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Milestone 15 Protocols for semi-field and field experiments

Matthew J Allan, Robin Dean, Alexandra-Maria Klein, Dimitry Wintermantel, Matthias Albrecht, Janine Schwarz, Anina Knauer

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Protocols for semi-field and field experiments

Milestone 15

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Authors

Matthew J Allan[1*], Robin Dean[2], Alexandra Klein[3], Dimitry Wintermantel[3], Matthias Albrecht[4], Janine Schwartz[4], Anina Knauer[4]

Author affiliations

[1] Atlantic Pollination Ltd, Eastleigh, United Kingdom; matt@atlanticpollination.com

[2] Red Beehive Company, Bishops Waltham, United Kingdom

[3] University of Freiburg, Nature Conservation and Landscape Ecology, Freiburg, Germany

[4] Agroscope, Agroecology and Environment, Zurich, Switzerland

PoshBee

Pan-European assessment, monitoring, and mitigation of stressors on the health of bees

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Preface

This document comprises Milestone 15, which is a set of protocols for use in investigating impacts of multiple stressors on different types of bees in semi-field and field situations. It includes:

a) notes applicable to all studies
b) protocols common to all semi-field studies
c) protocols for semi-field studies on honey bees
d) protocols for semi-field studies on bumble bees
e) protocols for semi-field studies on solitary bees
f) protocols common to all field studies
g) protocols for field studies on honey bees
h) protocols for field studies on bumble bees
i) protocols for field studies on solitary bees

Summary

Under the terms of the PoshBee agreement, the members tasked with delivering Work Package 7 carried out semi-field and field studies on honey bees, bumble bees and solitary bees. These studies involved the exposure of the bees to more than one stressor, for example a fungicide and an insecticide or a fungicide and a nutritional deficiency. This is in contrast to the conventional study design where one stressor or product alone is assessed.

These studies built upon recommendations by the European and Mediterranean Plant Protection Organisation (EPPO), European Food Safety Authority (EFSA), Prevention of Honeybee Colony Losses (COLOSS), and the Organisation of Economic Co-operation and Development (OECD). The team of researchers incorporated several innovative approaches in designing and carrying out the studies. In the authors' view both the quality and quantity of data required for ecotoxicology studies can be improved by adoption of novel methods, including electronic means and artificial intelligence, and the design, manufacture and use of equipment specifically for such studies. Several examples are described.

It is the intention of the team to include in this document practical advice for workers who are not familiar with such studies.

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1. Notes applicable to all studies

1.1 The Principles of the Studies

It is common for researchers to study the impacts of a single plant protection product on bees. The studies discussed here are designed to examine the impacts of multiple stressors, as reported by many authors (e.g. Siviter, 2021; Stuligross, 2020) on honey bees and/or bumble bees and/or solitary bees. The bees will typically be *Apis mellifera, Bombus terrestris,* and *Osmia bicornis*. Different species and subspecies may be specified and studied.

The terminology used for ecotoxicology studies will be used here – i.e. Tier 1 refers to lab-based studies; Tier 2, otherwise known as semi-field studies, refers to research carried out on free-flying bees in enclosures; Tier 3, otherwise known as field studies, refers to research carried out on bees free to fly unconstrained in the environment.

One or more stressors selected from (a) a chemical and (b) a nutritional deficiency will be applied; consideration may be given to including one or more of the following: (c) a pathogen stressor and (d) a parasite or parasitoid stressor. These latter two stressors introduce considerable additional complexity, and are not recommended for regulatory purposes. Depending on the stressors, the study may involve an exposure phase and a post-exposure or monitoring phase. Assessments in eusocial species will include adult bee behaviour and mortality, colony strength and weight, and juvenile mortality; assessments in solitary species will include adult bee behaviour and mortality, reproductive success and juvenile mortality.

The fundamental principles are as laid out in various guidance documents, including the European Food Safety Authority (EFSA 2013), Organisation for Economic Cooperation and Development (OECD 2014) European and Mediterranean Plant Protection Organization (EPPO 2010), and COLOSS Bee Book (COLOSS 2013) with amendments to include multiple stressors. The authors recommend refinements and modifications that take advantage of emerging technology to give improved data collection and analytical power.

The protocols described here will not cover Tier 2 feeding studies such as the Oomen study (Lueckmann 2019) or the EPA (US EPA, 2016) feeding study, which are not easily adaptable to examining the impact of multiple stressors.

The recommendations for forage crops to be grown for the semi-field and field studies are appropriate for temperate areas of Europe and North America. Researchers may utilise other plant species to suit local conditions. The principles of the studies are widely applicable in many parts of the world.

The techniques described here open up the possibility of examining the impacts of adjuvants in plant protection products, which have largely been overlooked (Straw, 2021).

1.2 The Study Design

The study design will consider and record the following:

- the test species
- the life stages
- the providers of the bees
- data on management and history of bees prior to study, including health status and treatment for diseases and parasites

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- data on hives and nests
- description of special equipment
- endpoints
- selection and application of stressors
- justification for doses
- regulatory issues such as approval to apply non-registered products
- adherence to standards such as Good Laboratory Practice
- duration of exposure phase
- duration of monitoring post-exposure phase
- number of treatment groups
- replicates per treatment group
- statistical methods
- crops
- location
- map reference
- information on pesticide history
- information on maintenance history
- soil data
- weather data
- risk assessments: responsibility
- health and safety: accident and first aid procedures
- field crew training and verification of skills

2. Protocols applicable to semi-field studies on all bee species

2.1 Training of observers

a. Records will be kept of training sessions for field staff.
b. Training sessions will include formal risk assessments and safety measures (including possibility of anaphylactic shock if stung, and exposure to Lyme disease by tick bite); assessment techniques; observation techniques; and recording techniques.

2.2 The Principles of the study

The test bees will be placed in enclosures in which a crop is grown to provide nectar and pollen to foraging bees. The chemical stressors will be applied in accordance with the label instructions in order to create the maximum realistic field exposure. Other stressors will be applied as specified by the researcher.

2.3 General considerations

a. Selection of crop. Several criteria influence the choice of crop. For example, it may be that a product in question can only be applied to specific crops (for example apples, in which case studying impact on an alternative crop, e.g., grapes, would be meaningless). Otherwise a surrogate crop such as *Phacelia tanacetifolia* or oil seed rape may be considered (Gradish, 2016). The advantages of *Phacelia* are that it is a robust plant, highly attractive to bees yielding abundant nectar and pollen over a long period, and is largely unaffected by pests. Other

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Brassicas besides oil seed rape may be considered such as radish and mustard. These are more prone to attack by aphids and other insects, which cannot be controlled before or during the study by insecticides. In certain circumstances *Fagopyrum esculentum* (buckwheat) may be used where a pollen of less nutritional value is required for comparison.

b. Use of actual crop. In some cases it may be appropriate to use the actual crop for which the plant protection product is formulated, such as apples or cherries, instead of the surrogate crops mentioned above. In such a case it should be noted that, as above, these crops cannot be protected by chemical application prior to or during the study.

c. Manipulation of crop to influence flowering. A useful trait of *Phacelia* is the potential to manage the flowering period with cutting, giving researchers the ability to manipulate the duration of the flowering period. The timing of this operation is critical: too late in the plant's growth cycle and the plant will not regenerate. If it is done early enough, the plants rebound with more flowers and a tighter canopy. The height of the cut is also a factor, as the closer to the ground, the longer the period to start producing flowers again. None of the other crops mentioned above respond to cutting back in the same way.

d. Crop management. The crop should be planted and maintained in accordance with good agricultural practice with the exception that no plant protection products can be applied.

e. Seed. The sowing rates in Table 1 are suitable for inside enclosures; for full field experiments, advice from a local agronomist should be sought.

Table 1: Recommended seed sowing rates

Crop	Sowing rate
Phacelia	5kg/ha
Oil seed rape	5kg/ha
Buckwheat	15-25kg/ha
Radish	12kg/ha
Mustard	10kg/ha

f. **Excessive sowing rates should be avoided**, as plants growing close together have a tendency to lodge, i.e. collapse, particularly in heavy rain.

g. Sowing seed. The researcher may choose to have the crop sown over an entire field, which confers the advantage that the selection of plots may be postponed until the crop can be evaluated for rate of growth and density of plants. The plots can therefore be selected with a degree of uniformity. The alternative is to mark out the plots, then sow only within the outline of the enclosures. If this method is selected, it would be advisable to sow extra plots so that the poorest plots in terms of plant growth may be discarded. If the plots are hand-sown, it is advantageous to mix the seed thoroughly with sand or compost before scattering to make the distribution of plants more uniform. The ground should be well prepared, preferably being cultivated several times in order to destroy weeds which would compete with the target crop. If possible the seed bed should be rolled after sowing to improve germination.

h. Growth rate. Estimates of the growth rate of the crop should be made in order to synchronise the date on which the bees will be ready for the study with the flowering of the crop.

i. Multiple plant species. It may be advantageous for more than one plant species to be grown together for a study, for example a *Brassica* sp. plus *Phacelia*. This can extend the period of flowering and hence the duration of the study. However, growing two or more species together increases the challenges of getting all plants to thrive, and achieving near-simultaneous

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flowering. The provision of combinations of flowers will add complexities to analysis and interpretation. Professional agronomy assistance may be valuable at this point.

j. Application. Products should be applied according to label instructions, which may give broad descriptions such as 'before flowering' or 'not during flowering'. The instructions may be more precise, such as 'before BBCH 55' (Meier, 2001), in which case the researcher must have a clear understanding of such terminology. It should be noted that (a) specific guidance is provided for the recognition of BBCH stages in agricultural crops such as oil seed rape, but not for *Phacelia* for example, and (b) not all plants in a crop are at identical stages, so if for example the product is to be applied at BBCH 61 (start of flowering), a proportion of plants will be more advanced, and a proportion will be less advanced.

k. Enclosures. Enclosures shall be tunnels or cages covered with netting of mesh size sufficient to prevent the passage of males and females of the target bee species (Medrzycki, 2013). The size of the enclosures will vary according to target bee species. The following sizes may be regarded as sufficient for the numbers of bees described in the following protocols:- honey bee enclosures $\geq 72m^2$; bumble bee enclosures $\geq 54m^2$; solitary bee enclosures $\geq 36m^2$. If the selected crop is oil seed rape, consideration may be given to protecting the crop from common pollen beetle by using a small enough mesh size.

l. Wind damage. Particular attention should be paid to the fixing down of the enclosures as high winds can cause substantial damage. Preferably a trench will be dug close to the corners of the enclosure using a mechanical digger; the edge of the net can then be buried for security. The net should also be fixed at low level to the structure of the enclosure, using cable ties or similar.

m. Access. Each enclosure should be provided with a zipped doorway.

n. Location. The enclosures should be erected to a uniform orientation, preferably at some distance from natural features (trees, hedgerows, topography, uneven or wet ground) or man-made features (buildings, walls, tracks) which may cause variability in the growth of the crop(s).

o. Signposting. Each enclosure will be clearly identified so that field technicians can move quickly and confidently between plots.

p. Separation. The minimum separation between enclosures should be 4m, and the minimum distance from any enclosure to the nearest adjacent crop should be 7m, to minimise spray drift and contamination.

q. Allocation. The different treatments will be allocated randomly to the enclosures. This may either be completely random (if plant cover is very uniform) or with a stratified random allocation approach that takes crop abundance and/or colony strength (if relevant) into account.

r. Interior considerations. The internal layout of the enclosure will be such that the spray application will not be impeded by structural members or equipment when applying product. For example, a typical spray apparatus may include a 3m boom so structural members must be at least 3m apart along the route taken by the spray contractor.

s. **Paths inside enclosures.** The provision of covered paths inside the enclosures is not essential, as field technicians walking in the enclosures will keep paths clear. For study observations, however, two pieces of ground cover fabric should be fitted in diagonally opposite corners as described later to facilitate the finding and identification of dead adults and juveniles.

t. Agronomy. The following data will be recorded - (a) the history of pesticide applications on the study site over the previous two years, and (b) the current application of pesticides on adjacent land.

u. Irrigation. In most studies, irrigation will not be required. In some circumstances, however, it may be advantageous, for example after sowing if the soil is too dry for seeds to germinate, or during flowering when hot and dry conditions can kill plants. The research team should be aware

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of what options are available. The ideal situation would be if irrigation equipment is already in place. The quantity of water required should not be underestimated. Several tonnes will be necessary for even a medium-sized study. Watering by hand is impractical and time-consuming. It would be valuable to know in advance of a contractor who could irrigate the plots mechanically at short notice, plus the cost, quality and availability of water on site.

v. Duration of study. The study may be carried out in two phases, i.e. the exposure phase in which the bees are in contact with the selected stressors, followed by the optional monitoring phase in which the bees may be removed from contact with stressors. Consideration of the study bee species and the selected stressors will determine the duration of each phase.

2.4 Mode of application of chemical stressors.

Plant protection products, otherwise known as pesticides (insecticides, miticides, fungicides and herbicides) are applied to crops as solid seed coatings, as powders and as liquid sprays, depending on the usual application mode of the plant protection product. Spraying is the most common.

