Validation of a New Bed Bug Repellency Bioassay

Connor Brescia

ENTM Capstone

Purdue University

Study Questions:

1. Determine whether a newly developed bed bug bioassay can indicate insecticide repellency in adult males of the Harlan susceptible strain

Test Species & Strain:

Cimex lectularius L.; Harlan susceptible strain that has been maintained in the laboratory without insecticide exposure for >40 years.

Life Stage:

Adult males

Treatments:

1. Exposure of 15 male bed bugs to partially untreated and partially water-treated glazed ceramic wall tiles for 24 hours with replicates both with and without a darkened treatment area
2. Exposure of 15 male bed bugs to partially untreated and partially ecoraider (essential oils) treated glazed ceramic wall tiles for 24 hours with replicates both with and without a darkened treatment area
3. Exposure of 15 male bed bugs to partially untreated and partially phantom (chlorfenapyr) treated glazed ceramic wall tiles for 24 hours with replicates both with and without a darkened treatment area
4. Exposure of 15 male bed bugs to partially untreated and partially transport (bifenthrin + acetamiprid) treated glazed ceramic wall tiles for 24 hours with replicates both with and without a darkened treatment area

Background Information:

Given the nocturnal nature of bed bugs, they display behavioral propensity to hide in dark and tight areas with low intensity (Ameya Gondhalekar; personal observation). Examples of such hiding areas include, harborages deep inside a couch, recliner, and any other household furniture. A video associated with this project shows the movement of bed bugs from a well-lit area of a bioassay arena to a darker side (General Video 1). In corroboration with this observation, bed bugs are attracted to darker-colored objects and pitfall traps when given a choice (Singh et al. 2015). Although the preference of bed bugs to harbor in darker areas with less light intensity has not been exploited to develop insecticide repellency bioassays, an identical behavior displayed by German cockroaches (Blattella germanica L.) has been utilized to develop choice experiments (Ebeling et al. 1966). These bioassay experiments termed as the "Ebeling choice-box bioassays" provide information on whether German cockroaches display repellent behavior or behavioral resistance when exposed to residues of any insecticide (Ebeling et al. 1966). The Ebeling choice box and its modified versions consists of two connected chambers, one of which is painted with a dark color (usually black) and the other chamber is not painted black and is thus clear or well lit (Fardisi et al. 2019). Cockroaches are free to move in and out of both light and dark chambers of the Ebeling choice box through one or more openings in the wall or barrier that separates the two compartments. Under control Given the nocturnal nature of bed bugs, they display a behavioral propensity to hide in dark and tight areas with low intensity (Ameya Gondhalekar; personal observation). Examples of such hiding areas include harborages deep inside a couch, recliner and any other household furniture. In corroboration with this observation, bed bugs are attracted to darker-colored objects and pitfall traps when given a choice (Singh et al. 2015). Although the preference of bed bugs to harbor in darker areas with less light intensity has not been exploited to develop insecticide repellency bioassays, an identical behavior displayed by German cockroaches (Blattella germanica L.) has

been utilized to develop choice experiments (Ebeling et al. 1966). These bioassay experiments termed as the "Ebeling choice-box bioassays" provide information on whether German cockroaches display repellent behavior or behavioral resistance when exposed to residues of any insecticide (Ebeling et al. 1966). The Ebeling choice box and its modified versions consists of two connected chambers, one of which is painted with a dark color (usually black) and the other chamber is not painted black and is thus clear or well lit (Fardisi et al. 2019). Cockroaches are free to move in and out of both light and dark chambers of the Ebeling choice box through one or more openings in the wall or barrier that separates the two compartments. Under control conditions when both chambers are not treated with insecticides, cockroaches released on the light side of the choice-box move over to the dark side of the box as per their behavioral preference. However, when the darker side of the choice box is treated with an insecticide, they may show a preference to harbor or aggregate on the well-lit side of the box if they are repelled by the insecticide. In contrast, if the insecticide is not repellent, they would still harbor on the dark side until they become intoxicated or start dying. In this assay design it is essential to keep the lights in the bioassay room turned on for 24 h (24:0 light:dark cycle) to maintain the brighter light intensity on the unpainted/untreated side of the choice bioassay arena.
The concept of the repellency bioassay described in this bed bug study is similar to the Ebeling choice box bioassay for German cockroaches. More specifically, the bioassay arena consists of an unreactive treated surface (ceramic squares) that has an untreated area (one of the halves) and an insecticide-treated area (the other half). The two areas of the ceramic substrate are demarcated with a line and bed bugs have a choice to stay in the treated or untreated area. To confine bed bugs to the substrate, a modified base of the lid of a 100 x 15 mm clear or transparent Petri dish is used. This base or lid is modified by covering one half of its outer side with a dark blue-colored painter's tape. The Petri dish base or lid is placed on the ceramic substrate in a position that aligns the insecticide-treated side of the ceramic tile with the masked or darker side of the Petri dish and the clear portion of the dish is aligned with the untreated side of the substrate. To ensure that bed bugs do not find an insecticide untreated area on the dark side, even the inner rim of the masked side of the Petri dish is treated with the same concentration of insecticide that is used to treat the substrate. When bed bugs are released on the untreated or well-lit side of the substrate, most of them move to the darker or insecticide-treated side of the tile because of their behavioral propensity to settle in dark areas. However, if the insecticide under question has a repellent effect, they would be forced out to the untreated and well-lit side of the substrate over time.

