COLLEGE OF AGRICULTURE AND LIFE SCIENCES
ENTOMOLOGY
VIRGINIA TECH

Impact of E90 Rechargeable Mosquito Repeller on Honey Bee Foraging and Recruitment

Protocol

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1 ADMINISTRATIVE INFORMATION

1.1 Funding Sources

Sources	**Type of Support**
Thermacell	Financial
Department of Entomology	Materials / Facilities

1.2 Budget Justification

The project will be run as a series of independent field trials for the College of Agriculture and Life Sciences. We anticipate three trials in total, but final cost for the series will depend on the number of trials conducted. Of the anticipated three trials, one will be conducted by Dr. Couvillon, one will be conducted by Dr. Schürch, and one will be conducted by both. The total cost for the three trials will be USD 74,997 (USD 24,999 each trial). We are grateful for the support to Dr. Couvillon's and Dr. Schürch's research budget, respectively.

1.3 Roles and Responsibilities

1.3.1 Names, Affiliations, and Roles of Protocol Contributors

	Affiliation	**Role**
Margaret Jane Couvillon	Department of Entomology, Virginia Tech	Co-Principle Investigator
Roger Schürch	Department of Entomology, Virginia Tech	Co-Principle Investigator
John Hainze	Thermacell	Co-Sponsor
Benjamin Mcmillan	Thermacell	Co-Sponsor

1.3.2 Sponsor

John Hainze
VP of Science & Research
Thermacell
26 Crosby Drive
Bedford, MA 01730

1.3.3 Role of Study Sponsor in Protocol Development, Data Analysis, and Academic Publication

The study sponsor does not claim authority over analysis and interpretation of data; writing of the report; and the decision to submit the report for publication. The sponsor provides:

- input on usage of E90 Rechargeable Mosquito Repeller
- comments on protocols

2 INTRODUCTION

2.1 Background and Rationale

Honey bees are a widely used, tractable organism commonly deployed to investigate the impact of pest control measures on bee health (regulated in 40 CFR 158; see Cresswell (2010) for a meta-analysis). Conveniently, honey bees can be trained to collect food at an artificial feeder (Couvillon et al., 2015), which allows investigators first to monitor foraging and recruitment behaviors and then to determine if these behaviors are affected by a treatment. For example, we have used this methodology to determine that the addition of caffeine to the sugar solution increases foraging and recruitment (Couvillon et al., 2015), whereas the addition of the neonicotinoid imidacloprid decreases foraging and recruitment (Ohlinger et al., 2022). However, what is not known is if the presence of a nearby pest control device (like E90 Rechargeable Mosquito Repeller) that emits volatiles might negatively impact behaviors. This question was investigated in a previous study that used another pest control device (Couvillon et al. submitted).

Here we investigate the effect of a E90 Rechargeable Mosquito Repeller on the foraging and recruitment behaviors of honey bees visiting either a control feeder containing a sucrose solution with a nearby untreated device, or a treatment feeder containing the same sucrose solution with a nearby working E90 Rechargeable Mosquito Repeller. To reflect how these devices are used by consumers, we will study the effect of the E90 Rechargeable Mosquito Repeller while the device is on and while the device is off for the next morning.

2.2 Objectives

To study the impact of active ingredient in Thermacell devices on honey bee foraging, recruitment and persistency.

2.3 Trial Design

The trials are designed as a randomized paired parallel group trials with an 1:1 allocation ratio between treated and untreated bees.

3 METHODS

3.1 Interventions and Outcomes

3.1.1 Study Setting

The trials will be conducted at the Prices Fork Research Station close to the Blacksburg campus of Virginia Tech, Virginia, USA (37.211462°N, 80.489366°W, Fig. 3.1).

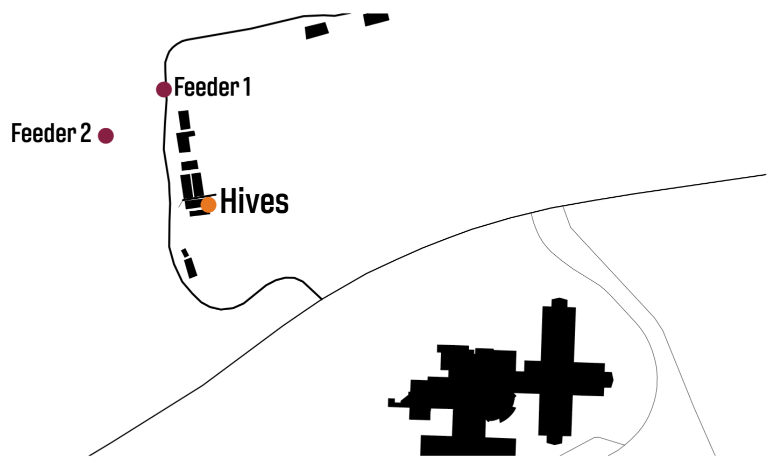

Figure 3.1: Prices Fork Research Station with suggested hive locations in orange and suggested feeder locations in maroon. Both feeders are about 100 m from the hive entrances, and about 60 m from each other.