2.5 Chemical stressors applied as spray

a. The chemical should be applied as formulated product, not active ingredient.
b. The product should be applied as the maximum realistic field dose (EFSA, 2013).
c. The product will be applied in accordance with the timings on the label. For example, it may be before flowering, or after flowering, or during flowering when bees are flying.
d. The presence and identities of adjuvants (extenders, wetting agents, sticking agents and fogging agents) should be identified if possible. No adjuvants should be added when making up the tank mix.
e. It may be advantageous for the application to be carried out by a professional spray contractor, preferably with research experience and equipment appropriate for use in research enclosures. If the work is to be done by field technicians, training should be provided, and preferably professional qualifications obtained.
f. The researcher and contractor will jointly confirm that the product is as specified.
g. Each enclosure and the treatment to be used in that enclosure should be clearly identified. A system of verification of correct applications should be put in place. For instance, a tag on the doorway may be removed by the contractor after spraying for checking. Alternatively, a tag, flag or other marker may be left near the doorway of each enclosure to indicate application has been carried out.
h. Plastic spray sheets may be temporarily fixed round enclosures to prevent spray drift reaching adjacent enclosures.
i. The wind speed during application will be recorded periodically using a vane anemometer. A mean wind speed of not over 2m/s (EFSA, 2013) is desirable during application. If wind speed is excessive, the researcher may justify a decision to continue, or postpone application.
j. No-one will enter an enclosure that has been sprayed earlier than the recommendations on the product label.
k. Clean water will be available on site for the spray applications. The spray equipment will be calibrated on site.
l. The performance of the spray will be checked periodically to ensure the volume of liquid dispensed is accurate; it is desirable to do this after each enclosure, but this can be time-consuming; it will be acceptable to do so after three or four enclosures, and calculate the mean volume applied.

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m. Two 100ml samples of solution will be taken from the spray tank, and two 100ml samples of undiluted product will be taken from the product container, and placed in a freezer for later shipping to an analytical laboratory to verify the quantity of active ingredient present.
n. The following data will be recorded in connection with spray application: weather data including mean and peak wind speed; BBCH stage of crop development; product name; product batch number; product use by date; dilution rate, application rate per hectare; theoretical application rate per enclosure; actual application per enclosure.
o. For 48 hours after application, field technicians will avoid contaminating non-sprayed enclosures by one or both of the following means: (a) wearing disposable overalls, gloves and shoe coverings, (b) sequencing tasks so that no person enters a non-sprayed enclosure after being in a sprayed enclosure.
p. Where a study requires the application of two products simultaneously to the same crop, they may be mixed together in the tank.
q. The unused products will be disposed of legally and properly.
r. Enclosures that are acting as negative controls, i.e., are not to be sprayed with products, will be sprayed with the same volume of water only, at approximately the same time as enclosures sprayed with product.

2.6 Chemical stressors applied as seed coating

Seed coatings are available for a limited number of crops. A study is therefore limited to those species. Since drilling requires large specialised machinery, it is inconvenient to conduct studies in enclosures. Should such a study be considered, the whole site would have to be cultivated, and then the plots marked out and enclosures erected over the growing crop.

2.7 Chemical stressors applied as powders and granules

Replicating field conditions inside enclosures using solid plant protection products is challenging, as conventional machinery is too large. Given that the proportion of plant protection products applied as solids is very low in comparison with liquid sprays, the authors do not advocate the use of Tier 2 caged studies for powder and granule applications.

2.8 Nutritional stressors.

a. Bees rely entirely on nectar and pollen for nutrition. Nectar is primarily a solution of simple sugars (substantially fructose and glucose with minute proportions of vitamins and minerals in water) (Crane, 1990). The concentration varies considerably, but the nutritional value of the sugars is not highly variable. Nectar is converted to honey by honey bees by evaporation of water and addition of enzymes, and stored in large quantities (>20kg in many cases). Bumble bees and solitary bees collect much less nectar and consume it more directly as a source of fuel for flight and metabolic heat. A shortage of nectar will result in starvation.
b. The nutritional value of pollen on the other hand varies widely (Vanderplanck, 2014) depending on the protein content, the lipid content, and the amino acid profile. Pollen plays a more complex role in that it enables the growth of young bees either by direct consumption or in the case of honey bees, by indirect consumption, whereby nurse bees digest pollen and feed immature bees by secretions from the hypopharyngeal glands.
c. The term ‘nutritional stressor’ in the context of these protocols refers to the nutritional quality of the pollen available to the bees, and is applied by growing crops that yield pollens of different nutritional values (Stuligross ,2020).

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d. Low nutritional value pollens include maize *Zea maiz,* sunflower *Helianthus anuus,* rock rose *Cistus* spp. and buckwheat *Fagopyrum esculentum* (Vaudo, 2020). Of these, buckwheat has advantages in the speed of growth once germinated, plus the dense stand the plant produces. It is however susceptible to cold and easily killed by frost. *Phacelia* on the other hand yields a pollen that is of high value (Cawoy, 2009).
e. If buckwheat is used as a low nutritional value pollen in comparison with *Phacelia*, a rough rule of thumb for planning a study is that in good conditions *Phacelia* may start to flower six weeks after sowing and buckwheat may take four weeks.
f. The researcher should be satisfied that impacts are due to the nutritional value of the pollen, and not the quantity of the pollen available. This question may be addressed by measuring the pollen available on one flower or plant and calculating how much is available on the plot. Alternatively, in the case of honey bee studies, the mass of pollen available on a plot may be assessed by the use of a small pollen trap placed over the entrance of the hive for a short period, for example one hour, repeated on several days.
g. The nutritional value of the pollens should be evaluated by analysis of pollen collected by bees to determine at least the mean protein content, but also preferably the lipid content and the amino acid profile (Vanderplanck, 2014).

2.9 Pathogen and Parasite Stressors

1. Social and non-social bees suffer from pathogens; these are well documented in the case of honey bees, less so for other test species (Morse, 1997). It seems self-evident that bees with these conditions would be further compromised if also exposed to chemical or nutritional stress. The maintenance of numerous colonies of bumble bees or honey bees, or populations of solitary bees, which exhibit low variability in the levels of parasites and pathogens is extremely challenging however. Studies examining these factors are more suited to research rather than regulatory processes.
2. Similarly, test bee species are attacked by parasites and parasitoids. While it may be slightly easier to distribute these among the test populations, nevertheless the complexity of controlling and assessing such variables at Tier 2 level limits the value of such a study at least as a regulatory tool.

2.10 Residues

1. The choices of samples to be taken for chemical analyses depends on the routes by which the chemicals reach the bees, both adults and juveniles, and by the expertise and resources of the laboratory. For example, the following substrates may yield useful information when analysed: leaves, flowers, nectar, pollen, honey, bee bread or stored provisions, old bees, young bees, juveniles, nest materials, faeces, wax, propolis, etc.
2. Protocols are to be agreed in advance with the laboratory regarding collecting samples, packaging, labelling, transporting, freezing and shipping.

2.11 Controls

1. Negative controls are required. In the case where chemical stressors are applied by spraying, the untreated enclosures will be sprayed with an equal volume of water.
2. Positive controls, such as exposing bees in enclosures to fenoxycarb (EFSA, 2013) are not essential, but may be carried out if required to ascertain that the plant protection product is reaching the bees in the study.

2.12 Verification of exposure

1. Samples of the spraying solution should be taken from the mixing tank or from the spray boom nozzles, and transported to the laboratory for analysis. The measured concentration should be within the range 80 to 120% of the nominal concentration.
2. If laboratory resources permit, verification of exposure can additionally be measured by sampling plant tissues, such as flowers or leaves. In this case further sampling of the crop throughout the study may be taken to assess the stability of the product.

Recording, Reporting and Data Storage will be in accordance with current good practice (Pirk, 2013).

3. Protocols for semi-field studies on honey bees

3.1. Outline

Honey bee semi-field studies rely on a balance being maintained in enclosures between the forage available and the nutritional needs of adult and juvenile bees. The creation of small but stable colonies is facilitated by the use of small (Mini Plus Beuten) hives. Manipulations and data collection may be enhanced by equipment such as study frames, pollen traps and dead bee traps which are specifically designed and manufactured for such studies.

3.2 Duration of the semi-field honey bee study

The study will comprise an exposure phase and a monitoring phase. The exposure phase will cease before the forage from the crop(s) is considered to be inadequate to support each colony. The exposure phase will last a minimum of seven days. The study will extend over at least one brood cycle (21+ days) and preferably over two brood cycles (42+ days).

The timings given relate to the first exposure of honey bee colonies to the stressors. For example, D0 would be the day of a spray application of product; D-7 would be seven days before application; D+4 would be 4 days after application.

3.3 Colony Specification

1. The following will be specified: colony type (full-size/nucleus/miniature); type of hive (for example Langstroth, Dadant, Deutsche Normal, etc); and colony origin.
2. Colonies which have previously been part of a study will not be selected as study subjects within the following year.
3. For semi-field studies, the size of the colony should be matched to the forage resources available. If too many foragers are in competition for too little food, there is a danger that hive bees will discard or cannibalise young brood, particularly eggs, leading to excessive brood loss even in control colonies.
4. To reduce the disparity between colony demands for food and the nectar and pollen resources in an enclosure it is recommended that the Mini Plus Beute (MPB) be used for ease of creating and sustaining small stable colonies. This is a small well-insulated hive with frames approximately 200 x 160mm.
5. MPB study colonies may be created by selecting frames of brood and stores from larger colonies, and weighing the number of adults to be added. The queen should be from the same colony. It is recommended that a MPB study colony should be made up seven to ten days prior to the study, and comprise 3,000 adult workers with one and a half full frames of

sealed brood. An enclosure of $\geq 72m^2$ with a good crop can sustain a colony of this size for a week with only moderate loss of weight.

6. All queens in a study should be of the same age, either born in the current year of the study or one year previously. The queens should be closely related if possible, preferably sisters. The queens should be marked. The subspecies or variety of the queens should be noted, although these data are hard to verify given that so much cross-breeding has occurred over a long period. In particular it should be noted that the term 'Buckfast-type' has little meaning now.
7. Queens may be open-mated or artificially inseminated.
8. The queens may or may not have clipped wings.
9. The colony history prior to the study will be recorded, including health status and medications/treatments.
10. All colonies will be examined in the field by an experienced beekeeper prior to the study to identify the presence of bacterial diseases (American and European foul brood); fungal disease (chalk brood); parasitic mites (*Varroa destructor*); and wax moth (*Achroia grisella* or *Galleria mellonella*). Field workers will be trained to detect the presence of the predators small hive beetle (*Aethina tumida*) and Asian hornet (*Vespa velutina*). Diseases and conditions that require laboratory identification will not be investigated.
11. Routine treatment for *Varroa* mites (*Varroa destructor*) will be maintained as normal.
12. A record of medications of each colony over the two years prior to the study will be maintained.

3.4 The PoshBee study frame

Fig 1: The PoshBee study frame

1. The PoshBee study frame is a key component in such studies. It incorporates a number of unique features.
2. It is fitted with beeswax-coated black plastic foundation which (a) improves photographic images for brood assessments, (b) prevents workers making passages and the queen escaping.

3. Each frame is coded with a unique identifier on each face which is laser etched (if it is covered with wax or propolis, it can be scraped with a hive tool to reveal the code burned into the timber).
4. Each side of the study frame can be selectively covered with a machined queen excluder which is held in place with magnets, yet can be removed easily and quickly; this enables the queen to be trapped on one side of the frame, or alternatively for the queen to be prevented from entering the cage and laying on the frame.
5. The queen may be held on one side of the cage, then a few days later held on the other side; this enables the impacts of stressors to be observed on eggs or on larvae in the same experiment.
6. The queen excluder cover may be replaced by a perforated cover which prevents any bee accessing the frame, or alternatively prevents any bee escaping from that frame; this facilitates transport of samples from the field to the lab.
7. The profile of the sides, top and bottom of the PoshBee Study Frame ensures that the queen cannot escape when a cover is fitted, but when two such frames are placed side by side, the profiles align to ensure that bee passages are formed to allow free movement of workers to tend brood.
8. The PoshBee Study Frame is constructed of thicker timber than conventional frames which appears to encourage building of comb closer to the edges of the frame which is advantageous for brood studies.

3.5 Colony strength assessments

1. Adult bee populations will be estimated on site by observers who have been trained in visual assessments using library photographs of many frames in accordance with published methods, such as the Liebefeld method (Bargen, 2020) or ColEval (Hernandez, 2020). The number of adult bees on each side of each frame will be estimated and recorded. The number of adult bees on the inside walls, the floor, and the cover of the hive will be estimated and recorded.
2. The amount of brood in a colony can also be estimated using the ColEval method, by brushing adult bees from every frame. The observer estimates the proportion of each frame containing (a) sealed brood, (b) open brood, (c) nectar/honey, and (d) pollen. However it should be noted that shaking or brushing bees from all frames in a colony can be disruptive, and that the loss of adults and brood due to the beekeeper's handling may substantially exceed the impact that the study is designed to detect. It is suggested therefore that brood populations are assessed prior to the study, at the end of the study, and possibly once during the study. In order to minimise loss of bees during the procedure, it is recommended that a special wide hopper that fits over the open brood box be made. Bees brushed off the frames can safely drop into the hopper and down into the brood box.

