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Protocols No.3

Deliverable D1.7

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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. This deliverable is the third and last in the protocol series and follow up on Deliverable 1.3 and 1.6 (Protocols No1 and 2, respectively).

Summary

In this deliverable, a set of scientific protocols and manuals is presented for the upcoming field season (2022) of Tier 1, Tier 2 and Tier 3 of the B-GOOD project, including information on the implementation of protocols onto inspection sheets 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 with a total amount of 120 colonies). Tier 3 aims at 58 beekeepers selected from an open call scattered through the EU, with a total amount of around 174 colonies. The original Tier 1 and Tier 2 protocols, which have been already used for data collection in the process, have been slightly updated. Protocols for Tier 3 have been created and will be used starting from the current season. Protocol updates and changes for all Tiers were based on the outcome of feedback meetings with Tier 1 and Tier 2 coordinators, and evaluation surveys distributed to Tier 1 apiary managers and Tier 2 beekeepers. All Tier 1 and 2 protocols are uploaded to the consortium communication channel, Microsoft TEAMS, accessible to all BGOOD partners. To beekeepers, protocols are distributed via e-mail. Validated and evaluated versions will become publicly available at the end of the project, and will be published on the B-GOOD Data portal. 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 honeybee 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 the project. At this stage, the support documents 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 Tier 1 Pilot A, Tier 2 and Tier 3 within WP1 (not presented in this deliverable as they are working/living documents). A Tier 1 workplan was written in the first few months of the project. The workplan includes (weblinks to) protocols and manuals providing guidelines about beekeeping; it also provides information on when to use the different protocols and manuals. This workplan has been updated after several rounds of evaluations to incorporate a new set of protocols and changes in the frequency of data collection for 2021 and 2022. For Tier 2, a workplan was written at the end of 2020 and data were collected by beekeepers in 2021. The workplan included protocols and manuals, but guidelines for beekeeping were not provided. The Tier 2 workplan will be updated accordingly, with the addition of new protocols, that were selected after being tested in Tier 1. For Tier 3, a workplan was written in the beginning of 2022, and we aim to collect data in 2022. Preparation of workplans and protocols occurred conform the DoA planning.

The first set of manuals and protocols (D1.1 Protocol No.1) was prepared solely for use in Tier 1 in WP1. Those protocols were tested, used (hence validated) and evaluated in WP1 in 2020 and after an update again in 2021 for their ability to give insight into the health status of the colonies. Protocols for Tier 2 (first published in D1.6 Protocols No 2) were selected based on the following criteria:

1) adequate insights of the (health) status of a colony,
2) refrain from disturbance of the bees, and
3) be user friendly for use by beekeepers/end-users.

These criteria for Tier 2 were based on the evaluation of Tier 1 protocols. Some adaptations were made if deemed necessary to fit the listed criteria. For example, the Liebefeld method protocol was not put through to the beekeepers in Tier 2 as it was found during evaluation to be too disturbing for the bees and very labour-intensive. In 2021, Tier 2 protocols were tested, used (hence validated) and evaluated by the beekeepers for their ability to give insight into the health status of the colonies, but also for bee-disturbance and user-friendliness. The protocols prepared for Tier 3 are selected based on the same criteria as Tier 2 and additionally aiming to reduce 'classical' data collection actions to a minimum. Data collection of Tier 3 participants will therefore be even more sensor dependent.

In this document we present the updated protocols for Tier 1 and Tier 2 after validation and evaluation in 2021. We also present the new Tier 3 protocols, based on the most recent protocols and manuals of the previous Tiers, but tailor made for the Tier 3 group of end-users.

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. For Tier 2 and Tier 3, protocols are communicated to beekeepers via e-mail; translation in local language have occurred in Italy, Finland, Germany, Switzerland and The Netherlands. At the end of the project the protocols that meet all criteria (i.e. give adequate insights of the (health) status of a colony, refrain from disturbance of the bees, and are user friendly), will be incorporated into the BEEP app as much as possible, and published on the B-GOOD data portal. Where possible, data collection conforming to the latest versions of the protocols has already been incorporated in specific (seasonal) inspection sheets 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, according to a data collection schedule that is season dependent and was provided to the users within the workplan.

In parallel to evaluation sessions of protocols and workplan, feedback is being continuously gathered by Tier 1 and Tier 2 participants via the helpdesk (D.1.2 Helpdesk). Based on the feedback throughout 2020 and 2021, protocols have been updated (from Tier 1 to 2 and from Tier 2 to 3) 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 (D1.7). Beekeepers in Tier 3 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) that are responsible for these analyses, have not been included in the protocols distributed to the beekeepers, nor is this document. This is to prevent confusion for the partners involved in the Tiers, 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 that are ready to implement to current end-users.

