

FEB 26, 2024

OPEN  ACCESS**DOI:**

dx.doi.org/10.17504/protocols.io.eq2lyj4qplx9/v1

Protocol Citation: Mary-Kate F. Williams, Natalie K. Boyle, Robert N. Schaeffer, Diana L. Cox-Foster 2024. *Osmia lignaria* laboratory rearing protocol. [protocols.io](https://dx.doi.org/10.17504/protocols.io.eq2lyj4qplx9/v1) <https://dx.doi.org/10.17504/protocols.io.eq2lyj4qplx9/v1>

MANUSCRIPT CITATION:

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Osmia lignaria laboratory rearing protocol

Mary-Kate F. Williams^{1,2}, Natalie K. Boyle³, Robert N. Schaeffer¹, Diana L. Cox-Foster²

¹Department of Biology, Utah State University, Logan, Utah, USA;

²USDA-ARS-PWA Pollinating Insect Research Unit, Logan, Utah, USA;

³Department of Entomology, The Pennsylvania State University, University Park, Pennsylvania, USA

Mary-Kate F. Williams: Corresponding author;



Mary-Kate F. Williams

Department of Biology, Utah State University, Logan, Utah, U...

ABSTRACT

Our protocol was designed to rear *Osmia lignaria* Say (Hymenoptera: Megachilidae) from immature stages to adult emergence following their natural phenology in northern Utah. Many aspects of the bee's biology can be analyzed using this protocol, including ecology, genetics, microbiome, pathology, toxicology, and pollinator management. An individual bee can be observed as a response to a treatment group, and its development and survival can be measured. The interaction of various stressors can help define synergistic, antagonistic, and additive effects, including acute, sublethal, and chronic treatment responses. *Osmia lignaria* was used as a model to rear solitary bees in a laboratory setting.

ATTACHMENTS

[Oligonaria lab protocol - 3D print file.stl](#)

[Oligonaria lab protocol Blank datasheet examples.pdf](#)

[Oligonaria lab protocol Materials.xlsx](#)

[Oligonaria lab protocol Randomizing eggs and first instars.pdf](#)

[Oligonaria lab protocol Recipes.pdf](#)

IMAGE ATTRIBUTION

M-K. F. Williams took all photographs and X-ray images. Rendered images were created with BioRender.com. Protocol image was created with BioRender.com by M-K. F. Williams.

Protocol status: Working
We use this protocol and it's working

Created: Dec 19, 2023

Last Modified: Feb 26, 2024

PROTOCOL integer ID: 92523

Keywords: rearing, laboratory, solitary, bee, feeding, assay, development, survival, insect, well plate

Funders Acknowledgement:

National Science Foundation
Grant ID: 2211232

USDA-ARS
Grant ID: Base funds

GUIDELINES

- Use sterilized (autoclaved or otherwise unopened or new) glass, plastic, and stainless steel items.
- Check data loggers and incubators once a week while in use; ensure that programmed temperatures and RH do not deviate and safeguard against machine malfunction.

MATERIALS

Details for suppliers, manufacturers, brands, catalog and reference numbers, and product links used to develop our methods can be found in the attached Excel sheet. The Excel sheet provides additional or alternative links for discontinued or unavailable items we used to create our protocol.

Preliminary steps

1. Autoclave

Propagation and acquiring solitary bee nests

1. *O. lignaria* cocoons (adults inside cocoons) (for release)
2. Apple orchard (or target crop of research)
3. Corrugated plastic nesting box (in navy blue)
4. Nest block with drilled holes (wooden block with drilled holes)
5. Cardboard tubes (spiral wound three-ply cardboard outer tube, 8 mm inner diameter)
6. Straws (spiral wound white glassine paper insert, 8 mm outer diameter); one end of the straws should be plugged with black hot glue to mark an "end" to the nest to provisioning mothers
7. Zip ties
8. Wooden release box(es) (made of wood with drilled ventilation holes, ventilation holes covered in mesh, and lid left ajar to allow for bee exit)
9. Release tube(s) (made from a black ABS pipe and white PVC end cap slips, small holes drilled for ventilation on the ABS tube, and one large hole to allow for bee exit on one of the PVC end caps)
10. Twine (if using wooden release boxes)
11. Hemostat (6 inches [in])

Retain eggs/first instars and nest provisions

1. Completed solitary bee nests (after propagation/pollination, no older than 24 hours past completion)
2. Single-edge razor blade
3. Size 7 insect pins
4. Insect pinning board (or foamboard, or hard-backed item)
5. Stainless steel grafting tool
6. Sterilized Ringer's Solution
7. Porcelain or glass spot plate
8. 1.7 milliliter (mL) microcentrifuge tubes
9. 2% agarose gel inside of a large Petri dish (cooled 100 mL agarose in a large Petri dish)
10. Featherweight forceps
11. Weigh boats

12. Scale (capable of measuring 0.0000 gram [g])
13. 70% ethyl alcohol
14. ExsporTM

Homogenization of provisions and treatment administration

1. *Osmia lignaria*-collected provisions (separated from mud plug, mud partitions, and eggs/first instars)
2. Weigh boats
3. Stainless steel laboratory spatula with scoop (one per treatment group)
4. Large glass beaker (between 500-1000 mL)
5. 50 mL Falcon screw cap conical tubes (one per treatment group)
6. 15 mL Falcon screw cap conical tubes (one per treatment group)
7. Ultra fine tip point sharpie
8. -80°C freezer

Distribution of treated provisions into 3-D printed well plates

1. Homogenized treated provisions in 50 mL Falcon tubes
2. Small glass Petri dishes (one per treatment group)
3. Laboratory labeling tape
4. Ultra fine tip point sharpie
5. 3-D printed well plates (131.2 millimeters [mm] Length x 55.6 mm Width x 17.1 mm Height, 8.5 mm well diameter, 15.1 mm well depth; see attached 3-D printing file); alternatively, plastic mold cast well plates can be used
6. Straws (same as propagation straws) cut to well depth of well plates (15.1 mm)
7. Weigh paper cut into quarters (4x4 in paper cut to approximately 2x2 in sizes)
8. Scale (capable of 0.0000 g)
9. Featherweight forceps
10. Disposable nitrile gloves

Grafting eggs/first instars onto treated provisions

1. Retained eggs/first instars on 2% agarose gel in large Petri dish
2. Sterilized Ringer's Solution
3. Porcelain or glass spot plate (use new well per treatment group)
4. Stainless steel grafting tool (one per treatment group if able)
5. 1.7 mL microcentrifuge tubes
6. Alcohol burner
7. 70% ethyl alcohol
8. Sterilized Reverse osmosis (RO) water
9. Labeled 3-D printed well plates with distributed homogenized treated provisions
10. Plastic trays (10x14 in)

11. Dissection microscope with external light source
12. Transparency film (cut to fit top of well plates)
13. Incubator capable of temperature and relative humidity (RH) programming
14. Plastic container (1.89 liters [L])
15. Data logger capable of recording temperature and RH measurements

Observing development and monitoring survival

1. Dissection microscope with external light source
2. 1.7 mL microcentrifuge tubes
3. Hemostat (6 in)
4. Specimen radiography system with digital imager
5. Size 000 gelatin capsules
6. Sticky board (plastic board covered with masking tape with sticky side exposed)
7. Disposable nitrile gloves
8. Counter or shelf space (to incubate samples at room temperature, approximately 20–22°C)
9. Container to store sticky boards
10. Data logger capable of recording temperature and RH measurements
11. -80°C freezer

Winter diapause

1. Incubator capable of temperature RH programming
2. Weigh paper cut into quarters (4x4 inch paper cut to approximately 2x2 inch sizes)
3. Scale (capable of 0.0000 g)
4. Disposable nitrile gloves
5. Size 000 gelatin capsules
6. Container with samples on stored sticky boards
7. Data logger capable of recording temperature and RH measurements
8. 4°C walk-in cooler or incubator
9. Plastic container (1.89 L)
10. Sterile RO water
11. Glass scintillation vials (without lids and label before use)
12. Ultra fine point sharpie
13. Container to keep vials upright
14. Cotton balls
15. -80°C freezer
16. 1.7 mL microcentrifuge tubes

Emergency

1. Samples (cocoons) inside of glass scintillation vials with cotton ball plugs
2. Incubator capable of temperature and RH programming

3. Ultra fine point sharpie
4. 1.7 mL microcentrifuge tubes
5. -80°C freezer
6. 70% ethyl alcohol
7. Plastic container (1.89 L)
8. Sterile RO water
9. Disposable nitrile gloves
10. Single-edge razor blade
11. Autoclave

SAFETY WARNINGS

 Before using chemical reagents, please refer to the manufacturer's SDS (Safety Data Sheet) for safety and hazard information.

ETHICS STATEMENT

No approval of research ethics committees was required to create this protocol because experimental work was conducted with an unregulated invertebrate species.

