A large-scale field experiment to quantify the impacts of neonicotinoids (NNIs) on honeybees and wild bees.

NERC Centre for Ecology and Hydrology
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Revision notes: *This version contains a revised power analysis using data from the first sample year of the Pilling et al (2013) honeybee study and derives variance components based on a mixed model approach. This document has been revised following the 2014 revision of the power analysis presented in EFSA (2013). Document updated following start of the experimental component of the study in Autumn 2014.*

Background

Neonicotinoid (NNI) seed dressings have been used extensively across Europe and elsewhere since the early 2000s to protect oilseed rape and other important crops against pests[1]. They have recently been implicated in the decline of wild bees and harm to domesticated honeybees, and this led to a moratorium with respect to some uses in the EU whilst more data are generated. However, evidence for NNI impacts on bees is inconclusive and so the ban remains controversial[2]. Laboratory studies suggest some toxicity to bees[3], but these experiments are highly artificial and their relevance to the real world is unclear. Field trials provide a more realistic test of impacts on bees of NNI use by farmers, but some of these studies have proven inconclusive[4,5] although there is strong evidence in spring sown oilseed raps of field scale effects on bumblebees and solitary bees[10]. However, many studies have been criticised for a range of reasons including contamination of the no-NNI control, low replication and small plot sizes.

Aim

NERC Centre for Ecology and Hydrology is working with Bayer CropScience AG and Syngenta Crop Protection to implement a large-scale field experiment to quantify the impact on honeybees of two commercial neonicotinoids (NNI) seed treatments in commercially grown crops of oilseed rape ('Clothianidin' Bayer CropScience and 'Thiamethoxam' Syngenta).

Budget

The indicative budget available for this project from Bayer and Syngenta is currently $3M (£1.8M).

Experimental design

For the design of the experiment and associated monitoring the guidance provided by the European Food Safety Agency[6] on the risk assessment of plant protection products on bees[5] has been taken into consideration where feasible.

a) Location and timing

The field experiment has been established in the United Kingdom, Germany and Hungary as of autumn 2014 for monitoring and harvesting in 2015. This will take full advantage of the European moratorium on NNI use by improving the chances of NNI-free control treatments (a major criticism of some previous studies). The selection of these three countries was based on three factors: i) to provide the experiment with good bio-geographic spread across Europe; ii) represent countries and landscapes where intensive cultivation of oilseed rape is a typical and increasing practice; and iii) there was a strong likelihood of being granted an experimental licence to undertake a regulatory experiment of such an unprecedented scale.

Ideally the experiment would be conducted for more than one year in order to understand temporal effects. However, the current budget and requirements for replication only allows for a single year study.

b) Statistical power testing and threshold for effect size detection

The specific protection goals defined by the EFSA in their bee risk assessment of plant protection products require that studies should have sufficient replication to identify a 7 % detrimental effect on bee colony strength (i.e. number of bees in a hive) with this to be detected with an 80 % confidence in a one-tailed test[5] To put this into perspective, small, medium, and large effect sizes have conventionally been defined as 20, 50 and 80 % by Cohen[7] – a key work on power analysis – and so the likelihood of detection of 7% changes in colony sizes are small. The exact origin of the requirement to be able to detect a 7 % effect size is unclear from the EFSA draft guidance. However, in the comprehensive meta-analysis of both lethal and sub-lethal impacts of the NNI Imidacloprid[7] demonstrated that field realistic exposure rates for honeybees in oilseed rape (acute dose applied at a single time point = 0.023 – 0.03 ng; chronic dose applied over several time periods = 0.7 1.3 µg L^{-1}) would have sub-lethal effects on honeybees that reduced performance by between 6 and 11 %. These sub-lethal effects included impacts on gustatory thresholds, success in returning to colonies and learning and memory. Based on this, Cresswell[7] makes the reasonable assertion that the replication of studies aiming to detect non-lethal effects of NNIs on bees should be sufficient to detect such small effect sizes, i.e. in the range of 6 – 11 %.

