Video Article

Video Tracking Protocol to Screen Deterrent Chemistries for Honey Bees

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Abstract

The European honey bee, *Apis mellifera L.*, is an economically and agriculturally important pollinator that generates billions of dollars annually. Honey bee colony numbers have been declining in the United States and many European countries since 1947. A number of factors play a role in this decline, including the unintentional exposure of honey bees to pesticides. The development of new methods and regulations are warranted to reduce pesticide exposures to these pollinators. One approach is the use of repellent chemistries that deter honey bees from a recently pesticide-treated crop. Here, we describe a protocol to discern the deterrence of honey bees exposed to select repellent chemistries. Honey bee foragers are collected and starved overnight in an incubator 15 h prior to testing. Individual honey bees are placed into Petri dishes that have either a sugar-agarose cube (control treatment) or sugar-agarose-compound cube (repellent treatment) placed into the middle of the dish. The Petri dish serves as the arena that is placed under a camera in a light box to record the honey bee locomotor activities using video tracking software. A total of 8 control and 8 repellent treatments were analyzed for a 10 min period with each treatment was duplicated with new honey bees. Here, we demonstrate that honey bees are deterred from the sugar-agarose cubes with a compound treatment whereas honey bees are attracted to the sugar-agarose cubes without an added compound.

Video Link

The video component of this article can be found at https://www.jove.com/video/55603/

Introduction

The European honey bee, *Apis melliferaL.*, is an economic and agriculturally important insect that provides pollination services that are valued at more than $200 billion globally[1]. In the United States and Europe, honey bee colony numbers have been declining. The United States has lost *ca.* 60% of managed honey bee colonies from 1947-2008 whereas Europe has lost *ca.* 27% from 1961-2007[2,3]. There are a number of factors that might be responsible for the increased number of colony losses, including but not limited to, parasite infestations, pathogen infections, beekeeping practices, and pesticide use[2-4].

Honey bees may be exposed to pesticides via two main pathways. Pesticide exposure outside of the hive can occur when foraging individuals come into contact with crops that have been sprayed with chemicals for protection from pests. Pesticide exposure within the hive can occur when beekeepers utilize chemicals to control in-hive pests and pathogens, such as mites, bacteria, and microsporidia[4]. Pesticide residues have been identified within wax, pollen, and honey bee samples from 24 apiaries in the United States and Canada[5,6]. Effects of pesticide contact to honey bees include acute toxicity as well as sub-lethal effects such as paralysis, disorientation, and behavioral and health changes[1,7]. As modern agriculture requires the use of pesticides to maintain high crop yields, these chemicals will continue to be relied upon in the future[2]. In order to better protect honey bees from pesticide exposures, there is a need for the development of new protocols and regulations[5]. One possible approach for protection is the use of repellents to reduce the exposure of honey bees to pesticides while foraging for food.

Insect repellents (IRs) have typically been used as personal bite protection measures against arthropod disease vectors[8]. The most widely used and successful IR, developed more than 60 years ago, is DEET[8,9]. It is considered to be the gold standard for insect repellent testing and is used by the World Health Organization and Environmental Protection Agency as a positive control for novel repellent screening[10]. Additionally, DEET has been found to disperse honey bees from a threat to their colony[11]. Current attributes associated with personal IRs include: (1) lasting effect against a broad number of arthropods; (2) non-irritating to the user when applied to the skin or clothing; (3) odorless or pleasant odor; (4) no effect on clothing; (5) no oily appearance when applied to skin and to withstand sweating, washing, and wiping by the user; (6) no effect on commonly used plastics; and (7) chemically stable and affordable for widespread use[12]. A repellent used for honey bees would only need a few of these attributes such as lasting effects, non-irritating to applicators, odorless or pleasant odor, chemically stable and affordable for widespread use, and non-toxic to honey bees. However, before exploring these attributes in depth, a method for screening compounds for repellency/deterrence in a high-throughput manner is needed. Here, we describe a protocol for a laboratory assay to screen compounds for the deterrence of honey bees, an important step in determining repellency. The following protocol is modified from a previous study describing a visual tracking method to assess the sublethal effects of pesticides on honey bees[13]. However, this protocol differs in that it is designed to measure the effects

of candidate repellents that might deter honey bees from pesticide-treated crops. There are no recommended protocols for the laboratory testing of chemical deterrents for honey bees and, thus, this protocol provides a simple approach to screen such compounds.