BEFORE START INSTRUCTIONS

Preliminary steps

1. Purchase or otherwise obtain *Osmia lignaria* Say (Hymenoptera: Megachilidae) (adults in cocoons) from a supplier near the researcher's location. The Orchard Bee Association provides guidelines for purchasing and obtaining *O. lignaria* (Orchard Bee Association [2021]. Guidelines for Sustainably Sourcing and Managing Mason Bees. Link: <https://www.orchardbee.org/certification-guidelines-1>).
2. Check bloom for the desired fruit or nut crop as often as needed to prepare for the release of *O. lignaria* using release boxes or tubes.
3. *Osmia lignaria* requires mud to complete nest construction. Ensure *O. lignaria* can access moist soil (mud) within the orchard during pollination (Bosch and Kemp 2001; full reference in protocol).
4. Obtain 3-D printed well plates (see 3-D print file) or create Smooth-Cast™ 305 well plates using a silicon mold (see "Recipes" PDF file).
5. Sterilize glass and stainless steel items for the experiment (autoclave at  121 °C for  00:15:00). Repeat sterilization if more than one grafting date is needed.

Propagation and acquiring solitary bee nests (Fig. 1)

- 1 Deploy corrugated plastic nesting boxes (Artz et al. 2013, 2014) into the orchard before bloom. Place nest blocks with straw inserts or bundles of cardboard tubes with straw inserts into nest boxes.

Note

Nest boxes can be zip-tied to branches of a tree, two per tree, on different branches. Nest boxes should be attached to alternating trees in the orchard. Nest boxes should face south-southeast when placed in the orchard (Artz et al. 2014).

Note

Bundles of cardboard tubes with straw inserts should be 40-50 per bundle, and two bundles per nest box (80-100 tubes with straw inserts).

CITATION

Artz DR, Allan MJ, Wardell GI, Pitts-Singer TL (2013). Nesting site density and distribution affect *Osmia lignaria* (Hymenoptera: Megachilidae) reproductive success and almond yield in a commercial orchard. *Insect Conservation and Diversity*.

LINK

<https://doi.org/10.1111/icad.12026>

CITATION

Artz DR, Allan MJ, Wardell GI, Pitts-Singer TL (2014). Influence of nest box color and release sites on *Osmia lignaria* (Hymenoptera: Megachilidae) reproductive success in a commercial almond orchard. *Journal of Economic Entomology*.

LINK

<https://doi.org/10.1603/EC14237>

- 2 Once bloom has begun, place release boxes or release tubes containing cocoons into the orchard on trees that do not have nest boxes.

Note

Release boxes should fit snugly at the base of where branches come out of the trunk, while release tubes can be secured on branches with zip ties.

Note

Approximately 250 nesting females per acre is sufficient to pollinate apples, and 300 nesting females per acre is sufficient to pollinate almonds. Potential dispersal and mortality of pre-nesting females should be considered for final release numbers. If using loose cocoons, 40-50% more females should be for release. The number of males released should be 1.5 to 2 times more than the number of females.

- Bosch J, Kemp WP (2001). How to Manage the Blue Orchard Bee As an Orchard Pollinator, Sustainable Agriculture Network handbook series; bk. 5.

- 3 Once *O. lignaria* cocoons are placed in the orchard for release, check nest blocks or bundles of tubes daily for completed nests.

Note

Nesting should be checked during the late morning or early afternoon.

Note

If there is a completed nest (signified by a mud plug at the entrance of the straw), remove the completed nest by pulling the straw insert with hemostats. Replace with an empty straw, plugged at one end with black hot glue, to encourage population retention.

- 4 Carefully transport completed nests to the laboratory to retrieve provisions and eggs/first instars.

Note

Avoid dropping or rough handling of nests.

Note

Orient nests with the plugged entrance (mud) facing upward during transportation to avoid injuring or dislodging eggs and/or first instars from provisions.

- 5 Continue checking for completed nests daily, and processing nests until the ideal amount of provisions and sample size (eggs/first instars) is obtained for the experiment.

Note

The researcher can continue to check for nests until the end of bloom. Unused nests no more than  48:00:00 old can be retained and used if more than one grafting event is required for the experiment. Nests older than  48:00:00 old may contain larvae beyond the first instar stage (beyond use for the experiment).

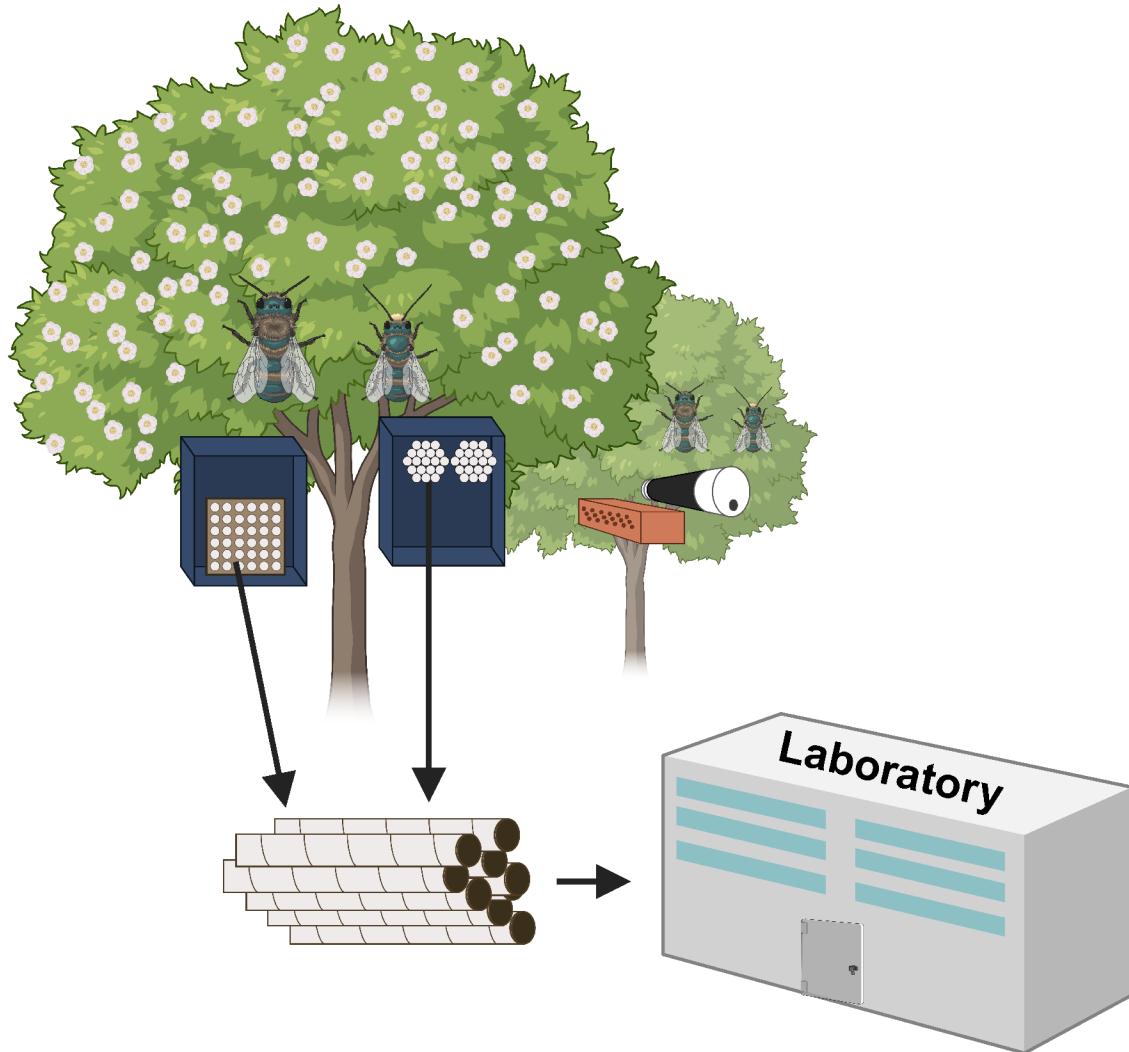


Figure 1. Propagation and acquisition of solitary bee nests in an apple orchard. Completed nests should be carefully transferred to the laboratory within 24 hours. Rendered image created with BioRender.com.

Separating eggs/first instars from provisions (Fig. 5)

- 6 Take a completed nest and shallowly cut along the straw laterally with a single-edge razor blade. Using size 7 insect pins, pin the nest open against an insect pinning board (or foamboard, or hard-backed item). This serves to secure the straw while retrieving provisions and eggs/first instars.

Note

To avoid injuring eggs/first instars, do not dig razor blades too deep into the nest while cutting it open.

- 7 Using a stainless steel grafting tool, dip the flattened head into sterile Ringer's Solution, then gently pick up the egg/first instar from the provision and deposit it onto a 2% agarose gel in a large Petri dish. Cover the large Petri dish with the dish lid to prevent egg/first instar desiccation. Keep the large Petri dish at room temperature. Set aside eggs/first instars for later.

Note

Dipping the tool into Ringer's Solution allows eggs/first instars to adhere to it. Ringer's Solution can be placed into wells of a spot plate or aliquoted into a  1.7 mL microcentrifuge tube.

CITATION

Cold Spring Harbor Laboratory Press (2008). Ringer's solution (pH 7.3-7.4). Cold Spring Harbor Protocols.

LINK

<https://doi.org/10.1101/pdb.rec11273>

Note

Apply enough pressure to deposit eggs/first instars onto the gel surface. Avoid breaking or damaging the egg/first instar when depositing it onto the gel.