3. Protocols and manuals

Data collection methods throughout the experiment and data entry onto the BEEP app are detailed below. Subsets of the Tier 1 protocols were used in Tier 2, and Tier 3. Please see Table 1 for an overview of measurements over time, protocols used for all three Tiers, 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).

Table 1: Overview of experimental observations over time. The protocol number, the timing (frequency) of measurements and the months in which they are to be performed are provided for each activity. Coloured cells show in which months experimental observations are expected to be performed. The Tier 1 and Tier 2 tables have been updated with protocols and additional related inspection sheets since the start of Tier 1, publication in D1.1 Protocols No 1 and D1.6 Protocols No 2. Tier 3 table has been prepared for use in 2022. The inspections for Tier 2 are a subset of the inspections in Tier 1, and Tier 3 is a subset of Tier 2.

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

Experimental observation	Protocol	Timing	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Presence of queen & brood	P1	every 20 days*				█	█	█	█	█	█	█		
EFSA/Liebefeld	P8/P2	every 20 days*				█	█	█	█	█	█	█		
Top photo analysis	P3	1 x month**	█	█	█	█	█	█	█	█	█	█	█	█
Mite infestation level	P3	1 x week				█	█	█	█	█	█	█		
Sampling for lab analyses	P5	3 x year *				█			█			█		
Atypical worker behaviour	P6	every 20 days*				█	█	█	█	█	█	█		
Clinical signs of disease	P7	every 20 days*				█	█	█	█	█	█	█		
Sampling drone brood eggs	P9	1 x year *				█								
Queen cell presence	P10	every 20 days*				█	█	█	█	█	█	█		
Brood pattern consistency	P11	every 20 days*				█	█	█	█	█	█	█		
Overall impression	NA	1 x month **	█	█	█	█	█	█	█	█	█	█	█	█
Data annotation	NA	1 x month **	█	█	█	█	█	█	█	█	█	█	█	█
Colony mortality	NA	1 x month**	█	█	█	█	█	█	█	█	█	█	█	█
Data quality and cleaning	P12	1 x month	█	█	█	█	█	█	█	█	█	█	█	█
BEEP app inspection sheets to be used			1, 5	1, 5	1, 5	2, 3 5	2, 4 5	2, 4 5	2, 3 5	2, 4 5	2, 4 5	2, 3 5	1, 5	1, 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.
** Should be done 1 x a month during the winter period, but should preferably be reduced to every 20 days during the bee season.

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

Experimental observation	Protocol	Timing	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Presence of queen & brood	P1.2	1 x month *				■	■	■	■	■	■	■		
Top photo analysis	P3.2	1 x month	■	■	■	■	■	■	■	■	■	■	■	■
Sampling for lab analyses	P5.2	3 x year *				■			■			■		
Clinical signs of disease	P7.2	1 x month *				■	■	■	■	■	■	■		
Queen cell presence	P10.2	1 x month *							■	■	■	■		
Drone presence	P13.2	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	6	6	8	7	7	8	7	7	8	6	6

* 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.

Tier 3 Two inspection sheets were prepared for use in the BEEP app, covering the different protocols: 9 Health; 10 Sampling.

Experimental observation	Protocol	Timing	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Presence of queen & brood	P1.3	1 x month *				■	■	■	■	■	■	■		
Sampling for lab analyses	P5.2	3 x year *				■			■			■		
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			9	9	9	9, 10	9	9	9, 10	9	9	9, 10	9, 10	9, 10

* 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

Via the BEEP app, data are collected from research institutes and selected beekeepers. On the app itself, field methods are described and the data entry sections are provided for each scientific protocol. Methods are described for all of the three Tiers. There are deviations in some protocols between tiers. If there are differences, protocols for each of the tiers are provided. Several protocols in Tier 1 are not applicable for Tier 2, as are some of Tier 1 and Tier 2 protocols are not applicable for Tier 3. A complete overview of all the protocols in the different Tiers are shown in the table 2.

Table 2: Overview of experimental protocols in all the Tiers. In each subsequent Tier, protocols are fine-tuned and updated based on evaluations of previous Tiers. The P-numbers coincide with the protocol numbers in Table 1.

Experimental observation	Tier 1	Tier 2	Tier 3	Remarks
Presence of queen & brood	P1	P1.2	P1.3	
EFSA/Liebefeld	P8/P2			Tier 1 participants can choose either EFSA or Liebefeld method
Top photo analysis	P3	P3.2		
Mite infestation level	P4			
Sampling for lab analyses	P5	P5.2	P5.3	
Atypical worker behavior	P6			
Clinical signs of disease	P7	P7.2		
Sampling drone brood eggs	P9			
Queen cell presence	P10	P10.2		
Brood pattern consistency	P11			
Data quality and cleaning	P12			
Drone presence		P13		Information of drone brood is collected within P8 / P2 in Tier 1

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.

