**Processing *Osmia lignaria* provisions**

Lab Safety

*Required PPE*

* Gloves
* Lab coat

*Materials of concern*

* Ethanol - flammable; causes serious eye irritation
* Razor blade - sharp
* Ethylene oxide - flammable; toxic if inhaled; causes eye and skin irritation; may cause genetic defects, cancer, respiratory irritation, and frostbite

Post-collection processing

*NOTE: Work in batches to ensure that the eggs and provisions do not dehydrate.*

*Materials*

* Fine-tipped sharpie
* DNeasy spray
* 70% ethanol
* 5% bleach
* Filled nesting tubes
* Razor blade
* Lab spatula
* Forceps
* Ringer’s solution
* Reagent reservoir
* 2% agarose gels
* 50 mL Falcon tubes
* Sterile beaker
* Microcentrifuge tube
* Scale (measures to milligrams)
* Datasheet

*Methods*

1. Sanitize the biosafety fume hood with DNeasy spray and 70% ethanol.
2. Sterilize a razor blade, forceps and a lab spatula by submerging them into 5% bleach and then 70% ethanol.
3. Pour sterile Ringer’s solution into a brand new reagent reservoir.
4. Use the sterile razor blade to cut the nesting tube longitudinally. Sterilize the razor blade between nesting tubes.  
   *NOTE:* Do not dig the razor blade too deep into the nest to avoid injuring the bees.
5. Dip the sterile lab spatula into the sterile Ringer’s solution.
6. Using the sterile lab spatula, gently pick up and then deposit the eggs from the first (male; nearest the entrance of the nest) and last two (female; furthest from the entrance of the nest) brood cells onto the 2% agarose gel. Sterilize the lab spatula between each egg.  
   *NOTE:* Try not to grab any provision material as you pick up each egg.

*NOTE:* Do not let the eggs stay inside the covered Petri dish longer than 12-24 hrs, as the humidity could encourage fungal growth.

1. Label each 2% agarose gel with the date and time the eggs were grafted.
2. Place Petri dishes containing eggs into the ‘ambient’ incubator running the ‘June’ program.
3. Using sterile forceps, weigh male and female provisions (*N* = 10 per sex) to determine how much provision material to reallocate in the later steps. Sterile the forceps between each provision.
4. Using sterile forceps, transfer all provisions from each nest into its respective sterile Falcon tube. Sterile the forceps between each provision.
5. Homogenize the provision material using the sterile lab spatula. Sterilize the lab spatula between each nest.
6. Tare a microcentrifuge tube on a scale.
7. Subsample 100 mg of provision material from three randomly chosen Falcon tubes from each box. These samples will be used for comparisons between the provision microbiome and nutritional content at the nest-level versus the homogenized provision material from all nests created in the steps below.
8. Homogenize provision material from all nests into a large sterile beaker.
9. Tare a microcentrifuge tube on a scale.
10. Subsample 300 mg (*N* = 3) of provision material from the homogenized mass to sample the microbiome.

Preparing microbiome treatments

*Materials*

* Fine-tipped sharpie
* DNeasy spray
* 70% ethanol
* 5% bleach
* Sterile Petri dishes
* Sterile large beakers
* Sterile Falcon tubes
* Parafilm
* Lab spatula
* Ethylene oxide
* Sterile DI water
* Sterile 1x PBS-0.15% Tween
* Scale (measures to milligrams)
* Mesh bags
* Plastic bag
* R2A plates
* YM plates

