1µg/L in the laboratory (Laycock et al 2012).

The common variable at all the sites was exposure to residues of thiamethoxam in pollen and nectar. However, when the relationship between these and colony mass at 3-4 weeks, at the end of the study and with queen production were assessed, within the limitations of this study, no clear and consistent relationships were identified (Figures 7A and 7B). There were also no consistent relationships with residues of clothianidin in nectar (Figure 7C). Wider assessments are, however, warranted to determine the representativeness of the residues detected in this study of the range of exposures of bumble bee colonies in the field.

The study did not show that neonicotinoids used within a normal agricultural setting have a major effect on bumble bee colonies. Even when there was a large area of oilseed rape present it is clear that bumble bees have diverse foraging strategies. Exposure to treated crops was diluted by foraging on a range of sources of pollen and residues of imidacloprid and clothiandin in nectar were either close to or below the LOQ or were diluted by foraging on a wider variety of plants. Pollen and nectar were clearly collected from thiamethoxam treated crops but there were no major effects of exposure to thiamethoxam or clothianidin residues on queen production or mass gain at the time the residues were taken or at the end of the study.

This study was not a formal statistical test of the hypothesis that neonicotinoid insecticides reduce the health of bumble bee colonies. Nevertheless, were neonicotinoids in pollen and nectar from treated oilseed rape to be a major source of field mortality and morbidity to bumblebee colonies, we would have expected to find a greater contribution of insecticide residues from the nearby treated crops and for there to have been a clear relationship between observed neonicotinoid levels and measures of colony success. The absence of these effects is reassuring but not definitive. The study underlines the importance of taking care in extrapolating laboratory toxicology studies to the field, as well as the great need of further studies under natural conditions.

6 References

Benton, T. (2006) Bumblebees. The New Naturalist Library, Collins, London

Cook, S.M., Awmack, C.S., Murray, D.A. & Williams, I.H. (2003). Are honey bees' foraging preferences affected by pollen amino acid composition? *Ecological Entomology* **28**, 622-627.

Corbet, S.A., Fussell, M., Ake, R., Fraser, A., Gunson, C., Savage, A. and Smith, K. (1993). Temperature and pollinating activity of social bees. *Ecol*. *Entomol*. **18**, 17-30.

EFSA (2013a) European Food Safety Authority; Conclusion on the peer review of the pesticide risk assessment for bees for the active substance imidacloprid. EFSA Journal 2013;11(1):3068. [55 pp.] doi:10.2903/j.efsa.2013.3068. Available online: www.efsa.europa.eu/efsajournal

EFSA (2013b) European Food Safety Authority; Conclusion on the peer review of the pesticide risk assessment for bees for the active substance clothianidin. EFSA Journal 2013;11(1):3066. [58 pp.] doi:10.2903/j.efsa.2013.3066. Available online: www.efsa.europa.eu/efsajournal

EFSA (2013c) European Food Safety Authority; Conclusion on the peer review of the pesticide risk assessment for bees for the active substance thiamethoxam. EFSA Journal 2013;11(1):3067. [68 pp.] doi:10.2903/j.efsa.2013.3067. Available online: www.efsa.europa.eu/efsajournal

EPPO (2010) Efficacy evaluation of Plant Protection Products PP1/170(4) Side Effects on honeybees *Bulletin OEPP/EPPO Bulletin* **40**, 313–319

Farkas, A and Zajacz, E (2007) Nectar Production for the Hungarian Honey Industry *The European Journal of Plant Science and Biotechnology* **1**(2), 125-151

Goulson D, Hughes, W.O.H., Derwent, L.C., and Stout, J.C. (2002) Colony Growth of the Bumblebee, Bombus terrestris, in Improved and ConventionalAgricultural and Suburban Habitats, *Oecologia,* **130** (2), 267-273

Gill, R.J., Ramos-Rodriguez, O., and Raine, N.E. (2012) Combined pesticide exposure severely affects individual- and colony-level traits in bees *Nature* doi:10.1038/nature11585

Laycock I, Lenthall KM, Barratt AT and Cresswell JE (2012) Effects of imidacloprid, a neonicotinoid pesticide, on reproduction in worker bumble bees (Bombus terrestris). *Ecotoxicology* **21**(7), 1937–1945

Mommaerts V, Reynders S, Boulet J, Besard L, Sterk G and Smagghe G (2010). Risk assessment for side-effects of neonicotinoids against bumble bees with and without impairing foraging behaviour. *Ecotoxicology*, **19**, 207-215.

Osborne J.L., Martin, A.P., Carreck, N.L., Swain, J.L., Knight, M.E., Goulson, D., Hale, R.J. and Sanderson, R.A, (2008) Bumblebee flight distances in relation to the forage landscape *Journal of Animal Ecology* **77**, 406–415

Sawyer, R. (1981) Pollen Identification for Beekeepers Ed Pickard R University College Cardiff Press.

Schoning R, Schmuck R (2003) Analytical determination of imidacloprid and relevant metabolite residues by LC MS/MS Bulletin of Insectology 56 (1): 41-50

Shykoff, J.A., Muller, C.B. (1995) Reproductive Decisions in Bumble-Bee Colonies: The Influence of Worker Mortality in Bombus terrestris (Hymenoptera, Apidae)*Functional Ecology* , Vol. 9, No. 1 106-112

Tasei JN, Lerin J and Ripault G (2000). Sub-lethal effects of imidacloprid on bumblebees, *Bombus terrestris* (Hymenoptera: Apidae), during a laboratory feeding test. *Pest Management Science*, **56**, 784-788.

Thompson, H.M. and Hunt, L.V. (1999) Extrapolating from honeybees to bumble bees in pesticide risk assessment *Ecotoxicology* **8** (3), 147-166

Waddington, K.D. (1979). Flight patterns of foraging bees relative to density of artificial flowers and distribution of nectar. *Oecologia* **44**, 199-204.

