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Identification of a Sex Attractant Pheromone for Male Winterform Pear Psylla, *Cacopsylla pyricola*

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Abstract Pear psylla, *Cacopsylla pyricola* (Förster) (Hemiptera: Psyllidae), a major economic pest of pears, uses a female-produced sex attractant pheromone. We compared the chemical profiles obtained from cuticular extracts of diapausing and post-diapause winterform males and females to isolate and identify the pheromone. Post-diapause females produced significantly more of the cuticular hydrocarbon, 13-methylheptacosane, than post-diapause males and diapausing females. In olfactometer assays, conspecific males were attracted to synthetic racemic 13-methylheptacosane, whereas females were not, indicating that the behavioral response to this chemical is sex-specific. Furthermore, 13-methylheptacosane was as attractive to males as a cuticular extract of females, suggesting that this chemical was largely responsible for the female attractiveness. A field study showed that males but not females were attracted to 13-methylheptacosane, confirming the olfactometer results. This study provides evidence that 13-methylheptacosane is a sex attractant pheromone for *C. pyricola* winterform males. This is the first identification of a sex pheromone in the Psylloidea. Our results open the path to developing monitoring tools and possibly new strategies for integrated pest management of this insect.

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Introduction

Psylloidea is a superfamily of sternorrhynchous Hemiptera with approximately 2,500 described species (Burckhardt 1994). Over 150 species of psylloids have been reported as potential pests of cultivated temperate and subtropical plants (Burckhardt 1994). Of particular commercial importance are *Diaphorina citri* (Kuwayama) and *Trioza erytreae* (Del Guercio) on citrus; *Cacopsylla pyricola* (Förster), *C. pyri* (Linnaeus), *C. bidens* (Šulc), and *C. pyrisuga* (Förster) on pear; *C. mali* (Schmidberger) on apple; and *Bactericera cockerelli* (Šulc) on potato. Current management practices for these pests rely mainly on pesticide applications. Alternative control methods for these species are needed, and the identification of attractant semiochemicals could provide a basis for new integrated pest management strategies.

Pear psylla, *C. pyricola*, is a major pest of commercial pears, *Pyrus* L. (Rosaceae), in North America and Europe. Pear psylla is a multivoltine, seasonally dimorphic species, with a small, light-colored adult occurring during the growing season ("summerform"), and a darker and larger overwintering morphotype ("winterform") that appears in late summer or early autumn in response to decreasing photoperiod (Oldfield 1970). The winterform, characterized by lack of mating and immature ovaries, overwinters in reproductive diapause (Krysan and Higbee 1990). Diapause ends in December and January, but cold temperatures prevent mating and ovarian development (Krysan and Higbee 1990). In the central Washington state study area,

mating by post-diapause winterforms begins in mid-February as ambient temperatures begin to increase (Krysan and Higbee 1990; Horton et al. 1998, 2007).

The role of chemical signals in psyllid mate location has only recently begun to be investigated. Behavioral evidence for a female-produced volatile sex attractant was reported for the pear psylla *C. bidens* by Soroker et al. (2004). More recently, similar results indicating male attraction to female-produced volatiles were obtained for both post-diapause winterform and summerform pear psylla, *C. pyricola* (Horton and Landolt 2007; Horton et al. 2007, 2008; Guédot et al. 2009), and for the citrus psyllid, *D. citri* (Wenninger et al. 2008).

Previous work with *C. pyricola* post-diapause winterforms showed that cuticular extracts of females were attractive to males (Guédot et al. 2009). These extracts were at least as attractive as a comparable number of live females, suggesting that cuticular extraction might be a suitable procedure for collecting the components of the attractant for analysis and identification (Guédot et al. 2009). Additionally, males were repelled by live males and extracts of males, further evidence that extracts contained sex-specific compounds or blends of compounds (Guédot et al. 2009). Thus, our first objective was to compare the chemical profiles of extracts from post-diapause winterform males and females, to look for differences between the sexes that might indicate putative pheromone components.

Olfactometer bioassays showed that field-collected winterform females did not become attractive to males until late February when ovarian maturation and mating begins in the field (Horton et al. 2007). Thus, our second objective was to compare the chemical profiles of extracts from post-diapause vs. diapausing winterforms. We were particularly interested in identifying chemicals that were exclusively or more abundantly present in post-diapause females than diapausing females, so as to pinpoint the chemicals that might be responsible for female attractiveness.

Several studies have suggested that, although psyllid males are attracted to female-produced pheromone, females are not attracted by volatiles from either sex. For example, females of the pear psylla *C. bidens* and the citrus psyllid *D. citri* did not exhibit attraction to either male or female conspecifics in bioassays (Soroker et al. 2004; Wenninger et al. 2008). With *C. pyricola*, field experiments that used live psylla indicated that females did not show a preference for either male- or female-baited traps compared to unbaited traps (Brown et al. 2009).

Our third objective was to determine if response by *C. pyricola* to a putative pheromone was sex-specific. To achieve this objective, we compared the behavioral responses of post-diapause males and females to an isolated chemical found to be more abundant in extracts of post-diapause females than post-diapause males. Because cuticular extracts of females were as attractive to males as live females (Guédot et al. 2009), we also compared attraction of males to the identified chemical vs. extracts of females, to determine whether this single chemical might be the major or even sole compound comprising the female-produced attractant.

For practical purposes, the development of a synthetic pheromone lure for pear psylla would provide a useful tool for monitoring and management strategies. Field experiments using live *C. pyricola* as attractants had shown that males exhibited a clear preference for female-baited traps vs. unbaited traps or traps baited with males (Brown et al. 2009). Thus, our final objective of this study was to field test the effectiveness of the chemical identified as a potential sex attractant for male pear psylla.

