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A Novel Acute Toxicity Bioassay and Field Trial to Evaluate Compounds for Small Hive Beetle Control

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Abstract: Beekeepers need new registered products to control the small hive beetle (SHB), *Aethina tumida*, a significant pest of western honey bee (*Apis mellifera*) colonies. Few approved chemical controls exist, and there is no standardized system for screening compounds against SHBs. We outline a detailed method for an acute toxicity bioassay that delivers compounds via pollen. We provide a detailed method for a field trial that delivers treated pollen in traps made from modified compact disc cases. We applied both methods in proof-of-concept experiments to assess acetamiprid as a SHB control agent. Using the laboratory bioassay, we found acetamiprid (LC_{50} = 20.5 µg/g) to be more toxic to SHBs than coumaphos was (LC_{50} = 1250 µg/g), yet less toxic to SHBs than fipronil was (LC_{50} = 1.78 µg/g). In our field trial, colonies treated with acetamiprid and fipronil significantly reduced ($p < 0.001$) live SHB populations over those of control colonies. Traps containing acetamiprid retained significantly higher ($p < 0.001$) numbers of dead SHBs than did traps containing fipronil. We outline the first detailed methods to assess the toxicity of compounds delivered in pollen for adult SHB control. Our proof-of-concept experiments showed acetamiprid to be a promising control agent for SHBs.

Keywords: *Apis mellifera*; *Aethina tumida*; honey bee; fipronil; acetamiprid; coumaphos

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1. Introduction

The small hive beetle (Coleoptera: Nitidulidae; *Aethina tumida* Murray, SHB) can threaten western honey bee (Hymenoptera: Apidae; *Apis mellifera* L.) colony health by destroying brood and resources in hives, sometimes leading to complete colony collapse [1,2]. Beekeepers utilize a variety of control options, often as a part of an informal Integrated Pest Management (IPM) strategy, to manage SHB populations [3]. These control strategies include physically killing SHBs, trapping SHBs inside hives [4], using entomopathogenic nematodes [5], modifying hive structure to make them less accommodating to SHBs [6], and using chemical controls [7]. In IPM, chemical controls are used to reduce pest populations below an economic threshold [8]. Ideally, chemical controls are employed as a last resort and rotated to avoid the development of pest resistance [9].

Only two compounds, coumaphos and permethrin, are labeled for use against SHBs in the United States [10], and neither demonstrate high efficacy. Coumaphos (Check-Mite+™), also used to control the parasitic mite *Varroa destructor*, is an organophosphate delivered in a plastic strip placed between brood frames in a honey bee colony [11,12]. Permethrin (GardStar®) is a pyrethroid delivered in a soil drench to target pupating SHBs in the ground surrounding a hive [10]. Over time, SHBs have developed resistance to coumaphos [13]. This has led beekeepers to use off-label chemical control methods for in-hive treatments, including the use of fipronil-laced baits that are added to various, often

homemade, traps [14,15]. To date, there are no compounds labeled to be mixed with pollen, a SHB food, and delivered in traps placed within hives.

An ideal chemical control will demonstrate high toxicity to SHBs while maintaining low toxicity to honey bees. Extensive laboratory and field testing is necessary to identify such compounds. Laboratory bioassays allow one to screen compounds by measuring toxicity, while field trials permit scientists to examine the effectiveness of potential chemical controls in field-realistic settings. Herein, we outline methods for (1) a SHB acute toxicity bioassay and (2) a follow-up field trial utilizing traps derived from compact disc (CD) cases. In the laboratory, we measured adult SHB mortality at a variety of concentrations for a given compound to calculate a lethal concentration that kills 50% of the adults (LC_{50}). In the field, we measured colony changes in SHB populations as a proxy for the effectiveness of chemical controls. Additionally, we utilized these methods in proof-of-concept experiments to determine the effectiveness of acetamiprid, a neonicotinoid that primarily binds to the nicotinic acetylcholine receptor (nAChR) [16], as a potential control agent for SHBs. We hypothesized acetamiprid would demonstrate higher toxicity to SHBs than would coumaphos. Overall, our goal was to create standardized methods that can be used to screen new compounds for efficacy against SHBs.

2. Standard Methods

We outline standard methods for an acute toxicity bioassay and corresponding field trial below. For all procedures, we recommend following appropriate laboratory safety protocols, such as wearing personal protective equipment (PPE), disposing of hazardous materials safely, and sterilizing equipment and workspaces.

2.1. Acute Toxicity Bioassay

This bioassay measures the toxicity of compounds delivered in pollen to adult SHBs. To accomplish this, compounds are mixed into pollen balls, placed into experimental cages with a known number of adult SHBs, and the mortality of SHBs is measured for a set length of time. A variety of controls, replicates, and multiple concentrations of each compound help generate a reliable Lethal Concentration 50 (LC_{50}) value for each compound of interest. We cannot classify this as an acute oral toxicity bioassay exclusively, neither can we calculate an oral LC_{50} value, given SHBs consume and are topically exposed to the treated pollen during the assay. Additionally, this bioassay does not include individual administration of treated pollen to SHBs to generate a Lethal Dose 50 (LD_{50}) value.

1. Prepare supplies and equipment.
    - 1.1 Pollen
        - 1.1.1 Weigh 1 ± 0.1 g of firm bee-collected pollen or commercial pollen supplement (Table 1A) on a calibrated scale (Table 1B). Roll this pollen into balls with gloved hands.
        - 1.1.2 Wrap individual pollen balls in plastic wrap (Table 1C). These can be stored in a refrigerator (+4 ± 1 °C) up to two weeks before use.
    - 1.2 SHBs
        - 1.2.1 Rear SHBs following the procedures outlined by Neumann et al. [17].
        - 1.2.2 Separate adult SHBs of the same age into a clean (wash with soap and water), ventilated container [17] with water-soaked cotton available to the SHBs ad libitum. The adult SHBs can be kept alive by providing a pollen food source [17]. Remove all food sources 24 h before distributing the SHBs into experimental cages. Note: We used tap water to wash containers and hydrate SHBs.
    - 1.3 Experimental cages
        - 1.3.1 Prepare cages by removing the liner from the lid (Table 1D). Screw the lid onto the 118.29 mL cup (Table 1D). Heat a metal fork over a Bunsen burner inside a fume hood. Create 16 air holes on each side of the cup

by puncturing the plastic with the fork four times (Figure 1A). The hot fork should melt holes into the sides of the cups. Note: Avoid creating air holes near the cage lid. Do not make them large enough for SHBs to escape (i.e., ≤2 mm) [18].