Tier 3
No deviations from Tier 1.

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

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

Protocol 2 Liebefeld method (v20220204)

Estimation of colony strength by assessing the amount of comb surface covered by bees/brood/honey/pollen with a grid.

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 to estimate colony traits. Preferably, the same person(s) should conduct the Liebefeld measurement to keep the experimenter bias variability 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 estimated how much of a 5x5 cm square would be covered with bees, brood, etc.
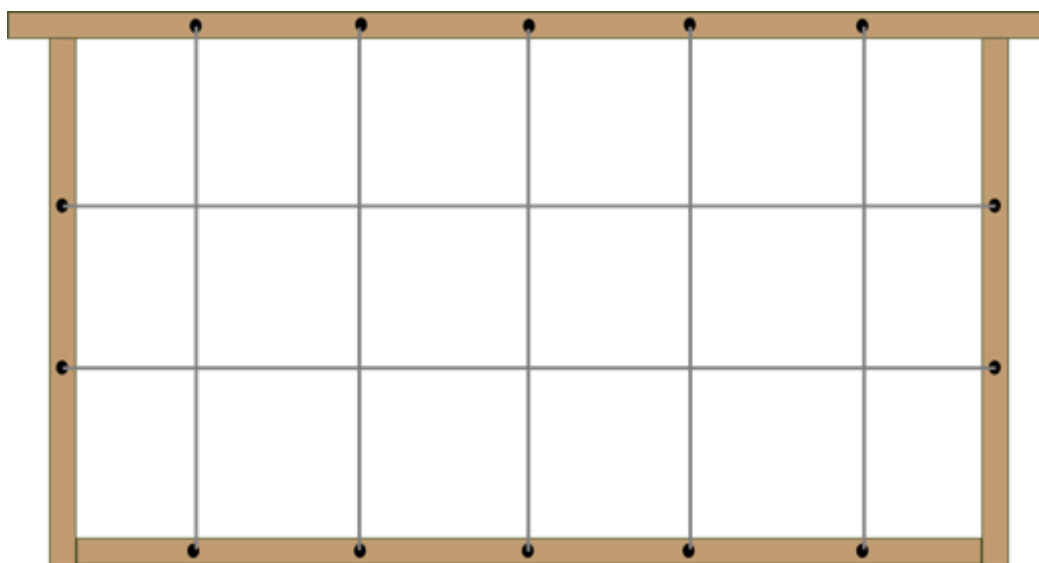
Figure 2: Scheme of grid for Liebefeld method. Squares should be 5x5 cm^2.

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 cm^2 (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.
- In case of honey super(s):
o   Colony traits that need to be measured in the supers are: bees and capped honey
    o   The same procedure with brood box applies for estimation of these parameters in the honey super. The only difference is that, in the BEEP app instead of recording information per side of frame, record total amount of honey and/or bees for each super.

Tier 2 and 3
Not applicable for Tier 2 and 3.

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.

3.1.2   Protocol 3 Top photo analysis (v20220204)

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).
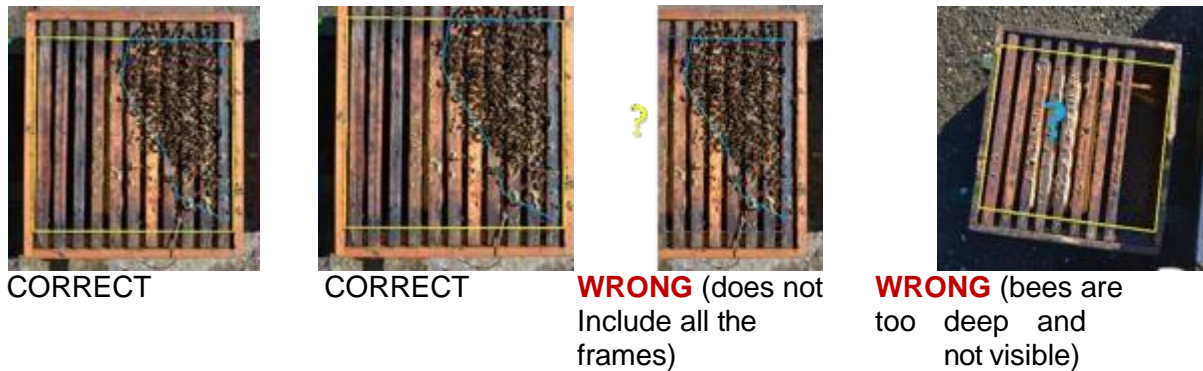
CORRECT   CORRECT   **WRONG** (does not   **WRONG** (bees are
                                  Include all the   too   deep   and
                                  frames)   not visible)

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. Both lines are necessary to 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 1).
- Please see 'TPA_tutorial' for a how-to video. (click here for tutorial)
- Enter the information of 'pixels with bees' and 'pixels total top' into the BEEP app.
- Upload the photo when entering data onto the BEEP app.