Westphal, C., Steffan-Dewenter, I. and Tscharntke, T. (2009) Mass flowering oilseed rape improves early colony growth but not sexual reproduction of bumblebees *Journal of Applied Ecology*, **46**, 187–193

Whitehorn, P.R., O'Connor, S., Wackers, F.L., and Goulson, D. (2012) Neonicotinoid pesticide reduces bumble bee colony growth and queen production Science 29 March 2012 / 10.1126/science.1215025

7 Appendix

Table A1. Residues in samples of nectar and pollen from bumble bee colonies on site A (neither the olefin or the 5-hydroxy imidacloprid metabolite were above the LOD (0.1-0.2 µg/Kg) (C1-C20 are colony numbers) (honeybee data are shown in Table 6). *Founding queen absent

Recovery Corrected Residue (µg/kg)

			Nectar			Pollen		
Colony	Queens present	Max wt gain (g)	Thiamethoxam	Clothianidin	Imidacloprid	Thiamethoxam	Clothianidin	Imidacloprid
C1	17	443	-	0.075	<0.025	<0.5	<0.5	<0.5
C2	47	341	1.517	0.098	<0.025	0.701	<0.5	<0.5
C3	177	654	0.724	0.026	<0.025	<0.5	<0.5	<0.5
C4	1	222	-	0.063	<0.025	1.145	<0.5	<0.5
C5	53	882	0.685	0.033	<0.025	0.893	<0.5	<0.5
C6	3	398	1.037	0.108	<0.025	0.604	<0.5	<0.5
C7	4*	811	1.186	0.055	<0.025	<0.5	<0.5	<0.5
C8	6	626	-	0.062	<0.025	<0.5	<0.5	<0.5
C9	33	840	0.593	<0.025	<0.025	0.545	<0.5	<0.5
C10	1*	897	1.343	0.055	<0.025	0.531	<0.5	<0.5
C11	5*	525	<0.5	0.045	<0.025	<0.5	<0.5	<0.5
C12	5	607	0.994	0.025	<0.025	<0.5	<0.5	<0.5
C13	3	335	0.995	0.037	<0.025	0.521	<0.5	<0.5
C14	146	884	<0.5	<0.025	<0.025	<0.5	<0.5	<0.5
C15	3	596	0.537	0.027	<0.025	<0.5	<0.5	<0.5
C16	44*	490	<0.5	<0.025	<0.025	<0.5	<0.5	<0.5
C17	2	217	1.384	0.076	<0.025	0.977	<0.5	<0.5
C18	1	344	1.048	0.050	<0.025	0.641	<0.5	<0.5
C19	52*	827	<0.5	<0.025	<0.025	<0.5	<0.5	<0.5
C20	22*	539	1.534	0.073	<0.025	<0.5	<0.5	<0.5

Table A2. Residues in samples of nectar and pollen from bumble bee colonies on site B (neither the olefin or the 5-hydroxy imidacloprid metabolite were above the LOD (0.1-0.2 µg/Kg) (M1-M20 are colony numbers) (honeybee data are shown in Table 6). *Founding queen absent

Recovery Corrected Residue (µg/kg)

			Nectar			Pollen		
Colony	Queens present	Max wt gain (g)	Thiamethoxam	Clothianidin	Imidacloprid	Thiamethoxam	Clothianidin	Imidacloprid
M1	7	981	-	0.102	<0.025	0.768	<0.5	<0.5
M2	12	435	2.860	0.205	<0.025	0.550	<0.5	<0.5
M3	48	577	3.877	0.178	<0.025	0.623	<0.5	<0.5
M4	44*	697	3.143	0.186	<0.025	0.520	<0.5	<0.5
M5	5	267	3.747	0.106	<0.025	0.534	<0.5	<0.5
M6	2	148	2.900	0.199	<0.025	0.840	<0.5	<0.5
M7	1	86	2.235	0.193	<0.025	1.550	<0.5	<0.5
M8	42	763	2.310	0.172	<0.025	0.556	<0.5	<0.5
M9	15*	765	3.168	0.283	<0.025	0.636	<0.5	<0.5
M10	144	827	2.037	0.093	<0.025	0.606	<0.5	<0.5
M11	0*	956	1.817	0.143	<0.05	0.593	<0.5	<0.5
M12	23*	270	2.118	0.254	<0.05	0.570	<0.5	<0.5
M13	6	516	1.104	0.152	<0.05	0.606	<0.5	<0.5
M14	0*	378	1.618	0.199	<0.05	0.776	<0.5	<0.5
M15	15	726	1.990	0.226	<0.05	0.999	<0.5	<0.5
M16	46	568	2.492	0.208	<0.025	1.027	<0.5	<0.5
M17	15	347	2.122	0.203	<0.025	0.762	<0.5	<0.5
M18	58*	562	2.283	0.180	<0.025	<0.5	<0.5	<0.5
M19	102	1028	1.947	0.190	<0.025	0.615	<0.5	<0.5
M20	24	842	1.860	0.144	<0.025	0.508	<0.5	<0.5

Table A3. Residues in samples of nectar and pollen from bumble bee colonies on site C. (neither the olefin or the 5-hydroxy imidacloprid metabolite were above the LOD (0.1-0.2 µg/Kg) (I1-I20 are colony numbers) where no data are shown no sample was available (honeybee data are shown in Table 6). *Founding queen absent

Recovery Corrected Residue (µg/kg)

			Nectar			Pollen		
Colony	Queens produced	Max wt gain (g)	Thiamethoxam	Clothianidin	Imidacloprid	Thiamethoxam	Clothianidin	Imidacloprid
I1	5	395	-	<0.025	0.080	<0.5	<0.5	<0.5
I2	8	421	-	<0.025	<0.025	<0.5	<0.5	<0.5
I3	6	399	-	<0.025	<0.025	<0.5	<0.5	<0.5
I4	1	177	-	-	-	-	-	-
I5	4	562	<0.05	<0.025	<0.025	<0.5	<0.5	<0.5
I6	24	526	-	<0.025	<0.025	<0.5	<0.5	<0.5
I7	1	9	-	0.043	0.089	<0.5	<0.5	<0.5
I8	19	503	-	-	-	<0.5	<0.5	<0.5
I9	64	338	-	<0.025	0.039	-	-	-
I10	15	598	<0.05	<0.025	<0.025	<0.5	<0.5	<0.5
I11	26	374	-	-	-	<0.5	<0.5	<0.5
I12	44	264	<0.05	<0.025	<0.025	-	-	-
I13	8	272	-	<0.025	0.084	<0.5	<0.5	<0.5
I14	2	164	-	0.029	0.083	<0.5	<0.5	<0.5
I15	0*	47	-	-	-	-	-	-
I16	33	631	-	<0.025	0.057	<0.5	<0.5	<0.5
I17	1*	337	<0.05	<0.025	0.029	<0.5	<0.5	<0.5
I18	2	18	-	-	-	-	-	-
I19	3*	494	-	<0.025	<0.025	<0.5	<0.5	<0.5
I20	58	364	<0.05	<0.025	0.030	<0.5	<0.5	<0.5