We have run a statistical power analysis (Table 1) following the EFSA 2013 recommendations (using the revised formula of 2014, see Appendix A for details) and found that for many basic honeybee population metrics (peak & mean colony strength, rate of increase in colony size, peak & overwintering colony weight as well as various metrics of overwintering colony strength) between 10 and 20 % effect size would be detectable with the current replication used in the study of 11 replicate blocks (see below for details of experimental design). However, for the current replication and expected variances for population metrics (based on a previous study by Pilling et al, 2013) we would be unable to detect effect sizes of under 20 % for the average number of dead bees per hive, transect counts of bees on the crop and hive weight gain. Note we focus here on honeybees for the power analysis as limited data is available on variance components for other wild bee model systems. However, during the final analysis of the current study, we will repeat the power analysis for any response variable where non-significant effect are found using directly derived experiment estimates variance. This will be used to provide caveats on any non-significant response reported to neonicotinoid exposure.

	Effect sizes for N replicate blocks of a control and NNI treated field.				Replicate blocks (N) required to detect threshold effects sizes (ES)				Variance components for EFSA power analysis	
	N=3*	***N=4****	***N=11****		***7 % ES***	***10 % ES***	***15 % ES***	***20 % ES***	***Site = τ^2***	***Hive = σ^2***
Peak colony strength	14.1 %	12.5%	7.7%		N=13.4	N=6.3	N=2.6	N=1.4	0.0025	0.0193
Mean colony strength	11.6%	10.0%	6.1%		N=8.4	N=10.0	N=1.7	N=0.9	0.0008	0.0168
Rate of increase in colony strength	24.0%	21.0%	13.2%		N=42.2	N=20.0	N=8.4	N=4.5	0.0076	0.0625
Average dead bees per hive	50.7%	45.6%	30.6%		N=280.2	N=132.9	N=55.9	N=29.6	0.0985	0.1260
Transect counts of bees on crop	64.5%	59.1%	41.8%		N=610.4	N=289.6	N=121.7	N=64.5	0.2508	0.0570
Peak colony weight	14.0%	12.1%	7.5%		N=12.8	N=6.1	N=2.6	N=1.3	0.0043	0.0070
Maximum weight gain of colony (max - start weight)	47.1%	42.2%	28.2%		N=229.4	N=108.8	N=45.7	N=24.2	0.0688	0.1741
Overwintering colony weight	18.1%	16%	10%		N=23.1	N=11	N=4.6	N=2.4	0.0086	0.0076
Overwintering colony strength	15.2%	13.5%	8.4%		N=15.9	N=7.5	N=3.2	N=1.7	0.0034	0.0203
Overwintering % nectar cell area	22.5%	19.8%	12.5%		N=37.0	N=17.5	N=7.4	N=3.9	0.0128	0.0179
Overwintering % pollen cell area	33.7%	29.8%	19.2%		N=94.7	N=44.9	N=18.9	N=10.0	0.0113	0.1746

*Note in the experimental design there are 11 replicate blocks split between three countries, Germany (N=3), Hungary (N=4) and the UK (N=4)

Table 1. Power analyses indicating the effect size that can be detected for a range of commonly-measured population-level parameters recorded as part of honeybees monitoring for a given number (N) of replicate blocks. The power analysis is derived from EFSA (2013) and are described in detail in Appendix A. Variance parameters are determined from establishment year data (2005) described in Pilling et al. (2013: PLoS ONE **8**:e77193) and focus on oilseed rape associated bee colonies, where τ^2 = between site variance in response parameter, equivalent to the CV^2 and σ^2 = within site between colony variance, equivalent to $ln(1+CV^2)$. Limitations on available data restrict the power analysis at present to Honeybees. Power analysis is based on the revised power analysis presented in a revision of EFSA (2013: EFSA Journal **11:3295**:266) released in 2014. Note that all measures of colony strength for honeybees are recorded using the Liebefeld method and the rate of increase in colony strength is based on a Pearsons correlation coefficient of the increase in Liebefeld colony strength over the first 5 weeks of monitoring in a given year. Variables with the suffix T1 are considered to be core Tier 1 parameters of key importance in assessing honeybee responses to Neonicotinoids.

c) Experimental treatments

Within each country (UK, Ger, Hun) we established either three (Germany) or four (UK and Hungary) replicates of three experimental treatments applied to commercial winter sown oilseed rape crops (Figures 1 & 2), namely:
a) without Neonicotinoid (CONTROL);
b) with Clothianidin seed treatment; and
c) with Thiamethoxam seed treatment.

Note soil contamination of Neonicotinoid residues in Germany prevented four replicate blocks being established in this country.

