bio-protocol

Published: Jun 20, 2022

Protocol for Initiating and Monitoring Bumble Bee Microcolonies with *Bombus impatiens* (Hymenoptera: Apidae)

David M. Lehmann*

Center for Public Health and Environmental Assessment (CPHEA), Health and Environmental Effects Assessment Division, Integrated Health Assessment Branch, U.S. - Environmental Protection Agency, Research Triangle Park, NC, 27711, USA
*For correspondence: lehmann.david@epa.gov

Abstract

Populations of some bumble bee species are in decline, prompting the need to better understand bumble bee biology and for assessing the effects of environmental stressors on these important pollinators. Microcolonies have been successfully used for investigating a range of endpoints, including behavior, gut microbiome, nutrition, development, pathogens, and the effects of pesticide exposure on bumble bee health. Here, we present a step-by-step protocol for initiating, maintaining, and monitoring microcolonies with *Bombus impatiens*. This protocol has been successfully used in two pesticide exposure-effects studies and can be easily expanded to investigate other aspects of bumble bee biology.

Keywords: Bumble bee, *Bombus*, Microcolony, Pollinators, Pesticides

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This protocol was validated in: PLoS One (2020), DOI: 10.1371/journal.pone.0241111

Background

Bumble bees are valuable pollinators in agricultural and natural settings (Kleijn *et al.*, 2015). Disconcertingly, populations of some bumble bee species are in serious decline (Cameron *et al*., 2011). Many factors are believed to contribute to the reported population declines, including poor nutrition, parasites, pathogens, and pesticides (Brown and Paxton, 2009; Goulson, 2005, 2013, 2015; Meeus *et al.*, 2011; Wood *et al*., 2019). Recognizing their importance and the number and complexity of factors affecting their populations, there is a need to better understand bumble bee biology and the effects of environmental stressors on bumble bees.

Microcolonies are formed when a small group of bumble bee workers is isolated in a queenless environment. Under these conditions, the workers self-organize to build nest structures and lay unfertilized eggs that produce drones (Free, 1955). The model is versatile, offering the ability to investigate a range of endpoints, including behavior, the gut microbiome, nutrition, development, pathogens, and pesticide exposure (reviewed in Klinger *et al*., 2019).

Currently, there are no detailed protocols for initiating and monitoring microcolonies published for any bumble bee species, only condensed protocols in the methods sections of research publications (Gradish *et al*., 2012, 2013; Smagghe *et al*., 2007). Here, we detail a step-by-step protocol for initiating and monitoring bumble bee microcolonies with the common eastern bumble bee (*Bombus impatiens* Creson) (Hymenoptera: Apidae). We also provide detailed instructions for preparing microcolony food provisions. An overview of the procedures for initiating and monitoring microcolonies can be found in Figure 1. The protocols presented here were originally described in two peer-reviewed publications (Camp *et al*., 2020a, 2020c) and a subsequent publication comparing these two studies (Weitekamp *et al.*, 2022). While these protocols were designed for assessing the effects of pesticide exposure on bumble bees, they can be easily expanded to investigate other aspects of bumble bee biology, including behavior, nutrition, development, pathogens, and gut microbiome (reviewed in Klinger *et al*., 2019).