Table A4 Identification of pollen sources c= corbicular load, n = in colony

colony	source	Cruciferae *Brassica napus*	Rosaceae *Prunus/ pyrus*	*rubus*	Salicaceae *Salix caprea*	Hippocastanaceae *Aesculus hippocastanum*	Paplionaceae *Trifolium*	*Vicia*	*Ulex*	Compositae *sunflower/aster*	Umbelliferae *anthriscus*	Labiate	<5%
c1	c	50	50	0	0	0	0	0	0	0	0	0	0
c2	c	0	98	0	0	0	0	0	0	0	0	0	2
c3	c	50	50	0	0	0	0	0	0	0	0	0	0
c5	c	70	30	0	0	0	0	0	0	0	0	0	0
c6	c	0	96	0	0	0	0	0	0	0	0	0	4
c7	c	50	50	0	0	0	0	0	0	0	0	0	0
c8	c	20	80	0	0	0	0	0	0	0	0	0	0
c9	c	10	30	0	60	0	0	0	0	0	0	0	0
c10	c	20	80	0	0	0	0	0	0	0	0	0	0
c11	c	20	80	0	0	0	0	0	0	0	0	0	0
c12	c	10	30	0	60	0	0	0	0	0	0	0	0
c14	c	0	96	0	0	0	0	0	0	0	0	0	4
c15	c	0	99	0	0	0	0	0	0	0	0	0	1
c16	c	10	90	0	0	0	0	0	0	0	0	0	0
c18	c	40	60	0	0	0	0	0	0	0	0	0	0
c19	c	0	99	0	0	0	0	0	0	0	0	0	1
c20	c	20	80	0	0	0	0	0	0	0	0	0	0
c4	n	65	35	0	0	0	0	0	0	0	0	0	0
c13	n	14	80	0	0	6	0	0	0	0	0	0	0
c17	n	70	26	0	0	0	0	0	0	0	0	0	4
mean		26.0	66.95	0	6	0.3	0	0	0	0	0	0	0.80
se		5.6	6.0	0	4.1	0.3	0	0	0	0	0	0	
max		70	99	0	60	6	0	0	0	0	0	0	

colony	source	Cruciferae *Brassica napus*	Rosaceae *Prunus/ pyrus*	*rubus*	Salicaceae *Salix caprea*	Hippocastanaceae *Aesculus hippocastanum*	Paplionaceae *Trifolium*	*Vicia*	*Ulex*	Compositae *sunflower/aster*	Umbelliferae *anthriscus*	Labiate	<5%
m1	c	5	45	0	50	0	0	0	0	0	0	0	0
m2	c	50	50	0	0	0	0	0	0	0	0	0	0
m3	c	70	30	0	0	0	0	0	0	0	0	0	0
m4	c	0	98	0	0	0	0	0	0	0	0	0	2
m5	c	0	99	0	0	0	0	0	0	0	0	0	1
m6	c	0	99	0	0	0	0	0	0	0	0	0	1
m7	c	0	99	0	0	0	0	0	0	0	0	0	1
m8	c	10	90	0	0	0	0	0	0	0	0	0	0
m9	c	0	0	0	15	0	60	0	0	12	12	0	1
m10	c	20	80	0	0	0	0	0	0	0	0	0	0
m11	c	10	90	0	0	0	0	0	0	0	0	0	0
m12	c	0	0	0	100	0	0	0	0	0	0	0	0
m13	c	80	20	0	0	0	0	0	0	0	0	0	0
m14	c	10	90	0	0	0	0	0	0	0	0	0	0
m15	c	0	30	0	70	0	0	0	0	0	0	0	0
m16	c	50	50	0	0	0	0	0	0	0	0	0	0
m18	c	5	95	0	0	0	0	0	0	0	0	0	0
m19	c	92	8	0	0	0	0	0	0	0	0	0	0
m20	c	0	97	0	0	0	0	0	0	0	0	0	3
m17	n	0	98	0	0	0	0	0	0	0	0	0	2
mean		20.1	63.4	0	11.8	0	3.0	0	0	0.6	0.6	0	0.55
se		6.8	8.4	0	6.3	0	3.0	0	0	0.6	0.6	0	
max		92	99	0	100	0	60	0	0	12	12	0	

colony	source	Cruciferae *Brassica napus*	Rosaceae *Prunus/ pyrus*	*rubus*	Salicaceae *Salix caprea*	Hippocastanaceae *Aesculus hippocastanum*	Paplionaceae *Trifolium*	*Vicia*	*Ulex*	Compositae *sunflower/aster*	Umbelliferae *anthriscus*	Labiate	<5%
i7		-											
i15		-											
i18		-											
i2	c	20	80	0	0	0	0	0	0	0	0	0	0
i3	c	0	20	80	0	0	0	0	0	0	0	0	0
i6	c	0	100	0	0	0	0	0	0	0	0	0	0
i8	c	20	20	0	0	0	60	0	0	0	0	0	0
i13	c	0	7	0	0	0	85	7	0	0	0	0	1
i20	c	0	50	0	0	0	50	0	0	0	0	0	0
i1	n	0	16	0	0	0	80	0	0	0	0	0	4
i4	n	0	20	0	0	40	0	30	0	0	0	10	0
i5	n	0	0	0	0	70	0	30	0	0	0	0	0
i9	n	0	0	0	0	85	10	0	0	0	0	0	5
i10	n	0	0	0	0	15	0	20	0	0	0	60	5
i11	n	0	10	0	0	0	0	20	70	0	0	0	0
i12	n	45	45	0	0	0	0	0	0	0	0	0	10
i14	n	70	15	0	0	0	10	0	0	0	0	0	5
i16	n	0	0	0	0	48	0	0	0	0	0	48	4
i17	n	5	5	0	0	45	0	45	0	0	0	0	0
i19	n	60	0	0	0	0	30	0	0	0	0	0	10
mean		12.9	22.8	4.7	0	17.8	19.1	8.9	4.1	0	0	6.9	2.59
se		5.6	7.2	4.7	0	6.9	7.3	3.5	4.1	0	0	4.4	
max		70	100	80	0	85	85	45	70	0	0	60	

Figure A1 Plan Site B (Modesto = clothianidin treated oilseed rape)