Each treatment was applied to contiguous patches (although sometimes split across multiple fields) of oilseed rape of between 40-70 ha in size. The size of the treated patch is a compromise between the area of crop likely to be approved under experimental licence and the foraging distance of honeybees
to ensure a realistic field exposure to the pesticide, i.e. there will be fewer opportunities to forage on neighbouring habitats and possibly untreated oilseed rape (a criticism of some previous field trials). The exact size sown at an individual site depended on local configurations of fields and as such it was not possible to have a standard area of crop sown.

The basic experimental unit (or replicate block) is the triplet of these 40-70 ha patches (treatments a, b, c), each separated by a minimum of 4 km following EFSA guidelines[6]. Each replicate or triplet was chosen to be (as far as is practical) in the same general landscape, on the same soil type with similar drainage and otherwise show as few differences as possible in other environmental and biophysical conditions (e.g. altitude). Note that each replicate block was separated by at least 10 km. In all cases farms were be located in relatively intensively managed agricultural landscapes currently associated with moderate to high levels of oilseed rape production, as far as this was feasible under the requirement of plot isolation. At each site model bee systems will be established, represented by six honeybee hives, 12 bumblebee colonies (B. terrestris) and 50 cocoons of the solitary bee Osmia bicornis which will be encouraged to nest in artificial trap nests.

The treated and untreated oilseed rape will be the same variety in each country. The selected variety was chose as the most widely grown hybrid for a given country and is grown according to uniform best practice agronomy.