1.4 Incubator and desiccator

1.4.1 Clean all interior surfaces of the incubator (Table 1E) and desiccators (Table 1F) with a 10% solution of bleach/H_2O (v/v) and let dry. Always clean before each round of the bioassay.

1.4.2 Place desiccators inside an incubator set to 33 ± 1 °C. Confirm the temperature using a data logger (Table 1G).

1.4.3 Maintain ~70% humidity inside the desiccators by filling the leak-proof tray in the desiccator with a saturated salt solution.

1.4.3.1 Making the solution: Mix 400 ± 0.1 g NaCl into 1 L of H_2O.

1.4.3.2 Fill the tray midway with the solution and monitor solution levels daily. Add solution as needed and replace completely weekly to avoid mold growth.

1.4.3.3 Regularly monitor the humidity and temperature inside the desiccators by placing a hygrometer (Table 1H) probe inside the desiccators. Run the cord outside the incubator to monitor without needing to open the incubator.

Table 1. Tools and supplies used for the acute toxicity laboratory bioassay and field trial.

Item Letter Corresponds to the First Mention of the Item in the Text	Item and Description
A	Pollen patties with 4% pollen (Global Patties, Butte, MT, USA)
B	Analytical Balance (Mettler Toledo, AL 204)
C	Plastic wrap (Glad Food Wrap)
D	118.29 mL (4 oz) clear cup with white lid (Uline, #S-9934)
E	Binder incubator (Hogentogler, #BD400UL-120V), or equivalent. The incubator must maintain temperature within ±0.5 °C.
F	30.5 cm × 30.5 cm × 45.7 cm desiccators (Thermo Scientific, #08-642-23C)
G	Data loggers (Onset, HOBO #UX100-011)
H	RH/Temperature Monitoring Hygrometer (Traceable, 4154)
I	Amber Glass Threaded Vials, 1 dram (Fisherbrand, 03-339-23B)
J	Stainless steel laboratory scoopula (Fisherbrand, #14357Q)
K	47 mm Petri dish (Fisher Scientific, 09-720-501)
L	Insect collection aspirator, with aluminum intake and outtake valves and plastic tubing (Bartovation, #0649985933202)
M	Cannister vacuum (Dewalt, DCV580H)
N	Maxell CD-365 Slimline Jewel Cases (Office Depot, #981294)
O	Professional Hot knife (Walnut Hollow, WH29639)