Site B

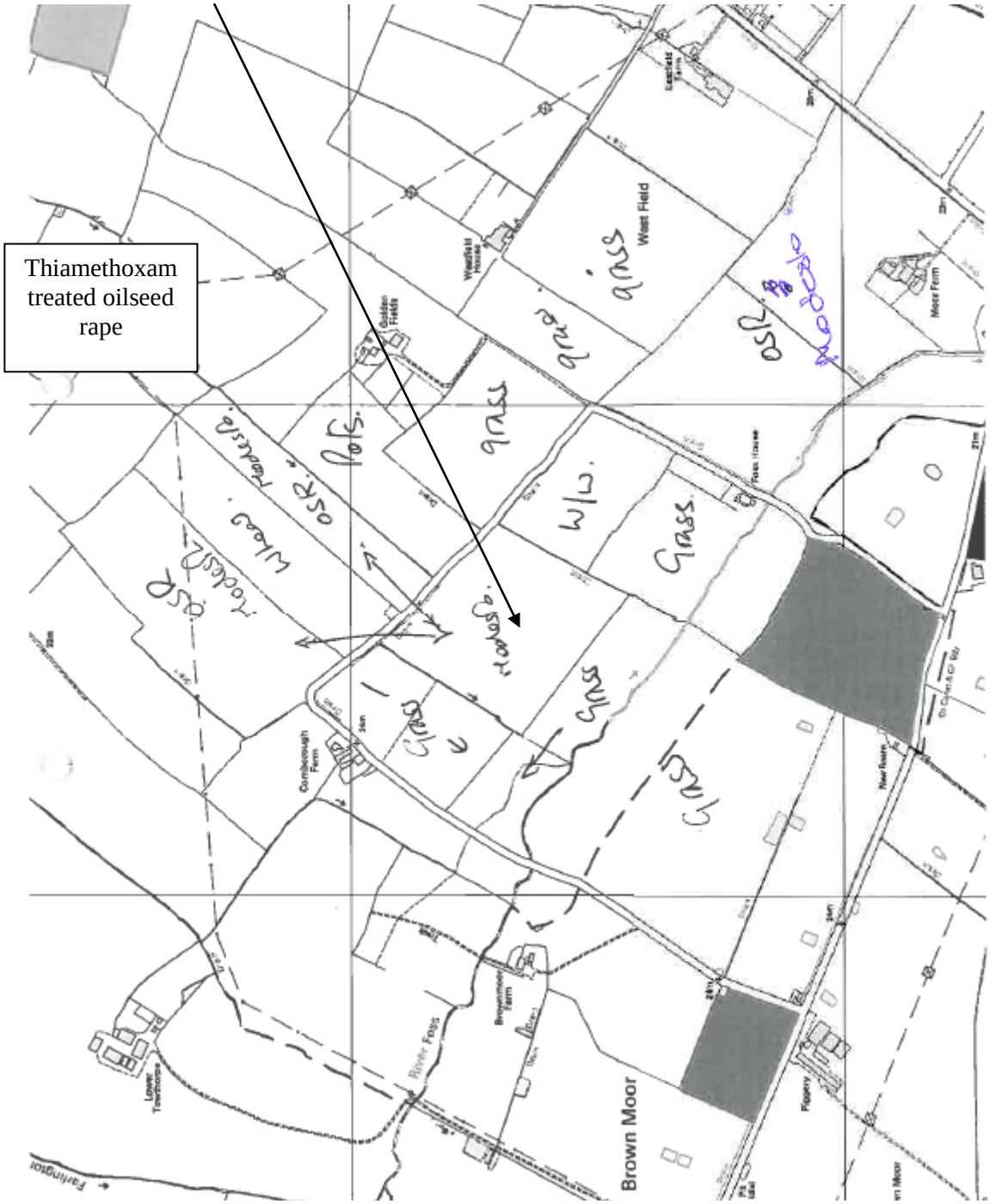


Figure A2 Showing honeybee tent in place on site B

Figure A3 Showing bumble bee colony in outer box

Figure A4 Plan Site C

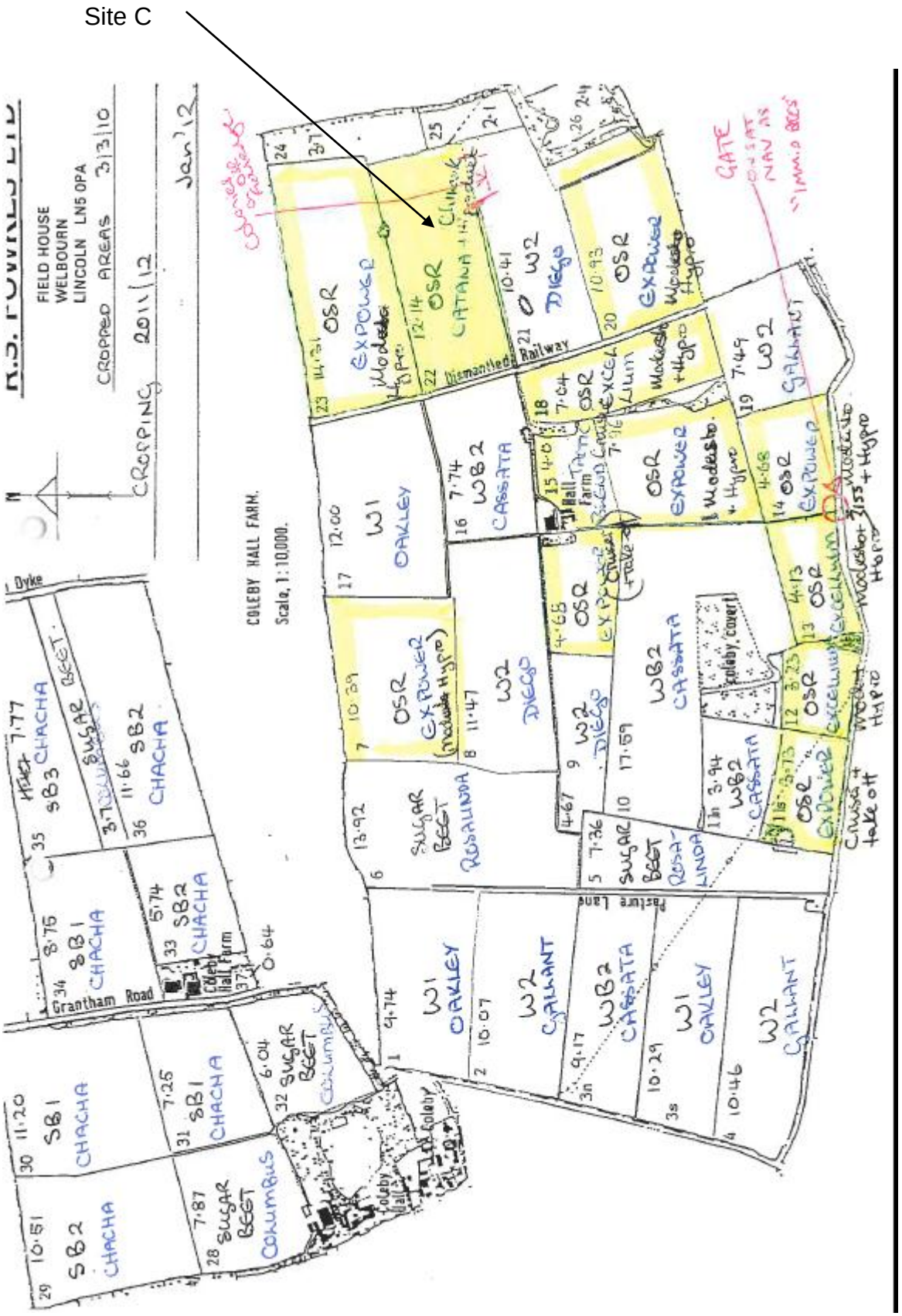


Figure A5 Site C

Figure A6 Plan Site A

Site A

Figure A7 Site A

» Weight

Message: units are assumed to be in the same order at each time.

Box's tests for symmetry of the covariance matrix

Chi-square 328.22 on 53 degrees of freedom: probability <0.001

F-test 6.06 on 53 and 3091 degrees of freedom: probability <0.001

Greenhouse-Geisser epsilon

epsilon 0.2143

Analysis of variance (adjusted for covariate)

Variate: Weight
Covariate: Bee_number

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	cov.ef.	F pr.
Subject stratum							
Treatment	2		3107723.	1553861.	11.54	0.91	<.001
Covariate	1		462206.	462206.	3.43		0.069
Residual	56		7541816.	134675.	11.93	1.04	
Subject.Time stratum							
d.f. correction factor 0.2143							
Time	9		17528027.	1947559.	172.46	1.00	<.001
Time.Treatment	16	(2)	1709541.	106846.	9.46	1.00	<.001
Residual	474	(39)	5352680.	11293.		1.00	
Total	558	(41)	35928469.				

(d.f. are multiplied by the correction factors before calculating F probabilities)

Information summary

All terms orthogonal, none aliased.

Message: the following units have large residuals.

Subject 21 Time T8	324.4	s.e. 94.5
Subject 39 Time T7	303.1	s.e. 94.5

Covariate regressions

Variate: Weight

Covariate	coefficient	s.e.
Subject stratum		
Bee_number	3.9	2.10

Combined estimates

Bee_number	3.9	2.10

Tables of means (adjusted for covariate)

Variate: Weight
Covariate: Bee_number

Grand mean 812.5

Time	T0	T1	T2	T3	T4	T5	T6
	567.6	593.3	632.6	682.0	831.3	960.3	1041.8

Time	T7	T8	T9
	1012.3	947.5	855.8

Treatment	Control	Modesto	Chinook
	892.7	834.1	710.6

Time	Treatment	Control	Modesto	Chinook
T0		576.2	564.5	562.3
T1		608.3	593.5	578.2
T2		692.0	611.3	594.6
T3		839.7	683.0	523.3
T4		1009.7	786.2	697.9
T5		1087.4	969.7	823.8
T6		1129.6	1119.2	876.4
T7		1034.6	1117.0	885.3
T8		1015.3	1025.7	801.5
T9		934.2	870.3	763.0

Least significant differences of means (5% level)

Table	Time	Treatment	Time Treatment
rep.	60	200	20
l.s.d.	46.98	77.18	117.92
d.f.	101.59	56	131.22
Except when comparing means with the same level(s) of			
Treatment			81.65
d.f.			101.59

(Not adjusted for missing values)