Note

Label the large Petri dish with the date the eggs/first instars were deposited on the 2% agarose gel to prevent mixing with incoming nests. Do not leave eggs/first instars inside the covered dish longer than  12:00:00 to  24:00:00 at room temperature, as humidity in the dish could encourage bacteria or fungus growth (Fig. 2).

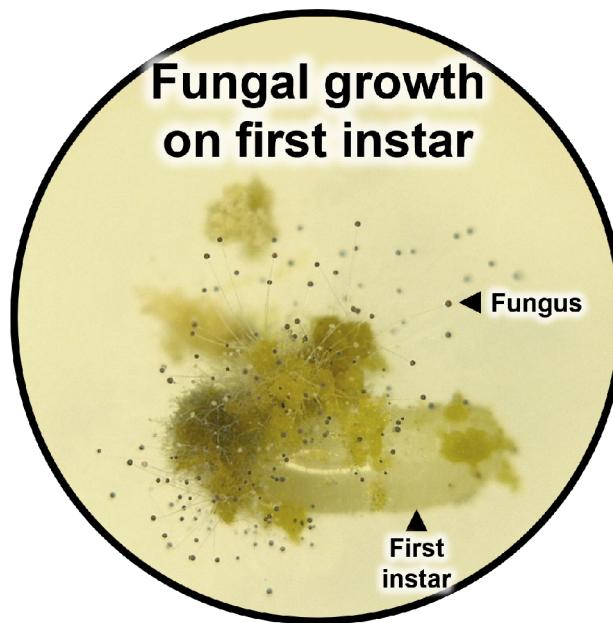


Figure 2. Example of first instar left inside a covered dish longer than 24 hours. Fungus began to grow on the first instar, rendering it impractical for the experiment. Fungal contamination is a potential cause for concern, which can stem from the provision, nesting materials, or lab environment. Here, a fungus can be observed with spore-bearing structures. Photo by M-K. F. Williams.

Note

Randomize eggs/first instars to avoid nest biases or lineage biases. Offspring sex can be selected if desired. Females are laid first (farthest from the plugged entrance) and males are laid last (closest to the plugged entrance). Consult the "Randomizing eggs and first instars" PDF file for instructions.

Note

If any eggs/first instars remain and are no longer needed for the experiment, place them in a 1.7 mL microcentrifuge tube and freeze at -80 °C if desired for molecular analyses.

Note

Use eggs/first instars collected on the provision homogenization date. However, if the researcher requires more eggs/first instars, those left over from the previous day could be used. Do not use offspring that have progressed past the first instar stage. Any unopened leftover nests may contain offspring that have hatched to the second instar stage.

Note

Do not chill eggs/first instars in the large Petri dish to delay development. Our experience has shown chilling can lead to egg/first instar failure (death).

Note

Descriptions of egg, first instar, and second instar of *O. lignaria*.

- **Egg:** white, shiny, and sausage-shaped (not pictured)
- **First instar:** encased in the chorion of the egg, feeds on egg fluids inside the egg's split chorion (Bosch and Kemp 2001), recognized by clear poles at the apical and posterior end of the sausage shape (Fig. 3); if first instar larvae hatch or leave the chorion they have developed into second instar stage
- **Second instar:** larva hatches from the chorion and begins to eat the provision with a pronounced head capsule (Fig. 4)

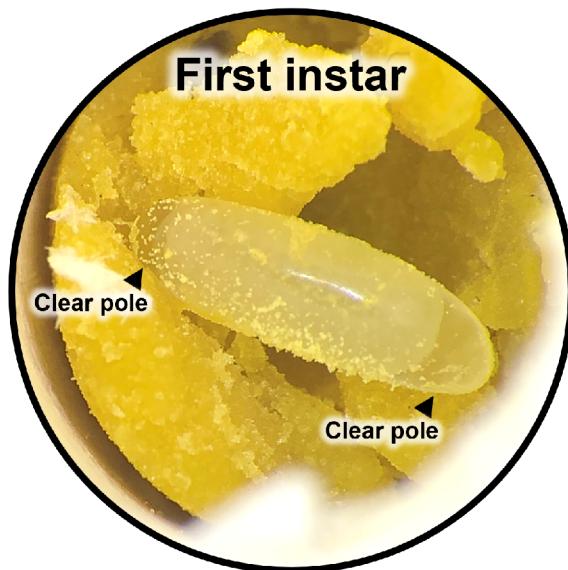


Figure 3. First instar of *O. lignaria*. Black arrows indicate clear poles at the apical and posterior ends of the first instar. Photo by M-K. F. Williams.

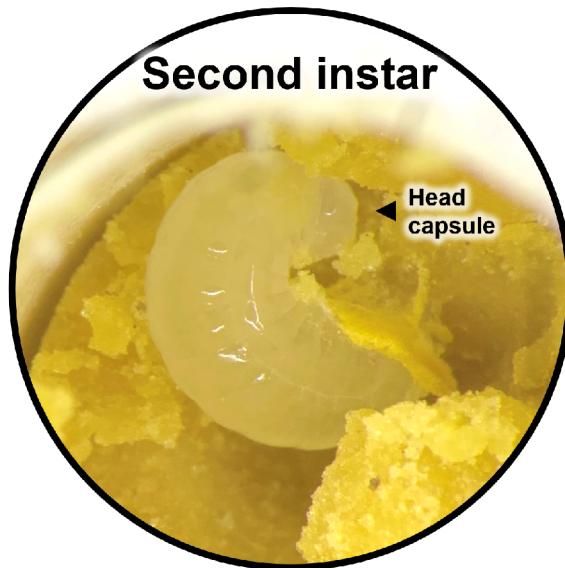


Figure 4. Second instar of *O. lignaria*. Head capsule is indicated by a black arrow. Photo by M-K. F. Williams.

- 8 With the egg/first instar separated from the provision, use featherweight forceps to pool the *O. lignaria*-collected provisions into a weigh boat.

Note

Avoid incorporating wet or dried mud partitions into the provisions.

Note

Record the number of cells and weight of the pooled provisions after each nest is opened.

Note

Excess provisions can be frozen at  -80 °C for molecular analyses and pesticide analyses if desired.

Note

After handling a nest, clean and sterilize the area with 70% ethyl alcohol and Expor™. Repeat for every additional nest.

Note

When all provisions and eggs/first instars are retrieved from a nest, discard emptied straws.

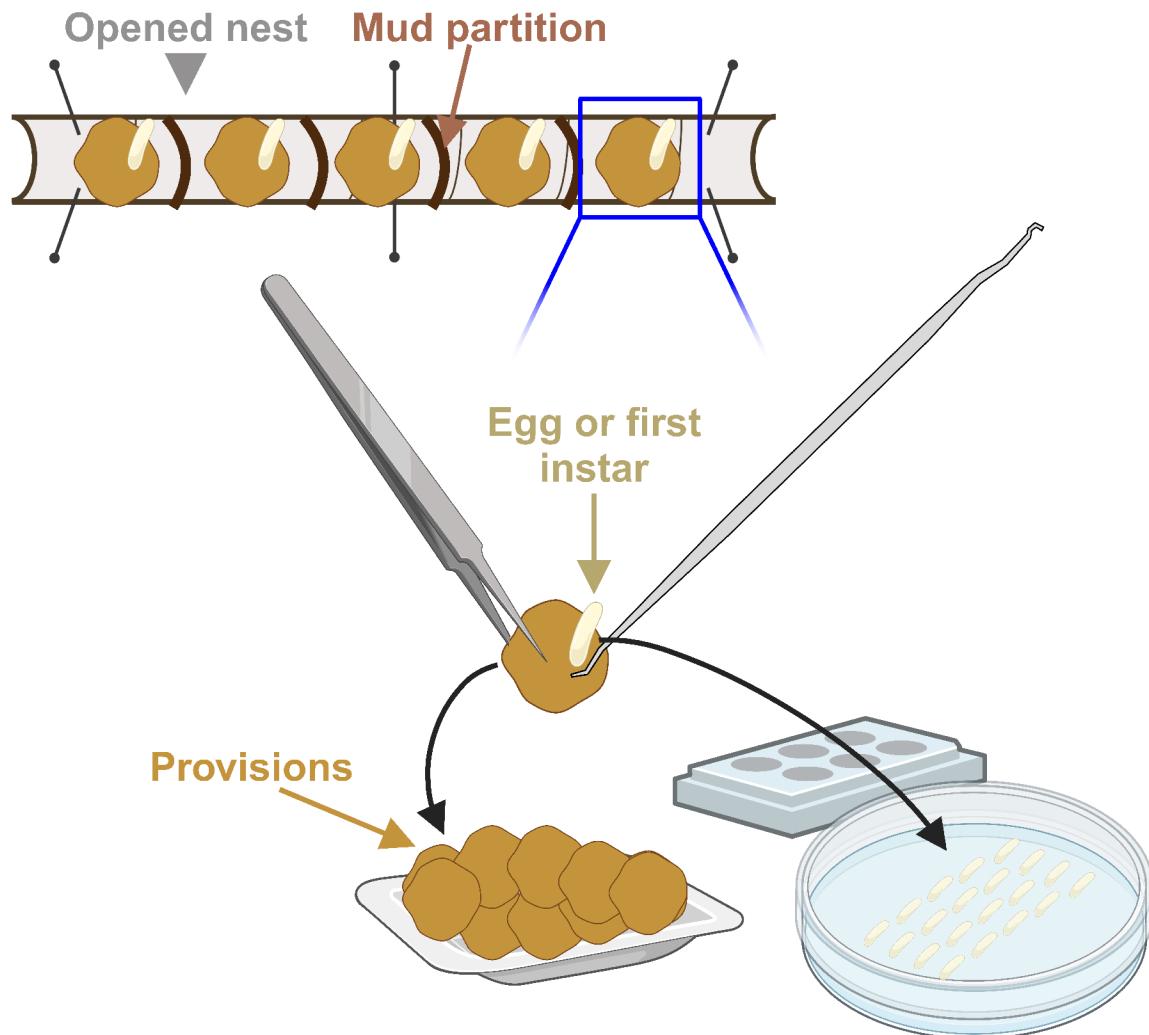


Figure 5. Separate eggs/first instars from provisions. Collect provisions in a weigh boat and eggs/first instars onto an agarose gel in a large Petri dish. Rendered image created with BioRender.com.

