Project Number: 96-NOW

FINAL REPORT TO THE ALMOND BOARD OF CALIFORNIA,

FISCAL YEAR 1997

Project Title: **Pheromone-based Monitoring and Mating Disruption of Navel Orangeworm.**

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BACKGROUND:

The navel orangeworm (NOW), Amyelois transitella, is a key pest of nut crops in California. Despite intensive research in a number of areas, this insect continues to be a significant problem for many growers. The major component of the female-produced sex pheromone for this insect was identified as Z11,Z13-hexadecadienal in 1979 (Coffelt et al. 1979), but due to inconsistencies in its efficacy, the pheromone has not been used to any extent either as a trap bait or for mating disruption. The goal of this project is to determine the cause of and resolve these inconsistencies, so that the NOW pheromone can be developed into a useful and efficacious tool for both monitoring and control of NOW.

PROCEDURES:

1. **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) and wind tunnel studies at UCR. All insect cultures were maintained at 20-25° C.

2. **Preparation, analysis and testing of pheromone extracts**: Virgin female insects were put on a reverse light cycle, as they normally call just before and around 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 several thousand 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 in our 1995 report.

An extract containing ~1000 pheromone gland equivalents was fractionated by high pressure liquid chromatography, giving a fraction containing all compounds eluting before the known pheromone, a fraction containing the pheromone, and a fraction containing all compounds eluting after the pheromone. Fractions were concentrated by fractional distillation of the solvent under nitrogen atmosphere, checked by GC and GC-EAD, and will be tested during the remainder of the 1996 grant period in wind tunnel tests.

3. **Wind tunnel tests**. A small plexiglass wind tunnel was built and tested by us at UCR, for conducting tests with pheromone extract fractions. However, we were not able to obtain consistent behavior from navel orangworm male moths, even using virgin females as lures. Consequently, we have arranged for Dr. Ring Carde, who recently joined the Entomology department at UCR, to take over this part of the project, as described in more detail in results.

4. **Syntheses and testing of pheromone compounds and analogs**: The syntheses of most of the analogs and the known pheromone component were described in last year's report. The synthesis of the major component was repeated to provide fresh, pure material. Briefly, the technical grade material (84% pure; Hercon Environmental) was cleaned up by reduction to the alcohol (Z11,Z13-16:OH) with sodium borohydride in ethanol, followed by low temperature (-20° C) recrystallization 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, and purified by column chromatography folllowed by flash distillation.

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

Z11,E13-16:OH and E11,E13-16:OH were also prepared as a mixture of isomers and then separated by silver-ion chromatography, followed by oxidation to the aldehydes and purification as described above.

To test the effects of the various isomers and analogs as synergists or inhibitors of the major pheromone component, grey rubber septum lures were loaded with mixtures of the major component with 10% of each analog, with a total load of 110 micrograms per septum (Table 1). Septa were then placed in Pherocon 1C sticky traps, with 5 replicates per treatment, and traps were deployed in almond orchards for periods of 2-4 weeks. Traps were counted once or twice weekly. The test was repeated three times (June/July, July/August and October) due to low moth populations in the test area.

5. **Development of an Aerosol-Can Filling Facility at Kearney Agricultural Center.**

In the process of developing our puffer pheromone dispenser capabilities, and to make possible high quality experimental work with aerosol cans filled with pheromone blends that are varied depending on the pest complex that is to be controlled, an aerosol can filling facility was contructed at Kearney Agricultural Center, The Center management dedicated a 20 X 40 ft. building for this purpose and outfitted it as necessary, including the addition of a heavy duty compressor. We purchased and installed state-of-the-art aerosol can filling equipment. This is the only facility of its kind that we know of in a University laboratory setting in the U.S. It has the capacity to fill about 300 cans per hour with any specified blend of pheromone chemicals, neutral carriers and stabilizers, and propellants. The heart of each puffer-activated aerosol can is a 60 microliter valve, which is a new development which we have adopted before it became commercially available for other uses. The valve allows precise metering of 60 microliters of pressurized material per puff and due to its small capacity, enables us to dispense up to 4000 puffs per can from a

single can, giving a seasonal range of 60 to 150 days per can, depending on the details of when and at what frequency pheromone is dispensed.

