A large-scale field experiment to quantify the impacts of neonicotinoid (NNI) seed dressings on honeybees in the UK, Germany and Hungary

Field protocol for additional measures of wild pollinators (*Bombus terrestris, Osmia bicornis*) in UK, Germany and Hungary

Version 2.0

01 Mar 2015

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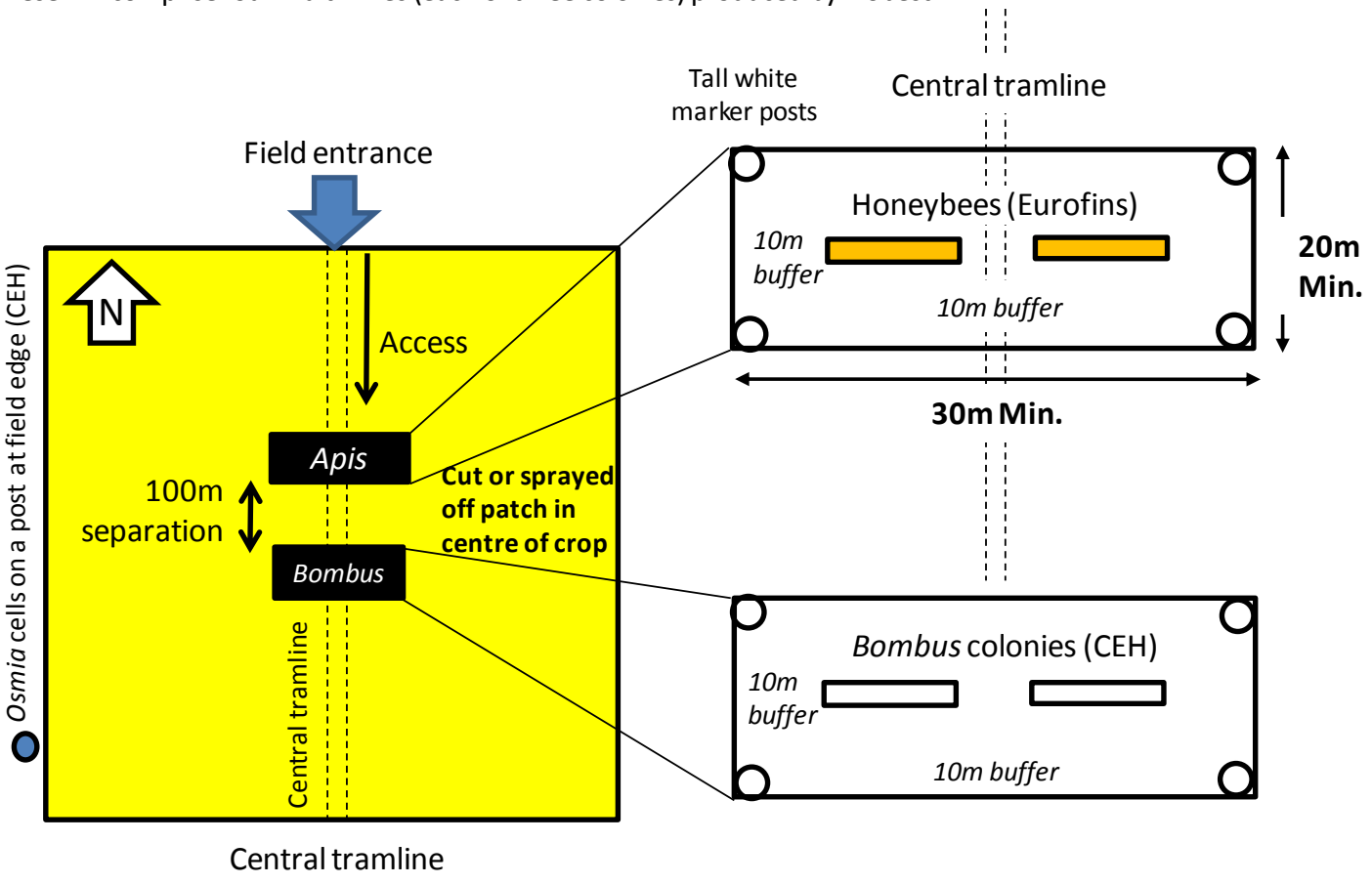
See current version of the main experiment study plans for full details of the rationale and experimental design

1. Field set-up

Within each study site 12 commercially produced *Bombus terrestris* colonies will be placed in a central location (either a central patch of cut crop or a central headland). These will comprise four multi-hives (each of three colonies) produced by Biobest.



In addition, 50 commercially produced *Osmia bicornis* cocoons will be placed in a release tin on a 2 m high metal pole along a field boundary with tall vegetation (the most like site for nesting) at each site.

