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Coby van Dooremalen, Zeynep Ülgezen

WR

B-GOOD

Giving Beekeeping Guidance by cOmputatiOnal-assisted Decision making



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Preface

This deliverable consists of a set of open access protocols offered with IT functionality to ensure harmonization of data and sample collection.

Summary

In this deliverable, a set of scientific protocols and manuals is presented for the upcoming field season (2021) of Tier 1 Pilot A and Tier 2 of the B-GOOD project, including information on the implementation of protocols onto the BEEP app. Tier 1 of WP1 consists of apiaries in eight B-GOOD partner institutes, and Tier 2 consists of 40 selected beekeepers by five B-GOOD partners in different countries (eight beekeepers per country). Tier 1 protocols have been updated and Tier 2 protocols have been created after an evaluation and discussion process that took place between WP1 partners in 2020, following several months of data collection. Several changes and additions were made in data collection methods for Tier 1. For Tier 2, some protocols were dropped or adjusted when necessary to accommodate beekeepers. At this stage, protocols are shared with partners and beekeepers through restricted online channels. Validated and evaluated versions will become publicly available at the end of the project. The latest versions of the protocols and manuals are provided in this document.

1. The need for protocols and manuals

WP1 facilitates the collection of standardized and large-scale data on honey bee health indicators across the EU, preferentially in an automated or semi-automated way. Protocols and manuals for (end-)users ensure harmonization of the data and sample collection during and after the project. At this stage, the protocols can be divided in two groups: the manuals to use the BEEP base and BEEP app, and the scientific protocols to perform additional field measurements of the colonies.

Workplans were written for both Tier 1 Pilot A and Tier 2 within WP1 (not presented in this deliverable as they are working/living documents). Pilot A workplan was written in the first few months of the project. The workplan includes protocols and manuals providing guidelines about beekeeping and provides information on when to use the different protocols and manuals. This workplan has been updated after the first round of evaluation to appropriate the new set of protocols and frequencies of data collection for 2021. For Tier 2, a workplan was written at the end of 2020 to start data collection by beekeepers in 2021. The workplan includes protocols and manuals, but guidelines for beekeeping are not provided. Beekeepers in Tier 2 will continue beekeeping according to their usual practices. Preparing workplans and protocols conform with DoA planning.

This first set of manuals and protocols (protocol No.1) was prepared solely for use in Tier 1 in WP1 (D1.1). These documents have now been validated and evaluated in WP1 for their ability to give insight into the health status of the colonies, but also for bee-disturbance and user-friendliness. Protocols that are considered for Tier 2 should 1) give adequate insights of the (health) status of a colony, 2) refrain from disturbance of the bees, and 3) be user friendly. Based on these criteria and evaluation of Tier 1 protocols, a set of protocols was selected for Tier 2, and some adaptations were made if deemed necessary to fit the criteria. For example, the Liebefeld method protocol will not be put through to the beekeepers in Tier 2 as it was found to be too disturbing for the bees and very labour-intensive during evaluation. This second set of protocols (D1.6) are for use in Tier 1, by research institutes, and Tier 2, by selected beekeepers only, in WP1.

2. Publication of manuals and protocols

2.1 Scientific protocols

Where possible, existing protocols from the literature were used and adapted for B-GOOD use. The protocols are published (for the time being) on the consortium communication channel *Microsoft Teams*. The plan for Tier 2 and Tier 3 is to incorporate the protocols that meet all criteria (give adequate insights of the (health) status of a colony; refrain from disturbance of the bees, and be user friendly) for throughput to the next Tier in the BEEP app. Where possible, data collection conforming to the latest versions of the protocols has already been incorporated in specific inspection sheets (checklists) in the BEEP app. The inspection sheets will be used by the users that collect data on the colony (health) status in the BEEP app.

In parallel to evaluation sessions of protocols and workplan, feedback is being continuously gathered by Tier 1 participants via the helpdesk (D.1.2 Helpdesk). Based on the feedback throughout 2020, protocols have been updated and uploaded on *Teams* for immediate implementation. This feedback process was also used for final decisions made in the new set of adapted protocols that are a part of this deliverable (D1.6). Beekeepers in Tier 2 will be encouraged to use the helpdesk as well.

The laboratory protocols that are being used from May 2020 onwards for disease analyses by the National Reference Laboratories (FLI and SCIEN), those responsible for these analyses, have not been included. This is to prevent confusion for the partners involved in Tier 1 and Tier 2, as neither the partners nor the beekeepers have to perform these analyses.

2.2 BEEP app and BEEP base

The open source BEEP base and BEEP app are already publicly available. For more information see: <https://beep.nl/home-english>. Manuals and other support documentation are also made publicly available, see <https://beepsupport.freshdesk.com/>. If updates are needed, this will be shared online. This includes extensions to the standardized BEEP data category structure. In this way, current end-users, not related to B-GOOD, but using the BEEP app and BEEP base, will have instant benefits from BEEP app and base improvements derived from the B-GOOD project. This strategy ensures instant communication, exploitation and dissemination of knowledge and improvements to current end-users.

3. Protocols and manuals

Data collection methods throughout the experiment and data entry onto the BEEP app are detailed below. Not all protocols prepared for Tier 1 will be used in Tier 2. Please see Table 1 for an overview of measurements over time, protocols used for Tier 1 and Tier 2, the relevant protocol for each method and more information on data collection. Detailed information about methods for data collection are provided in the protocols. When possible, activities are combined to minimize colony disturbance. As documents will be updated throughout the project, a version number is added to them (e.g. v20200220, i.e. version 2020 February 20th).

Tier 1 Five inspection sheets were prepared for use in the BEEP app, covering the different protocols: 1 Winter; 2 Varroa, 3 Summer+, 4 Summer, 5 Health.

Tier 2 Four inspection sheets were prepared for use in the BEEP app, covering the different protocols: 5 Health; 6 Overwintering; 7 Bee Season; 8 Bee Season+

Experimental observation	Protocol	Timing	Months											
			Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Presence of queen & brood	P1	1 x month *												
Top photo analysis	P3	1 x month												
Sampling for lab analyses	P5	3 x year *												
Clinical signs of disease	P7	1 x month *												
Overall impression	NA	1 x week **												
Colony Health	NA	1 x week **												
Colony mortality	NA	1 x month *												
BEEP app inspection sheets to be used			6, 5	6, 5	6, 5	8, 5	7, 5	7, 5	8, 5	7, 5	7, 5	8, 5	6, 5	6, 5

* The months may vary between institutes, dates are only provided as an outline, and to represent activities that can be combined. Participants should make 'local' decisions on timing of data collection, depending on colony status, phenological state and climate of country. See workplan section on experimental observations for more details.

** Preferably should be done once a week, but acceptable to reduce to once a month in case apiary is in a remote area or has difficult access.

3.1 Scientific protocols

For each scientific protocol, field methods are described and the data entry section on BEEP app is provided. Data are collected from research institutes and selected beekeepers via the BEEP app. Methods are described both for Tier 1 and Tier 2. There are deviations in some protocols between tiers. If there are differences, protocols for both tiers are provided. Several protocols in Tier 1 are not applicable for Tier 2.

3.1.1 Protocol 1 Presence of the queen and brood (v20210129)

The presence of worker brood gives information on queen fecundity, the future worker force and the ability of the colony to rear eggs through to adulthood.