Figure 1. Schematic representation of the proposed experimental design

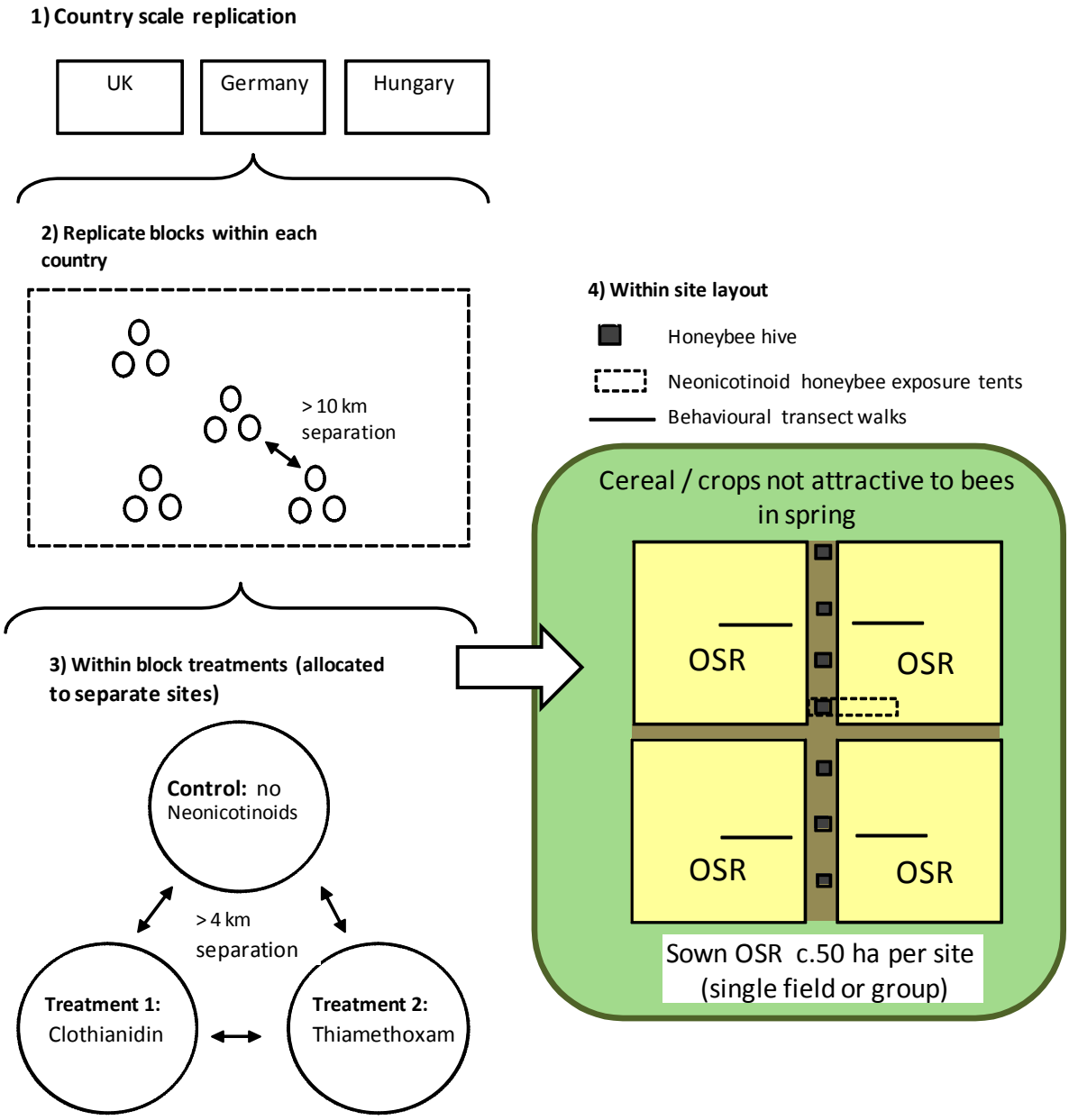
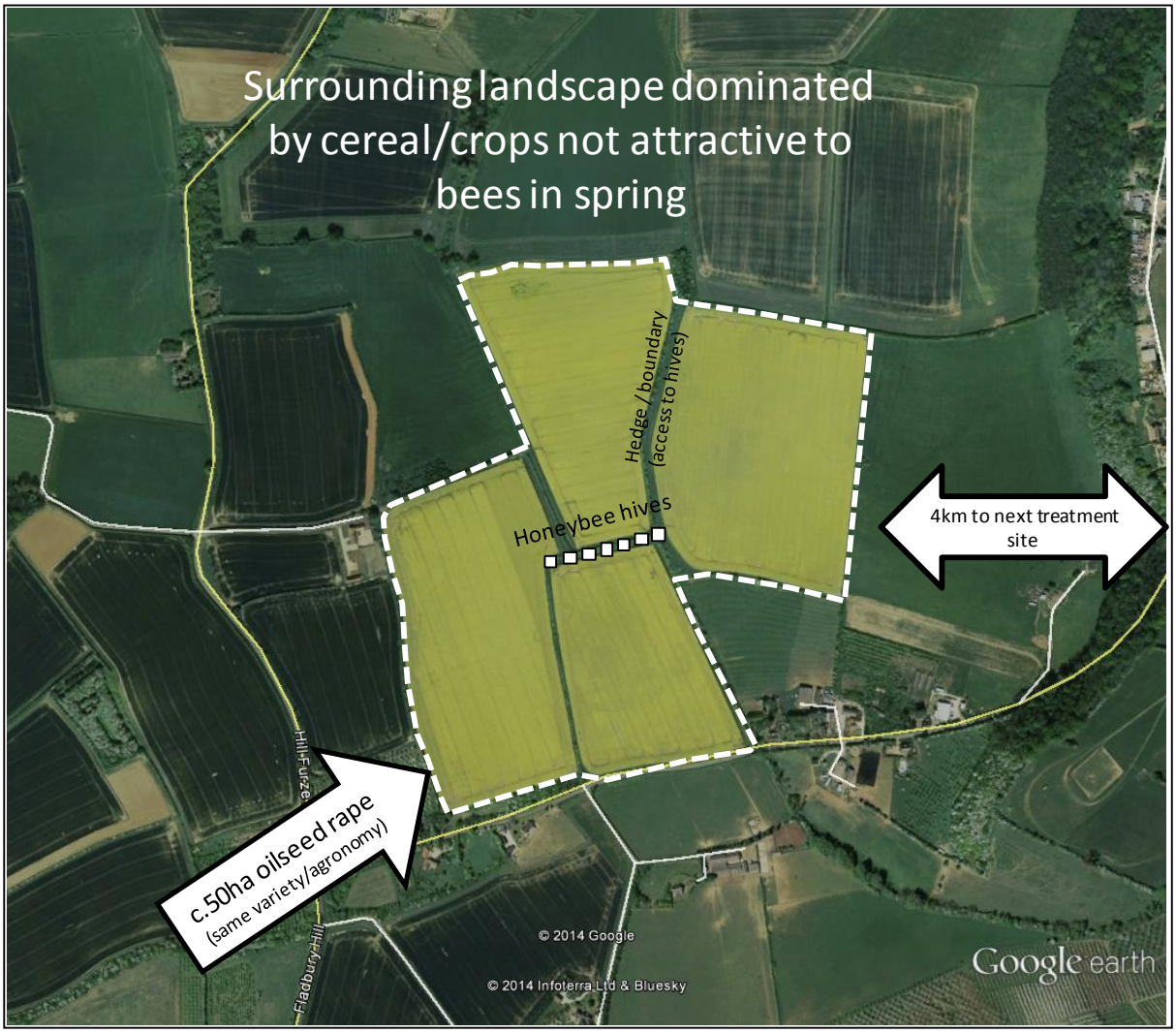


