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Protocols No.1

Deliverable D1.1

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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 and instructive videos offered in an IT functionality to ensure harmonization of data and sample collection.

Summary

In this deliverable, a set of scientific protocols and manuals are presented that will be validated and optimized in TIER1 of the B-GOOD project Work Package 1 (WP1), including support for using the BEEP app and BEEP base. At this stage, the scientific protocols have restricted access for B-GOOD partners. Validated and evaluated versions will become publicly available at a later stage. BEEP app and BEEP base manuals are immediately open access to ensure instant dissemination of improvements to current end users of BEEP. The latest versions of the protocols and manuals are provided in this document.

1. The need for protocols and manuals

WP1 will facilitate 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.

A work plan was written in the first months of the project for TIER 1 Pilot A within WP1 (not presented in this deliverable as it is a working/living document). The work plan includes protocols and manuals providing guidelines about beekeeping (these guidelines will not be provided within TIER 2 or TIER 3), and provides information on when to use the different protocols and manuals.

This first set of manuals and protocols (protocol No.1) is prepared solely for use in TIER1 in WP1. These documents will be 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. Some of the protocols are primarily developed for validation of the newly developed tools. Such protocols are only expected to be used in TIER1 of WP1. 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.

2. Publication of manuals and protocols

2.1. Scientific protocols

As the scientific protocols at this stage are prepared only for TIER1 in the B-GOOD project and adjustments are to be expected, the latest versions of the protocols will continually be made available to the B-GOOD consortium, in particular to WP1, TIER 1 participants. 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 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 the implementation of the protocols, feedback will be gathered of the TIER 1 participants via the helpdesk (D.1.2 Helpdesk). Based on the feedbacks, protocols will be updated and uploaded on Teams for immediate implementation.

The laboratory protocols that will be 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 this set. This is to prevent confusion for the partners involved in TIER 1.

2.2. BEEP app and BEEP base

As the BEEP base and BEEP app are already publicly available, the manuals are made publicly available using the digital BEEP app environment. If updates are needed, this will be performed online. 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 coming forwards from the B-GOOD project. I.e. this strategy ensures instant dissemination of knowledge and improvements to current end-users.

3. Protocols and manuals

Data collection methods throughout the experiment are detailed below. Please see Table 1 for an overview of measurements over time, 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 (e.g. v20200220).

Table 1**:** Overview of experimental observations over time (v20200220). 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.

Experimental observation	Protocol	Timing	Months *Jan*	*Feb*	*Mar*	*Apr*	*May*	*Jun*	*Jul*	*Aug*	*Sep*	*Oct*	*Nov*	*Dec*
Presence of queen & brood	P001	1 x month *												
Liebefeld method	P002	3 x year *												
Top photo analysis	P003	1 x month												
Mite Infestation level	P004	1 x week **												
Sampling for lab analyses	P005	3 x year *												
Atypical worker behaviour	P006	3 x year *												
Clinical signs of disease	P007	1 x month *												
Overall impression	NA	1 x week **												
Colony mortality	NA	1 x month *												
BEEP app inspection sheet			1 & 2	1 & 2	1 & 2	2 & 3	2 & 4	2 & 4	2 & 3	2 & 4	2 & 4	2 & 3	1 & 2	1 & 2

** 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

3.1.1. Protocol 1 Presence of the queen and brood (v20200220)

Every four weeks starting from the end of the winter period till the end of the beekeeping season (essentially, the period of honey bee foraging activity), check the hive comb surface for the presence of queen and of all stages of brood. The presence of worker brood gives information on queen fecundity, viability of worker force and the ability of the colony to rear the eggs until adulthood.

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 no open brood (eggs and larvae) is present, queen failure is assumed (after rechecking in 1 week). The missing queen needs to be replaced by a laying queen.
- 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.

3.1.2. Protocol 2 Liebefeld method (v20200220)

The amount of food resources (honey and pollen), brood size and colony size are key determinants of colony development and survival. For the estimation of these parameters we will use the Liebefeld method (manual estimation by using a grid). It is less laborious and less invasive compared to alternative methods such as photo analysis of frames.

Measurements should be done three times a year. First in spring, when the bees start to forage; second time in summer, when the colonies have reached their maximum size; and a third time in autumn, before the over-wintering.

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 1).

- Drill small holes at 5 cm distance 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.