Tier 1

Field methods

- Open a colony and sequentially remove frames from the hive.
- Check the hive comb surface until the presence of queen and of all life stages of brood – eggs, larvae, pupae – are verified. The queen should be labelled for easy detection.
- If queen is not found and/or no open brood is present, queen failure is assumed (after rechecking in 1 week). Queen presence can be presumed if eggs are present.
- Please be aware that the queen might stop laying eggs prior to swarming, in early winter and during extreme weather events.
- Record queen presence, brood presence and any replacement queens in the BEEP app.

Tier 2

No deviations from Tier 1.

BEEP app data entry

Data is entered onto the BEEP app on the presence or absence of queen and brood in all stages (eggs, larvae, pupae) (Figure 1).

The screenshot shows the BEEP app interface for data entry. At the top, it says 'Bee colony'. Below this, there are two main sections. The first section is labeled 'Queen' and contains a 'Presence' label with two radio buttons: 'Yes' (which is selected) and 'No'. The second section is labeled 'Brood' and contains a 'Status' label. Under 'Status', there are three columns: 'Eggs', 'Larvae', and 'Pupae'. Each column has two radio buttons: 'Yes' (which is selected in all three) and 'No'.

Figure 1: Screenshot from BEEP app on data entry of P1.

3.1.2 Protocol 2 Liebefeld method (v20210129)

The amount of food resources (honey and pollen), brood size and colony size are key determinants of colony development and survival. As an alternative to the digital photography and analysis method (P8), the Liebefeld method can be used (if there is a lack of financial resources) to estimate these colony traits. Preferably, the same person(s) should conduct the Liebefeld measurement to keep the experimenter bias at a minimum.

Tier 1

Materials

- Pre-marked grid

Making the pre-marked grid

For convenience, we recommend to make an 'open' grid with metal wires by using a standard hive frame of the same dimensions with the frames used in the apiary (Figure 2).

- Drill small holes 5 cm apart on the wooden frame on all four sides.
- Put wires through the holes and stretch them across the frame to connect holes and create squares.
- Measure the squares that are not 5x5 cm, so you know the surface area and are better able to estimate how much of a 5x5 cm square would be covered with bees, brood, etc.

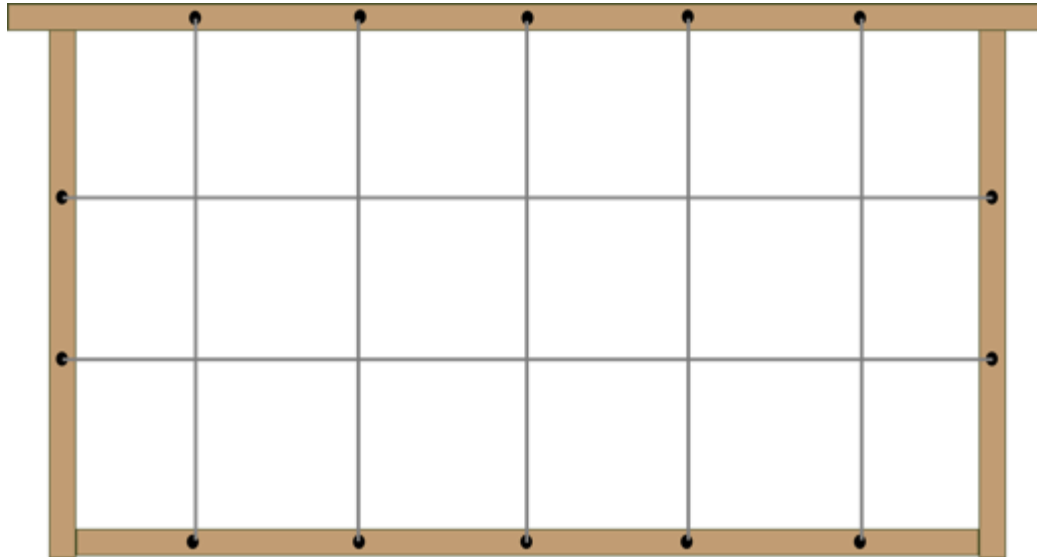


Figure 2: Scheme of grid for Liebefeld method. Squares should be 5x5 cm².

Field methods

Estimation of comb surface covered by bees/brood/honey/pollen with a grid

- Colony traits that need to be measured are: colony size (bees), capped brood (pupae), open brood (larvae), eggs, drone brood, pollen stores, and honey (sealed only). When estimating, the separation between capped brood, open brood and eggs only applies to worker bees. When estimating drone brood, include 'brood in all stages (eggs, larvae and pupae).
- Open a colony and sequentially remove combs of bees (frames).
- Overlay each side of every comb in a hive with a grid pre-marked in 5x5 cm² (Figure 2).
 - 1) Measure the area covered with bees: count the total number of squares covered with bees per frame side. This includes the number of squares fully covered and the ones partially covered. The partially covered squares should be estimated as the proportion covered, up to one decimal point. Record the number of squares in the BEEP app for each side of all frames in the hive. The BEEP app will automatically calculate the sum of bees.
 - 2) Remove the bees from the frame and estimate the other parameters. For removing the bees, hold a frame above (or half in) the brood box and remove the bees by 1) moving the frame downwards with a sudden stop, 2) holding the frame by one 'ear' and tapping with your free hand on the hand holding the ear (a little rough on the eggs), or use a feather or soft brush (not very hygienic). Frames do not need to be free of bees completely. It is fine if bees stay on the frame as long as the brood and/or food reserves are visible and can be estimated. Repeat the counting of squares for all colony parameters and record the information in the BEEP app.
- In the BEEP app, take care to record the number of squares covered on both sides of each frame in the hive. If a parameter is not present, score it as 0. When entering the data, please keep the same sequence of frames for all parameters, such that in theory we could 'rebuild' the colony frame by frame.

Tier 2

Not applicable for Tier 2.

BEEP app data entry

Data on the number of squares on the Liebefeld grid covered by bees, pollen, honey, capped brood, open brood, drone brood and is entered in the BEEP app Figure 3. Based on the hive dimensions, the number of frames and brood boxes, the BEEP app calculates colony size and the total amount of other colony resources (pollen, brood etc.)

Figure 3: Screenshot from BEEP app on data entry of P2.

3.1.3 Protocol 3 Top photo analysis (v20210129)

Colony size will also be estimated by taking a photo of the top-side of the hive. There are several benefits of this method over Protocol 2 as it is less invasive, faster, and easier, but particularly: (1) it can be used during winter when temperatures are too low for removing frames, (2) it may be easy enough for beekeepers to apply in Tier 2. However, it is a relatively rough and subjective estimation of the number of bees and should be interpreted accordingly.

This method was first published in: van Dooremalen, C., B. Cornelissen, C. Poleij-Hok-Ahin, and T. Blacquiere. 2018. Single and interactive effects of *Varroa destructor*, *Nosema* spp., and imidacloprid on honey bee colonies (*Apis mellifera*). Ecosphere 9(8), e02378. 10.1002/ecs2.2378

Tier 1

Materials

- Camera – preferably DSLR
- Bee smoker

Field methods

- Blow a puff of smoke into the hive from below.
- After a minute, remove the lid and take first a high-resolution photo of your label/number/code of the hive and then of the top-side. For accuracy of the photo analysis,

take care to include the entire set of top frames in the photo, and use a standard angle lens (Figure 4).