Methods and Materials

Source of Insects Winterform pear psylla were collected from a commercial pear orchard located near Yakima, Yakima Co., Washington, USA in December 2008 (diapausing winterform) and in February-March 2009 (post-diapause winterform) using a beat tray and aspirator. In December 2008, adults were separated by sex in the field, and placed in groups of ca. 350 insects on pear shoots (excised from branches in the field and placed in water vials) in 10-liter ventilated plastic containers. The containers and insects were stored at ~16°C under a L8:D16 photoperiod for 24 h before the insects were used for extractions. In February and March 2009, adults were separated by sex in the field and placed in groups of ca. 200 insects on pear shoots in 1-liter glass jars. The jars and insects were kept at ~24°C under a L16:D8 photoperiod for 72 h before the insects were extracted. Insects for olfactometer bioassays were held in glass jars under the same conditions for 3–7 d. On each collection date, a subsample of 10 post-diapause females collected in February-March 2009 was dissected to determine ovarian maturity (an indicator of diapause status), and the number of spermatophores (an indicator of the number of times a female had mated) (Krysan and Higbee 1990; Horton et al. 2007). Behavioral assays and extractions were not conducted until dissected females in the subsample had reached an average ovarian score of 5 or higher (Krysan and Higbee 1990), at which stage females are attractive to males in olfactometer assays (Horton et al. 2007). Although field collected psylla were mated, the mating status has no effect on female attractiveness in pear psylla (Horton et al. 2008).

Cuticular Extracts Extractions were performed between 12:00 and 15:00 h (P.S.T.). For each extraction, 50 psylla of one sex were transferred into an 11-ml glass vial containing 300 µl pentane for 5 min, during which the glass vial was agitated by hand. The solvent was then

transferred to a clean glass vial (extract). Simultaneously with each extraction, a control treatment was prepared using the same procedure with 300 µl pentane without psylla. Treatment and control extracts were stored for up to 5 d at 0°C until about 1 h before experiments were conducted. These samples were used in both chemical and behavioral assays. Samples used for chemical analyses were spiked with 10 µl of a 500-µg/ml solution of octadecane as an internal standard (Matheson Coleman & Bell, Cincinnati, OH, USA). In bioassays, each olfactometer consisted of paired 1-liter glass jars containing either the extract or a solvent control, applied with glass syringes (Hamilton Company®, Reno, NV, USA) to filter paper disks (55 mm diam; Whatman #1 Cat. No. 1001 055; Whatman®, Maidstone, UK), and allowed to evaporate in a fume hood for 1 min. Each filter paper disk was folded to prevent it from laying flat on the bottom of the jar. The disks were placed in the jars, and the jars were immediately attached to an olfactometer (described below).

Analysis of Extracts One-µl aliquots of extracts and associated solvent controls were analyzed by coupled gas chromatography-mass spectrometry (GC-MS) using an Agilent 6890 GC with a 5973 mass selective detector with electron impact ionization (70 eV). The GC was equipped with a DB-1MS fused silica capillary column, 0.25 mm ID×60 m, 0.25 µm film thickness (J&W Scientific, Folsom, CA, USA), programmed at 60°C for 1 min, increasing to 240°C at 30°C per min, then at 10°C per min to 340°C, and held for 13 min. Hydrocarbons were quantified using the integrated peak area data from the GC-MS response to increasing quantities (5–50 ng) of the authentic standards heptacosane, nonacosane, and hentriacontane (Sigma Aldrich®, St. Louis, MO, USA).

Quantitative GC-MS analyses were conducted to identify compounds present specifically or in greater abundance in females than males, comparing 1) diapausing winterform females with diapausing winterform males and 2) post-diapause winterform females with post-diapause winterform males. For each set, five extracts of females, five extracts of males, plus solvent controls, were analyzed. Because differences in chemical profiles between males and females of diapausing and post-diapause psylla appeared to be quantitative rather than qualitative, we calculated the female to male ratio for each identified chemical. Measurements of wing length and scutellum width of male and female pear psylla have shown that females are slightly larger than males, with a female to male ratio of less than 1.2 for both of these measurements (Wong and Madsen 1967). Thus, compounds that were at least twice as abundant in females compared to males were selected to be tested in the behavioral bioassays for attractiveness.

Compounds in extracts were identified by analysis on a Hewlett-Packard (HP) 6890 GC interfaced to a HP 5973 mass selective detector (electron impact ionization, 70 eV). The GC was fitted with a DB5-MS column (30 m× 0.25 mm i.d., 0.25 micron film thickness; J&W Scientific, Folsom, CA, USA), programmed from 100°C/1 min, 10°C/min to 280°C, and held for 20 min. One-µl aliquots were injected in splitless mode, with injector and transfer line temperatures at 280°C. Helium carrier gas was used in constant pressure mode.

Cuticular hydrocarbons were identified by a combination of retention time comparisons vs. straight-chain alkane standards (Carlson et al. 1998) and interpretation of their mass spectra. Where visible, the molecular ion indicated the total number of carbons in the molecule, methyl-branched hydrocarbons gave enhanced diagnostic ions at branch points that allowed the positions of the methyl branches to be determined, and the presence of methyl branches resulted in diagnostic shifts in retention times vs. straight-chain standards (Nelson 1993; Nelson and Blomquist 1995; Carlson et al. 1998). Long-chain aldehydes were identified tentatively by interpretation of their mass spectra and retention index comparisons with straight-chain alkanes, and identifications were confirmed where possible by comparisons of retention times and mass spectra with those of authentic standards.