In addition, six uniform solitary bee trap nests will be placed along this boundary on 2m metal poles. Each nest will comprise an11×11×28cm wooden block with an equal number of 4, 6, 8 and 10mm diameter holes drilled in it.

Actions in date order:

1. **Labelling the nests**

Each *Bombus* colony and trap nest will be labelled with a unique number provided by CEH using an indelible marker pen. *Bombus* multi-hives will be marked on the outer and inner boxes.

2. **Disease sampling of Bumblebees, pre- setting out on sample fields.**

Five worker Bombus per nest should be sampled and immediately deep frozen (min 18°C, freezer to 80°C, dry ice). Do this by opening the individual nest entrance at the same time holding an opened polythene bag over the entrance. Once the entrance is open worker bees will move towards the light. The first 5 to go into the bag are your sample. Close the entrance and transfer the bees in the bag to the freezer. Do not leave the bees in the bag for long before freezing as they can bite their way out of the bag.

This can be done with the nest removed from the multihive as it will make manipulation easier. Once sampled, return the nest to the multihive unit. The bag should be marked with the nest identification code on the outside and in pencil on the inside.

3. **Setting out *Bombus* and *Osmia* in the field**

Osmia

You will be supplied with:

- Two wooden trap nests and *Osmia* release tin on a 2m high metal pole
- Four single trap nests each on 2m high metal poles

- A map showing the preferred location of the six trap nests and *Osmia* release tin for each site.

The trap nests should be set up along the same margin. These should be set back against the linear woody feature so that potential occupants find the nests as they fly along the edge. They need to have the open end of the trap pointing into the field. They set out at 20-25m intervals, with the double one with the black release tube for *Osmia* in the middle. It is more important to place the nest against a tree or shrub, where possible, than to keep exact distances.
The posts should be driven about 0.60m into the ground with the enclosed post-banger so as to make them very firm, before the trap nests and U bolts are attached. A socket and adapter which can be used with a battery-powered drill is included in the equipment, as well as a cranked socket-driver (for those times when the battery runs out in the wrong place!). (See instruction sheet for details).
The trays for mud should be
set up under the *Osmia* trap nests. Take a bottle of water and a spade with you (to get the soil from the field beside the trap nest).

Bombus multihives

These should be placed in their own area as indicated on the site map. They should be set out such that the nest entrances face east or west (2 open one way, one the other). Each multihive should be 15-20m from the next one (max 60m overall). They should be set back a minimum of 1m from the crop edge, preferably 2m if there is room.

Bombus colonies will be set up (4 multihives per field) by Eurofins before the monitoring begins. They will be set out with pollen and biogluc turned on. These should be turned of 5 days after setting out, or at OSR 'yellow haze' stage, which ever is sooner. This will allow the colonies to establish in the landscape, without a pinch point in supplies. The date of turning of should be noted for each field. Each multihive should be marked with the field number and an individual multihive number (1-4).

Weather station

The weather station, one per set of three fields, is set up within the Honeybee hive or Bombus nest area. It needs to be well out in the open and in an area which has been cleared of the crop, the manufacturer suggests at least as far away as 10 times the height of the nearest tree. Get the best clearance you can, particularly to the south. The bottom of the platform should be 1.5m above the ground. If this can be done before the honeybee hives are moved in it will be easier. See instruction sheet for detail.

The sensor platforms should be marked for each locality as they are set out. They are already numbered. Lodge a copy of this information at CEH. The posts have a single hole at the top. This is used to bolt the platform to the post. The ring terminal (red) MUST be fixed to the post with the mounting bolt as this is the earth for the pyranometer. A flexible socket-drive and the required sockets for setting up are in the crate.

The platform should be a level as possible, with final adjustment of the pyranometer by the legs of the unit. The bubble should be central. NOTE the open side of the platform should point north. This

is marked on the platform. The pyranometer may need slightly releasing on its mounting bolts for adjustment. Don't forget to re-tighten - GENTLY!

Photo instruction sheet included.

4. Monitoring

Visit 1 - Set up

a) Set up margin nests (as described above)
b) Set out trap nests for *Osmia*, add *Osmia* cocoons to release chamber and set up mud supply.
c) Set up weather sensor/logger at one field in each main set.

Visit 2 Monitoring

Monitoring visits should be made at a minimum ambient temperature of 14°C and 50% sun.

a) *Bombus* pollen samples - Take pollen samples from returning workers at the *Bombus* multihives. These need to be caught in a net and the pollen load removed from the legs to a sample tube using a Queen Honey-bee marking cage (you learn the technique quickly- without getting stung) use pollen from both legs as a load. The aim is to get a minimum of 5, maximum of 15, bee-loads in 15 minutes from a set of 3 nests in a multihive. Keep these as 4 separate samples please, 4 multihives per field.

