# Protocol to measure pesticide effects on *Bombus terrestris* colonies in a dose-response in-hive feeding with declining pesticide concentrations

## Study design

In order to determine the impact of a pesticide on bumblebees, colonies will be exposed to different concentrations of this pesticide through a sucrose solution (syrup) that is accessible from within the nest. The colonies will be monitored during a pre-exposure, exposure and post-exposure phase. The pre-exposure phase may consist of a single measurement if only one baseline value is required or two or more measurements if the pesticide impact on the change of bumblebee parameters is desired. The exposure period will be 10 days (the duration can be adapted to realistic exposure periods for the pesticide). The pesticide degradation should be mimicked by lowering the pesticide concentration daily at least during the first 4-5 days. One group of colonies should be exposed to the highest realistic sequence of pesticide concentrations and the other groups will be exposed to a multiple or a fraction of this sequence. Syrup consumption and number of adult bees shall be monitored daily in order to determine the amount of syrup and pesticide consumed per bee.

## Study site

All colonies will be placed at the same study site to minimize confounding effects due to differences in the surrounding landscape. There are no specific requirements on the study site except that sufficient floral resources should be available to sustain the bees and particularly to meet their pollen requirements. Highly attractive nectar sources may be avoided to maximize syrup consumption.

## Sample preparation

A 50% w/w sucrose solution should be prepared. A small part (required amount to be calculated) of the sucrose solution should be spiked with the pesticide to obtain a concentration that is at least as high as the highest concentration required for the experiment. This standard shall then be diluted with untreated syrup to obtain the different required pesticide concentrations. The syrup shall be distributed to small feeding containers labelled according to the concentration. 20 mL should be provided to the colonies per day. The supplied amounts may be adapted depending on the overall syrup consumption of the colonies.

## Syrup feeding container

Different designs of syrup feeders can be used. However, the typical gravity feeders may cause contamination as they may be dropping. Therefore, feeders that use capillary force are preferable. Even though placing these under the nest would be convenient, it should be avoided as the syrup will contaminate the ground of the nest and therefore bees will likely still be exposed to the higher concentration when the syrup container is replaced with a container containing syrup with a lower concentration of the pesticide. Therefore, capillary feeders should be placed inside the nest. These may be fitted with a stick for easy removal.

## Experimental colonies

At the start of the experiment, the *Bombus terrestris* colonies shall have one queen and a standardized number of workers (in our case colonies with 30-35 bees will be ordered). Small colonies will allow for a longer study duration. The colonies should be delivered in nest boxes containing no cotton to ensure that bees can easily be spotted and counted. The nest boxes should be provided with a queen excluder and with a one-way gate for bees entering the nest (if the automatic bee activity monitors by Atlantic Pollination Ltd. are to be used).

Some spare colonies shall be ordered as colonies may be replaced when they die early in the study (particularly in the pre-exposure period). Nest boxes shall be placed inside polysterene or wooden boxes (housing units) to protect colonies from rain and direct sunlight.

## Study phases

### Pre-exposure period

After delivery, the colonies shall be moved to a dark room and opened under a red light. Then the following steps should be followed:

1.) Label the nest box (with a hive ID).

2.) Close Biogluc syrup container underneath the nest (if BioBest colonies are used).

3.) Replace the nest lid by a transparent cover (acrylic glass) with ventilation holes.

4.) Note number of bees that exited if some escaped through the temporary opening on top.

5.) Photograph nest (so that adult bees and cocoons (the latter only in the pre-exposure period) can be counted and nest ID be seen) and note this.

6.) Weigh nest box (including weight of internal syrup container and bees).

7.) Remove, weigh and return internal syrup container. To facilitate removing or exchanging the syrup container, additional plastic covers should be prepared with a hole big enough to move the syrup container through. These can then be slid under the lid before opening it, when putting syrup containers in or out of the nest although this may not necessarily be required in the dark room with red light only.

8.) Count the number of dead adults and dead juveniles (larvae + pupae).

