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FINAL REPORT TO THE ALMOND BOARD OF CALIFORNIA

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Project Title: **Pheromone-based monitoring and mating disruption of navel orangeworm**

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OBJECTIVES:

- Work out practical, field details of pheromone-based monitoring systems for NOW, and mating disruption tactics for NOW.
- Determine whether there are previously unidentifed components in the NOW pheromone blend.
- Synthesize and test potential synergists and antagonists of the NOW pheromone.
- Develop methods of stablizing NOW pheromone undr field conditions.

BACKGROUND.

The female-produced sex pheromone of the navel orangeworm (NOW) moth and the development of applications for the pheromone have been studied by several research groups. The single-known major component of the pheromone, (*Z*,*Z*)-11,13-hexadecadienal, was identified by Coffelt et al. (1979). Considerable effort has been expended to determine whether that component could be used for monitoring or for direct control through pheromone-communication disruption. The research afforded mixed results (Landolt et al., 1981; Curtis et al., 1985). Monitoring of NOW through the attracting of male moths to traps baited with (*Z*,*Z*)-11,13-hexadecadienal was rarely efficacious as compared to male attraction to female-baited traps, suggesting that

additional pheromone components may be present in the female moths. In small-scale pheromone-communication-disruption trials, orientation of male moths to traps containing living virgin females as bait was effectively disrupted, but in large-scale trials in almond orchards, levels of egg deposition by female moths in the centers of pheromone-permeated blocks were unacceptably high. Possibly, this failure was due in part to the lability of (*Z,Z*)-11,13-hexadecadienal in the presence of oxygen and ultraviolet light, causing it to have degraded during the course of the trials. Possibly, also, the size of the pheromone-permeated blocks (8 ha) was too small to prevent mated females from flying into the area of evaluation from nearby, non-pheromone-permeated areas, thereby nullifying the intention of the experiments. In this regard, it is known that NOW female moths have a potential flight range of at least 375 to 500 m (Andrews et al. 1980, Andrews & Barnes 1982), a capacity for movement that could have enabled significant numbers of mated females to move the 150 m from the borders to the centers of the pheromone-permeated plots (Curtis et al. 1985).

In recent research, we have developed a novel method for distributing large amounts of pheromone from widely separated machines, which we call "puffers" (Shorey et al. 1996). The machines contain timer-activated mechanisms for releasing puffs of pheromone components from pressurized canisters. The rationale for the use of puffers derives from our finding that, for certain moth species, the concentration of pheromone maintained in the atmosphere above a certain area of crop appears to be the critical factor determining the extent of communication disruption that is obtained, and this desired concentration of pheromone can either be produced from a large number of evaporators spaced relatively close together, each releasing a small amount of pheromone, or from a smaller number of evaporators spaced further apart, each releasing a correspondingly larger amount of pheromone.

Puffers have a number of potential advantages for atmospheric permeation with pheromones (Shorey et al. 1996), with the fact that the pheromone chemicals are protected from oxidative degradation until after they are released into the air being especially pertinent to the needs of NOW pheromone-communication disruption work.

In this report, we present: 1) the results of field trials testing the puffer technology for mating disruption of NOW in several crops, and 2) the results of laboratory studies aimed at identifying potential synergists and/or antogonists of the NOW pheromone, so that highly attractive lures for monitoring purposes can be developed.

METHODS AND MATERIALS.

Field trials:

The NOW pheromone component, (*Z,Z*)-11,13-hexadecadienal, obtained from Hercon Laboratories, Inc., was 85 % pure technical grade material. Chemicals were loaded into canisters by Technical Concepts, Inc. Chicago, IL, providing the following amounts per canister: (*Z,Z*)-11,13-hexadecadienal, 1.44 g; (*Z*)-5-tetradecenyl acetate, 7.31 g; (*E*)-5-tetradecenyl acetate, 7.31 g; absolute ethyl alcohol , 103.30 g; propane, 60.00 g; and butane 40.00 g. The latter two materials were provided as propellants. The (*Z*)-5-tetradecenyl acetate, (*E*)-5-tetradecenyl acetate, and ethyl alcohol were considered as neutral carriers for the (*Z,Z*)-11,13-hexadecadienal, although further experimentation will be conducted to ensure that they do not influence the behavior of NOW male moths. Puffer machines were obtained from Technical Concepts, Inc. They are designed for periodic release of puffs of atomized liquid from pressurized canisters. An internal, battery-powered clock in each machine allowed programming the release of puffs of the above-described blend of chemicals at 30- or 10-min intervals, depending of the experiment. The total amount of chemicals released in each puff averaged 60.6 mg, resulting in a release of 2.91 or 8.73 g per puffer/d when the timer was set for a 30- or 10-min interval, respectively. Each puffer was provided with a 15-cm-diam circular cotton-cloth target positioned on a wire frame so that it was located 10 cm in front of and perpendicular to the canister orifice. The target was intended for the capture of most of the chemicals directed at it, serving as a large evaporation surface for the continual release of (*Z,Z*)-11,13-hexadecadienal during the intervals between puffs.