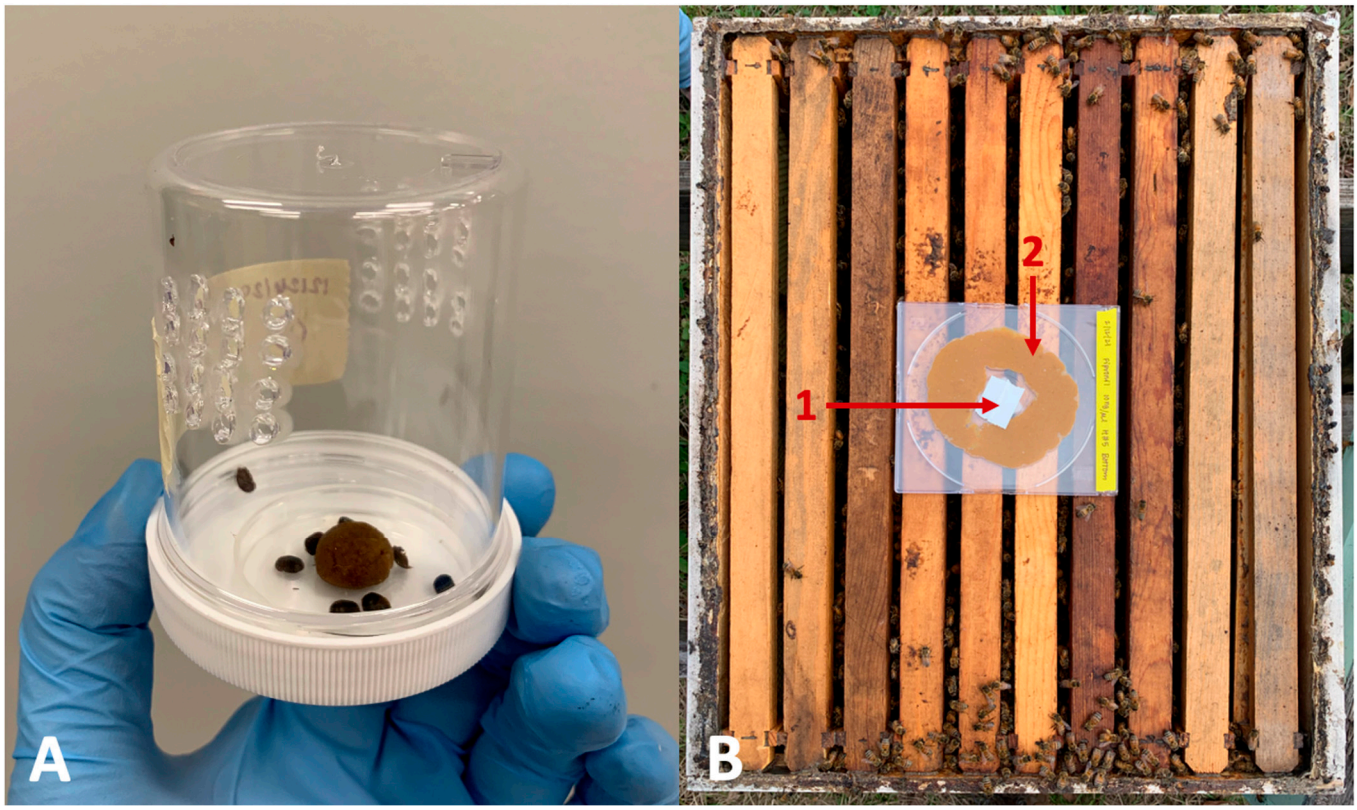

Figure 1. (**A**) Experimental cage design used for acute toxicity bioassays. Ten adult small hive beetles (SHBs) are placed in a ventilated cage with a 1 g ball of treated pollen. (**B**) Trap placed in the center of the hive between the lid and frames for the field trial. Traps were made from CD cases with three entrances to allow adult SHBs to access 25 g of treated pollen in the center (denoted by arrow 2). All other access to the pollen was prevented by covering small holes in the center of the CD case with tape (denoted by arrow 1).

2. Prepare cages with SHBs.
   - 2.1 Randomly place ten unsexed adult SHBs into each experimental cage using gloved hands. Provide water [we used tap water] on cotton balls ad libitum in each cage and place all cages inside the desiccators in the incubator. Note: Prepare at least five cages for each control group (negative, solvent, positive) and concentration of each compound to be tested. It is best to prepare 1–3 extra cages.
   - 2.2 To ensure SHB health and control for mishandling during experimental establishment, wait 24 h and replace all dead SHBs with those housed in the extra cages. Each cage should begin the study with ten living adult SHBs.
   - 2.3 After the 24-h survival period, randomly assign cages to treatment groups. At least five replicates are needed for each of the three controls (negative, solvent, positive) and each concentration of all compounds. At least five concentrations are needed for each compound to graph a dose–response curve accurately. The typical replicate schedule: (3 control groups (negative, solvent, positive) + 5 concentration groups of compound X) × 5 replicate cages/group = 40 cages (or 400 SHBs = 40 cages × 10 SHBs/cage). Every new compound tested adds 25 cages (5 concentration groups × 5 replicate cages/group) to the study.
   - 2.4 Clearly label each cage with the date, compound or control group, concentration, and replicate number.
3. Prepare solutions.
   - 3.1 Clearly label amber vials (Table 1I) with the date, compound, and concentration of compound that will be added.

3.2 Prepare 50 µL of test solution for each experimental cage. It is best to prepare at least 10% excess to account for evaporation.

3.2.1 Weigh the total amount of compound needed into the vial of the highest concentration using a sterilized metal scoopula (Table 1J) and calibrated scale. Note: Rinse the metal scoopula with 200 proof ethanol and deionized water three times to sterilize it.

3.2.2 Pipette the necessary amount of solvent (i.e., acetone) into a vial to create the stock solution. Perform serial dilutions to prepare all other lower concentrations. Note: Be sure to use a vortex mixer and centrifuge all solutions, especially the stock solution, before performing the serial dilutions. Wear proper PPE and work in a fume hood.

4. Manually mix compounds into pollen balls.

4.1 Begin by preparing the pollen used for the negative control group. Place each unadulterated pollen ball into half of a 47 mm Petri dish (Table 1K).

4.2 Flatten all pollen balls in the palm of a gloved hand for all other treatments.

4.3 Pipette 50 µL of test solution into the center of the flattened pollen ball. Manually massage the pollen by folding for 30 s, then place the ball into half of a Petri dish. Note: It is best if two individuals assist in this process—one to pipette and one to mix the pollen.

4.3.1 After preparing the negative controls, prepare the solvent controls by pipetting 50 µL of solvent onto a flattened pollen ball.

4.3.2 Be sure to keep pollen refrigerated (+4 ± 1 °C) before mixing to avoid the pollen sticking to the gloves.

4.3.3 Work from lowest to highest concentration and change gloves between compounds to avoid contamination.

4.3.4 This procedure involves manually folding pollen containing pesticides. Always utilize proper PPE and work in a fume hood. Wear gloves and ensure the compound being tested cannot move through the glove material.

4.4 Repeat this process until the number of pollen balls needed for each control and compound replicate are made.

5. Add treated pollen to corresponding SHB cages.

5.1 Remove water-soaked cotton balls from the SHB cages.

5.2 Orient the cage so that the lid is facing upwards and gently tap the cage on the counter to drop the SHBs to the bottom of the cage.

5.3 Remove the lid. Place the lid on the counter and position the Petri dish containing a pollen ball in the center of the lid. Note: Be sure to insert the correct treatment of pollen.

5.4 Keep the lid flat and invert the cup onto the lid to send the SHBs onto the pollen. Screw on the lid securely to close the experimental cage (Figure 1A).

5.4.1 Watch carefully when screwing on the lid to avoid injuring or killing any SHBs around the edge of the lid.

5.4.2 Work from lowest to highest compound concentration and change gloves between compounds to avoid contamination.

6. Randomly place experimental cages of SHBs into the desiccator housed in the incubator. Note: If multiple desiccators are being used, ensure cages from a single treatment are evenly or randomly distributed among all desiccators to account for slight changes in conditions that may impact SHB morality.

7. Measure mortality.

7.1 Count SHB mortality at the 4, 24, 48, and 72 h after providing the test pollen.

7.2 Consider a SHB dead when it remains motionless when probed.

7.3 Dispose of cages with 100% mortality to maintain a clean desiccator environment.

7.4 At the end of the bioassay (after recording the final time point of SHB mortality), freeze all cages to kill any remaining SHBs.