Protocol

1. Prepare Sugar-agarose Cubes

1. Weigh out 8 g of sugar and place into a 50 mL Erlenmeyer flask.
2. Fill the Erlenmeyer flask with 20 mL of de-ionized water. Dissolve the sugar by swirling the flask.
3. Weigh 170 mg of agarose and add it to the sugar solution.
4. Heat the sugar-agarose solution in a microwave on high for 25 s. Dissolve the agarose into the sugar solution.
5. **Allow the flask and sugar-agarose solution to cool.**
   NOTE: The flask should be cool to the touch, but do not allow the solution to solidify.
   1. To prepare a sugar-agarose cube for the control treatment, pour the semi-cooled sugar-agarose solution into a weigh boat mold.
      NOTE: The weigh boat mold has the dimensions 1.5 x 1.5 x 0.3 cm^3.
   2. To prepare a sugar-agarose-compound cube for the repellent treatment, add the desired amount of compound to the semi-cooled solution (*e.g.*, 1% DEET in sugar-agarose solution (v/v)). Swirl the flask to mix in the compound and then pour the solution into a weigh boat mold.
      NOTE: Eight control molds and eight test molds will be prepared at this point. Control molds do not contain repellent.
6. Cool the sugar-agarose cubes in the molds in a refrigerator for 5-10 min. Remove the solidified sugar-agarose cubes from the weigh boat molds and place them into a plastic container with a moistened paper towel.
7. Place the containers into a refrigerator for storage. The sugar-agarose cubes should be used within 7 days of preparation.

2. Programming the Video Tracking Software and Experimental Setup

1. **Under Experiment Settings in the Experiment Explorer bar (in the tracking software, see Materials Table), located on the left of the screen, make sure that the correct camera is selected and that the video recording is centered on the petri dish arenas.**
   1. If the video recording needs to be centered, go into the settings of the camera and under the AOI controls and select the center X and center Y options.
2. Under Experiment Settings, change the number of arenas to 16. Under Tracked Features select Center-point detection.
3. **Select Arena Settings to setup the arena desired for the assay.**
   1. Place the 16 Petri dishes in a 4 x 4 pattern on top of a light box, positioned under the camera (**Figure 1**).
      NOTE: These dishes are used to create the recording arena and are empty.

Figure 1: Petri dish arrangement on the light box. Petri dishes are arranged in a 4 x 4 block on top of the light box. This arrangement provides easy identification of the control and repellent treatments for the visual tracking protocol. Please click here to view a larger version of this figure.

4. In the Arena Settings, use the grab background button, located on the right side tool panel, to take a picture of the 16 Petri dishes. This will be used as the template to setup the arena.
5. Select "Create ellipse" from the tool bar at the top of the Arena Settings screen and create a circle that matches the diameter of one of the petri dishes in the image grabbed. Place the Arena 1 marker into the circle (**Figure 2**).