Tier 2
No deviations from Tier 1

Tier 3
Not applicable for Tier 3

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.

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

3.1.3   Protocol 4 Counting Varroa mites after (natural) fall (v20200220)

Mite infestation level of each colony will be estimated 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.

[Before, during and after Varroa treatments, we suggest to increase the number of measurements as such that they will give you a good indication of the effects of the mite treatment. In such cases, frequency of counting should depend on the treatment method, the duration of the treatment used, but perhaps also on the remoteness of the apiary.]

When you apply varroa control methods, please record the date and treatment type in the BEEP app.

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.
- Clean and Reset the sticky board, if measurement have to be continued

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)

Tier 2 and Tier 3
Not applicable for Tier 2 and Tier 3

BEEP app data entry
The number of mites per day is entered onto the BEEP app (Figure 7)

Figure 7: Screenshot from BEEP app on data entry of P4.

3.1.4   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 (before eventual summer mites treatment); 3) and a third in autumn before over-wintering.

Tier 1 (v20220204)

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 freezer proof sample and ensure correct identification in case 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
- 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 (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 cup of 100ml 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 on the lid.
- It is fine if drones are included in the sample but less 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 labs for lab analysis must be done on dry ice, by courier services that enable fast transport. Preferably, samples will arrive within 72h after sampling in the field at FLI/SCIEN institutes. Arrival of samples should be within 48h 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.
- The Institutes to which the samples need to be sent to 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 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 8).
- Carton queen shipping box (with ventilation openings).
- 3 x queen cages with sugar dough in them.

Figure 8: 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 preferably -80°C until dry-ice shipping. If no -80°C is available, the samples can be stored at -20°C alternatively.
- 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

Tier 3 (v20220208)

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 third in autumn before the over-wintering.

BEEP app
- For anonymization purposes, the BEEP app will generate a unique identifier per sample. 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 freezer proof label and ensure 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 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 freezer containers with drilled holes (Figure 1)
- 3 x halved queen cages with sugar dough in it

Figure 9: Example of freezer container with holes.

Collection of samples

- Before sampling, place the halved queen cage with sugar dough into the container to equip bees with some food prior to transport.
- 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 (preferably one 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 (64g = sample container incl. bees (but without queen cage) are 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.
- 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°.
- It is fine if drones are included in the sample but less is better.

Transport
- Place the freezer containers with bees into a shipping box to prepare for transport. Make sure the transport box has ventilation holes as well. Please make sure that the containers are firmly closed and cannot open during transport.
- The samples should be sent the same day of sampling in the field because the bees should arrive alive.
- Transport details will be communicated to you personally as these may differ between countries.
- Coordinate the dates of transfer for samples with the B-GOOD partner organisation in your country before arranging transport. This in order to make sure the receiving person is available for the package and further processing

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).
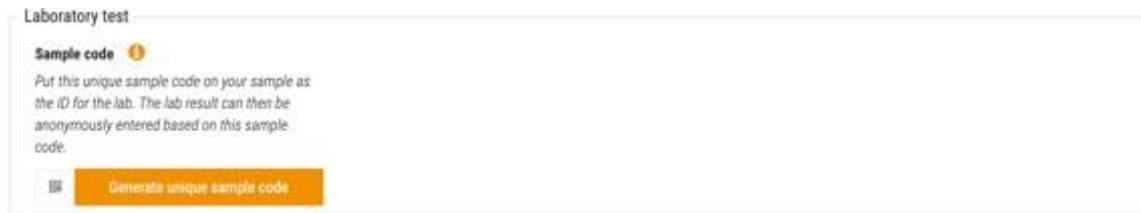

Figure 10: Screenshot from BEEP app on data entry of P5.

3.1.5   Protocol 6 Atypical worker behaviour (v20220204)

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.
- Each frame side should be observed for 1 min for atypical behaviour.
- See Table 3 for honey bee behaviours; any behaviour outside of the normal repertoire of 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 3: 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 donators mouthpart, donator 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 and Tier 3
Not applicable for Tier 2 and Tier 3

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.

3.1.6   Protocol 7 Clinical signs (v20220204)

Tier 1
Check the hive for clinical signs of disease.