Homogenization of provisions and treatment administration (Fig. 6)

6m

- 9 Deposit all collected provisions into a sterilized large glass beaker. Mix the provisions thoroughly for 3m
⌚ 00:01:00 to ⌚ 00:02:00 using a sterile stainless steel laboratory spatula.

Note

Provision material may stick to the side of the beaker. Make sure to scrape provisions on the beaker walls to incorporate them in the homogenization. Alternatively, a ceramic mortar and pestle can be used instead of a large beaker.

- 10 Distribute the homogenized provisions into a labeled **50 mL Falcon tube** (one per treatment group). Ensure the same amount of homogenized provisions is distributed among the **50 mL Falcon tubes** before treatment administration.

Note

Tare the weight of the empty tube before weighing homogenized provisions. Before adding treatments, the final weight of homogenized provisions in **50 mL Falcon tubes** should be within **0.1 g** of the desired homogenized provision weight.

Note

Leftover untreated homogenized provisions can be frozen in a **15 mL Falcon tube** at **-80 °C** and can be used for pollen identification and/or pesticide analysis.

- 11 Add treatments to homogenized provisions. Homogenize provisions again by mixing the treated provisions in their respective tubes for 3m
⌚ 00:01:00 to ⌚ 00:02:00 using a stainless steel laboratory spatula.

Note

Use a different spatula per treatment to avoid cross-contamination. Treatments that can be investigated include addition of pesticides, pathogens, or a combination of both.

Note

Addition of liquid treatments are based on the weight of homogenized provisions distributed in the  50 mL Falcon tubes.

Note

Provision material may stick to the side of the tube. Make sure to scrape provisions on the tube walls to incorporate all material into the homogenate. Alternatively, a ceramic mortar and pestle can be used instead of a large beaker.

Note

This step guarantees that the added treatment is evenly distributed throughout the homogenized provisions and that all bees on a given graft date receive the same combined provisions. Additionally, this step will ensure uniformity among provisions provided to all eggs/first instars on a given date.

Note

Provisions will contain a natural microbiome that will vary by location and floral source of pollen and nectar (Rothman et al. 2019). Consider ethylene oxide sterilization if sterilized provisions are desired (Strange et al. 2023).

CITATION

Rothman JA, Andrikopoulos C, Cox-Foster D, McFrederick QS (2019). Floral and foliar source affect the bee nest microbial community.

LINK

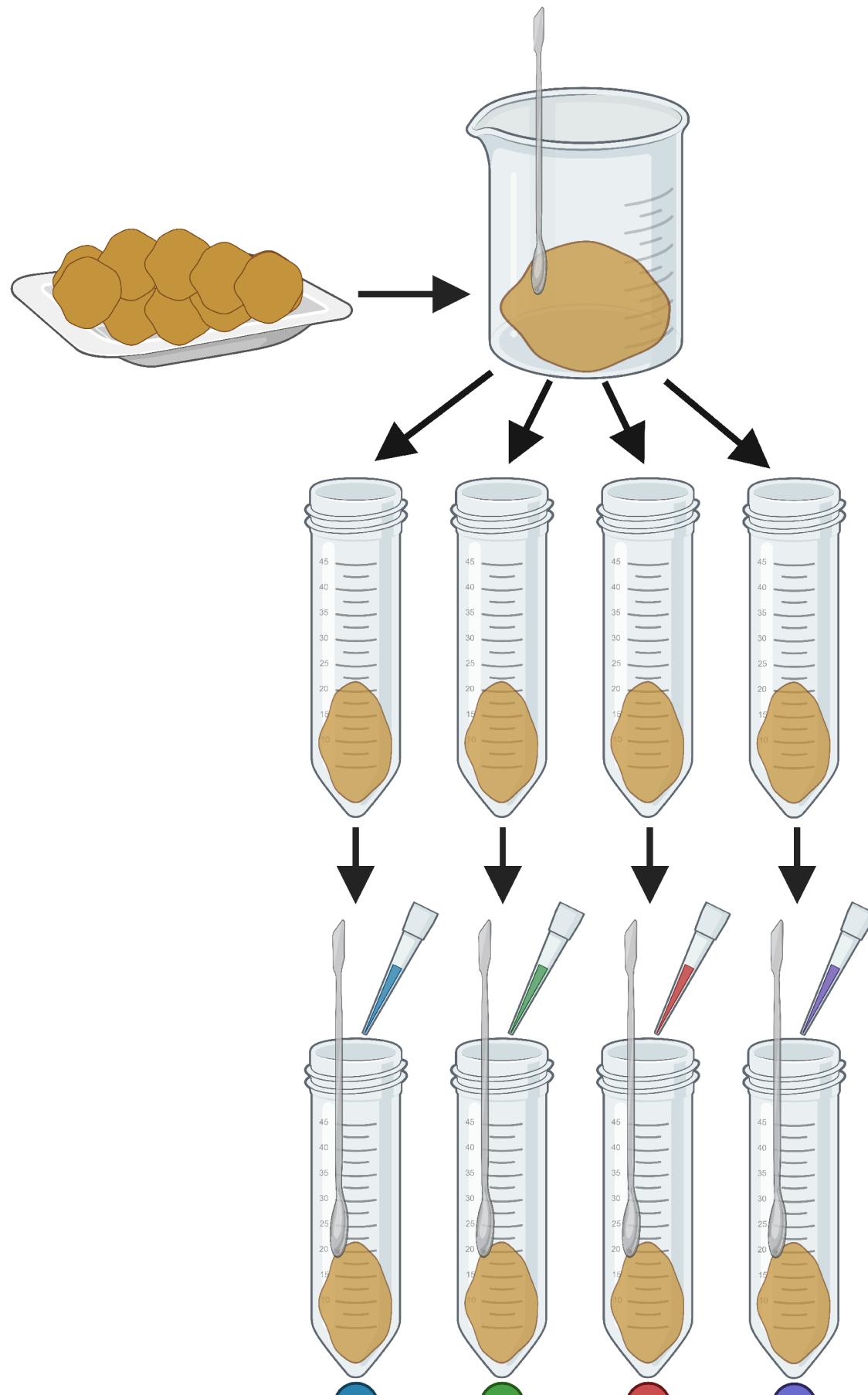
<https://doi.org/10.1007/s00248-018-1300-3>

CITATION

Strange JP, Tripodi AD, Huntzinger C, Knoblett J, Klinger E, Herndon JD, Vuong HQ, McFrederick QS, Irwin RE, Evans JD, Giacomini JJ, Ward R, Adler LS (2023). Comparative analysis of 3 pollen sterilization methods for feeding bumble bees. *Journal of Economic Entomology*.

LINK

<https://doi.org/10.1093/jee/toad036>



A

B

C

D

Figure 6. Homogenization of provisions and treatment administration. Each letter represents a different treatment group. Rendered image created with BioRender.com.

Distribution of treated provisions into 3-D printed well plates (Fig. 7)

- 12 Carefully remove the homogenized treated provisions from their respective  50 mL Falcon tubes using each treatment group's respective stainless steel laboratory spatula. Place the treated provisions into a small, labeled Petri dish.

Note

Use one small Petri dish per treatment to avoid cross-contamination. Label small Petri dishes with one of the treatment group names using laboratory labeling tape and a fine tip sharpie.

- 13 Label the 3-D printed well plates with the plate number and orientation of treatments according to columns.

Note

Plates can be labeled with laboratory labeling tape and a sharpie.

- 14 Cut straws, the same utilized during propagation, to the same length as the 3-D printed plate well depth ( 15.1 mm).

- 15 Using the cut straws as a cookie-cutter, weigh and punch  0.35 g of homogenized treated provisions. Use weigh paper cut into quarter sizes (4x4 in sheets cut to approximately 2x2 in sizes), to keep the scale clean.

Note

Change the weigh paper and disposable nitrile gloves between treatment groups to avoid cross-contamination.

Note

Provisions should be as close to the bottom of the straw (and well) as possible. This will prevent developing bees from being exposed to the external environment and simulate being inside a closed environment as it would happen in nature.