Tests were conducted with different blends of pheromone components, solvents/diluents, and propellants to optimize the physical properties of blends (e.g., miscibility of mixtures and vapor pressures over the range of temperatures expected to be encountered in the field).

RESULTS AND DISCUSSION

Much of the work planned for the 1996 season had to be postponed due to the failure of two separate chemical companies to provide NOW pheromone. Each company had contracted to provide a kilo of the pheromone by April 1, 1996. In the event, neither company has been able to provide any material by April 1, 1997! One contract has ben cancelled completely, and we still hope to get some material from the second company shortly. We have also put out another contract to an English company which has some experience in insect pheromone synthesis, and they are currently partway through the synthesis. Finally, we are actively trying to interest two other companies, one in the U.S. and one in Japan, in carrying out the synthesis, for this and future years. Both of these companies also have considerable experience in pheromone synthesis.

Without having pheromone to work with the limited studies that we were able to carry out are described below.

1. Developing a Protocol for Filling Aerosol Cans with NOW Pheromone. Our aerosol cans contain 4 ingredients - pheromone chemicals, neutral carriers or solvents, stabilizers and/or antioxidants, and propellant. All of these had to be tested in order to develop a system that could be used for dispensing differing blends of one of more pheromone components in reproducible amounts. We adopted Dupont 134A as the propellant for several reasons. First, this material is the next generation replacement for environmentally harmful freon-type propellants, and it is completely environmentally benign. Second, Dupont 134A is nonexplosive and nonflammable, increasing the safety factor while loading and working with the cans. Third, using this material allowed us to avoid the high

costs which would be associated with building a fireproof and explosion-proof aerosol can facility, which would have been required with more traditional, hydrocarbon type propellants (e.g., propane-butane mixtures).

Although it is a gas at room temperature and pressure, Dupont 134A is loaded into the cans as a pressurized liquid. This ensures a constant pressure in the can at a given temperature as the amount remaining in the can is reduced through being dispensed. Practically, this means that the last puff from a can contains the same amount of pheromone as the first puff, assuring a constant release rate, which is not available with any of the other types of dispenser which are currently available, such as the hand-applied plastic tubes or pouches.

Dupont collaborated with us in conducting tests to ensure that the range of pheromone chemicals that we proposed to dispense from the cans, including the NOW pheromone, is soluble in the propellant at all anticipated concentrations, and under all environmental conditions. In our first loading, cans were loaded only with pheromone and propellant. However, it became apparent that under the high summer temperatures encountered in orchards in mid-summer, the propellant over-pressurized the cans (internal pressure in any aerosol can increases with temperature), so that the puffers failed to fire in the hottest part of the day. Consequently, we undertook a series of experiments in which we filled cans with different concentrations of pheromone in two neutral diluents (ethanol and acetone), and varied the proportion of diluent to propellant from 10 to 90%. Depending on which diluent is used and in what ratio, the physical properties (such as vapor pressure) of the blend of diluent, pheromone, and propellant can be modified and fine-tuned. Cans filled with these blends were tested for effective pheromone release at a range of temperatures from 50 to 110°F. The best blend of ingredients for filling aersol cans for field use was found to be 40% Dupont 134A to 60% pheromone chemicals in ethanol. This combination, which was used in all further 1996 aerosol can filling, allows reproducible puffs of pheromone to be emitted at all temperatures between 50 and 110°.

However, in trials with another pheromone, we did experience a problem with ethanol as diluent, with the ethanol reacting with and degrading the pheromone. Consequently, we are continuing to investigate other possible diluents with Dupont, with

the hopes of finding one which will be completely inert with any pheromone, no matter how sensitive. Currently methyl t-butyl ether is looking promising.