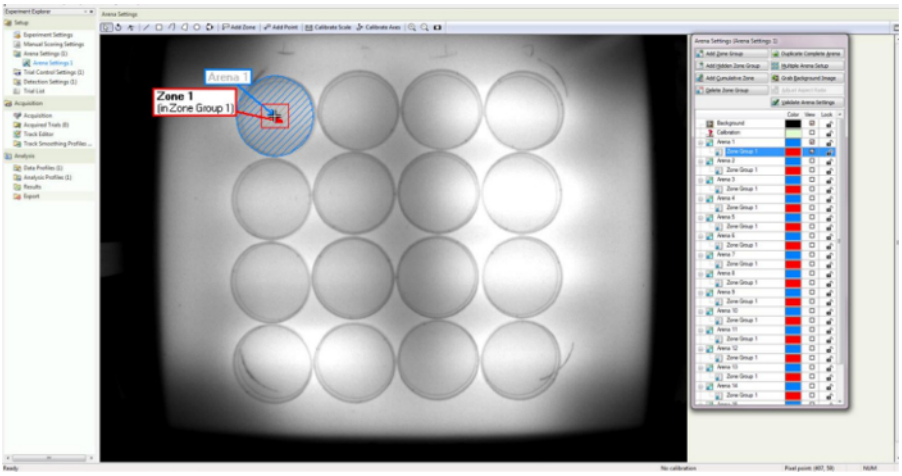

Figure 2: Screenshot of the Visual Tracking Software Arena Settings. The observation of diagonal stripes within the circle provides confirmation of the detection area in the circle. A Zone 1 marker is provided for each square and defines the target zone for each Petri dish. Please click here to view a larger version of this figure.

6. Select "Zone Group 1" located under Arena 1 in the right side tool panel. Select "Create Rectangle" from the tool bar at the top of the screen. Create a 30 x 30 pixel square using this and then position it at the center of the circle that was created in the previous step. Select "Add Zone" from the top tool bar and then click in the middle of the square. Move the Zone 1 marker so that it is in the square.
   NOTE: The square created is the feeding zone and it is not a requirement that the feeding zone be exactly the same size as listed as long as it is slightly larger than the sugar-agarose cube.

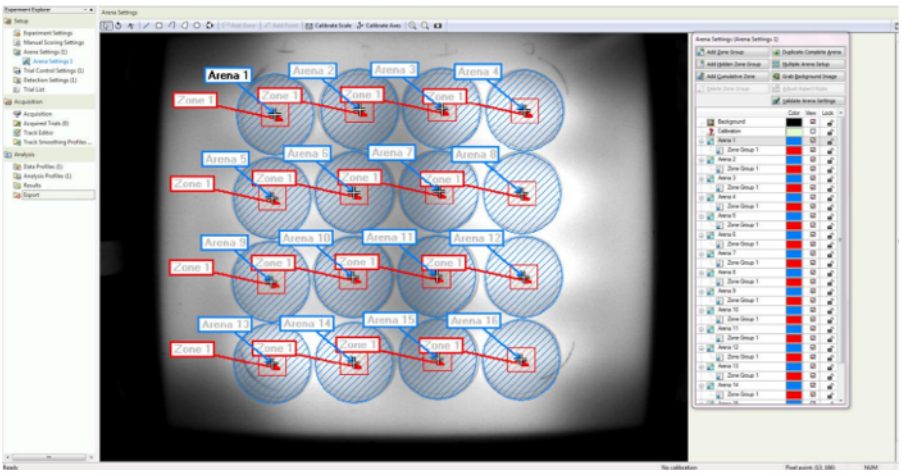

Figure 3: Screenshot of Completed Arena Settings. The completed arena settings should look like this example. Please click here to view a larger version of this figure.

7. **Select "Arena 1" in the right side tool panel and then click duplicate complete arena. From the drop down menu select "All Other Arenas" and then click ok. Move the duplicated arena setups to the remaining petri dishes in the grabbed image (Figure 3).**
   1. Select "Arena 1" and then select "Calibrate Scale" on the top tool bar. Draw the calibration line across the diameter of the Arena 1 Petri dish. Change the diameter measurement to 9 cm (diameter of the Petri dishes being used). Select "Validate Arena Settings" on the right side tool panel.

8. Select "Detection Settings" on the control bar on the left.
9. Select "Detection Settings 1" and then select "Grey Scaling" in the drop down menu located on the right side tool box. Under detection, set the range so that it is 0 to 83 (**Figure 4A**).
10. Right click "Detection Settings" and make a new setting named "Detection Settings 2". Make sure "Grey Scaling" is still selected, but do not change the other parameters.
    NOTE: There will be large yellow circles in the video window over the arenas. This will aid in arranging the Petri dishes into the correct positions before recording (**Figure 4B**).