8. Calculate LC_{50} values.
   8.1 Calculate the LC_{50} values for each compound. LC_{50} values should be calculated as the amount of active ingredient per gram of pollen (i.e., µg/g). The lethal dose that kills 50% of the adult SHBs (LD_{50}) values cannot be calculated because the amount of compound to which each SHB was exposed remains unknown.
   8.2 Exclude any round with <80% adult SHB survival in the negative and solvent control groups. Be sure to account for any death in the negative and solvent controls when calculating LC_{50} values.
   8.3 Use data from only one complete trail when creating dose–response curves for a compound of interest. A trial constitutes an entire replicate schedule (see step 2.3 in the bioassay standard method) conducted at the same time. Do not combine data from multiple trials (i.e., over multiple time points) given conditions vary between trials, possibly impacting the endpoints generated.

2.2. Field Trial

This field trial involves standardizing the number of adult SHBs in hives, administering treatments (in this study: in modified CD cases for a set time), and then counting the number of living adult SHBs remaining in the hive and dead adult SHBs in traps to calculate treatment efficacy. To accomplish this, hives are made SHB-free (to standardize SHB populations) and then a known number of adult SHBs is added to each hive. The treatments and concentrations used are selected from data generated from the acute toxicity bioassay.

1. Prepare honey bee hives.
   1.1 Ensure all hives have approximately the same colony strength (adult bees and brood) and size/equipment configuration (i.e., number of boxes and frames).
   1.2 Standardize adult SHBs in each hive (t = 0 d).
      1.2.1 Use the "bounce method" described in Section 3.2.3.1 of Neumann et al. [17] to remove all adult SHBs from hives. Find and set aside or cage the queen before using this method.
      1.2.2 Use an electrically powered aspirator to collect SHBs quickly.
      1.2.3 If needed, create an electrically powered aspirator by connecting the tubing of an insect aspirator (Table 1L) to a compact vacuum (Table 1M).
   1.3 Number all hives and assign them evenly/randomly into treatments.
      1.3.1 Include a minimum of three treatments: solvent control, positive control, and experimental compound of interest.
2. Prepare traps.
   2.1 Cut three entrances into the closed CD case (Table 1N) using a hot knife (Table 1O) inside a fume hood. The openings should be 3.5 cm long and 0.5 cm wide (Figure 2). Note: Ensure the openings lead directly into the center of the case without any obstructions. Center the openings along each of the three unhinged sides to avoid the ring of plastic inside the case.
   2.2 Cover the holes on the center of the top and bottom of the CD case with tape (Figure 1B, Arrow 1). This will ensure the treated pollen remains inside the trap.
   2.3 Prepare one trap for every hive box in the field trial. For example, a hive consisting of a deep brood box and medium super would receive two traps. Note: Clearly label each trap with the date that treated pollen is inserted, the treatment ID, the treatment concentration, hive number, and trap position in the hive.

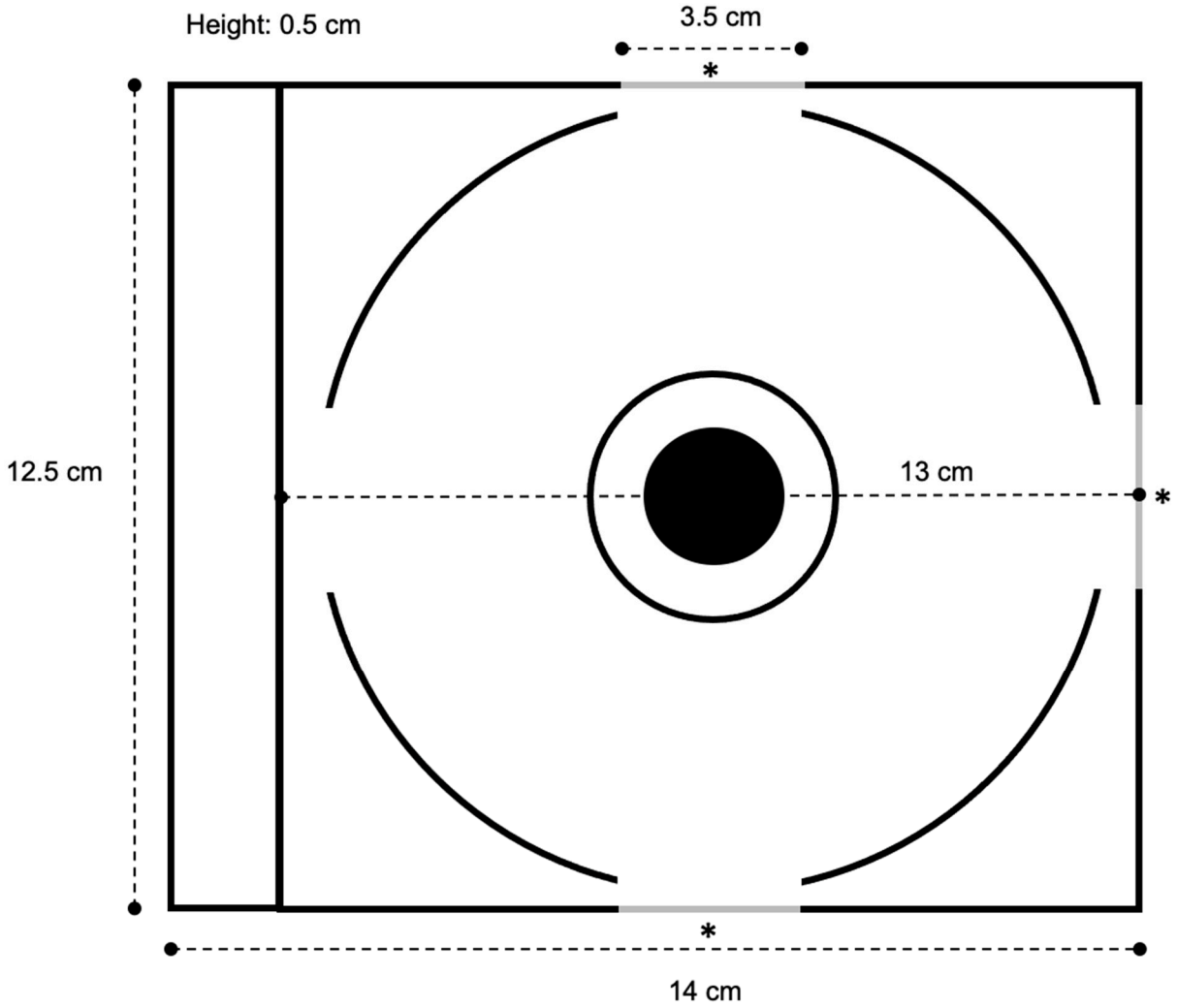

Figure 2. Trap design used in the field trial. The CD cases were modified by cutting a 3.5 cm entrance on 3 sides (denoted by *) to allow small hive beetles access to treated pollen inside the cage.