3.6 Assembly of study colonies

1. Study colonies may be created by use of converter hives. These contain conventional frames longitudinally in the bottom box and MPB frames transversely in the top box (alternative methods of managing bees to create study colonies are acceptable). To prepare for the study, a number of colonies on conventional frames are established in converter hives and allowed to expand. The number of colonies prepared should be in excess of that required for the study, in order to discard unsatisfactory colonies. During this phase the colonies will be managed by conventional means, i.e. they will be fed if necessary, swarm prevention will be carried out, etc.

2. At approximately D-28 the queen in each colony will be allowed access to the top box and allowed to lay in the MPB frames. A queen excluder may be used to restrict the queen to the top box. At approximately D-24 the queen will be returned to the bottom box and confined there by a queen excluder.
3. At approximately D-14 to D-7 study colonies should be created as follows: from each converter hive the queen will be removed and caged; two MPB frames full of brood on each side will be placed in an MPB brood box on a screened floor with integral dead bee trap and pollen trap; two frames of drawn comb with no brood will be added; one study frame (see below) with a queen excluder cover on each side will be added; and one frame of foundation will be added. Adult bees will be shaken from frames and a weighed quantity (preferably 300g) introduced to the MPB hive. The queen will be released from the cage and observed as she walks down between the frames. A queen excluder will be placed over the brood box, and a second box containing honey stores will be added. The boxes of honey stores will be prepared in advance and the weights will be equalised so that each colony receives approximately the same amount of food. 2kg of honey stores or fondant equivalent is recommended.
4. The study colonies should be allowed to stabilise for a few days, before being assessed by ColEval; any colonies with non-laying queens should be discarded; any colonies which are clearly smaller should be discarded. The remaining colonies should be allocated to treatments at random, unless there is significant variation in populations, in which case the colonies should be grouped according to populations, and the members of each group allocated at random to the treatments.
5. To ensure that there is sufficient brood available for assessments on brood fixing day (BFD), the queen in each colony will be manipulated as follows: (a) three days before BFD, the queen will be placed on the Study Frame, and a queen excluder placed over her; (b) two days or one day before BFD, the queen will be released, and the queen excluder put back in place so that nurse bees can care for the brood, but the queen cannot lay more eggs.
6. An optional manipulation provides the opportunity to observe differences in response to exposure by larvae in contrast to eggs. As an illustration, the queen may be (a) caged on the Study Frame five days before BFD; (b) released on the following day; (c) caged on the reverse side of the Study Frame one day before BFD; (d) released on BFD.

3.7 Photography work

1. Photos of brood are to be taken in line with OECD Guidance Document 75 (OECD, 2014).
2. Photographic assessments of brood may be extended to two brood cycles, i.e. 42 days using day 22 as the second brood fixing day. The brood assessment days would therefore be BFD; BFD+5; BFD+10; BFD+16; BFD+22: BFD+27; BFD+32; BFD+ 38; BFD+44; plus or minus one day in each case.
3. Taking high quality photographs of brood in the field is challenging. Apparatus and techniques should be tested and refined prior to the study (Jeker, 2012). A combination of a quality digital SLR with an autofocus 80mm macro lens (preferably with vibration reduction function) will give good results providing the lighting is correct. The lights may be floodlamps or flash. In order to get even lighting at the base of an open cell (particularly when surrounded by sealed cells) more than one light source is advantageous, four being preferable. The light may be directed directly at the cells, but the authors recommend that the illumination should be directed at an angle into the cells, or reflected off the housing

interior or other reflectors. Diffusers may be used to soften the illumination and reduce reflections from nectar and honey.

4. Cells built by bees to accommodate brood are built with an upward slope of approximately 13°. In order to obtain the clearest view to the base of occupied cells to detect eggs and first instar larvae, the camera should be tilted at 13° with respect to the frame (or vice versa).
5. To provide consistent lighting conditions, the photographic apparatus is fitted within a housing which blocks most external light. For speed and ease of use, the housing should be on wheels and small enough to fit in a car.
6. The housing should incorporate power packs (including spares) and control switches.
7. Photographs should be taken with maximum depth of field. To achieve this a shutter speed of up to 0.5sec is acceptable, providing the housing is stable and the technician uses a remote shutter release.
8. It is advantageous but not necessary to use a camera with high dynamic range (HDR), which takes multiple shots at different shutter settings and constructs a composite photograph from the different images.
9. It is sufficient to save the images as high quality (FINE) jpegs. Alternatively or additionally, the images may be saved as raw data files (RAW). Saving as RAW files allows the user to enhance the images using commercial photo manipulation software, although this function is not essential.
10. Photographs should be regularly downloaded, backed up and securely archived.

3.8 Image recognition systems

1. The value of high quality photographs of honey bee brood is that they can be easily analysed using image recognition software, allowing the life cycle of thousands of individual bees to be easily tracked from egg to adult, delivering accurate, verifiable, objective and abundant data sets. Commercial systems can be leased (e.g. visionalytics.de) or free systems with limited functionality may be accessed such as DeepBee (Alves, 2020).
2. Image recognition systems operate in brief as follows: each side of each frame is identified and dated; each photo of a specific frame taken at different times is overlaid onto every other photo of that frame, and each cell given a unique identifier; the system ‘reads’ the contents of the cell (empty, egg, young larva, etc.) and determines whether the bee in that cell survives to emerge as an adult.
3. The system automatically calculates the following ratios: (a) brood termination rate (BTR) (the percentage of eggs that do not survive to adult; (b) brood compensation index (BCI) (a measure of how many cells in which bees died are subsequently refilled); (c) brood index (BI) (an indicator of mean bee brood development).
4. Such systems can easily analyse a sample size of 500 or more in each study colony, up to a maximum of approximately 900 using the PoshBee Study Frame, although there is little statistical advantage in the latter number compared with the former.

3.9 Introduction of colonies into enclosures

1. Colonies should be introduced when sufficient forage is available, preferably, but not necessarily, both nectar and pollen.
2. Wild birds and mammals such as hares may need to be removed from the enclosures.
3. The colony location should be in one corner of the enclosure, not adjacent to the door, as shown in Figure 2.

4. The hive should be placed on a hive stand raised off the ground by 200 to 400mm for ease of handling and to reduce access for beetles which may eat bees in the dead bee trap.
5. Water should be provided in a commercial bee feeder or alternative arrangement and topped up regularly. Water feeders should be removed or covered during application of product by spray. The number of foragers collecting water should be recorded at each assessment visit. The value of these data is that it gives an indication of the availability of nectar within the enclosure. In most circumstances, honey bees will not collect water if nectar is available (Lipinski, 2019).
6. The hive should be placed on a piece of ground cover fabric measuring 2m x 2m placed and secured in one corner of the enclosure, from which dead bees can be picked up during mortality counts. A similar piece of fabric will be placed and secured in the diagonally opposite position within the enclosure. A challenging aspect of semi-field honeybee studies, particularly with larger colonies, is the disorientation of foragers when the colonies are first introduced to the enclosures, and the excessive loss of bees which fly into corners and die without returning to the hive. This mortality occurs in treated and control colonies, and on occasion the mortality among control colonies is so great that the statistical validity of a study may be called into question. By counting dead bees on two pieces of fabric in opposite corners of the enclosure, the researcher has additional data on this phenomenon. If, for example, the counts of dead bees in each corner are substantially different, such data may indicate different causes of mortality at the hive and on the netting.
7. Each hive should be secured against being blown over by placing weights on the roof or strapping it to the hive stand.

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8.

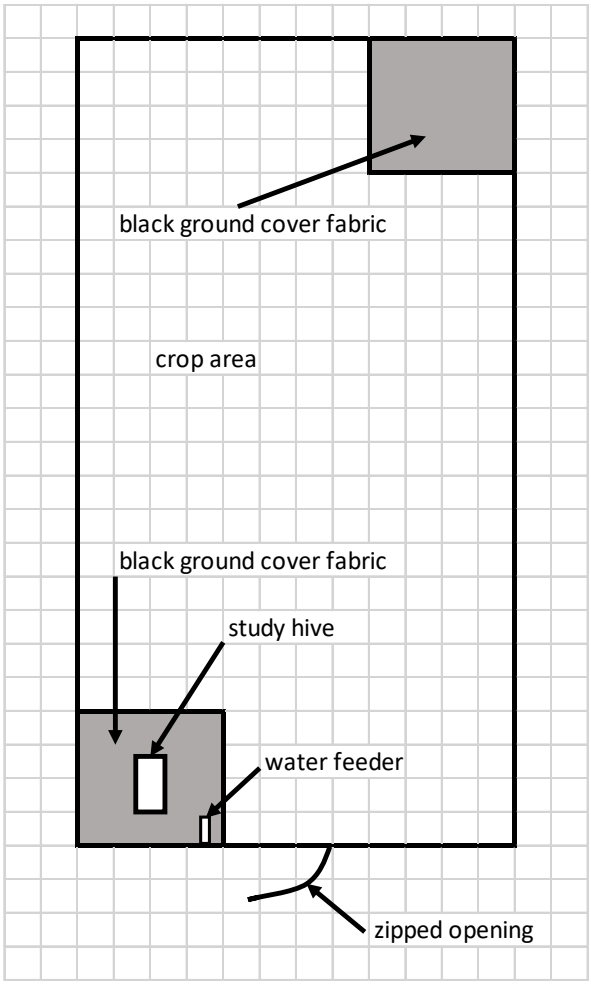

Figure 2: Layout of enclosure for semi-field honeybee study

3.10 Techniques for handling honey bee colonies

Bee handlers should (a) use slow movements when manipulating bees; (b) use limited amounts of smoke; (c) brush bees off frames (not shake); (d) be alert for presence of queen at each examination; (e) ensure that queens are marked in advance; (f) ensure that the queen's location on frame is noted and that queens are not dislodged or lost.

3.11 Food resource issues

1. The steps in the transmission of food (and hence product) from the flower to the colony are: pollen and nectar are collected by foragers; pollen and nectar are transported to the hive; pollen is packed into cells immediately adjacent to brood while nectar is transferred to young hive bees which deposit it in cells immediately adjacent to the brood; the subsequent transfer of food to juvenile bees is either via direct feeding of pollen and nectar or via secretions from nurse bees' hypopharyngeal glands.
2. In the setting of the study enclosure, the food stored as honey or bee bread is not utilised unless external sources are inadequate; the presence of honey in the upper box does not affect the flow of food from outside into the colony, and by trophallaxis and nursing, into the young bees; the presence of honey and bee bread in the upper box will not reduce the collection of food that has been exposed to plant protection products (Gruter, 2007).

3.12 Assessments

1. Colonies will be weighed by placing hives on scales on predetermined days.
2. The number of bees entering the hive will be counted over a period of 120 seconds on predetermined days.
3. The number of bees carrying pollen loads will be counted over a period of 120 seconds on predetermined days.
4. The number of foragers will be counted by placing a 1m square frame at random on the crop and counting the number of bees actually foraging on flowers within the frame, or investigating the flowers in the frame while in flight. The procedure will be repeated three times, and will be carried out on predetermined days.
5. Adult mortality in dead bee trap will be counted by removing and discarding dead adult bees. Juvenile mortality will be counted by removing and discarding dead pupae, prepupae, old larvae (4^{th} and 5^{th} instars), and young larvae (1st, 2^{nd} and 3^{rd} instars). These counts will be carried out on predetermined days.
6. Adult mortality will also be assessed by counting and discarding dead adult bees on the ground cover fabric surrounding the hive plus the ground cover fabric in the opposite corner of the enclosure, as described above. The counts should be recorded separately as the former is indicative of impact at the hive, while the second is indicative of disorientation which would affect control and treated colonies. Juvenile mortality will be assessed in the same way. The procedure will be repeated on the predetermined days.
7. Assessments of the adult bee population of each colony will be made by technicians trained in ColEval assessments. One bee handler will display in turn each side of each frame in the bottom box of each hive. The observer will record the number of adult bees on the frame. Assessments will also be made of the number of adults (a) on the floor, (b) on the walls of the hive, and (c) in the top (food storage) box. The bees will not be shaken or brushed off the frames, in order to avoid bees being counted multiple times. This assessment will be carried out on the predetermined days.
8. The assessment of brood will also be carried out using the ColEval method. This may be carried out on the same day as the adult assessment, the day before or the day after. To assess the brood properly it is necessary to first brush the adult bees from the frames. It is crucial that the queen is not brushed off and lost in this process. This manipulation can be quite disruptive of the colony and may result in bee losses greater than the impact of the stressors. It should therefore be carried out with care by experienced operatives.
9. Brood mortality will be assessed by an image recognition system. Photographs of each study frame will be taken on in accordance with the schedule previously described in this document.
10. The image recognition system will generate the following endpoints as defined in OECD75 – brood termination rate (BTR), brood compensation index (BCI), and brood index (BI). These analyses may be extended from a period of one brood cycle (21 days) to two or over (42+ days).
11. Counting pollen loads carried into the hive over a short period is an inadequate measure of foraging activity. By fitting small cartridge-type pollen traps for a few hours, a precise measure of pollen collection rate of a colony can be obtained, without causing a harmful shortage. Pollen samples will be taken on predetermined days. Pollen samples will be labelled and placed in a freezer for subsequent transport to a laboratory for chemical analysis.