The first set of tests was set up as a paired comparison, with one block of walnuts, almonds, or pistachios being permeated by (*Z,Z*)-11,13-hexadecadienal released from puffers arranged around its perimeter, and a comparison, untreated block being situated 2

to 10 km distant in a general upwind direction. Two tests each in pistachios and almonds and one test in walnuts were conducted in square 16-ha blocks, with 40 puffers spaced around the perimeter of each block at separations of about 40 m. The puffers were placed in trees along the block perimeter, at approximately 2/3 the elevation of the trees. This elevation was selected because it gives the greatest likelihood of mixing of a released vapor in all vertical strata of the trees during night-time hours when air movement in tree canopies most approaches laminar flow. Tree heights were approximately 2.5 m for pistachios, 6 m for almonds, and 12 m for walnuts. Each puffer was equipped with a large S-shaped hook which allowed its suspension from a tree branch. Placement of puffers in trees was aided by a telescoping pole that enabled installation at elevations up to 8 m above the ground. For this set of tests, the puff interval was always set at 30 min

An additional test in walnuts was conducted to evaluate a smaller square block of 4.4 ha, which was surrounded by 28 puffers spaced 30 m apart along the perimeter. In this test, puff intervals of 30 and 10 min were evaluated.

Evaluation of communication-disruption efficacy.

On the day that each perimeter treatment with puffers was deployed, from two to four stickem-coated wing traps, each baited with four laboratory-reared virgin female NOW moths, were positioned in the block at the center of the array and at the same elevation as the puffers (2/3 tree height). The untreated control blocks associated with each treatment were similarly equipped with female-baited traps. In these untreated blocks, the numbers of males that were found in the traps the following morning were used as an index of normal female-to-male communication. Lesser numbers of males captured in treated blocks surrounded by puffers indicated the degree of success of communication disruption in those blocks.

Statistical treatment of data.

Because the data collected were obviously multiplicative in nature, with treatment standard errors being proportional to their means, the data from the individual trap collections were transformed to $\ln(x + 1)$ for analysis. Means and standard errors were calculated on this transformed scale, although for presentation, the data are calculated back to the original scale of measurement.

Laboratory studies.

Insects: Navel orangeworm cultures are maintained on a bran and honey diet in the laboratory at both UC Riverside and Kearney Ag. Center, as previously described (Coffelt et al. 1979a). Male and female pupae were separated, and the emerging adults were maintained in 30 cm square screen cages until needed. Virgin females were used either as trap baits in field tests, or for preparation of pheromone gland extracts. Male insects were used for conducting coupled gas chromatography-electroantennogram detection (GC-EAD) studies at UCR. All insect cultures were maintained at 20-25° C.

Preparation and analysis of pheromone extracts: Virgin female insects were put on a reverse light cycle, as they normally call at the end in the couple of hours before dawn. Pheromone glands were dissected out of 1-3 day old virgin females at the end of the dark cycle. Briefly, the abdomen of the female was gently squeezed to extrude the gland on the end end of the ovipositor, and the gland was clipped off with iris scissors. The gland was soaked in pentane (25 microliters) for 10 min, and the pentane was then transferred to a clean vial. Extracts from about 1000 females were consolidated and concentrated by passive evaporation of most of the pentane from the open vial in a fume hood.

Pheromone gland extracts were analyzed by gas chromatography on several capillary columns of differing polarity (DB-5, DB-WAX, DB-17), and by coupled GC-EAD, using DB-5 and DB-WAX columns, and by GC-mass spectrometry. Compounds were identified by comparison of retention times and mass spectra with those of synthetic standards, prepared as described below.

Consolidated extracts containing several hundred pheromone gland equivalents were fractionated by high pressure liquid chromatography, using a Supelcosil LC-SI column (3 mm I.D. x 15 cm, particle size 5 microns) eluted with a gradient of 0-50 % tetrahydrofuran in hexane. Fractions were concentrated by fractional distillation of the solvent under nitrogen atmosphere.

3. **Syntheses of pheromone compounds and analogs**: Technical grade Z11,Z13-hexadecadienal (Z11,Z13-16:Ald, approx 84% pure), the major component of NOW pheromone, was purchased from Hercon Environmental, from company archives. The

technical grade material was cleaned up by reduction to the alcohol (Z11,Z13-16:OH) with sodium borohydride, followed by low temperature (-20° C) reduction of the corresponding alcohol from hexane, giving material of >99% purity. The purified alcohol was then reoxidised to the aldehyde using the Swern oxidation protocol.

