Another look at pheromonal or related attractants for leaffooted bugs (*Leptoglossus* spp.) infesting California nut crops

Project No.: **ENTO18.Millar**

Project Leaders: **Jocelyn G. Millar, Dept. of Entomology, University of California, Riverside CA 92521. Ph 951-827-5821. Email millar@ucr.edu**
Houston Wilson, Dept. of Entomology, UC Riverside.

Project Co-Investigators and Personnel:
Kent Daane, Division of Environmental Science, Policy, and Management, UC Berkeley.
Sean Halloran, Dept. of Entomology, UC Riverside.

A. Summary *(In laymen's terms – emphasize key findings and recommendations)*
To date, we have shown that sexually mature *Leptoglossus zonatus* produce a blend of 9 compounds which are likely to be their aggregation pheromone. *L. clypealis* males produce a similar blend. We have identified 8 of the 9 compounds, and are working on the identification of the 9^{th} and final compound. A first attempt at identification was not successful because of an insufficient amount of the purified compound. Thus, much of this past year has involved preparing and combining ~100 headspace extracts from groups fo sexually mature males to collect a larger amount of the 9^{th} compound, and purifying it for nuclear magnetic resonance analyses, which were run in the first 2 weeks of December. The resulting spectral data files are still being interpreted. Once it has been identified, the compound will be synthesized in multigram quantities for field trials. We are also developing a more efficient synthesis of another of the compounds, *cis*-α-bergamotene, which is not commercially available. In other studies, bioassays intended to determine whether differences in the cuticular hydrocarbon profiles of summer and winterform bugs contribute to winter aggregations were not successful, probably due to the assays being conducted in relatively small petri dishes so that they could be video taped. These assays will be repeated in the coming year, using larger bioassay chambers so that the bugs will be more likely to exhibit natural behaviors.

Field studies were conducted in parallel to identify and develop the use of a suitable trap for leaffooted bugs (LFB). Comparison of multiple trap types in 2017 indicated that a black hanging panel-trap was relatively attractive to LFB adults. This trap could likely be even more effective if paired with a lure or bait, which is in the process of being developed (see above). Until then, subsequent work has evaluated the effect of trap color and position across multiple orchard types, as LFB are known to move across almond, pistachio, and pomegranate orchards over the course of the season. Field studies in 2018 and 2019 indicated that in some cases yellow, blue, and green traps were more attractive than a black trap. Furthermore, use of unbaited traps at the orchard edge were ineffective at detecting LFB colonization of almond trees. While our work to date demonstrates that the hanging-panel trap itself has some valence on LFB, the addition of a pheromone or other attractant lure could greatly increase its efficacy as a tool within an IPM program for LFB.

Finally, there is some evidence from *L. occidentalis* that LFB adults respond to infrared (IR) cues and may use them to locate suitable hosts. As such, laboratory assays were conducted to evaluate the response of adult *L. zonatus* to a strong and weak IR cue. Response to the IR

cues were variable, but this may have to do with the strength and positioning of the cue itself. Additional studies are planned in 2020 to further evaluate this.

B. Objectives

1. Finish identification and synthesis of the 9th and final compound produced by summerform male *L. zonatus (*LZ*)*. (Millar)
2. Bioassay the reconstructed blend of compounds produced by summerform male LZ. (Millar and Wilson)
3. Identify, synthesize, and bioassay the analogous blend of compounds produced by summerform male *L. clypealis*. (LC) (Millar and Wilson)
4. Bioassay summer- and winterform bugs against each other, to verify that cuticular hydrocarbons are involved in maintaining overwintering aggregations. (Millar)
5. Continue optimizing trap characteristics, including color, with and without attractant lures. (Wilson).
6. Test attraction of LZ to infrared radiation. (Wilson)