METHODOLOGY: Push the pollen-laden bee in the tube towards the grid using the plunger. As you push, gently spin the plunger and you will find that you can make the bee move against the grid. When a leg with pollen load is against the grid, knock the pollen off (a cocktail stick is ideal) into a small tube (in crate). All samples from one multihive in one tube please. Please mark tube with field number and multihive number. Place a pencil label inside the tube and mark the outside with marker pen. Use a clean stick for each field and wash the plunger and tube in approximately 50ml of clean 100% industrial Ethanol between fields. This is to minimize transfer of pollen traces between fields. Discard the washings and dry the plunger on clean paper towel each time. This should be air-dried (evaporates fairly quickly) before being used on bees again.

b) *Bombus* colony weights: Six of the hives (those to be left out until mid-June 2015, see below) will be weighed every ~7 days following field exposure to the crop using a digital balance to the nearest 1 g. On each occasion individual hives will be removed from the multi-hive for weighing.

c) Pollinator transect - Make a 30 minute observational transect within the flowering crop recording all visits to crop flowers by bees at the greatest level of taxonomic definition you can, without catching the bees. (This is what the German surveyor is happy with, I am asking the Hungarian surveyor to catch, put in a pot and freeze for later identification. You can do any combination from these which you are happy with, depending on experience, but of course collecting will add to the eventual processing time.) The transect runs along the same edge as the trap nests for 100m, looking 1m into

the crop. Pace out and mark with tape (marker tape in crate, tie to suitable point on margin) as you set it up the first time. Do not expect to get a lot of bees as there is an awful lot of flower and your sample area is small.

d) Crop flowering - Record the stage of crop flower cover as one of:
1. Yellow haze - buds opening
2. All yellow - open flowers dominant
3. Green appearing - Upper branches with seed pods visible, but still a lot of flower on the lower branches of the plants.
4. Green dominant - Most of plants in seed, most flower along tractor wheel-marks.

Visit 3 Monitoring, as above

Visit 4 Monitoring, as above

Shutting up of Bumblebee nests for sampling

The bumblebee nests and *Osmia* trap nests will be sampled in two stages, mid-May (coinciding with the end of oilseed rape flowering and so the period of maximum exposure) and mid-June (corresponding with the maximum production of reproductive stages, e.g. new queens). The bumblebees will be frozen at Wallingford. For each collection round it will be necessary to ask you to shut up the nests to be sampled the evening before. The details of this procedure will need to be worked out for each site, but shutting up should be as late as possible with collection/freezing the next morning.

The *Osmia* nest sampled in mid-May needs to be the one with the largest number of filled tubes. Both samples will be kept at ambient temperature.

Mid-May B. terrestris sample round monitoring
This sampling period is intended to coincide with the period of oilseed rape flowering and so stored products are expected to be dominated by pollen and nectar foraged from this crop. It is likely that the mid-June sampling round (important for determining queen production) may have few stored products. On this sample round six colonies from each site will be sent back to CEH Wallingford for dissection and counting of workers, removal of stored products for neonicotinoid residue analysis (pollen, nectar and wax) and removal of workers for disease analysis. Where queen cells are identified they will be counted, but it is expected that this sample round will be too early for queen production from most colonies. In each case colonies will be dissected and:

1) Colony will be weighed.
2) Total counts of all live workers and other casts presents will be made. Five workers will be collected for disease analysis and frozen. All individual will be collected and frozen for long term archiving.
3) Samples for at least 10 cells will be used to collect c. 1- 1.5 g of stored nectar, pollen and wax for residue analysis.
4) Five larvae will be collected and frozen for residue analysis.
5) Number of cells containing stored products and larvae will be counted. Distinction will be made between worker (<12m cells) and reproductive queen cells (>12mm). Note few queens are expected in this sample round.

Mid-June B. terrestris sample round monitoring

This sampling period is intended to coincide with the period of maximum reproductive output of the colonies, but will be significantly after the end of the flowering of the oilseed rape crop. It is likely many stored products collected from the oilseed rape will have been used by this time. On this sample round six colonies from each site will be sent back to CEH Wallingford for dissection and counting of workers and reproductive stages, removal of stored products for neonicotinoid residue analysis (pollen, nectar and wax) and removal of workers for disease analysis. In each case colonies will be dissected and:

1) Colony will be weighed.
2) Total counts of all live workers and other casts including queens and drones presents will be made. As queen excluders have been fitted to colonies no emerging queen will have been able to escape the colony. All individual will be collected and frozen for long term archiving.
3) Samples for at least 10 cells will be used to collect c. 1- 1.5 g of stored nectar, pollen and wax for residue analysis. This sample will be archived.
4) Five larvae will be collected and frozen for residue analysis. This sample will be archived.
5) Number of cells containing stored products and larvae will be counted. Distinction will be made between worker (<12m cells) and reproductive queen cells (>12mm).