E11,Z13-16:OH and E11,E13-16:OH were prepared as a mixture of isomers using a multistep synthesis. The alcohols were separated by liquid chromatography on a silver-ion coated ion exchange column eluted with methanol (Houx et al. 1974). The separated alcohols were then oxidised to the aldehydes by the Swern protocol.

Z11,E13-16:OH and E11,E13-16:OH were prepared as a mixture of isomers which were then separated by silver-ion chromatography, and oxidised by the Swern protocol to the aldehydes.

RESULTS.

Field trials.

When 16-ha blocks of pistachios, almonds, or walnuts were permeated by pheromone released from 40 puffers operating on a 30-min puff interval, 100% disruption of communication, based on numbers of male moths captured in female-baited traps in puffer-protected vs untreated plots, was obtained (Table 1). On several of the evaluations, the numbers of males captured were determined on the first night following set-up of the puffers, as well as 3 to 4 days thereafter. In all cases, 100% disruption of communication was occurred on the first night of pheromone release from the puffers.

Based on measurements and calculations that showed that total expenditure of material from the puffers was 60.6 mg/puff, and that the amount of (*Z,Z*)-11,13-hexadecadienal was 0.66% of the total ingredients in the canister, the amount of (*Z,Z*)-11,13-hexadecadienal expended was 0.40 mg/puff, or 19.2 mg in the 48 puffs that occurred each 24 hrs. With 40 puffers set up on the 4 sides of a square block, only 19 of the puffers usually have their pheromone plumes extending across that block at any one time (Shorey et al. 1996). The 16 ha in these plots, then, were served at any one time by 19 puffers, each releasing 19.2 mg of (*Z,Z*)-11,13-hexadecadienal /d, so the effective

release rate of (*Z,Z*)-11,13-hexadecadienal flowing in the air over the 16 ha was 365 mg/d (22.8 mg per ha/d).

Results of an additional test in walnuts, with canisters filled with ingredients as specified above and activated by 28 puffers arrayed around the perimeter of a 4.4-ha block, are shown in Table 2. In this case, when puffers were activated at 30-min intervals, some male moths were detected in the female-baited traps in the centers of the treated blocks. Based on a calculation that at any one time 13 of the puffers were serving the interior of the protected block, and with the release rate of (*Z,Z*)-11,13-hexadecadienal being as calculated above, then the effective release rate of (*Z,Z*)-11,13-hexadecadienal flowing in the air over the 4.4 ha was 250 mg/d (56.7 mg/ha/d). After 5 days of experimentation with a puff interval of 30 min, the puff interval was reduced to 10 min (Table 2). This now gave an effective release rate of 170 mg/ha/d. During the following 4 days, no male moths were captured in the female-baited traps in the puffer-protected block, although a mean of 5.9 males was captured per trap in the untreated control block.

Laboratory studies:

Syntheses of chemicals for testing. The syntheses of all four isomers of the navel orangeworm pheromone were completed on a small scale (Schemes 1 and 2) for testing in wind tunnel experiments, and as baits in field traps. However, the compounds proved to be less stable than anticipated, and interconverted readily, even when stored at -20°. Thus, three of the four isomers rapidly developed isomeric impurities at levels of 1-7%, and cannot be used for the field tests we had planned. These tests involve adding small amounts of the various isomers to the major component of the pheromone to see whether attraction is enhanced or inhibited. The syntheses will be repeated using modified routes to generate the high purity materials required for testing next spring. However, the materials that we have on hand have been used to examine female pheromone gland extracts, and to identify and determine the amounts of the minor isomers which are present in the extracts.

Analyses of pheromone glands. We have dissected and extracted approximately 1000 NOW pheromone glands, preparing both extracts from individual females to determine individual variability, and pooled extracts for fractionation and wind tunnel testing.

Because of the demonstrated instability of the pheromone compounds, we conducted a preliminary gas chromatography (GC) study to ensure that the compounds were not interconverted during analysis. It was found that GC injector temperatures above 200° C resulted in detectable interconversion. Consequently, all GC analyses of insect extracts were conducted at or below 200°.

Individual and pooled extracts have been analyzed by gas chromatography on two GC columns, and by coupled gas chromatography-electroantennography, which uses a live male moth antenna as the detector. We have not detected antennal responses to any compounds other than Z11,Z13-hexadecadienal and its isomers in the extracts, suggesting that these may be the only compounds constituting the pheromone. However, we cannot yet rule out the possibility that the extracts may contain unstable compounds which do not survive gas chromatography, even at reduced temperatures. There are several precedents for such unstable pheromone components in other moth species.

