COMPREHENSIVE FINAL REPORT 05-WL-02

Attractants for Navel Orangeworm

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Introduction. The Navel Orangeworm, *Amyelois transitella*, is the most serious insect pest of almond and pistachios in California and a commercial pest of a number of other crops, including but not limited to walnuts and figs. The navel orangeworm is primarily controlled with organophosphate (OP) and pyrethroid insecticides, but alternative methods of control are sorely needed given the regulations regarding applications of OP and secondary pest problems caused by pyrethroids. Sex pheromones and other semiochemicals are invaluable tools for monitoring and control of insect pest populations. When these chemicals, produced by insects (for their own communication), are accurately identified and synthesized, they may be employed in IPM strategies to monitor populations and determine treatment timing or to reduce populations by mass trapping, attract-and-kill, or mating disruption. A number of economically important lepidopteran species have been successfully controlled using synthetic sex pheromone in mating disruption. This technique has many advantages over conventional insecticides as pheromones are non-toxic chemicals and it is highly unlikely that resistance can be developed. Non-toxicity is not only desired because it leaves no residues, but also there is no effect on natural enemies that keep populations of secondary species under control. For the successful application of pheromones in mating disruption, however, it is essential that the synthetic and natural sex pheromone be identical, i.e., the chemicals emitted by female to lure males must be accurately identified. Minimal modifications in the chemical structures of pheromones render them completely inactive. Largely, the pheromone systems of moths contain multiple constituents in a precisely defined ratio. Despite the tremendous effort by leading scientists in the field of chemical ecology over two decades, only one single constituent (Z11Z13-16Ald) from the navel orangeworm pheromone system (Coffelt et al., 1979) was known until recently. We have taken a molecular-based strategy to study chemical communication in this species and to identify a complete pheromone system. In our approach, potential pheromone constituents and attractants are screened with proteins involved in the reception of pheromones. The reception of pheromones and other semiochemicals in insect antennae is initiated by binding to pheromone-binding proteins (PBPs) or odorant-binding proteins (OBPs) that transport the hydrophobic (water insoluble) pheromones through the aqueous environment inside the antennae towards the pheromone receptors (Leal, 2005; Leal, 2005; Leal, 2003; Leal et al., 2005). In this project's first year, we reported (1) isolating odorant- and pheromone-binding proteins from the navel orangeworm, (2) obtaining amino acid sequences of the isolated OBPs and PBPs, and (3) cloning the cDNAs (genes) encoding these OBPs and PBPs. In the second year of this project we (i) constructed recombinant vectors for the cloned OBPs and

PBPs, (ii) developed expression systems for the navel orangeworm OBPs and PBPs, and (iii) produced, characterized, and purified recombinant OBPs and PBPs. With a recently developed binding assay, we began screening attractants that led to the discovery a multi-component sex pheromone system which is now being tested in the field and should become commercially available in the near future.

Results. We have generated a large sample of recombinant olfactory proteins from the navel orangeworm utilizing a previously developed expression protocol. We have now studied the secondary structure of the recombinant AtraPBP1 by circular dichroism (CD). As indicated by a maximum at 193 nm and two minima at 208 and 224 nm (Figure 1), AtraPBP1 is a helix-rich protein, a structure previously observed for the pheromone-binding protein from the silkworm moth, *Bombyx mori* (Horst et al., 2001; Lautenschlager et al., 2005; Sandler et al., 2000). Also, AtraPBP1 undergoes a pH-dependent conformational change, as indicated by the change in the second minimum (224 nm) at pH 5 (Figure 1).