We will house honey bee colonies (N = 1 per trial; N = 3 for the series) in glass-walled observation hives at the field laboratory of Dr. Couvillon and work with each colony sequentially. On Day 0, the first day of the experiment, we will set up two feeders, each offering high quality sucrose solution, 60 m apart from one another and about 100 m to the east of the hives (Fig. 3.1).

3.1.2 Timeline for Hives

The timeline for hives is summarized in Figure 3.2. We will *pre-train* a set of bees on days leading up to the experiment (pre-training). Bees used in *pre-training* will be discarded from analyses. On the day of the experiment, during the *training phase*, we will train ca. 20 bees from the observation hive to forage at one of the two feeders, equidistant from the hive, using standard procedures (Couvillon et al., 2015). The duration of the *training phase* varies, as it continues until enough bees are trained to

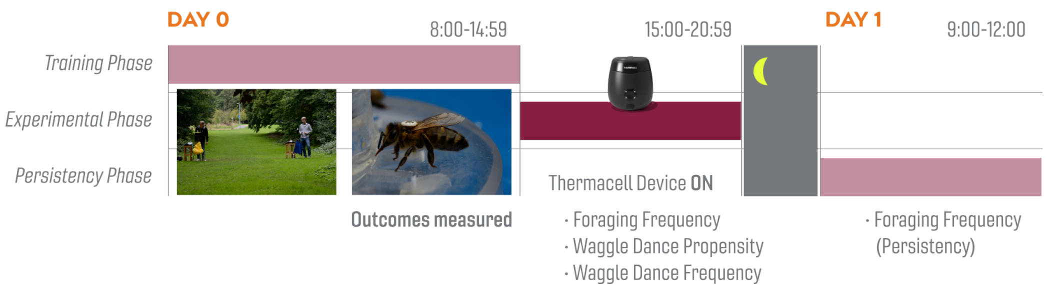

Figure 3.2: Timeline for each hive, with initial *training phase*, *experimental phase* and *persistency phase*. The pruple bars indicate the times hives spend in each phase.

each feeder, but it typically takes a few hours. Once bees are foraging at and recruiting reliably to their designated feeder, we will begin the *experimental phase* at 15:00.

On the pre-training day (day -1), we will prepare two E90 Rechargeable Mosquito Repeller devices with active ingredient or no active ingredient refills in the lab, respectively. The allocation of the two treatment modalities to the devices will be done through coin toss. The two devices will then be allowed to *pre-burn* for 2 hours on the pre-training day, which helps to burn off any stored material in the wicks. On day 0, prior to the *experimental phase*, refills will be weighed to the nearest 1/100 g with the cap on. After weighing the refills they will be loaded back into the devices. The devices will be set atop a tripod and placed near the allocated feeder: We will establish within 3 m (10 ft) of each feeder either a E90 Rechargeable Mosquito Repeller with no active ingredient or active ingredient (see section 3.2 below for details on interventions). The choice of treatment versus control feeder allocation will be randomized for each trial. These devices will allow us to study the impact of active ingredient on honey bee foraging and recruitment.

During the *experimental phase*, we shall allow the trained bees to forage and to recruit freely to their designated, trained feeder while the devices are turned on. The *experimental phase* will last 6 hours, simulating the maximum length of time a consumer is likely to run the device. Across that time, we shall monitor (1) foraging frequency and, back at the observation hive, (2) waggle dance propensity and (3) waggle dance frequency via video recordings and later analysis (see below). Although the trial is set to end at 21:00, if there is no foraging activity for 5 minutes, which is likely to occur after 20:00, then the trial may conclude and not go until 21:00. At the end of this phase, the devices and feeders will be removed from the field and refills weighed again, concluding Day 0.

On Day 1, we will conduct a mortality census early in the morning. We will then conduct observations during the *persistency phase*. This will involve returning the identical feeders and treated/untreated E90 Rechargeable Mosquito Repeller (with devices turned off) to the field by 9:00. For the next 3 hours, we shall monitor (4) foraging persistency of reactivated foragers that remember and return to their designated feeder.

Videos recorded to SD cards during the *experimental phase* will be brought to the Couvillon Lab on the main Virginia Tech campus. We shall save each SD card to an online, cloud-based repository. Dance propensity and dance frequency shall be determined via video analysis on computers (Couvillon et al., 2015).