12. The presence of the queen will be established at each examination of the hive by either (a) visual detection of the queen or (b) the presence of eggs.

3.13 Residues

1. The choices of samples to be taken for chemical analyses depends on the routes by which the chemicals reach the bees, both adults and juveniles, and by the expertise and resources of the laboratory. For example, the following substrates may yield useful information when analysed: leaves, flowers, nectar, pollen, honey, bee bread or stored provisions, old bees, young bees, juveniles, nest materials, faeces, wax, propolis, etc.
2. The foraging and storage behaviours of honey bees make sample collection simple. For example pollen can easily be collected from pollen traps, and nectar/honey may be withdrawn from combs. To verify exposure, various plant matrices may be tested such as leaves, petals, nectar or pollen. Additionally, larval or pupal tissue may be easily collected and analysed.
3. It is desirable that residues be individually sampled from each enclosure; if resources do not permit this, samples may be pooled and homogenised so that for example all controls are amalgamated, all of Treatment A are polled, etc.
4. Protocols are to be agreed in advance with the laboratory regarding collecting samples, packaging, labelling, transporting, freezing and shipping.

3.14 Behavioural assessments

The study may be designed to incorporate observations on behavioural abnormalities, such as aggression, lethargy, erratic movement, excessive guard bee response, excessive grooming, immobility, trembling, clustering and so forth. Details of such observations are given in OECD 75 (OECD, 2014). Such assessments should be scheduled, and should preferably be carried out by a single nominated field technician to ensure consistency.

3.15 Statistical analysis

Data will be statistically analysed in accordance with OECD (OECD, 2014) and EFSA (2014) guidance.

4. Protocols for semi-field studies on bumble bees

4.1 Outline

Bumble bee semi-field studies require enclosures in which appropriate crops are grown that will sustain the growing colony populations with adequate provision of pollen and nectar. Small colonies will be introduced and allowed to build up. When a defined proportion of the colonies have started producing sexual individuals (i.e. the ‘switch point’ has been reached) the colonies will be frozen for later analysis in the lab to assess the study endpoints (Knaebe, 2019).

4.2 Colony specification

a. The suppliers should be made aware of the specific requirements of the study.
b. When ordering colonies for bumble bee studies the researcher should specify the date the bees are required; the number of workers required; the feed to be provided (sugar syrup/pollen mass); the nest details (with/without one-way flap? with/without fibre insulation?).

c. The supplier must state the species and subspecies, whether *Bombus terrestris audax, B. t. terrestris,* or *B. t. dalmatinus* (Owen, 2016).
d. For security additional colonies should be ordered.
e. The researcher should liaise with the supplier to ensure delivery of colonies matches expected start of flowering of crops in test enclosures.
f. It is advantageous to start the study with the smallest practical colony (for example one queen and 10 workers, plus developing larvae) to allow greatest duration of the study, as once the colony reaches switch point, activities are curtailed with consequent reduction of data.

4.3 Nest boxes

1. The nest boxes should be provided with (a) one-way gate for bees entering, and (b) queen excluder on exit to prevent queens escaping; field technicians should be careful to ensure that the one-way gate is in position after each examination.
2. The nests should be provided with sucrose syrup and may be provided with pollen, either as removed from honey bee corbiculae, or in mass; these resources should be made available to the bees prior to the study, but removed when the study commences.
3. The nest boxes should not be provided with cotton wool-type material that is conventionally included to provide insulation to the nest, as the presence of this material hinders observations; *Bombus terrestris* colonies create a covering of wax and vegetable matter to cover the nest; this can be easily pulled back to make observations and it is rapidly replaced.

4.4 Handling colonies prior to study

1. Colonies may be stored in cool conditions if necessary to wait for the start of study (if, for example, there is lack of bloom, poor weather conditions, etc).
2. It is desirable to mark the mother queens of the colonies, so that if mortality occurs it is correctly identified, as loss of foundress queens has implications for studies. Numbered discs and queen marking paint are readily available from beekeeping suppliers.

4.5 Specialised equipment

1. A bumble bee handling unit (optional) which fits over the bumblebee nest enables bees to be handled with substantially reduced risk of stinging and prevents escape of bees, particularly queens, during handling.
2. To avoid risk of product cross-contamination between enclosures, one handling unit should be identified for use in treated enclosures, and one for use in control enclosures.
3. An electronic bumble bee monitoring unit (optional) may be used to assess number of flights (described below).
4. If electronic bumble bee monitoring units are used it is important to verify that the one-way gate remains attached throughout the experiment.
5. Innovative video recording systems are available that record and interpret bee movements. These could provide substantially improved endpoints.

4.6 Introduction of colonies to enclosures

1. The nests should be placed within larger boxes (housing units) made of wood or Styrofoam to prevent sunlight or rainfall from entering. Provision should be made to enable the lid of the cardboard outer to be raised as normal to facilitate ventilation.

2. The housing units should be placed on stands, at a convenient height for handling and observations; the arrangement should allow for easy placement and removal of the bumble bee handling unit if these are used.
3. Overheating is a danger in extreme weather; additional holes may be created in the (plastic) nest box and the surrounding housing unit to improve ventilation. Cold packs may be placed inside the housing unit during extreme heat.
4. Bumble bees do not usually collect water (nectar is typically their only source of water). However, it may be advantageous to install water feeders in enclosures, in case truly critical shortages of water occur, in which case very dilute sucrose syrup (1:10 w:w) may be offered.
5. Colonies should be introduced when sufficient forage is available.
6. If possible, wild birds and mammals such as hares should be removed from the enclosures.
7. Each nest may be placed in any consistent location in the enclosures; near a doorway is convenient for access and avoids damaging the crop.
8. The nest boxes should be strapped or weighed down on a stand. The nests should be attached in a way that ensures that the entrances and exits align with the corresponding holes of the housing unit.
9. Food supplements should be removed or closed when colonies are placed inside the enclosures.
10. The colonies should be placed inside the enclosures two or three days prior to application of test plant protection products in order to assess the colonies prior to exposure. This ensures that the colonies have time to adapt to their environment and start foraging.

4.7 Techniques for handling bumble bee colonies

1. Bumble bees possess powerful stings; these are not barbed, so a bumble bee worker or queen can sting rapidly and repeatedly; if disturbed in the nest they will respond aggressively, but in the enclosure, they are exceedingly unlikely to sting if more than a few metres from the nest.
2. It is advisable to wear gloves when handling bumble bees; there will be a compromise between protection and sensitivity – thick leather gloves with little sensitivity or lab-type rubber gloves with abundant sensitivity but little protection.
3. Slow hand movements aggravate bumble bees less than rapid jerky movements.

4.8 Assessments

1. **Colony weight.** The nests will be taken out of the housing unit and placed on scales.
2. **Adult mortality in nest.** The number of dead adults inside the nest will be counted. Once a week these should be removed from the colony to avoid accumulating numbers of bee bodies and their degradation which will make it difficult to accurately estimate mortality.
3. **Juvenile mortality in nest.** The number of dead pupae and larvae will be counted and removed regularly. As juveniles degrade quickly this should be done at least twice a week.
4. **Number of adults in nest (and enclosure).** The number of adults will be estimated by taking a photo of the nest and subsequently counting adults. The number of dead adults should be subtracted from the total count to obtain the number of living adults. To account for differences in activity that may influence the number of bees that are inside the nest, the number of bees within the enclosure (but outside the nest) may also be estimated and added to the number of living adults.
5. **Foraging activity.** Unless electronic devices are used that continuously track bee activity, parameters related to foraging activity should only be assessed during adequate weather

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conditions: ≥13°C, no rain, no/low wind (<2 m/s). Especially on hot days, assessments in the middle of the day when activity is low should be avoided. To avoid confounding effects of daytime, different treatments should be assessed simultaneously if possible (i.e. if there are multiple observers). If parallel assessment is not possible, assessment of different treatments should be alternated. Also, to limit a potential confounding observer effect, observers should to some extent alternate between treatments (this could mean that they alternate after every enclosure if there are as many observers as treatments or that they alternate only every nth time or that they alternate only between different assessment days. The latter is necessary when there is a considerable risk of cross-contamination, e.g., in the morning after spray applications).

6. **Number of flying bees at entrance.** Counting may be done manually using a hand tally counter during a period of 3 min or preferably automatically and continuously using an electronic bee activity monitor.
7. **Number of pollen loads.** Bees entering with visible pollen loads may be counted within a defined timeframe (such as 3 min). Alternatively or additionally, pollen may be stripped from the legs of foragers returning to the colony. Because the size of foragers varies, this is not as straightforward as collecting pollen from honey bees. A pollen trap suitable for bumble bees made by 3D printer has been described (Judd, 2020).
8. **Individual foraging performance.** The number of individual flowers a bee visits during a time period of 120 seconds will be recorded for three bumble bees per enclosure (note the definition of a flower: two different flowers visited on the same inflorescence/floral unit still counts as two visited flowers, they do not have to be on different plants or inflorescences). If multiple plant species are present in the enclosures, the number of visited flowers should be distinguished between plant species. In this case, it is advisable to use multiple click-counters or a mobile app that allows for counting of multiple different items. If the observed bee is lost before the two minutes are over (i.e. the bee cannot be seen anymore or returned to the nest), the time will be stopped. If the observation time is greater than 90 seconds, the observation is valid and can be noted down as one of the three observations per cage. If the time is between 60 and 90 seconds, another bee has to be observed for at least 60 seconds to compensate. Another 120 second observation then has to be conducted. If the observation time is less than 60 seconds, the observation will be discarded.
9. **Flower visitation rate and number of foragers in quadrats.** The enclosures shall be divided into subsections (e.g. the space between different uprights). At each observation day one subsection on the left and one subsection on the right will be randomly sampled without replacement (i.e. subsections will only be repeatedly observed after all subsections have been observed once). Inside the selected subsection a quadrat of 1m × 1m shall be placed in a position that contains a representative floral cover. Within the quadrats, the number of bees and the number of visited flowers shall be counted within 3 min. If the subsections are small enough to be easily monitored by one observer the whole subsection rather than a quadrat may be monitored. This may reduce disturbance but will be tricky if activity is high. This assessment is less relevant if the previous foraging activity parameters (number of flying bees at the hive entrance and individual foraging performance) are assessed, especially if monocultures are grown inside the enclosures.
10. **Floral cover.** The number of inflorescences with open fresh flowers (i.e. flowers that have not yet produced fruits or seeds) will be counted in the previously mentioned quadrats (i.e. if flower visitation is not assessed it is still advisable to record floral cover as other parameters are also affected by the number of available flowers). On three representative inflorescences, the number of open flowers will be counted. The mean value of open flowers

per inflorescence will be multiplied with the number of inflorescences to obtain the total number of flowers. If multiple flower species are grown inside an enclosure, the process is repeated for all flower species.

11. **Number of cocoons.** The number of cocoons is assessed in the beginning of the experiment by taking a photo and counting cocoons. As the colonies grow this method gets increasingly difficult to use especially if the wax cover that some colonies create is not removed. A more accurate assessment will be done during the dissection of the colonies after they have been freeze-killed.
12. **Pesticide residues.** Pesticide residues may be quantified in pollen, nectar and/or bees. It is only necessary to test for the target active ingredients as there is little risk of substantial exposure to other pesticides. Therefore, we believe it is better to invest in residue analyses on multiple days to identify how exposure changes over the experiment than to invest in multi-residue analyses. Most important are residue analyses on the day after application for pesticides applied during bloom or on the day after colony placement for pesticides applied before bloom as exposure is expected to be highest then. To obtain sufficiently large sample sizes, samples may be pooled across enclosures of the same treatment. To catch foragers, different sweep nets should be used for different treatments. The choice of the matrix to test may depend on the available screening method, the required amount of sample, the ease in obtaining the samples (it may be difficult to collect large amounts of bumble bee-collected pollen or nectar) and/or the minimum colony size (in small colonies less intrusive methods may be preferred over intrusive ones).

4.9 Samples for analysis may include the following.

a) Bee-collected pollen: Pollen foragers may be caught using sweep nets to collect their pollen for residue analyses. Afterwards, the foragers can be released. The pollen should be sorted by plant species and weighed.
b) Bee-collected nectar: Nectar foraging bees may be caught to extract their nectar immediately or later in the lab. If the nectar is to be extracted in the lab, bees should not be freeze-killed. Both the bees and the nectar will be stored for residue analyses in this case. If multiple plants are grown inside the enclosures, it should be noted on which plant species the bee has been seen foraging.
c) Nectar from flowering plants may be collected using micro-capillaries.
d) Bees for residue analysis may be sampled using sweep nets.