Field methods

- Visually observe colonies to assess the presence of clinical signs in brood and adult bees. A decision-making tree for clinical signs of diseases observed in European honey bee colonies can be found below (figure 12 for brood and figure 13 for adult bees). If you have the suspicion that a disease is present in the hive, record the type of disease in the BEEP app.
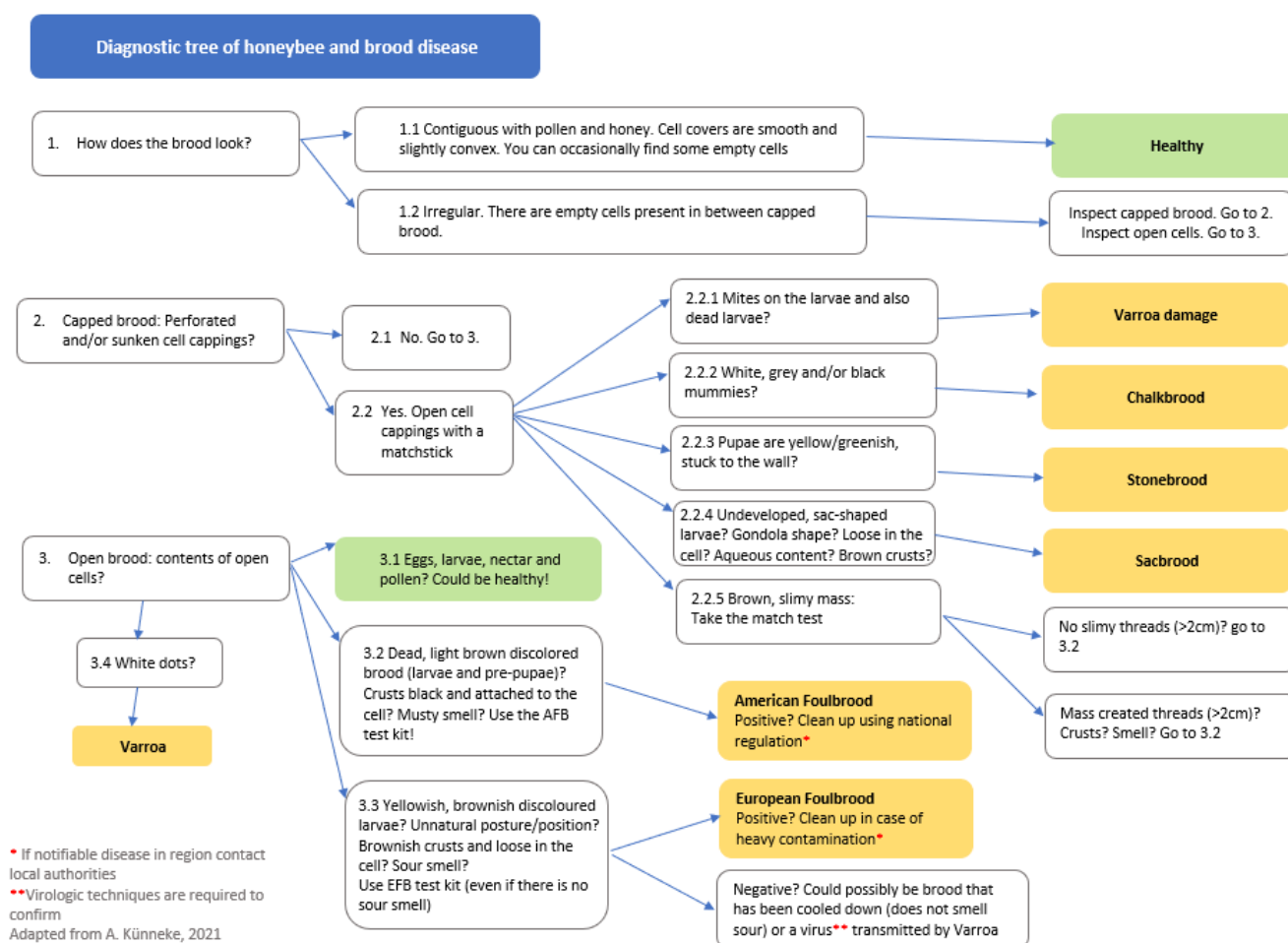