*Methods*

1. Sanitize the biosafety fume hood with DNeasy spray and 70% ethanol.
2. Sterilize a lab spatula by submerging it into 5% bleach and then 70% ethanol contained within sterile Falcon tubes.
3. Tare a large beaker on a scale.
4. Weigh all provision material collected in the protocol above.
5. Divide provision material in half: one half will remain unmanipulated, while the microbiome from the other half will be heat-killed via ethylene oxide treatment.
6. Weigh each half of the provision material. Record this data, as it will be used to determine how much sterile DI water is needed to rehydrate half of the provision material after ethylene oxide treatment.
7. Place one half of the provision material into sterile Petri dishes.
8. Label the Petri dishes with the date and “Natural Microbiome.” Place Petri dishes containing eggs into the ‘ambient’ incubator running the ‘June’ program with the lids on until you are ready to graft eggs.
9. Sterilize the lab spatula again as described above.
10. Transfer the other half of the provision material into mesh bags. Place these mesh bags into a plastic bag to prevent contamination.
11. Sterilize the provision material overnight with ethylene oxide gas at the USDA-ARS Pollinating Insect-Biology, Management, Systematics Research unit.
12. Tare a sterile Falcon tube on a scale.
13. Sterilize the lab spatula again as described above.
14. Transfer the sterilized half of the provision material to the tared Falcon tube.
15. Weigh the dry weight of this half of the provision material.
16. To replicate the natural moisture content of the provisions, add enough sterile DI water to make up the difference between the fresh and dry weight of the sterilized provision material (1 µL water = 1 mg).
17. In a labeled sterile microcentrifuge tube, pipette sterile DI water as a control for sequencing.
18. Homogenize with a sterile lab spatula to distribute the sterile DI water.
19. Plate 75 mg of the sterilized, rehydrated provision material suspended in 350 µL 1x PBS - 0.15% Tween solution onto R2A and YM plates (*N* = 3 each) to ensure that any remaining microbes were effectively killed. In addition, plate the sterile DI water, 1x PBS-0.15% Tween and beads as controls.
20. Once the sterilized provision material is thoroughly mixed, place this half into sterile Petri dishes.
21. Subsample 300 mg (*N* = 3) of treated provision material for future microbiome and nutritional analyses.
22. Label the Petri dishes with the date and “Ethylene Oxide Treated.” Place Petri dishes into the ‘ambient’ incubator running the ‘June’ program with the lids on until you are ready to graft eggs.

Grafting eggs onto treated provisions

*Materials*

* Fine-tipped sharpie
* DNeasy spray
* 70% ethanol
* 5% bleach
* Lab spatula
* Sterile small beakers
* Nesting straws
* Sterile well plates
* Eggs
* Provision material
* Parafilm
* Scissors
* Scale (measures to milligrams)
* Dissecting microscope
* Datasheet

*Methods*

1. Sanitize the biosafety fume hood with DNeasy spray and 70% ethanol.
2. Sterilize a lab spatula by submerging it into 5% bleach and then 70% ethanol within small, sterile beakers.
3. In the reagent/media hood, cut:
   1. Clean nesting straws (25 mm long, a little taller than the height of the well plates) - as many bees that you will be rearing
   2. Parafilm strips - as many bees that you will be rearing
   3. Parafilm rectangles (130 mm x 16 mm) - as well plates you are using
4. UV sterilize the cut nesting straws and parafilm for 15 min.
5. Wrap a strip of parafilm around the bottom of each straw to help it adhere to the side of the well plate. This will prevent the straws from falling over and knocking the bees off of the provision, which could kill them.
6. Tare a cut straw with wrapped parafilm on the scale.
7. Using the cut straws as a cookie-cutter, weigh the appropriate amount of provision material ± 2 mg of the provision material as determined by the averages you found above.
8. Repeat steps 5-7 for every sample.
9. Place weighed provisions into their respective place in the well plates. Arrange samples in a checkered pattern to ensure cross contamination does not occur.
10. Graft randomized eggs back onto provision materials using the methods described above, while tracking their position and assigned provision treatment.
11. Once eggs are grafted onto treated provisions, use a dissecting microscope to check for damaged eggs. If any eggs were damaged (deflated appearance), immediately regraft a new egg.
12. Mark the top left corner of the parafilm rectangles with its unique plate number and assigned treatment to ensure it is consistently placed back on each respective well plate in the correct orientation.
13. Drape parafilm rectangles on top of the well plates to prevent desiccation of the developing bees and provisions.
14. Place the well plates into their respective incubators. Note the date and time you place them into the temperature treatments.
15. To have a baseline measure of provision microbiome composition in response to temperature, subject provisions without bees to treatments.  
    *NOTE: In 2023, 351 mg ± 2 mg of provision material (N = 8 per treatment) was subjected to thermal regimes on June 7th.*
16. Continue onto the following protocol: “Measuring bee life-history traits & health.”