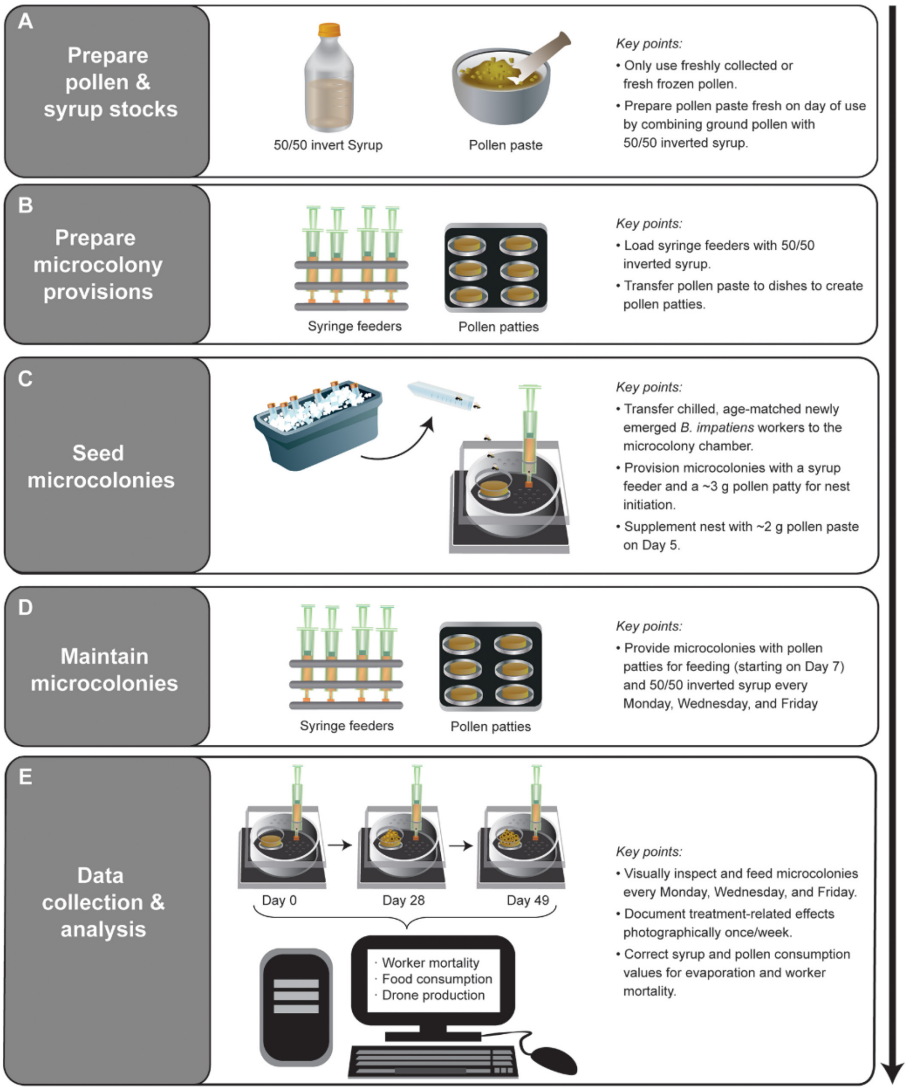

Figure 1. Overview of procedures for initiating and monitoring microcolonies.
(A and B) Prepare syrup and pollen stocks for provisioning the microcolonies. Although syrup can be prepared in advance and stored at 4°C, pollen patties should be made fresh on the day of use. Transfer pollen to dishes and collect the weight. **(C)** Use only age-matched, newly emerged *B. impatiens* workers when using this protocol. To facilitate experimental manipulation, chill workers on ice. Distribute five bees to each microcolony chamber. Provision microcolony chambers with a ~3 g pollen patty for nest building and a syringe feeder filled with 50/50 inverted syrup. Supplement the nest with ~2 g of pollen paste on day 5. **(D)** Provide microcolonies with pollen patties for feeding (starting on Day 7) and 50/50 inverted syrup every Monday, Wednesday, and Friday for the duration of the experiment (recommend no more than 49 days). Collect the weight of the old syringe feeders and pollen dishes to use when calculating food consumption. **(E)** Investigators are encouraged to collect data on worker mortality and drone production (*i.e.*, timing to emergence of 1st drone, number of drones emerged, and drone weight). Syrup and pollen consumption values should be corrected for evaporation and worker mortality. The black vertical arrow on the righthand side indicates the order of operations for initiating and monitoring microcolonies.

Part I: Protocol for microcolony food preparation

Materials and Reagents

1. Fresh or fresh-frozen honey bee-collected corbicular pollen (see Protocol for Microcolony Food Preparation Notes #1) either sourced from investigator-maintained honey bee colonies or a commercial vendor (Swarmbustin' Honey, catalog number: BP-DKLB).
2. Sorbic acid (Amresco, catalog number: 0667-500G)
3. Citric acid anhydrous (Fisher, catalog number: A940-500)
4. Pure cane sugar (*e.g.*, Domino Sugar)
5. Distilled water (Gibco, catalog number: 15230)
6. Potassium Sorbate Solution (see Recipes)

Equipment

1. Laminar flow hood
2. 4°C laboratory refrigerator (Thermo Scientific, catalog number: TSV18CPSA)
3. -20°C laboratory freezer (Thermo Scientific, catalog number: TSX3020FARP)
4. Basic coffee grinder or (ideally) commercial blender (Waring, catalog number: 7010S)
5. Vacuum food sealer (FoodSaver, catalog number: FM2100)
6. Freezer storage bags for vacuum food sealer (FoodSaver, catalog number: FSFSBF0226NP)
7. Analytical top loading scale/balance (Ohaus, catalog number: AX2202/E)
8. Analytical balance standards: 200 mg, 500 mg, 1 g, 2 g, 10 g, 20 g, 30 g, 100 g, 200 g, 300 g, 500 g, and 1 kg
9. Hand-operated, electronic pipet for large volumes (Drummond Pipet-Aid, catalog number: 4-000-101)
10. Hot plate with stir function (2) (Cimarec, catalog number: SP195025)
11. pH meter (Orion Star, catalog number: STARA2110)
12. pH meter calibration standards: pH 4.0 and pH 7.0 (VWR, catalog number: E452-500ML and E459-500ML)

General supplies

1. N95 disposable respirator (VWR, catalog number: 89201-508)
2. Mortar and pestle (VWR, catalog number: 470019-978)
3. Sterile bottletop 0.45 µm filters (VWR, catalog number: 10042-462)
4. 60 mL Luer slip syringe with tips cut off (Exel International, catalog number: ES60)
5. 25 mL graduated glass pipet (VWR, catalog number: 76003-570)
6. Aluminum foil
7. Disposable paper mats for covering working surfaces (Versi-Dry Lab Table Soakers, catalog number: 62080-00)
8. 2 L Pyrex bottle (Corning, catalog number: 1395-2L)
9. Magnetic stir bars (Komet, catalog number: 50087909)
10. 35 mm × 10 mm disposable Petri dish lids (Falcon, catalog number: 351008)
11. 43 mm aluminum weigh dishes (QORPAK, catalog number: MET-03105)
12. 1 L Pyrex beakers (2) (Corning, catalog number: 1000-1L)

Procedure

A. 50/50 Inverted Syrup: 1 L Bottles (1.5 L produced)

1. For easier clean-up, cover the hot plate with aluminum foil prior to use.
2. Combine 1,000 mL distilled water with 850 g pure cane sugar and stir on a hot plate until all sugar granules

are dissolved.