Figure 12: Diagnostic decision-making tree based on honeybee brood diseases.
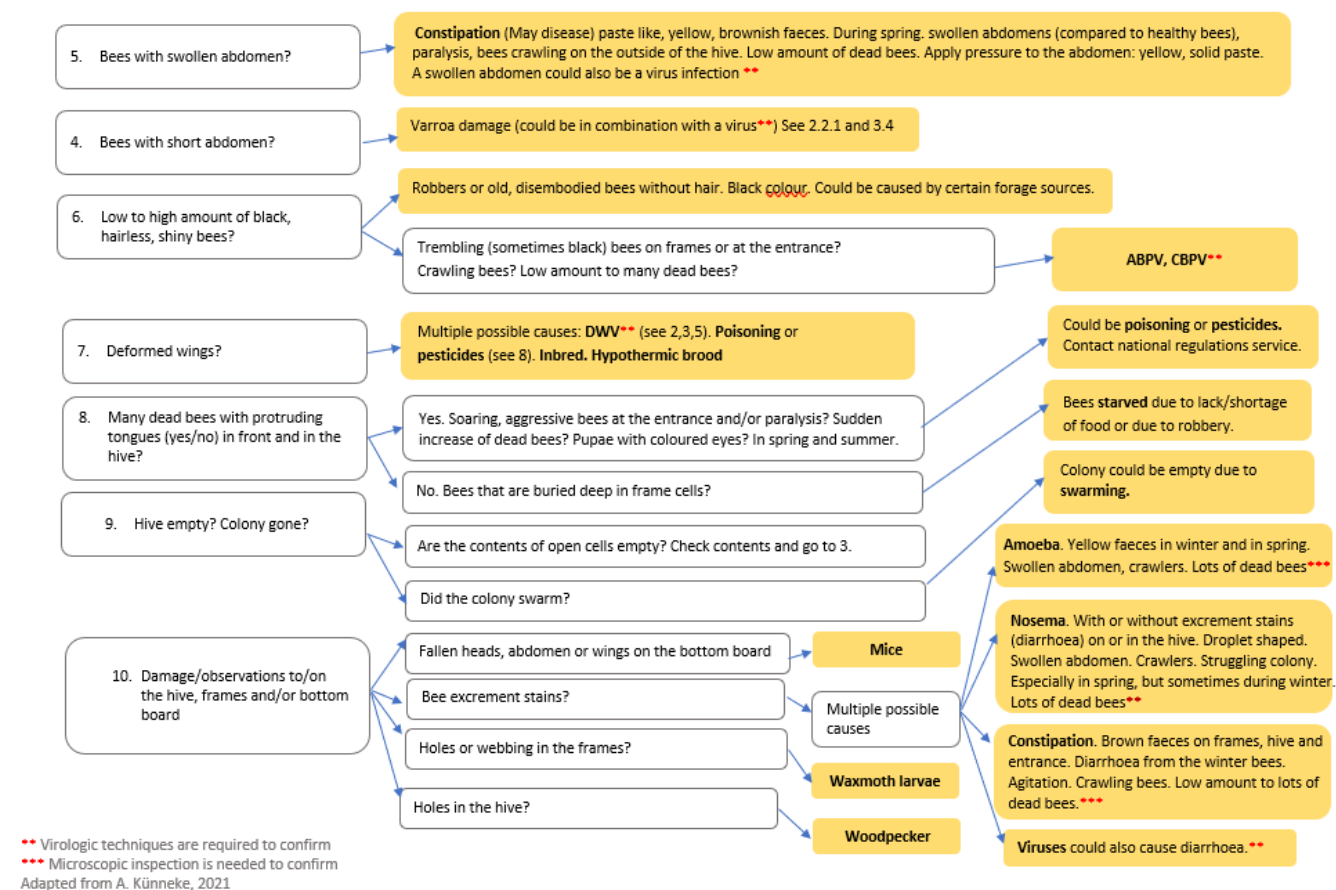
Figure 13: Diagnostic decision-making tree based on adult honeybee diseases.

Tier 2
No deviations from Tier 1.

Tier 3
Not applicable for Tier 3

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 14).

Figure 14: Screenshot from BEEP app on data entry of P7.

3.1.7   Protocol 8 EFSA protocol (v20220204)

Tier 1 (Tier 1 only, not applicable to Tier 2 or Tier 3)

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.

Materials
Experimental material
- Field forms
- Empty super
- Scale (max. weight 5kg, d=5g), 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
- Smoker
- Lighter
- Fumigator's fuel
- Beekeeping protective equipment

Experimental setup

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, 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 20 days. Monitoring should be done once every 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 is 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 degrees).

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 be 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 the monitoring, the 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.

1 Monitoring of experimental hives

1.1 Photographing of combs

1.1.1 Material installation

Prepare the material as follows:

- Install the camera in the tunnel and turn on the lights (set the LED lights into 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

1.1.2 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.

