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Project Title: Characterization and chemistry of sexual communication in two psyllid pests of pears (Homoptera: Psyllidae)

Investigators

Principal Investigator (PI): David Horton

Co-Principal Investigator (Co-PI): Victoria Soroker

Collaborating Investigators:

Peter Landolt

Anat Zada

Institutions

USDA-ARS, Wapato, WA

Volcani Center, Bet-Dagan

USDA-ARS, Wapato, WA

Volcani Center, Bet-Dagan

Keywords *not* appearing in the title and in order of importance.

Sex attractant pheromone, monitoring, cuticular compounds

Abbreviations commonly used in the report, in alphabetical order:

GC-MS: gas chromatography-mass spectrometry

PC: principal components

SF: summerform

WF: winterform

Budget: IS: \$170,000

US: \$103,000

Total: \$273,000

Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

EVALUATION OF THE RESEARCH ACHIEVEMENTS

UNITED STATES

Significant findings:

- Demonstrated that surface extracts from females are as attractive to males in olfactometer trials as an equivalent number of live females
- Used GC-MS to identify chemicals present in cuticular washes of diapausing and post-diapause male and female winterforms
- Synthesized one chemical (13-methylheptacosane) that was found to occur at levels 3-fold higher in post-diapause female winterforms than male winterforms
- Demonstrated attractiveness of 13-methylheptacosane to male winterform psylla in olfactometer trials and in field trials
- Identified 3 chemicals (including 13-methylheptacosane) that were found to occur at levels 2.7- to 8.6-fold higher in female summerforms than male summerforms

Details of Results:

Olfactometer trials with cuticular extracts (winterform). Pentane extracts of post-diapause winterform female psylla were attractive to male psylla in olfactometer trials (**Figure 1: top chart**). Conversely, males were repelled by extracts of other males (**Figure 1: middle chart**), which appears to be the first example of male-male repellency in any psyllid species. The female extract was statistically as attractive to males as an equivalent number of live females (**Figure 1: bottom chart**).

GC-MS analysis of cuticular extracts from winterform psylla. A GC-MS analysis of cuticular extract from male and female diapausing and post-diapause winterform psylla was done (**Figure 2: upper panel – postdiapause psylla, lower panel – diapause psylla**). We identified many of the peaks in both traces (**Table 1**), and determined quantities of the chemicals in females and males (shown as female:male ratios in **Table 1**). One chemical (13-methylheptacosane) occurred at a level 3-fold higher in females than males for post-diapause winterforms, but was found to occur at similar levels between sexes in diapausing winterforms (**Table 1**).

Olfactometer trials with post-diapause winterforms and 13-methylheptacosane.

Olfactometer trials were conducted with post-diapause winterform psylla and synthesized 13-methylheptacosane (**Figure 3**). Our first assay assessed attractiveness of the chemical (vs. solvent blank) to male and female psylla. Male psylla (but not female psylla) were significantly attracted to the chemical (**Figure 3, top panel**). A second series of assays was then done to compare attractiveness of the synthesized 13-

Figure 2. GC-MS traces of solvent extracts from diapausing female and male psylla (Panel A), and post-diapause female and male psylla (Panel B); in both panels, upper trace is female wash and lower trace is male wash. Peak #11 corresponds to 13-methylheptacosane.

methylheptacosane versus the crude cuticular extract (**Figure 3, bottom panel**). Again, the synthesized chemical was attractive to male psylla when assayed against a solvent control. The crude extract was also attractive. Lastly, male psylla did not discriminate between 13-methylheptacosane and the crude extract, which suggests that the individual chemical was as attractive to male psylla as the full cuticular wash.