3. Add 0.85 g citric acid anhydrous and continue stirring while heating to a rolling boil.
4. Cover beaker with aluminum foil and boil for 20 min (see Figure 2).

Figure 2. Syrup at rolling boil and treated with citric acid.

5. Allow to cool on a room temperature stir plate, while covered and stirring with a stir bar.
6. Once cooled, add 7.5 mL (5 mL per 1 L produced) Potassium Sorbate Solution (see Recipes below).
7. Record the pH and label the container appropriately (see Protocol for Microcolony Food Preparation Notes #2).
8. Parafilm bottle cap and store 50/50 inverted syrup at 4°C for up to 14 days once opened (30 days unopened).

B. Preparing Pollen (see Protocol for Microcolony Food Preparation Notes #3 and 4)

1. Grind frozen pollen to a fine powder with a coffee grinder or (ideally) commercial blender (Figure 3A-C).

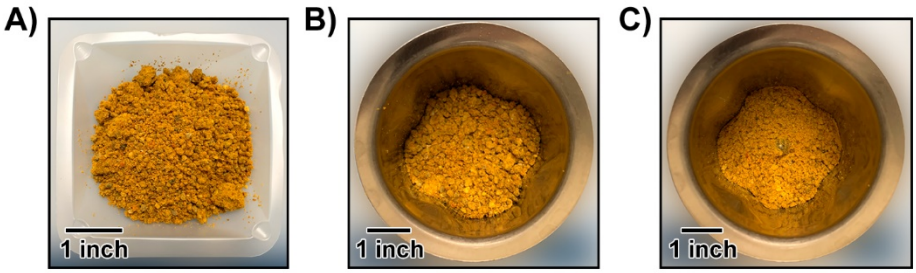

Figure 3. Pollen consistency.
(A) Frozen fresh collected honey bee corbicular pollen. **(B)** Honey bee orbicular pollen in a blender cup. **(C)** Honey bee corbicular pollen ground to a fine powder for making patties and paste.

2. Calibrate the analytical balance prior to use.
3. Store ground pollen in vacuum seal freezer bags at a weight of 500 g per bag.

4. Store ground pollen at -20°C until ready to use.

C. Preparing Pollen Paste for Nest Initiation and Routine Feeding

1. Remove one vacuum-sealed bag of freshly collected honey bee pollen from the -20°C freezer when ready to use.
2. Weigh out the desired amount of frozen pollen in 100 g increments at a time.
3. Reseal any unused ground pollen in a new vacuum seal bag, label appropriately, and store at -20°C.
4. Use a commercial blender to blend the pollen to a fine powder consistency (Figure 4A–C).
5. Add 38.5 mL of 50/50 inverted syrup to 100 g of ground pollen and mix with a spoon to a peanut butter-like consistency (Figure 4A).
6. Place a damp paper towel over the pollen paste while working to prevent evaporation.
7. Transfer the pollen paste to disposable 43 mm aluminum weigh dishes. For nest initiation, weigh out 3.0–3.25 g of pollen paste and place offset to one side in the lid of a pre-weighed 35 mm × 10 mm disposable Petri dish (Figure 4B). For routine feeding, fill to the top of the dish, but leave a small space on one side for transfer with forceps to the microcolony chambers (Figure 4C). If using pollen as the dosing vehicle for a pesticide exposure-effects study, either work from the lowest concentration to the highest concentration or use clean forceps when switching to a new dose group.

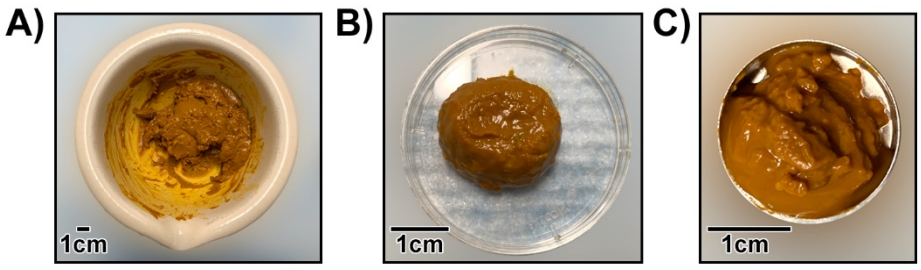

Figure 4. Pollen processing.
(A) Pollen paste with peanut butter-like consistency. **(B)** Pollen paste for routine feeding in a feeding dish. A small void is left to allow manipulating the feeding dishes with forceps while minimizing the risk of cross-contaminating exposure groups. **(C)** Nest dish with initiation patty offset to allow space for adding additional pollen paste on day 5.

8. Pollen patties for nest initiation and paste for routine feeding and nest initiation should be made fresh on the day of initiation or feeding.

Protocol for Microcolony Food Preparation Notes

1. Fresh-frozen pollen should be stored in vacuum sealer bags or other air-tight containers at -20°C for up to 2 years. If stored longer, investigators should confirm palatability relative to freshly collected pollen before committing to a large, time-consuming experiment.
2. The pH of 50/50 inverted syrup should be between 4 and 5.
3. To maximize continuity within an experiment, all pollen needed for one study should be pooled and blended to produce a single, uniform food stock.
4. Grinding pollen in advance and distributing it into either single-use bags or bags sufficient to cover experimental needs for one week at a time will save a significant amount of time during the experiment.