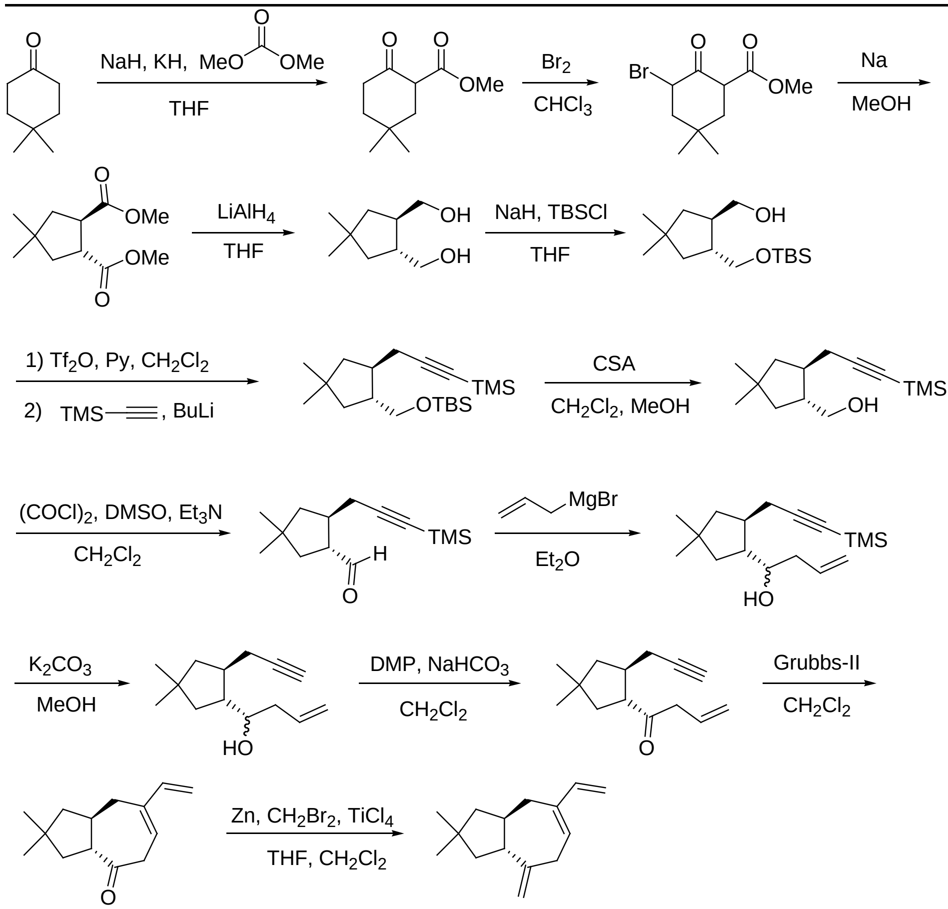
C. Annual Results and Discussion

1. Isolation, identification, and synthesis of the 9th compound produced by sexually mature males:

In work to date, we have shown that sexually mature summerform males of both LZ and LC produce blends of compounds that are the likely attractant pheromones of these species. The two species produce blends which are similar but not identical. However, we have not yet identified the component in extracts from males that produces the strongest antennal responses in electrophysiological assays, i.e., the compound that is most likely to be a crucial component of the active blend. Importantly, the same compound is produced by males of both species. In 2018, we were able to isolate a few micrograms of this compound, and by a combination of spectroscopic methods and microchemical tests, we were able to narrow the structural possibilities down to less than 10 compounds, but because of the small amount available, we were able to pinpoint the specific compound. At that point, we decided to make what we considered the most likely compound, which turned out to be substantially more difficult than anticipated. The resulting multistep synthesis is shown in Scheme 1. To our frustration, the spectra of this compound were similar to but did not exactly match those of the insect-produced compound. Because of the difficulty and length of time required to make this compound, we have suspended work on making additional possibilities until we can get more detailed spectra from a second sample of the unknown compound (see point 2 below).

2. Isolation of a larger sample of the unknown compound, to obtain more detailed nuclear magnetic resonance (NMR) spectra.