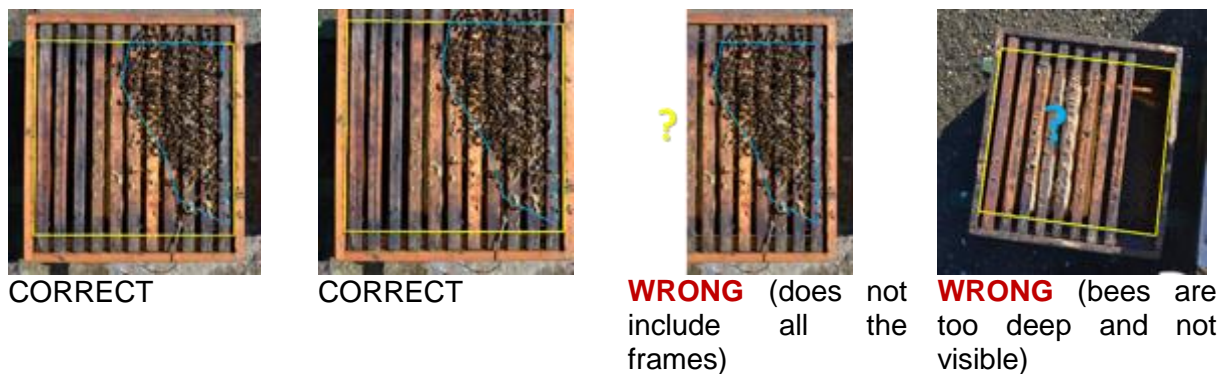


Figure 4: Correct position of hive top in photograph and potential mistakes to avoid. Yellow lines show size of the top side of the box and blue lines show subjective estimation of top side of the bee cluster, if possible to estimate. Only with both can one estimate the number of bees in the colony.

- If there is more than one brood box:
 - Only take a photo of the top box and make sure that the number of boxes entered in the BEEP app is correct.
 - If you suspect that one of the brood boxes is empty of bees, then reduce and adjust the number accordingly in the app.

Computer Analysis:

- Measure the available area and area occupied by bees using the software ImageJ. (<https://imagej.nih.gov/ij/>). Calculate the fraction covered in bees based on the number of pixels in a colony. To do so, calculate the ratio between the number of pixels of the area covered with bees (on top and visible between the frames) and the overall number of pixels of the top area of the box that represents the inner side of the box (see also yellow markings in Figure 4).
- Please see 'TPA_tutorial' (by B. Cornelissen) on Teams WP1 channel files for a how-to video.
- Enter the information of 'pixels with bees' (pixels_colony) and 'pixels total top' (pixels_box) in the BEEP app.
- Upload the photo when entering data in the BEEP app.

Tier 2

No deviations from Tier 1.

BEEP app data entry

Number of pixels of the area covered by bees (pixels with bees) and the overall number of pixels of the top area of the brood box (pixels total hive top) are entered on the BEEP app (Figure 5). Based on the frame dimensions and number of brood boxes and frames, the BEEP app calculates the colony size.

MA 1

Brood layers: 1

Frames per layer: 10

Frame width (cm): 34.9 cm

Frame height (cm): 19.8 cm

Total colony size: -

Photo from top brood box: Select/drop image

Pixels total hive top: 0

Pixels with bees: 0

Colony size (bzz): 0

Figure 5: Screenshot from BEEP app on data entry of P3.

3.1.4 Protocol 4 Counting *Varroa* mites after natural fall (v20200220)

The mite infestation level of each colony will be measured by quantifying naturally falling mites. Although we apply standard *Varroa* control in the mini-apiaries and no Integrated Pest Management, it is still important to measure mite infestation levels of the hive as *Varroa* is considered to be one of the most harmful stressors for honey bees and treatments against it are not 100% effective.

Tier 1

Materials

- Sticky board
- Guide for counting mites (Figure 6)

Field methods

- Use a screened bottom board for the hive, and place a sticky surface on the upper side of the board (the sticky surface should entirely cover the bottom of the hive).
- After one week, remove the sticky surface and count the number of mites. Place a guide above the board (Figure 6) to avoid counting the same mites. If dead bees are present on the board, check them as they act as magnets to fallen live mites.
- Divide the number of mites over the days that the sticky surface is left underneath the hive to obtain the daily mite fall.



Figure 6: A guide (wires across a frame of wood) placed on top of the *Varroa* sticky trap to help with mite counts (taken from Dietemann et al. 2013)

Subsample

- Alternatively, if high numbers of mites are present ($>1,000$), mite counting can be sub-sampled.
- To sub-sample, print a grid with 2 cm² cells on the sticky board (Figure 7)
- Group cells into blocks of nine and randomly select three cells per block. Count the number of mites in each selected cell.
- It is sufficient to count 22% of the cells to obtain a highly reliable estimate (Ostiguy and Sammartaro, 2000).
- Divide the total number of mites in the sub-sample by the number of counted cells, and then multiply by the total number of cells to obtain the number of mites over the 1-week period.

- Divide the number of mites over the days that the sticky surface is left underneath the hive to obtain the daily mite fall.
- Record this in the BEEP app (mite fall per day, method: natural fall).

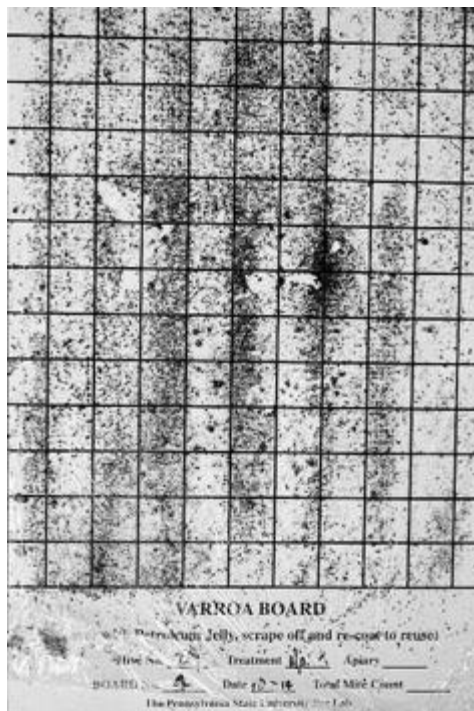


Figure 7: Example of sticky board with grid (taken from Ostiguy and Sammataro 2000)

Tier 2

Not applicable for Tier 2.

BEEP app data entry

The number of mites per day is entered onto the BEEP app (Figure 8)

Figure 8: Screenshot from BEEP app on data entry of P4.

3.1.5 Protocol 5 Sampling bees for lab analyses

For diagnosis of bee diseases, each colony will be sampled three times per year: 1) first in spring, when the bees start to forage; 2) second in summer, when the colonies have reached their maximum size; 3) and a third in autumn before over-wintering.

