Identification, Synthesis, and Field Evaluation of the Sex Pheromone from the Ten-Lined June Beetle

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Interpretive Summary:

Isolation and identification of the TLJB pheromone is underway. Females produce such minute amounts of pheromone that samples have to be pooled for chemical analysis. Insects were field-collected throughout the summer, pheromone was extracted by multiple approaches, and the collected extracts pooled and fractionated by silica gel column. The peak of the active compound was identified by a combination of antennal activity (electroantennogram) and field behavioral assays. While isolation of the pure compound for chemical identification is slow and tedious, the retention time of the active peak was marked by standards so that isolation could be continued off-season. A sample of the active peak was collected by preparative GC from thousands of injections of the active fractions which were obtained during the last field season. The isolated compound was analyzed by GC-MS. Detailed analysis of the mass spectrum suggested a novel structure. As usual, elucidation of novel compounds requires further analytical and chemical analysis which will be continued with samples collected in the next season(s).

Objectives:

1. Isolation and identification of the pheromone produced by TLJB Females
   a. Obtain a large number of field-collected females.
   b. Extract natural pheromone from field-collected females.
   c. Isolate the active fraction.
   d. Isolate pheromone to purity by Preparative Fraction Collection.
   e. Determine the chemical structure of the pheromone by GC-MS, GC-FTIR, and microanalytical chemistry.

2. Synthesize new pheromone.

3. Evaluate synthetic pheromone in the field.
   a. Develop pheromone traps with slow-release devices.
   b. Conduct field evaluation and exploratory tests of control by mass trapping and mating disruption.

Materials and Methods:

Females were collected by hand in the field during the short flight activity period (about a half hour per night for 2 months) in summer, 2006. Males were captured in black light traps. To obtain more females, we also collected grubs and pupae by digging them from the soil around almond trees weakened by TLJB. These immature insects were brought to the lab, and kept at 25°C in individual ice cream cups filled with moisten sand. The larvae were fed on fresh carrot and inspected every other day until they pupate. The ice cream cups containing pupae were handled carefully to avoid damage while keeping soil moist. Emerged adults were sexed and placed on large plastic cases. After completing development (7-10 days), females were used for pheromone extraction.

Pheromone was extracted by three different methods: solid-phase micro extraction (SPME), airborne volatile collections (aeration), and whole-body extraction. Extracts were evaluated by behavioral field tests (male attraction and sexual response) and/or antennal activity (EAD or GC-EAD response). Whole-body extracts and aeration collections were fractionated by open silica gel column by eluting with mixtures of hexane and ether of increasing polarities. Active fractions were pooled, injected multiple times into a gas chromatograph system in which the EAD-active peak was isolated and pooled for chemical identification (Preparative Fraction Collection).

In general, structure elucidation of pheromones is approached first by gas chromatography-mass spectrometry (GC-MS) with pooled samples. If a pheromone structure is not too complex or novel, it may by possible to determine it by GC-MS analysis alone or in combination with chemical derivatization (small scale chemical reactions). If the structure is complex, unusual, and/or novel, further analytical tools (e.g.: GC-IR) may be necessary. While the PI's lab is equipped for in-depth chemical analysis, GC-IR and additional techniques are less sensitive than GC-MS and thus require even more isolated pheromone sample. Once a chemical structure is determined, identification is completed by synthesis and field evaluation.

Results and Discussion:

The PI, co-PI, and a team of 11 collaborators, including postdocs, undergraduate students, graduate students, and volunteers took over 20 night trips to infested almond orchards near Manteca to collect females. Catching females is labor intensive as they are collected by hand after following males flying towards a calling female. This has to be done during a very short time window of flight activity that occurs each evening that lasts for about 30 min sometime between 8 and 10 PM. The flight activity in 2006 started effectively in July and ended in early August, which was shorter than in previous years, probably because of the cooler and wetter than normal Spring. TLJB population was also very low, with a maximum of 20 females captured per night in total by all individuals, as compared to almost 80 per night in the previous year. While we collected over 700 females in the flight season of 2005, this year we were able to collect only 198 females.

In an attempt to obtain more females, we took multiple trips to Fresno Co. prior to the flight season to dig soil close to damaged almond trees and collect grubs (larvae) and pupae. Mortality of grubs and pupae collected in the field was so high that only a handful of adults have emerged. Only 8 adults emerged from 290 field-collected grubs. The availability of grubs, however, let us test the hypothesis that pheromone is also produced by immature insects, as it has been previously observed with two other scarabs in the genus *Cyclocephalla*, the Southern and Northern masked chaffer. When stimulated with grub extracts, male antennae of the TLJB showed no EAG activity thus indicating that there is no trace of pheromone produced by TLJB grubs. Previously, the PI has tested this hypothesis with over 10 species of scarab beetles and found that it is true only for *Cyclocephalla* species.

We observed that the yield of pheromone extraction was the highest with whole-body extraction, followed by aeration and SPME. While SPME was a clean procedure, the entire pheromone collection is used up in one single injection. To compensate for the fewer number of females available this year, we intensively collected pheromone from the field-collected females by aeration before extracting the same individuals with hexane. Although the yield of pheromone extraction is lower in aeration than in whole-body extraction, the former is non-invasive while the latter allows only one extraction. Aeration and whole-body extracts were pooled and tested for pheromonal activity in the field and for EAD activity by GC-EAD. These crude extracts were pooled and fractionated by open column chromatography. Active fractions were tested by GC-EAD and pooled for preparative GC.

In order to continue the work off-season, we marked the retention time of the active peak by using hydrocarbon standards. In that way, we could continue the isolation of the active compound when males were not available for GC-EAD measurements. We have acquired an automated fraction collector that allows the effluent from GC to divert at a given retention time to a U-shaped glass tube on which the target peak (pheromone in our case) is trapped. Once enough material was pooled, the compound was extracted from the collector with hexane, the concentrated material was recovered and injected into our newly refurbished GC-MS. Detailed analysis of the mass spectrum obtained

with all samples pooled indicated that the pheromone has a chemical structure new to science. This will require further chemical analysis (microderivatizations) and additional chemical analysis. It may be necessary to obtain infrared spectrum of the compound by GC-FTIR to determine its chemical structure before the pheromone can be synthesized.