Figure 2. An example of an individual site.



Summary of measurements
All assessments will be undertaken in both treated and control sites to ensure both adequate exposure to NNIs has occurred in the treated crops, and that no contamination has occurred in the controls. Full experimental protocols are also available online for both honeybees, bumblebees and solitary bees. However, we summarise monitoring undertaken below which represent.

1) Chemical analysis of NNI concentrations

Chemical residue analyses will be undertaken by an accredited CEH laboratory. A sample archive will be established to underpin the project by providing additional samples for analysis should unexpected results emerge, and for QA and audit purposes.

The following measures will be taken:

- Oilseed rape nectar and pollen NNI concentrations (using caged honeybees to sample the crop on two occasions during flowering.

- Typical forager nectar and pollen NNI concentrations (by sampling free-flying honeybees and bumblebees)
- To supplement this information on the actual exposure, additional pollen will be collected from foraging bees subsequent examination under microscope to quantify the ratio of oilseed rape to non-oilseed rape pollen.
- Stored products from honeybees, B. terrestris colonies and Osmia bicornis (pollen, nectar, wax sampled on three occasions during exposure)
- Additional material for archived chemical analysis if required:
    - Soil samples
    - Foliage samples
    - Foraging workers (honeybees and B. terrestris)

2) Honeybee biological measurements - Colony size and mortality

Biological measurements will focus on measurements of population size and breeding success of commercial honeybee colonies (six per field plus one reserve). All hives will receive standard *Varroa* treatment. These will be augmented by standardised observations to quantify bee behavioural effects. For both, the EFSA guidelines will be taken into consideration as far as feasible, but measurements will be slightly less frequent for reasons of cost and practicality.

Primary assessment endpoints:

- Colony size and success assessment (Liebefeld method & hive weight).
- Forager mortality (hive mortality assessment during exposure period days (using dead bee traps); also difference in colony size between the controls and NNI treatments)
- Disease & viruses – sampling on three occasions (at start of the study, before overwintering and after overwintering)
- Overwintering of the test colonies (including feeding, disease treatment, frequent checks during winter)
- Overwintering survival and success (honeybees - Liebefeld method & hive weight in spring)

Secondary assessment endpoints - Behaviour:

- Standardised observations of bee activity during exposure period.
- Observations of bee behaviour using a standard protocol (creation of pollen pellets and nectar collecting; signs of clinical intoxication, motionless bees, cleaning behaviour etc)

3) Bumblebee biological measurements

Biological measurements will focus on measurements of population size and breeding success of Bombus terrestris colonies (12 per field). All hives will be fitted with queen excluders to reproductive queens from leaving the colonies.

Primary assessment endpoints:

- Colony weights and counts of workers, queens and drones based on colony dissections.
- Disease & viruses – sampling on three occasions (at start of the study, before overwintering and after overwintering)

Secondary assessment endpoints - Behaviour:

- Standardised observations of bee activity during exposure period.

4) Solitary bee (O. bicornis) biological measurements -

Biological measurements will focus on measurements of population size and breeding success of Osmia bicornis establishing in artificial trap nests after being released at each site.

Primary assessment endpoints:

- Dissections of trap nests to assess breeding population sizes (number of provisioned cells) combined with removal of stored products for residue analysis.
- Breeding success of O. bicornis and other solitary bees using trap nests combined with assessment of parasitism rates.
- Disease & viruses

5) Agronomic measurements

Each crop (control, NNI treated will be grown to a uniform, best practice agronomy protocol to ensure consistency of inputs. This information will be recorded, together with crop yield and quality.