Tier 1 (v20210129)*BEEP app*

- For anonymization purposes, the BEEP app will generate a unique identifier per sample for laboratory analysis. The samples should be labelled with these IDs. Please label the samples on the container and additionally place a label inside the container (on paper with a pencil), to ensure correct identification, even if the outside label gets damaged or disappears during storage or transport.
- Make sure the samples collected are correctly linked to the colony ID to ensure correct feedback from the lab.
- Record the date of sample collection and the date that samples are sent to the reference labs in the BEEP app.
- Check that the date and time of the inspection containing the sample code in the BEEP app corresponds with the actual sample collection date and time.

Materials

- 8 x 100 ml cups
- 8 x perforated lids
- 8 x solid lid (no holes)

Collection of samples

- To collect samples, first open the colony and check the combs starting with the frames on the outer edge.
- Remove the first frame fully occupied by bees on both sides (most likely this will be a frame at the periphery of the brood nest).
- Make sure that the queen is not present on the comb; if present, return her to the hive (or take another frame).
- Fill a 100 ml cup with bees; it will be around 300 bees when the cup is full.
- One of the best methods to do this is to shake the bees from the frame onto a sheet, then bend or fold the sheet and use it to fill the bees into the cup. Many colonies have a covering foil on top underneath the lid. One can use this sheet but you can also bring a sheet or use a sheet of newspaper. The cup should be placed on the top of the open colony so all bees that spill out of the cup fall directly back into the colony. With this method, you can easily fill the cup to the top.
- Alternatively, if the bees are calm, fill the cup by scraping the bees off the comb, holding the cup vertically and the comb at 45°.
- To keep bees alive until freezing, make sure that there are holes in the lid.
- It is fine if drones are included in the sample, but fewer is better.
- Once in a lab, freeze bees as quickly as possible, by either using liquid nitrogen, dry ice or placing cups in a freezer at -80°C.
- After freezing, place a solid lid (no holes) on the cup to prepare samples for storage and transport.

Storage and transport

- As soon as the sample is frozen, it should never defrost until the moment of RNA or DNA extraction. Therefore, it is important to establish a continuous cold chain for the samples.
- The transfer of samples to the B-GOOD lab for lab analysis must be done on dry ice, by courier services that enable fast transport. Preferably, samples will arrive within 72 h after sampling in the field at FLI/SCIEN institutes. Arrival of samples should be within 48 h from the moment that they are sent by courier.
- Apiaries should coordinate the sending date of samples with reference labs before arranging transport. Make sure that samples do not arrive at the labs on weekends. Suggested planning:
- Sampling on Monday/Tuesday

- Send samples to FLI/SCIEN on Monday/Tuesday/Wednesday (latest)
- Samples should be sent together with the signed MTAs and a list of sample codes.
- If drone eggs have been sampled as well (P9), do not forget to include them when sending samples. Please also include a list of sample codes for drone eggs.
- The Institutes to which the samples need to be sent are:
SCIEN for: UGENT, UCOI, TNTU, INRA
FLI for: WR, UCLUJ, UBERN, MLU

Dead colonies

A colony is considered dead if 1) the hive is absent of any living bees 2) the colony is too weak to recover in spring because (i) less than two frames are occupied by winter bees or (ii) the queen is dead and the hive cannot replace the queen by building emergency cells (no brood).

- If colonies are dead, and there are still bees present in the hive, collect samples. Store them until the moment of regular sampling and send them to the reference labs with the same batch. Indicate on the sample that it involves a dead colony.
- If possible, collect and store the queen separately.
- Record in the BEEP app under 'Loss'

Tier 2 – Beekeepers (v20201016)

BEEP app

- For anonymization purposes, the BEEP app will generate a unique identifier per sample for laboratory analysis. The samples should be labelled with these IDs. Please label the samples on the container and additionally place a label inside the container (on paper with a pencil), to ensure correct identification of the sample, even if the outside label gets damaged or disappears during transport.
- Make sure the samples collected are correctly linked to the IDs to ensure correct feedback from the lab.
- Record the date of sample collection and the date that samples are sent to the reference labs in the BEEP app.
- Check that the date and time of the inspection containing the sample code in the BEEP app corresponds with the actual sample collection date and time.

Materials

- 3 x 0.5 L freezer containers with drilled holes (max. 2 mm) (Figure 9).
- Carton queen shipping box (with ventilation openings).
- 3 x queen cages with sugar dough in them.



Figure 9: Example of freezer container with holes

Collection of samples

- To collect samples, first open the colony and check the combs starting with the frames on the outer edge.
- Remove the first frame fully occupied by bees (most likely this will be a frame at the periphery of the brood nest).
- Make sure that the queen is not present on the comb; if present, return her to the hive (or take another frame).
- Fill the freezer container with about 300 bees (a 100 ml cup or 40g without container contains about 300 bees).
- One of the best methods to do this is to shake the bees from the frame onto a sheet, then bend or fold the sheet and use it to fill the bees into the cup. Many colonies have a covering foil on top underneath the lid. One can use this sheet but you can also bring a sheet or use a sheet of newspaper. The cup should be placed on the top of the open colony so all bees that spill out of the cup fall directly back into the colony. With this method, you can easily fill the cup to the top.
- Alternatively, if the bees are calm, fill the cup by scraping the bees off the comb, holding the cup vertically and the comb at 45°.
- To keep bees alive, make sure that there are holes in the container and/or on the lid.
- It is fine if drones are included in the sample, but less is better.
- Place the queen cage with sugar dough into the container to equip bees with some food prior to transport.

Transport

- Place the freezer containers with bees into a queen shipping box to prepare for transport. Please make sure that the containers are firmly closed and cannot open during transport.
- Coordinate the dates of transfer for samples with the B-GOOD partner organisation in your country before arranging transport.
- The samples should be sent the same day of sampling in the field.

Tier 2 – Partners (v20201016)

Storage & transport

- Once you receive samples from a beekeeper, note if the shipping was successful and whether the bees are still alive.
- Immediately freeze the bees by either using liquid nitrogen, dry ice or placing containers in a freezer at -80°C.
- After freezing, place a solid lid (no holes) on the container or exchange the container for one with a solid lid to prepare samples for storage and transport. Please make sure that the labelling is correct and will withstand the freezing (best is label inside the container on paper with identification code written in pencil).
- As soon as the sample is frozen, it should never defrost until the moment of RNA or DNA extraction. Therefore, it is important to establish a continuous cold chain for the samples.
- The transfer of samples for lab analysis must be done on dry ice, by courier services that enable fast transport. Arrival of samples should be within 48 h from the moment that they are sent.
- Send all samples (8 beekeepers x 3 colonies = 24 samples) from each sampling period together.
- Partners should coordinate the sending date of samples with reference labs before arranging transport. Make sure that samples do not arrive at the labs on weekends.
- Samples should be sent together with the signed MTAs.
- The Institutes to which the samples need to be sent are:

SCIEN for: WR (50%), SML, BSOUR

FLI for: WR (50%), UBERN, MLU

BEEP app data entry

A unique Sample ID code is generated by the BEEP app in a new inspection for each colony to be sampled, which is then used to label samples collected for disease analysis (Figure 10).

