Hivebench Electronic Laboratory Notebook

Protocol

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Drone Quality

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Behavior Protocol
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Abstract

A honey bee colony primary vehicle to pass along genes is via drones. Because colonies produce thousands of drones per year, this provides colonies an opportunity to pass along genes. Because of this, colonies not only want to raise and maintain high quality drones. However, drones quality is condition dependent

Honey bee drones live in eusocial colonies. Immediately after emergence, workers feed and care for drones until they begin mating flights. Because of this care, workers may influence drone mating strategies.

Objectives

1. Do drones have an "honest" sexual quality signal?
    1. Hyphothesis: Workers detect sperm concentration
    2. Alternative hypothesis: Workers detect a non-sperm signal that is associated with sperm production.
        1. If so: Hypothesis: this signal is either a morphometric cue or hydrocarbon cue
        2. Alternative: The signal is an unknown cue
2. Do worker change drone interaction based on drone quality?
    1. Hypothesis: Workers give low sperm producing males more beneficial care, i.e. trophallaxis, grooming, vibrations
    2. Alternative Hypothesis: Workers give low sperm producing males more aggressive care, i.e. aggression
3. Do worker behavior change flight behavior?
    1. Hypothesis: Worker interaction is associated with age of first flight and mean flight duration
    2. Alterantive Hypothesis: Worker interaction is not associated with age of first flight and mean flight duration
4. Do drones invest more resources into sperm rather than seminal fluids when stressed?
    1. Hypothesis: Amitraz and heat stressed drones will produce less seminal fluid proteins than the control, but simlar sperm concentration numbers
    2. Alternative Hypothesis: Amitraz and heat stressed drones will produce similar seminal fluid proteins and sperm as the control'
5. Do drones change flight behavior based on sperm quality?
    1. Hypothesis: Drones will begin flights earlier and undergo shorter mean flight durations when producing lower sperm concentrations
    2. Alternatives Hypothesis: Drones will not begin flights earlier and will not undergo shorter mean flight durations when producing lower sperm concentrations

Procedure

Protocol #1: Observation Hive - Drone behaviors

HIve Setup

Modern experimental observation hives usually consist of either two or four standard Langstroth "deep super" frames.

1. Build a rectangular hive framing of parallel wooden bars with notches to hold the Langstroth comb-frame lips.
2. Space the comb frames ~0.95 cm apart; this leaves enough space to allow bees to pass between the edges of the frames, but not so much that the bees are encouraged to build additional comb between frames.
3. Leave the same space between the sides of the comb frames and the hive side bars
4. Include a wooden hive top bar to connect the hive sidebars together.
5. Include wire-mesh covered ventilation holes in the top and/or side bars of the hive to allow free flow of fresh air in the nest.
6. When bees cover these holes with propolis from time to time, clean it away.
7. Attach removable glass or Plexiglas® front and back walls to the parallel wooden side-bars to allow for viewing of the frame surfaces; again, leave ~0.95 cm space between the comb surface and the viewing glass to allow bees to move freely on the comb surface, but minimize additional comb construction, which can obstruct comb viewing.
8. Complete the hive with a tube, port, or runway connecting the lower part of the hive to the outside in order to allow bees to forage naturally.
9. Install a "background" colony in the observation hive approximately one week before beginning an experiment to give the bees time to adjust to their new environment.
10. Include one comb frame containing brood (50%-75% capped) and pollen, and one empty frame (drawn or foundation) for honey storage.
11. Add a queen and 1,330-1,530 workers from a queen-right colony to the hive. A total of 200 drones will be places per colony
12. Regardless of size, a hive should be installed in a highly regulated environment
    1. Maintain the observation room at 21-30°C.
    2. Because natural hives are dark inside, the room should be kept dark or the hives should be kept covered when no observation is being performed
    3. Red light can simulate a dark environment, as honey bees do not perceive red light (Backhaus, 1993)
13. Drones must be caged within the observation colony for at least 12-24 to maximize acceptance. If not, the drones will be evicted or wander out.