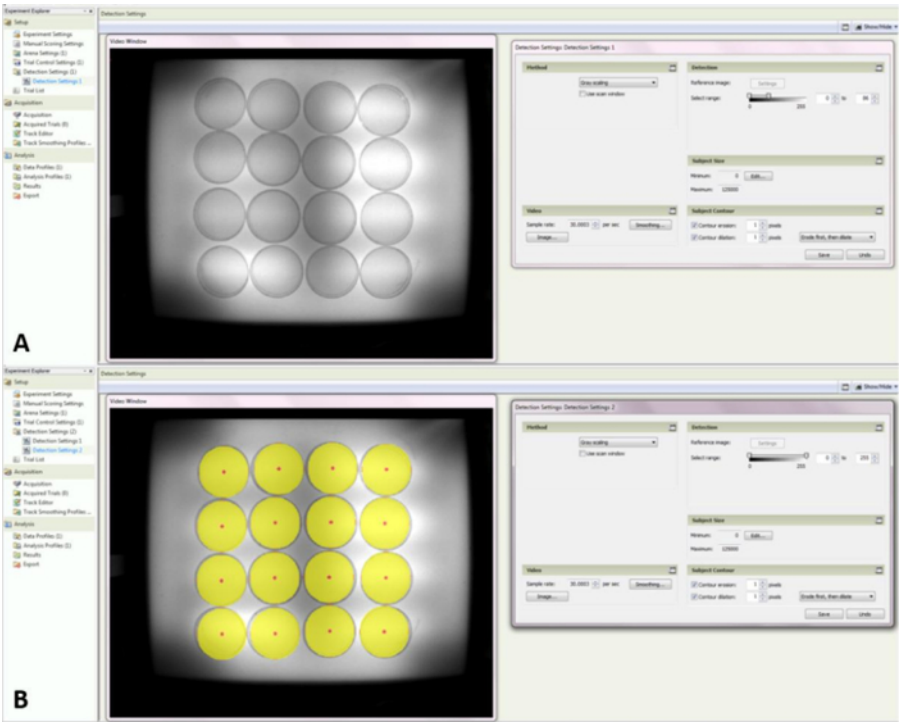

Figure 4: Screenshot of the Detection Settings 1 and 2. (**A**) Shows what the arena will look like with the grey scaling corrected for a honey bee subject in a petri dish. (**B**) Shows the arena detection area so that positioning of the petri dishes can be done between trials. Please click here to view a larger version of this figure.

11. Select "Trial List" in the Experimental Explorer and click "Add Variable" in the top tool bar. Name the user defined variable as "Treatment". Select the "Predefined Values" drop down bar in the user-defined Treatment column and add C (control) and T (treatment) as Predefined Values. Select the "Add Trials" button located on the top tool bar. Add two trials, then for each arena select whether it is a control arena (C) or treatment arena (T) (**Figure 5**).

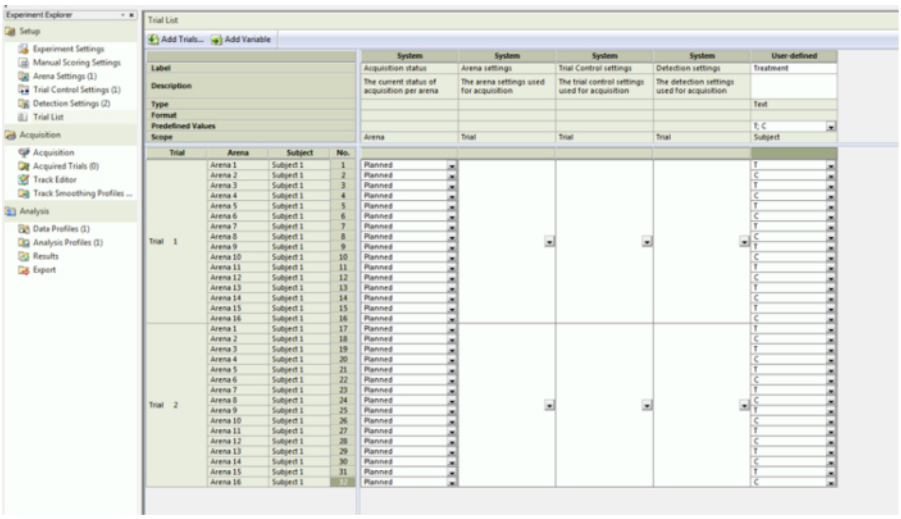

Figure 5: Screenshot of the Trial List. Labeling the arenas correctly is important here as the program uses the information here to separate the data to statistically analyze it. Please click here to view a larger version of this figure.