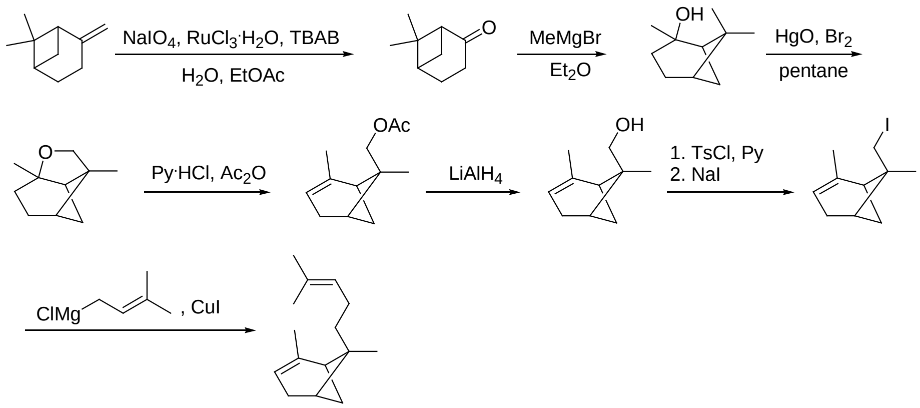
In parallel with the synthetic efforts described above, and to hedge our bets, we continued to collect and stockpile the volatiles produced by cohorts of sexually mature summerform LZ, to make another effort at purifying an amount sufficient to get full NMR spectroscopic data, and allow us to definitively identify the compound. Of these extracts, 82 contained detectable amounts of the unknown. These 82 extracts were pooled, carefully concentrated so as not to lose the volatile unknown, and fractionated by liquid chromatography. Multiple types of NMR spectra were taken on the purified compound during the first two weeks of December, and we are still working on the interpretation of the resulting spectral data files at the time of writing.



Scheme 1. Synthesis of one of the possible structures of the unknown component produced by sexually mature male *L. zonatus*.

3. Develop a more efficient synthesis of *cis*-α-bergamotene, another key component in the blend produced by LZ males.

In 2017-18, we tried twice to use a previously published synthetic route to prepare *cis*-α-bergamotene, one of the major components in the blend produced by sexually mature male LZ which is not commercially available. This route did indeed produce the desired compound, but it was both lengthly and very low-yielding, producing only a couple of hundred milligrams of impure product after several months effort. Thus, we have just finished a pilot scale synthesis of an improved route to *cis*-α-bergamotene (Scheme 2). This route will now be scaled up to provide several grams of the stereoisomerically pure compound for field bioassays next year.



Scheme 2. Improved synthesis of *cis*-α-bergamotene

4. Mating bioassays with mixed winterform and summerform bugs.

Typically, leaffooted bugs in winter aggregations do not mate, whereas sexually mature summerform bugs mate repeatedly. The bugs are coated with blends of waxy cuticular hydrocarbons, and these blends are both species- and sex-specific. Thus, when a male bug touches a female bug with his antennae, these compounds tell him immediately if she is the same species as him, and that she is a female. When we analyzed the cuticular hydrocarbons (CHCs), we found differences between the CHCs of summer- and winterform bugs, and these differences may be at least partly responsible for the lack of mating in winterform bugs. As a first test of this hypothesis, we wanted to pair winter- and summerform males and females in all possible combinations, for example, to see whether summerform males recognized winterform females as being females, and vice versa. Thus, mating trials were carried out between January and March, using winterform LZ collected in Fresno in winter, and summerfrom insects from our lab colonies. Pairs of males and females were placed in petri dishes, which were lined up in groups of 10 under a video camera programmed to take a photo once per minute for 8 hours. The photos were then examined for mating activity.

A total of 17 bioassays were carried out over this three month period, 14 running from 11:00-19:00, and 3 starting at 16:00 and running to 24:00 to include 4 hours of darkness. For 16 of the bioassays each dish contained only one mixed sex pair, whereas one bioassay was run with 3 males and 3 females in each dish. For three assays, a wooden dowl was added to each dish in case additional substrate was needed for insects to transmit vibrational signals. Across the bioassays, different combinations of insect sex and seasonal-stage were combined. The majority of the bioassays were winter males vs. summer females or vice versa. During each bioassay at least two dishes contained a pair of summerform bugs as positive controls (mating would be expected), and during several of the assays a pair of winterform insects were used, as negative controls (mating would <u>not</u> be expected).