Field trial with winterform psylla and 13-methylheptacosane. Field trials were done in late March and early April at the USDA-ARS research farm. Sticky traps, composed of nylon mesh coated with tanglefoot (**Figure 4**), were used to assess attractiveness of 13-methylheptacosane to male and female winterform psylla. Gray septa were used to dispense 3 concentrations of the chemical (10, 100, or 1000 μg of the product); solvent-loaded septa were used as controls. Each treatment was replicated 11 times. Numbers of males and females per trap were determined after 5 days in the field. There was marginally significant evidence that males preferentially accumulated on the 13-methylheptacosane traps during the late March test (**Figure 5, top panel**). More conclusively, our early April trial provided very strong and highly significant evidence that males were attracted to the chemical (**Figure 5, bottom panel**). Females exhibited no obvious patterns in trap catch, other than some weak evidence for avoidance of higher concentrations of the chemical in the first trial.

GC-MS analysis of cuticular extracts from summerform psylla. A GC-MS analysis of cuticular extracts from male and female summerform psylla identified 3 chemicals that occurred at higher levels in the female extract than the male extract (2.7- to 8.6-fold higher in females). The chemical with the largest female:male ratio was again 13-methylheptacosane (data not shown).

Figure 3. Olfactometer trials with 13-methylheptacosane

Table 1. Identification of hydrocarbons and related compounds in cuticular extracts of winterform *Cacopsylla pyricola*. Bold font designates the 13-methylheptacosane.

Peak #	Retention time (min)	Identification	Ratio female/male	
			Diapause	Post-diap
1	16.99	tricosane	1.1	1.0
2	17.86	tetracosane	tr	tr
3	18.15	docosanal	1.1	1.0
4	18.70	pentacosane	1.1	1.0
5	19.21	2-methylpentacosane	1.2	0.6
6	19.36	3-methylpentacosane	1.3	1.1
7	19.51	hexacosane	1.0	tr
8	19.81	tetracosanal	1.2	0.8
9	20.00	2-methylhexacosane	1.0	1.0
10	20.29	heptacosane	1.1	1.2
11	20.53	13-methylheptacosane	1.5	3.2
12	20.59	pentacosanal	1.0	1.1
13	20.77	2-methylheptacosane	1.1	1.2
14	20.85	3-methylheptacosane	1.0	1.1
15	21.04	octacosane	1.0	1.2
16	21.07	unidentified	1.2	1.1
17	21.31	unidentified	1.1	0.9
18	21.36	hexacosanal	1.1	1.0
19	21.51	2-methyloctacosane	0.9	1.0
20	21.81	nonacosane	1.1	1.1
21	22.08	11-, 13-, and 15-methylnonacosane	1.1	1.2
22	22.18	heptacosanal	1.0	1.1
23	22.35	2-methylnonacosane	1.1	1.0
24	22.44	3-methylnonacosane	1.0	1.1
25	22.67	triacontane	1.0	1.1
26	22.75	unidentified	1.2	1.2
27	23.11	octacosanal	1.2	1.1
28	23.66	hentriacontane	1.1	1.1
29	23.97	11-, 13-, and 15-methyl- hentriacontane	1.0	0.8
30	24.31	11,15- and 13,17-dimethyl- hentriacontane	1.2	0.8
31	24.55	unidentified	1.0	1.0
32	24.61	unidentified	1.0	1.0
33	24.76	dotriacontane	1.0	1.1
34	25.40	triacontanal	1.1	1.1
35	26.08	tritriacontane	1.1	1.1
36	26.54	11-methyltritriacontane	1.1	0.9
37	26.97	unidentified	1.3	0.9
38	27.66	tetratriacontane	1.0	1.0
39	29.52	pentatriacontane	1.0	1.0

Figure 4. Trap design for field test

Figure 5. Results of field test

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Significant findings:

- The age of maximal attractiveness in females and behavioral response in males was determined.
- GC-MS was used to identify chemicals present in cuticular washes of females and males summer, winter and "post diapause" form females.
- Significant differences in cuticular semi-volatiles were found between male and female summerforms. The main differences were in quantities of 7-methylheptacosane and 11, 15-dimethylheptacosane that were found to occur at levels 2.5- and 6-fold (respectively) higher in female summerforms than male summerforms. 2-methylpentacosane and 2-methylheptacosane were 3.4 and 2 (respectively) fold higher in summer males.
- Significant seasonal changes in cuticular profiles were observed. The profile of females at three phenological stages was clearly distinct from each other. The profile of "diapausing" winterform females was similar to that of winterform males.
- The cuticular profile of laboratory reared summerforms was similar to that of field collected summer forms. However, significant age associated changes in female cuticular profile were observed.
- In laboratory assays, among the identified compounds, 7-methylheptacosane was attractive to male winterforms but usually not to summer forms. Summerform males were attracted to a mixture of 7- and 13- methylheptacosane. Summer form females were repelled by 13-methylheptacosane.
- Field experiments were inconclusive, but some attraction was observed to a mixture of 7- and 13-methylheptacosane by summer forms.

Details of Results:

In attempts to identify the pheromone of *Cacopsylla bidens* we compared cuticular chemical cues among sexes and physiological stages, reproductive state and sexual attractiveness of summer and winter forms, and behavior of different ages of summer forms.

GC-MS analysis of cuticular extracts from summer and winterform psylla.

Table 2 lists chemicals that have been identified in extracts. It appears that psylla epicuticle contains a series of hydrocarbons, straight chain and branch alkanes, and long chain aldehydes. Retention times of methylalkanes, long chain alkenes and aldehydes, relative to straight chain compounds and diagnostic MS fragments, demonstrated unequivocally the position of methyl branches. To confirm our analysis, we gathered MS data about these compounds from the literature and prepared our own MS database, including retention indices. Nine minor compounds (<5% relative quantity) remained unidentified.

Using the composition percentages of the different elements obtained from the GCMS, 6 principal components (PC) were extracted from the data. Each PC is an independent weighted sum of the original chemical components, and together the PCs explained 67% of the variation in the original data. Although most of the chemicals could be found in all extracts, the PC values summarizing the chemical components were used in discriminant analysis, identifying each extract as belonging to one of the 5 groups (winterform males (WM) and females (WF), summerform males (SM) and females (SF) and "post diapause" winter form females (TF)). For each extract, four (number of groups minus 1) canonical discriminant scores were calculated. Prediction of group allocation based on these scores was correct for 85.7% of all the extracts. In **Figure 6**, we show that the first two canonical scores clearly distinguish between the Male-SF and Female-SF extracts; considerable overlap between Male-WF and Female-WF extracts was noted. On the other hand, the three groups of females (SF, WF and TF) are clearly distinct. These data are consistent with the observation that winterform females are not so attractive to males. As can be seen from psylla dissections most of the mating occurs from January onwards. **Figure 7** shows relative quantities of selected compounds in the 4 groups (winterform females (WF), summerform males (SM) and females (SF) and "post diapause" winter form females (TF)). Discriminant analysis of summerform field collected and laboratory reared psylla (**Figure 8**) reveals high similarity between the two groups, but also difference between 1-4 day old females and the rest of the females' groups.

Table 2. Chemical compounds identified in cuticular extracts of *Cacopsylla bidens* of both sexes and three forms of females (summer form, SM & SF), winter form (WM & WF) and post diapauses winterform females (TF).

Components in bold were significantly higher in groups specified in brackets.