Preparation of 13-Methylheptacosane Butyllithium (2.7 M in hexane, 1.1 ml, 3 mmol) was added to a slurry of tetradecyltriphenylphosphonium bromide (1.53 g, 2.8 mmol, Lancaster Research Chemicals, Windham, NH, USA) in 20 ml anhydrous ether under argon at room temperature. After stirring for 30 min, a solution of 2-tetradecanone (0.30 g, 1.4 mmol) in 5 ml ether was added, the resulting slurry was stirred 30 min, then poured into 1 M aqueous HCl. The mixture was extracted with hexane, and the hexane extract was washed with saturated aqueous $NaHCO_3$ and brine, then dried over anhydrous Na_2SO_4 and concentrated. The residue was taken up in ~10 ml hexane, and left overnight to precipitate the bulk of the triphenylphosphine oxide. The resulting solution was purified by vacuum flash chromatography on silica gel, eluting with hexane. The mixture of purified alkenes in hexane was then hydrogenated with 100 mg of 5% palladium on charcoal catalyst in a septum-sealed flask fitted with a balloon full of hydrogen for 3 h. After filtration, the crude product (0.43 g, ~95% pure by GC) was recrystallized from 12 ml acetone at −20°C, yielding 0.21 g 13-methylheptacosane as a low-melting white solid, mp 26°C. The ^{1}H NMR spectrum matched that previously reported (Marukawa et al. 2001). The mass spectrum showed *m/z* (abundance) 394 (trace, M^+), 379 (1), 365 (1), 225 (8), 224 (12), 197 (9), 196 (19), 168 (4), 155 (5),

141 (7), 127 (10), 113 (15), 99 (22), 85 (62), 71 (80), 57 (100), 43 (53).

Behavioral Bioassays A Y-tube olfactometer was used to assess the response of male and female post-diapause winterform psylla to olfactory signals. The olfactometer, fully described in Horton and Landolt (2007), consisted of a 27-cm long, 2.5 cm diam glass tube forming the stem of the Y, joined to two arms at 135° to one another, each 7 cm in length. The Y-tube was positioned horizontally with a ~15° incline. Commercial compressed air (Oxarc Inc., Spokane, WA, USA) was passed through a charcoal filter, an air humidifier, and 1-liter glass jars containing odor sources. A 25 cm × 2 mm diam polytetrafluoroethylene hose (PTFE) (Cole-Parmer Instrument Company, Vernon Hills, IL, USA) connected each jar to an arm of the Y-tube. Airflow through each arm of the olfactometer was maintained at 50 ml per min during the assays. Before each bioassay, air was passed through the whole system at 50 ml/min in each arm, including the jars containing the odor sources, for 15 min. For all assays, a replicate consisted of 10 psylla of the same sex assayed individually. For each replicate, after five psylla were assayed, the arms of the olfactometer were rotated 180° horizontally, and the other 5 males were assayed. Approximately 30 min preceding an assay, psylla to be assayed were placed in a 50 ml vial. During the assays, a single psylla was allowed to enter the olfactometer from the vial and was then given 10 min to enter an arm of the Y-tube. Psylla that did not enter an arm within 10 min were discarded. Choice was defined by a psylla contacting the upwind end of an arm, at the insertion point of the PTFE hose. After each replicate, the olfactometer was dismantled, soaked in hot soapy water, rinsed with water, acetone and hexane, and then baked in an oven at 150°C for at least 2 h. Two sets of assays were carried out, with comparisons within each set being randomized.

Bioassays were conducted using the Y-tube olfactometer to address response of post-diapause winterform male and female psylla to 13-methylheptacosane [hereafter, 13-MeC27], which was found in the chemical analyses to be substantially more abundant in extracts from post-diapause females than post-diapause males. In a first experiment, two comparisons were made in which either males or females were given a choice between 500 ng of 13-MeC27 and solvent controls. Each comparison consisted of 10 replicates. In a second experiment, three comparisons were conducted of post-diapause males to 13-MeC27 vs. cuticular extracts of post-diapause females: (a) 13-MeC27 vs. control; (b) cuticular extract of females vs. control; and (c) 13-MeC27 vs. cuticular extract of females. Each comparison consisted of 15 replicates. In the first and third comparisons, 250 ng of 13-MeC27 (equivalent to ~25 females) were applied to a filter paper. Each cuticular extract consisted of 25 females extracted in pentane for 5 min.

Field Trial A field assay was conducted 2–7 April 2009 in a pear psylla-infested pear orchard at the United States Department of Agriculture's Experimental Farm near Moxee, Yakima Co., WA, USA (46°30′ 18.60″ N 120°10′ 06.64″ W). The orchard is a mixture of Bartlett and Anjou pear trees approximately 3 m in height, with ~3 m spacing between tree trunks. Sticky traps were used to assess the attraction of male and female winterform pear psylla to 13-MeC27. Each trap was constructed of a 30×30 cm section of light brown Lumite® fabric (No. 5006304, Chicopee Mills Inc., New York, NY, USA) with 1 mm^2 sized mesh. Two opposite edges of a fabric section were each stapled to a 30 × 1.5 × 0.5 cm piece of hemlock. A ~30 cm section of twisty tie was stapled to the center of each hemlock piece, and traps were hung between two branches using the twisty ties. The fabric section was coated with a thin layer of Tanglefoot Tangle-Trap Sticky Coating (Grand Rapids, MI, USA). Gray halobutyl rubber septa (West Pharmaceutical Services, Lyonville, PA, USA) were used as dispensers. Septa were pre-extracted with methylene chloride, and then aired for 24 h at 24°C in a fume hood before use. Septa were loaded with 10, 100, or 1,000 μg of 13-MeC27 in 100 μl aliquots of pentane, followed by an additional 200 μl of pentane to adsorb the test compound into the rubber matrix. Control lures were treated with 100 μl of pentane, followed by 200 μl of pentane. Lures were aired at room temperature in a fume hood overnight, then stored in a freezer at 0°C overnight until use in field traps. Lures were attached to the center of traps using safety pins. Treatments were deployed in a randomized complete block design with eleven blocks. Each block consisted of four treatments: control, and 10, 100, and 1,000 μg doses of 13-MeC27 per septum. Traps were placed one per tree at ~1.5 m above ground; two trees separated adjacent traps. Traps were collected from the field on 7 April, and returned to the laboratory where captured male and female psylla on each trap were counted.