Note

Tare straw weight before using it as a cookie-cutter. Press straw into the homogenized treated provision group, then weigh the straw with homogenized treated provision inside of it. If the weight is less than $\text{ } \pm 0.35 \text{ g} - 0.01 \text{ g}$ take the straw and use it as a cookie cutter again to ensure final weight is not above $\text{ } \pm 0.35 \text{ g} + 0.01 \text{ g}$ before placing the straw into the well plate. If the weight exceeds $\text{ } \pm 0.35 \text{ g} + 0.01 \text{ g}$, remove provision from the bottom side of the straw.

Note

Plate one treatment group at a time for all well plates. Start with control group, then move on to another treatment group (ex., low concentration to high concentration, single pathogen inoculation to multiple pathogen inoculations, etc.).

Note

Do not deposit provisions without straw. The straw serves as a liner for when fifth instars begin to spin silk (cocoon initiation to cocoon completion). Additionally, the straw gives the researcher the ability to transfer bees safely to gelatin capsules.

- 16 Place the straw containing the homogenized treated provision into its designated place in the well plate.
- 17 Repeat these steps for each treatment group, plating one treatment at a time, until the target sample size is reached. Each treatment group should have an equal number of samples represented on the 3-D printed plates.

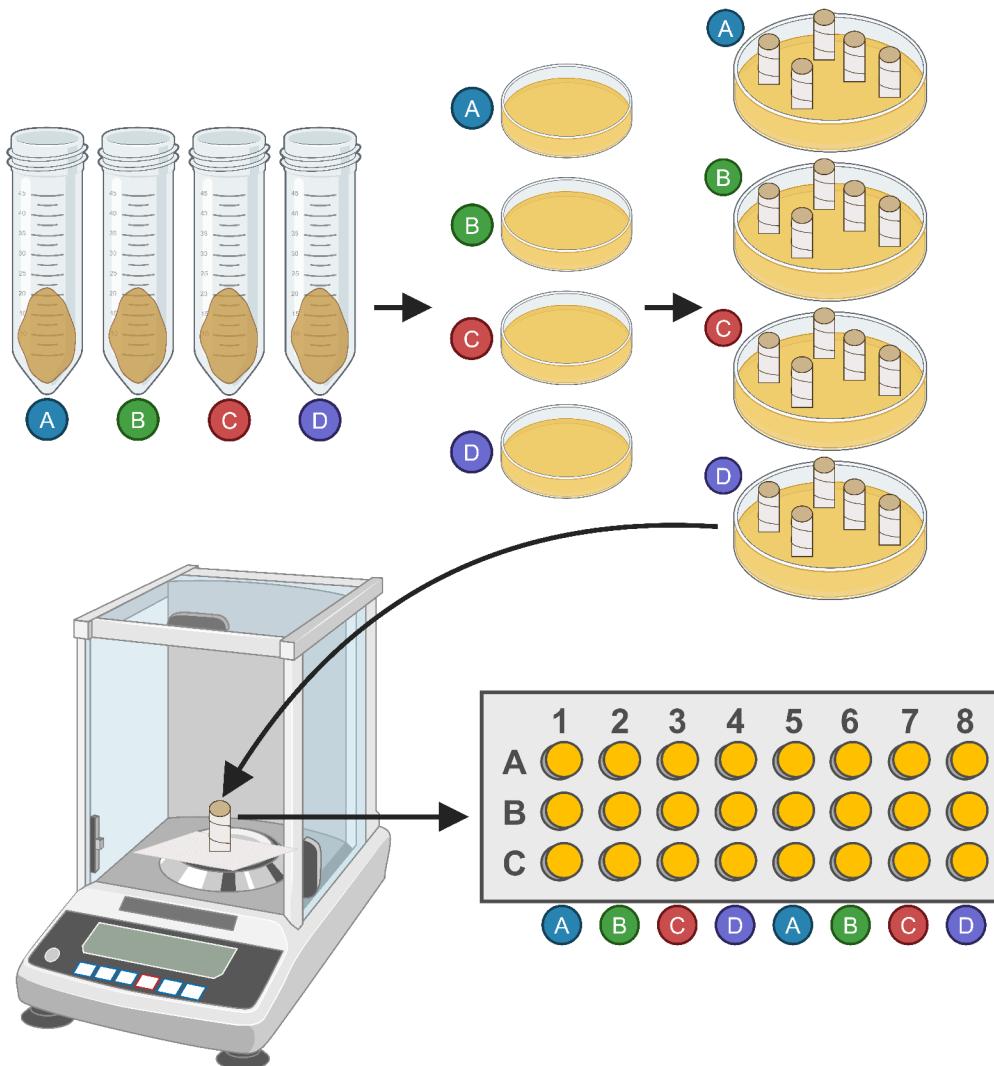


Figure 7. Distribution of treated provisions into 3-D printed well plates. Rendered image created with BioRender.com.

Grafting eggs/first instars onto treated provisions (Fig. 9)

- 18 Using the stainless steel grafting tool dipped in Ringer's Solution, gently pick up an egg/first instar from the 2% agarose gel inside the large Petri dish to transfer onto a treated provision inside a well.

Note

Perform this step on the same day as treatment administration. Use one stainless steel grafting tool per treatment. Ringer's Solution can be placed into wells of a spot plate (use one well per treatment group to avoid cross-contamination) or aliquoted into 1.7 mL microcentrifuge tubes (use one tube per treatment group to avoid cross-contamination). If the researcher does not have enough stainless steel grafting tools to have one per treatment, sterilize the tool between treatments using flame sterilization.

- 19** Gently deposit the eggs/first instars onto homogenized treated provisions using the stainless steel grafting tool.

Note

Once eggs/first instars are grafted onto treated provisions in the designated wells, use a dissection microscope to check for damaged eggs/first instars. If eggs/first instars are damaged, regraft immediately with leftover eggs/first instars. Damaged eggs/first instars will look dull (less glossy, Fig. 8) compared to undamaged ones (Fig. 3). Some damaged eggs/first instars may deflate over the next few days if not noticeable on the graft date (Fig. 8). If the researcher questions the current state of the egg/first instar, it is advisable to regraft immediately to maximize the desired sample size. Do not regraft more than 24:00:00 to 48:00:00 after treatment administration.



Figure 8. Examples of damaged (left) and deflated (right) first instars after grafting onto homogenized plated provisions. Photos by M-K. F. Williams.

- 20 Once eggs/first instars have been checked, document the bee's graft date (considered as "Day 0") and starting development life stage (eggs or first instars only).

Note

Example of datasheets we used can be found in the attached "Blank datasheet examples" PDF file.

Note

The second instar stage will standardize development and survival throughout an experiment.

- 21 Cover the 3-D printed well plates with a piece of transparency film (sized to cover the surface of the plate) to prevent egg/first instar and provision desiccation.

Note

Label the top left corner of the transparency film with the letter "A" to signify the correct position of it going back on top of the well plate after being taken off to observe development.

Note

If desired, the transparency film can be fastened loosely to the plate with laboratory labeling tape.

- 22 When grafting is complete, place the 3-D printed well plates with transparency film covers onto a plastic tray and into an incubator set to  26 °C with  60 % .

Note

A plastic container with RO water can be placed at the bottom of the incubator to aid in humidity.

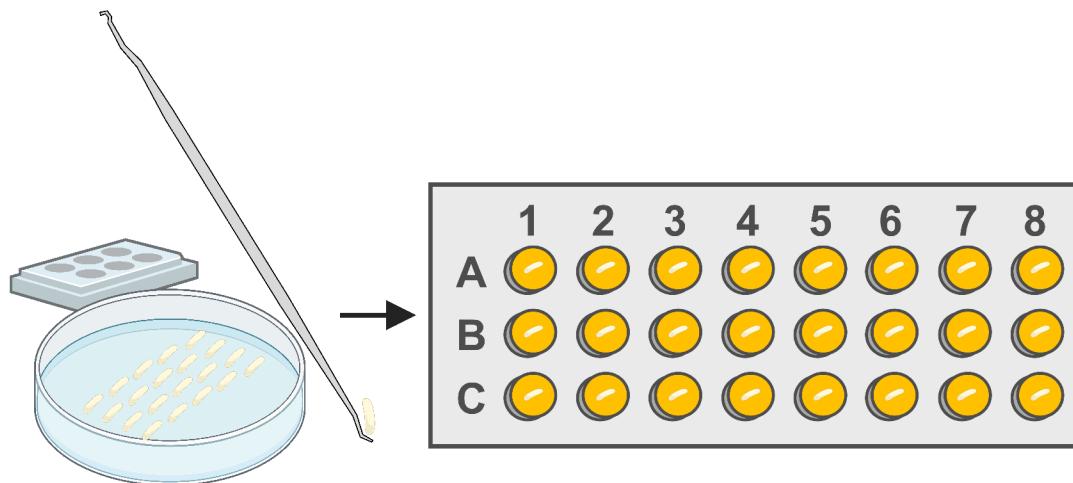


Figure 9. Grafting eggs/first instars onto treated provisions distributed into 3-D printed well plates.
Rendered image created with BioRender.com.

Observing development and monitoring survival (Fig. 15)

12s

- 23 Beginning on the graft date, monitor bees once a day for changes in development using a dissection microscope between 12 PM and nightfall. Document date of observed development life stage.