9.) Mark the (foundress) queen (and note this). If the queen has already been marked note whether the queen was seen alive, seen dead or not seen.

10.) Note the number of bees that escaped during the second opening (if any bees escaped when marking the queen).

The next day, the colonies shall be placed at the study site. This may be done completely randomly or based on a stratified random sampling approach that attempts to obtain similar colony weights for each row (in both directions). The colonies shall be re-assessed 1-2 days after colony placement i.e. steps 5-10 shall be followed.

The colonies shall then be allocated to the different treatment groups (i.e. concentrations) using a stratified random sampling approach. Right before starting the exposure phase, the colonies shall be assessed for a third time in the pre-exposure phase.

## Stratified random allocation of colonies

This may be done in different ways. A convenient way is using the R package 'anticlust'. The function 'anticlustering' can be used to form groups with large in-group variance and low between-group variance in several parameters, such as a) colony weight (mean of both assessments), b) number of adults (mean of both assessments), c) number of dead adults (second assessment), d) brood production (difference in number of cocoons between first and second assessment), e) change in adults (difference in number of adults between first and second assessment) and f) syrup consumption (difference in weight between first and second assessment). These groups are then randomly assigned to different treatments (i.e. pesticide concentrations). If there are more colonies than required for the experiment, the function 'matching' can be used to select sets of colonies that are most equal. A set contains as many colonies as there are treatment groups as the colonies shall be distributed to the different treatment groups. The sets of colonies with the lowest in-set variance are used as experimental colonies, the remaining ones as spare colonies. The function 'anticlustering' is then used to form groups with minimal between-group variance that are randomly allocated to the different treatments (i.e. pesticide concentrations).

Alternatively, spare colonies may be selected based on specific criteria e.g. low syrup consumption per bee. An alternative method of allocating colonies to treatments is ranking the experimental colonies based on (a) mean number of adults (over the two measurements), (b) mean increase in adults (between the two measurements) (c) mean number of cocoons (d) number of dead adults in last measurement (reverse, i.e. ascending order). Then, calculate the sum of these ranks and order them to obtain a colony strength rank. Divide colonies into groups twice as large as the number of treatment groups, as these groups will then further be divided into colonies with high and low syrup consumption per bee. Randomly allocate the colonies of these subgroups to the different treatment groups.

The anticlustering approach is however generally preferable as it is superior in minimizing between-group variance. A combination between the anticlustering approach and the hierarchical approach is conceivable to give different weight to different covariates.

Consider attaching a sticker on the nest boxes that is color-coded according to treatment group.

## Exposure period

During the exposure period the colonies shall be assessed daily and syrup containers will be exchanged daily (at least during the first few days when pesticide concentrations decline to mimic degradation). That means steps 5-10 shall be repeated except that syrup containers are being exchanged for samples with pesticide concentration (where applicable). The sample weight must also be recorded prior to placing it inside the nests. New queens shall be counted, caught and frozen in falcon tubes (with indication of hive ID, and date).

### Post-exposure period

In the post-exposure period, assessments shall be done weekly.

### Colony termination

The colonies shall be freeze-killed at the end of the experiment to allow dissecting the colonies afterwards. Colonies will be freeze-killed when (a) they lose their foundress queen (b) they produced queens (c) they produced queen cocoons 3 weeks earlier. Two weeks after 10% of the colonies were killed due to reasons b or c, all remaining colonies shall be terminated.

## Parameters

### Number of dead adults, pupae and larvae

The number of dead adults, pupae and larvae shall be counted during every field assessment. In the first post-exposure assessment and the last assessment as well as every 2 weeks during the post-exposure monitoring period, dead bodies shall be removed, and where possible be sexed (depending on the developmental stage) to obtain numbers per caste.

### Number of living adults

During every field assessment, a photo shall be taken of the colonies. The number of living adults will be estimated by counting the adults on the photo and subtracting the number of dead adults.