Overall, this repellency bioassay design utilizes visual cues of bed bugs to determine insecticide repellency, which is similar to the chemosensory or host cues (e.g. carbon dioxide and heat) that are exploited in other repellency bioassay. However, the advantage of the bioassay protocol used here is that it allows utilization of test substrates (e.g., carpet, plywood, ceramic etc.) which are more commonly used in residual insecticide efficacy bioassays. Also, the assay design mimics the set-up used for efficacy testing. Details on how this repellency bioassay was conducted with bed bugs can be found in the "Experimental procedures" section below.

Experimental Procedures:

Bed bug feeding and rearing: The Harlan strain bed bugs were fed defibrinated rabbit blood purchased from Hemostat Laboratories (Dixon, CA) using the membrane feeding method (ChinHeady et al. 2013). Meshed plastic jars containing various bed bug life stages were

maintained in a reach-in environmental chamber (Percival Scientific, Perry, IA) at 25°C temperature, 25– 40% relative humidity and 12:12 h light: dark period. Adult males used for bioassays were less 4 than one month old post-eclosion to adulthood and were fed rabbit blood 7-10 days prior to conducting repellency bioassays

Preparation of ceramic tiles for insecticide treatments: White ceramic wall tiles (6 x 6 inches) were used as test substrates for this bioassay. To enable partial treatment of ceramic tiles with the desired insecticide a line was drawn through the center of the substrate and then one of the sides was covered with aluminum foil, which was secured in place with a masking tape and by wrapping or folding the foil around the edges of the tile. The masking tape on the aluminum foil near the center of the substrate prevented water or insecticide from drifting under the foil and thereby prevented contamination of the untreated side of the ceramic surface.

Treatment of half-covered ceramic tiles: All treatments were carried out in a fume hood. All ceramic tiles were treated one at a time by placing them on absorbent paper, which prevented contamination of the fume hood with insecticide residues. The insecticides being tested were diluted into distilled water at manufacturer-specified concentrations. Distilled water was used to spray control tiles. Spraying of tiles was accomplished via the use of small handheld spray bottles. Water treatments were conducted first, followed by insecticide treatments. A separate hand sprayer was used for water and insecticide treatments. During treatment or spraying, the spray bottle was held 5–10 cm away from the ceramic tile. The spray bottle was calibrated to determine the number of pumps necessary to dispense ~0.95–1.00 ml of water or insecticide dilution per ceramic tile (i.e. equivalent to 1 gallon per 1000 square it was observed that some spray solution drifted away from the ceramic. So, although the weight of the spray bottle decreased by 0.9–1.1 gram after spraying the substrate, the weight of the treated ceramic did not increase by ~1 gram. To resolve this issue, spraying time was slightly increased (but not measured), which then resulted in ~1.4–1.7 ml of spray solution being dispensed on the ceramic square (determined from reduction in weight of the spray bottle) and accompanied by ~1.00 gram increase in weight of the ceramic. The spray volume adjustment to 1. 4–1. 7 ml per ceramic tile allowed an increase in weight by ~1 gram or equivalent to 1 gallon per 1000 square feet.

Preparation and treatment of Petri dishes:
When conducting residual insecticide bioassays, bed bugs are confined to a substrate by placing a plastic Petri dish base or lid over them. Since bed bugs are not able to walk or climb the smooth sidewalls of a Petri dish base or lid, they remain confined to the treated substrate. A similar Petri dish enclosure was used for the repellency bioassay; however, one-half of the outer side of the Petri dish lid or base was masked with a dark blue colored painter's tape. This was achieved by initially drawing a line through the center of the dish (on the outer side) and covering one side as well as the side walls on that side with a painter's tape. Additionally, before masking one side of the Petri dish, the inner rim or side wall of the taped side of the Petri dish was sprayed with water or the insecticide treatment for the intended replicate using the spray bottle. The other half of the Petri dish lid or base that was not masked was left untreated. The reason for treating the inner rim of the Petri dish side wall that was masked, was to ensure that bed bugs that reside in the treated or dark side of the bioassay arena were not able to partially escape insecticide exposure by resting snugly in contact with the untreated rim of the Petri

dish. As explained in the next section, the masked side of the Petri dish enclosure was always aligned with the water or insecticide-treated side of the substrate.