12. **Select "Data Profile" under the Experiment Explorer panel. On the left hand column select "Treatment" located under the "User-defined Independent Variables" heading. This will add a "Filter" box into the area with the flow chart. Select "C" in the pop up box and then place the newly created filter in between the "Start" box and "Result 1" box. The flowchart arrows should adjust so that they point from the start box to the filter and finally to the result 1.**
    1. Repeat step 2.12, however this time select "T" for the filter and then select "Result" under the "Common Elements" section. Move the newly created boxes into the flowchart area and connect the boxes with arrows (**Figure 6**).

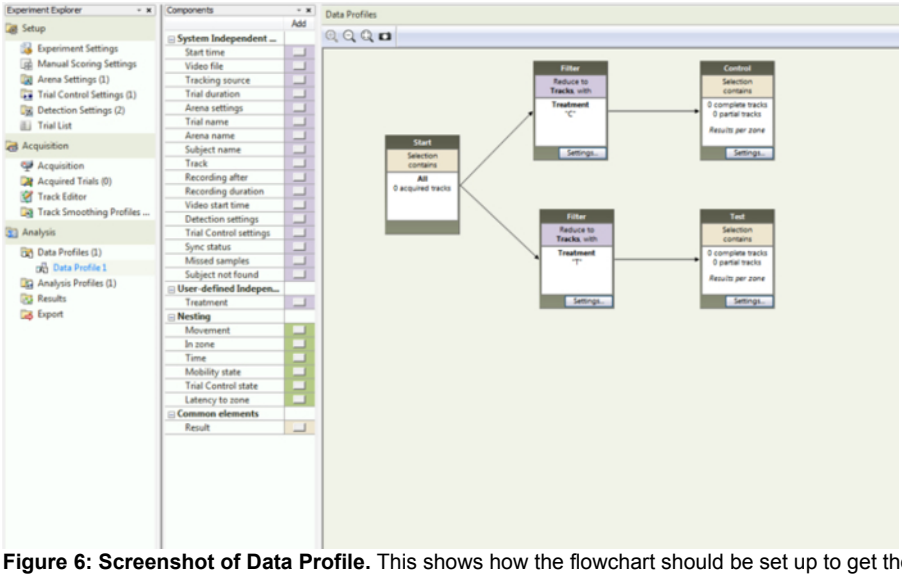

Figure 6: Screenshot of Data Profile. This shows how the flowchart should be set up to get the appropriate separation in the statistical analysis. Please click here to view a larger version of this figure.

13. Save the file.

3. Collect Honey Bee Individuals

1. Put on protective clothing and select a hive to collect honey bees (mostly foragers).
2. Remove the outer and inner cover of the hive. Use a hive tool to select a frame from the top hive body that does not contain brood. Gently lift the frame out of the hive box.
3. Inspect the frame for the honey bee queen. If she is not there, sweep the honey bee workers from the frame into a container for transport. Collect enough individuals to be able to run two complete trials for each compound to be tested (16 individuals are utilized per trial with controls).
   NOTE: The collected individuals should primarily be foragers; however, there may be other aged individuals such as nurse bees included in the collection.
4. Make a note of the time when the honey bees were removed. Transfer them into a 9 cm x 7 cm x 9 cm plastic box containing air holes and place them into an incubator at 32 °C and 70% relative humidity.
5. Starve the honey bees for 15 h.
   NOTE: This usually works best if the honey bees are collected the evening prior to being tested.