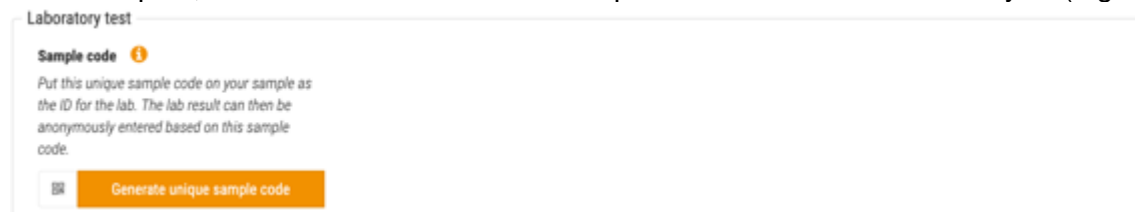


Figure 10: Screenshot from BEEP app on data entry of P5.

3.1.6 Protocol 6 Atypical worker behaviour (v20210129)

Atypical behaviour by workers is one of the first signs of diminished health within the colony. It does assume a basic knowledge of normal typical behaviour of honey bees.

Tier 1

Field methods

- Visually assess several combs inside of the hive for the presence/absence of worker bees showing atypical behaviour.
- A majority (> 60%, including brood comb) of frames containing workers inside the hive should be inspected.
- See Table 2 for honey bee behaviours; any behaviour outside of the normal repertoire for bees can be considered as atypical
- Some examples of atypical behaviours include: running quickly over the comb for long periods, trembling (aside from the trembling dance) or shaking.

Table 2: Honey bee worker behaviour catalogue (Scheiner et al. 2013)

Task	Description
cell cleaning	removing debris from used brood cells (cocoons, larvae excretion), cleaning cell walls. Takes place in a cell not currently being used
general nest sanitation	removing debris from nest (mouldy pollen, old cappings, dead brood, and dead adults)
brood care	feeding larvae (head in brood cell > 1.3 min), attending queen
construction	smoothing wooden hive parts with mandibles and manipulating wax and propolis in cracks and corners of the hive
fanning wings	flapping wings while standing in hive/at entrance
food care	insertion of head into a cell containing nectar, receiving nectar-on bridge
grooming a nestmate	running nest mate body parts through mandibles
grooming self	running own body parts through mandibles
inspecting a cell	momentary insertion of the anterior portion of the head into an empty cell
nest care	manipulating wax of cells (not cappings), building new empty cells
patrolling	walking around nest
standing and chaining	standing stationary or hanging while stationary on nestmates
brood cap manipulation	trimming or smoothing wax cappings on brood cells and capping brood with wax
honey cap manipulation	trimming or smoothing wax cappings on cells of honey and capping honey with wax
trophallaxis	nestmate exchange of food (not near entrance), receiver thrusts tongue at donor's mouthpart, donor opens mouthparts, pushes tongue forward, and regurgitates a drop which is lapped up
vibrating	fast rhythmic body vibrations (non-dance)
head in pollen	insertion of head into a cell containing pollen
inspecting brood	head in brood cell, < 1.3 min
dancing	dancing without/with pollen
washboarding/ plaining	standing and rocking back and forth with mouthparts open
attending dance	dance attendance without/with pollen

Tier 2

Not applicable for Tier 2.

BEEP app data entry

Data is entered in the BEEP app on the presence or absence of atypical behaviour (Figure 11).

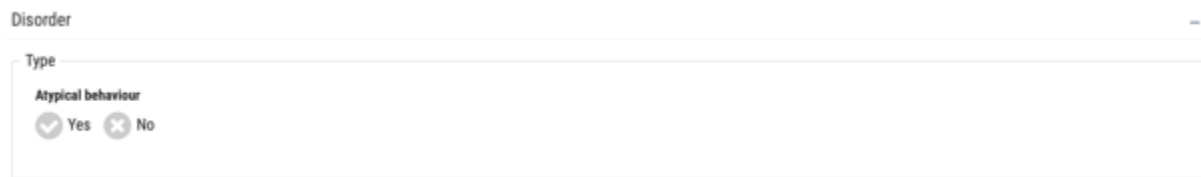


Figure 11: Screenshot from BEEP app on data entry of P6.

3.1.7 Protocol 7 Clinical signs of disease (v20210129)

Tier 1

Field methods

- Visually observe colonies to assess the presence of clinical signs of disease in brood and adult bees. A detailed list of the clinical signs of diseases observed in European honey bee colonies can be found below. If you have the suspicion that a disease is present in the hive, record the type of disease in the BEEP app.

VARROOSIS ('Varroa mites' in BEEP app)

Clinical signs

Colony:

- Reduced colony population and/or altered demography
- Collapsed colony/empty hive

Adult honey bees:

- Presence of phoretic mites on honey bees
- Smaller bees
- Crawling bees
- Honey bees with deformed/atrophied wings
- Honey bees with a small abdomen

Brood:

- Mosaic brood/spotty pattern
- Abnormal perforations in caps (small holes)
- Abnormal sealing of cells
- Presence of *Varroa* in brood cells (brown mature females/white immature stages)
- Light brown to brown dead larvae (check for absence of AFB as 'ropey' larvae/sealed cells)
- Cannibalised/slumped/dicoloured brood
- Dried or otherwise dead larvae
- Deformed/dead pupae/ Dead honey bees with deformed wings in sealed cells
- Dead emerging bees in brood with extended proboscis (only the head emerges with the tongue sticking out)
- Mite faeces

AMERICAN FOULBROOD

Clinical signals

- "Spotty" brood pattern / mosaic brood
- Capping with different colour/ dark sunken cell cappings/ holes in cappings
- Ropy larvae (use the match test)
- Coffee brown coloured larvae
- Tongue of dead larvae pointing upwards

- Sticky scales
- Specific 'fishy' odour of sick larvae

EUROPEAN FOULBROOD

Clinical signals

- Mosaic brood/spotty brood pattern
- Capping with holes
- Slumped larva
- Larvae with a yellowish to brown colour (generally in unsealed brood)
- Vinegar or putrefaction odour of the larvae

NOSEMOSIS ('Nosema' in BEEP app)

Clinical signals

- Dead honey bees in front of the hive
- Crawling honey bees, bees clinging to the grass
- Traces of diarrhoea outside and/or within the hive
- Reduction in colony demography and/or population

CHRONIC BEE PARALYSIS VIRUS

Clinical signals

- Trembling and/or crawling honey bees
- Dead honey bees in front of the hive
- Small black honey bees, shiny and hairless
- Honey bees rejected by guards
- Abnormal behaviour at the flight board
- Bees with bloated abdomens, sometimes with diarrhoea

ACUTE BEE PARALYSIS VIRUS

Clinical signals

- Brood and adult mortality
- Presence of *Varroa destructor*

BLACK QUEEN CELL VIRUS

Clinical signals

- Dead queen larvae and prepupae sealed in queen cells with blackened walls

DEFORMED WING VIRUS

Clinical signals

- Brood and adult mortality
- Vestigial and crumpled wings
- Bloated abdomens
- Severely shortened adult life span for emerging worker and drone bees
- Presence of *Varroa destructor*

SMALL HIVE BEETLE

Clinical signals

- Observation of adult beetles, larvae or eggs
- Galleries inside the combs
- Brood destruction
- Modification of the honey colour and honey fermentation

SACBROOD

Clinical signals

- Uneven brood pattern with discoloured, sunken or perforated cappings scattered through the brood cells
- Infected larvae die shortly after capping (with appearance of a Chinese slipper) and fail to pupate

Tier 2

No deviations from TIER 1.

