BARD Research Grants
Guidelines and Regulations for Grant Management (Annex A)

Final Scientific Report – Cover Page (G6a)

BARD Project Number: US-4410-11C

Title: Optimization and field-testing of synthetic sex attractants for two psyllid pests of pears
(Hemiptera: Psyllidae)

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Investigators	Affiliated Institutions
David R. Horton (PI)	USDA-ARS
Victoria Soroker (Co-PI)	ARO, Volcani Center
Peter Landolt	USDA-ARS
Jocelyn Millar	University of California, Riverside

Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.
Cacopsylla, sex pheromone, hydrocarbon, monitoring, mating effects

Abbreviations commonly used in the report, in alphabetical order:

Budget: IS: \$ 117,000

US: \$ 163,000

Total: \$ 280,000

Signature
Principal Investigator (PI)

Signature
Principal Institution's Authorizing Official

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Final Scientific Report - Publication Summary (numbers) (G6b)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged				
Submitted, in review, in preparation	1 (in prep.)		2 (in prep)	
Invited review papers				
Book chapters				
Books				
Master theses			1	
Ph.D. theses				
Abstracts			1	
Not refereed (proceedings, reports, etc.)		3		

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings			5	5
Longer Visits (Sabbaticals)				

Description of Cooperation:

Cooperation focused on two advances: (1) the realization (prompted by results from Israel) that compounds in addition to the previously identified compound (13Me-C27) might be part of the sex pheromone of the U.S. psyllid (*C. pyricola*); and (2) the realization (prompted by results from the U.S.) that the pheromone components would be soluble in oil and thus possibly amenable to dispensing as a sprayable formulation. These details were discussed between cooperators through e-mails and telephone conversations. These conversations led to U.S. tests of new compounds; and, tests in both countries of a sprayable formulation.

Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted				
Issued (allowed)				
Licensed				

EVALUATION OF THE RESEARCH ACHIEVEMENTS

UNITED STATES

OBJECTIVES OVERVIEW

1. Improve field activity of pheromone
 - a. Evaluate seasonality of male response to pheromone
 - b. Evaluate new compounds suggested by (a) new chemical extractions; (b) observations suggested by V. Soroker (cooperator in Israel)
 - c. Test competing trap designs
2. Develop practical use for pheromone
 - a. Develop and test a sprayable formulation of pheromone
 - b. Test whether pheromone disrupts psyllid mating success
 - i. Laboratory: proof-of-concept
 - ii. Large plot trials

SIGNIFICANT FINDINGS

- (a) Laboratory study showed that post-diapause males failed to respond to post-diapause females until ovaries of females had developed, even while males did respond to the synthetic pheromone; (b) field trial confirmed laboratory trial
- Evaluation of 3 new compounds (prompted by results in Israel as well as new GC-MS work in US) indicated 2 of those compounds were attractive to male psyllids
- Showed that a clear panel trap baited with pheromone captured substantially more psyllids than delta traps baited with pheromone
- Showed that the pheromone mixed with oil and water retained attractiveness to male psyllids in laboratory and field trials
- Proof-of-concept trial in cages suggested that saturation of air with pheromone reduced male success at locating and mating females; however, saturation of potted trees with the sprayable formulation led to the opposite results, with mating rates increasing in the presence of pheromone.
- Efforts to use the sprayable formulation to reduce winterform mating in large field plots were not successful in each of two trials; there was evidence that the sprayable formulation actually led to increased rates of mating

LEVERAGING OF BARD FUNDING

The U.S. team used research results obtained from this BARD project to leverage grant funds from two other sources:

- Washington Tree Fruit Research Commission (FY 2014-2015): Tests of a sprayable pheromone formulation against winterform psylla (\$19,000)
- Western Region IPM Grants Program (FY 2015): Disruption of pear psylla with a sprayable sex attractant: new technology for pear IPM (\$23,844)

These funds were used to support work with a sprayable formulation of the pear psylla pheromone (**Objective 2b**).