Marking Bees

To tag newly emerged bees (< 24-hour-old adults) using plastic tags:

1. Remove all adult bees from combs of mature pupae
2. Place the combs in individual sealed cages in an incubator (34.5ºC, 60-70% relative humidity) overnight
3. After 12-24 hours, shake all adult bees found on the combs into plastic bins with a thin layer of petroleum jelly applied around the upper edge of the bin (at this age, most workers are unable to fly or sting; petroleum jelly will keep them in the bin).
4. Gently pick up individuals using soft forceps or fingers, and glue a numbered/RFID tag to the centre of the thorax using fine forceps or a small-moistened stick. The numbered tags will differ in color based on treatment.
5. Wood-glue (Elmer's Glue) and shellac (Honig Müngersdorff) are appropriate glues for bees. However, UV-resin glue is best
6. Do not impede neck or wing movement with the tag.
7. Do not damage the bee while handling; the abdomen is particularly delicate at this age.
8. Orient tags in the same direction.

Behavior Assay

List of Behaviors

Aggression	a drone was considered to be receiving aggression if workers were biting or rapidly and frantically chewing on the drone's thorax and abdomen, which typically caused the drone to adopt a ''flinching'' posture or run away.
Eviction	A drone was considered to be receiving an eviction attempt if it was drug by its legs or wings across the combs or along the bottom of the observation hive toward the queen excluder
Trophallaxis	nestmate exchange of food (not near entrance), receiver thrusts tongue at donators mouthpart, donator opens mouthparts pushes tongue forward, and regurgitates a drop which is lapped up
Grooming	Grooming was distinguished from aggression in that the interaction consisted of gentle nibbling on a drone's thorax and abdomen and the drone did not show the flinching posture associated with aggression
Vibration	fast rhythmic body vibrations (non-dance)

Individual Drones

1. Monitor worker–drone interactions for a week from 9 a.m. to 4 p.m. throughout the study period. During every day of observation, each colony was monitored by a minimum of one observers simultaneously, and the observers rotated between colonies hourly to minimize observer bias.
2. Monitor for 3 min each a randomly selected drones that had been accepted into the colonies. The user will randomly selected drones alternating between control and treatment drones.
    1. The user will also choose drones in a specific grid based on a random number generator. The numbers will be generated before the experiment to reduce bias
    2. The color markers, whcih indicated treatment/control, will be hidden by the user to reduce bias
3. During the 3-min observation periods, record each time a drone received grooming, trophallaxis, aggression and vibration signals from workers.

Protocol #2: Phenotyping

Sperm Dissections from newly emerged drones

1. Before dissection, and to prevent sperm from migrating into the penis bulb, the drones were not killed. Instead, they were dissected alive by pinning them on a bee wax plate (Taber 1999).
2. From each drone, the right testis, mucus gland, seminal vesicle, ejaculatory duct and penis (endophallus) were taken out with a fine forceps and put in 0.9% saline solution.
3. The lengths of the right testis and mucus gland were measured by using a dissecting binocular microscope supplied with a micrometer lens.
4. The semen from the seminal vesicle, testis, ejaculatory duct and penis was separately discharged in a small Petri dish with a fine insect needle and fine forceps in a 1-mL saline solution (0.9%) (Mackensen & Tucker 1970).
5. Tap water was added to make three dilution ratios. The dilution ratios used for counting the spermatozoa in the seminal vesicle were 1:2, 1:5 and 1:10 in the first two, third, and last two ages, respectively. On the contrary, the dilution ratios for counting spermatozoa in the testes were 1:10, 1:5 and 1:2 for ages 1-6, 7-9 and >10, respectively. The dilution ratio used for counting the contents of the ejaculatory duct and penis was 1:2 in all drone ages.

I could try this from Jaycox 1961

Drones to be dissected were chilled to immobility to prevent their ejaculation when being dissected. The seminal vesicles and vasa deferentia were placed in 1 ml. of a 2-percent starch solution and macerated with a fine pair of needles. This mixture was thoroughly agitated with an eyedropper before being further diluted

with either 4 or 9 ml. of a spermatozoal counting fluid (Levinson and MacFate 1956).The final mixture was agitated and used to fill the counting chambers of a hemocytometer. The starch solution dispersed the spermatozoa well and caused them to become coiled temporarily. The second fluid acted as a fixa- tive which made the counting easier by preventing the spermatozoa from uncoiling.
Citation: Mazeed, A. M., & Mohanny, K. M. (2010). Some reproductive characteristics of honeybee drones in relation to their ages. *Entomological Research, 40*(5), 245-250.