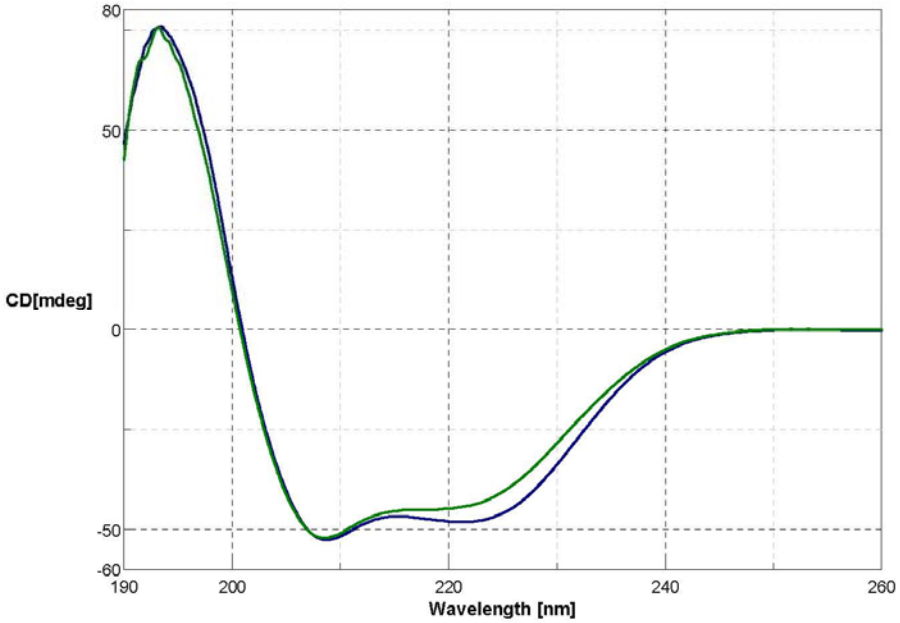

Figure 1. CD spectra of AtraPBP1 at pH 7 (bottom) and pH 5 (upper trace).

This pH-dependent conformational change is characteristic of moth PBPs and it is reflected in one of the two minima in the CD spectra because the C-terminus of the protein folds into a α-helix, whereas the helix in the N-terminus unwinds (Damberger et al., 2000; Horst et al., 2001; Lautenschlager et al., 2005; Wojtasek and Leal, 1999). pH-Titration by intrinsic fluorescence also suggest that AtraPBP1 has two conformations one at the sensillar lymph pH (high pH) and the other at low pH (Figure 2).

We have developed a new binding assay based on the separation of bound and free ligands by a centrifugal device. After incubation of a test compound with a PBP, the free ligand is removed by filtration, whereas the ligand bound to the protein is extracted with organic solvent and analyzed by GC and GC-MS (Leal et al., 2005). Using this novel binding assay, we have observed that binding of the major pheromone constituent, Z11Z13-16Ald to AtraPBP1 is pH dependent, with high binding affinity at high pH and no binding at low pH. These experiments confirmed that this male-specific olfactory protein binds a

constituent of the navel orangeworm pheromone and is, therefore, a pheromone-binding protein.

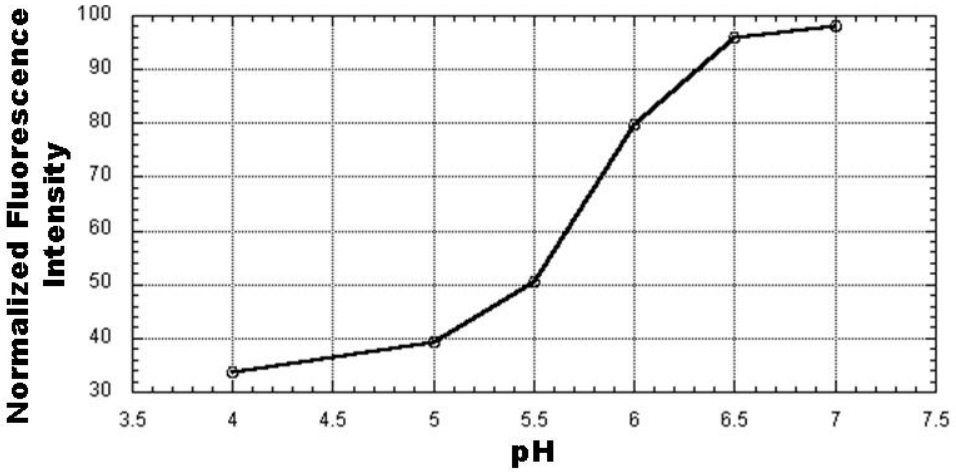

Figure 2. Effect of pH on the intrinsic fluorescence of AtraPBP1.

Having validated AtraPBP1 as a pheromone-binding protein, we used this "molecular target" to fish out other ligands, i.e., other potential attractants for the navel orangeworm. We observed that AtraPBP1 binds (Z,Z)-11,13-hexadecadienyl acetate (NOWOAc, in short) with the same apparent affinity as that observed for (Z,Z)-11,13-hexadecadienal (ZZNOW, in short) (Figure 3).