3.1.3 Interventions

The device to be tested here will be the E90 Rechargeable Mosquito Repeller. The device consists of a battery powered heat source that will activate insecticide-treated refills. Running the device is scent free for humans.

The two interventions in the experiments are:

Treatment The refill in the device **is** treated with active ingredient.

Control The refill in the device **is not** treated with active ingredient.

Devices with treated and untreated refills will be placed within 3 m (10 ft) of their designated feeder during the *experimental* and *persistency phases*. The devices will only be on during the *experimental phase*.

Thermacell will provide 2 devices, 6 fuel cartridges, and 6 treated and 6 untreated refills.

3.1.4 Outcomes

1. Primary Outcome: Foraging Frequency at Feeder During *Experimental Phase*

   foraging frequency during experimental phase Bees will initially be trained to forage at two designated artificial sucrose solution feeders. Once we have trained the desired number of bees to each feeder (approx. 20), we will start the exposure to untreated or treated devices in the *experimental phase*. We will count for each bee how often she will visit the feeder across the up to 6 hour period. We term this discrete, positive count the *foraging frequency during the experimental phase*.

2. Secondary Outcomes: Recruitment Dances and Foraging on Follow-up Days

   waggle dance propensity during experimental phase During the *experimental phase*, we will record the *waggle dance propensity* of individual bees. In other words, we will assess an individual forager's propensity to perform waggle dances (yes / no, binomial) during the up to 6 hour period. Bees only perform dances for resources they value highly (von Frisch, 1967).

   waggle dance frequency during experimental phase We will also record waggle dance frequency, which involves counting for each bee how many independent dances an actively foraging bee will perform in the up to 6 hour *experimental phase*. We term this discrete, positive count the waggle dance frequency during the *experimental phase*.

   foraging persistency during the persistency phase After the *experimental phase*, sucrose solution feeders will be removed for the night. The next morning, empty feeders will be returned to the original spots, and we will count visitation of marked bees to these feeders during the 3 hour *persistency phase*. We term this discrete, positive count the *foraging persistency during the persistence phase*.

3.1.5 Potential confounding variables

number of visits during training phase bees marked early in the *training phase* have longer time to train to the respective feeder and form a tighter commitment (Couvillon et al., 2015). This might lead to more visits during the *experimental* and *persistency phases*. Therefore, we add number of visits to feeder during *training phase* to statistical models to control for the individual commitment of a bee, irregardless of treatment.

3.1.6 Sample Size

Sample size in respect to hives is largely determined by the available time to train bees and conduct the experiments. Typically, 3–4 hives can be trained during an experimental summer season. Correlation among bees within hives is very low for many aspects of their behavior and comparable to between hive correlations (Schürch et al., 2016). Therefore, we will view each bee as an independent replicate when considering statistical power. However, to balance treated/untreated devices in respect to feeder position, we will aim for 3 runs. We will aim to train about 20 bees from each hive to each of the feeders (treated and untreated, so total n = 40 bees per trial) before starting the *experimental phase* for a total of 120 bees.

3.1.7 Colony sourcing

Colonies will be bought from the Virginia Tech apiary.

3.2 Assignment of Interventions

3.2.1 Blinding

MJC and RS and any additional investigators from Virginia Tech will be blind to treated/untreated modality of the individual devices. Instead, treated and untreated refill provided by Thermacell will be labeled with spelled out greek letters (i.e., "GAMMA" and "DELTA"). Unblinding will occur after the primary analysis is complete.

3.2.2 Allocation Sequence Generation

Allocation of the interventions to Feeder 1 (see Fig. 3.1, BLUE feeder) will be determined by virtual coin toss in *R* using the following code:

```
set.seed(20230608)
c("GAMMA", "DELTA")[rbinom(size = 1, n = 3, prob = 0.5) + 1]
```

3.2.3 Allocation Implementation & Concealment

Allocation will be implemented by Roger Schürch, Margaret Couvillon or Bradley Ohlinger. Greek letter labels will be covered and invisible to field workers.

3.3 Data Collection, Management, and Analysis

3.3.1 Data Collection Methods

We will collect data using the Android App *ODK Collect*. Figure 3.3 shows the forms deployed on a smartphone. In the field, we will issue dedicated tablets to student workers.

3.3.2 Data Management

We will upload case report forms from *ODK Collect* into a Google Sheet on the Project's Virginia Tech Google Drive for longterm storage.

We will download the data for analysis through *R* scripts using the `googledrive` package. Any corrections to the data will be made in scripts prior to analysis (i.e., the raw data will not be touched).