2. Analyses and fractionation of NOW pheromone. Extracts of several thousand individually dissected female pheromone glands were prepared and pooled, and analyzed by GC-MS and coupled GC-electroantennogram detection. The male antennae responded strongly only to the major, known component of the pheromone, and to traces of two isomers of that component. However, it is not uncommon for compounds which give little or no response in electroantennogram studies (due to being present in small amounts, or due to small numbers of antennal receptors for that compound) to have strong synergistic or antagonistic effects. Consequently, we planned a second check for these types of compounds, using bioassays. First, a pooled extract from a large number of female pheromone glands was fractionated by liquid chromatography, giving a fraction containing everything eluting before the known pheromone, a fraction with the known pheromone component and its isomers, and a fraction with everything eluting after the known pheromone. These fractions will be tested in wind tunnel bioassays, both alone and in combination, through the winter months, to determine whether there are other as-yet unidentified components to the navel orangeworm pheromone.

3. Testing of pheromone fractions, and potential synergists and antagonists in the laboratory.

We had set up and begun experimenting with a small wind tunnel for carrying out bioassays with the fractions of the NOW pheromone, and with the synthetic compounds. However, NOW are notoriously difficult to work with in wind tunnel bioassays, and we were not able to obtain reproducible flights, even to virgin female moths. Fortuitously, Dr. Ring Carde, one of the world's experts on using wind tunnels to study pheromone-mediated moth behavior, recently joined our department, and he and his group have agreed to carry out these assays for us. Dr. Carde has some 20 years of accumulated experience with moths in wind tunnel bioassays, so we are confident that he will be able to

resolve the problems which we were having, and determine whether there are any synergists in the fractions of the female-produced NOW pheromone.

4. Field screening of potential synergists and antagonists. As a further check for pheromone synergists and antagonists, a series of compounds with structures related to the known pheromone component were synthesized and tested in 3 separate field screening trials, using blends of compounds loaded on rubber septa as baits for sticky traps. However, attraction to all baits used, including virgin females, was very weak, indicating that moth populations were low at the times and places that trials were conducted. The total numbers of moths caught are listed in Table 1, but this data should be interpreted with caution, because these numbers represent the summed trap captures of 5 traps over a period of about 6 weeks. These trials will be repeated next year at several sites and at several times until the effects of the various additives on the attractiveness of lures is clear and unambiguous.

5. Degradation of NOW pheromone under field conditions. To determine whether isomerization and degradation of the technical grade pheromone loaded into the puffer cans was a problem, the contents of puffers which had been used in field tests for 4 weeks in 1995 and stored at -20° since then were analyzed. Isomerization of the pheromone in the can proved to be minimal (1.2%) indicating that the formulated pheromone is adequately protected inside the can until the moment of release, and that loaded cans can be stored for many months without problems, i.e., they have a reasonable shelf life, which will be crucial for commercialization. It must be emphasized that this represents a major improvement over the formulations used in the late 1970's- early 1980's, in which degradation of the pheromone almost certainly contributed to control failure.

We were not able to carry out trials with various antioxidants and stabilizers as protectants for pheromone in monitoring lures because of the failure of the chemical companies to provide pheromone as contracted, as mentioned above. These trials will be carried out as soon as pheromone is available in 1997.

Acknowledgments:

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Table 1. Cumulative catches from 3 field trials of male navel orangeworm moths in traps baited with the major component of the NOW pheromone, the major component plus various additives (10%), and virgin female moths.

LURE BLEND (μG)	TOTAL MOTHS CAUGHT[1]
Z11,Z13-16:Ald (100)	25
Z11,Z13-16:Ald (100) + Z11,E13-16:Ald (10)	14
Z11,Z13-16:Ald (100) + E11,Z13-16:Ald (10)	5
Z11,Z13-16:Ald (100) + E11,E13-16:Ald (10)	15
Z11,Z13-16:Ald (100) + Z11,Z13-16:OH (10)	18
Z11,Z13-16:Ald (100) + Z11,Z13-16:Ac (10)	4
Z11,Z13-16:Ald (100) + Z11-16:Ald (10)	20
Z11,Z13-16:Ald (100) + Z13-16:Ald (10)	35
Virgin females (3/trap)	40
Untreated control	1

[1] Each treatment was replicated 5 times in each trial. Trials were conducted between June and October, 1996, in almonds.

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April 2, 1997.

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Dear Mr. Heintz,

Please find enclosed a copy of our final report for the 1996 year; my apologies that it is a couple of days late. Please call me should you have any further questions, and thank you for your continued support of our efforts.

Yours sincerely,

Jocelyn G. Millar
Assoc. Professor