1.1.3 Frames weight with bees
Dispense smoke at the entrance before you start 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**
- Weigh 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 15**).
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 position.
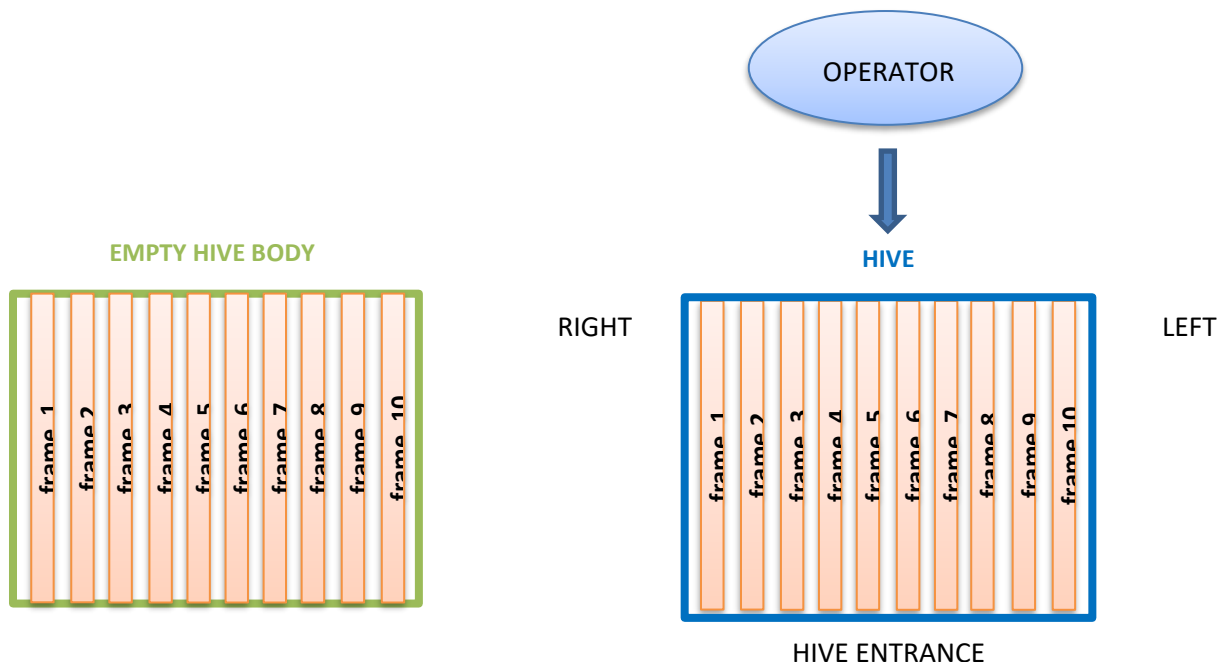
Figure 15: Hive's scheme

All frames should be labelled properly before you start taking photos

1.1.4 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 in the end (after the hive is closed) if in focus. If the tunnel moves, the focus should be checked again.

1.2 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 platform

3.1.8   Protocol 9 Sampling drone brood eggs (v20220204)

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, 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.
- Unscrew the Eppendorf tube and keep it ready.
- To collect the eggs, scoop the toothpick under the egg and lift it up (Figure 16.1). If it is difficult to see the eggs, cutting out a part of the wax can help (Figure 16.2).
- Take the Eppendorf tube and place the eggs approximately 1 cm deep in the tube (Figure 16.3 and 16.4).
- Place the egg flat against the tube and turn the toothpick a quarter stroke to release the egg from the toothpick (Figure 16.6).
- 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 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 to the reference labs along with the bee samples for disease analysis.
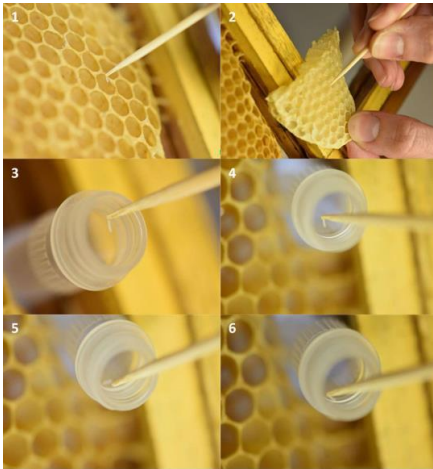

Figure 16: Sampling drone eggs from a frame, in six steps (image 1-6).

Tier 2 and Tier 3
Not applicable for Tier 2 and Tier 3

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.

3.1.9   Protocol 10 Queen cell presence

Tier 1 (v20220224)

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, supersedure 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 (Figure 17.A). 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 (Figure 17.B).
- Supersedure cells: Are built when the colony wants to replace the current queen. These cells are generally found on the centre of the comb (Figure 17.C.), but are not created from modified worker cells. Commonly the colony raises 1-3 supersedure cells.
- Emergency cell: Are built if the old queen is dead. Like supersedure cells, they are usually found on the centre of the comb (Figure 17.D), but these cells are created from modified worker cells. Usually, the colony raises a high number of emergency cells (especially compared to supersedure cells).