Statistical Analyses Statistical analyses were performed using SAS Version 9.1 for Windows (SAS Institute 2002). Behavioral bioassay data were analyzed with paired sample *t*-tests in PROC TTEST as described in Horton et al. (2007, 2008). The *t*-test assumes a normal distribution of the arithmetic differences between paired observations (Zar 1999). The normality assumption was tested using the Shapiro-Wilk statistic in PROC UNIVARIATE. When the normality assumption was not met, the paired differences were analyzed using a signed-ranks test in PROC UNIVARIATE (Zar 1999; Horton et al. 2007, 2008). Because the signed-ranks test yielded significant differences in all comparisons, possibly leading to the commission of a type I error, we opted for a more conservative approach and used results obtained with paired sample *t*-tests.

For the field assays, count data were square-root transformed to normalize the distributions for analysis. The data were analyzed as a randomized block, two-factor (sex × treatment) repeated measures analysis of variance using PROC MIXED, with sex being the repeated factor because both sexes were counted on each trap. The means of the three doses (10 μg, 100 μg, and 1,000 μg) were compared to the control mean to assess whether the compound led to an increase in trap catches vs. the unbaited controls. In the event of a significant treatment by sex interaction, the SLICE statement in PROC MIXED was used to examine treatment effects for each sex separately. Following a significant *F* value for the ANOVA, differences between means were separated by Tukey's test.

Results

Analysis of Extracts Analyses of cuticular extracts from male and female winterform *C. pyricola* revealed the presence of a number of long chain hydrocarbons and related compounds (Fig. 1; Tables 1 and 2). The identified cuticular hydrocarbons of winterform pear psylla comprised 36.4±1.8% (mean ± S.E.M.) C_{23}-C_{35} straight-chain alkanes, 28.8±0.5% C_{25}-C_{33} monomethyl-branched alkanes, 7.1± 0.6% C_{31} dimethyl-branched alkanes, and 15.0±0.4% C_{22}-C_{30} aldehydes. The most abundant compounds found in all extractions were heptacosane, 2-methylheptacosane, nonacosane, 2-methylnonacosane, hentriacontane, and a mixture of 11,15- and 13,17-dimethylhentriacontanes.

Comparisons of the chemical profiles of males and females of either diapausing or post-diapause psylla revealed quantitative rather than qualitative differences, with every chemical identified being present, although in different amounts, in every extract. Total amounts of identified hydrocarbons were significantly lower in diapausing vs. post-diapause winterforms (t=4.1; df=18; P<0.001), varying from 528.8±21.7 (mean ± S.E.M.) ng and 581.7±36.3 ng for diapausing males and females, respectively, to 690.6± 47.0 ng and 712.7±36.0 ng for post-diapause males and females, respectively. Diapausing winterforms had no chemicals that were at least twice as abundant in females as in males. By contrast, in post-diapause winterforms, 13-MeC27 was 3.2 times more abundant in females than males. The 13-MeC27 was also the most abundant compound in diapausing females compared to diapausing males, although it was only 1.5 times more abundant in females than males.

Three chemicals, 13-MeC27, tetracosanal, and hexacosanal, were at least two times more abundant in post-diapause females than diapausing females. Two of these chemicals (tetracosanal and hexacosanal) were also more abundant in post-diapause males compared to diapausing males.

Behavioral Bioassays In the first experiment on the response of post-diapause winterform males and females to 13-MeC27, all 100 males and 100 females assayed made a choice within the 10 min cutoff time. Males most often chose the filter paper that had been treated with the 13-MeC27 when paired with a solvent control filter paper; 62.0% of males choosing the 13-MeC27 (t=4.81; df=9; P=0.001; Fig. 2a). Females showed no preference when presented with the 13-MeC27 vs. the solvent control (t=1.18; df=9; P=0.27; Fig. 2a).

In the second experiment on the response of winterform males to 13-MeC27 vs. cuticular extract of winterform females, all 450 males assayed made a choice within 10 min. Males most often chose the filter paper treated with the 13-MeC27 when it was paired with a solvent control filter paper; 67.3% of males chose the 13-MeC27 (t=11.31; df=14; P<0.0001; Fig. 2b). Similar results were obtained when assaying males to filter papers treated with extracts of females vs. solvent controls; 63.3% of males chose the extract of females (t=5.74; df = 14; P<0.001; Fig. 2b). Males did not show a preference when exposed to the 13-MeC27 vs. the extracts of females, with 50.7% of males selecting the 13-MeC27 (t=0.27; df=14; P=0.79; Fig. 2b).