3. Prepare treated pollen and place into traps.
   - 3.1 Weigh pollen balls.
     - 3.1.1 Weigh 25 ± 0.1 g of bee-collected pollen or commercial pollen supplement (Table 1A) on a calibrated scale (Table 1B). Roll 25 ± 0.1 g portions of pollen into balls with gloved hands. Prepare one pollen ball for each trap needed. Note: If the pollen mixture is sticky, place it in the freezer (−20 ± 1 °C) to harden before handling.
     - 3.1.2 Wrap individual pollen balls in plastic wrap (Table 1C). Store in refrigerator (+4 ± 1 °C) for up to two weeks before discarding.
   - 3.2 Prepare solutions.
     - 3.2.1 Select one concentration for each compound. The concentration is chosen based on the results of the laboratory bioassays. At a minimum, include a solvent (i.e., acetone) control, positive control, and one experimental compound. Note: Be sure to select a concentration that kills 100% of the adult SHBs for the positive control. We used fipronil and thiamethoxam as positive controls, but any compound with proven 100% efficacy can be used.
     - 3.2.2 Clearly label amber vials (Table 1I) with the date, compound, and concentration of compound that will be added.

3.2.3 Prepare 1375 µL of solution for each trap. This follows the 50 µL:1 g pollen ratio used in laboratory bioassays (25 g pollen × 50 µL = 1250 µL) and includes 10% of the original volume excess to account for evaporation (1250 µL × 0.10 = 125 µL).

3.2.3.1 Weigh the total amount of compound needed and place it into the vial of the highest concentration using a sterilized metal scoopula (Table 1J) and calibrated scale. Note: Rinse with 200 proof ethanol and deionized water three times to sterilize the metal scoopulas.

3.2.3.2 Pipette solvent into the vial to create the desired concentration of each compound. Note: Cap vials to avoid evaporation and vortex centrifuge all solutions. We used our solutions within 12 h to avoid evaporation of the solvent.

3.3 Mix pollen balls and place in CD cases.

3.3.1 Divide a 25 ± 0.1 g pollen ball into two 12.5 g halves. Flatten the first half in the palm of a gloved hand.

3.3.2 Pipette 625 µL of solution into the center of the flattened pollen ball half. Manually massage the pollen by folding for 1 min, then set aside. Note: It is best if two individuals assist in this process—one to pipette and one to mix the pollen. Be sure to keep pollen refrigerated (+4 ± 1 °C) before mixing to avoid the pollen sticking to the gloves.

3.3.3 Repeat steps 3.3.1–3.3.2 for the second half of the 25 g pollen ball. Manually combine the two halves by folding them together for 1 min. This ensures the compound is evenly distributed throughout the entire 25 g pollen ball.

3.3.4 Roll the treated pollen ball between two gloved hands to create a cylindrical shape. Place the treated pollen in the open CD case (Figure 1B, Arrow 2).

3.3.5 Using new gloves, close the CD case on the pollen. Slowly press down on both sides of the case to evenly distribute the pollen inside the center of the case and avoid cracks (Figure 1B, Arrow 2). Note: Be sure to maintain at least a 1 cm gap between the edge of the treated pollen and the CD case entrances. This ensures bees cannot access the treated pollen from outside the trap.

3.4 Wrap traps in plastic wrap (Table 1C), grouping those of the same compound and concentration. Place them in the refrigerator (+4 ± 1 °C) for up to two weeks until use.

4. Inoculate hives with SHBs.

4.1 Prepare one sealed vial of 100 adult SHBs for each hive in the field trial. Note: Rear SHBs following the procedures outlined in Neumann et al. [17].

4.2 Inoculate each hive with adult SHBs at t = 2 d from treating the hives. Open the hive lid. Gently tap the bottom of the vial on the hive box to knock all SHBs to the bottom and prevent SHBs from escaping. Release the SHBs from the prepared vials into the uppermost hive box and quickly close the hive lid.

4.2.1 Do not place traps before inoculating hives with SHB to avoid releasing SHBs directly onto a trap in the uppermost hive box.

4.2.2 SHB should be released in the evening (1–2 h before sunset) or at night (1–2 h after sunset) to simulate preferred adult SHB hive invasion behavior [17,19].

5. Insert traps into hives.

5.1 Place baited traps into each box at t = 4 d (t = 2 d since hives were inoculated with SHBs). Place the trap on top of the frames in the center of the box (Figure 1B). Note: In a hive with two boxes, one trap should be placed between the hive lid and top box, and another between the two boxes.