Correction factors have been applied to residual d.f.(see analysis-of-variance table for details)

» Total number of queens

Regression analysis

Response variate:	Queens
Distribution:	Poisson
Link function:	Log
Fitted terms:	Constant + Bee_number + Treatment

Summary of analysis

Source	d.f.	deviance	mean deviance	deviance ratio	approx F pr.
Regression	3	142.	47.20	1.22	0.312
Residual	56	2170.	38.76		
Total	59	2312.	39.19		

Dispersion parameter is estimated to be 38.8 from the residual deviance.

Message: the following units have large standardized residuals.

Unit	Response	Residual
3	177.00	3.04
14	146.00	2.62

Message: the following units have high leverage.

Unit	Response	Leverage
12	5.00	0.478
21	7.00	0.158

Estimates of parameters

Parameter	estimate	s.e.	t(56)	t pr.	antilog of estimate
Constant	3.110	0.500	6.22	<.001	22.42
Bee_number	0.0151	0.0191	0.79	0.433	1.015
Treatment Modesto	-0.066	0.357	-0.18	0.854	0.9363
Treatment Chinook	-0.572	0.441	-1.30	0.200	0.5645

Message: s.e.s are based on the residual deviance.

Parameters for factors are differences compared with the reference level:

Factor	Reference level
Treatment	Control

□ 95% confidence intervals

Treatments	Low	Predi	Upp
Control	18.48	30.62	50.73
Modesto	16.83	28.67	48.84
Chinook	8.52	17.29	35.09

» Various assessments

»» Queen numbers (emerged)

Regression analysis

Response variate:	Queen_num
Distribution:	Poisson
Link function:	Log
Fitted terms:	Constant + Bee_number + Treatment

Summary of analysis

Source	d.f.	deviance	mean deviance	deviance ratio	approx F pr.
Regression	3	41.2	13.72	1.36	0.265
Residual	56	565.3	10.09		
Total	59	606.5	10.28		

Dispersion parameter is estimated to be 10.1 from the residual deviance.

Message: the following units have large standardized residuals.

Unit	Response	Residual
36	43.00	2.88
49	52.00	3.54

Message: the error variance does not appear to be constant; large responses are more variable than small responses.

Message: the following units have high leverage.

Unit	Response	Leverage
12	1.00	0.240
21	4.00	0.184

Estimates of parameters

Parameter	estimate	s.e.	t(56)	t pr.	antilog of estimate
Constant	1.177	0.663	1.78	0.081	3.243
Bee_number	0.0001	0.0250	0.00	0.997	1.000
Treatment Modesto	0.856	0.476	1.80	0.078	2.353
Treatment Chinook	0.753	0.494	1.53	0.133	2.124

Message: s.e.s are based on the residual deviance.