Field Trial A total of 6,974 male and 3,958 female psylla were caught over the 6 d of the study. A significant interaction between sex and treatment was observed (F=8.76; df=3, 70; P<0.001) (Fig. 3). The mean catch of females on traps baited with 13-MeC27 was not significantly different from catch on the control traps, regardless of the dose of 13-MeC27 (F=0.25; df=3, 70; P=0.86). In contrast, the mean trap capture of males was higher on traps baited with 13-MeC27 than control traps (F=20.31; df=3, 70; P<0.001) (Fig. 3). The control traps did not capture more males than females (F=1.49; df=1, 70; P=0.23). More males than females were caught on traps baited with the 10 μg dose (F= 21.58; df=1, 70; P<0.001), with the 100 μg dose (F=28.95; df=1, 70; P<0.001), and with the 1,000 μg dose (F=6.79; df=1, 70; P=0.01). Furthermore, more males were caught on traps baited with the 10 μg dose (t=-6.28; df=70; adjusted P<0.001), with the 100 μg dose (t=−7.15; df=70; adjusted P<0.001), and with the 1,000 μg dose (t=−4.69; df=70; adjusted P<0.001) than on the control traps.

Discussion

The role of chemical signals in mate location is well established for many species in numerous insect orders. Recently, studies have shown that males are attracted to female-produced volatiles in three species of Psyllidae: *C. bidens* (Soroker et al., 2004), *C. pyricola* (Horton and

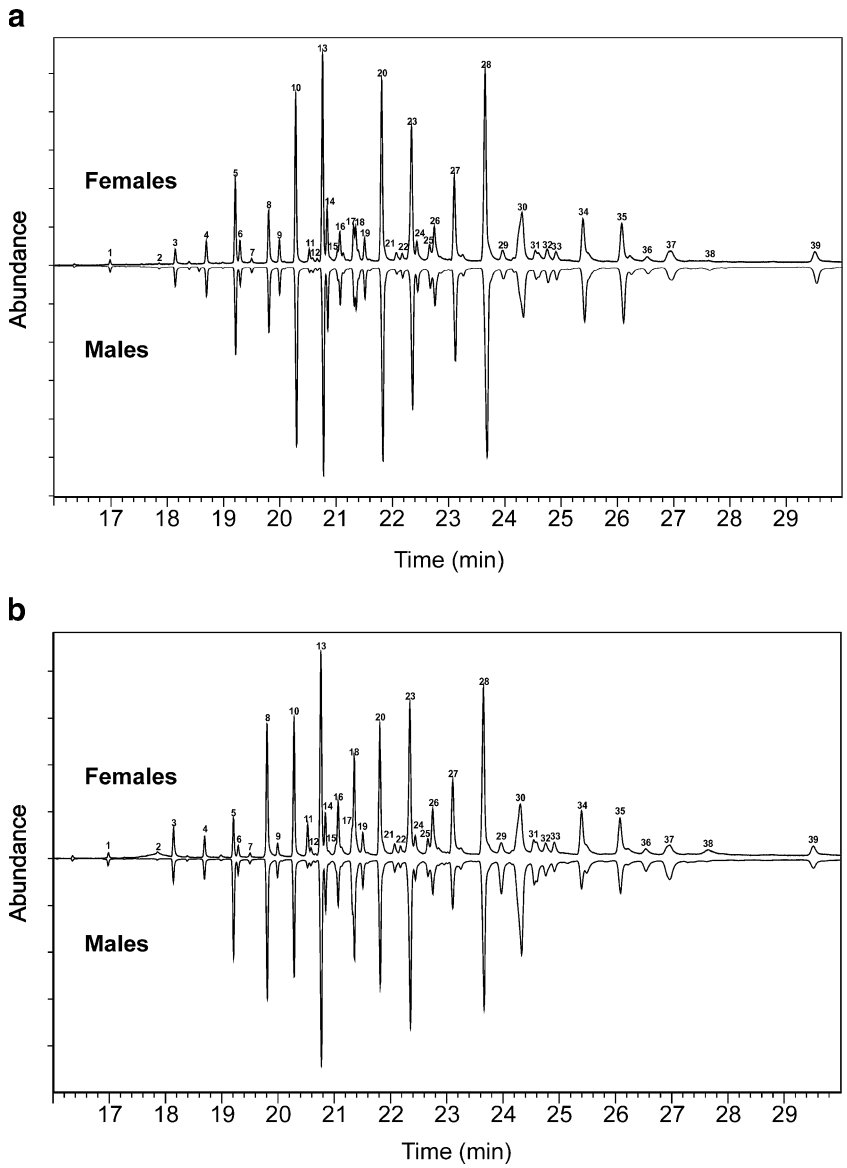

Fig. 1 Gas chromatography-mass spectrometry (GC-MSD) profiles of representative solvent extract of (**a**) diapausing winterform females (upper trace) and males (lower trace) and (**b**) post-diapause winterform females (upper trace) and males (lower trace), run on the DB5-MS column. Identifications of the numbered peaks are reported in Table 1

Landolt, 2007; Horton et al., 2007, 2008; Guédot et al. 2009), and *D. citri* (Wenninger et al., 2008). Despite the evidence and interest in the role of olfactory signals in mate location in the Psyllidae, the chemical(s) involved in the sex attractants had not been identified for any of these species.

The primary role of insect cuticular hydrocarbons is to provide a hydrophobic barrier that minimizes transpiration and that prevents desiccation (Nelson 1978; Lockey 1988; Howard 1993). However, components of the cuticular lipids often have important secondary roles as intraspecific recognition signals that communicate information such as sex, species, and physiological state (for review see Singer, 1998; Howard and Blomquist 1982, 2005). In the present study, the chemical analysis of cuticular extracts of male and female winterform *C. pyricola* revealed the presence of long chain (C22 to C35) *n*-alkanes, methyl- and dimethyl-branched alkanes, and aldehydes. To our knowledge, this is the first study that describes the cuticular lipid composition of any species in the Psyllidae.