Note

Observe development from the egg/first instar stage until the cocoon completion stage:

- **Second instar:** larva hatches from the chorion and begins to eat the provision with a pronounced head capsule (Fig. 4)
- **Fifth instar:** indicated by the first sign of frass (brown fecal pellets), frass is brown in color, rod-shaped, and slightly flat, the fifth instar stage is also determined by development of setae (often hard to see with respect to larva orientation within a well) (Fig. 10)
- **Cocoon initiation:** first sign of silk spun by a late fifth instar, initial silk spin from the mouth of the larva is hard to see, larva performs a "chewing" motion without eating anything (Fig. 11)
- **Cocoon completion:** dark brown color, opaque brown structure with delicate silk outer layer (prepupa is a late fifth instar larva inside a completed cocoon) (Fig. 12)

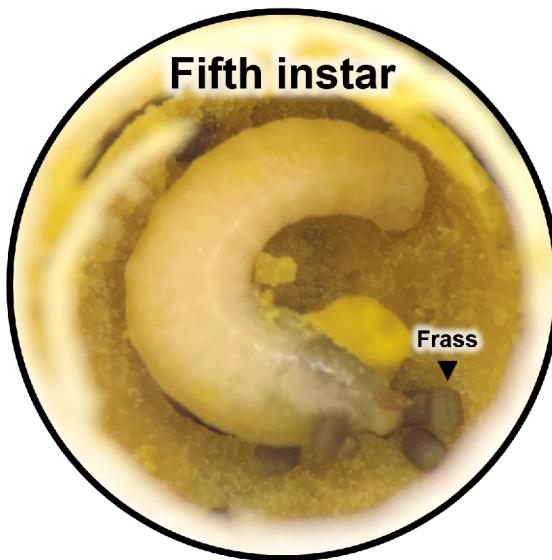


Figure 10. Fifth instar of *O. lignaria*. Frass is indicated by a black arrow. Photo by M-K. F. Williams.

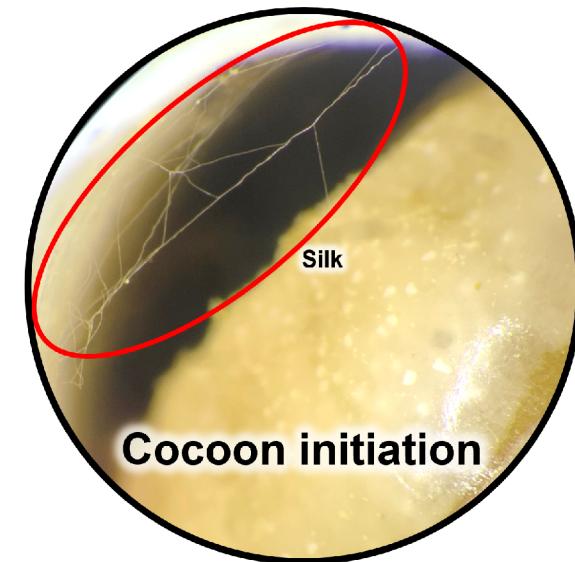


Figure 11. Cocoon initiation, the first signs of silk produced by late fifth instar of *O. lignaria*. Imaged are thin strands of silk produced by the late fifth instar. The image was taken with an increased magnification using the dissection microscope. Photo by M-K. F. Williams.

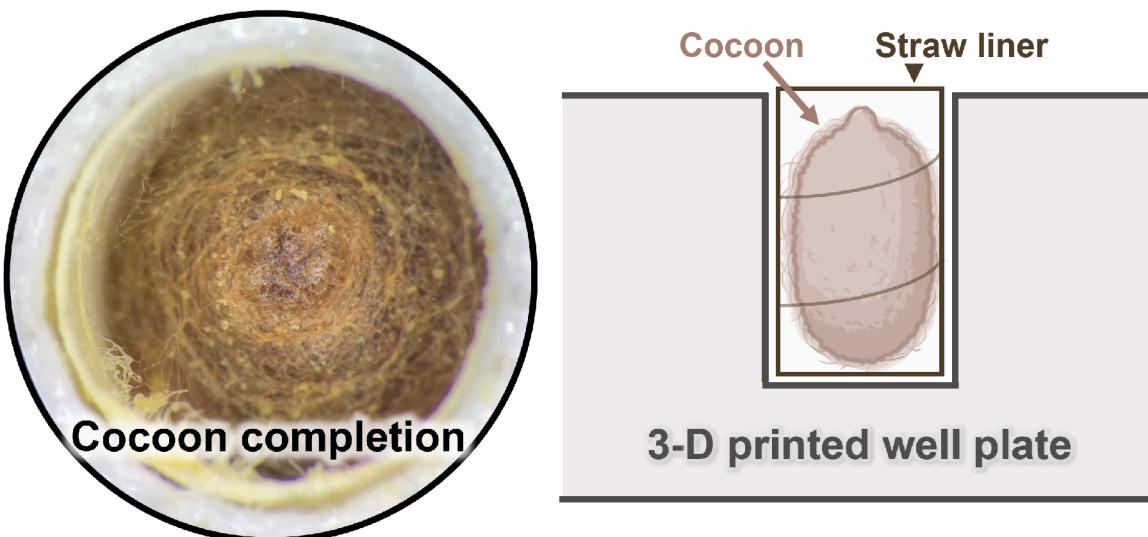


Figure 12. Cocoon completion corresponds to the prepupa stage of *O. lignaria*. Cocoon is pictured as it appears looking into the well plate (left). Cocoon orientation should be with the apical end (termed nipple; a protruding piece of cocoon) exposed to the researcher (right). Photo by M-K. F. Williams. Rendered image made with BioRender.com.

24 If a bee dies, excise the dead bee from the well and freeze in a  1.7 mL microcentrifuge tube at  -80 °C .

Note

Use hemostats to pull the straw containing the bee and provision from the well plate and place it into a **1.7 mL** microcentrifuge tube. Record the date the bee was taken from the experiment. The researcher may also want to freeze any remaining provisions in a separate **1.7 mL** microcentrifuge tube. Dead bees and provisions could be analyzed for pathogens.

Note

Photograph and record descriptions of dead bees. These observations may aid in determining how treatments physiologically impact development and survival.

- 25** Once cocoons have darkened, and the nipple (apical end of the cocoon visible to the researcher) (Fig. 12) has become dark with white silk surrounding it, transfer the cocoon to a gelatin capsule and adhere to a sticky board.

Note

Organize samples by their well plate number in rows (Fig. 13). Label the sticky board with plate number and well position in a space above the gelatin capsule. Sample labels can be written directly on the sticky side of the tape with an ultra fine tip sharpie.

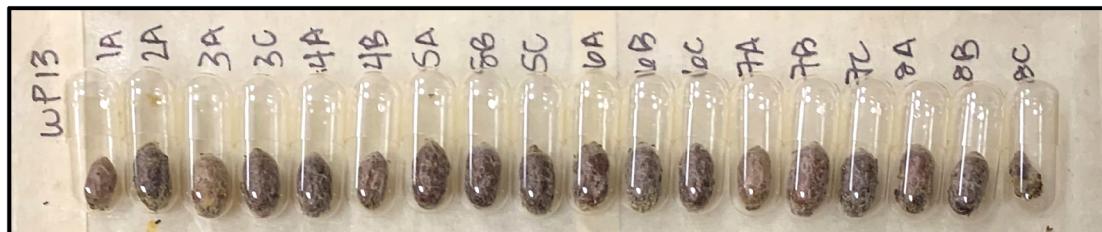


Figure 13. Cocoons in gelatin capsules adhered to a sticky board. Samples were organized in rows according to well plate number (WP13). The well plate identifier (WP13) and position identifiers (above gelatin capsules) create the sample identifier (13-1A, 13-2A, etc.). Straw inserts and frass were removed for clearer X-rays of developing bees. Photo by M-K. F. Williams.

Note

Cocoons can remain inside the straw liner or be removed from the straw liner when placed into a gelatin capsule.

- 26 Transfer the sticky boards with developing bees to room temperature.

Note

Place sticky boards in a box to keep them contained. The box can be placed on a countertop or shelf in the laboratory. The box should have a data logger capable of recording temperature and relative humidity (RH) inside of it.

Note

Alternatively, an incubator set at  20 °C with  70 % can be programmed instead of using a laboratory countertop (Bosch and Kemp 2001).

- 27 Use an X-radiograph (hereby X-ray) imaging system and machine to document the prepupal, pupal, and adult stages. Perform X-rays  00:00:12 at 24 kilovolts (kV) once a day, between 12 PM and nightfall, until ramp-down conditions.