Part II: Protocol for microcolony initiation and monitoring

Materials and Reagents

1. Newly emerged *B. impatiens* workers (Biobest (Romulus, MI), Koppert Biological Systems (Howell, MI) or another commercial vendor; see Protocol for Microcolony Initiation and Monitoring Notes #3).
2. 50/50 inverted syrup and pollen paste/patties prepared as described in Part I (above)
3. Data sheets for recording data (see Supporting information figures 1–6)
4. Head lamp (*e.g.*, Petzl Tactikka) and/or small handheld flashlight with red light filter (*e.g.*, Mini Maglight PRO LED)
5. Parafilm wrap (Masterflex, catalog number: PM992)
6. Mortar and pestle (VWR, catalog number: 470019-978)
7. Disposable paper mats for covering working surfaces (Versi-Dry Lab Table Soakers, catalog number: 62080-00)
8. 20 mL oral dosing syringes (Medi-dose, catalog number: NAW-2000) with manufacturer-supplied tight-fitting caps (drill a hole in each syringe 1/8” hole located at the 2 mL mark prior to use)
9. 43 mm aluminum weigh dishes (Qorpak, catalog number: MET-03105)
10. Specimen forceps (12” length; VWR, catalog number: 82027-382)
11. General-purpose laboratory tape (VWR, catalog number: 89097-912)
12. Rectangular ice pan, approximate dimensions 15” L × 10” W × 6” D (VWR, catalog number: 10146-216)
13. Rectangular plastic container, approximate dimensions 7.5” L × 5” W × 2.5” D (Cambro, catalog number: 42PP190)
14. 50 mL conical tubes (Corning, catalog number: 352070)
15. Disposable 35 mm × 10 mm Petri dishes (Falcon, catalog number: 351008)
16. 43 mm aluminum weigh dishes (QORPAK, catalog number: MET-03105)

Equipment

1. 4°C laboratory refrigerator (Thermo Scientific, catalog number: TSV18CPSA)
2. -20°C laboratory freezer (Thermo Scientific, catalog number: TSX3020FARP)
3. Analytical top loading scale/balance, 0.1 mg (OHAUS, catalog number: 30100604)
4. Analytical balance standards: 10 g, 20 g, and 30 g
5. 5” stainless-steel geology sieve (1.57” depth × 5” diameter), #10 mesh (SciOptic, ASTM 10, catalog number: 305 stainless steel)
6. Clear observation tops for geology sieves with access lids (see Protocol for Microcolony Initiation and Monitoring Notes #1).
7. Bottom plates with holes drilled for ventilation for geology sieves to sit on top of (see Protocol for Microcolony Initiation and Monitoring Notes #2).
8. Environmental chamber with temperature and humidity controls

Software

1. Microsoft Excel Spreadsheet Software® (v16.0; Redmond, WA)
2. GraphPad Prism® (v6; La Jolla, CA)

Procedure

A. Prepare nest provisions and feeders (see Protocol for Microcolony Initiation and Monitoring Notes #4)

1. Prepare syrup feeders using 20 mL oral dosing syringes with a pre-drilled 1/8” hole located at the 2 mL mark. Holes should be parafilmed to facilitate filling syringes with either control 50/50 inverted syrup or, if the experiment calls for it, test article-containing 50/50 inverted syrup.
2. Fill syringe feeders by submerging the syringe tip into a beaker or conical tube containing 50/50 inverted syrup or test article-containing 50/50 inverted syrup by pulling up on the plunger. Cap syringes after filling with syrup.
3. Remove the parafilm and record the syrup weight in the datasheet for the experiment (see Supporting information figure 1 for an example data collection sheet).
4. Prepare pollen patties as described in Part I (above).

B. Prepare microcolony chambers for nest initiation

1. Label each microcolony observation top with the date of initiation and assigned microcolony number.
2. Place stainless steel sieve on the bottom plate with an absorbent paper towel placed in between the pieces, and clear observation lid on top.
3. Place the lid of a 35 mm × 10 mm disposable Petri dish marked with the microcolony number on the bottom, in the microcolony chamber with a 3–3.25 g nest initiation patty for nest building (Figure 5).

Figure 5. Microcolony chamber components.
Stainless steel geology sieve (1.57” depth × 5” diameter) with pass-through floor (#10 mesh), bottom plate with holes drilled for ventilation, see-through top for collecting observations, removable lid for accessing chamber interior, and syringe feeder. Design adopted from Bayer CropSciences.

C. Weighing and seeding microcolonies with newly emerged *B. impatiens* workers (Protocol for Microcolony Initiation and Monitoring Notes #5)

1. Fill the ice pan and place the rectangular plastic container in the center of the ice pan with 50 mL conical tubes positioned around the inside edge of the ice pan for storing collected newly emerged workers.