Parameters for factors are differences compared with the reference level:

Factor	Reference level
Treatment	Control

□ 95% confidence intervals

Treatments	Low	Predi	Upp
Control	1.47	3.25	7.16
Modesto	4.43	7.65	13.19
Chinook	3.86	6.90	12.36

»» Worker numbers

Regression analysis

Response variate: Workers_num
Distribution: Poisson
Link function: Log
Fitted terms: Constant + Bee_number + Treatment

Summary of analysis

Source	d.f.	deviance	mean deviance	deviance ratio	approx F pr.
Regression	3	313.	104.24	3.76	0.016
Residual	56	1551.	27.70		
Total	59	1864.	31.59		

Dispersion parameter is estimated to be 27.7 from the residual deviance.

Message: the following units have large standardized residuals.

Unit	Response	Residual
10	224.00	3.17

Message: the following units have high leverage.

Unit	Response	Leverage
12	6.00	0.416

Estimates of parameters

Parameter	estimate	s.e.	t(56)	t pr.	antilog of estimate
Constant	4.204	0.348	12.08	<.001	66.94
Bee_number	-0.0065	0.0150	-0.44	0.665	0.9935
Treatment Modesto	-0.875	0.289	-3.02	0.004	0.4168
Treatment Chinook	-0.511	0.258	-1.98	0.053	0.5997

Message: s.e.s are based on the residual deviance.

Parameters for factors are differences compared with the reference level:

Factor	Reference level
Treatment	Control

95% confidence intervals

Treatments	Low	Predi	Upp
Control	42.99	58.52	79.65
Modesto	14.91	24.39	39.90
Chinook	23.20	35.09	53.08

Pairwise testing: homogeneous groups in tpro, P=0.05

Control 4.069 . b
Modesto 3.194 a .
Chinook 3.558 a b

»» Drone numbers

Regression analysis

Response variate: Drones_num
Distribution: Poisson
Link function: Log
Fitted terms: Constant + Bee_number + Treatment

Summary of analysis

Source	d.f.	deviance	mean deviance	deviance ratio	approx F pr.
Regression	3	882.	294.10	9.64	<.001
Residual	56	1709.	30.52		
Total	59	2591.	43.92		

Dispersion parameter is estimated to be 30.5 from the residual deviance.

Message: the following units have large standardized residuals.

Unit	Response	Residual
12	258.00	2.63
31	161.00	2.50

Message: the following units have high leverage.

Unit	Response	Leverage
12	258.00	0.534
21	126.00	0.226

Estimates of parameters

Parameter	estimate	s.e.	t(56)	t pr.	antilog of estimate
Constant	2.767	0.353	7.84	<.001	15.92
Bee_number	0.0403	0.0114	3.54	<.001	1.041
Treatment Modesto	0.560	0.239	2.34	0.023	1.751
Treatment Chinook	-0.160	0.322	-0.50	0.622	0.8525

Message: s.e.s are based on the residual deviance.

Parameters for factors are differences compared with the reference level:

Factor	Reference level
Treatment	Control

□ 95% confidence intervals

Treatments	Low	Predi	Upp
Control	24.36	36.54	54.81
Modesto	46.86	63.98	87.34
Chinook	19.21	31.15	50.53

Pairwise testing: homogeneous groups in tpro, P=0.05

Control	3.598	a	.
Modesto	4.159	.	b
Chinook	3.439	a	.

»» Egg numbers

Regression analysis

Response variate: eggs_num
Distribution: Poisson
Link function: Log
Fitted terms: Constant + Bee_number + Treatment

Summary of analysis

Source	d.f.	deviance	mean deviance	deviance ratio	approx F pr.
Regression	3	571.	190.42	3.89	0.014
Residual	55	2691.	48.92		
Total	58	3262.	56.24		

Dispersion parameter is estimated to be 48.9 from the residual deviance.

Message: the following units have large standardized residuals.

Unit	Response	Residual
35	339.00	4.17

Message: the following units have high leverage.

Unit	Response	Leverage
31	232.00	0.216

Estimates of parameters

Parameter	estimate	s.e.	t(55)	t pr.	antilog of estimate
Constant	4.653	0.557	8.35	<.001	104.9
Bee_number	-0.0566	0.0268	-2.12	0.039	0.9449
Treatment Modesto	0.529	0.359	1.47	0.147	1.696
Treatment Chinook	-0.939	0.481	-1.95	0.056	0.3909

Message: s.e.s are based on the residual deviance.

Parameters for factors are differences compared with the reference level:

Factor	Reference level
Treatment	Control

□ 95% confidence intervals

Treatments	Low	Predi	Upp
Control	18.76	32.47	56.19
Modesto	35.06	55.08	86.55
Chinook	5.49	12.69	29.35

Pairwise testing: homogeneous groups in tpro, P=0.05

Control 3.480 a b
Modesto 4.009 . b
Chinook 2.541 a .

»» Larvae multi-occupancy numbers

Regression analysis

Response variate:	larvae_multi_num
Distribution:	Poisson
Link function:	Log
Fitted terms:	Constant + Bee_number + Treatment

Summary of analysis

Source	d.f.	deviance	mean deviance	deviance ratio	approx F pr.
Regression	3	718.	239.22	5.92	0.001
Residual	56	2263.	40.41		
Total	59	2980.	50.51		

Dispersion parameter is estimated to be 40.4 from the residual deviance.

Message: the following units have large standardized residuals.

Unit	Response	Residual
27	172.00	2.51
42	114.00	2.69

Message: the following units have high leverage.

Unit	Response	Leverage
12	0.00	0.305

Estimates of parameters

Parameter	estimate	s.e.	t(56)	t pr.	antilog of estimate
Constant	4.652	0.431	10.78	<.001	104.7
Bee_number	-0.0258	0.0200	-1.29	0.201	0.9745
Treatment Modesto	-0.518	0.311	-1.67	0.101	0.5955
Treatment Chinook	-1.569	0.422	-3.72	<.001	0.2082

Message: s.e.s are based on the residual deviance.

Parameters for factors are differences compared with the reference level:

Factor	Reference level
Treatment	Control

□ 95% confidence intervals

Treatments	Low	Predi	Upp
Control	42.74	61.52	88.54
Modesto	22.28	36.63	60.25
Chinook	5.91	12.81	27.73

Pairwise testing: homogeneous groups in tpro, P=0.05

Control 4.119 . b
Modesto 3.601 . b
Chinook 2.550 a .