6. Monitor traps.
   - 6.1 Remove any propolis deposits on the CD case entrances every 2–3 d using a toothpick.
   - 6.2 Record observations of SHB activity, mortality, and reproduction.
7. Remove CD traps and collect remaining SHBs when t = 19 d (t = 14 d since inserting traps). Note: We left the traps in the hives for 14 d. This time can vary based on the specific research question being asked.
   - 7.1 Work one hive at a time, beginning with the negative and/or solvent controls, followed by the experimental group, and finally the positive control. This will avoid contamination and confusion of samples.
   - 7.2 Collect dead SHBs by removing traps.
     - 7.2.1 Place each trap in a sealable plastic bag. You can collect other dead SHBs in the hive. Do not include them in the count of SHBs that died due to exposure to the test compound. Instead, they can be saved for residue analysis or other research purposes.
     - 7.2.2 Record the number of live SHBs in each trap.
   - 7.3 Collect live SHBs from the hive via aspiration.
     - 7.3.1 Remove all live SHBs from each piece of equipment and frame using the "bump method" described in Section 3.2.3.1 of Neumann et al. [17].
       - 7.3.1.1 Use an electrically powered aspirator rather than a mouth aspirator and avoid inhaling compounds throughout the field trial. Wear appropriate PPE.
       - 7.3.1.2 Use a new collection vial for each hive. Clearly label each vial with the corresponding hive number.
       - 7.3.1.3 Do not aspirate any dead SHBs, since the cause of death is unknown. Collect dead SHBs outside of traps separately.
     - 7.3.2 Place traps and sealed vials of live SHBs into a freezer (-20 ± 1 °C) to euthanize all remaining live SHBs and preserve the samples until processing.
8. Process SHB samples.
   - 8.1 Open each trap and count the number of dead SHBs found inside the trap. Note: The trap may contain SHB fragments. When this occurs, only count one body segment (for example, only SHB abdomens) to estimate the number of dead SHBs. Subtract the number of live SHBs recorded, if any, inside traps on the date of data collection to create an accurate number of dead SHBs inside each trap due to compound exposure.
   - 8.2 Count and record the number of live SHBs aspirated from each hive.
   - 8.3 For each hive calculate: Live SHBs recovered = # of live SHBs aspirated (include from multiple aspirators, if applicable) + # of live SHBs recorded inside traps during data collection (include from multiple traps, if applicable). Dead SHBs inside traps = # of frozen SHBs in traps (include from multiple traps, if applicable)—live SHBs recorded inside traps during data collection (include from multiple traps, if applicable). Dead SHBs outside traps = # of dead SHBs found in the hive, outside the traps. Average live SHBs recovered per treatment = sum of the number of live SHBs recovered for all hives/total number of hives. Average dead SHBs inside traps per treatment = sum of the number of dead SHBs inside traps for all hives/the total number of hives.

3. Proof-of-Concept Methodology

3.1. Acute Toxicity Bioassay

We performed bioassays utilizing the standard methods described above. The assays were conducted from December 2020 to January 2021 at the University of Florida Honey Bee Research and Extension Laboratory (UF HBREL) in Gainesville, FL, USA. Containers of adult SHBs feeding on pollen or pupating SHBs in damp soil were provided from a

lab-reared colony maintained at the USDA Center for Medical, Agricultural, and Veterinary Entomology (USDA-CMAVE) in Gainesville, FL, USA.

We used 100% acetone as a solvent control to dilute all compounds tested in our bioassays (all the compounds were soluble in acetone). We performed preliminary range finding bioassays by testing three concentrations of each test compound, with each concentration being five times higher than the proceeding one (coumaphos—100, 500, 2500 µg/g; acetamiprid and fipronil—10, 50, 250 µg/g; thiamethoxam—500, 2500, 12,500 µg/µL). We used knowledge from previous assays and existing literature reporting the toxicity of these compounds on other beetle species and honey bees to select the initial range-finding concentrations [15,20–22]. With this information, we calculated the LC_{50} values for coumaphos, acetamiprid, and fipronil by determining SHB mortality at ranges of 500–2500 µg/g, 12.5–300 µg/g, and 1.25–2.75 µg/g compound, respectively. Every round included at least five negative (nothing added), five solvent (acetone), and three positive (thiamethoxam/fipronil) controls. We used thiamethoxam as an alternative positive control (2500 µg/g) during the refinement process but did not generate an LC_{50} value.

3.2. Field Trial

In February and March 2021, we conducted a field trial utilizing the standard method outlined above. The field trial included 15 hives located at the USDA-CMAVE. Each hive consisted of two boxes without queen excluders, with 13 hives consisting of a deep box with a medium super and the remaining two hives consisting of two deep boxes. A minimum of nine frames was in each of the 30 boxes. COVID-19 regulations at the site where we conducted the field trial restricted the time we could spend in the apiary. Thus, we were not able to remove all SHBs from each hive as outlined in step 1.2 of the field trial standard method. Instead, we standardized the number of SHBs in each hive to ten seen during an inspection of ten frames. We saw more than ten SHBs in two of the 15 hives. We used the bounce method in the top box of these two hives to lower SHB populations below our 10-SHB threshold. We inoculated the hives with 100 SHBs from a lab colony maintained at the USDA-CMAVE.

We divided the 15 hives into the following three treatments of five hives/treatment: solvent control—100% acetone; experimental compound—300 µg/g acetamiprid; and positive control—5 µg/g fipronil. We selected the lowest concentrations of each compound that exhibited 100% mortality in laboratory bioassays. We selected these high concentrations to validate our method and reduce the chances that SHBs would develop resistance to the compounds. We prepared all pesticide solutions at the UF HBREL.

3.3. Data Analysis

For the laboratory bioassays, we used the software PoloPlus (version 1.0, LeOra Software Company, Petaluma, CA) to create log concentration-probit curves and calculate LC_{50} values with 95% confidence intervals. Any death in the negative and solvent controls was accounted for in the program and any round with <80% adult SHB survival in these two groups was excluded. Rounds with <100% mortality of the positive controls were also excluded from the analysis. We excluded two concentrations of coumaphos and one concentration of fipronil as outliers when calculating LC_{50} values due to pipetting errors and abnormally high mortality.

We constructed a generalized linear model for our field trial with a negative binomial distribution for both live and dead SHB counts. In both models, we selected SHB counts (either live or dead) as our response variable and treatment type (acetone, acetamiprid, fipronil) as our predictor fixed effect variable. All models were constructed in R (version 4.1.1) with glmmTMB::glmmTMB [23]. We visually assessed various model assumptions using simulated residuals in DHARMa::simulateResidals [24]. We calculated the significance of our models with a Type II Wald chi-square test using car::Anova [25]. Additionally, we constructed pairwise comparisons of estimated marginal means (EMMs) with a Tukey adjustment using emmeans::emmeans [26]. For treatments with only zero values, we com-

pared the overlap of 95% confidence intervals based on a negative binomial distribution to determine significance [27].