2. Transfer enough newly emerged workers to the 4°C refrigerator for 10–15 min to support the experiment (*i.e.*, Number of workers needed = [(5 workers/microcolony × the number of microcolonies desired) + 10% extra bees to account for dead/injured bees]).
3. Remove bees from 4°C and transfer them to the shallow rectangular plastic container on ice.
4. Next, transfer five randomly selected bees at a time to the 50 mL conical tubes. Transfer the conical tubes to the lab bench to allow the bees to become active again.
5. Check the conical tubes for any dead or damaged bees prior to weighing; replace dead or damaged bees as needed.
6. Weigh the conical tube with the five newly emerged bees for the first microcolony. Add the bees to their microcolony and then weigh the empty conical tube to determine the weight of the bees. Record these numbers in the datasheet for the experiment (see Supporting Information figure 2 for an example data collection sheet).

D. Supplementing the nest initiation patty

1. Five days after microcolony initiation, supplement the nest initiation patty with an additional ~2 g of pollen paste.

E. Microcolony routine feeding (see Protocol for Microcolony Initiation and Monitoring Notes #6–9)

1. Seven days after microcolony initiation, give each microcolony ~2 g pollen paste for feeding in a disposable 43 mm aluminum weigh dish.
2. Provide microcolonies with fresh, pre-weighed ~3.5 g pollen paste and either control 50/50 inverted syrup or test article-containing 50/50 inverted syrup every Monday, Wednesday, and Friday starting one-week post initiation.
3. Record the weight of the new dish with new pollen paste and new syrup feeder into the datasheet for the experiment (see Supporting information figure 1 for an example data collection sheet).
4. Record the weight of the old pollen paste with the original dish and previous syrup feeder to determine consumption.
5. To more accurately quantify pollen/syrup consumption levels, include two evaporation controls in the study design. These controls should be set up and processed exactly like all other microcolonies, except they do not contain bees (see Supporting information figures 3 and 4 for an example data collection sheet).

F. Monitoring microcolonies and data collection (see Protocol for Microcolony Initiation and Monitoring Notes #10–12)

1. Using an environmental chamber, maintain microcolonies in darkness at 25°C ± 0.5°C and 50% ± 5% relative humidity throughout the duration of the study. Red light may be used when microcolonies are outside of the environmental chamber on the bench. Avoid white light where possible.
2. Evaluate each microcolony from initiation to study termination (see Supporting information figure 5 for an example data collection sheet).
3. During each observation, collect the following information: 1) number of dead workers; 2) days to first drone emergence; 3) number of drones emerged; and 4) drone weight.
4. Optional, additional information can be collected, including 1) time to first uncapped egg chamber; 2) days to first capped egg chamber; 3) days to first larval mass; and 4) days to first pupal cell.
5. Any drones that emerge should be removed when the microcolonies are fed (*i.e.*, Monday, Wednesday, and Friday). After removal, weigh each drone individually (see Supporting information figure 6 for an example data collection sheet).
6. Optional but encouraged: randomly select and photographically track at least one microcolony from each experimental group (Wednesdays recommended) to capture microcolony progression and developmental milestones.

G. Terminating microcolonies

1. When the experiment is complete, after 42 or 49 days, either proceed to additional assay endpoints or euthanize the workers, drones, and remaining brood by CO_2 narcosis followed by transfer to -80°C.

Protocol for Microcolony Initiation and Monitoring Notes

1. The observation tops can be made according to the specifications detailed in Figure 6A and 6B. As designed, the tops have a recessed 5" diameter ring that prevents the lids from slipping off the top of the sieve. However, other designs for the top can be used, provided they 1) are large enough to cover the sieve [bees will be housed in the space between the mesh floor of the sieve and the observation top (see Figure 7)], have an opening for the syringe feeder, and have ventilation holes to allow air circulation.

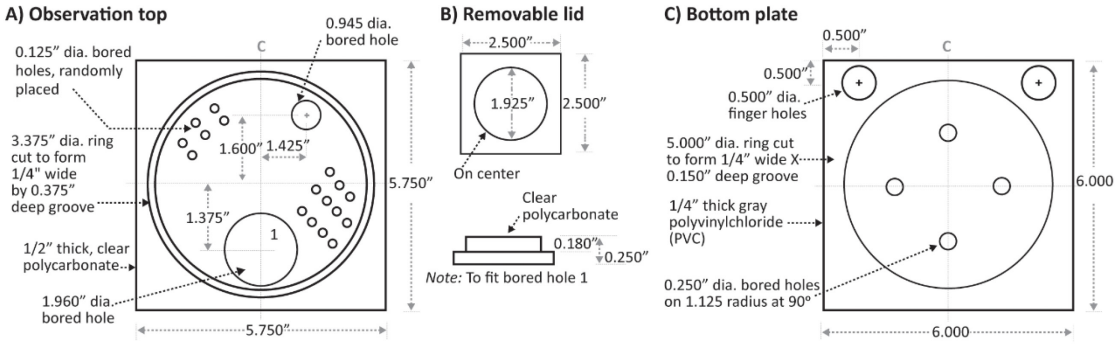

Figure 6. Materials and measurements for the microcolony observation top, removable lid, and bottom plate.
Microcolony chamber observation tops **(A)**, removable lids **(B)**, and bottom plates and **(C)** used previously (Camp *et al*., 2020a, 2020c; Weitekamp *et al.*, 2022) can be reconstructed according to the specifications shown. dia. = diameter.