»» Larvae single-occupancy numbers

Regression analysis

Response variate:	larvae_single_num
Distribution:	Poisson
Link function:	Log
Fitted terms:	Constant + Bee_number + Treatment

Summary of analysis

Source	d.f.	deviance	mean deviance	deviance ratio	approx F pr.
Regression	3	227.	75.68	3.40	0.024
Residual	56	1246.	22.25		
Total	59	1473.	24.97		

Dispersion parameter is estimated to be 22.2 from the residual deviance.

Message: the following units have large standardized residuals.

Unit	Response	Residual
8	126.00	2.46
21	155.00	2.63
42	123.00	3.39

Message: the following units have high leverage.

Unit	Response	Leverage
12	20.00	0.452
21	155.00	0.163

Estimates of parameters

Parameter	estimate	s.e.	t(56)	t pr.	antilog of estimate
Constant	3.411	0.332	10.27	<.001	30.28
Bee_number	0.0129	0.0128	1.01	0.319	1.013
Treatment Modesto	0.031	0.234	0.13	0.896	1.031
Treatment Chinook	-0.644	0.298	-2.16	0.035	0.5250

Message: s.e.s are based on the residual deviance.

Parameters for factors are differences compared with the reference level:

Factor	Reference level
Treatment	Control

□ 95% confidence intervals

Treatments	Low	Predi	Upp
Control	28.18	39.47	55.27
Modesto	28.94	40.70	57.23
Chinook	12.77	20.72	33.62

Pairwise testing: homogeneous groups in tpro, P=0.05

Control	3.676	.	b
Modesto	3.706	.	b
Chinook	3.031	a	.

»» Large pupae numbers

Regression analysis

Response variate: pupae_large_num
Distribution: Poisson
Link function: Log
Fitted terms: Constant + Bee_number + Treatment

Summary of analysis

Source	d.f.	deviance	mean deviance	deviance ratio	approx F pr.
Regression	3	269.	89.83	2.40	0.077
Residual	56	2096.	37.42		
Total	59	2365.	40.09		

Dispersion parameter is estimated to be 37.4 from the residual deviance.

Message: the following units have large standardized residuals.

Unit	Response	Residual
3	170.00	3.31
30	127.00	2.92

Message: the following units have high leverage.

Unit	Response	Leverage
12	0.00	0.517

Estimates of parameters

Parameter	estimate	s.e.	t(56)	t pr.	antilog of estimate
Constant	2.946	0.607	4.86	<.001	19.03
Bee_number	0.0101	0.0242	0.42	0.678	1.010
Treatment Modesto	-0.472	0.454	-1.04	0.303	0.6239
Treatment Chinook	-1.493	0.684	-2.18	0.033	0.2247

Message: s.e.s are based on the residual deviance.

Parameters for factors are differences compared with the reference level:

Factor	Reference level
Treatment	Control

□ 95% confidence intervals

Treatments	Low	Predi	Upp
Control	13.28	23.44	41.35
Modesto	7.04	14.62	30.36
Chinook	1.53	5.27	18.11

»» Small pupae numbers

Regression analysis

Response variate:	pupae_small_num
Distribution:	Poisson
Link function:	Log
Fitted terms:	Constant + Bee_number + Treatment

Summary of analysis

Source	d.f.	deviance	mean deviance	deviance ratio	approx F pr.
Regression	3	2109.	702.94	12.98	<.001
Residual	56	3033.	54.16		
Total	59	5142.	87.15		

Dispersion parameter is estimated to be 54.2 from the residual deviance.

Message: the following units have high leverage.

Unit	Response	Leverage
12	224.00	0.380
21	515.00	0.168

Estimates of parameters

Parameter	estimate	s.e.	t(56)	t pr.	antilog of estimate
Constant	5.194	0.238	21.86	<.001	180.1
Bee_number	0.00279	0.00955	0.29	0.772	1.003
Treatment Modesto	0.199	0.162	1.23	0.225	1.221
Treatment Chinook	-1.032	0.238	-4.33	<.001	0.3564

Message: s.e.s are based on the residual deviance.

Parameters for factors are differences compared with the reference level:

Factor	Reference level
Treatment	Control

□ 95% confidence intervals

Treatments	Low	Predi	Upp
Control	150.22	190.78	242.29
Modesto	185.68	232.87	292.05
Chinook	45.13	68.00	102.46

Pairwise testing: homogeneous groups in tpro, P=0.05

Control	5.251	.	b
Modesto	5.450	.	b
Chinook	4.219	a	.

»» Nectar cell numbers

Regression analysis

Response variate: nectar_cells
Distribution: Poisson
Link function: Log
Fitted terms: Constant + Bee_number + Treatment

Summary of analysis

Source	d.f.	deviance	mean deviance	deviance ratio	approx F pr.
Regression	3	769.	256.45	12.50	<.001
Residual	56	1149.	20.51		
Total	59	1918.	32.51		

Dispersion parameter is estimated to be 20.5 from the residual deviance.

Message: the following units have large standardized residuals.

Unit	Response	Residual
1	99.00	2.45
8	134.00	3.00
16	126.00	2.81

Message: the error variance does not appear to be constant; large responses are more variable than small responses.

Message: the following units have high leverage.

Unit	Response	Leverage
12	0.00	0.504

Estimates of parameters

Parameter	estimate	s.e.	t(56)	t pr.	antilog of estimate
Constant	3.681	0.463	7.95	<.001	39.70
Bee_number	-0.0109	0.0208	-0.53	0.601	0.9891
Treatment Modesto	-1.961	0.524	-3.74	<.001	0.1407
Treatment Chinook	-2.422	0.623	-3.89	<.001	0.08872

Message: s.e.s are based on the residual deviance.

Parameters for factors are differences compared with the reference level:

Factor	Reference level
Treatment	Control

□ 95% confidence intervals

Treatments	Low	Predi	Upp
Control	22.10	31.70	45.46
Modesto	1.66	4.46	11.95
Chinook	0.85	2.81	9.29

Pairwise testing: homogeneous groups in tpro, P=0.05

Control	3.456	.	b
Modesto	1.495	a	.
Chinook	1.034	a	.