4.10 Date of switch point

Bumble bee colonies switch from the production of workers to the production of reproductives (males and queens). The date when this happens should be determined by the presence of queen cocoons (queen cocoon width > 12 mm (Rundlöf, 2015). As a second criterion the presence of males can be used.

4.11 Colony termination

Each colony will be removed from the site and frozen in its entirety, 21 days after the first queen pupae have been observed or after the foundress queen is noticed to be dead. When the last control colonies are frozen, the remaining pesticide-exposed colonies will also be frozen.

4.12 Colony dissection after colony termination

1. A standardised photo of the nest will be taken, showing colony identification, enclosure identification and date.
2. The colony weight will be measured (nest box + colony).
3. The empty nest box will be weighed (nest box without colony).
4. The number of workers will be counted; the sex will be determined by the presence of a sting. Workers should be separated in different 50ml Falcon tubes into those that were probably alive until the colony was frozen and those that had died prior to freezing. Bees already dead are drier and crispier, especially on the underside of their abdomen.
5. Queens will be identified by size.
6. The number of males will be counted. They will be identified by the lack of a sting and the presence of male genitalia. They should also be divided into different 50ml Falcon tubes depending on whether alive or dead at the point of colony termination.
7. The number of gynes (new queens) will be counted. New queens should also be separated into those that were alive and those that were dead at the point of colony termination.
8. The presence or absence of the foundress queen presence will be noted. The foundress queen should be recognised by the marking done prior to the experiment. If the marking is no longer visible it should be noted that the foundress queen is not present. However, it is possible that the marking has been lost, in which case it may be possible to identify the foundress queen by her dull and worn appearance with ragged wings.
9. Number of worker and male cocoons (< 12 mm width), intact and eclosed, will be counted.
10. The number of intact and eclosed worker/male cocoons should be counted and stored separately.
11. Number of queen cocoons (> 12 mm width), intact and eclosed, will be counted.
12. The number of intact and eclosed queen cocoons will be counted and stored separately.
13. Number of wax cups will be noted.
14. Number of pollen storage cells will be noted.
15. The amount of stored pollen will be estimated by dismantling pollen cells and weighing contents.
16. The presence of parasites, kleptoparasites and commensals (such as wax moth webs/larvae/cocoons/adults, cuckoo bees, flies, wasps, parasitoids, ants, spiders, mites etc) will be noted.
17. The number of wax cells with egg clumps will be noted.
18. The number of wax cells with larval clumps will be noted.
19. The number of wax cells with separated larvae will be noted.
20. Developmental stage of pupae will be noted in accordance with the following Table 2, based on eye colour, body colour and presence of wings. (Wintermantel, 2018)

Table 2: Bumble bee development stages

Developmental stage	Eye color	Body color	Wings
1	White	White	No
2	Pink	White	No
3	Brown	White	No
4	Brown	Brown	No
5	Brown	Black	No
6	Brown	Black	Yes

21. The pupal body mass per caste and developmental stage will be measured.
22. The adult body mass per caste will be measured.

23. The intertegular distance of adult bees per caste will be measured.

5. Protocols for semi-field studies on solitary bees

5.1 Outline

Solitary bees have a short active period compared with honey bees and bumble bees. It is important that the researcher is able to synchronise the emergence and subsequent nesting of the bees with the availability of flowers in the enclosures. This requires controlled incubation of cocoons to ensure that sufficient numbers of female bees are emerged, mated and ready to lay eggs

5.2 Ordering bees

a. In Europe the following species may be obtained from commercial suppliers - *Osmia cornuta, Osmia bicornis* and *Megachile rotundata*. In addition it may be possible to obtain *Anthophora plumipes* and *Colletes hederae*. The most convenient species to work with are *O. bicornis,* followed by *O. cornuta;* both species have very similar behaviours, but are active at different times of the year. *O. cornuta* precedes *O. bicornis,* and is therefore preferable for studies on early-flowering crops such as almonds. Unless otherwise specified, this document refers to the use of *Osmia* species.
b. *Osmia* bees are generally provided as dormant adults.
c. Solitary bees should be ordered well in advance, preferably the year before required.
d. When ordering, it can be advantageous to have the male and female cocoons separated, as this simplifies the control of emergence of the adults and the synchronisation of bee emergence and bloom of the target crop.
e. The order should be for substantially more cocoons than anticipated as emergence can be spread over many days, in which case some emerged bees will be too early and some too late for inclusion in the study, and some will not emerge at all. As a rule of thumb at least twice as many cocoons should be provided in order to be confident that the correct number of viable adults can be put into the study. A good crop of *Phacelia* in an enclosure can provide sufficient pollen and nectar to fully support the mating activities of two females per square metre. On this basis an enclosure of 36m^2 would support 72 females, in which case double the number, i.e. at least 144 females, should be allocated to each enclosure; the number of associated male cocoons would be 200 or more.
f. The ratio of female to male cocoons should be determined to ensure that sufficient females are present for the study.
g. On delivery, the cocoons should be stored in a refrigerator at 4°C, in cardboard, not plastic containers.

5.3 Preparation

a. *Osmia* bees are normally supplied as dormant adults overwintering in cocoons, and should be separated into male and female cocoons, based primarily on size and

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secondarily on shape. Where there is uncertainty, the cocoon should be allocated to the males.

b. On receipt a small number of male cocoons should be opened to examine the bee inside and check its viability. If the bee is alive, it will soon show signs of motion, particularly near the end of overwintering. One end of the cocoon has a slightly raised nipple. The cocoon should be cut open with a pair of surgical scissors, or sliced open with a scalpel. This will expose the face of a male (identifiable by the white hairs on the frons). If no movement is detectable, the cocoon can be opened more and the bee removed from it. As the bee warms up, it will show signs of life. If the bee exhibits no movement, more cocoons should be opened. If the bee's mouthparts are extended and motionless, it is dead. In this case, more bees should be examined to get an indication of the extent of the mortality. If there is even a small proportion of dead bees, it may indicate a wider problem, as other bees in the batch may be alive, but with limited viability and vigour. Such an issue should be discussed with the supplier.

c. When examining cocoons, note should be made of the presence of parasites, specifically the mite *Chaetodactylus osmiae*, and the wasp *Monodontomerus obscurus*. In the case of the mite, cocoons can be washed (Bosch, 2001). In the case of the wasp, affected cocoons may be detected, in which case they should be removed to prevent re-infestation. The processing procedures by the supplier should have eliminated these issues however. Other pests are unlikely to be present in sorted cocoons.

5.4 Handling cocoons prior to study

a. To ensure that sufficient individuals have hatched at the correct time for the study, it is necessary to assess the response of individuals to incubation; a typical incubation trial may be as follows:

Table 3: Determining incubation times to emergence

	Day approx.	Action
A	D-28	Transfer 20 male cocoons to 10°C
B	D-26	Incubate at 28°C; plot numbers of individuals emerged against time
C	D-21	Repeat A and B using 10 females and 20 males
D	D-19	Repeat A and B using 10 females and 20 males
E	D-12	Repeat A and B using 10 females and 20 males

b. The charts will show a steadily reducing period of incubation required to provoke emergence. After the final incubation, the data can be extrapolated to give predicted times for emergence of both males and females. Since the time for males to emerge is less than females, it can be advantageous to start incubating females before males so they emerge more or less simultaneously.

c. The above procedures will identify in advance of the study what proportion of the population is dead so that mitigation actions can be taken if necessary.

d. Cocoons from which adults have not emerged during the incubation process may contain considerable numbers of the parasitic wasp *M. obscurus*. Such cocoons should be removed from the study because if the wasps get established, they can destroy solitary bee populations and invalidate many of the longer-term endpoints (Bosch, 2001).

5.5 Nest boxes

a. Conventional cardboard tube or timber laminates may be used although data collection is restricted and observations of developing bees are limited. Superior data collection is achievable using nests with cavities arranged in trays, each with an acetate cover. Such nest boxes enable each tray to be removed for photography or examination without damaging juveniles or disturbing nesting mothers. Post-study lab analysis of the development of young bees is simplified and enhanced.
b. In the semi-field situation, the nest boxes should be mounted on stands so that the front of the nests are easy to observe. A height of 1m to 1.5m above the ground is convenient. Shade should be provided to prevent juvenile forms overheating in the nest boxes.

5.6 Release chambers

Bees are most easily introduced into enclosures as adults in release chambers which protect them from weather and predators. These may be card or timber boxes which have slots or holes for adults to exit. Multiple openings should be provided to avoid bees being trapped by congested exits. When release chambers are placed inside enclosures, provision should be made for them to be supported off the ground where they are vulnerable to predation by beetles. Preferably the release chambers should be adjacent to the nests.

5.7 Introduction to enclosures

1. In caged studies, where there is no risk of bees absconding, unlike in field studies, it is more convenient and reliable to incubate cocoons in the lab or at other remote facilities and transport them to the study as emerged adults or as adults still in the cocoon, on the point of emergence.
2. Some researchers may prefer to place unemerged cocoons in the enclosures and permit the adults to emerge in response to ambient temperatures. While this approach is feasible, the disadvantage is that the emergence period is longer and less predictable.
3. The sequence of activities carried out by adult female *Osmia* bees is: emerge, defecate, mate, feed, search out nest site, adopt nest site, gather mud to construct a cell, gather pollen to provision the cell, lay an egg, and seal the cell (O'Toole, 2000). It may take several days from emergence to gathering pollen. It is important therefore that a significant number of the females in the enclosures are known to be nesting before commencing the study. The simple way to determine how many females are nesting is to view each layer of each nest in the evening and count the females that are roosting overnight.
4. *Osmia* bees construct cell walls with mud collected in a wet state. In each enclosure a mud reservoir will be created by digging a shallow (10cm to 30cm deep) depression into which sufficient water (preferably 5 to 10l) is poured (Krunic, 2006). This reservoir should be refilled with water regularly and not allowed to dry out. Care should be taken when refilling mud reservoirs not to drown burrowing mothers as they may not be visible. Collection of mud may be made easier by poking a few cavities into the sides of the depression.

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5.8 Study phases

1. Unlike semi-field studies with *Bombus* and *Apis*, there is no need to remove the *Osmia* nests and bees from the enclosure for transfer to a monitoring site. There should be adequate forage, with possible management by trimming of the crop, to sustain all the females for the duration of nesting.
2. The study will begin when a predetermined number of females are actively nesting, i.e. have started to apply mud to the ends and sides of nesting cavities.
3. On D-1 each layer of nest cavities will be removed from its cabinet, and the extent of nest construction up to that point will be marked with a permanent marker. It is assumed that the contents of each cell sealed prior to that point will not be exposed to the applied product.
4. Subsequently, at regular intervals each layer of nest cavities will be photographed by removing it from the nest cabinet and placing it on a stand attached to a camera stand or similar. The acetate sheet covering the nest cavities will be annotated with the enclosure identification, the nest identification, the layer identification and the individual cavity identification. The date will also be shown on the image.
5. When floral resources diminish to the level of being inadequate, or bee flight has effectively ceased, the entrances to the study nests may be covered by a fine mesh (less than 0.5mm x 0.5mm) to prevent parasitism by *M. obscurus*.
6. Nests at this stage may be moved a short distance to safe storage, but it should be noted that young larvae are vulnerable to damage by rough handling or road transport. It is preferable to keep the nests on site for approximately four weeks. Nests in these circumstances should be well shaded and protected from rain, as overheating and damp are both dangerous to the juveniles.
7. When convenient the nests should be removed to lab or other facilities for storage and examination, where they should be held at ambient conditions, or air-conditioned office conditions.
8. Several assessments are made by examination of the nest contents in the lab by comparing the cells, pollen loads and juvenile bees with the images captured during the field phase. This work may commence approximately one month after removal of the nests from the study site.
9. Guidance from the bee suppliers will be sought, regarding the cold storage of the cocoons recovered from the study nests, in order to facilitate assessments of offspring vigour and/or hatching success.

5.9 Application of stressors

1. Prior to a group decision being made by the appropriate members of the research consortium, the combination of stressors to be assessed is unknown. The options under consideration include pesticides in different forms, pathogens, parasites, and nutritional stress. Some options will be briefly considered here: a sprayed insecticide and a sprayed fungicide; a seed treatment and a sprayed fungicide; a sprayed pesticide and a pathogen stress. The current limited knowledge of the biology of solitary bees, particularly with regard to pathogens and predators, is a barrier to the inclusion of diseases and pests as stressors in this study.
2. **Sprayed insecticide plus sprayed fungicide**: at 3 days (± 1 day) following the commencement of nesting, the treatments will be applied by a specialist spray contractor with expertise in precise application of small quantities of product in enclosed area. The treatments will be

applied in the following sequence – water only (controls); product A only; product B only; products A and B. Where insecticide and fungicide are being jointly applied, they may be mixed in the same tank.