Note

Observe development from the prepupal stage to adult molt (Fig. 14):

- **Prepupa:** late fifth instar larva inside of a completed cocoon (stage corresponds to cocoon completion)
- **Pupa:** division of the body into three segments from the larval form (separation of head, thorax, and abdomen), noted by legs and opaque wings close to the body
- **Adult molt:** noted by legs loose and translucent wings away from the body

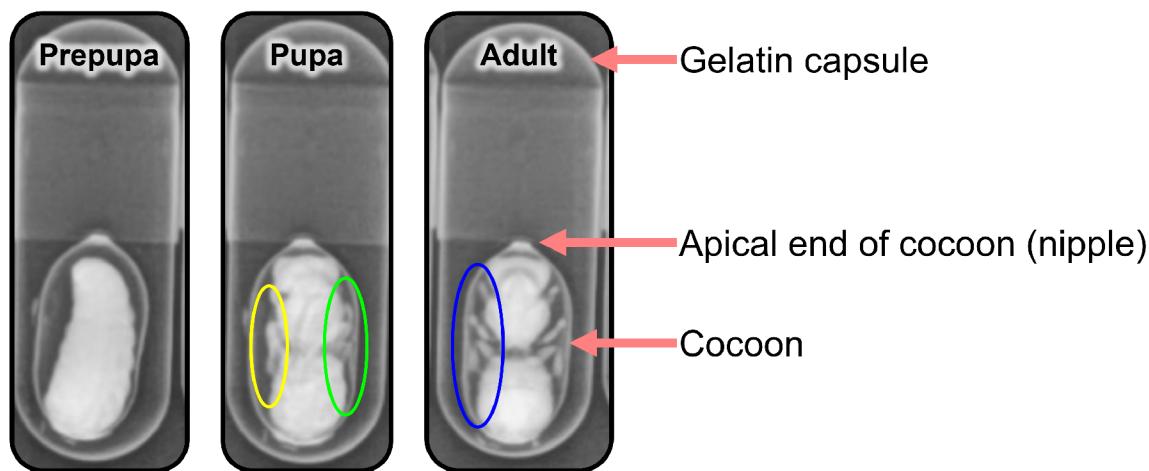


Figure 14. From left to right: prepupa, pupa, and molted adult of *O. lignaria*. Prepupae are late fifth instar larvae inside a completed cocoon. Pupa of *O. lignaria* are noted by opaque wings (yellow circle) and legs (green circle) close to the body. Molted adults of *O. lignaria* are noted by legs loose (blue circle) and translucent wings away from the body. All X-ray images were taken by M-K. F. Williams.

Note

When performing X-rays, handle the sticky boards with developing bees carefully. If dropped, expect high mortality and interrupted development (Williams et al. *in prep*).

- Williams MKF, Schaeffer RN, Cox-Foster DL (*in prep*). Discovery of a critical time point in the development and handling of an orchard pollinator, the blue orchard bee (*Osmia lignaria* Say, Hymenoptera: Megachilidae).

Note

Daily X-rays should be performed at a consistent time each day. X-rays provide a non-invasive way to observe development without risk to the bee. This step is possible if the researcher can access a specimen radiography system with a digital imager.

Note

X-rays can be performed until August 1 if the adult molt date is not desired, or X-rays can continue until September 1 if the adult molt date is desired.

Note

If the researcher wants a clearer X-ray image of the developing bee, the cocoon can be pulled from the straw liner by gently pulling it with hemostats at the nipple (apical end of the cocoon). Frass can be gently pulled off the cocoon using disposable gloves; then the cocoon can be placed into a gelatin capsule. Change disposable gloves between treatment groups or between samples. If the researcher decides to remove frass, take caution and; then be gentle with the cocoon not to harm the developing bee inside.

Note

Once a sample on the sticky board leaves the experiment, it creates a blank space on the X-ray. Cross out that sample's identifier on the physical board to avoid mixing up sample identifiers later in the experiment. Place an empty gelatin capsule in a pulled sample's place to avoid shifting from its original space.

Note

On August 1 of the study year, if bees have not pupated, they should be frozen at  -80 °C as it is unlikely those bees will survive. Open by slicing off the apical end of the cocoon (nipple) with a single-edge razor blade, dissecting scissors, or scalpel blade (#5 or 7). Take the razor blade edge and slice away from the developing prepupae, starting where the nipple is sliced off, to open the side of the cocoon. Pull the cocoon open after cutting, not harming the prepupa inside. Use a new blade for each sample or flame sterilize between samples. Use forceps to retrieve the bee and sterilize between samples.

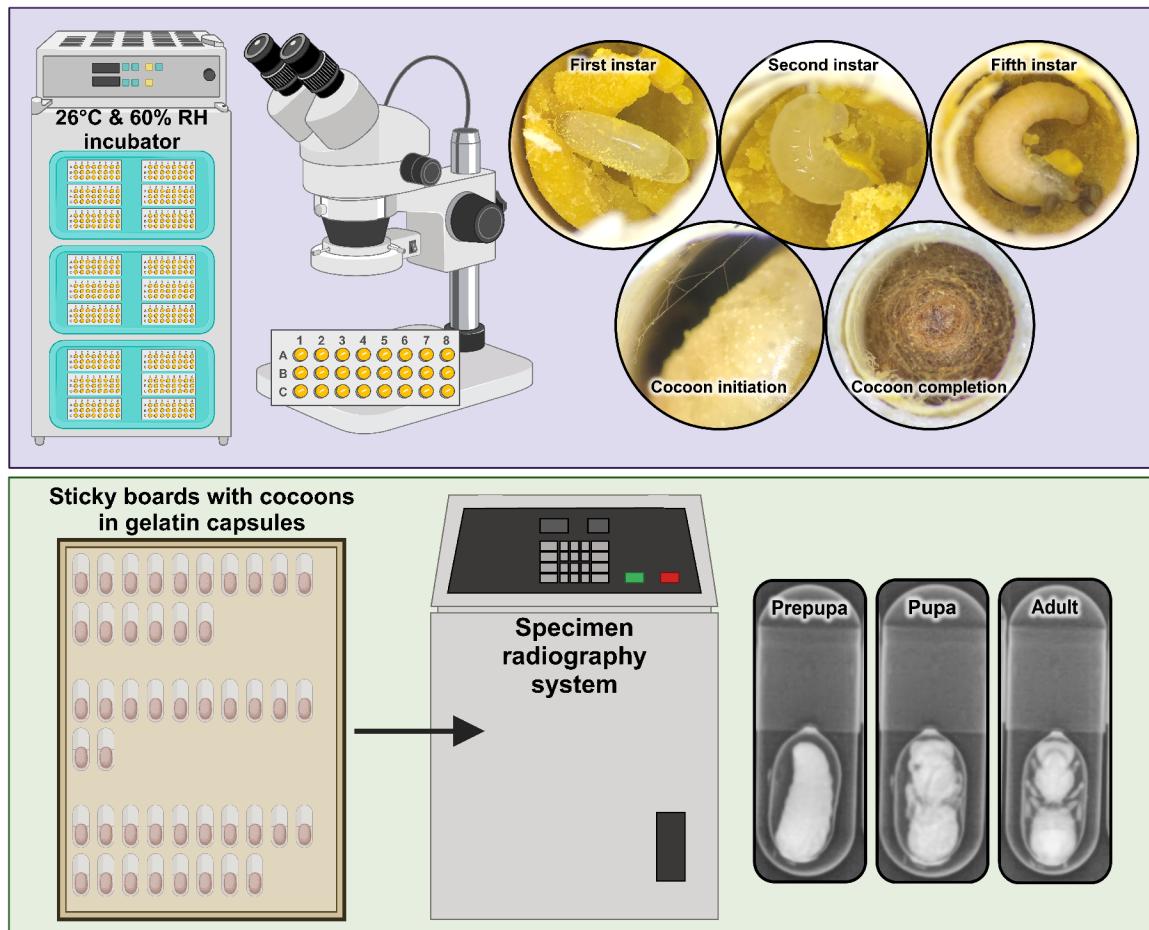


Figure 15. Observing development and monitoring survival using a dissection microscope and specimen radiography system. Photos by M-K. F. Williams. Rendered images created with BioRender.com.

Winter diapause (Fig. 16)

- 28 On September 21 or 22 of the study year, perform a final X-ray then, if able, identify bees that are still at the pupal stage (have not molted into an adult).

Note

If bee has not made it to the adult stage at this time, it will not survive. Freeze samples in a 1.7 mL microcentrifuge tube at -80 °C. If the researcher is unsure if the bee is a pupa or adult, we recommend leaving the sample alone until the end of emergence. If the researcher feels comfortable, dead adults can be pulled at this time. For examples of a dead adult's appearance in an X-ray image, consult Pitts-Singer et al. (2014).

CITATION

Pitts-Singer TL, Cane JH, Trostle G (2014). Progeny of Osmia lignaria from distinct regions differ in developmental phenology and survival under a common thermal regime. *Journal of Insect Physiology*.

LINK

<https://doi.org/10.1016/j.jinsphys.2014.05.018>

- 29 After performing the X-ray in Step 28, weigh cocoons without frass to obtain a pre-winter diapause weight in grams (milligrams are also acceptable).

Note

Use weigh paper cut into quarters (4x4 in paper cut to approximately 2x2 in sizes) and change between each sample.

- 30 Once the pre-winter diapause weight is obtained in Step 29, place the cocoon it into a new gelatin capsule and back onto its original spot on the sticky board.

Note

Handle one sample at a time to avoid mixing up sample identifiers.

- 31 **Ramp-down conditions for diapause induction:** On September 23 of the study year, place box containing sticky boards and a data logger into an incubator programmed at 15 °C with 50 % for one week.

Note

A plastic container with RO water can be placed at the bottom of the incubator to aid in humidity.