DETAILS OF RESULTS

Objective 1a. Evaluate seasonality of male response. Field observations prior to this grant suggested that post-diapause winterform psylla (*C. pyricola*) became attractive to males once

ovaries had matured to stage 4 (stage preceding first mature egg). This hypothesis was examined in the laboratory in January-April 2011-2012, and was confirmed (Figure 1; upper panel shows the 2011 data, lower panel shows the 2012 data). The left panels show olfactometer results using field-collected females as the attractant; numbers in bars show mean ovarian development and spermatophores per female. The seasonality in attractiveness was due to seasonality in female production of pheromone and not to seasonal changes in male behavior, given that early-season males were attracted to the synthetic form of the pheromone (right panels in Fig. 1).

Objective 1b. Evaluate new compounds. Re-examination of GC-MS profiles for summerform psyllids (prompted by results from the Israeli cooperator) led to the discovery that two other chemicals (11-methylheptacosane, 9-methylheptacosane) co-occurred with the 13-methylheptacosane peak (the latter being the previously identified pheromone). We conducted olfactometer trials to examine attractiveness of these additional compounds. The 11-MeC27 compound was indeed attractive to summerform psyllids (Figure 2, second bar from top); the 9-MeC27 compound was not attractive. Addition of the 11-MeC27 compound to the 13-MeC27 compound, however, did not improve attractiveness of the pheromone over the 13-MeC27 compound alone (Figure 2: bottom bar).

Objective 1c. Test competing trap designs. A field trial was done in March 2012 to examine effects of trap

Figure 1. Response of field-collected males to field-collected females (left panels) or synthetic pheromone (right panels) in the 2011 (upper) and 2012 (lower) seasons. Numbers in bars indicate mean ovarian scores and spermatophores per female.

Figure 2. Olfactometer results with synthetic compounds. Red indicates significant preference.

type (Figure 3: photographs) on catch of male psyllids. An interception trap composed of clear screen plus pheromone captured more male psyllids than the other traps (Figure 3: bar chart). The delta trap did very poorly, despite presence of the pheromone lure.

Objective 2a. Develop a sprayable formulation.

Because 13-MeC27 is a hydrocarbon, it is fully soluble in oil, suggesting that field application of the pheromone might be possible by spraying the compound rather than by using pheromone-saturated septa or other release-dispensers. We examined in a series of assays whether 13-MeC27 in oil was biologically active. Olfactometer trial. We examined response of male winterform psyllids to oil-treated shoots vs oil+pheromone-treated shoots. Shoots were treated with 1% oil or 1% oil+pheromone, with rates chosen to provide a signal of about 50 female equivalents on the oil+pheromone side of the olfactometer. Two trials, each with 100 males (10 runs x 10 males per run) were conducted. In both trials, a statistically significant number of males chose the pheromone side of the olfactometer (Figure 4).

Physical contact assay. We next examined whether treatment of shoots with oil+pheromone led to increased time males spend in physical contact with the shoot. Shoots treated with 1% oil or 1% oil+pheromone (concentration of pheromone as in the olfactometer trial) were paired in small circular arenas (15 cm in diameter). Shoots were placed with cut ends in water, 5 cm apart. Five winterform males were added to each chamber, and location of each male was recorded at 10 minute intervals over a 2 hour assay period. The assay included 20 replications of 5 males each. Mean cumulative number of male psyllids in contact with the pheromone-treated shoot was significantly larger than number of males in contact with the oil-only shoot (Fig. 5).

Field trials with oil+pheromone formulation. Field trials were done twice each in the winterform generation (April) and summerform generation (June and July). Paired shoots were sprayed with 10 ml of 1% oil in water or 1% oil+pheromone (n=10 pairs). Volume of pheromone per shoot was enough to provide a signal equivalent to about 50 females. Shoots were each about 18 inches in length. Each shoot was then encircled with a mesh cylinder that had been coated with tangletrap (Figure 6). Traps were left in the field for 3 days. The winterform trial was done at mid-generation (April 1) and late in the generation

Figure 3. Photographs of trap types (upper panel), and number of winterform males per trap in March 2012 field trial (lower panel).