2. Bottom plates can be made according to the specifications detailed in Figure 6C. The bottom plates were designed to prevent bee waste and other debris that pass through the perforated floor of the sieve from contaminating other microcolonies and from fouling the environmental chamber. The design of the bottom plates includes a recessed 5" diameter ring that the sieve drops into. However, investigators can use any bottom plate design, provided the bottoms have ventilation holes to allow air circulation and are large enough for the sieve to sit on top of it.
3. Microcolonies should not be initiated with randomly aged workers when using this protocol. Using age-matched, newly emerged workers reduces the possibility of confusing age-related deaths for treatment effects and promotes consistency across microcolonies within an experiment and across experiments. When using this protocol, investigators should use fresh or fresh-frozen honey bee-collected corbicular pollen with this protocol. Provisioning microcolonies with old (*i.e*., >2 years old) or improperly stored pollen may impact microcolony progression and productivity.
4. Pollen patties for nest initiation and paste for routine feeding should be made fresh on the day of initiation or feeding.
5. Since worker size can impact microcolony nest development and food consumption rates by workers (Peat and Goulson, 2005; Couvillon and Dornhaus, 2010; Amsalem and Hefetz, 2011; Roger *et al*., 2017a, 2017b), protocol users are encouraged to seed microcolonies with bees of a similar mass.
6. Microcolonies must be provided *ad libitum* access to pollen and syrup for the duration of the experiment. Restricting access to food provisions will disrupt microcolony development, reduce productivity, and complicate the interpretation of experimental results.
7. The delay in providing pollen specifically for feeding is to reduce the likelihood that the workers will attempt to lay eggs on both the pollen for feeding and nest initiation patty.

8. Microcolonies consume significantly more pollen when feeding developing larvae. Therefore, it is best to give productive microcolonies ~3.5 g of pollen paste to minimize the risk that they will run out of pollen.
9. Unless retention is required for additional analysis, dispose of the old pollen dish.
10. To help keep the bees calm when manipulating the microcolonies, place the chambers on a disposable paper mat that will absorb vibrations.
11. To promote consistency across microcolonies, rotate the position of individual microcolonies within the environmental chamber.
12. If a founding worker dies in the first 24 h, replace it with a new newly emerged worker (obtain weight of new worker).
13. Because the number of worker bees in a microcolony can impact nest productivity (Gradish *et al*., 2013) and food consumption rates, protocol users are encouraged to track the number of dead workers throughout the experiment.
14. Production of drones is a key metric of microcolony success. The time to first drone emergence, the number of drones emerged, and drone weight can all be readily quantified. Importantly, all these measures can be affected by experimental treatments providing insights into how a test material impacted the microcolony.

Data analysis

This pair of protocols was developed to explore the effects of pesticide exposure on microcolony progression and productivity (see Camp *et al*., 2020a, 2020c; Eitekamp *et al.*, 2022). While that is the case, these protocols can easily be used to address a variety of research questions, thereby enabling investigators to gain significant insight into other aspects of bumble bee biology. Based on experience using this system, 8–10 microcolonies should be used per experimental group. Below is an overview of how to process and analyze data collected from pesticide exposure-effects studies using this protocol. Detailed methods for processing and analyzing data for these endpoints can be found in Camp *et al*. (2020a, 2020c) and Weitekamp *et al.* (2022). To aid new investigators, sample data collection sheets along with guidance on how to record and process microcolony data are provided as supplementary information at the end of this protocol.

Analysis of microcolony data can be broken down into two interrelated parts. For the first part, microcolonies should be visually inspected and photographed to identify treatment-related effects on nest progression. Microcolonies established according to this protocol will progress according to the timeline shown in Figure 7. When collecting observations weekly, uncapped egg chambers will appear by the end of week 1. Capped egg chambers sometimes appear late in week 1, but most often by the end of week 2. Larval masses can be detected during weeks 2 and 3. Pupal cells will appear during weeks 3 and 4. The first drones appear during week 5 and will continue to emerge for the remainder of the study. If desired, observations can be collected more frequently to tease out subtle effects on microcolony development. However, collecting observations is time-consuming, and frequent disruptions may impact worker behavior and ultimately study outcome.

