

Protocol for field assessments of *Bombus terrestris* colonies

Colony specifications

Purchase Biobest colonies in a standard-size nest (23x30x20 cm) from a local supplier (not Biobest) that have

- a queen, cocoons and 30-35 adult workers
- a queen excluder
- a one-way gate on the entrance
- no cotton cover
- pollen and nectar supplement for transport.

(this is all standard, except for the number of adult workers which is lower than in a standard Biobest colony)

Special equipment

- Transparent acrylic/plexiglass covers with the following dimensions: (to be purchased by your organisation).
- Electronic bee counting units (prepared by ATPOLL and delivered from ALU-FR).

Timeline

- 1) Pre-placement assessment (in the lab; about one week before apple/almond bloom)
- 2) Allocation of colonies to study sites (as soon as data from the pre-placement assessment is available)
- 3) Colony placement at study sites (about 4 days before treatment (i.e. mechanical weeding or glyphosate application))
- 4) In-field assessment (first: 1 day after colony placement, then weekly)
- 5) Treatment
- 6) Sampling of bees for haemolymph extraction (1 day after Roundup application)
- 7) Sampling of bees and pollen for residue analysis (1 day after Roundup application and when all apple/almond fields started flowering; pollen traps need to be activated the day before)

Pre-placement assessment

The pre-placement assessment shall be done in a dark room under red light. As bees cannot see red light, they (typically) do not fly when opening the nest. Steps to be taken are

- 1) Label the nest box with a unique colony ID.
- 2) Remove nectar reservoir from the interior of the nest (if present).
- 3) Exchange lit by a transparent cover and close it.
- 4) Weigh the nest box.
- 5) Count the number of dead adults and pupae and remove them.

- 6) Mark the foundress queen and note if the marking was successful and if not if the queen was seen.
- 7) Take a sample of ten adults and store them in RNA later (about 1 ml per bumblebee).
- 8) Take a photo of the nest. Make sure the nest number can be seen on the photo. If possible turn normal light on for the photo. Turn it off again afterwards.
- 9) Count number of cocoons visually (not from photo).
- 10) Open Biogluc syrup container underneath the nest and close it shortly before transporting the colonies to the sites.

Allocation of colonies to study sites

Send the excel file with the pre-placement assessment data to Dmitry (dimitry.wintermantel@nature.uni-freiburg.de) so that he can run an R script that will allocate the colonies to the study sites. Allocation will be done in a semi-random fashion as

The R script will rely on the package 'antclust'. First, groups with large in-group variance and low between-group variance in several parameters will be formed.

The function 'antclustering' will 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 'antclustering' is then used to form groups with minimal between-group variance that are randomly allocated to the different treatments (i.e. pesticide concentrations).

Colony placement at study sites

The colonies shall be placed at a minimum distance of 3 m from each other at the northern end of the study site (facing the study site to the south or south-east). Any nectar supplement should be removed and the large Biogluc reservoir underneath the colony should be closed if this wasn't done already before. One of the six experimental colonies shall be equipped with an automatic flight activity monitor that continuously records the number of bees entering the nest. It is important, that the one-way gate that prevents bees from exiting through the entrance tube remains in place throughout the whole study. Another of the six colonies shall be equipped with a pollen trap that shall be activated on the day of Roundup application (immediately after) and when apple/almond fields start to be in full bloom.

In-field colony assessments

The colonies shall be assessed 5-6 times in the field. If there is sufficient time between placement and treatment, the colonies shall be assessed once before treatment and 5 times after treatment. A full assessment, which shall be done 3 days after treatment comprises of the following steps:

- 1) Take a photo of the nest. Make sure the nest number can be seen on the photo.
- 2) Weigh the nest box.
- 3) Remove the wax cover very gently. And weigh the removed wax. Record the weight in g.
- 4) Count the number of dead adults and ejected larvae (or dead brood in general) and remove them.
- 5) Indicate if the foundress queen was seen alive, seen dead or not seen.
- 6) Indicate whether queen brood is present (diameter over 1.2 cm).
- 7) Take another picture. This time place your finger and the wax in one cover to distinguish it from the first picture.
- 8) Remember to take a third picture at night.