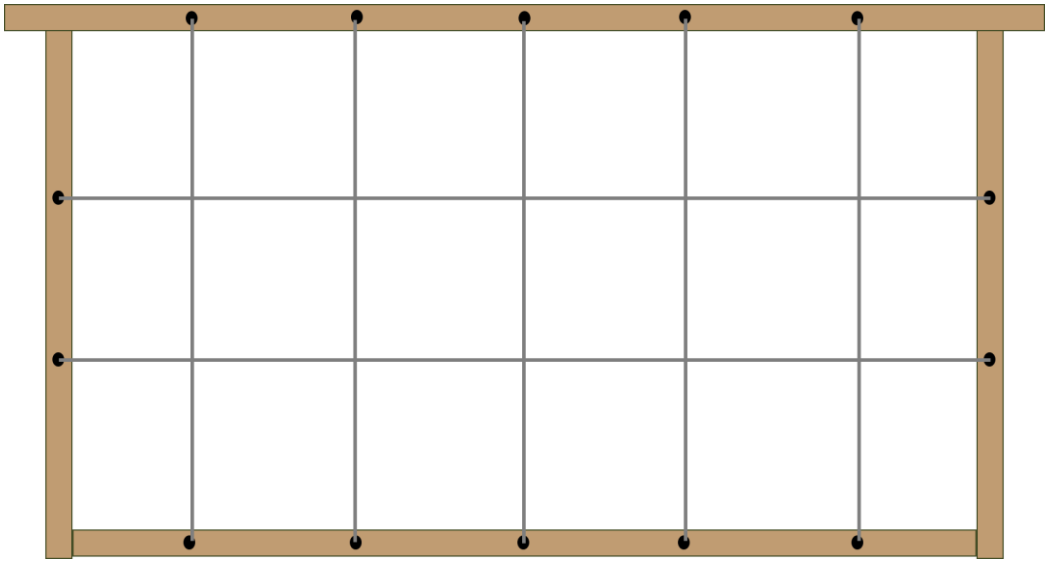

Figure 1: Scheme of grid for Liebefeld method. Squares should be 5x5 cm^2.

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 1).
  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 fraction covered, up to one decimal. 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 for 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.

3.1.3. Protocol 3 Top photo analysis (v20200220)

Colony size will also be estimated by taking a photo of the topside of the hive. This will be done every first week of each month throughout the whole year. There are several benefits of this method as it is less invasive, faster, and easier, mainly: (1) it can be used during winter when temperatures are too low for removing frames (2) correlations can be made with the weight of the hive as it can be used more often (more data points), (3) it may be easy enough for beekeepers to apply in TIER2. 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

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 the 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 2).

Figure 2: Correct position of hive top in photograph and potential mistakes to avoid. Yellow lines show size of the top side of the box anblue lines show subjective estimation of top side of the bee cluster, if possible to estimate. Only with both, you can 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 2).
- 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) into the BEEP app.

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

Mite infestation level of each colony will be measured by quantifying naturally falling mites. The infestation levels will be counted preferably once a week, or at least once a month, throughout the whole year. 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.

Materials

- *Sticky board*
- *Guide for counting mites (Figure 3)*

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 3) 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 daily mite fall.

Figure 3: A guide 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) the mite counting can be sub-sampled.
- To sub-sample, print a grid with 2 cm^2 cells on the sticky board (Figure 4).
- 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 Sammataro, 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 daily mite fall.
- Record this in the BEEP app (mite fall per day, method: natural fall).

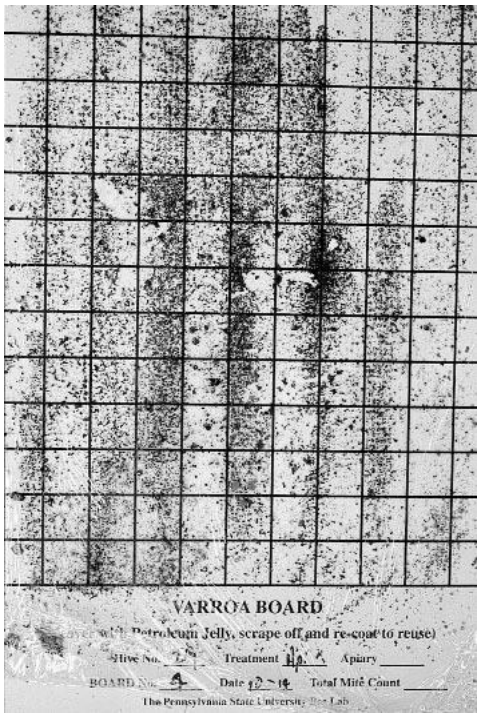

Figure 4: Example of sticky board with grid (taken from Ostiguy and Sammataro 2000)

3.1.5. Protocol 5 Sampling bees for lab analyses (v20200220)

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 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. 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.

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 (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 a cup of 100ml with bees, it will be around 300 bees when the cup is full.
- To keep bees alive until freezing, make sure that there are holes on the lid.
- If the bees are calm, fill the cup by scraping the bees off the comb, holding the cup vertically and the comb at 45°. Alternatively, tap the frame onto a covering sheet, then pour/scoop the bees that have dropped onto the sheet into the cup.
- 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 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.
- 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)
- The Institutes that 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’

*All legal requirements for the transport of materials across the EU-border will be set in place before sending the samples. This transfer of material requires appropriate MTAs, just like they are needed for the exchange of the same material between partners located within EU Member States.