4. Results

4.1. Acute Toxicity Bioassay

Coumaphos had the highest LC_{50} value (LC_{50} = 1250 µg/g) to adult SHBs of all the tested compounds (Table 2), making it the least toxic compound tested for SHBs. Acetamiprid (LC_{50} = 20.5 µg/g) was 61× more toxic to adult SHBs than coumaphos was, yet 11.5× less toxic to adult SHBs than fipronil was (LC_{50} = 1.78 µg/g), the most toxic compound we tested. All SHBs died when exposed to ≥2500 µg/g of thiamethoxam, a positive control.

Table 2. Small hive beetle acute toxicity Lethal Concentration 50 (LC_{50}) values with 95% confidence intervals and chi-square values generated in PoloPlus.

Compound	n	LC_{50} (µg/g)	95% CI	χ^2 (df)
Coumaphos	488	1250	1159–1328	1.672 (4)
Acetamiprid	300	20.5	10–31	4.939 (3)
Fipronil	398	1.78	1.58–2	11.312 (5)

4.2. Field Trial

Treatment significantly affected the number of live SHBs recovered from hives at the end of the two-week trial (χ^2 = 44.05 (2), $p < 0.001$) (Table 3). The greatest number of live adult SHBs was recovered in hives with acetone traps (86.8 live SHB/hive) (Table 3). The number of live SHBs recovered in hives with acetamiprid traps (11.6 live SHB/hive) was 7.48× lower than that recovered from hives with acetone traps ($t = -5.50$, $p < 0.001$) (Table 3; Figure 3A). Comparatively, the number of live SHBs recovered in hives with fipronil traps (10.4 live SHB/hive) was 8.35× lower than that recovered from hives with acetone traps (t = 5.76, $p < 0.001$) (Table 3; Figure 3A), but not different from that recovered in hives with acetamiprid traps (t = 0.28, p = 0.958) (Figure 3A).

Table 3. Total number, average, 95% confidence intervals, and standard error of live and dead small hive beetles (SHBs) collected after two weeks of treatment exposure via CD trap.

Treatment	Live SHBs			Dead SHBs in Traps		
	Total # SHBs	Avg. # SHBs/Hive (95% CI)	SE	Total # SHBs	Avg. # SHBs/Hive (95% CI)	SE
Acetone	434	86.8 (50.8–148.4) [a]	21.6	0	0 [b]	0
Acetamiprid	58	11.6 (6.4–21.1) [b]	3.59	357	71.4 (60.6–84) [a]	5.8
Fipronil	52	10.4 (5.7–19.1) [b]	2.93	8	1.6 (0.7–4) [b]	2.93

Columnar means with different letters are different from visually assessing confidence intervals (see Figure 3) or at $p < 0.001$ from linear contrasts with Tukey adjustment.

The average number of dead SHBs in traps varied significantly by treatment (χ^2 = 108.23 (2), $p < 0.001$) (Table 3). The number of dead SHBs found in acetamiprid traps (71.4 dead SHB/hive) was 44.63× higher than that found in fipronil traps (1.6 dead SHB/hive; t = 10.40, $p < 0.001$) (Table 3; Figure 3B). No dead SHBs were found inside the acetone traps (Table 3; Figure 3B). Based on the lack of overlap of confidence intervals, the number of dead SHBs in acetone traps was significantly lower than that found in acetamiprid traps, but not different from that in fipronil traps (Figure 3B).

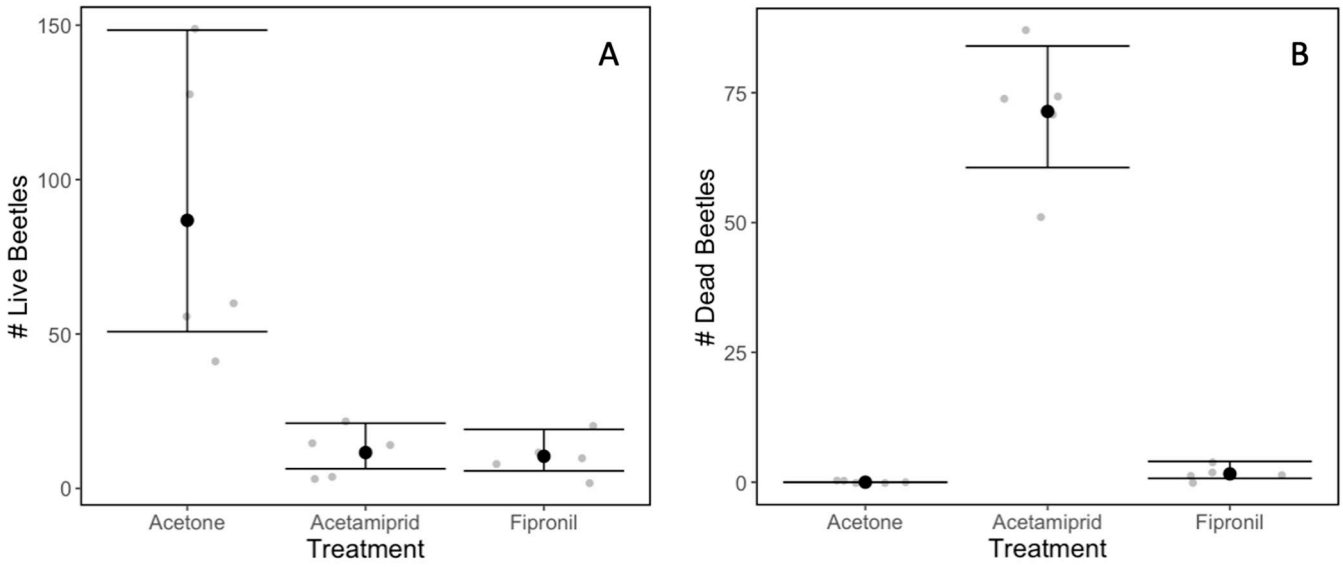

Figure 3. The 95% confidence intervals constructed from a generalized linear model with a negative binomial distribution for the average number of live small hive beetles (SHBs) aspirated from hives (**A**) and dead SHBs collected from traps (**B**) in each treatment. The averages for each treatment are depicted by a large dot with the confidence interval extending above and below it. Smaller dots represent the number of SHBs collected from the individual hives. Treatment means without overlapping confidence intervals are significantly different from one another.