BEEP app data entry

If clinical signs of disease are observed in the colony, the type of disease is entered in the BEEP app (Figure 12).

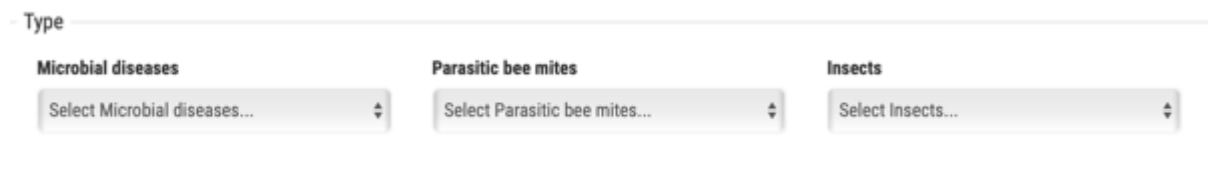


Figure 12: Screenshot from BEEP app on data entry of P7.

3.1.8 Protocol 8 EFSA protocol (v20210129)

This field protocol describes the data collection used in the EFSA project (OC/EFSA/SCER/2017/02) during the last two years. The protocol was re-adapted for the B-GOOD mini-apiaries.

Tier 1

Experimental material

- Field forms
- Empty super
- Scale (max. weight 5 kg, d=5 g), e.g. a hook scale with a support (for a good working height)
- Extra batteries for scale
- Camera (for photographing combs)
- Tunnel + LED lights + support
- Battery for LEDs
- Electrical wire/cable drum
- Marker (for marking frames)

Beekeeping material

- Queen cage
- Queen marker
- Hive tool
- Bee brush
- Fumigator
- Lighter
- Fumigator fuel
- Beekeeping protective equipment

Experimental set up

All hives are marked with permanent numbers. Each super is numbered (bottom super =1), and each frame is marked (with a thick marker on the wood) on both sides (side A and B) and

on top with a unique code. For instance, in Portugal, hive 1, super 2, frame 7 the code will be: PT.1.2.7.A

Timings and frequency of in-hive monitoring

Each site will be visited once every 18-20 days. Monitoring should be done once every 18-20 days starting in early spring (data from hive scales can be used to decide when to start the monitoring). It is extremely important that data are collected within 21 days of the last visit in order for the sampling frequency to cover the developmental time of workers (21 days from egg to adult). However, hive monitoring should be adapted to weather conditions (not possible when raining, and precautions should be taken when temperatures are low ($<14^{\circ}\text{C}$)).

Timing of observations may be important because some foragers will be in the field. Sequence of hive monitoring and visits to study sites should be randomized.

Considerations before starting the in-hive monitoring

Precautions during cold conditions in spring:

- It is important that the brood and queen will not become cold, because they are very sensitive to cooling down, and in spring the colonies contain only few worker bees to generate heat. Hence, food combs should be photographed first, and bees on the brood combs should only be removed as the last task before closing the hive. Adult bees should be brushed directly into the original nest. Handling should be as fast as possible during the photographing of brood in order for the colony not to lose heat.

Precautions during hot periods:

- It is important that the brood and queen will not be overheated. Hence, the caged queen should be kept in the shade.

Precautions during late summer:

- Opening the colonies for a prolonged period, particularly during late summer, may result in honey robbing. In order not to leave the experimental colonies vulnerable to honey robbing during monitoring, assessment should be done as early in the morning as possible.
- Another option could be closing the entrance of the colonies that are not being evaluated with sponge.

Monitoring of experimental hives

- Photographing of combs:
 - Material installation

Prepare the material as follows:

- Install the camera in the tunnel and turn on the lights (set the LED lights to the maximum).
- Put the empty super next to the hive that will be measured
- Turn the scale on and tare it
- Wear latex gloves (like those we use in the lab) on top of the thick gloves. Operators should change the latex gloves between hives. This prevents cross contamination between hives if there is a diseased colony.



Important:

- Always check the scale tare between each weighing

- Hive opening

Record the time the hive is handled (i.e. starting time and ending time), as this will affect the automatically collected hive scale data.

- Frames weighed with bees

Dispense smoke at the entrance before you start so as to keep bees calm and on the frame. Most likely, you would want to combine this analysis with Protocol P3 TPA; if so, we suggest to first take a picture from the top.

Start with top super

Take out the frames **one by one** from the hive.

- First grab **frame 1**
- Weight the frame with bees (record the weight of the frame **with bees**)
- Rest it **softly** in the empty super (without dropping the bees)
- Afterwards, take out the **frame 2**
- Proceed the same way as in frame 1 (as well as for all the remaining frames from **frame 2 to frame 10**)
- if bees are left in the super, also weigh the super with and without bees

Start measuring from the top box and repeat the process until you weight all the boxes from that colony. This way, your **bottom box** will be on the **top** when you finish this first task.



Important:

- Always start from the frame at your **left (frame 1)** and finish with the **rightmost (frame 10)** knowing that right and left are defined by the operator's position, who will be positioned behind the hive (**see Figure 13**).
- It is essential to put the frames in the empty super in exactly the **same order and direction** as in the hive. To do this, put a mark on every frame always on the same side.
- Encage the queen as soon as possible to protect it from potential clumsiness (and mark it if necessary).
 - It is important to know the weight of "frame 5" with bees and without them. So, if we exchange the position of the frames, we need to be careful writing the data because "frame 5" could be in the position of the 7th frame.

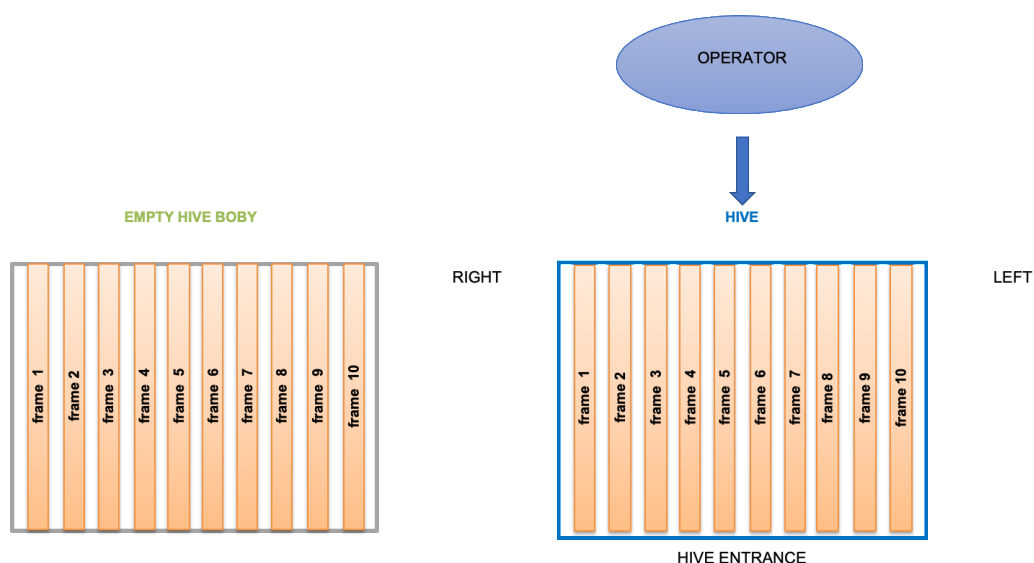


Figure 13: Hive's scheme

All frames should be labelled properly before you start taking photos

- Frames weight 'without bees' and brood measurements

Once the hive is empty, weight the remaining bees on the hive bottom if possible. If not possible, make an estimation on the number of bees considering that a full side of the box is equivalent to approximately 1000 bees.

