**Sampling Event 2 - Final Provision & Larvae**

Lab Safety

*Required PPE*

* Gloves
* Glasses
* Lab coat

*Materials of concern*

* Ethanol – flammable, toxic, health hazard, irritant
* Bleach – corrosive and irritant to eyes and skin
* Razor blade - sharp

Post-collection processing

*Materials*

* DNase spray
* 70% ethanol
* Sharpie
* Beakers (2)
* Razor blade
* Forceps
* Vortexer
* Sterile microcentrifuge tubes
* Sterile well-plate
* Nesting tubes
* Scale (must measure in milligrams)

*Methods*

1. Sanitize the biosafety fume hood with DNeasy spray and 70% ethanol.
2. Sterilize a razor blade and forceps by submerging them into 70% ethanol and then 5% bleach.
3. Using the sterile razor blade, cut the nesting tube longitudinally.
4. Label two sets of microcentrifuge tubes for each of your samples (one for provisions and another for larvae). Record which nesting tube and brood cell the bees came from on the label.
5. Weigh the microcentrifuge tubes in milligrams.
6. With the sterile forceps, gently clasp the larvae and place into a correspondingly labeled sterile well plate.
7. Pour enough 95% ethanol into each well plate to submerge the larva. This will humanely euthanize the larvae so that the body size and microbiomes of larvae can be determined in the protocols below.
8. Sterilize the forceps again before handling the provision.
9. Pick up the provisions with forceps and place into a correspondingly labeled sterile microcentrifuge tube.
10. Repeat steps 3-9 for every nesting tube, sterilizing the razor blade and forceps between every sampling effort.
11. Weigh each microcentrifuge tube containing the sampled provisions and larvae.
12. Subtract the sample weight from step 11 from step 5 to determine the weight of each provision.

Culturing the provision microbiome

*Materials*

* DNase spray
* 70% ethanol
* P1000 micropipette
* P100 micropipette
* Sterile 1000 µL micropipette tips
* Sterile 100 µL micropipette tips
* Sterile 1x PBS-0.15% Tween 20
* Sterile glass beads
* Beaker
* Vortexer
* YM agar plates (100 mg/mL chloramphenicol)
* R2A agar plates (100 mg/mL cycloheximide)
* Parafilm
* Incubator (27°C)

*Methods*

1. Sanitize the biosafety fume hood with DNeasy spray and 70% ethanol.
2. Pipette 1000 µL of 1x PBS-0.15% Tween 20 into each provision sample.

*NOTE: For every new bottle of PBS-Tween solution, pipette 1000 µL into a sterile microcentrifuge tube as a control for sequencing. This will help you identify potential contaminants.*

1. Label a YM and R2A media plate for each provision sample.
2. Vortex the suspension immediately before plating.
3. Pipette 100 µL of the sample onto the correspondingly labeled R2A and YM plates for each provision sample. Plate the samples within 48 hrs.
4. Using ~5 sterile glass beads, spread the sample over the plate.
5. Empty used glass beads into a beaker.
6. Once all samples have been plated, seal the plates with parafilm and place into an incubator set at 27°C.
7. Store the samples into the 4°C fridge until you are satisfied with your plated samples (i.e., no replating is needed).
8. After 5 days, count the total number of colonies on each plate.
9. Record the data in your lab notebook and Excel spreadsheet.

Determining larval body size

*Materials*

* Larvae
* Forceps
* Weigh boat
* Digital calipers
* Scale (must measure in milligrams)
* Light microscope

*Methods*

1. Place a weight boat onto a scale. Tare the scale.
2. Using forceps, carefully pick up a larva and place it into a weight boat.
3. Weigh each larva to the nearest milligram.
4. Under a light microscope, use digital calipers to measure both the length and width of the larvae.
5. Record the data in your lab notebook and Excel spreadsheet.

Isolating and culturing the larval microbiome

*Materials*

* DNase spray
* 70% ethanol
* 75% ethanol
* 5% bleach
* Sharpie
* Larvae
* Beakers (6)
* Physiological saline solution (see ‘Stock Solution’ document)
* Sterile microcentrifuge tubes
* P1000 micropipette
* Sterile 1000 µL micropipette tips
* Forceps
* Laboratory spatula

*Methods*

1. Sanitize the biosafety fume hood with DNeasy spray and 70% ethanol.
2. Sterilize forceps and a laboratory spatula by submerging them into 70% ethanol and then 5% bleach.
3. Label sterile microcentrifuge tubes for each sample
4. Using the sterile forceps, surface sterilize larvae by submerging them in 75% ethanol three times for 5-10 sec per wash.
5. Wash larva a final time in physiological saline for 5-10 sec to remove residual ethanol.

*NOTE: For every new bottle of physiological saline solution, pipette 1000 µL into a sterile microcentrifuge tube as a control for sequencing. This will help you identify potential contaminants.*

1. Place the larva into a correspondingly labeled microcentrifuge tube.
2. Repeat steps 2-5 for each larva, sterilizing the forceps between each sample.
3. Pipette 500 µL physiological saline into each microcentrifuge tube.
4. With a sterile laboratory spatula, pulverize the larva. Sterilize the spatula between each sample. Plate the samples within 48 hrs.
5. Pipette 50 µL of the sample onto the correspondingly labeled R2A and YM plates for each provision sample.
6. Using ~5 sterile glass beads, spread the sample over the plate.
7. Empty used glass beads into a beaker.
8. Once all samples have been plated, seal the plates with parafilm and place into an incubator set at 27°C.
9. Store the samples into the 4°C fridge until you are satisfied with your plated samples (i.e., no replating is needed).
10. After 5 days, count the total number of colonies on each plate.

**Sources**

Vojvodic, S., Rehan, S.M. & Anderson, K.E. (2013). Microbial gut diversity of Africanized and European honey bee larval instars. *PLoS ONE, 8*(8): e72106. <https://doi.org/10.1371/journal.pone.0072106/>

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