5. Discussion

We outlined a novel standard method for a SHB acute toxicity bioassay and corresponding field trial. To our knowledge, this is the first detailed method to assess the toxicity of compounds delivered in pollen for adult SHB control. Kim et al. [28] utilized pollen delivery methods in laboratory bioassays and a CD trap field study to evaluate the efficacy of coumaphos as a SHB control agent, yet they did not propose detailed methods. Utilizing pollen, a known preferred food source of SHBs [17], may create a variety of new control methods for beekeepers to implement in IPM systems to manage SHB populations.

We also completed a proof-of-concept experiment examining the potential of acetamiprid as a SHB control agent. This compound has been tested previously as a control for SHB larvae [29] and a variety of other beetles, including the asparagus beetle (*Crioceris asparagi*) [30], cabbage seed weevil (*Ceutorchynhus assimilis*) [31], and Colorado potato beetle (*Leptinotarsa decemlineata*) [20]. To our knowledge, this is the first use of acetamiprid against adult SHBs. Furthermore, our results suggest acetamiprid may be a good candidate compound for SHB control.

5.1. Standard Methods

While standard methods for pollinator bioassays exist [32], such guidelines are still needed for SHBs. Topical bioassays have been used for evaluating the toxicity of potential control methods against adult SHBs [12,33]. This allows the calculation of acute contact LD_{50}s for SHBs. To do this, a compound is administered directly to the exoskeleton of each SHB and then the SHB is monitored regularly to determine mortality. To calculate an oral LD_{50} value, adult SHBs should be individually fed treated pollen and monitored regularly to measure mortality. A potential critique of our acute toxicity bioassay is the lack of certainty regarding oral or topical exposure of SHBs to the test compounds, though we argue that it is likely both. In our acute toxicity bioassay, groups of adult SHBs crawl throughout the pollen and feed on it, as seen when rearing SHBs with pollen [17]. As a result, adult SHB mortality may be caused by topical exposure, oral exposure, or a combination of both. Thus, we only calculate an LC_{50} value using our assay. Nevertheless, our standard method is valuable in that it is field-realistic. The adult SHBs would be

exposed topically and orally to treated pollen if beekeepers delivered SHB controls via pollen or other foodstuffs.

A future SHB control agent should exhibit high toxicity to SHBs while maintaining relatively low toxicity to honey bees. While our proposed methods effectively assess the effectiveness of compounds against adult SHBs, they do not assess honey bee health. Thus, they cannot be used alone to determine the potential success of a future SHB control agent. These methods should be utilized alongside existing methodologies to assess bee health. These assessments include acute toxicity bioassays for bees [34], bee and brood scores taken at all stages of field trials [35], and residue analyses conducted on honey/pollen/wax samples [36]. The focus of our study was on SHBs specifically. Consequently, we opted to exclude piloting honey bee health assessments and advocate for using those that already exist [32,34]. It is also possible that the method itself can impact the bees, given our reliance on the bounce method to catch and count SHBs. Nevertheless, this technique is essential for the accurate estimation of live beetle populations. The inclusion of negative controls can help one distinguish between adverse effects brought on by the bounce method and/or the compounds being tested.

We list specific equipment to make the assay reproducible in Table 1. However, many specific items can be replaced with like items and still achieve the same desired outcome of assessing SHB control agents. For example, we choose to modify a CD case as traps for our field trial. The CD cases were easy to open and close for pollen placement, as well as facilitate the quantification of dead SHBs at the end of the trial. Other SHB trap designs could be used in place of the CD case and still effectively deliver treated pollen to adult SHBs. The most important aspect of the trap of choice is to ensure bees do not have access to the treated pollen. Finally, we identified thiamethoxam as an effective positive control for SHB acute toxicity bioassays.

5.2. Proof-of-Concept Experiments

Acetamiprid shows promise as a potential control agent for adult SHBs. This neonicotinoid demonstrated higher efficacy than coumaphos in our acute toxicity bioassay. Furthermore, traps with fipronil and acetamiprid treated pollen significantly reduced live SHB populations in our field trial. Interestingly, traps containing acetamiprid retained more dead SHBs than did those with fipronil, perhaps due to a faster mode of action. The lack of dead SHBs retained in the fipronil traps suggest SHBs consumed small amounts of the fipronil-treated pollen and then crawled out of the trap to die. This potentially transports the compound out of the trap, thereby increasing the risk of bee and hive product exposure to the compound. Alternatively, SHBs may have been repelled by the fipronil traps and never consumed it.

It is important to note the potential movement of SHBs between hives in our field trial. At the end of the two-week study, we aspirated >100 live SHBs from two of the five hives administered acetone-treated pollen. This is evidence of SHB movement between hives or the emergence of adult SHBs from the soil during the trial. We randomly assigned treatments to account for hive strength rather than blocking by treatment. Ideally, SHB populations would be eliminated in all hives before inoculation, but this is not always possible due to the hiding behavior of SHBs in hives. Additional variabilities could be eliminated by treating the soil to kill pupating SHBs and/or moving the test hives to a new apiary site that did not host colonies the preceding two months. Our field trial was heavily restricted by COVID-19 policies that made these practices not possible at the time the study was conducted.

6. Conclusions

There is an urgent need for new chemical control methods that can be incorporated into an IPM management program for SHBs. We proposed detailed standard methods to evaluate the success of potential compounds against adult SHBs in the laboratory and field. Our proof-of-concept experiments support the need for the further testing of acetamiprid

as a potential control agent. Future research is necessary to refine our standard methods, assess honey bee health, and continue serving beekeepers by identifying effective SHB control methods.

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