Alkanes	Monomethyl- Alkanes	Dimethyl-Alkanes	Aldehydes
Heneicosane- C21	5-Me-C27 (SF)	5,15 –DiMe-C27	19:Ald
Dodecosane- C22	7-Me-C27 (SF)	5,17 –DiMe-C27	20:Ald
- C21Tricosane	9-Me-C27 (SF?)	11,15 -DiMeC27 (SF)	21:Ald
- C24Tetracosane	11-Me-C27 (SF?)	10,14 –DiMe-C28	22:Ald (TF)
ane- C25Pentacos	13-Me-C27 (SF?)	11,15 –DiMe-C28	23:Ald
- C26Hexacosane	2-Me-C28	12,16 –DiMe-C28	24:Ald (TF)
- C27Heptacosane	11-Me-C28	5,15 –DiMe-C29	25:Ald (SF?)
- C28 (SF)Octacosane	13-Me-C28	5,17 –DiMe-C29	26:Ald (TF)
e- C29Nonacosan	15-Me-C28	11,15 –DiMe-C29	27:Ald (TF?)
Triacontane-C30	17-Me-C28	13,17-DiMe-C29	28:Ald
Hentriacontane-C31	2-Me-C29	12,16 –DiMe-C30	30:Ald
Dotriacontane-C32	3-Me-C29	13,17 –DiMe-C30	
Tritriacontane-C33	7-Me-C29 (TF?)	8,18 –DiMe-C31	
anetriaconTetra -C34	11-Me-C29	11,15 –DiMe-C31	Acetates
Pentatriacontane-C35	13-Me-C29	13,17–DiMe-C31	26:Ac?
	15-Me-C29	10,14–DiMe-C32	
Alkenes	4-Me-C30	11,15–DiMe-C32	
$\Delta(?)$ -C22	12-Me-C30	12,16–DiMe-C32	
$\Delta(?)$ -C25	13-Me-C30	14,18–DiMe-C32	
$\Delta(9)$ -C27	14-Me-C30	5,9 –DiMe-C33	
$\Delta(7)$ -C27	15-Me-C30	5,11 –DiMe-C33	
	2-Me-C31	5,15 –DiMe-C33	
Monomethyl- Alkanes	3-Me-C31	5,17 –DiMe-C33	
2-Me-C23	11-Me-C31	9,13 –DiMe-C33	
7-Me-C23	13-Me-C31	13,17 –DiMe-C33	
11-Me-C23	15-Me-C31	15,19 –DiMe-C33	
2-Me-C24	16-Me-C31	11,15 –DiMe-C33	
2-Me-C25 (SM)	11-Me-C33	11,21 –DiMe-C33	
3-Me-C25	13-Me-C33		
7-Me-C25	17-Me-C33	Trimethyl Alkanes	
9-Me-C25		9,13,17 –TriMe-C33	
11-Me-C25		11,15,19 –TriMe-C33	
13-Me-C25		13,17,21 –TriMe-C33	
2-Me-C26			
3-Me-C26		Tetramethyl Alkanes	
2-Me-C27(SM)		7,11,15,19 –TetraMe-C31	
3-Me-C27 (SF)

Figure 6. Discriminant analysis of cuticular profiles of summer and winter form males (SM and WM) and females (SF and WF) and post diapauses winterform females (TF). Each of the 5 circles gives a 95% confidence interval for the mean of the two scores for the specific group. Points within the circle are clearly predicted as belonging to that group by the discriminant analysis. Each group is composed of 7-9 samples prepared from 20-40 individuals. SF- collected between May and Sep; WF-21 Nov - 7 Feb; TF –"Post diapause" Mid Feb-March.

Figure 7. Selected peaks in cuticular profiles of summer males (SM) and summer females (SF), winter (WF) and post diapause females (TF). Columns related to several compounds means co-eluting peaks in a non-polar GC-MS column.

Figure 8. Discriminant analysis of cuticular profiles of laboratory reared summer form males and females (of different ages) and field collected summer form females (SF). Each of the 6 circles gives a 95% confidence interval for the mean of the two scores for the specific group. Points within the circle are clearly predicted as belonging to that group by the discriminant analysis. Each group composed of 6-9 samples prepared from 20-40 individuals.

Ovarian development of laboratory reared summerform females of different age groups was compared by dissecting at least 20 females per group and categorizing them into 5 stages. **Table 3** shows changes in ovarian development with psylla age. Females 1-4 days old were immature, while both 5-8 and 10-13 days old females contained mature oocytes.

Table 3. The ovarian stage of summer form females of different ages. Data are percent of at least 20 females per group.