Collecting solitary bee margin trap nests.

As for the bumblbebees collection of trap nests will occur under a two phase process intended to determine population key metrics describing potential exposure to neonicotinoids as well as population level metrics. Again these collections will occur in mid-May early June (coinciding with the end of oilseed rape flowering and so the period of maximum exposure) and September (coinciding with the full development of nesting wild bees within the trap nests). All trap nests from all countries will be returned to CEH Wallingford for processing.

Mid-May trap nest sample round monitoring

On this sample round two of the six trap nests from each site will be sent back to CEH Wallingford for dissection and counting of *O. bicornis* cells, removal of stored products from these cells (pollen and nectar) and removal of larvae and pure pollen for microscopy analysis. This round will focus specifically on the model system *O. bicornis* and is intended to identify the size of the breeding population (occupied cells) and exposure to neonicotinoids (residue in pollen and nectar ball). Assessments of breeding success and parasitism rates for *O. bicornis* and other breeding wild bees in the trap nests will be made from the second collection of the trap nests. This reflects the destructive nature of the sampling (removal of stored products to reach minimum sizes for residue analysis) combined with the early and variable developmental stages of the *O. bicornis* making them highly susceptible to disturbance related mortality effects. In addition it is necessary to chill the trap nests to prevent the consumption of the pollen and nectar provisioning ball during processing to avoid its consumption, a factor that may also unduly affect mortality. This early sample however corresponds with the exposure period of the bees to the crop (i.e. this is taken at the end of crop flowering, not after). The breaking apart of the , where it is assumed all cells with a clay partitioning wall and a pollen / nectar ball provisioning a cell are of this species (pers. Com. Mike Edwards).

Each trap nest will be broken apart.

1) The total number of drilled hole in the trap nest (assumed to be used by one breeding female) occupied by *O. bicornis* will be counted. The number of provisioned cells in each of these occupied drilled hole will be recorded. Differences in the colour of the provisioning pollen and nectar ball in each cell will be noted as this may reflect differences in the types of pollen collected by females (e.g. powder yellow or dark orange).
2) A sample of c. 1-1.5 g of the stored product of each nectar colour will be collected with larvae removed. This will be taken from different cells aiming to maximize the number of separate drilled holes it was collected from (this will be recorded). This sample will be used for neonicotinoid residue analysis.
3) A separate sample of pure pollen will also be collected from pollen and nectar balls of an individual colour. This will be retained for microscopy analysis to determine what plants the *O. bicornis* have been using to provision the cells.
4) Larvae will be counted and stored for residue and disease analysis.

September trap nest sample round monitoring

Although counts of O. bicornis cells were undertaken in the first sample round this round is intended to provide more detailed population level assessments across all species by rearing out cells. On this sample the remaining four trap nests will be collected sent back to CEH Wallingford for dissection and counting. They will be kept at ambient temperature. At this later stage in the year it expected that larvae will be in cocoons and so are less susceptible to disturbance resulting from dissection and so can be more easily be sampled for subsequent rearing of bees and parasitoids without undue mortality effects.

1) The total number of drilled hole in the trap nest (assumed to be used by one breeding female) will be counted. The number of provisioned cells in each of these occupied drilled hole will be recorded. Individual cocoons will be removed and stored in Gelatin capsules. These will go through a forced cold period (c. 4 °C) in controlled Temperature rooms to simulate winter diapause and encourage emergence. All parasitoids and bees will be collected and identified.

5. Preliminary assessment of expected timing of sampling

It looks as if the OSR will be flowering here at a similar time as Germany and Hungary, perhaps a week later. This assumes 13th April for start of field work, but Osmia can go out several weeks before this to suit yourselves.

Overall timetable

Mar 14th Pallets delivered to sites				
Nominal start date OSR flowering			UK	Sample
Start Week 1			13th April	
Start Week 2			20th April	Monitor 1
Start Week 3			27th April	Monitor 2 (latter part)

Start Week 4			4th May	
Start Week 5			11th May	Monitor 3
Collect first set of Bumblebee boxes and Osmia in-field trap nest			15th May	
Start Week 10			15th June	
Collect second set of Bumblebee boxes and Osmia in-field trap nest			19th June	
Retrieval of field edge trap nests			7th-14th September	