- Cropping history
- Crop inputs (pesticide, fertiliser etc)
- Crop yield and quality

References

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Appendix A. EFSA (2013) power analysis (as revised in 2014) and data sets used to determine replication and effect sizes for the field study.

The below equation defines the EFSA (2014) power equation used to determine the replication for a field based trial assessing the impacts of pesticides on honeybee colonies.

Eq. 1

$$N = \frac{2.\left(z_{1-\alpha}+z_{1-\beta}\right)^2.\left[1+(n-1)\frac{\tau^2}{\sigma^2+\tau^2}\right]}{n.p^2/(\sigma^2+\tau^2)}$$

Where:

N Replication required (treated and control sites) to detect a particular effect size.

n Number of honeybee colonies within each site.

α The significance level of the test, set at 0.05.

$z_{1-\alpha}$ α-quantile of standard normal distribution *N*(0,1) where 1-α = 0.95 and $z_{1-\alpha}$ = 1.644. this defines the significance level of the text..

$z_{1-\beta}$ β-quantile of standard normal distribution *N*(0,1) where 1-β = 0.80 and $z_{1-\beta}$ = 0.841. This parameter defines the power of the test, i.e. and 80 % probability of detecting a given effect size (e.g. 7 %) with a given significance level (α).

τ^2 Variation between sites, defined as CV^2 or (standard deviation between sites / mean)^2. Note that standard deviation is equivalent to the square root of the Mean Square presented in the ANOVA table based on the (Pilling *et al.* 2013) peak colony size data (Table S2).

σ^2 Variation between colonies, defined as $\log_e(1+CV^2)$ or $\log_e$ (1+(standard deviation between colonies / mean)^2). Note that standard deviation is equivalent to the square root of the Mean Square presented in the ANOVA table based on the (Pilling *et al.* 2013) peak colony size data (Table S2).

$\sigma^2 + \tau^2$ Total variation (between colonies and sites).

ρ logarithmic treatment effect For a 7 % effect size difference between the treated and control this would be equivalent to $\log_e(0.93)$ = -0.0726, for a 15 % effect size this would be ln(0.85) = -0.1625.

To parameterize this power equation we use data derived from honeybee monitoring undertaken as part of the Pilling et al (2013) study investigating the impact of neonicotinoid field treated oilseed rape. We use data from the first year of monitoring of this study (comparable to the single monitoring year of the current presented study). We run the power analysis for the following population metrics of the honeybee colonies:

1) Within season exposure to treated crop metrics of population size: a) peak colony strength (number of bees in hive described suing the Liberfeld method), b) Mean colony strength, c) rate of increase in colony strength (first 5 weeks), d) average number of dead bees per hive, e) transect counts of bees on the crop, f) peak colony weight and g) maximum weight gain of colony (max - start weight).
2) Metrics of colony strength describing overwintering success of hives: a) Overwintering colony weight, b) Overwintering colony strength, c) Overwintering percentage area of nectar storage cells (determined using the Liebefeld Method) and d) overwintering percentage area of pollen storage cells.

Data for this study is derived from sampling undertaken in 2005 in two regions (Alsace and Picardie) where each region containing two sites, one with oilseed rape treated with neonicotinoids and the other an untreated control site (a total of four sites). On each site 6 honeybee hives were monitored. To this data we apply the power analysis (eq.1) proposed by EFSA (2013) described above which incorporates into its derivation two sources of variance, variance between sites (τ^2) and variance between the individual colonies (σ^2) where each site will have multiple colonies. To identify variance components we ran a mixed effects models in SAS 9.3 using PROC MIXED. For each of the explanatory variables we fit a null model (intercept only) with SITE as a random factor. From this model we use random effects covariance parameter estimates to derive variances used in the calculation of τ^2 (based on the covariance parameter estimate for the residual variance).

Raw data derived from Piling et al (2013) used in the derivation of the variance components for the EFSA power analysis are presented below.