Age (days)	no visible oocytes (stage 1)	oocytes are small and white (stage 2)	some oocytes are larger (stage 3)	< 5 developed oocytes (stage 4)	>5 developed oocytes (stage 5)
1-4	28	28	24	16	4
5-8	0	0	0	5	95
10-13	0	0	0	35	65

Ovarian analysis of field collected females from samples that were taken for cuticular extractions, indicates dramatic seasonal changes in reproductive maturity (**Figure 9**). The most pronounced changes were for the winter form, especially between mid-November and January, when most of the females are apparently in rather short reproductive diapause (ovaries not developed and females not mated; **Figure 9**). Subsequently, mating increases and reproductive development commences. By February, all females were found to be mated and had mature ovaries.

Figure 9. The ovarian stage and the mating rate of females field collected from May to March. Females at stages four and five were considered to contain developed ovaries. Data are percent of at least 20 females per group.

Chemicals

R and S13-methylheptacosane were obtained as a gift from Prof. Ryohei Yamaoka and Prof. Kenji Mori. Racemic 7-Me-C27 and 9-Me-C27 and 13-Me-C27 were synthesized by the Israeli partners.

Olfactometer trials

All studies were conducted in a system similar to that operated by the US-partners.

Olfactometer trials with laboratory reared female and male C. bidens. The effect of male and female age on female attractiveness and male response was studied. Females were found to be most attractive to males at 5-8 days old, but were not significantly attractive before and after this period (**Figure 10**). Attractiveness at 5-8 days is apparently correlated with ovarian development (**Table 3**). As was previously shown at this stage most of the females are ready for oviposition and thus also mating.

Figure 10. The effect of female summerform age on the attractiveness to 5-8 day old summer form males in the olfactometer. N= number of replicates of 10 males each. The results are average % males attracted + SEM. * X^2 , $p < 0.05$. The volatile source consists of 5 females each.

The effect of male age on their attraction to females was not very clear. As can be seen in **Figure 11**, significant attraction to females was attained at 5-8 days old and maintained thereafter.

Figure 11. The effect of male age on attraction to 5-8 day old females. N= number of replicates of 10 males each. The volatile source consists of 5 females each. The results are average % males attracted + SEM. * $p < 0.05$; # $p < 0.01$, X^2 .

Olfactometer trials with synthetic compounds using field collected male C. bidens.

Direct evaluation of attractiveness of some the identified female prominent summerform compounds on winter form and summer form *C. bidens* (**Table 4**) shows sex and morph specificity. Since the dose of 40ng was found to be optimal for 7- Me-C27 in winterform males (**Figure 12**), all the other compounds were tested with the same dose.

Male winterforms were attracted to 7-Me-C27 and somewhat repelled by 13-Me-C27 while the summerform males were attracted to the mixture of 7-Me-C27 and 13-Me-C27 and the S enantiomer of 13-Me-C27. Male summerforms, were significantly attractive to 7-Me-C27 ($62 \pm 4.8\%$, X^2 , $p=0.049$) but only when the latter were collected in April (1st generation summerforms), subsequent generations of summerform were indifferent to this compound. Female summerforms were attracted to 7-Me-C27 but repelled by 13-Me-C27.

Table 4. The attractiveness of synthetic compounds (40 ng) to field collected summer and winter form *C. bidens*. Table shows % individuals that chose the arm of the tested compound. Data are average + SEM (n). X^2 , ***= $p<0.001$; **= $p<0.01$; *= $p<0.05$; #= $p<0.1$. NT-not tested.

Compound	Male Winterform	Male Summerform	Female Winterform	Female Summerform
7-Me-C27	66 ± 2.7 (20) ***	52 ± 5.6 (28) ns ^a	NT	57 ± 9.4 (10) *
9-Me-C27	48 ± 5.2 (21) ns	48 ± 4.1 (12) ns	NT	54 ± 6.0 (14) *
13-Me-C27	38 ± 5.5 (12) #	53 ± 5.7 (10) ns	43 ± 12.4 (6) ns	33 ± 13.2 (6) **
(S)-13-Me-C27	63 ± 8.7 (10) **	64 ± 2.38 (6) *	NT	NT
7-Me+ 13-Me-C27	35 ± 9.4 (6) ns	61 ± 5.6 (14) *	NT	58 ± 7.3 (10) ns
7-Me+ 9-Me+ 13-Me-C27	NT	53 ± 2.7 (10) ns	NT	45 ± 8.0 (6) ns

a- Significant attraction was observed in males of first generation summer forms but not thereafter.