»» Pollen cell numbers

Regression analysis

Response variate:	pollen_cells
Distribution:	Poisson
Link function:	Log
Fitted terms:	Constant + Bee_number + Treatment

Summary of analysis

Source	d.f.	deviance	mean deviance	deviance ratio	approx F pr.
Regression	3	54.6	18.22	1.70	0.178
Residual	56	600.7	10.73		
Total	59	655.3	11.11		

Dispersion parameter is estimated to be 10.7 from the residual deviance.

Message: the following units have large standardized residuals.

Unit	Response	Residual
24	56.00	3.72

Message: the following units have high leverage.

Unit	Response	Leverage
12	0.00	0.357
21	9.00	0.199

Estimates of parameters

Parameter	estimate	s.e.	t(56)	t pr.	antilog of estimate
Constant	1.124	0.682	1.65	0.105	3.077
Bee_number	0.0103	0.0257	0.40	0.690	1.010
Treatment Modesto	0.598	0.467	1.28	0.205	1.818
Treatment Chinook	-0.418	0.619	-0.68	0.502	0.6581

Message: s.e.s are based on the residual deviance.

Parameters for factors are differences compared with the reference level:

Factor	Reference level
Treatment	Control

□ 95% confidence intervals

Treatments	Low	Predi	Upp
Control	1.79	3.81	8.07
Modesto	3.85	6.92	12.43
Chinook	0.95	2.50	6.60

»» Weight bees and brood

Regression analysis

Response variate: weight_bees_brood
Fitted terms: Constant + Bee_number + Treatment

Summary of analysis

Source	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	3	345891.	115297.	8.19	<.001
Residual	54	760026.	14075.		
Total	57	1105917.	19402.		

Percentage variance accounted for 27.5
Standard error of observations is estimated to be 119.

Message: the following units have large standardized residuals.

Unit	Response	Residual
5	1083.	2.69

Message: the error variance does not appear to be constant: intermediate responses are more variable than small or large responses.

Message: the following units have high leverage.

Unit	Response	Leverage
12	771.	0.360

Estimates of parameters

Parameter	estimate	s.e.	t(54)	t pr.
Constant	712.4	54.4	13.10	<.001
Bee_number	3.89	2.17	1.79	0.079
Treatment Modesto	11.0	38.4	0.29	0.775
Treatment Chinook	-119.9	40.4	-2.97	0.004

Parameters for factors are differences compared with the reference level:

Factor	Reference level
Treatment	Control

□ 95% confidence intervals

Treatments	Low	Predi	Upp
Control	738.3	793.1	847.8
Modesto	748.8	804.1	859.4
Chinook	615.0	673.1	731.3

Pairwise testing: homogeneous groups in tpro, P=0.05

Control 793.1 . b
Modesto 804.1 . b
Chinook 673.1 a .

» Flying activity

Message: units are assumed to be in the same order at each time.

Box's tests for symmetry of the covariance matrix

Chi-square 22.38 on 13 degrees of freedom: probability 0.050

F-test 1.72 on 13 and 32230 degrees of freedom: probability 0.050

Greenhouse-Geisser epsilon

epsilon 0.9021

Analysis of variance (adjusted for covariate)

Variate: Ratio
Covariate: Bee_number

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	cov.ef.	F pr.
Subject stratum							
Treatment	2		0.89920	0.44960	7.23	0.91	0.002
Covariate	1		0.00442	0.00442	0.07		0.791
Residual	56		3.48211	0.06218	1.34	0.98	
Subject.Time stratum							
d.f. correction factor 0.9021							
Time	4		0.72179	0.18045	3.90	1.00	0.006
Time.Treatment	8		1.19329	0.14916	3.22	1.00	0.003
Residual	209	(19)	9.66680	0.04625		1.00	
Total	280	(19)	14.82343				

(d.f. are multiplied by the correction factors before calculating F probabilities)

Information summary

All terms orthogonal, none aliased.

Message: the following units have large residuals.

Subject 58	-0.401	s.e.	0.108
Subject 33 Time T3	-0.516	s.e.	0.180
Subject 47 Time T3	0.547	s.e.	0.180
Subject 49 Time T2	0.538	s.e.	0.180
Subject 50 Time T1	0.647	s.e.	0.180
Subject 59 Time T1	0.882	s.e.	0.180

Covariate regressions

Variate: Ratio

Covariate	coefficient	s.e.
Subject stratum		
Bee_number	-0.0005	0.00201
Combined estimates		
Bee_number	-0.0005	0.00201

Tables of means (adjusted for covariate)

Variate: Ratio
Covariate: Bee_number

Grand mean 0.426

Time	T1	T2	T3	T4	T5
	0.358	0.487	0.379	0.445	0.460

Treatment	Control	Modesto	Chinook
	0.480	0.453	0.345

Time	Treatment	Control	Modesto	Chinook
T1		0.438	0.467	0.169
T2		0.544	0.509	0.407
T3		0.390	0.486	0.261
T4		0.472	0.448	0.416
T5		0.554	0.356	0.470

Least significant differences of means (5% level)

Table	Time	Treatment	Time Treatment
rep.	60	100	20
l.s.d.	0.0789	0.0742	0.1421
d.f.	188.54	56	243.84
Except when comparing means with the same level(s) of			
Treatment			0.1376
d.f.			188.54

(Not adjusted for missing values)
Correction factors have been applied to residual d.f.(see analysis-of-variance table for details)

DEFRA hereby excludes all liability for any claim, loss, demands or damages of any kind whatsoever (whether such claims, loss, demands or damages were foreseeable, known or otherwise) arising out of or in connection with the preparation of any technical or scientific report , including without limitation, indirect or consequential loss or damage; loss of actual or anticipated profits (including loss of profits on contracts); loss of revenue; loss of business; loss of opportunity; loss of anticipated savings; loss of goodwill; loss of reputation; loss of damage to or corruption of data; loss of use of money or otherwise, and whether or not advised of the possibility of such claim, loss demand or damages and whether arising in tort (including negligence), contract or otherwise. This statement does not affect your statutory rights.

Nothing in this disclaimer excludes or limits DEFRA's liability for: (a) death or personal injury caused by DEFRA's negligence (or that of its employees, agents or directors); or (b) the tort of deceit; [or (c) any breach of the obligations implied by Sale of Goods Act 1979 or Supply of Goods and Services Act 1982 (including those relating to the title, fitness for purpose and satisfactory quality of goods);] or (d) any liability which may not be limited or excluded by law (e) fraud or fraudulent misrepresentation.

The parties agree that any matters are governed by English law and irrevocably submit to the non-exclusive jurisdiction of the English courts.