4. Conduct Visual Tracking Assay

1. Start up the visual tracking program and open the saved experimental file that was created for this assay.
2. Select "Detection Settings 2".
3. Place control sugar-agarose cubes into the centers of 8 of the 16 Petri dishes. Repeat this process with the sugar-agarose-compound (deterrent) cubes in the remaining 8 dishes.
4. Use forceps to remove a single honey bee from the plastic box and place it into one of the control petri dishes.
5. Repeat step 4.4 for the remaining 15 Petri dishes.
6. Place all of the dishes on to the light box and position them so that the arena detection areas (shown on the computer screen when detection setting 2 is selected) fit into each of the petri dish arenas. Illuminate the light box from below by an array of LED lights set to the red spectrum. Surrounded the entire box and camera with a black plastic sheet to eliminate external light and reduce shadows within the arenas.
7. After the petri dishes have been set, make sure "Detection Settings 1" is the selected setting.
   NOTE: At this point the visual tracking software should be picking up only the honey bees within the petri dish arenas.
8. Select "Acquisition" from the Experiment Explorer. Press the green "Start Trial" button located in the Acquisition Control pop up box on the right to begin recording.
9. Run the assay for 10 min and then click the red "Stop Trial" button on the Acquisition Control pop up box to stop recording.
10. Remove the individuals from the petri dishes and place them into a separate container so that they are not tested twice.
11. Repeat steps 4.4-4.10 for the second trial on the same compound.
    NOTE: This method can be modified to increase the number of honey bees per treatment as needed by the user.
12. Select Statistics in the Experiment Explorer and then click calculate.
13. Export the data to a data manager software and then analyze the data for significance using a using a one-way analysis of variance with a Tukey's post-test, and an unpaired t-test using preferred statistical software program.

Representative Results

A visual tracking protocol was developed to record the amount of time the honey bees spent in a target zone with either sugar-agarose (control treatment) or sugar-agarose-compound cube (deterrent treatment). The recorded time was analyzed using a statistical software program and the mean time spent ± standard error in the target zone is reported as a bar graph. DEET, the gold standard for insect repellent/deterrent testing, was used in this protocol as a positive control. The honey bees provided with a sugar-agarose cube (negative control) spent 343 ± 26 s in the target zone whereas the honey bees provided with a sugar-agarose-DEET (repellent) spent 16 ± 4 s in the target zone (**Figure 7**). DEET significantly reduced the amount of time spent by the honey bees in the target zone by *ca.* 95% compared to that of the control treatment.

Compounds that were of interest to determine deterrence to honey bees were then screened through this protocol. Figure 8A represents a compound that does not deter honey bees from the food source in the target zone. The mean time spent by honey bees in the target zone in control dishes was *ca.* 352 ± 60 s, compared to *ca.* 282 ± 43 s for honey bees within petri dishes that had sugar-agarose cubes infused with compound A. **Figure 8B** represents a compound other than DEET having similar deterrence effects on the individual honey bees. Honey bees within in the control petri dishes remained in the target zone for a mean time of *ca.* 493 ± 31 s, compared to *ca.* 23 ± 3 s for honey bees within petri dishes containing a sugar-agarose cube infused with compound B. These results validate the use of this protocol for screening of chemical deterrents for honey bees. Prior to running this protocol with compounds of interest, it may be necessary to conduct a time-course trial to determine the amount of starvation time for the honey bees.

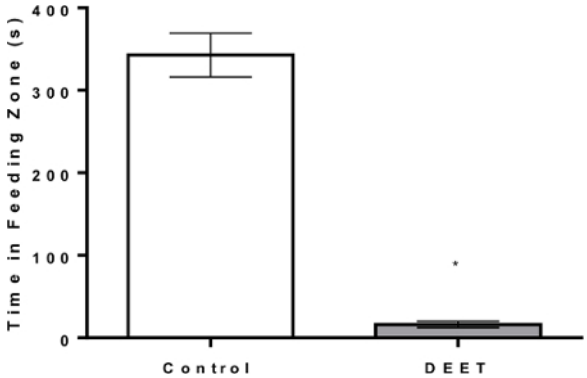

Figure 7: Example Results from the Visual Tracking Software Deterrence Protocol Positive Control DEET. Honey bees are collected from a hive in the evening and removal time is recorded. They are then transferred into a plastic container containing air holes. The box is placed into an incubator set at 32 °C and held overnight for 15 h. The following morning, individuals are placed into petri dishes containing either a control sugar-agarose cube or a DEET-infused sugar-agarose cube. The deterrence protocol described is then run. The results shown in this figure are typical for the repellency gold standard-DEET. From this figure we see that the average amount of time a starved honey bee will spend in the feeding zone with a control cube (*ca.* 343 s) is significantly greater (P <0.0001) than the average time an individual spends in the feeding zone with a cube impregnated with DEET (*ca.* 16 s). Please click here to view a larger version of this figure.