The chemical profiles of extracts from diapausing and post-diapause winterforms were similar, with quantitative rather than qualitative differences. Post-diapause winterforms had greater amounts of cuticular hydrocarbons than diapausing winterforms. Similarly, hydrocarbon profiles of the adult face flies *Musca autumnalis* De Geer indicated a small increase in the total amount of hydrocarbons on the cuticle of reproductive females compared to diapausing females (Jurenka et al. 1998). In contrast, diapausing mosquitoes, *Culex pipiens* Linnaeus, produced more cuticular hydrocarbons than non-diapausing mosquitoes, purportedly as a mechanism to reduce water loss (Benoit and Denlinger 2007). Overall, the diapause syndrome is a

Table 1 Identification of hydrocarbons and related compounds in cuticular extracts of *Cacopsylla pyricola*. Identifications of compounds in boldface type have been confirmed by matches of retention indices and mass spectra with those of authentic standards

Peak #[a]	Retention index[b]	Identification	Diagnostic ions[c]
1	2300	**tricosane**	324 [M^+]
2	2400	**tetracosane**	338 [M^+]
3	2435	**docosanal**	278, 306 (324, M^+)
4	2500	**pentacosane**	352 [M^+]
5	2563	2-methylpentacosane	323, 351 (366, M^+)
6	2581	3-methylpentacosane	337 (366, M^+)
7	2600	**hexacosane**	366 [M^+]
8	2638	**tetracosanal**	306, 334, 352 [M^+]
9	2663	2-methylhexacosane	337, 365 (380, M^+)
10	2700	**heptacosane**	380 [M^+]
11	2736	**13-methylheptacosane**	196/224 (394, M^+)
12	2740	**pentacosanal**	348 (366, M^+)
13	2764	2-methylheptacosane	351, 379, 394 [M^+]
14	2775	3-methylheptacosane	365, 379 (394, M^+)
15	2800	**octacosane**	394 [M^+]
16	2804	unidentified	
17	2835	unidentified	
18	2842	**hexacosanal**	334, 362, 380 [M^+]
19	2861	2-methyloctacosane	365, 393 (408, M^+)
20	2900	**nonacosane**	408 [M^+]
21	2931	11-, 13- and 15-methyl-nonacosane	168/280; 196/252; 224, (422, M^+)
22	2943	**heptacosanal**	376 (394, M^+)
23	2963	2-methylnonacosane	379, 407 (422, M^+)
24	2973	3-methylnonacosane	393 (422, M^+)
25	3000	**triacontane**	422 [M^+]
26	3012	unidentified	
27	3044	**octacosanal**	362, 390, 408 [M^+]
28	3100	hentriacontane	436 [M^+]
29	3128	11-, 13- and 15-methyl-hentriacontane	168/308; 196/280; 224/252, (450, M^+)
30	3159	11,15- and 13,17-dimethyl-hentriacontane	168/239, 252/323 (464, M^+); 196/267, 224/295 (464, M^+)
31	3181	unidentified	
32	3186	unidentified	
33	3200	**dotriacontane**	450 [M^+]
34	3248	triacontanal	390, 418, 436 [M^+]
35	3300	tritriacontane	464 [M^+]
36	3329	11-methyltritriacontane	168/336 (478, M^+)
37	3356	unidentified	
38	3400	**tetratriacontane**	478 [M^+]
39	3500	pentatriacontane	(506, M^+)

[a] Numbers correspond to peaks in chromatograms in Fig. 1a and b.

[b] Retention indices calculated versus straight chain alkanes on DB5-MS column.

[c] [M^+] indicates a visible molecular ion of the mass shown. Values in round brackets indicate molecular ions which were not visible, but which could be inferred from the diagnostic fragments and the retention time.

complex and often poorly understood process in which changes in cuticular lipids form but one small part of the overall changes in the physiological state of an insect.

The sexual dimorphism observed in the cuticular lipid profiles in both diapausing and post-diapause winterforms was quantitative rather than qualitative, with the same hydrocarbons being present in both sexes for both diapause states. Comparisons of the relative amounts of cuticular hydrocarbons in the post-diapause winterform revealed that only one chemical, 13-methylheptacosane (13-MeC27), was at least two times more abundant in females than males. Furthermore, comparisons of winterforms by sex revealed that post-diapause females produced relatively larger amounts of two other compounds, tetracosanal and

Table 2 Mean (± S.E.M.) amounts (ng) of cuticular hydrocarbons and related compounds per psylla for females and males of the diapause and post-diapause winterform of *Cacopsylla pyricola*, and female to male ratio (F:M) for each hydrocarbon. Peak in boldface type is 13-methylheptacosane. *N*=5 replicates