Afterwards, grab **frame 10** (from the 1st box – it is the **original bottom box**):

- Brush the bees into the beehive in order to remove all the bees
 - Weight the frame (record the frame weight **without bees**)
 - Take two pictures using remote control of the camera from both sides for honey, beebread, capped brood, larvae and eggs analysis. **Check if the first photos are in focus and capturing the whole frame!**
 - Put it back in the hive in the initial location and side
 - Grab **frame 9** and proceed the same way as in the frame 10 (as well as for all the remaining frames from **frame 9 to frame 1**)
 - Photos should be checked at the end (after the hive is closed) if in focus. If the tunnel moves, the focus should be checked again.
- End of operations:

When all the frames are placed identically in the hive:

- release the queen in the hive
- close the hive while taking care not to crush the queen
- register the ending time of operations for each hive
- all field sheets should be kept in a folder
- check photos to see if they are out of focus

When back in lab:

- Transfer image files to computer. Organizing image files; it is important to keep track of date, locality, hive number and frame number.
- Data from field data sheets are entered and stored in a digital platform.

Tier 2

Not applicable for Tier 2.

BEEP app data entry

Currently not incorporated into the BEEP app.

3.1.9 Protocol 9 Sampling drone brood eggs (v20210129)

Samples will be collected once a year, combined with the spring visit for sampling bees for lab analyses. If drone brood is not present in some colonies during the spring visit, these colonies should be sampled the next visit that drones are present. In case the queen is replaced, new samples should be collected from the moment that drone eggs are present.

Tier 1

Materials

- 8 toothpicks
- 8 Eppendorf tubes with screw cap
- Fridge with cold packs or freezer

Field methods

- Open a hive and search for freshly laid drone eggs.
- Screw the Eppendorf tube open and keep it ready.
- To collect the eggs, scoop the toothpick under the egg and lift it up (Figure 14.1). If it is difficult to see the eggs, cutting out a part of the wax comb can help (Figure 14.2).
- Take the Eppendorf tube and place the eggs approximately 1 cm deep in the tube (Figure 14.3 and 14.4).
- Place the egg flat against the tube and turn the toothpick a quarter stroke to release the egg from the toothpick (Figure 14.5 and 14.6).
- When inserting the toothpick, pay attention not to damage previously collected eggs.
- Repeat these steps until 10 drone eggs are collected and firmly close the Eppendorf tube.
- Write the unique ID of the hive on a sticker taped on the Eppendorf tube. This unique ID is the same ID given to the bee samples collected from each hive for disease diagnostics. If drone eggs are sampled at a later date than disease analyses sampling, please use the last code generated by the BEEP app for disease analysis. Make sure you clearly state the sampling date on the samples, in addition to the code
- Collect the required 10 drone eggs for each hive in the mini-apiary. Use a new toothpick and Eppendorf tube for each hive.
- Place samples in a -20°C freezer as quickly as possible. For remote apiaries, samples can be transported in a fridge with cold packs but should be placed in the freezer as soon as possible.
- Samples should be sent on dry ice to the reference labs along with the bee samples for disease analysis.

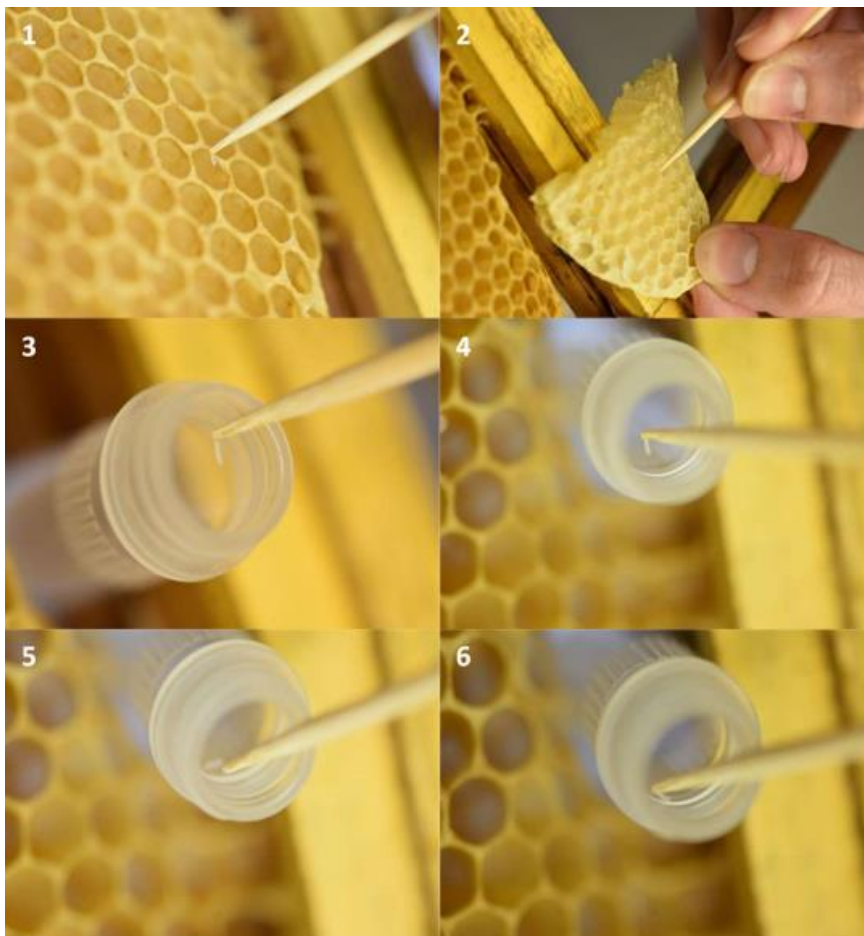


Figure 14: Sampling drone eggs from a frame, in six steps (image 1-6).

Tier 2

Not applicable for Tier 2 as yet.

BEEP app data entry

The same code generated by the BEEP app is used for P5 and P8. See Figure 10 for BEEP app code.

3.1.10 Protocol 10 Queen cell presence (v20210129)**Tier 1***Field methods*

- Open a colony and sequentially remove frames from the hive.
- Shake or brush every brood comb free of bees and examine carefully for the presence of queen cells.
- Record the presence and type of queen cell in the BEEP app (queen cup, emergency cell, swarm cell, supercedure cell).
- If queen cells are removed, please include this information as a management action.

Queen cell types:

- Queen cup: It is a small cup, with an opening on the bottom. For the purposes of this experiment, we define queen cups as empty queen cells (without eggs or larvae)
- Swarm cells: Are built when the colony is preparing to reproduce and swarm. These cells are usually present on the edges of the comb.
- Supersedure cells: Are built when the colony wants to replace the current queen. These cells are generally found on the centre of the comb.
- Emergency cell: Are built if the old queen is dead. Like supersedure cells, they are usually found on the centre of the comb.