However, overall the results were indeterminate because of the 54 “summer male vs summer female” bioassays only 8 pairs (15%) of *L. zonatus* were observed mating. Only a single pair of “winter male vs summer female” were observed mating, and two pairs of “summer males vs winter females” were observed mating. The relatively low level of mating observed in the positive control summer vs summer pairings indicates that the petri dishes were unsatisfactory arenas for these assays. It was not possible to conduct further assays because by the end of March, the winterworm *L. zonatus* began to revert back to the summerform. These bioassays will be repeated in the coming year, using more appropriate bioassay cages.

5. Establishment of a colony of LZ from Florida, to check for the presence of cryptic species.

There is some question as to whether or not *Leptoglossus zonatus* is one species, or two cryptic species, as suggested by recent genetic work by Joyce et al. (2017). The case for two cryptic species was further supported by a recent publication on the putative pheromone of *L. zonatus* from Brazil, which reported that Brazilian LZ produced a different blend of volatiles (Inoue et al. 2019) than we are seeing with our California colonies. Thus, we did not want to get blindsided by identifying pheromones for one LZ species, when there may actually be two. As a check on this, Russ Mizell (U. of Florida) sent us LZ collected from Florida citrus. We obtained the appropriate USDA permits, established a colony of the Florida bugs in the UCR quarantine facility, and reared them through a generation to produce sexually mature summerform males. Volatiles were collected 12 different groups of these males, and proved to be identical to those produced by our California LZ, providing some reassurance that there may be only one pheromone strain in the US, even if there are different genetically identifiable haplotypes or strains.

6. Optimizing trap characteristics, including color, with and without attractant lures.

Work in 2017 determined that an unbaited black hanging-panel trap was attractive to LFB and thus could be a useful tool to evaluate candidate pheromone lures as those were developed. Additional efforts in 2018 and 2019 attempted to see just how useful an unbaited trap could be by (1) comparing LFB capture across an array of different trap colors and (2) comparing LFB capture in traps at the orchard edge and interior with populations in the tree canopy. The idea is to see (1) if trap color increases attractiveness and (2) can unbaited traps at the orchard edge provide any information about the timing of orchard colonization and/or LFB populations in the crop canopy.

In 2018, LFB capture in unbaited black hanging panel traps positioned at the edge of multiple almond, pistachio and pomegranate orchards did not reflect populations in the crop canopy. In 2019, pairs of unbaited black hanging-panel traps were setup at the edge and 50 yards away from five almond orchards with persistent spring LFB populations. The idea here was to see if a trap 50 yards out from the edge (in an open field) could detect LFB as they moved towards the orchard edge. Trap capture was overall very low, even though there were notable LFB populations in the crop canopy at all sites. Thus, unbaited traps did not reflect the timing of LFB colonization of almond trees.

Trap color preference was first evaluated in a pomegranate orchard in fall 2018 (Oct. – Dec.). Here, LFB adults exhibited a strong preference for yellow traps, followed by blue and green traps. In 2019 the colored traps were set out sequentially in almond (Mar. – May), pistachio (Jun. – Sept.) and pomegranate (Sept. – Dec.) blocks. LFB capture was very low in almonds and so no preferences could be determined. Many more LFB adults were subsequently recovered in pistachio and pomegranate. Surprisingly, there was no clear color preference observed in the pistachio orchard. In the pomegranate block, LFB catch was numerically higher in yellow and blue traps, but there was not a statistically significant difference amongst the different trap colors.

Test attraction of LZ to infrared radiation

A previous study documented that another species of *Leptoglossus* responded to infrared

(IR) cues and appeared to utilize them to locate host plants. As such, a preliminary trial was run to evaluate LZ response to IR. A modified two-choice chamber was constructed based on the experimental design used in the other study, and LZ adults were given the opportunity to choose between a low and high IR source. Response to IR was unclear, but this may be related to the design of the assay and what levels of IR are most biologically relevant to LZ. Additional work is now underway to better understand these factors and follow-up experiments are planned for 2020.