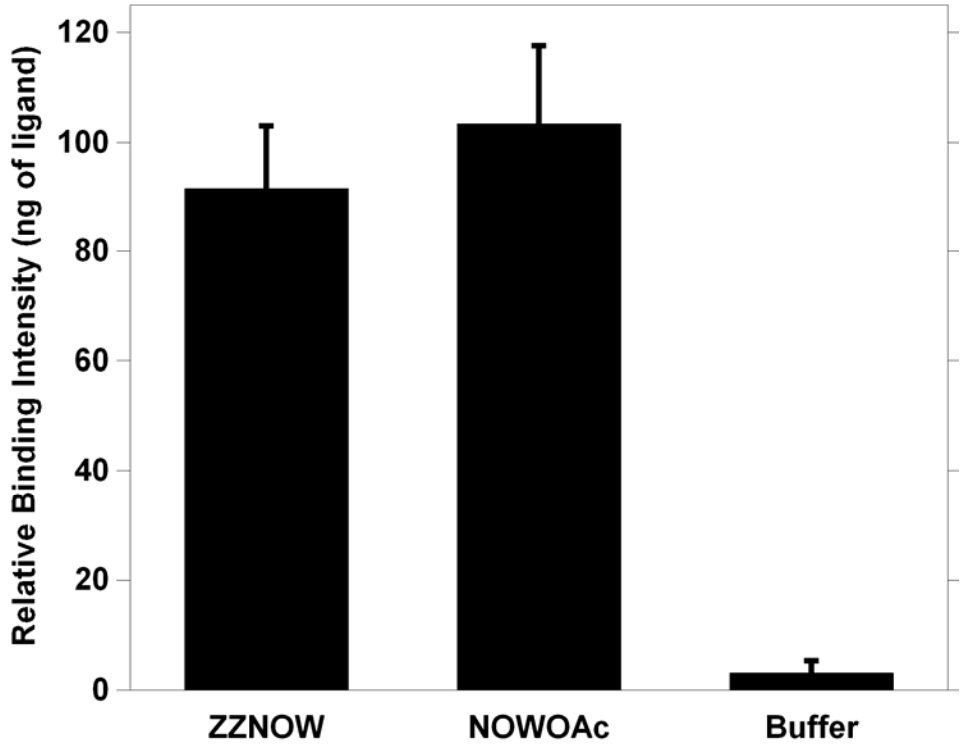

Figure 3. Binding of (Z,Z)-11,13-hexadecadienal [ZZNOW] and (Z,Z)-11,13-hexadecadienyl acetate [NOWOAc] to AtraPBP1 at pH 7. Only traces of these ligands were detected when protein was replaced by buffer (control).

The molecular evidence supporting that (Z,Z)-11,13-hexadecadienyl acetate binds to AtraPBP1 prompted us to investigate the occurrence of this semiochemical in the female pheromone gland. To determine the optimum time for extraction of pheromone glands, we studied initially the sexual behavior of the navel orangeworm. We then extracted with hexane pheromone glands excised from virgin females at the time of the peak of pheromone production and calling behavior. Crude extracts were analyzed by an electrophysiology technique named single sensillum recordings (SSR), in which a response from a single pheromone detector in the antennae is recorded. SSR indicated that the pheromone gland extracts contained multiple components as multiple neurons were activated. Flash chromatography on the crude extract generated one fraction (3%) in which esters and acetates are normally eluted. This 3% fraction showed also electrophysiological activity by SSR. When analyzed by GC-EAD, a small peak corresponding to (Z,Z)-11,13-hexadecadienyl acetate (NOWOAc) was detected (Figure 4). The retention time was confirmed by an authentic sample of NOWOAc both in a non-polar (Figure 4C) and a polar column (data not shown). Also, GC-MS and GC-FTIR data from the peak confirmed that the navel orangeworm produces (Z,Z)-11,13-hexadecadienyl acetate.