Figure 17 Different types of queen cells in honeybee colonies: A) Queen cups, B) Swarm cells, C) Supersedure cell if the queen is alive or emergency cell if the queen is dead, D) Emergency cells. Photo credits: C van Dooremalen (A,B,D), M. Schoonman (C).

Tier 2
No deviations from Tier 1.

Tier 3
Not applicable for Tier 3

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 18).

Figure 18: Screenshot from BEEP app on data entry of P10.

3.1.10 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 and Tier 3
Not applicable for Tier 2 and Tier 3

BEEP app data entry
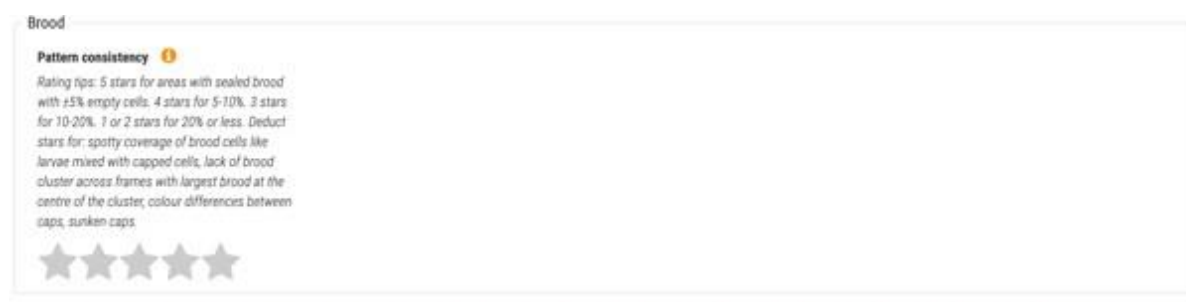Brood pattern consistency is rated on a 5-point scale in the BEEP app based on estimates (Figure 19).

Figure 19: Screenshot from BEEP app on data entry of P11.

3.1.11 Protocol 12 Data cleaning and quality

Tier 1

To evaluate the data entry in the BEEPapp, minimize discrepancies in sensor data and revise as necessary to ensure workable data.

Each month, to carry out the following:

- Within one week of the end of each month the inspection data for that month needs to be uploaded in the BEEP App by apiary managers.
- One week following the end of each month INRAE will generate an Excel report for each apiary manager for each of their colonies to describe the discrepancies, if any present, between automated data and inspection data. The report covers all data entered via the BEEPapp as well as the automated data collected via the BEEPbase.
- 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 Excel 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 status.
- 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 BEEPapp as necessary.
- We ask that the apiary managers to check their own data weekly via the BEEPapp to see if there are hardware or network errors that can be caught on a faster than monthly cycle.

Tier 2 and Tier 3
Not applicable for Tier 2 and Tier 3

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

3.1.12 Protocol 13 Drone presence

Tier 1
Not applicable for Tier 1. Drone brood presence included in Liebefeld or EFSA method

Tier 2

During swarming season, beginning in spring from the start of bee foraging activity, check colonies for presence of drone brood. The presence of drone brood gives an indication on swarming tendency of colonies. More importantly, it also gives an indication of the colony health. Healthy colonies are willing to reproduce, and are able to invest resources in drones. Hence, colonies should be checked for drone brood at least 1x a month or each time the colony is opened.
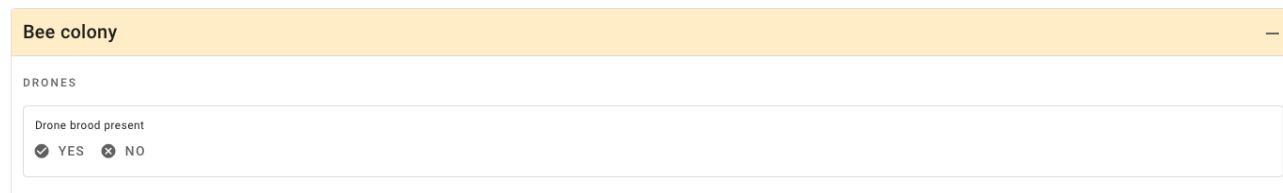
Field methods
- Open a colony and sequentially remove frames from the hive.
- Check the hive comb surface to see for the presence of drone brood.
- If drone brood present, record it in the BEEP app.

Tier 3
Not applicable for Tier 3

BEEP app data entry
Data on the presence or absence of drone brood is entered into the BEEP app (figure 20).

Figure 20: Screenshot from BEEP app on data entry of P13.

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/. In order to increase user-friendliness for the participants of the three tiers, a registration guide for the BEEP app was directly entered into the workplan.

Acknowledgements

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