Exposure of adult male bed bugs to bioassay substrates:
After the ceramic tiles were completely dry (overnight drying at room temperature), aluminum foil covering one side of the tile was removed and discarded in an insecticide waste collection box. The side of the tiles from which the aluminum foil was removed served as the untreated area of the substrate that was pre-demarcated by a line in the center. Inner rims or sidewalls of Petri dish lids or bases that were also treated with water or insecticide on the masked side, were also allowed to dry overnight. Next, 10 adult male bed bugs placed in a 0.5 oz./ 15 ml clear Portion cup were released on the untreated side of the substrate by inverting the Portion cup. Once all the bed bugs from the inverted Portion cup were on the substrate, it was removed and quickly replaced with a Petri dish enclosure. This bioassay was continued for 24 h and observations on distribution of bed bugs on the substrate were periodically recorded as described in the next section. To ensure that the untreated area of the Petri dish was continuously well-lit, the fluorescent light bulbs in the room were kept on for 24 hours (24:0 light:dark cycle). Bioassays were conducted at ambient room temperature of 22 ±1° C and relative humidity levels of 50–60%

Distribution and mortality observations:
30 minutes after releasing bed bugs on the choice bioassay substrate observations on their position within the arena were made along with general qualitative observations on their movement behavior. Next, at 1, 2, 3, 4 and 24 hours after releasing bed bugs, their positions along with notable qualitative observations were recorded. Since one of the sides of the Petri dish enclosure was masked with a painter's tape, these observations included only recording bed bugs on the unmasked side of the dish or the untreated well-lit side of the substrate and assuming that the remaining bed bugs were on the masked side that was treated with insecticide or water. However, during the last observation at 24 h, the Petri dish enclosure was removed and position of bed bugs on the treated or dark side was ascertained. Additionally, at 24 h, observations on alive, moribund, and dead bed bugs were also made. Bed bugs that were actively moving without any signs of intoxication were scored as alive. Intoxicated bed bugs that could not walk actively after prodding with a toothpick or knocked down bed bugs that showed uncoordinated body, leg and antenna movements were scored as alive but denoted as moribund. Lastly, bed bugs that did not walk or show any body part movement upon prodding with a toothpick were recorded as dead.

Statistical analysis of data:
The percent repellency values for 0.5, 1, 2, 3, 4 and 24 hour observation intervals were calculated by using the following formula:

$$\text{Percent repellency} = [(Nc-Nt)/(Nc+Nt)] \times 100$$

In this formula, NC is number of bed bugs on the untreated and well-lit side of the substrate and Nt is number of bed bugs on the dark / water or insecticide treated side of the arena. If an

insecticide or treatment is non-repellent, this formula would yield negative values indicative of lack of repellency. The magnitude of non-repellency would depend on the actual value with -100% indicative of complete absence of repellency.

Percent repellency values were then compared across masking tape blacked-out petri dishes and untaped petri dishes. This was done within the separate data sets for each spray treatment using ANOVA calculated within JMP. I then used this to determine whether differences in repellency were significant between with and without tape treatments for each insecticide.

Results and Discussion:

Data: Percent repellency data yielded results varying from highly non-repellent to distinctly repellent depending on the combination of treatments being tested. Differences in repellency across with and without tape treatments were significant in three out of four treatments (fig 1, 2, 3). Ecoraider possessed the greatest difference in repellency due to the addition of tape, while phantom was the lowest and the difference observed with the addition of tape was insignificant. The with tape water-treated control experienced the strongest non-repellent response with 100% of bed bugs moving to the tape-covered side after a period of 24 hours had passed. The control additionally saw slight repellency when tape was not provisioned, which decreased over the course of 24 hours (fig 3.). Ecoraider tape-treated tiles experienced non-repellency that became even less repellent over 24 hours with tape, while without tape bed bugs exhibited repellent responses which decreased over the span of the bioassay (fig 1.). Transport again had a significant difference between with and without tape treatments. The with tape replicates using this insecticide were fairly nonrepellent (>40% non-repellency), and without tape was just barely nonrepellent initially, with trends moving the average to neither repellent or nonrepellent by the end of the 24 hour time period (fig 2.). Phantom was a slow-acting insecticide that generated nonrepellent responses regardless of the presence or absence of tape. The difference between the two was small and not statistically significant based on ANOVA. Mortality after 24 hours varied across treatments ranging from 0% in the water treated controls, 77% (with tape) + 62% (without tape) ecoraider, 8% (with tape) + 2% (without tape) phantom, to 100% in all transport replicates.