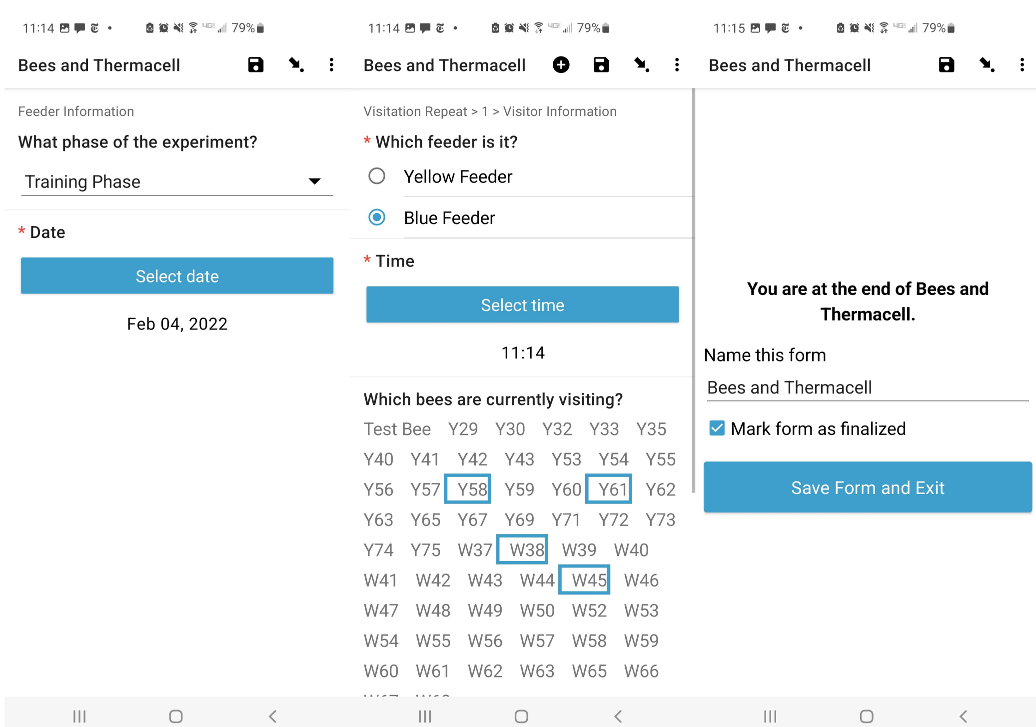

Figure 3.3: *ODK Collect* forms for the Thermacell field trials data collection.

3.3.3 Statistical Methods

The statistical analysis will follow Couvillon et al. (2015). In particular, we will analyze the data using mixed-effect models to account for similarity within hives. Count data will be analyzed using Poisson regressions. Binomial data (dance propensity) will be analyzed using logistic regression.

3.4 Monitoring

3.4.1 Data Monitoring

We will not implement formal data monitoring. Data integrity checks will be performed at analysis time.

3.4.2 Auditing

We do not foresee any audits for these data, though our tool chain creates an audit trail.

4 ETHICS AND DISSEMINATION

4.1 Research Ethics Approval

The proposed work involves invertebrates and does not require ethics approval.

4.2 Protocol Amendments

Amendments to the protocol will be documented in the appendix.

4.3 Declaration of Interests

The investigators will get funding for the field trials from Thermacell. No additional conflict of interest is present.

4.4 Access to Data

Access to the raw data on the Virginia Tech Google Drive will be limited to the investigator team, though copies of the data may be distributed to all personnel involved in the trials, including sponsors.

After the publication of results, data and analysis code will be made available to the public.

4.5 Dissemination Policy

The dissemination of results will be done through a series of reports:

1. At the end of each of the 3 experiments, we will deliver an interim summary of the number of bees per sample and completion of that experiment.

2. At the end of the primary analysis before unblinding (section 3.2.1), we will deliver a statistical analysis report to Thermacell.

3. After the blinded primary analysis report, Thermacell will unblind us (see section 3.2.1), and we will use that information to generate the final analysis report with appropriate labels (i.e., labelled axes, tables and text). As supplementary material to this report we will make available the raw data to Thermacell. We will consider this the end of the trials.

MJC and RS will then work to produce the scientific publication based on the final analysis report. Thermacell may use the final report and the raw data for communicating results to regulatory authorities and modifying product label to reflect what is learned. Both of those scenarios will take place under confidentiality agreements. Any other use of data in a public forum will be restricted until a peer reviewed manuscript is accepted for publication. We will also make the raw data and analysis code publicly available upon publication of the peer reviewed manuscript (either on the VT or public data repositories).

5 AMENDMENTS

N/A

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