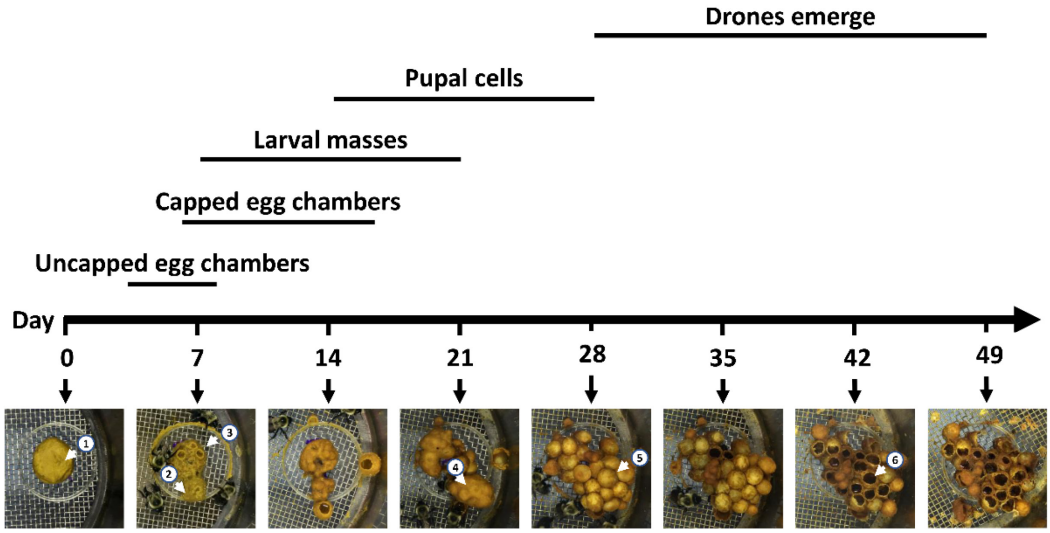

Figure 7. Microcolony progression through seven weeks of development.
Microcolonies were initiated with five newly emerged *B. impatiens* workers and provisioned with a nest initiation patty of pollen paste (3 g) and 50/50 inverted syrup to stimulate nest building. After 5 days, nests were given an additional 2 g of pollen paste. Starting on day 7, microcolonies were given fresh pollen paste and syrup every Monday, Wednesday, and Friday. Starting from Day 0 (*i.e.*, nest initiation), photos show microcolony progression from uncapped egg chambers to study termination on day 49. Bars with labels above indicate the range when feature typically appears. Numbered circles indicate features of the microcolony, including 1 = nest initiation patty, 2 = pollen supplement given on day 5, 3 = uncapped egg chamber, 4 = larval mass, 5 = pupal cell, and 6 = evacuated pupal cell.

In addition to qualitatively assessing microcolony status, data from various endpoints should be analyzed statistically, including microcolony development milestones (*i.e.*, time to first uncapped egg chamber, days to first capped egg chamber, days to first larval mass, and days to first pupal cell), syrup and pollen consumption, drone production and weight, and worker survival. These data should be expressed as mean ± standard deviation (STDEV). Using GraphPad Prism® (v6; La Jolla, CA), differences between the control and treatment groups can be assessed with One-way Analysis of Variance (ANOVA) and Dunn's multiple comparison test. If, according to the Brown-Forsythe or Bartlett's tests, variances are significantly different, instead use the non-parametric Kruskal-Wallis with Dunnett's multiple comparisons test.

Data reporting

To allow comparisons to be made between studies and to facilitate study replication, investigators are encouraged to report the 1) syrup formulation used for feeding, 2) pollen source and age, 3) age and source of the worker bees, and 4) environmental conditions (*i.e.*, temperature, relative humidity, and light regimen) used during the experiment. In addition, the start, end, and data collection dates should also be recorded and reported. All data should be made available to other investigators either as supplemental information or through a public repository for scientific data.

Data Analysis Notes

1. When investigating the effects of pesticide exposure on microcolony development and productivity, investigators should include an untreated control group, a positive control group, and, if applicable, a solvent control group in the study design. Evaporation controls should also be included to correct food consumption estimates when conducting pesticide exposure studies.
2. When desired, the pesticide can be delivered to the microcolony through the syrup and/or pollen provisions. Dosing pesticides via syrup is generally easier than with pollen. However, not all pesticides are water-soluble, and, for that reason, solvents may be required to solubilize the test material. In that event, investigators will need to empirically identify a suitable solvent concentration for use in their experiment [*e.g.*, 1% acetone (Camp *et al.*, 2020b)].

3. When evaluating the effects of pesticide exposure on microcolonies, the delivery vehicle (*i.e.*, pollen or syrup) can impact study outcome. Developing bumble bee brood consumes large amounts of pollen and, for that reason, delivering test material through the pollen may target the brood. To that point, acetamiprid delivered in the pollen, but not when delivered in the syrup, reduced average drone weight (Camp *et al.*, 2020a, 2020c; Weitekamp *et al.*, 2022). Consequently, important brood effects could be missed when only dosing through the syrup.
4. Syrup and pollen consumption values should be corrected for evaporation and, when determining consumption on a per bee basis, worker mortality. Also, if a syrup-filled syringe leaked, leading to an inaccurate consumption value, the value should be replaced with the average syrup consumption value for that treatment group and day.
5. Published results suggest that the microcolony model may only be appropriate for assessing brood effects for substances with low toxicity to adult workers (Krueger *et al.*, 2021).

Recipes

1. **Potassium Sorbate Solution**
   Prepare a 25% w/v sorbic acid and potassium salt solution by dissolving 25 g of sorbic acid in distilled water to achieve a final volume of 100 mL; sterile filter (0.45 µm) and store at 4°C for up to 90 days.

Acknowledgments

The author thanks Drs. J. E. Simmons, C. Weitekamp, and YH. Kim for thoughtful and critical review of this manuscript. The author is also grateful to Drs. D. Schmehl, A. Cabrera, J. Strange, and D. Cox-Foster for their generosity and guidance. This protocol was adapted from previous work published peer-reviewed journals (Camp *et al.*, 2020a, 2020c; Weitekamp *et al.*, 2022).

Competing interests

The authors declare no conflict of interest.

References

Amsalem, E. and Hefetz, A. (2011). The effect of group size on the interplay between dominance and reproduction in *Bombus terrestris*. *PLoS One* 6(3): e18238.

Brown, M. J. F. and Paxton, R. J. (2009). The conservation of bees: a global perspective. *Apidologie* 40(3): 410-416.