D. Outreach Activities

Wilson, January 9, 2019 "Pheromonal and Related Attractants for Leaffooted Bug in California", Orchard Pest and Disease Management Conference (Portland, OR) Presented an update on our research to date to approximately 60 scientists and PCAs who work in orchards across the West Coast.

Wilson, April 17, 2019 "Leaffooted Bug Pheromones and Related Attractants" , IPM Breakfast Meeting, UCCE North San Joaquin Valley (Modesto, CA). Presented an update on our research to date to approximately 45 growers and PCAs in the North San Joaquin Valley.

Wilson, July 25, 2019, "Biological Control of Navel Orangeworm and Leaffooted Bugs" Rivercamp Firebaugh, San Joaquin River Parkway Conservancy and Trust (Firebaugh, CA) Outreach and education event for approximately 70 youth campers aged 6-12. Provided an overview of LFB life-history, damage to almonds, and explained our on-going work with pheromones, traps and parasitoid ecology.

E. Materials and Methods:

Identification and synthesis work:

Colonies of *L. zonatus* were maintained continuously at UCR, and volatiles were collected from ~100 cohorts of sexually mature male bugs. 82 of these collections contained the compounds of interest, and were combined, concentrated, and fractionated by liquid chromatography. The purified 9th compound will be reanalyzed by microbore NMR spectrometry during the first week of December.

Standard synthetic methods were used to develop a synthesis of one of the ~6 possible candidates for the 9th compound, and a new route to another of the male-produced compounds, *cis*-α-bergamotene.

Bioassays of combinations of mature summer- and winter-form bugs were carried out in pairs, in glass petri dishes, using a video camera to record the number and duration of copulations between the various paired combinations.

Field Experiments – Orchard Colonization and Trap Color Preference

Almond Orchard Colonization

Early season LFB populations were monitored March-May in five almond orchards with consistently high spring populations of LFB. At each site, a black hanging panel trap was positioned at the orchard edge and in an open field 50 yards away. Traps were serviced weekly and all adult LFB were collected, identified and sexed.

Trap Color Preference

Influence of trap color was evaluated in almond, pistachio and pomegranate over the season. Trap colors included black, white, red, yellow, green, and blue. LFB adults were monitored sequentially over the growing season in five almond orchards (Mar. – May), then in one pistachio orchard (June – Sept.) and finally in one pomegranate orchard (Sept. – Dec.). Traps were serviced weekly and all adult LFB collected, identified and sexed. An improved version of this study would monitor colored traps in each orchard type over the entirety of the season, rather than sequentially, but these colored panel traps were customed ordered and there is only a limited supply of them for the 2019 season.

Laboratory Study – Response to Infrared Radiation

In the laboratory, adults were introduced into a modified two-choice chamber and given the option to choose between a low or high source of infrared radiation. Adults were acclimated to environmental conditions for 1-4 hours prior to being placed into the experimental chamber. Once in the chamber, adults were given 15 minutes to make a decision, at which point the trial for that individual was terminated. Results to date have been inconclusive, but may be related to the design of the experimental chamber and the strength of the IR source. Additional work is underway to better determine biologically-relevant levels of IR and to create an improved chamber for this work.

F. Publications that emerged from this work

The laboratory and field research described above is in progress, and not yet ready for publication.

References cited:

Inoue, K.M., Vidal, D.M., Saad, E.B., Martins, C.B. and Zarbin, P.H., 2019. Identificaton of the Alarm and Sex Pheromones of the Leaf-Footed Bug, Leptoglossus zonatus (Heteroptera: Coreidae). *Journal of the Brazilian Chemical Society*, *30*(5), pp.939-947.

Joyce, A.L., Higbee, B.S., Haviland, D.R. and Brailovsky, H., 2017. Genetic variability of two leaffooted bugs, *Leptoglossus clypealis* and *Leptoglossus zonatus* (Hemiptera: Coreidae) in the central valley of California. *Journal of Economic Entomology*, *110*(6), pp.2576-2589.