3.1.6. Protocol 6 Atypical worker behaviour (v20200220)

Atypical behaviour by workers is one of the first signals of diminished health within the colony. This indicator will be measured by visual inspection of the hive three times a year – spring, summer, and autumn. It does assume a basic level of normal typical behaviour of honeybees.

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 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 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 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

3.1.7. Protocol 7 Clinical signs of disease (v20200220)

During the beekeeping season, check the hive at least once a month for clinical signs of disease

Field methods

- Visually observe colonies to assess the presence of clinical signs 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 signals
Colony:
- Reduced colony population and/or altered demography
- Collapsed colony/empty hives

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 (absence of AFB 'ropey' larvae/sealed cells)
- Cannibalised/slumped/discoloured/cannibalized brood
- Dried 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 (match test)
- Coffee brown coloured larvae
- Tongue of dead larvae pointing upwards
- Sticky scales
- Specific 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 mortalities
- 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 mortalities
- 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 and fail to pupate

3.2. BEEP app and BEEP base

All manuals related to BEEP can be found here: https://beep.nl/app-home/manual-v2. In order to increase user-friendliness for the participants of TIER 1, WP1, a registration guide for the BEEP app was directly entered into the workplan.

Register in BEEP
To start using BEEP for the B-Good project, please follow these steps:
- REGISTER: Go to the BEEP app, using a web browser on a computer or alternatively on a mobile phone via this link for the English version. As a new user, click on the login screen on 'No account yet? Register as a new user'. Register with your work email address and follow the instructions. See the login support article for more information on this step.
- APIARY: When logging in for the first time, you will see the 'Create new apiary' screen where you can add the B-GOOD apiary details. When you are done, click on 'Create new apiary' button to save the data. See the Create new apiary article for more information on this step.

- HIVES: Open the apiary you created. You can change the settings per hive, by clicking on the hives. You can change the configuration and enter the details on the queens per hive. See this article for more information on this step.
- INSPECTIONS: By clicking on the pen icon under each hive, you can add inspections for that hive. This is also further described here.
- RESEARCH: An important step is to link your account to the B-GOOD research program. You only need to do this once. You can click on 'Research' in the menu on the left and select the B-GOOD program by following the on-screen instructions (Figure 4). This way the data can be accessed for analysis in WP1.

COLLABORATION: Multiple people can edit the data for the mini apiary. You can see here how you can set this up for your group.

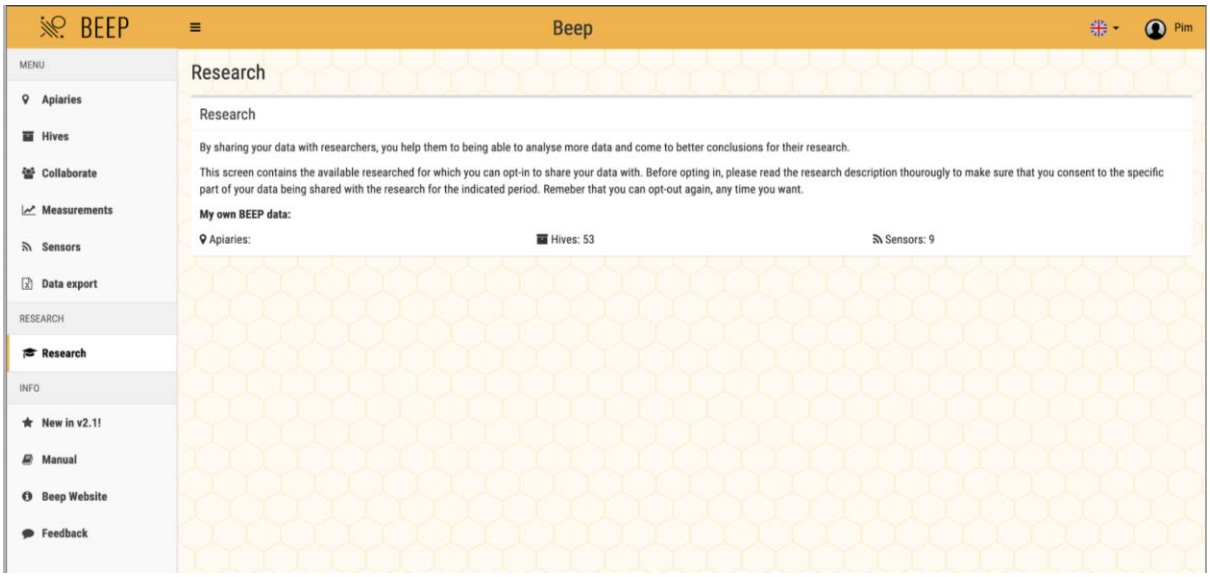

Figure 4: After creating your account on the BEEP platform, you can link your account to the B-GOOD programme by clicking on 'Research' in the left menu.

4. Acknowledgements

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