Figure 4. Olfactometer results

(April 13). In the early-April trial, we found a substantial and highly significant increase in numbers of male psyllids on pheromone-treated shoots compared to oil-only shoots (by almost 20 psylla per trap; Figure 6: top center panel). Females showed no preferences. Psyllid numbers declined substantially by the second trial in mid-April (Figure 6: bottom center panel), and males were only marginally more abundant in the pheromone treatment. For summerforms, numbers of males were significantly higher in the pheromone treatment than oil treatment in the June study (by about 15 psyllids per trap; Figure 6: top right panel), but showed no attraction to the pheromone-treatment in the July study (Figure 6: lower right panel). We had a large outbreak of defoliating sawflies in late June, and it is unclear what effect the extensive foliar damage had on psyllid behavior.

Figure 5. Physical contact with treated and control shoots

Figure 6. Mesh cylinder (with tanglefoot) encircling treated shoot. Bar charts show winterform (center chart) and summerform (right chart) attraction to oil+pheromone treated shoots vs oil-treated shoots.

Objective 2b(i). Proof-of-concept demonstration that saturation of air with pheromone volatiles slows mating success.

We examined whether saturation of a cage with 13-MeC27 slowed mating rates (numbers of spermatophores passed) of winterform *C. pyricola*. Unmated psyllids were collected from a pear orchard in late winter before mating had begun in the field. We used paired cages (2 x 2 x 2 foot) enclosing a mix of pear seedlings and pear cuttings (Figure 7). One cage received 3 septa, each loaded with 50 female-equivalents of 13-MeC27; septa were placed on the floor of the cage. The second cage received 3 solvent-treated septa. We added 50 females to each cage, and allowed them 24 hours to settle. After 24 hours, 50 males were added to each cage. After

another 24 hours, 25 females were randomly collected from each cage and dissected to determine numbers of spermatophores (=number of matings). The remaining 25 females were removed 24 hours following the initial removal. Four trials were done over a 3 week span of time. Results showed that saturation of cages with 13-MeC27 led to a statistical drop in number of spermatophores passed (compare red bars [=disruption cages] with gray bars [=control cages] in Figure 7). A second set of trials was done using the sprayable formulation of the pheromone. Two trials (one with summerforms, one with winterforms) were done. We show results for the summerform generation; the winterform trial produced similar results. Four pear trees (each approximately 5 foot tall) in pots were treated either with 1% oil or with 1% oil+pheromone. Each tree received approximately 50 ml of either oil or the oil+pheromone formulation. The trees were placed in large (8 x 8 x 8 foot) cages out-of-doors (Figure 8). The paired trees within each cage were of the same treatment. We added 100 virgin female summerforms to each cage, by attaching vials with the appropriate number of psyllids directly to the trunks of both trees in each cage (Figure 8). Females were allowed 6 hours to settle on the trees (confirmed by examination of cage walls at 6 hours); 100 male summerforms were then added to each cage. After 48 hours, we harvested all living psyllids from the walls of the cages and from both trees within each cage. Recovery was approximately 50% of females and 30% of males; recovery rates were identical in the two cages. Females were dissected to determine mating status (number of spermatophores). Results showed that spermatophore numbers summed across 50 females per cage were actually higher in the pheromone-treatment than the control treatment (Figure 8A); percentage of females that had been mated (Figure 8B) was also substantially higher in the pheromone cage (75%) than in the oil-only cage (50%). These results contradict our results with septa, suggesting that physical contact with the pheromone (in the trials with sprayable) had a different effect on male behavior than olfactory contact alone (trials with septa).

Figure 7. Cage saturation trial. Bar charts show spermatophore totals in females from "disrupted" cages (red) and pheromone-free cages (gray).

Objective 2b(ii). Large field plot trials with sprayable. We conducted a large field trial with the sprayable compound to explore effects on mating success under field conditions. The sprayable was chosen over septa due to its better fit with pear IPM programs, and to our concern that we would be unable to produce enough point sources to affect psylla behavior by using septa to dispense the pheromone. We additionally confirmed that the sprayable solution had activity for male winterforms under field conditions, re-conducting a mesh-trap study (Fig. 9). As with the