Site	Region	Treat	Hive number	Peak colony strength	Average colony strength	Rate of increase in colony strength	Overwintering hive strength	Overwintering % cover of nectar cells	Overwintering % cover of pollen cells
s1	Alsace	Control	1	21375	15276.33	0.570	16125	34.8	0.5
s1	Alsace	Control	5	21438	13375.17	0.773	12000	37.0	1.3
s1	Alsace	Control	9	19375	14573.17	0.954	15500	36.17	1.1
s1	Alsace	Control	13	17188	11047.1	0.586	14875	29.8	1.0
s1	Alsace	Control	17	20438	13135.5	0.828	12625	33.5	2.5
s1	Alsace	Control	21	19000	13692.9	0.823	13063	35.8	1.0
s2	Alsace	Treatment	2	23375	14864.7	0.793	13563	36.1	2.0
s2	Alsace	Treatment	6	21688	15140.8	0.513	12750	33.8	1.5
s2	Alsace	Treatment	10	16250	12745.0	0.375	10188	28.3	1.0
s2	Alsace	Treatment	14	20625	14786.6	0.477	13000	34.5	1.8
s2	Alsace	Treatment	18	23750	15026.2	0.845	14938	26.5	2.8
s2	Alsace	Treatment	22	22313	12323.2	0.735	13813	20.0	1.0
s3	Picardie	Control	3	20438	14797.0	0.957	14625	26.1	0.8
s3	Picardie	Control	7	22438	13901.2	0.982	13938	21.7	1.1
s3	Picardie	Control	11	17313	10864.8	0.764	10125	27.5	1.5
s3	Picardie	Control	15	20188	15484.7	0.780	12063	23.2	1.0
s3	Picardie	Control	19	17813	11573.0	0.727	11125	28.3	0.7
s3	Picardie	Control	23	28125	16156.3	0.605	11250	28.7	1.0
s4	Picardie	Treatment	4	21125	11479.5	0.983	8438	24.6	1.3
s4	Picardie	Treatment	8	17875	11271.0	0.734	13750	24.8	2.6
s4	Picardie	Treatment	12	14875	10948.2	0.445	14438	31.8	1.2
s4	Picardie	Treatment	16	16438	10536.8	0.576	14813	25.5	1.7
s4	Picardie	Treatment	20	17375	14312.7	0.404	14313	28.1	0.6
s4	Picardie	Treatment	24	20375	15250.3	0.547	13063	31.1	1.3

Site	Region	Treat	Hive number	Average number of dead bees per hive	Bee transect counts *	Overwintering colony weight	Peak colony weight	Colony weight gain (Max. – start weight) (Kg)
s1	Alsace	Control	1	14.5	5.2	.	.	.
s1	Alsace	Control	5	12.0	6.1	.	49.2	5.1
s1	Alsace	Control	9	9.4	3.7	.	46.5	2.6
s1	Alsace	Control	13	3.9	4.5	44.1	55.1	16.3
s1	Alsace	Control	17	8.8	6.0	42.8	51.7	11.5
s1	Alsace	Control	21	7.6	.	.	43.8	4.1
s2	Alsace	Treatment	2	10.8	5.1	45.9	53.9	11.6
s2	Alsace	Treatment	6	17.1	4.9	49.4	58.3	10.0
s2	Alsace	Treatment	10	8.9	2.9	42.6	52.3	9.3
s2	Alsace	Treatment	14	9.3	3.2	.	59.2	18.0
s2	Alsace	Treatment	18	8.6	4.0	41.7	53.2	15.8
s2	Alsace	Treatment	22	16.7	.	36.4	50.7	7.8
s3	Picardie	Control	3	3.5	1.3	49.8	62.7	21.4
s3	Picardie	Control	7	4.3	1.4	44.2	52.3	11.7
s3	Picardie	Control	11	3.5	1.7	46.5	53.2	12.4
s3	Picardie	Control	15	3.4	2.3	49.5	59.6	23.1
s3	Picardie	Control	19	6.9	2.3	.	50.4	11.3
s3	Picardie	Control	23	5.9	.	50.1	60.4	17.8
s4	Picardie	Treatment	4	7.1	1.5	46.5	51.8	10.9
s4	Picardie	Treatment	8	13.2	1.6	52	56.9	19.5
s4	Picardie	Treatment	12	4.8	2.5	60.3	66.5	25.7
s4	Picardie	Treatment	16	8.0	1.7	52.5	56.8	23.8
s4	Picardie	Treatment	20	12.2	2.0	.	59.1	5.3
s4	Picardie	Treatment	24	10.3	.	56.5	62.8	20.9

** Bee counts on transects were only undertaken at five locations in the crop at each site.*