Cameron, S. A., Lozier, J. D., Strange, J. P., Koch, J. B., Cordes, N., Solter, L. F. and Griswold, T. L. (2011). Patterns of widespread decline in North American bumble bees. *Proc Natl Acad Sci U S A* 108(2): 662-667.

Camp, A. A., Batres, M. A., Williams, W. C., Koethe, R. W., Stoner, K. A. and Lehmann, D. M. (2020a). Effects of the Neonicotinoid Acetamiprid in Pollen on Bombus impatiens Microcolony Development. *Environ Toxicol Chem* 39(12): 2560-2569.

Camp, A. A., Batres, M. A., Williams, W. C. and Lehmann, D. M. (2020b). Impact of Diflubenzuron on *Bombus impatiens* (Hymenoptera: Apidae) Microcolony Development. *Environ Entomol* 49(1): 203-210.

Camp, A. A., Williams, W. C., Eitzer, B. D., Koethe, R. W. and Lehmann, D. M. (2020c). Effects of the neonicotinoid acetamiprid in syrup on Bombus impatiens (Hymenoptera: Apidae) microcolony development. *PLoS One* 15(10): e0241111.

Couvillon, M. J. and Dornhaus, A. (2010). Small worker bumble bees (*Bombus impatiens*) are hardier against starvation than their larger sisters. *Insectes Soc* 57(2): 193-197.

Free, J. B. (1955). The behaviour of egg-laying workers of bumblebee colonies. *Brit J Anim Behav* 3(4): 147-153.

Goulson, D. (2013). An overview of the environmental risks posed by neonicotinoid insecticides. *J Appl Ecol* 50(4): 977-987.

Goulson, D., Hanley, M. E., Darvill, B., Ellis, J. S. and Knight, M. E. (2005). Causes of rarity in bumblebees. *Biol Conserv* 122(1): 1-8.

Goulson, D., Nicholls, E., Botias, C. and Rotheray, E. L. (2015). Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science* 347(6229): 1255957.

Gradish, A. E., Scott-Dupree, C. D., Frewin A. J. and Cutler G. C. (2012). Lethal and sublethal effects of some insecticides recommended for wild blueberry on the pollinator *Bombus impatiens*. *Can Entomol* 144(3): 478-486.

Gradish, A. E., Scott-Dupree, C. D., McFarlane, A. D. and Frewin, A. J. (2013). Too much work, not enough tarsi: Group size influences *Bombus impatiens* (Hymenoptera: Apidae) worker reproduction with implications for sublethal pesticide toxicity assessments. *J Econ Entomol* 106(2): 552-557.

Kleijn, D., Winfree, R., Bartomeus, I., Carvalheiro, L. G., Henry, M., Isaacs, R., Klein, A. M., Kremen, C., M'Gonigle, L. K., Rader, R., *et al*. (2015). Delivery of crop pollination services is an insufficient argument for wild pollinator conservation. *Nat Commun* 6: 7414.

Klinger, E. G., Camp, A. A., Strange, J. P., Cox-Foster, D. and Lehmann, D. M. (2019). *Bombus* (Hymenoptera: Apidae) Microcolonies as a Tool for Biological Understanding and Pesticide Risk Assessment. *Environ Entomol* 48(6): 1249-1259.

Krueger, A. J., Early, T. M., Ripperger, R. J., Cabrera, A. R. and Schmehl, D. R. (2021). The Utility of a Bumble Bee (*Bombus* spp. [Hymenoptera: Apidae]) Brood Test for Evaluating the Effects of Pesticides. *Environ Entomol* 50(5): 1105-1117.

Meeus, I., Brown, M. J., De Graaf, D. C. and Smagghe, G. (2011). Effects of invasive parasites on bumble bee declines. *Conserv Biol* 25(4): 662-671.

Peat, J. and Goulson, D. (2005). Effects of experience and weather on foraging efficiency and pollen versus nectar collection in the bumblebee, *Bombus terrestris*. *Behav Ecol Sociobiol* 58: 152-156.

Roger, N., Michez, D., Wattiez, R., Sheridan, C. and Vanderplanck, M. (2017a). Diet effects on bumblebee health. *J Insect Physiol* 96: 128-133.

Roger, N., Moerman, R., Carvalheiro, L. G., Aguirre-Guitierrez, J., Jacquemart, A. L., Kleijn, D., Lognay, G., Moquet, L., Quinet, M., Rasmont, P., *et al*. (2017b). Impact of pollen resources drift on common bumblebees in NW Europe. *Glob Chang Biol* 23(1): 68-76.

Smagghe, G., Reynders, S., Maurissen, I., Boulet, J., Cuvelier, X., Dewulf, E., *et al*. (2007). Analysis of side effects of diflubenzuron and tebufenozide in pollinating bumblebees *Bombus terrestris*. *Revue Synthese* 39-49.

Weitekamp, C. A., Koethe, R. W. and Lehmann, D. M. (2022). A Comparison of Pollen and Syrup Exposure Routes in Bombus impatiens (Hymenoptera: Apidae) Microcolonies: Implications for Pesticide Risk Assessment. *Environ Entomol*. doi:10.1093/ee/nvac026.

Wood, T. J., Gibbs, J., Graham, K. K. and Isaacs, R. (2019). Narrow pollen diets are associated with declining Midwestern bumble bee species. *Ecology* 100(6): e02697.