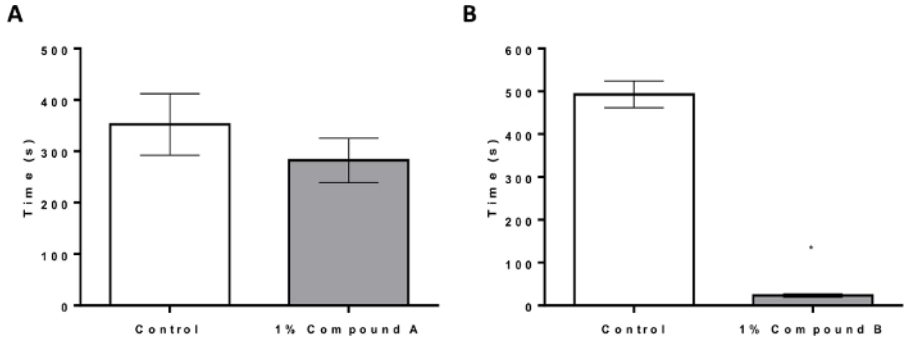

Figure 8: Example Results from Visual Tracking Software Tested Compounds. (**A**) Represents data that shows the mean time spent by individuals in the target zone in control dishes (*ca.* 352 s) is not significantly difference from the mean time spent in the target zone in tested dishes with compound A (*ca.* 282 s). (**B**) Represents data showing significant differences in mean time spent in the target zone between control cubes (*ca.* 493 s) and compound B-infused cubes (*ca.* 23 s). An unpaired t-test was run to determine significance (P <0.0001; DF 15). Please click here to view a larger version of this figure.

Discussion

This visual tracking protocol provides a simple approach to screen chemical deterrents for honey bees in a relatively quick and easy manner. There are no recommended protocols for the laboratory testing of chemical deterrents for honey bees. Previous semi- and full-field studies have examined honey bee repellents[14,15]; however the described protocols are time consuming, labor intensive, and require additional facility resources outside of a general laboratory. This protocol was designed as a pre-requisite evaluation of chemical deterrents prior to semi- or full-field testing of such compounds with honey bees.

There are challenges when screening individual to evaluating chemical deterrents for honey bees outside of the hive. For example, honey bees are social insects that that rely on pheromones within the hive that affect behavior[14]. This protocol requires the use of individuals that no longer receive pheromone cues, in addition to starvation. Starvation is required to standardize the feeding responses of individual honey bees. The starvation time was determined by a 24 h time course study. It should be noted that starvation can have detrimental effects on the individual honey bees. For example, the honey bees become lethargic at 18 h after collection from the hive. Based on these observations, the honey bees were starved for 15 h after collection from the hive.

The critical steps involved with this protocol to avoid unsuccessful screening include: (1) conducting the tests after at least 12 h of starvation; (2) avoid conducting tests after starvation exceeds 18-19 h, as this decreases honey bee vigor; (3) replace the control individuals for each trial; and (4) manage of external light and control shadows within the arenas. Additionally, the Petri dishes should be replaced before screening a new compound screen. Occasionally, a honey bee will defecate within the Petri dish during recording. This usually does not interfere with the recording or data collection. All Petri dishes should be washed thoroughly after each screen to remove sugar-agarose and compound residues as well as honey bee feces.

This protocol is primarily designed to screen compounds for deterrence to honey bees, but can be easily adapted to discern deterrence in other insect species. A major benefit of using the visual tracking software is that it makes a full video recording for the screening of each compound. If there is a need to review and analyze each recording, the investigator can select the file of interest and quickly conduct the screen again with the same or new parameters. The visual tracking software also has the capability to detect individual insects smaller than a honey bee. However, this may require a reduced field of vision for the camera field and fewer arenas to be recorded in a single screen. The strength of this protocol is the ability to screen compounds within minutes for deterrent effects in a laboratory setting. As such, time and money could be saved by reducing a compound library of interest to a select number of candidates for field testing.

Disclosures

The authors have nothing to disclose.

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