Discussion and Qualitative Observations: The percent repellency values calculated for the control are indicative of no repellency at all being present for the tape-treated replicates. This suggests the bed bugs were acting as expected and strongly preferring to hide on an untreated dark surface. The non-tape treated control was somewhat repellent and the results were significant, meaning some factors may have still been at play causing slight aversion to sprayed tile surfaces even without insecticide. If results for these replicates played out as expected of the control we would have seen values closer to 0, but we only saw a % repellency value close to this after 24 hours. It would be worth investigating why there was some degree of repellency for the first two important time samples. Ecoraider was repellent without tape, but nonrepellent with tape. This could mean that the insecticide is a compound bed bugs would tend to avoid, but their negative phototaxis is strong enough to override this aversion. Transport yielded similar results, but the reduction in repellency with tape was only enough to move results into the range of slightly repellent to neutral repellency. Transport results may have also been confounded by the fact mortality was 100% after 24 hours. Phantom was nonrepellent even without tape. The difference between with and without tape was insignificant in phantom suggesting bed bugs

don’t avoid contacting this insecticide regardless of lighting. The insecticides besides phantom were also less repellent than previous studies utilizing this bioassay, suggesting resistant field strain bed bugs are more capable of avoiding insecticide residues. At a broad scope, the insecticides varied from repellent to nonrepellent without the presence of tape, but with tape all were nonrepellent. This affirms that negative phototaxis plays a crucial role in the efficacy of insecticides since they are far less likely to be avoided by bed bugs if they are sprayed in tight dark refuges exterminators typically target.

Conclusion:

This experiment has made it clear the bed bug bioassay previously developed by Ameya Gonhalekar and Wenbo Li (Purdue University Urban Pests) functions properly with nonresistant lab strain bed bugs. This allows these bed bugs to serve as control when compared to resistant strains in future studies utilizing this bioassay technique. By utilizing this petri bioassay dish method we will be able to observe if resistant strains exhibit a higher capability to evade insecticides, how negative phototaxis influences the efficacy of commercial insecticides and the influence of negative phototaxis on overall repellency. With more repetition and analysis to work out any potential points of error in this assay, it could even replace arena and barrier test bioassays in many applications. Overall I can conclude this is an effective bioassay with low cost, space, and time expense. The bioassay offers itself as a reliable option for determining the repellent characteristics of insecticides and should offer a more realistic model of how well an insecticide will perform in the field where bed bugs can evade residues.

Figures

Avg %Repellency Ecoraider		
Time	with tape	without tape
1h	-18	32
4h	-30	22
24h	-38	4

Fig 1.

Avg % Repellency Transport		
Time	with tape	without tape
1h	-40	-2
4h	-42	-4
24h	-42	0

Fig 2.

Avg %Repellency Control		
Time	with tape	without tape
1h	-62.2	20
4h	-100	28.9
24h	-100	-4.4

Fig 3.

Avg %Repellency Phantom		
	with tape	without tape
1h	-90	-70
4h	-82	-68
24h	-78	-56

Fig 4.

References:

Chin-Heady, E., DeMark, J.J., Nolting, S., Bennett, G., Saltzmann, K. and Hamm, R.L., 2013. A quantitative analysis of a modified feeding method for rearing Cimex lectularius (Hemiptera: Cimicidae) in the laboratory. Pest management science, 69(10), pp.1115–1120.

Ebeling, W., Wagner, R. E. and Reierson, D.A., 1966. Influence of Repellency on the Efficacy of Blatticides. I. Learned Modification of Behavior of the German Cockroach, Journal of Economic Entomology, Volume 59, Issue 6, 1 December 1966, Pages 1374–1388, https://doi.org/10.1093/jee/59.6.1374

Fardisi, M., Gondhalekar, A.D., Ashbrook, A.R. and Scharf, M.E., 2019. Rapid evolutionary responses to insecticide resistance management interventions by the German cockroach (Blattella germanica L.). Scientific reports, 9(1), p.8292.

Singh, N., Wang, C. and Cooper, R., 2015. Role of vision and mechanoreception in bed bug, Cimex lectularius L. behavior. PLoS One, 10(3), p.e0118855