In all the other assessments, the removal of the wax cover (steps 3) and dead bees is omitted (dead adults are however always counted and dead brood are counted until 1 week after the full assessment).

Colony termination

Colonies shall be terminated if they lose their foundress queen or at the end of the experiment (i.e. approximately 1 month after treatment. If colonies start producing queens earlier this period may be reduced). Before removing colonies from the field, change if possible the gate settings so that bees can only enter and collect the colonies after some time - ideally at night (Inform night team to collect the colony and to place it in the basement). The next morning, count and remove dead bees under red light. Then freeze the colony.

In addition, all colonies shall be collected after the last field assessment that is about 1 month after treatment (if queen production commences earlier it can be decided to start this earlier). In a dark room under red light, remove and count dead adults and larvae. Remove ibuttons, too. Then freeze colonies. Terminated colonies shall be labelled with site ID, colony ID, date of removal from field, date of removal of dead bees.

Evaluation of photos

The colony photos shall be used to estimate (a) the wax cover area, (b) the initial number of cocoons, (c) the number of adults and the area covered by a wax ceiling. For this, the wax cover area will be estimated as the percentage of the nest that is covered by wax, whereby 100 % is defined as the area of the slightly elevated square within the nest box including the about 2 cm wide boarder to three sides (Fig. 1). This means values above 100% are not impossible. The number of cocoons shall be estimated in the pre-placement assessment and if possible (i.e. if the colonies haven't grown too much already) in the field assessment before application (if done) and in the first field assessment after application. The number of adults shall be estimated in all assessments and includes all adults including queens and dead adults. The number of living worker/male adults shall later be calculated.

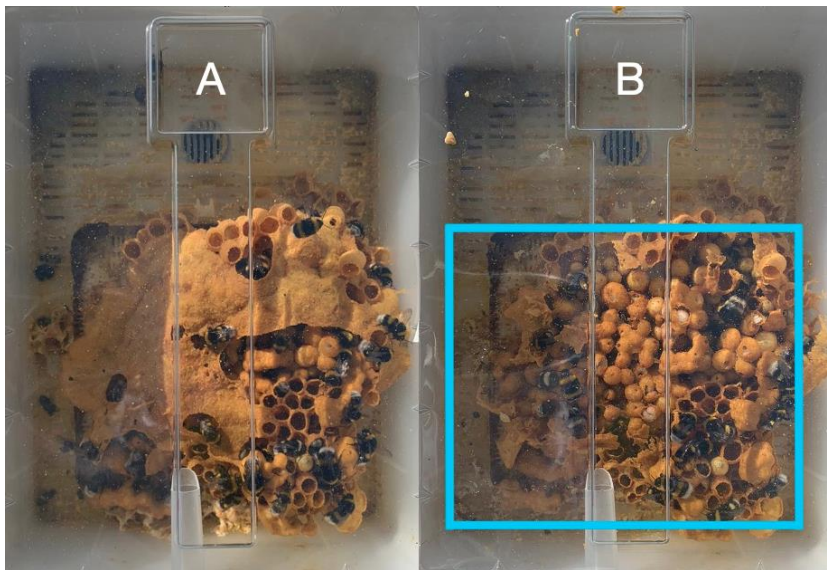


Figure 1: Bumblebee colony partially covered by a wax ceiling (A) and the same bumblebee colony after removal of the wax ceiling (B). The blue line delimits the area defined as the 100% reference area.

Data storage

A WhatsApp group designated only for photos of handwritten data sheets and photos of nests shall be created. The WhatsApp group is meant as a backup in case of data loss. The photos shall also be stored in separate folders per assessment date on a computer and a drive. The collected data shall timely be entered into excel files that can be accessed via a link (that will be sent before the beginning of the study) of which regularly local copies shall be made for back-up.