trial in previous years (Fig. 6), male psyllids preferentially accumulated on the oil + pheromone traps (red bars in Fig. 9) compared to the oil-alone traps (Fig. 9). We then used large field plot trials (16 tree plots; tree spacing of 16 x 16 foot; Fig. 10) to examine the effects of pheromone in oil on mating of post-diapause winterform psylla in March 2014 and March 2015. Trees were sprayed to drip (ca. 3 gallons of 1% oil + water, with or without pheromone, per plot; N = 6 plots per treatment; Fig. 10) in March of both years, timed to coincide with onset of mating in the field. Female psylla were collected from the interior four trees of each plot (Fig. 10) and dissected to determine mating status. Probability of being mated was higher both years in pheromone plots than control plots (Fig. 10), a result consistent with results using the sprayable formulation in our large cage trials (Fig. 8). At this time, we cannot explain the increased mating rates in the pheromone plots. One possibility is that presence of pheromone stimulated males to increase their search efforts on treated trees, leading to increased rates of contact with females. However, controlled behavioral trials will be needed to examine this hypothesis.

Figure 8. Large cage study showing pear trees (2 per cage) and psyllid release vial. Bar chart shows spermatophores per 50 females (A) and % of females mated (B) from oil+pheromone trees (red) and oil-only trees (gray).

Figure 9. Mesh traps encircling oil+pheromone treated shoots and oil-alone shoots. Bar charts show capture of male winterforms on pheromone mesh traps (red) and oil-alone mesh traps.

Figure 10. Design of large plot trial showing relationship of oil-alone plots (gray rectangles) and oil+pheromone plots (red). Small square within one of the plots shows the four trees within that plot from which females were collected for dissection. Bar chart shows percentage of females that had been mated in pheromone plots (red bars) and oil alone plots (gray bars).

ISRAEL

OBJECTIVES OVERVIEW

1. Improve field activity of pheromone
 - a. Evaluation of the seasonality of psylla reproduction
 - b. Evaluate the seasonality of female attractiveness (pheromone production)
 - c. Evaluate seasonality of male response to synthetic pheromone components
2. Develop practical use for pheromone
 - a. Test psylla trapping by synthetic pheromone impregnated in rubber septa (provided by J. Millar, cooperator in USA).
 - b. Test a sprayable formulation of pheromone developed by D. Horton (USA cooperator)

SIGNIFICANT FINDINGS

- No consistent seasonality in female attractiveness was observed. Female attractiveness was observed in winter forms in the end December and in May-June in summer forms.
- *C. bidens* has a very distinct but short period of low mating rate, that is associated with low ovarian development.
- This short period of low ovarian development and low mating rate cannot be considered as a reproductive diapause.
- Ovarian development is inhibited by short day photoperiod especially in presence of low temperatures.
- Male attraction to individual synthetic putative pheromone components is not strong and fluctuates seasonally. While 7MeC27 was attractive to males of the winter form, it was not attractive to the summer forms in the beginning of the summer. These data are in accordance with differences in cuticular profiles of winter and summer attractive females vs non attractive ones.
- Cuticular profile of females shows significant seasonality and difference according to their attractiveness to males especially in summer time.
- Differences between attractive and non-attractive females is based on several compounds .
- Female attractiveness in summer and winter is apparently based on a different combination of chemical cues, but 7meC27 is one of the common components to both mixtures.
- These two last findings are likely to be the main reason for our failure to have consistent success in trapping *C. bidens* in the field.
- It seems that a sprayable formulation of pheromone on the pear is better way of pheromone application to attract psyllid then the impregnated rubber septa, but additional trials are needed.

DETAILS OF RESULTS

Laboratory studies

To evaluate seasonality of male response to females two types of olfactometer experiments were conducted: exposure to field collected females around the year and evaluation of male response to synthetic putative pheromone components. Field collected males were exposed to field collected females in the olfactometer bioassay. We have followed male response to females for about 3 years. Between March and June the experiment rarely took place due to very low psylla populations in the field. Figure 11 shows olfactometer results using field collected males and females. Although we made an effort to keep the psyllid at natural conditions until the bioassay and kept females and males separate for at least 24 hours only on some of the dates was male attraction to females significant. In particular significant

attraction was observed in the periods of November-December 2013 and 2014. The timing of female attractiveness during the three consecutive seasons did not match well and in winter 2012-2013 the attraction was reported only in January in 2011. When these data are compared with the level of ovarian development and mating status of the field collected females (Fig. 12), it is clear that very dramatic changes in ovarian development and mating rate occur very consistently between November and January. This period is characterized by steep decreases in mating rate followed by steep increases. The timing of initiation of ovarian development was similar, starting around the 20th of December. Subsequently, males were not attracted to females except for one week in February 2012. This could indicate loss of attractiveness of females associated with mating. Additional periods of attractiveness were detected in the summerforms in May-June and September. Some changes in both mating and ovarian development observed during the beginning of the summer were also associated with some male attraction. During most of the year females collected in the field are mated with ovaries ready for oviposition (Fig. 12) and male attraction to females is therefore inconsistent.