Figure 12. Dose response of winterform males to 7-methylheptacosane in olfactometer. *** X^2 , $p<0.001$.

Field trapping data

A number of field experiments were conducted. Most were conducted using essentially the same trap as US partners. Results of summer form trapping conducted between August – October presented on **Figure 13**. Most of the trapped individuals were males and the most attractive traps were those containing a mixture of 7-Me-C27 and 13-Me-C27, thus supporting the laboratory olfactometer tests, though the differences are minor.

Figure 13: Psylla trapped between August 27 and October 6. Data are average and SEM of 7 traps per treatment. The data are a pool of 8 sampling dates.

Another field experiment started at 11 of October the same site with edition of 9-methyl heptacosane treatment. This experiment with a break during December continued until the beginning of March. **Figure 14A**, shows changes in trapping over the period also without significant preferences of specific lures. However when ratio of trapping relative to control are analyzed (**Figure 14B**), seasonal changes in psylla preference begin to occur in November. Initial preference of 9-methyl-C27 and mix of 7- and 13-methyl-C27 of summerform males in October was absent in winterforms from December onwards.

Taken together these data indicate that some attractive components of *C. bidens* female pheromone were identified from GCMS analysis, the key component/s for long range attraction remains to be identified. Moreover, it is also possible that female composition and male responsiveness change seasonally.

Figure 14. Psylla trapping by lures containing 100 μg of either 7-methyl-C27 (7); 9-methyl-C27 (9); 13-methyl-C27 (13), a mixture of 7- and 13-methyl C27 (7+13), or hexane as a control. **A:** Average total psylla trapped in each treatment. **B:** Psylla trapped in each treatment relative to control. All data are average of 7 traps per treatment.

Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged		3		3
Submitted, in review, in preparation			1	1
Invited review papers				
Book chapters				
Books				
Master theses				
Ph.D. theses				
Abstracts				
Not refereed (proceedings, reports, etc.)***		5	1	6

***Includes presentations at scientific meetings

Peer-reviewed publications from grant:

Guédot, C., D.R. Horton and P.J. Landolt. 2009. Attraction of male winterform pear psylla to female-produced volatiles and to female extracts and evidence of male-male repellency. *Entomologia Experimentalis et Applicata* 130: 191-197.

Guédot, C., J.G. Millar, D.R. Horton and P.J. Landolt. 2009. Identification of a sex attractant pheromone for male winterform pear psylla, *Cacopsylla pyricola*. *Journal of Chemical Ecology* 35: 1437-1447.

Guédot, C., D.R. Horton and P.J. Landolt. 2011. Response of summerform pear psylla (Hemiptera: Psyllidae) to male- and female-produced odors. *The Canadian Entomologist* 143: 245-253.

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant. NONE

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings		1	3	4
Longer Visits (Sabbaticals)				

Description of Cooperation:

A meeting was held between Israeli and U.S. cooperators during a visit by Dr. Soroker to the U.S. laboratory in year 2 of the project; cooperators also met informally at three international societal meetings (chemical ecology or entomology) to discuss progress and plans. Israeli and U.S. cooperators corresponded at regular intervals by telephone and e-mail.

Israeli and U.S. scientists co-developed methodology used in obtaining cuticular extracts from each country's respective psyllid, and in identifying female-specific compounds of interest for biological testing. The trap design that was developed by U.S. scientists was used by Israeli scientists in testing putative attractants. The multivariate statistical method developed by Israeli scientists for comparing chemical profiles of male and female psyllids is to be adopted in additional analyses of our GC-MS results by U.S. scientists. Data collected by both Israeli and U.S. scientists were used in writing a second (continuation) grant to BARD, to include the same investigators; that grant was funded (2012-2014).

Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted		1		1
Issued (allowed)				
Licensed				