5.10 Assessments

1. **Hatching success assessment.** The number of cocoons that emerged in time for inclusion in each enclosure of the study will be counted; the emergence of cocoons that emerged subsequently to being removed from the enclosures will also be counted.
2. **Establishment of females assessment.** The number of females that established in each enclosure will be counted.
3. **Flight activity assessment.** The number of female bees entering and leaving the nest in a fixed period (not less than five minutes) will be recorded.
4. **Foraging activity assessment.** The number of bees observed within a 1m square laid at random over the crop will be counted in a fixed period (not less than two minutes); this procedure will be carried out three times in each enclosure.
5. **Adult mortality assessment.** Adult mortality in a 24 hour period will be calculated as the number of females roosting inside the nest cavities one evening minus the females roosting the following evening.
6. **Larval mortality assessment.** During lab examination, each cell will be examined, and recorded in one of the following categories: successful cocoon; failed cocoon; dead pupa; dead larva; dead egg; pollen load with no egg; empty sealed cell with no pollen; incomplete cell. The date of pollen collection and the date of egg-laying relative to exposure to the stressors will be recorded. For each enclosure the total larval mortality will be reported, and the larval mortality relative to age of bee at exposure will be reported.
7. **Pupal mortality assessment.** The pupal mortality assessment will be carried out as above.
8. **Egg mortality assessment and failure to lay egg assessment.** Each pollen mass in cells with no bees will be examined and recorded as one of two categories, i.e. pollen provision without an egg or pollen provision with failed egg. The distinction will be determined by examination of the pollen mass. If an egg was laid that didn't hatch, the outline of the egg or an indentation are clearly visible.
9. **Offspring production assessment A.** The number of complete cells (with pollen provision, egg and mud seal) per enclosure will be counted. The mean number of cells per female bee at the start of the study will be calculated.
10. **Offspring production assessment B**. The count will be made as above. The mean number of cells completed per surviving female bee will be calculated.
11. **Mass of offspring assessment.** Each cocoon will be removed, sorted by size into male and female, and weighed. By charting the results, the distinction between male and female cocoons can be made.
12. **Offspring hatching assessment.** The cocoons will be stored either at external ambient conditions, or in cold storage, according to the guidance of the suppliers. In the following year, at the appropriate time, the cocoons can be allowed to emerge and tabulated under the headings male/female; control/treated; and date of emergence. It may be possible to detect differences in behaviour after emergence, such as vigour, response to food, eagerness to mate, etc. If desired, cocoons can be x-rayed to show the extent of the stored fat bodies. These are generally regarded as indicators of longevity, vigour and possibly fecundity.

13. **Sex ratio of offspring assessment.** By sorting the cocoons into male and female (as above) the sex ratio of the offspring can be calculated. It should be noted that the sex ratio can be no more than an indicator of effect as the sex ratio varies substantially in the wild.
14. Participants may consider the benefits of assessing: the duration of foraging flights for (a) nectar, (b) pollen and (c) mud; the time spent between foraging flights in the nest; and indicators of memory impairment such as mistaken entry to nest after foraging flight.
15. **Residue assessments.** The type and quantities of samples of bees, pollen, nectar, stored food, foliage etc., are to be agreed in advance with the relevant laboratory.

6. Notes applicable to field studies on all bee species

6.1 General considerations.

The following recommendations are supplementary to those detailed above for semi-field studies.

1. The researcher will consider and make provision for health and safety issues in the field; first aid equipment; emergency procedures; toilet facilities; washing facilities; and rest facilities.
2. Records will be kept of training sessions for field staff.
3. Training sessions will include formal risk assessments and safety measures.
4. Because of the area of plants required for field studies, it is probable that the selection will be limited to agricultural crops, for example apple, oil seed rape, cherry, almond, blueberries, borage, wild flowers, etc. The use of a surrogate crop such as *Phacelia* is unlikely.
5. The country selected for the study may depend on the crop being studied (for example almonds), or the time of year (for example New Zealand in December).
6. The selection of study site(s) is complex, in that the sites should be as similar as possible, with control site(s) that will not be treated.
7. Landscape evaluations will be essential prior to the study. The researcher will justify the extent of the landscape evaluation. For example 100m radius may be adequate for solitary bees; 1km radius for bumble bees; and 3km radius for honey bees. Note that these are typical and not extreme values.
8. Stressor combinations may be chemical + chemical, or chemical + nutrition, or chemical + pathogen/parasite.
9. Grower cooperation is essential. Clarity on grower inputs must be established, for example – land set aside for study; access tracks; damage to crops; security; agreement on grower inputs before and during study; potential reduction in crop by limiting product applications; date for bees and equipment to be removed from site.
10. Financial transactions must be clear, such as: agreement on payments for chemicals; fees for cultivation; agreement on schedule and details of application of products. The question of indemnity for crops that suffer because of lack of normal management must be addressed. The assistance of a professional agronomist may be beneficial.
11. If practical, data on products used on the wider landscape both historically and in the year of the study should be collected.
12. The choice of residues to be analysed, and the substrates (plant parts, bee parts, pollen, nectar, bee bread etc) should be determined prior to the study.
13. Procedures for collecting, packaging, freezing and transporting samples for analysis should be determined prior to the study.

6.2 The principles of the study

1. The test will be designed to study impacts on bees at field realistic doses. The alternative dose/response studies are not considered here. The impacts will be measured as loss of bees, in different ways for each test species.
2. The test will be carried out at a number of different sites. Each site will contain one or more fields. Each field may be treated or non-treated (control). Honey bees and/or bumble bees and/or solitary bees will be deployed at each field. The numbers of each test organism or colony will be derived from considerations of statistical power.
3. The test bees will be located adjacent to or within the crop in which bees are expected to forage for pollen and nectar. The test bees may be limited to one species such as honey bees, although it can be advantageous and relatively straightforward to include bumble bees and solitary bees in the same study.
4. In order to limit foraging away from the target crop, (a) the area of the crop should be as large as is practical, preferably over 2ha, and (b) the bee hives and nests should if practical be placed within the crop and not at the edge of the crop. An awareness of the relative attractiveness of the target crop to different bee species is useful. For example, *Osmia bicornis* does not appear to forage enthusiastically on oil seed rape, but it will fly long distances to oak trees, and collect high proportions of oak pollen.
5. The chemical stressors will be applied in accordance with the label instructions in order to create the maximum realistic field exposure. Other stressors will be applied as specified by the researcher.
6. The study duration will be decided by the researcher, and will preferably be as long as two honey bee brood cycles. The period may be varied for other test bee species.

6.3 Statistical validity

The researcher must justify the number of sites and the number of colonies (when considering honey bees or bumble bees) and individuals (when considering solitary bees) in order to detect the difference in effect between treated and control, for example to a significance level α= 5% and power β= 80% (EFSA, 2013).

6.4 Chemical stressors applied as spray

a. The chemical should be applied as formulated product, not active ingredient.
b. The product should be applied as the maximum realistic field dose (EFSA, 2013).
c. The product will be applied in accordance with the timings on the label. For example, it may be before flowering, or after flowering, or during flowering when bees are flying.
d. The presence and identities of adjuvants (extenders, wetting agents, sticking agents and fogging agents) in the product should be identified if possible (Straw, 2021). No additional adjuvants should be added when making up the tank mix.
e. The application should be carried out by the grower or spray contractor in accordance with their normal practice.
f. The researcher and contractor will jointly confirm that the product is as specified.
g. The spray equipment will be calibrated on site.
h. Two 100ml samples of solution will be taken from the spray tank, and two 100ml samples of undiluted product will be taken from the product container, and placed in a freezer for later shipping to an analytical laboratory to verify the quantity of active ingredient present.
i. The following data will be recorded in connection with spray application: weather data including wind speed; BBCH stage of crop development; product name; product batch

number; product use by date; dilution rate; application rate per hectare; theoretical application rate per enclosure; actual application per field.

j. Where a study requires the application of two products simultaneously to the same crop, they may be mixed together in the tank.

k. The unused products will be disposed of legally.

6.5 Chemical stressors applied as seed coating

1. The risk to bees from seed coatings is two-fold – (a) from dust containing chemicals released during the sowing process, and (b) from chemicals released by the seed coating passing systemically through the plant and being expressed in nectar and pollen.
2. Studies on such risk are relatively rare. A study design will have to be individually created.

6.6 Chemical stressors applied as powders

The proportion of plant protection products applied as powders in the agricultural setting is only 1% to 2% of that delivered as spray application. As above, such a study will be individually designed.

6.7 Nutritional stressors, pathogens and parasite stressors

The details given previously in the section of this document relating to semi-field studies are also applicable to field studies**.**

6.8 Controls

Negative controls are required; positive controls are not required.

6.9 Verification of exposure

1. Samples of the spraying solution should be taken from the mixing tank or from the spray boom nozzles, and transported to the laboratory for analysis. The measured concentration should be within the range 80 to 120% of the nominal concentration.
2. If laboratory resources permit, verification of exposure can additionally be measured by sampling plant tissues, such as flowers or leaves. In this case further sampling of the crop throughout the study may be taken to assess the stability of the product and residues.

Recording, reporting and data storage will be in accordance with current best practice.

7. Protocols for field studies on honey bees

Note – the following recommendations are supplementary to the protocols detailed above for semi-field studies.

7.1 Outline

1. In honey bee field studies colonies will be deployed in open landscape where they will be able to forage without restriction for pollen and nectar. For example, six study locations may be selected, at each of which six colonies may be deployed. In this illustration, at each site three colonies would be located in or near a crop which is being treated, and three would be in or near a crop which is not being treated. To support the decision-making process, surveys of each location need to be carried out to determine what forage will be available to test and control bees before, during and after the study. This is demanding in terms of time, experience and resources. Such work needs to be done in advance, when it may be difficult to predict when plants will flower, particularly when considering annuals.
2. Control colonies will be located in similar landscapes that are sufficiently far from the treated sites that the foraging ranges of test bees and control bees do not substantially overlap. We suggest a distance of 5km is sufficient. Selection of the control sites is demanding, as the researcher needs to be sure that crops and plants within reach of the control bees are not treated.
3. Stressors will be applied, primarily by application of plant protection products on crops surrounding or adjacent to the colonies.
4. To measure impact, the key endpoints are mortality of adults and mortality of brood. An assessment of overwintering success after treatment is desirable, but may not be practical.
5. Field studies demand a high degree of co-operation between expert beekeepers and expert scientists to harness the expertise of both.

7.2 Special equipment

It should be noted that equipment used in commercial beekeeping is not always convenient for research. Assessments may be much simplified by the use of custom-designed items such as pollen traps, dead bee traps, special queen frames, *Varroa* trays, weighing apparatus, sampling equipment, photographic equipment etc.

7.3 Duration of the honey bee field study

1. The study will commence a number of days before the application of a plant protection product or other stressor. The study will extend over at least one brood cycle (21+ days) and preferably over two brood cycles (42+ days).
2. The timings given relate to the first exposure of honey bee colonies to the stressors. For example, D0 would be the day of a spray application of product; D-7 would be seven days before application; D+4 would be 4 days after application, etc.

7.4 Colony specification

1. The following will be specified: colony type (full-size/nucleus/miniature); type of hive (for example Langstroth, Dadant, Deutsche Normal, Layens, British Standard, etc); and colony origin. All hives should be on two boxes. A queen excluder should be placed between the boxes and the queen restricted to the lower box.
2. The colonies will be manipulated prior to the study to equalise them as far as is practical. It is recommended that each colony comprises adult bees covering seven to ten frames; five to six frames of brood; two to three frames of food (nectar, honey, pollen and bee bread); and one or two empty frames to accommodate growth.
3. The colonies should be placed in their locations two to three weeks before application of the stressor, in order for the bees to familiarise themselves with the landscape.

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4. All queens in a study should be of the same age, either born in the current year of the study or one year previously. The queens should be closely related if possible, preferably sisters. The queens should be marked. The subspecies or variety of the queens should be noted, although these data are hard to verify given that so much cross-breeding has occurred over a long period. In particular it should be noted that the term 'Buckfast-type' has little meaning now.
5. The queens may or may not have clipped wings.
6. The colony history prior to the study will be recorded, including health status and medications/treatments.
7. All colonies will be examined in the field by an experienced beekeeper prior to the study to identify the presence of bacterial diseases (American and European foul brood); fungal disease (chalk brood); parasitic mites (*Varroa destructor*); and wax moth (*Achroia grisella* or *Galleria mellonella*). Field workers will be trained to detect the presence of the predators small hive beetle (*Aethina tumida*) and Asian hornet (*Vespa velutina*). Diseases and conditions that require laboratory identification will not be investigated.
8. A record of medications of each colony over the two years prior to the study will be maintained.
9. The entrance of each hive will be painted a light colour so that visual counts of bees entering are made easy.