Peak #[a]	diapausing winterform			post-diapause winterform		
	female	male	F:M[b]	female	male	F:M
1	4.1±0.2	3.8±0.1	1.1	4.3±0.3	4.3±0.2	1.0
2	t[c]	t	n/a[d]	t	t	n/a
3	4.1±0.2	3.7±0.1	1.1	4.6±0.3	4.8±0.4	1.0
4	6.5±0.5	6.0±0.4	1.1	6.4±0.4	6.2±0.4	1.0
5	13.7±0.9	11.9±0.7	1.2	9.8±0.9	17.2±1.2	0.6
6	6.3±0.3	4.7±0.2	1.3	4.8±0.2	4.6±0.2	1.1
7	3.5±0.1	3.4±0.0	1.0	t	t	n/a
8	7.7±0.7	6.3±0.4	1.2	18.8±2.0	22.6 ± 2.4	0.8
9	6.3±0.5	6.2±0.2	1.0	5.6±0.3	5.7±0.3	1.0
10	31.6±3.1	28.9±2.7	1.1	30.9±2.0	25.9 ± 2.0	1.2
11	**4.9±0.3**	**3.3±0.1**	**1.5**	**11.5±0.9**	**3.6±0.2**	**3.2**
12	3.0±0.0	3.0±0.0	1.0	2.9±0.0	2.7±0.0	1.1
13	34.0±1.8	31.5±1.8	1.1	50.6±3.8	43.7±3.5	1.2
14	11.2±0.5	10.7±0.6	1.0	12.3±0.8	10.7±0.7	1.1
15	7.5±0.2	7.2±0.1	1.0	5.3±0.2	4.5±0.2	1.2
16	6.1±0.4	5.3±0.2	1.2	9.5±0.8	8.3±0.8	1.1
17	10.9±0.4	10.1±0.5	1.1	12.9±0.6	14.7±0.7	0.9
18	8.8±0.5	7.9±0.4	1.1	21.4±1.8	22.0 ± 1.9	1.0
19	8.9±0.3	9.5±0.3	0.9	11.1±0.4	11.1 ± 0.5	1.0
20	39.3±2.7	37.0±2.1	1.1	39.4±2.3	34.9 ± 2.3	1.1
21	6.9±0.3	6.2±0.1	1.1	9.7±0.2	8.1 ± 0.3	1.2
22	6.2±0.1	6.1±0.1	1.0	7.2±0.2	6.8±0.2	1.1
23	32.6±1.7	29.7±1.2	1.1	49.5±2.6	47.2 ± 4.0	1.0
24	9.0±0.2	9.0±0.2	1.0	10.9±0.4	10.0±0.6	1.1
25	9.0±0.3	8.7±0.2	1.0	11.1±0.40	10.3±0.5	1.1
26	10.3±0.5	8.4±0.2	1.2	13.9±0.8	11.2 ± 0.7	1.2
27	28.0±3.1	23.1±1.9	1.2	29.6±2.4	26.2 ± 1.8	1.1
28	56.9±3.3	53.4±3.0	1.1	69.0±4.1	60.0±4.5	1.1
29	14.4±0.9	13.8±0.6	1.0	19.7±1.1	25.0 ± 1.7	0.8
30	37.8±2.4	31.9±1.4	1.2	50.4±2.9	61.3±5.6	0.8
31	13.5±0.6	12.9±0.5	1.0	19.6±1.1	20.1 ± 2.0	1.0
32	10.7±0.3	10.3±0.2	1.0	15.4±0.4	15.8±0.6	1.0
33	17.2±1.2	17.5±1.0	1.0	24.2±1.2	22.5 ± 2.3	1.1
34	26.7±4.0	24.1±2.2	1.1	26.9±2.7	24.1 ± 1.8	1.1
35	28.4±3.2	24.9±1.8	1.1	30.7±1.9	27.3 ± 2.1	1.1
36	13.0±1.2	12.3±1.0	1.1	15.4±1.0	17.1 ± 1.4	0.9
37	19.8±2.2	15.5±1.1	1.3	20.7±1.5	24.0 ± 2.1	0.9
38	8.5±0.0	8.7±0.0	1.0	11.2±0.1	11.5±0.1	1.0
39	13.2 ± 0.7	11.8±0.3	1.1	15.4±0.5	14.5±0.4	1.1

[a] Numbers correspond to peaks in Fig. 1

[b] Female to male ratio

[c] t = trace amounts (<0.5 ng per injection)

[d] n/a = not applicable

hexacosanal, compared to diapausing females, while post-diapause males produced relatively larger amounts of the two aldehydes than diapausing males. The two aldehydes were found in similar abundance in post-diapause females and males and are, therefore, probably not involved in mate attraction. Furthermore, in the Y-tube assays, 13-MeC27 alone was as attractive to males as the extract of females, which contained 13-MeC27 and a number of other hydrocarbons, including tetracosanal and hexacosanal. These results suggest that the relative and possibly absolute amount of 13-MeC27 produced by post-diapause females plays an important role in female attractiveness. Although 13-MeC27 is not specific to females, similar results have been obtained with other insects. For example, the contact sex pheromone produced by females of the locust borer, *Megacyllene robiniae* (Förster), also