Figure 11. Response of field collected males to five field collected females in olfactometer from October 2011 up to June 2014. The data are an average of 4 to 12 replicates of 10 males each. Stars above the bars indicate level of significance in X^2 test: *- $p < 0.05$.

Figure 12. Monthly changes in ovarian development and mating rate. The data are average ovarian state and percentage of mated females. N=459

Data confirmed once again that a period of reproductive diapause followed by post diapause male female attractive period do not occur in *C. bidens* under our environmental conditions. The observed sharp changes in reproductive state during winter at quite precise timing suggest it to be a relic of a reproductive diapause that is activated by a combination of photoperiod and temperature. Biological significance of this phenomenon under Mediterranean conditions is not clear. These patterns appear to be quite different from the response shown by other pear psyllids at higher latitudes, in which short-day induced diapause prevents both ovarian development and mating for a long interval of time (multiple months) beginning in early autumn. In the laboratory, by manipulating temperature and photoperiod during psylla rearing, we were able to show that short photoperiod mainly induces inhibition in ovarian development. Mating was less affected by these environmental conditions (Fig. 13 a&b).

Figure 13. The effect of photoperiod and temperature on ovarian development (a) and mating (b). The experiment was conducted on psyllids reared since first nymph instar at different controlled environmental conditions. SD15- 10h Dark: 14 h Light at 15°C; SD 25- 10 D: 14L at 25°C; EQ-12D: 12 L at 25°C; LD 25-14 D: 12L at 25°C; LD15-14 D: 10L at 15°C.

As female attractiveness is likely to change with age and reproductive status, response of wild collected males was tested against standard stimuli, cuticular components that were suspected as components of

female pheromones: synthetic hydrocarbons and one aldehyde (provided by US partners). In an olfactometer, males were presented with a choice of 40 nanogram of one of the compounds in hexane relative to hexane (control) at the same volume. More or less consistent attraction was reported in males to 7MeC27, although the male response did fluctuate during the year, to the extent that this compound was not attractive (or was even repellent) during the early summer month June–July (Fig. 14).

Figure 14. Response of field collected *C. bidens* males in Y-olfactometer to synthetic chemicals. The data are average percentage + SE of 6-8 replicates. * $P < 0.05$, # $P < 0.1$ Goodness of fit.

Interestingly, even during one month of December the response of males to 7MeC27 was not stable (Fig. 15). Maximal response is achieved in mid-December when, as was shown earlier, males are most attracted to females. Two other promising compounds in late autumn and winter were 11MeC27 and 13MeC27. None of the tested compounds were significantly attractive in June-July. The other tested compounds were not attractive.

Figure 15. Response of field collected *C. bidens* in Y-olfactometer to 7MeC27 during December 2012. The data are average percentage + SE of 6-8 replicates. * $P < 0.05$, Goodness of fit.

Characterization of seasonal changes in female chemical cues

To follow seasonal changes in female chemical cues cuticular extracts were prepared almost monthly by soaking 20-40 females in hexane for 10 min. A total of 368 extracts were prepared and analyzed by Trace GC 2000, using Restec rsi-5ms capillary column (30m x 0.25 μ m) and subsequently by GCMS Aligent 5975 GC (70 eV). The extract is composed of over 110 peaks. Only consistent components that represent over 1% of total extracts (69 peaks) were used for statistical analysis. We focused on 51 peaks that proved to be significant in PCA of a "chemical bouquet" of attractive vs. non-attractive females by test of communality. Components were identified by combination of retention time comparisons vs. straight-chain alkane standards, retention indices and interpretation of their mass spectra. In a principal component analysis, separation by season is clear (Fig 16). Subsequently, stepwise discriminant analysis

analysis was performed on selected cuticular components of attractive and non-attractive females. Very clear separation appears between attractive and non-attractive females in summer. These both appear very different from both attractive and non-attractive winterforms that are much less separated (Fig. 17a). Discriminant analysis of attractive and non-attractive females by season further emphasize the differences between seasons and the components contributing to the separation between the attractive and non-attractive females at each season (Fig. 17 b&c).