7.5 Colony strength assessments

1. Adult bee populations will be estimated on site by observers who have been trained in visual assessments using library photographs of many frames in accordance with published methods, such as the Liebefeld method or ColEval. The number of adult bees on each side of each frame will be estimated and recorded. The number of adult bees on the inside walls, the floor, and the cover of the hive will be estimated and recorded.
2. The amount of brood in a colony can also be estimated using the ColEval method, by brushing adult bees from every frame. The observer estimates the proportion of each frame containing (a) sealed brood, (b) open brood, (c) nectar/honey, and (d) pollen. However it should be noted that shaking or brushing bees from all frames in a colony can be disruptive, and that the loss of adults and brood – and crucially the queen - due to the beekeeper's handling may substantially exceed the impact that the study is designed to detect. It is suggested therefore that brood populations are assessed prior to the study, at the end of the study, and possibly once during the study. Two other pragmatic recommendations may be given - (a) the observer assesses ONLY adult bees and sealed brood on each frame, as both of these can be estimated fairly accurately without brushing bees off; and (b) no attempt is made during the ColEval assessment process to measure the amount of food in the frames. The hives are weighed regularly, and it can be reasonably assumed that a change in the hive weight is indicative of a change in the weight of the stored food, with some caveats.
3. The colonies will be distributed among the study sites by stratified random allocation, based on the estimated population (adult bees plus sealed brood).

7.6 Photography work

1. Impact on brood will be assessed by taking photos of the brood as detailed previously for semi-field work. Photos of brood are to be taken in line with the guidance contained in OECD Guidance Document 75 (OECD, 2014).

2. The researcher must make a decision whether to photograph the whole frame, or a central portion of the frame. The advantage of the latter is that the equipment is smaller and more mobile. To photograph a Dadant brood frame in its entirety while maintaining an angle of view sufficient to see the bases of cells requires a distance of over 2m between lens and frame. Handling such large equipment slows down the operation. Statistically there is little advantage in measuring impact on say 2,000 eggs on the whole frame compared with 500 on the central portion.
3. The use of image recognition software has been detailed previously in the section on semi-field studies. The image recognition system will generate the following analyses as defined in OECD75 – Brood Termination Rate (BTR), Brood Compensation Index (BCI), and Brood Index.

7.7 Techniques for handling honey bee colonies

Bee handlers should (a) use slow movements when manipulating bees; (b) use limited amounts of smoke; (c) brush bees off frames (not shake); (d) be alert for presence of queen at each examination; (e) ensure that queens are marked in advance; (f) ensure that the queen's location on frame is noted and that queens are not dislodged or lost

7.8 Assessments

1. Colonies will be weighed by placing hives on scales on predetermined days. The scales will be checked by using a calibrated weight periodically.
2. The number of bees entering the hive will be counted over a period of 120 seconds on predetermined days.
3. The number of bees carrying pollen loads will be counted over a period of 120 seconds on predetermined days.
4. Adult mortality in a dead bee trap will be counted by removing and discarding dead adult bees. Juvenile mortality will be counted by removing and discarding dead pupae, prepupae, old larvae (4^{th} and 5^{th} instars), and young larvae (1st, 2^{nd} and 3^{rd} instars). These counts will be carried out on predetermined days.
5. Assessments of the adult bee population of each colony will be made by technicians trained in the method known as ColEval (Hernandez, 2020). One bee handler will display in turn each side of each frame in the bottom box of each hive. The observer will record the number of adult bees on the frame. Assessments will also be made of the number of adults (a) on the floor, (b) on the walls of the hive, and (c) in the top (food storage) box. The bees will not be shaken or brushed off the frames, in order to avoid bees being counted multiple times. This assessment will be carried out on predetermined days.
6. The assessment of sealed brood will also be carried out using the ColEval method. The assessment will be made without shaking or brushing bees from the frame. It is easy to blow or stroke adults to one side to see the caps of sealed brood cells.
7. Pollen samples will be taken by inserting collectors into the pollen traps on predetermined days, and removing the pollen collection trays no more than 24 hours later.
8. Pollen samples will be labelled and placed in a freezer for subsequent transport to a laboratory for chemical analysis.
9. The presence of the queen will be established at each examination of the hive by either (a) visual detection of the queen or (b) the presence of eggs.

7.9 Residues

1. The choices of samples to be taken for chemical analyses depends on the routes by which the chemicals reach the bees, both adults and juveniles, and by the expertise and resources of the laboratory. For example, the following substrates may yield useful information when analysed: leaves, flowers, nectar, pollen, honey, bee bread or stored provisions, old bees, young bees, juveniles, nest materials, faeces, wax, propolis, etc.
2. The foraging and storage behaviours of honey bees make sample collection simple. For example pollen can easily be collected from pollen traps, and nectar/honey may be withdrawn from combs. To verify exposure, various plant matrices may be tested such as leaves, petals, nectar or pollen. Additionally, larval or pupal tissue may be easily collected and analysed.
3. It is desirable that residues be individually sampled from each enclosure; if resources do not permit this, samples may be pooled and homogenised so that for example all controls are amalgamated, all of Treatment A are amalgamated, etc.
4. Protocols are to be agreed in advance with the laboratory regarding collecting samples, packaging, labelling, transporting, freezing and shipping.

7.10 Statistical analysis

Data will be statistically analysed in accordance with current best practice.

8. Protocols for field studies on bumble bees

Note – the following recommendations are supplementary to the protocols detailed above for Semi-Field Studies.

8.1 Outline

In bumble bee field studies colonies will be deployed in open landscape where they will be able to forage without restriction for pollen and nectar. The study may involve bumble bees only or other bees such as honey bees and/or solitary bees. For example, six study locations may be selected, at each of which six colonies may be deployed. Stressors will be applied, primarily by application of plant protection products on crops surrounding or adjacent to the colonies. When a defined proportion of the colonies have started producing sexual individuals, the colonies will be frozen for later analysis in the lab to assess the study endpoints.

8.2 Colony specification

1. As discussed previously, liaison between supplier and researcher should include discussion of delivery date; number of workers; feed (syrup and pollen); one-way entrance; insulation; species. The authors recommend a minimum of 10 and a maximum of 35 workers. The colony should also contain worker larvae at the commencement of the study.
2. Additional colonies should be ordered to make up for potential loss or inadequate colonies.

8.3 Nest boxes

Commercial bumble bee nests are designed primarily for use in protected circumstances, for example glass houses. For field work, the nest boxes should be mounted on supports so they are easy to access and study. They should be securely fixed to prevent them being blown off, and protected from rain and from excessive heat.

8.4 Special equipment

Equipment designed specifically for research purposes as described previously may be advantageous, for example handling units that enable the nest to be manipulated without receiving stings; pollen traps that function with foragers of different sizes; electronic bee counters monitoring movements in and out of the nest; clear covers to replace lids supplied with commercial nest boxes, etc.

8.5 Handling colonies prior to study

1. Colonies may be stored in cool conditions if necessary to wait for the start of study if for example delayed because of lack of bloom, poor weather conditions, etc.
2. It is desirable to mark foundress queens prior to the study.

8.6 Placement of colonies on study sites

1. **Site layout**. For preference, colonies should be located in specially-created open spaces in the centre of fields, so that bees are surrounded by the target crop. If this is not practical, sites adjacent to the target crop are acceptable. For example, six study locations may be selected, at each of which six colonies may be deployed. Where the study involves more than one species of bee, the different species may be placed more or less adjacent.
2. The bumble bee colonies shall be distributed to sites by stratified random allocation, based on such parameters as colony weight, population, food consumption, mortality prior to the study, etc.
3. Each bumble bee colony will be located at least 3m from the adjacent bumble bee colony.
4. In order to avoid bumble bee colonies reaching the switch point too rapidly, the supplier should be made aware of the need for small colonies (sometimes known as research colonies). Ideally move colonies to sites when they are still small, providing there is sufficient forage. Unlike honey bee colonies, which may be moved on to site a considerable time prior to the study, bumble bee colonies should be released on site shortly before the study. The syrup reservoir should be removed or closed when the colony is put on site. Similarly, the pollen provision (if present) should be removed.

8.7 Techniques for handling bumble bees

1. Wear gloves, as bumble bees can sting rapidly and repeatedly. Thick gloves reduce sensitivity; thin gloves have limited protection. Beekeeping gloves are probably the best compromise.
2. Slow hand movements aggravate bumble bees less than rapid jerky movements.

8.8 Assessments

(These are to be read in conjunction with details given for semi-field studies above).

1. **Colony weight.** Each nest will be taken out of the housing unit and placed on scales.
2. **Adult mortality in nest.** The number of dead adults inside the nest will be counted and removed regularly.
3. **Juvenile mortality in nest.** The number of dead pupae and larvae will be counted and removed regularly.

4. **Number of adults in nest.** The number of adults will be estimated by taking a photo of the nest and subsequently counting adults on the photo.
5. **Number of flying bees at entrance.** Counting may be done manually using a click-counter during a period of 3 min or preferably automatically and continuously using an electronic bee activity monitor.
6. **Number of pollen loads.** Bees entering with visible pollen loads may be counted within a defined time frame (such as 3 min).
7. **Number of cocoons.** Assessed initially by photographing the nest without the wax cover, and later by dissection of the frozen nest.
8. **Pesticide residues.** Pesticide residues may be quantified in pollen, nectar, plant tissue and/or bees. Pollen foragers may be caught by sweep net.
9. Pollen foragers may be caught using sweep nets to collect their pollen for residue analyses. Nectar foraging bees may be caught to extract their nectar immediately or later in the lab. Nectar from flowering plants may be collected using microcapillaries. Bees for residue analysis may be sampled using sweep nets.

8.9 Date of switch point

Bumble bee colonies switch from the production of workers to the production of reproductives (males and queens). The date when this happens will be determined by the presence of queen cocoons or eclosed males.

8.10 Colony dissection after colony termination

Assessments will include:-

1. Photo of the nest.
2. Colony weight (nest box + colony).
3. Empty nest box weight (nest box without colony).
4. Numbers of workers, males and new queens at each stage of development.
5. Individual masses of workers, males and queens at each stage of development.
6. Foundress queen presence.
7. Amount of stored pollen.
8. Presence of parasites, kleptoparasites and commensals.
9. Intertegular distance of individual workers, males and queens.

9. Protocols for field studies on solitary bees

Note – the following recommendations are supplementary to the protocols detailed above for semi-field studies.

9.1 Outline

In solitary bee field studies male and female solitary bees will be released in open landscape where they will be able to forage without restriction for pollen (primarily) and nectar. Abundant nest resources will be made available. For example, six study locations may be selected, at each of which six to twelve nests may be deployed. Stressors will be applied, primarily by application of plant protection products on crops surrounding or adjacent to the nest sites.

9.2 Releasing solitary bees in the field

It is relatively straightforward to establish solitary bees, particularly *Osmia* species, inside enclosures. The process is more challenging in the field situation in that substantial numbers may abscond. Given the variability in time for solitary bees, both male and female, to emerge from cocoons and the possibility of a substantial proportion of the females absconding from the study sites, as a rule of thumb, the authors suggest that two to three times as many female bees as desired for the study be obtained, plus the appropriate number of males (i.e. approximately 1.5 times the number of females). Factors that affect the success of establishing bees at the study site include:

1. Males and females that emerge from cocoons at the study site are less likely to abscond; when released on site as already emerged adults a large proportion may be lost.
2. The disadvantage of allowing bees to emerge in response to ambient temperatures is that time to emergence may be unpredictable. Insufficient bees may emerge in time for the study.
3. The presence of female cocoons in the emergence chamber encourages males to remain in the vicinity in the expectation of females emerging. Alternatively a tincture of old cocoons in alcohol may be applied to the nest.
4. Males that remain mark nests with mandibular gland secretions to attract females.
5. Abundant nesting options should be offered, spaced around the study site. They should be brightly coloured and obvious. A small number of cocoons, either empty or occupied, may be inserted in each nest to attract occupants.
6. Abundant forage encourages females to establish.
7. The lack of mud reduces the chances of females establishing. *Osmia* bees construct cell walls with mud collected in a wet state. Unless there are reliable wet locations (streams, ditches or ponds) a number of mud reservoirs should be dug and watered before the bees emerge. They should not be allowed to dry out during the study. Collection of mud may be made easier by poking a few cavities into the sides of the depression. Care should be taken when adding water to the reservoirs to avoid drowning bees that are burrowing into tunnels and cavities.
8. The sequence of activities carried out by adult female *Osmia* bees is: emerge, defecate, mate, feed, search out nest site, adopt nest site, gather mud to construct cell, gather pollen to provision cell, lay egg, and seal cell. It may take several days from emergence to gathering pollen. It is important therefore that a significant number of the females in the enclosures are known to be nesting before commencing the study. The simple way to determine how many females are nesting is to view each layer of each nest in the evening and count the females that are roosting overnight.

9.3 Study phases

1. Ideally the study will begin when a predetermined number of females are actively nesting, i.e., have started to apply mud to the ends and sides of nesting cavities. However it should be acknowledged that synchronising emergence of bees from cocoons with flowering of forage crops and plants, possibly in conjunction with honey bees and bumble bees, is not straightforward.
2. On D-1 each layer of nest cavities will be removed from its cabinet, and the extent of nest construction up to that point will be marked with a permanent marker. It is assumed that the contents of each cell sealed prior to that point have not been exposed to applied product.
3. At regular intervals subsequently, each layer of nest cavities will be photographed by removing it from the nest cabinet and placing it on a stand attached to a camera stand or

similar. The acetate sheet covering the nest cavities will be annotated with the enclosure identification, the nest identification, the layer identification and the individual cavity identification. The date will also be shown on the image.