Sperm Concentration

Standard Curve

1. Semen obtained from four drones originated for control colonies was used. All semen samples were cream-colored and found at the tip of the genitalia on a bed of white mucus (Rousseau et al. 2015)
2. Ejaculates were first diluted 100 times, then 10 µl was sampled and further diluted 17, 20, 25, 30, 35, 40, 50, and 80 times (final dilutions were 1700, 2000, 2500, 3000, 3500, 4000, 5000, and 8000, respectively).
3. From these suspensions, 90 µl was transferred to UVette disposable cuvettes 50–2000 µl (Cat. No.: 952010069, Eppendorf AG, Hamburg Germany), and the absorbance at 600 nm was measured using a BioPhotometer (Eppendorf, Hamburg, Germany).
4. Sperm suspensions diluted 4000 times were counted using a hemocytometer.

Analysis
For routine sperm concentration analysis, the samples were diluted 4000 times, the absorbance at 600 nm was measured and the sperm concentration expressed as 109 spermatozoa per microliter was calculated from the regression equation.
Citation: 8p/Ciereszko, A., Wilde, J., Dietrich, G. J., Siuda, M., Bąk, B., Judycka, S., & Karol, H. (2017). Sperm parameters of honeybee drones exposed to imidacloprid. *Apidologie, 48*(2), 211-222.

Protein Analysis

1. For each drone, the paired accessory glands were dissected, the mucus was extruded from the glands by piercing with forceps, and stored individually at –20 °C,
2. The mucus of each pair of glands was dissolved in 25–400 µL distilled water, depending on protein concentration of the sample.
3. Aliquots of these extracts were used for total protein quantification by the method of Bradford (1976) using bovine serum albumin as standard.

Protocol #3:

Production of dsRNA
Since dsRNAs can cause off-target effects, you need to be careful in designing them. Nevertheless, RNAi efforts using dsRNA constructs have proven effective in honey bees. To avoid targets that might interfere with other honey bee genes, you need to compare your sequence with the honey bee genome during the design process using the Basic Local Alignment Tool (www.ncbi.nlm.nih.gov). Make sure none of the designed dsRNAs has 20-bp segments identical to any known bee sequence. As dsRNAs are processed by the dicer complex into a cocktail of siRNAs 19–21 nt in length, the absence of 20-nt stretches of homology minimizes the possibility of off-target effects.

1. Use the E-RNAi web application (Horn and Boutros, 2010) for optimal dsRNA design. Design of dsRNA sequences has to be stringent in order to avoid/ minimize off-target effects.
2. Set up appropriate negative controls. Other possible negative controls: e.g., Q-marker (Beye et al., 2002).
3. Amplify the chosen target fragment by using target specific T7 (TAA TAC GAC TCA CTA TAG GGC GAT) added primer in optimized PCRs using approximately 100-ng genomic DNA obtained by chloroform– phenol extraction (e.g. Maniatis et al., 1982).
4. Clone the amplified fragments into pGem-T easy vectors (Promega) according to the manufacturer's instructions. (Cloning eliminates the possibility of a dsRNA mixture due to a polymorphism of the PCR product).
5. Transform your plasmids into JM109 competent cells (Promega) following the instructions from the manufacturer.
6. Prepare the plasmids according to Del Sal (1988).
7. Analyse the identity of the cloned sequence by Sanger sequencing.
8. Once the right clone has been identified its insert needs to be amplified to serve as a template for dsRNA production by standard PCR using again T7 tailed primers.
    1. PCR protocol:
        1. 5 min DNA denaturation, and Taq activation, at 95°C
            1. 40 cycles of:
            2. 95°C for 30 sec,
            3. x°C (primer specific annealing temperature) 30 sec
            4. 72°C for 1 min.
        2. A final extension of 20 min at 72°C completes the protocol.
9. Purify the PCR-products with the QIAquick® PCR Purification Kit (Qiagen).
10. Use the T7 RibomaxTM Express RNAi System (Promega) for dsRNA production
11. Note: Time course experiments and experiments for optimizing the incubation temperature have to be conducted beforehand (e.g. Jarosch et al., 2011 used an extended transcription time of 5 h at 32°C).
12. Purify the dsRNA by a Trizol® (Invitrogen) - chloroformtreatment following the manufacturers´ instructions.
13. Resolve the pellet in nuclease free water.
14. Assess the dsRNA quality and quantity photometrically and by agarose gels or capillary gel electrophoresis.
    1. The photometric measurement of the OD260/OD280 ratio should be between 1.8 and 2. A lower ratio indicates contamination with proteins. As a contamination with DNA or dsRNA degradation cannot be detected by photometry, visualization of the dsRNA product is necessary. For this 1.5% agarose gels can be used, see section 3.2.1). A single distinct band should be visible.
15. Adjust dsRNA concentrations to 5 µg/µl by diluting with insect ringer (54 mM NaCl; 24 mM KCl; 7 mM CaCl2 x 2H2O) right before the injection.