Tier 2

Not applicable for TIER 2.

BEEP app data entry

Data on the queen cell presence or absence and if present, type of queen cell is entered onto the BEEP app (Figure 15).

Queen

Cells

☒ Yes ☐ No

Cells

Swarm cell

☒ Yes ☐ No

Queen cup

☒ Yes ☐ No

Emergency cell

☒ Yes ☐ No

Supercedure cell

☒ Yes ☐ No

Figure 15: Screenshot from BEEP app on data entry of P9.

3.1.11 Protocol 11 Brood pattern consistency (v20210129)

Brood pattern consistency is included as it is rated HH-H in the Healthy-B toolbox, and can provide support for data obtained through other measurements in acquiring a more complete view of the health status of a colony. Currently, we opted for this measurement for a qualitative or semi-quantitative assessment. Applying an accurate quantitative method is very labour intensive. Additionally, photo analysis of brood (P8) will already provide in-depth information on brood status.

Tier 1

Field method

- Open a colony and sequentially remove frames from the hive.
- Check the brood pattern consistency on each frame with brood.
- Estimate by eyesight the average percentage of empty brood cells within the area of the sealed worker and drone brood, compared to the total area of sealed brood cells in the colony.

[Alternative method (for people that prefer a more standardized, but labour-intensive method)]

- Overlay the 'Liebefeld grid' on the surface of the frame with brood.
- Select 4 adjacent squares from the Liebefeld grid, thereby creating a 10x10 grid, that encompasses the area on the comb covered by the brood. This area will cover about 100 cells.
- Calculate the percentage brood consistency (100 – estimated empty cells).
- Repeat this procedure 10 times on different patches of brood.
- Calculate the average percentage of brood consistency.]

BEEP app

- Rate the overall brood pattern consistency based on a 5-point scale in the BEEP app

Rating information:

- 5: for areas with sealed brood with <5% empty cells.
- 4: for 5-10% empty cells,
- 3: for 10-20% empty cells,
- 1 or 2: 20% or more.
- Deduct points for spotty coverage of brood cells like larvae mixed with capped cells, lack of brood cluster across frames with largest brood at the centre of cluster, colour differences between caps, sunken caps.

Tier 2

Not applicable for TIER 2.

BEEP app data entry

Brood pattern consistency is rated on a 5-point scale in the BEEP app based on estimates (Figure 16)

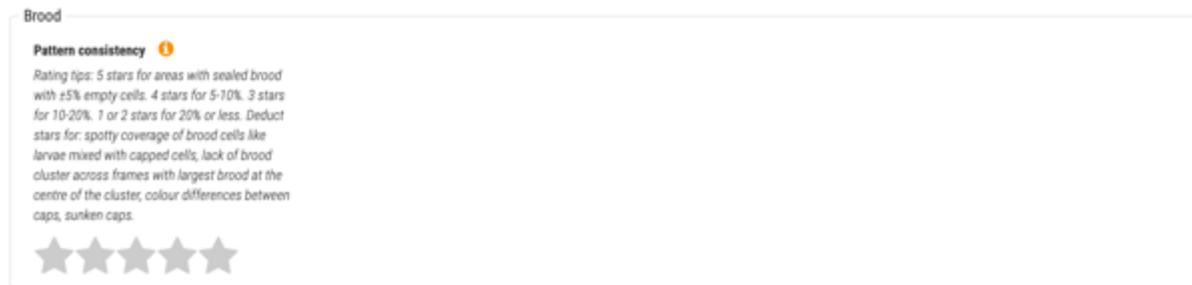


Figure 16: Screenshot from BEEP app on data entry of P10.

3.1.12 Protocol 12 Data cleaning and quality

Tier 1

As a one-off procedure, to evaluate the current data collection protocols for tier I apiaries and revise as necessary to ensure workable data for season 2 (meeting planned).

There is a prerequisite for the data portal to be operational and accessible.

Each month, to carry out the following:

- Within one week of the end of each month the inspection data for that month need to be entered in the BEEP app using the correct checklist and ensuring the date and time of the new inspection corresponds with the date and time of the actual inspection.
- One week following the end of each month AU will generate a pdf report for each apiary manager for each of their colonies to describe the data collected since the last report. The report covers all data entered manually in the BEEP app as well as the automated data collected via the BEEP base.
- INRAE will arrange an online meeting with all Apiary managers to go through any data issues they have experienced, or that are identified in the pdf report. If time allows, the same meeting will be used to log interesting/unusual notes coming from the beekeeper(s) with regards to any (of the 8) colony health statuses.
- INRAE will make corrections to each month's data as necessary and the data will then be uploaded to the data portal where it can be accessed for purposes of B-GOOD. The apiary managers are encouraged to correct the data in the BEEP app as necessary.
- Network of hardware errors can cause (potentially reversible) data loss. We ask that the apiary managers to check their own data weekly via the BEEP app to see if there are hardware or network errors that can be caught on a faster than monthly cycle. Version 3 of the BEEP app will contain a new feature to check this, namely an alerting feature.

We will initially carry out a trial of the procedure using the 2020 data starting from Feb. This way, we can iron out some of the issues we find before the first useful data of 2021.

In order to improve the quality of inspection data, the EFSA photo-based method is encouraged be used instead of the Liebefeld method. Both of the EFSA and Libefeld methods are conducted every 20 days from April to Oct instead 3 times per year.

Tier 2

Not applicable for Tier 2.

BEEP app data entry

Not applicable, as P12 is not a field method for data collection.

3.2 BEEP app and BEEP base

All manuals and other support documentation related to BEEP can be found here <https://beepsupport.freshdesk.com/en/>. [A support desk for questions is also provided.](#) In order to increase user-friendliness for the participants of Tier 1 and Tier 2, a registration guide for the BEEP app was directly entered into the workplan.

4. Acknowledgements

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5. References

Dietemann, V., F. Nazzi, S. J. Martin, D. L. Anderson, B. Locke, K. S. Delaplane, Q. Qauquiez, C. Tannahill, E. Frey, and B. Zielgelmann. 2013. Standard methods for varroa research. *Journal of Apicultural Research* 52, 1–54.

van Dooremalen, C., B. Cornelissen, C. Poleij-Hok-Ahin, and T. Blacquiére. 2018. Single and interactive effects of *Varroa destructor*, *Nosema* spp., and imidacloprid on honey bee colonies (*Apis mellifera*). *Ecosphere* 9(8), e02378.

Scheiner, R., Abramson, C. I., Brodschneider, R., Crailsheim, K., Farina, W. M., Fuchs, S., Grünwald, B., Hahshold, S., Karrer, M., Koeniger, G., Koeniger, N., Menzel, R., Mujagic, S., Radspieler, G., Schmickl, t., Schneider, C., Siegel, A.J., Szopek, M., Thenius M. 2013. Standard methods for behavioural studies of *Apis mellifera*. *Journal of Apicultural Research*, 52(4), 1-58.

Ostiguy, N. and Sammartaro, D., 2000. A simplified technique for counting *Varroa jacobsoni* Oud. on sticky boards. *Apidologie*, 31(6), 707-716.

<https://beep.nl/home-english>

<https://beepsupport.freshdesk.com/en/support/solutions>