### Number of cocoons

The number of cocoons is assessed during the pre-exposure period by taking a photo and counting cocoons. As the colonies grow this method gets increasingly difficult to use especially if the wax cover that some colonies create is not removed. Therefore, in the field it should be decided whether it makes sense to assess this parameter throughout the exposure period. In the post-exposure period, only a count on the first assessment day should be attempted. However, throughout the experiment queen cocoons should be looked out for. These are larger in size (>12 mm width; Rundlöf et al., 2015). A more accurate assessment will be done during the dissection of the colonies after they have been freeze-killed.

### Queen presence

In every colony assessment, it should be noted whether the queen was seen alive, seen dead or not seen.

### Queen production

Each nest box will be fitted with a queen excluder which will prevent new queens from leaving. At each assessment new queens will be caught, placed in labelled containers and transported preferably on dry ice or similar to a deep freeze. The number of queens and their size will also be analysed in the final colony dissection.

### Colony weight

In every assessment, the weight of the nest box including bees and nest should be weighed. It should be ensured that the syrup container is removed prior to weighing.

### Syrup consumption

Syrup containers should be weighed before and after they have been inside a colony and the difference in weight indicates the amount of syrup consumed.

### Pollen collection

Pollen traps should be attached on the colonies for 24 hours at least once during the exposure period and once right after the exposure period at the beginning of the post-exposure period. It is advisable that pollen collection will also be assessed once during the pre-exposure period and additional times during the post-exposure period.

### Flight activity

Flight activity may be monitored using electronic bee activity monitors. These should be attached and activated during the pre-exposure and exposure period and should only be temporarily removed on days where pollen traps are to be attached on the colonies

### Final colony dissection

In the final colony dissection, the number of adults per caste, their size (ITD, body mass) as well as the number of cocoons, sex ratio of pupae, pupal size and several other parameters will be recorded as described in a separate protocol (WP1.5.9) & the BSc thesis of Nadja Warth.

## Residue analysis

### Verification of concentrations in syrup

First, the concentration of active ingredient in the pesticide product should be measured to determine whether the actual concentration matches the label concentration. This is particularly important if the product container has been opened in a previous year. Then, the standard containing the highest concentration shall be examined preferably before the dilutions are prepared. Later on, if possible all dilutions shall be tested. To reduce costs, it is also possible to test only the highest and the lowest concentrations of a dilution sequence.

### Verification of pesticide exposure of bees

Adult workers shall be sampled ideally a first time shortly before the exposure period and then on several days during the exposure period and be screened for the active ingredient of the tested pesticide to verify exposure. In addition, multi-residue analysis may be conducted to identify exposure to other pesticides that the bees may be exposed to as they are foraging freely in the landscape. It may be advisable to test this on pollen to obtain values that can be easily compared to literature values and to quantify dietary exposure.

## Required materials

- Bumblebee colonies in commercial nest boxes
- Bumblebee housing unit (e.g. wooden box)
- Feeding containers
- Water dispensers
- Queen marker
- Automatic bee activity monitors with sufficient batteries (optional)
- Pollen traps
- Data-sheets, pens, folders and clipboards
- Plexiglass covers
- Camera or smartphone with camera
- Black umbrella
- Balances
- Pesticide
- Syrup
- Falcon tubes (for sampling for residue analysis)
- Cool box with (dry) ice (when queens are caught)
- Cool box with dry ice (when bee samples are taken for pesticide residue analysis)
- Protective beekeeping suits
- Single-use gloves
- Sweep nets
- Bee handling units that fit over bumblebee colonies and enable colonies to be handled with reduced risk of bees escaping and/or stinging (optional).
- Sharpies
- Single-use zip bags
- Color-coded stickers

## References

Rundlöf, M., Andersson, G.K.S., Bommarco, R., Fries, I., Hederström, V., Herbertsson, L., Jonsson, O., Klatt, B.K., Pedersen, T.R., Yourstone, J., Smith, H.G., 2015. Seed coating with a neonicotinoid insecticide negatively affects wild bees. Nature 521, 77–80. https://doi.org/10.1038/nature14420