Figure 16. PCA Separation of female cuticular profile from extracts prepared from females collected over 11 month from October 2012 till January 2014, each date is represented by 3-7 extracts prepared from 20-40 females.

Figure 17. Discriminant analysis (stepwise) of female cuticular profile from extracts prepared from dates when females were attractive or not attractive to males in: a. general comparison four groups are compared: SFAT-summer attractive; SFNT- summer not attractive of attractive; WFAT-autumn-winter attractive; WFNT- autumn-winter ; b & c discriminant analysis by season b: discriminant analysis between attractive and none attractive females in winter and autumn ;c. discriminant analysis in summer. Each point is represented by extracts prepared from 20-40 females. In b & c- red dots indicated extracts from attractive females, blue dots represent non-attractive.

Field experiments

Field trials were conducted in a Zova pear orchard. Initially we used "clear screen" traps similar to those suggested by our US partners. Subsequently we have used a spray formulation applied to the branches. All lures were prepared using the chemicals provided again by the US partner and according to their recipe. The same three hydrocarbons tested in the laboratory were evaluated in the field along with a hexane control (Picture 1).

Picture 1: sticky traps with pheromone loaded rubber septa at the center of the screen. The screens were placed between the branches (a) in the winter and (b) in the season; (c) application of spray formulation on the branch covered by sticky net.

Rubber septa were freshly loaded with 300 microliter of hexane containing 100 microgram of compounds each. The experiment started at December 10th and lasted till April 3rd. The traps with 4 different lures (7-MeC27, 11-MeC27, 13-MeC27 and hexane (control)) were randomly distributed along ten rows, spaced by at least one row and about three trees. The position of traps was rotated weekly within each row. Each treatment was replicated 10 times. No significant effect of any of the treatments was found, even though we recorded slightly more males after the first week trapping in traps with 7 or 13MeC27 than in those with 11MeC27 and control. It is possible that timing of attraction was missed as in laboratory experiments significant male attraction was recorded a week earlier. However, not much

Figure 18. Changes in psylla trapping on stick screen traps above branches of the same tree sprayed either with 7MeC27, 13Me C27 or Hexane. The data are average weekly trapping and SE of 10 replicates. A: females trapping data; B: male trapping data. Capital letters indicate significant differences between treatments. Different small letter indicate significant differences between dates Tukey's HSD test, $P < 0.05$.

better attraction of males was found in the next winter from mid Dec to third week of February with sprayable formulation of 7MeC27. However, surprisingly small but significant attraction to females was recorded (ANOVA, $F=5.68$, $p=0.0225$). Two summer experiments, one in July-August and the other in August-September with 7-MeC27 and 13-MeC27 resulted in contradictory outcomes. In the first one females were significantly more attracted to 7-MeC27 (Fig. 18), while in the second trial, males were significantly attracted to 7-MeC27 and females were not (Fig. 19).

Figure 19. Male and Female psylla trapped on sticky screen traps above branches of trees sprayed either with 7MeC27, 13Me C27 or Hexane as control. The data are average weekly trapping and SE of 10 replicates. Different letters indicate significant differences Tukey's HSD test, $P < 0.05$.

Conclusions

It appears that both females' attractiveness and males' responsiveness to synthetic cues change during the year. Female attractiveness apparently is due to a multi-component mixture of compounds that changes during the year. The 7MeC27 and 13Me C27 compounds are part of the profile of attractive psyllids in both seasons, although only small differences in catches of males and females in the traps loaded with the tested compounds vs control were observed with both formulations. This could be well a result of low volatility of the compounds especially in winter time; or, as was already suggested, the additional reason could be that one component is not competitive with the abundant field females. It is also possible that males and females are brought together by a combination of cues, such as chemical cues in combination with acoustic cues. This possibility should be explored. The fact that females are also attracted to sprayable formulation is very interesting and should be further investigated.