To determine whether there are thermally unstable components to the pheromone which are destroyed by GC, we have fractionated pooled extracts of several hundred pheromone glands by liquid chromatography. By bioassaying judiciously selected combinations of these fractions, we hope to determine whether or not there are any "hidden" components to the pheromone, which act to synergise Z11,Z13-16:Ald and its isomers. The fractionations have been carried out, and we anticipate carrying out wind tunnel bioassays of the fractions early in 1996.

DISCUSSION.

This research has shown that disruption of pheromone communication can be obtained in NOW by permeation of the atmosphere in 16-ha almond, pistachio, and walnut orchards with amounts of (*Z,Z*)-11,13-hexadecadienal as low as 23 mg/ha/d. This result is based on elimination of the ability of NOW males to orient to females used as bait in sticky traps, which were located more than 200 m from the closest puffer. Future research will

demonstrate whether the same procedure can effectively and practically prevent in-orchard mating of this species so as to prevent female moths from laying fertile eggs. In that research it will be essential that very large plot sizes or isolated plots are used at first so that in-flight of mated female moths does not obscure the results of the communication disruption that may be occurring inside the treated block.

Considering the differing vertical dimensions of the trees, and making the over-simplified assumption that the pheromone odor became uniformly distributed from soil surface to tree tops as it moved through the orchard, the volume of orchard space into which (*Z,Z*)-11,13-hexadecadienal mixed was in a ratio of 1:2:4 in pistachio, almond, and walnut orchards, respectively, based on tree height. Effective concentrations of (*Z,Z*)-11,13-hexadecadienal in the air of those blocks would be the inverse of these ratios, or 4:2:1, respectively. Therefore, it seems possible that the less-than complete pheromone communication disruption obtained in the second walnut test (Table 2), with the centrally located female-baited monitoring trap being only about 100 m from the border of the 4.4-ha plot, may have been caused in part by the vagaries of wind direction and dispersion of pheromone in the moving air. These vagaries could have caused the protection of the zone in the orchard containing the female-baited trap to be less complete than it was in the five tests of the first series, summarized in Table 1.

Further research will be necessary to determine the distribution of pheromone in orchards at varying distances downwind from puffers. We have presumed, partly based on prior knowledge of atmospheric mixing of air in tree canopies and partly based on our observations of the behavior of smoke plumes released at varying elevations in orchard air at night when air movement is most laminar, that the pheromone vapor will mix best into all strata of the orchard when it is released from about 2/3 the elevation of trees, and that it will remain as a distinct cloud, with little vertical loss, as it moves horizontally through the orchard. How much pheromone is actually lost during this progression through the orchard through absorption on foliage and other tree and soil surfaces and how much is lost thorough vertical mixing is very important to determine and should form the basis for future research.

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Table 1. Effect of releasing (*Z*,*Z*)-11,13-hexadecadienal from 40 puffers arrayed in trees around the perimeters of 16-ha blocks of almonds, pistachios, and walnuts on numbers of male moths of the navel orangeworm that oriented to and were captured in female-baited traps located in the centers of the blocks.

Crop	Test	Night after puffer deployment	Number of traps*	Mean (S.E. range)# male moths captured/trap: Puffer-protected block	Untreated block
Almonds	1	1	4	0.0	2.7 (xx,xx)
		2-4	4	0.0	2.3 (xx,xx)
	2	1-4	2	0.0	2.0 (xx,xx)
Pistachios	1	1	3	0.0	0.7 (1.9, 0.0)
		2-4	3	0.0	2.3 (5.3, 0.7)
	2	1	3	0.0	3.4 (5.5, 2.0)
		2,3	3	0.0	25.2 (37.9, 16.6)
Walnuts	1	1	4	0.0	1.4 (1.8, 1.1)
		2-4	4	0.0	5.8 (xx,xx)

* The indicated numbers of traps were hung on trees at approximately 2/3 of the elevation from the soil surface to the top of the foliage in each treated and untreated plot.

Original data were transformed to ln(x+1) for summarization of means and standard errors. Figures given here are converted back to the original scale.

Table 2. Effect of releasing (*Z,Z*)-11,13-hexadecadienal from 28 puffers arrayed in trees around the perimeters of a 4.4-ha block of walnuts on numbers of male moths of the navel orangeworm that oriented to and were captured in female-baited traps located in the center of the block.

Night after puffer deployment	Puff interval (min)	Number of traps*	Mean (S.E. range)# male moths captured/trap	
			Puffer-protected block	Untreated block
1-3	30	3	1.4 (3.3, 0.4)	63.6 (88.0, 45.6)
4-5	30	3	1.0 (2.0, 0.3)	6.6 (15.0, 2.6)
6-10	10	3	0.0	5.9 (12.0, 1.3)

* The indicated numbers of traps were hung on trees at approximately 2/3 of the elevation from the soil surface to the top of the foliage in both the treated and untreated plot.

Original data were transformed to ln(x+1) for summarization of means and standard errors. Figures given here are converted back to the original scale.