4. When floral resources diminish to the level of being inadequate, or bee flight has effectively ceased, the entrances to the study nests may be covered by a fine mesh (less than 0.5mm x 0.5mm) to prevent parasitism by *M. obscurus*.
5. Nests at this stage may be moved a short distance to safe storage, but it should be noted that young larvae are vulnerable to damage by rough handling or road transport. It is preferable to keep the nests on site for approximately four weeks. Nests in these circumstances should be well shaded and protected from rain, as overheating and damp are both dangerous to the juveniles.
6. When convenient the nests should be removed to lab or other facilities for storage and examination, where they should be held at ambient conditions, or air-conditioned office conditions.
7. Several assessments are made by examination of the nest contents in the lab by comparing the cells, pollen loads and juvenile bees with the images captured during the field phase. This work may commence approximately one month after removal of the nests from the study site.
8. The guidance of the suppliers of the bees will be sought, regarding the cold storage of the cocoons recovered from the study nests, in order to facilitate assessments of offspring vigour and/or hatching success.

9.4 Application of stressors

1. The options of stressors to be considered include pesticides in different forms, pathogens, parasites, and nutritional stress. Some options will be briefly considered here: a sprayed insecticide and a sprayed fungicide; a seed treatment and a sprayed fungicide; a sprayed pesticide and a pathogen stress; a sprayed pesticide and a nutrition stress. The current limited knowledge of the biology of solitary bees, particularly with regard to pathogens and predators, is a barrier to the inclusion of diseases and pests as stressors in this study.
2. Sprayed insecticide plus sprayed fungicide: at D+3 (± 1 day) following the commencement of nesting, the treatments will be applied preferably by a specialist spray contractor with expertise in precise application of small quantities of product in enclosed area. The treatments will be applied in the following sequence – water only (controls); product A only; product B only; products A and B. Where insecticide and fungicide are being jointly applied, they may be mixed in the same tank.
3. To apply a pesticide stress and a nutrition stress, it is necessary to acquire information on the forage available within flying distance of the nest location. In most cases the flying distance of *Osmia* females is modest, a few hundred metres perhaps. The task therefore of categorising a study site as suitable for an *Osmia* field study is considerably easier than for honey bees and bumble bees.

9.5 Assessments

1. **Hatching success assessment.** The number of cocoons that emerged in time for inclusion in each enclosure of the study will be counted; the emergence of cocoons that emerged subsequently to being removed from the enclosures will also be counted.

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2. **Establishment of females assessment.** The number of females that established in each enclosure will be counted.
3. **Flight activity assessment.** The number of female bees entering and leaving the nest in a fixed period (not less than five minutes) will be recorded.
4. **Foraging activity assessment.** The number of bees observed within a 1m square laid at random over the crop will be counted in a fixed period (not less than two minutes); this procedure will be carried out three times in each enclosure.
5. **Adult mortality assessment.** Adult mortality in a 24 hour period will be calculated as the number of females roosting inside the nest cavities one evening minus the females roosting the following evening.
6. **Larval mortality assessment.** During lab examination, each cell will be examined, and recorded in one of the following categories: successful cocoon; failed cocoon; dead pupa; dead larva; dead egg; pollen load with no egg; empty sealed cell with no pollen; incomplete cell. The date of pollen collection and the date of egg-laying relative to exposure to the stressors will be recorded. For each enclosure the total larval mortality will be reported, and the larval mortality relative to age of bee at exposure will be reported.
7. **Pupal mortality assessment.** The pupal mortality assessment will be carried out as above.
8. **Egg mortality assessment and failure to lay egg assessment.** Each pollen mass in cells with no bees will be examined and recorded as one of two categories, i.e. pollen provision without an egg or pollen provision with failed egg. The distinction will be determined by examination of the pollen mass. If an egg was laid that did not hatch, the outline of the egg or an indentation are clearly visible.
9. **Offspring production assessment A.** The number of complete cells (with pollen provision, egg and mud seal) per enclosure will be counted. The mean number of cells per female bee at the start of the study will be calculated.
10. **Offspring production assessment B.** The count will be made as above. The mean number of cells completed per surviving female bee will be calculated.
11. **Mass of offspring assessment.** Each cocoon will be removed, sorted by size into male and female, and weighed. By charting the results, the distinction between male and female cocoons can be made.
12. **Offspring hatching assessment.** The cocoons will be stored either at external ambient conditions, or in cold storage, according to the guidance of the suppliers. In the following year, at the appropriate time, the cocoons can be allowed to emerge and tabulated under the headings male/female; control/treated; and date of emergence. It may be possible to detect differences in behaviour after emergence, such as vigour, response to food, eagerness to mate, etc.. If desired, cocoons can be x-rayed to show the extent of the stored fat bodies. These are generally regarded as indicators of longevity, vigour and possibly fecundity.
13. **Sex ratio of offspring assessment.** By sorting the cocoons into male and female (as above) the sex ratio of the offspring can be calculated. It should be noted that the sex ratio can be no more than an indicator of effect as the sex ratio varies substantially in the wild**.**
14. Participants may consider the benefits of assessing: the duration of foraging flights for (a) nectar, (b) pollen and (c) mud; the time spent between foraging flights in the nest; and indicators of memory impairment such as mistaken entry to nest after foraging flight.

15. **Residues Assessments** – the type and quantities of samples of bees, pollen, nectar, stored food, foliage etc., are to be agreed in advance.

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10. Summary of endpoints

The endpoints discussed in this document are tabulated below. EFSA differentiates between primary and secondary endpoints. Primary endpoints (✔) are those that show direct evidence of impact, for example brood or adult mortality. Secondary effects (✔S), such as behavioural abnormalities, are regarded as being supportive of primary endpoints, in that they may assist to explain the primary effects. Secondary effects cannot be used as the sole basis for a regulatory decision.

These endpoints are not prescriptive but are intended as guidance to which endpoints are possible and which are desirable.

Table 4: Endpoints for semi-field and field studies (all species)

Endpoint	Honey bees	Bumble bees	Solitary bees
Colony population	✔	✔	
Adult mortality	✔	✔	✔
Queen mortality	✔	✔	
Juvenile mortality		✔	✔
Brood termination rate	✔		
Brood recovery rate	✔		
Brood index	✔		
Colony weight	✔	✔	
Overwintering success	✔		✔
Forager activity	✔S	✔S	✔S
Flight activity	✔S	✔S	✔S
Rate of pollen collection	✔S	✔S	
Rate of mud collection			✔S
Behavioural effects	✔S	✔S	✔S
Establishment of females			✔S
Offspring production		✔	✔
Offspring survival to diapause			✔
Mass of offspring		✔	✔
Offspring sex ratio			✔S
Impact on navigation		✔	✔S

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11. Tools for the improvement of field and semi-field studies

Table 5: Available and emerging technologies and techniques to enhance bee studies

Assessment	Species	Improved tools	Advantages
Multiple	*Apis*	Integrated system including study frame, pollen trap, dead bee trap and other features	Equipment designed specifically for research; ease and speed of handling, quality of data
Impacts on brood	*Apis*	Photographic methods with image recognition systems	Custom-built camera equipment is transportable; gives consistent results; analyses are rapid and accurate
Impacts on brood	*Apis*	Photographic methods provide data for enhanced OECD endpoints	OECD endpoints can be extended to two full brood cycles (42 days)
Population assessments	*Apis*	ColEval – improved method for counting bees and brood, compensating for observer bias	For assembling and selecting colonies as well as assessments during study
Pollen collection	*Apis*	Miniature pollen trap	Design permits quick and easy sampling of pollen without starving colony
Pollen collection	*Bombus*	Miniature pollen trap	Strips pollen from different sizes of workers - produced using 3D printer
Flight activity	*Bombus*	Electronic counter	Unit records every movement of bees into the nest
Multiple	*Bombus*	Handling unit	Colonies can be manipulated with few if any stings, and few escaping queens
Navigation and flight	*Osmia*	Automatic interpretation of video footage of nesting females using AI	Captures more data that is reliable and verifiable for less staff time (Schwartz et al)
Multiple	*Osmia*	Nest construction permits non-invasive examination of mothers and offspring	Many types of data that were previously unobtainable are now simple, quick and accurate
Landscape survey	Multiple	Drone photographic survey	Enables data gathering over wide area to augment foot surveys

12. Discussion

The aim of this document is to provide useful and practical information to researchers involved in semi-field and field studies on different types of bees, i.e., honey bees, bumble bees and cavity-nesting solitary bees, primarily *Osmia* species. The motivation for carrying out such studies may be research or it may be part of the legislative process by which a company seeks approval to release a product onto the market, in which case the study will be one of an escalating series, from Tier 1 (laboratory), Tier 2 (semi-field, i.e., in enclosures) to Tier 3 (open field). Both Tier 2 and Tier 3 are expensive, demanding and time-consuming. Semi-field studies require many enclosures to be erected in which plants are grown and bees introduced; pesticides are applied in an effort to simulate realistic conditions bees would encounter in an agricultural landscape. Field studies are simpler to set up in that no enclosures are needed, but the researcher has to contend with the varied landscape and inputs which the bees experience. Such studies are notoriously challenging.

The WP7 team in the PoshBee consortium have employed several new techniques and specially designed and built equipment, which are discussed in the preceding pages. These are in response to a number of factors. The first is that the amount of food (in the form of nectar and pollen) available to caged bees is limited. It is therefore important that the food demand of the bees is to a substantial degree matched by the food produced by the flowers, otherwise the test subjects will starve, with obvious consequences to the study. We therefore advocate the use of small honey bee hives, with modest populations (say 3,000 adults).

Secondly, it is clear that equipment designed for beekeeping and honey production is not necessarily the best for research purposes. We therefore designed and built an integrated system of apparatus specifically for research into honey bees. We also built equipment to improve the study of bumble bees and solitary bees with the intention that it would be easier to use and would deliver abundant high-quality data.

Thirdly, to be successful, this kind of work requires input from beekeepers and research scientists. The skillsets and expertise of these two expert groups do not necessarily overlap very much, even with possibly decades of experience. It has been our aim to include in this document information that will help scientists and beekeepers to liaise effectively.

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APPENDIX 1: Verification of MS15 (presented at AGM)

This milestone was also verified through reporting the protocols for WP7 semi-field and field studies at PoshBee AGM3 on 12th January 2021 (Figure 3) and AGM4 on 12th January 2022 (Figure 4). The presentation slides of the presentation 'Protocols for pesticide semi-field experiments' (AGM3) by Matthew Allan (AGM4) and 'WP7 introduction including verification of MS15: Protocol for semi-field and field experiments' by Alexandra Klein are available from the PoshBee website members library.

Figure 3: Presentation slide showing presentation of WP7 protocols at AGM3.

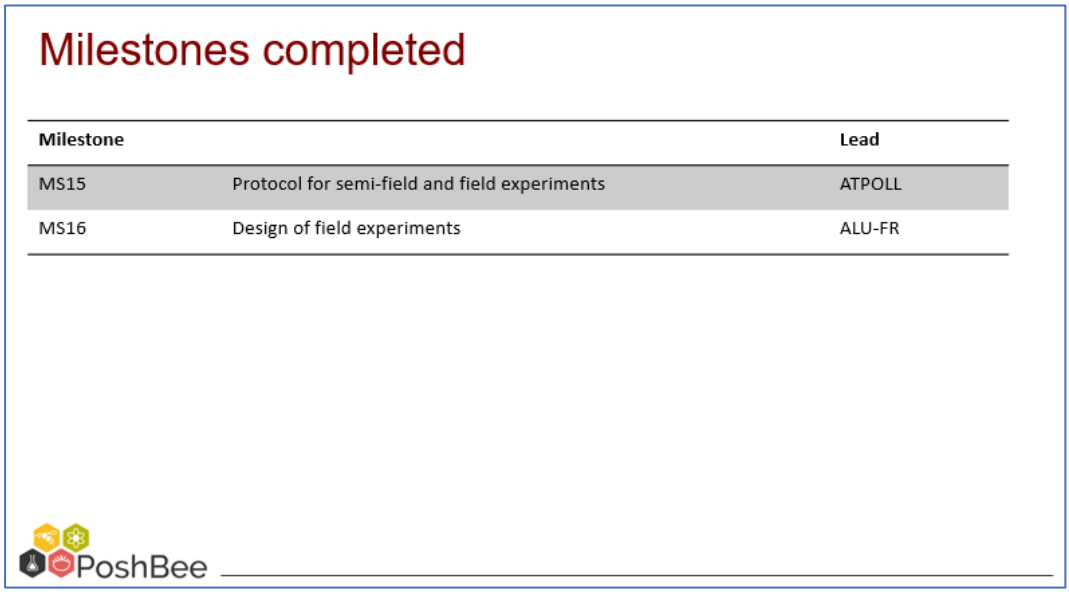

Figure 4: Presentation slide showing presentation of finalised WP7 protocols at AGM4.