- 32 **Winter diapause conditions:** On October 1 of the study year, in the early morning, place the box containing sticky boards and data logger into a walk-in cooler (or incubator) at 4 °C with 25 % until the

following spring. *Osmia lignaria* adults will enter diapause under these conditions.

Note

Alternatively, an incubator can produce diapause conditions.

Note

Monitor temperature carefully. Temperature changes will affect metabolism and nutrient utilization.

Note

We were unable to reprogram RH in the walk-in cooler. If using an incubator, winter diapause RH can also be set between  50 % to  70 % but not exceeding  70 % (Orchard Bee Association 2021).

- Orchard Bee Association (2021). Certified Best Practices for Sustainably Sourcing and Managing Orchard Bees: Management During Bee Development. Link: <http://tinyurl.com/OBAManagement>.

33 On February 26 or 27, the following spring, perform an X-ray on the winter diapause sticky boards before obtaining a post-winter diapause weight.

34 After obtaining the post-winter diapause weight, place cocoons into sterile glass scintillation vials without lids (one cocoon per vial). Label vials with the sample identifier and plug with a cotton ball.

Note

We recommend emerging bees in glass vials because adult *O. lignaria* may chew out of the gelatin capsules if not retrieved immediately. Once the cocoon is inside the vial, use enough of a cotton ball to plug the opening so that it is snug but not overly packed.

35 Place vials upright into a container and return to winter diapause conditions until ramp-up conditions.

Note

More than one container may be needed depending on sample size.

- 36 **Ramp-up conditions for emergence induction:** On February 28, place samples in winter diapause and a data logger into an incubator set at  15 °C with  50 % .

Note

A plastic container with RO water can be placed at the bottom of the incubator to aid in humidity.

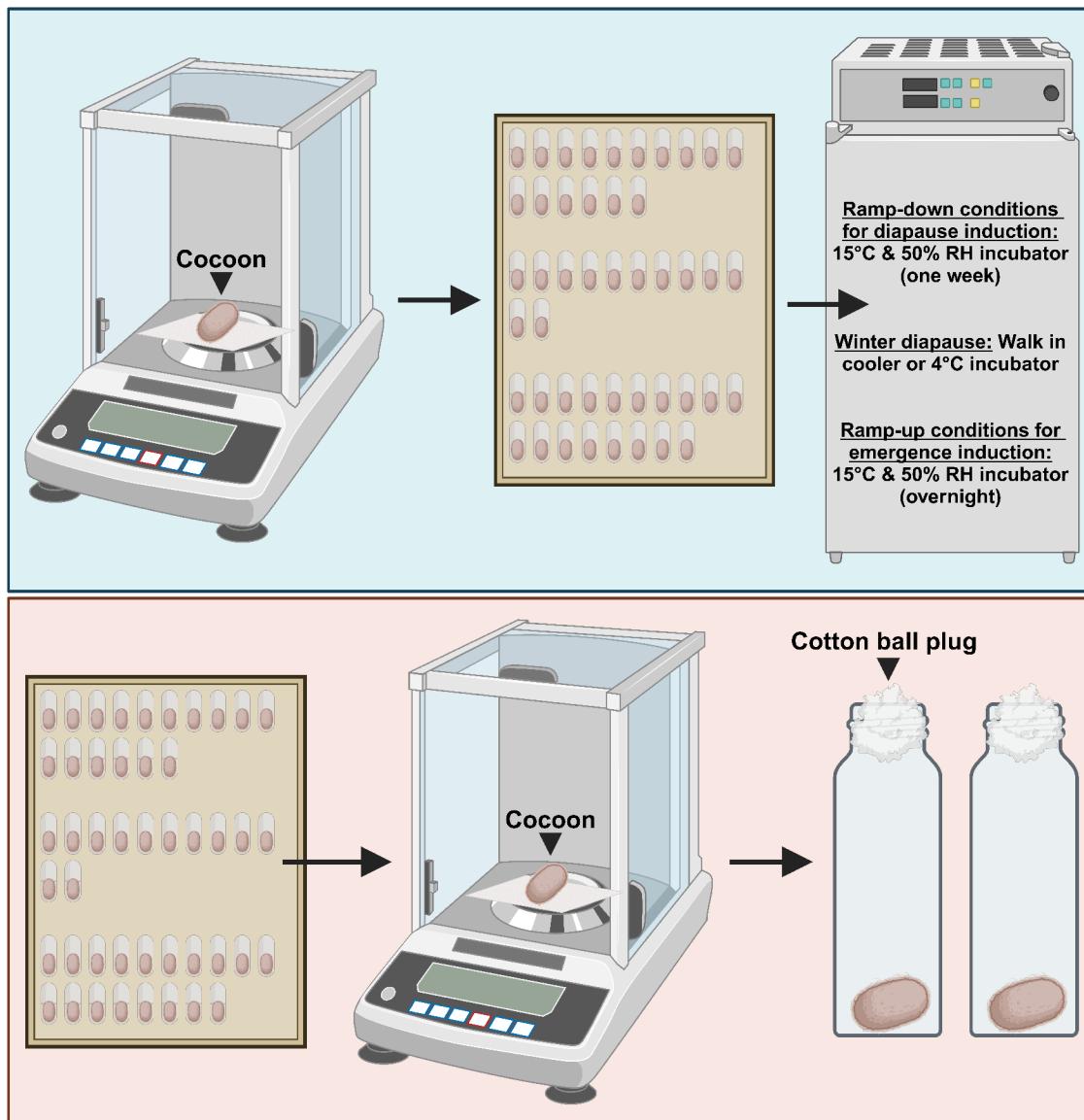


Figure 16. Winter diapause includes ramp-down incubation, 4°C incubation, and ramp-up incubation. Rendered images created with BioRender.com.

Emergence (Fig. 17)

30m

37 On March 1, change the incubator temperature to 26°C with 60% .

38 Check samples once a day between 12 PM and nightfall for two weeks (until March 15).

Note

Keep daily checks to a consistent time each day. Bees are anticipated to begin emergence within one day (starting March 2) and conclude fourteen days after being placed into emergence conditions (March 15). The emergence period can be extended if desired.

39

Once an adult emerges, record the emergence date and sex, then freeze the bee at  -80 °C in a labeled  1.7 mL microcentrifuge tube.

Note

Label side of tube with study name and sample identifier, and top of tube with sample identifier.

Note

Males are apt to emerge first, followed by females.

Note

Clean the surface of the checking area with 70% ethyl alcohol and ExsporTM after each emergence check.

40

On March 15 (nighttime), cut bees that failed to emerge out of their cocoons using a single-edge razorblade, dissecting scissors, or scalpel blade (#5 or 7). Record the final stage of bee development and determine the sex of adults. Freeze bees in  1.7 mL microcentrifuge tubes at  -80 °C .

Note

Label side of tubes with study name and sample identifier, and label top of tube with sample identifier.

Note

If the researcher desires, the emergence period can be extended for one to two more weeks.

41

Wash glass vials in 70% ethyl alcohol and water, and once dry autoclave vials (autoclave at

121 °C for 15m

(00:15:00) for future reuse.

Note

Scrub vials to clean before sterilization. This is needed if cocoons and excreted meconium are left behind in the vials

Note

Alternatively, vials can be rinsed, scrubbed, and cleaned with a 5% bleach-water solution.

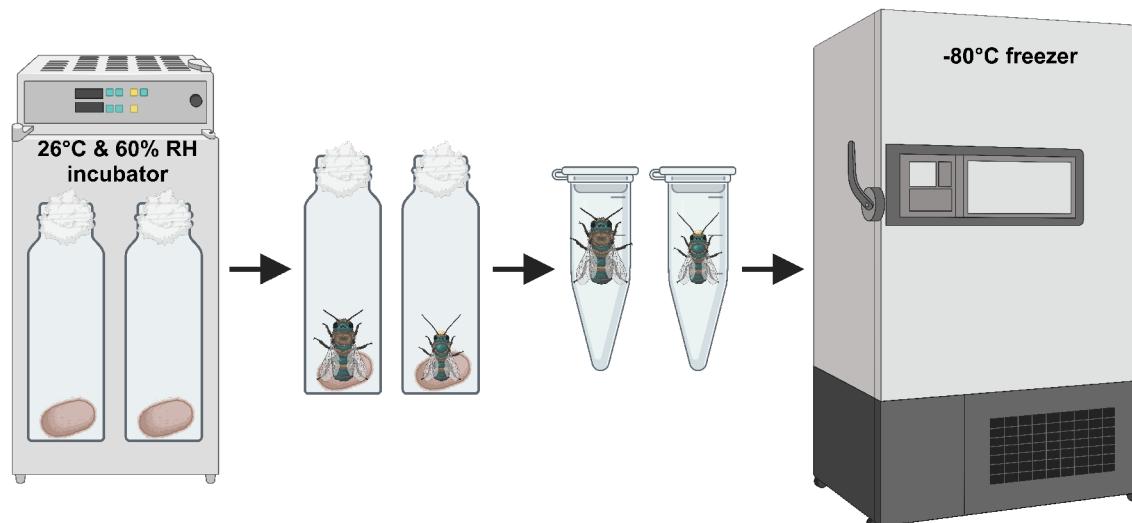


Figure 17. Emergence of adults after winter diapause. Rendered image created with BioRender.com.