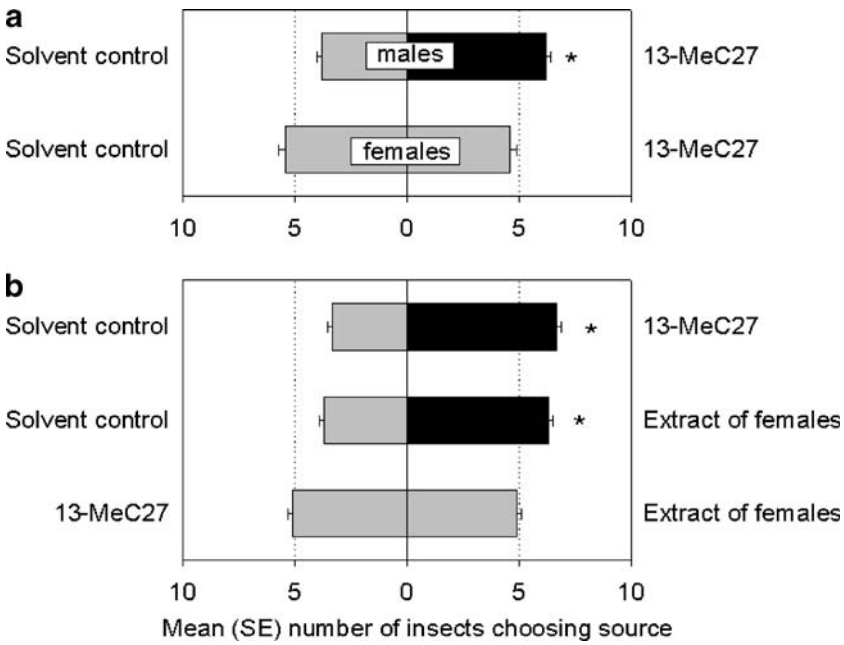

Fig. 2 Numbers of post-diapause winterform psylla (mean + S.E.M.) that chose paired odor sources. (**a**) Responses of males and females to 500 ng of 13-methylheptacosane (13-MeC27) vs. a solvent control (N=10). (**b**) Response of males to: 13-MeC27 (250 ng) vs. solvent control; extract of females (25 females) vs. solvent control; 13-MeC27 (250 ng) vs. extract of females (25 females) (N=15 per bar). * Choice is significant at P<0.05

was present, although in smaller quantities, on males (Ginzel et al. 2003).

The behavioral bioassays showed that responses to 13-MeC27 were sex-specific, with only males being attracted in both laboratory and field bioassays. In these assays, we tested the racemic blend of 13-MeC27, leaving male and female response to the individual stereoisomers unaddressed. The lack of responses by females to either female- or male-produced volatiles was also observed in the field with *C. pyricola* (Brown et al. 2009), and in the laboratory with *C. bidens* (Soroker et al. 2004) and *D. citri* (Wenninger et al. 2008). Males did not prefer the female extract over an equivalent amount of 13-MeC27, suggesting that 13-MeC27 may have been solely responsible for female attractiveness. These behavioral results are consistent with results described in the chemical analyses, and support the hypothesis that 13-MeC27 is a sex attractant pheromone for post-diapause males in *C. pyricola.* Further studies should address the attractiveness of the chiral forms of 13-MeC27.

13-Methylheptacosane is a relatively common constituent of insect lipids, and has been previously reported as a pheromone in insects. It is the major component of the contact recognition pheromone in males of the butterfly, *Colias eurytheme* (Boisduval) (Grula and Taylor 1979), and may be an aphrodisiac pheromone in that species (Grula et al. 1980). A mixture of three mono-methylheptacosanes, including 13-MeC27, was described as the major component of the post-pharyngeal gland secretions in the harvester ants *Pogonomyrmex salinus* Olsen and *Messor lobognathus* Andrews (Do Nascimento et al. 1993). As a cuticular hydrocarbon, 13-MeC27 occurs in the flesh fly, the pecan weevil, ants, grasshoppers, and crickets (reviewed in Nelson, 1978), and in termites (Haverty et al. 1996), wasps (Singer et

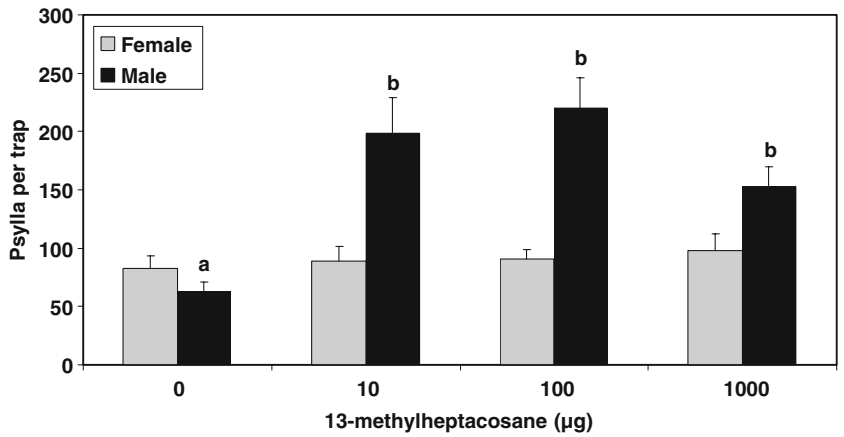

Fig. 3 Mean (+ S.E.M.) number of female and male winterform pear psylla captured on traps baited with 13-methylheptacosane dispensed from gray rubber septa at doses ranging from 0 to 1,000 µg (N=11 traps per dose). For male trap catches, treatments with different letters above them are significantly different (Tukey test, adjusted P≤0.05). Test conducted from 2 to 7 April 2009 near Moxee, WA

al. 1992; Liepert and Dettner 1996), and the moth *Scoliopteryx libatrix* (Linnaeus) (Subchev and Jurenka 2001).

Post-diapause winterform males of *C. pyricola*, but not females, were attracted to 13-MeC27 in the field. These results support field results with pear psylla indicating male attraction to females and no attraction between males, between females, and females to males (Brown et al. 2009), and support results of laboratory behavioral bioassays. This is also consistent with findings in two other psyllid species (Soroker et al. 2004; Wenninger et al. 2008). Our results indicate that males are attracted to a range of doses of 13-MeC27 covering two orders of magnitude, and that the doses tested were not critical for male attraction.

In summary, our results suggest that 13-MeC27 is a sex attractant pheromone for *C. pyricola* winterform males. Sex attractants are known to occur in non-psyllid Sternorrhyncha, including aphids, mealybugs, scale insects, and white flies (Yin and Maschwitz 1983; Lanier et al. 1989; Pickett et al. 1992; Hinkens et al. 2001; Rodriguez et al. 2005). This study provides the first identification of a sex pheromone in the Psylloidea. Further studies should address the optimization of this sex attractant for integrated pest management applications. Potential applications include a lure and trap useful to pear growers for monitoring pear psylla, lure and kill technologies, and mating disruption strategies for pear psylla management.

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