RNAi Feedings

1. Take a comb with fourth instar larvae out of the colony.
2. Transfer it to the lab.
3. Draw a map of the different treatment groups on the very same comb for future identification of the treated individuals. 4. Apply 1 µl of sugar solution containing the respective amount of dsRNA directly into the cells. Deposit it at the bottom of the worker brood cell that contains a drop of food. Avoid touching the larvae. Successful experiments used dsRNA concentrations between 0.5 µg (Nunes and Simões, 2009) and up to 1.26 µg (Aronstein et al., 2006).
4. In addition to the first dsRNA feeding, feed another µg of your dsRNA after 12 hours. This feeding cycle will be repeated for several days until the fifth instar is finished.
5. Place the comb back to its host colony two hours after treatment and take samples at the life stage you are interested in.

Protocol #4: Heat Stress

1. Cage queen for 24 hours on drone comb
2. Place drone comb in incubator at age 11 for 24 hours at the temperatures 35C (Control) and 40C (Treatment). Drones will also be left in the colony to show 35C is equivilent to the drones raised in the colony
    1. Drones will be collected before and after the heat stress treatment for qPCR to determined *Boule* gene expression
3. After 24 hours, the drone comb will be placed back into colony until emergence

Protocol #5: Amitraz Application

1. Larvae were fed a dimethoate-contaminated diet (45 mg/L) as a positive control, acetone- or methanol-contaminated diet as a solvent control, and no contaminated diet as a negative control.
2. The test concentrations were as follows: amitraz: 1.5 mg/L (10× mean residue level reported in pollen), 11 mg/L (10× maximum residue level reported in pollen), 25 mg/L (LC5) and 46 mg/L ($1/10^{th}$ the LC50)
    1. Chronic toxicity of amitraz, coumaphos and fluvalinate to *Apis mellifera* L. larvae reared *in vitro. Dai et al. 2018*
3. Feed on 4th instar to end of 5th instar

Experimental Design

1. Two Observation hive will be setup 7 days in advance to the experiment to allow the colony to adjust (See Protocol #1).
2. Amitraz application (Protocol #5), heat stress (Protocol #4), and RNAi knockout (Protocol #3) will be applied to during drone development. Each one will be done during seperate experiments.
3. The control and treatment drones will be marked with both RFID tags and number markings and added to the observation hives at 5-10%. The numbered tags will have different colors to easily distinguish between the control and treatment groups.
4. Drone observations will be done for 14 days post-emergence (See Protocol #1).
5. Drones will be collected and phenotyped (Protocol #2) at age 14. Drones will be immediately frozen (dry ice or liquid nitrogen) after sperm extraction in glass vials for future analysis (Protein analysis, morphometrics, hydrocarbons).
    1. 30 newly emerged drones will be phenotyped immediatly post-emergence
    2. All availible drones will be collected at age 14.

References

References
Link scientific articles.