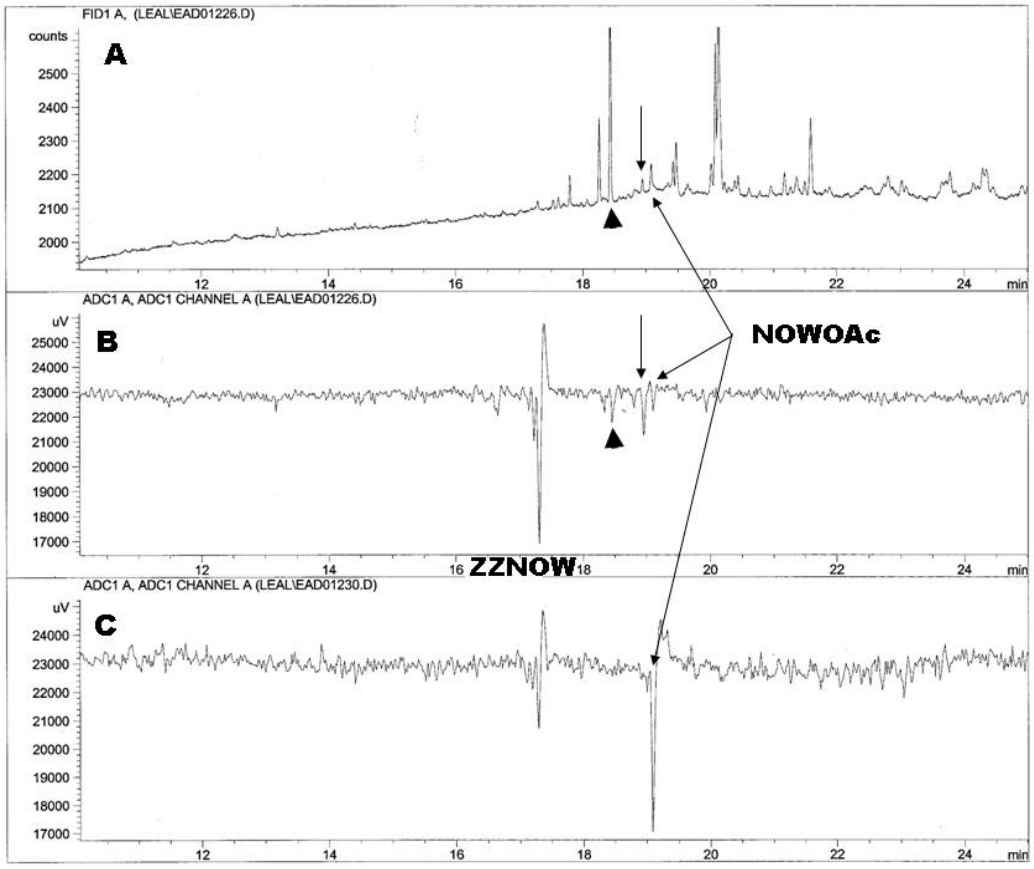

Figure 4. Gas chromatography (A) with simultaneous electroantennographic (B) recording (GC-EAD) obtained with gland extract from the navel orangeworm after separation on a silica gel column (3% fraction) and using male antenna as the detector. The peak of NOWOAc in the gland extract (A), and EAD (B) was confirmed with a mixture of a sample of NOWOAc (C) and ZZNOW for reference.

Preliminary field and indoor bioassays suggested that NOWOAc is neither an attractant nor an inhibitor for the navel orangeworm. However, field tests clearly indicated that NOWOAc is a behavioral antagonist for *P. farinalis*. Traps baited with the major constituent of the navel orangeworm sex pheromone (ZZNOW) caught males of *P. farinalis*, albeit in small numbers, but no males of the navel orangeworm. Interestingly, catches of male *P. farinalis* by traps baited with lures containing both ZZNOW and NOWOAc decreases significantly (Figure 5). These data indicate that NOWAc is a behavioral antagonist, which may be produced by the navel orangeworm to avoid attracting males of the wrong species.

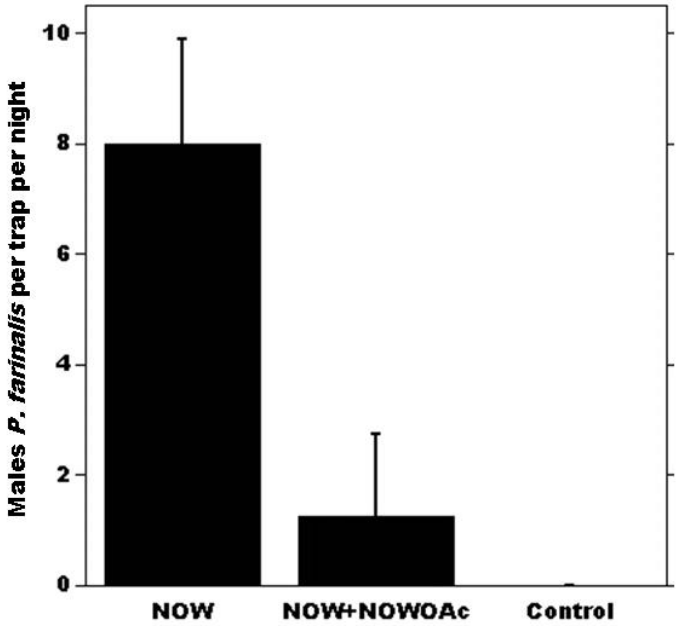

Figure 5. Effect of (Z,Z)-11,13-hexadecadienyl acetate [NOWOAc] on the capture of males *P. farinalis* with the previously known constituent of the navel orangeworm sex pheromone, (Z,Z)-11,13-hexadecadienal [NOW].

Similar fractions obtained from different gland extracts showed a consistent profile as the one shown in Figure 4. A strong EAD-peak for (Z,Z)-11,13-hexadecadienal (ZZNOW) was detected because small amounts of this compound eluted in the ester fraction (3%). Two other EAD-active peaks appear between ZZNOW and NOWAc, one indicated by an arrow and the other by an arrowhead (Figure 4). These compounds were identified as ethyl (Z,Z)-hexadecadienoate and ethyl palmitate, respectively.

An additional "molecular hint" was derived from our studies on the molecular basis of pheromone reception in the navel orangeworm. While screening for other potential ligands for AtraPBP1, we observed that (Z,Z)-11,13-hexadecadienol (NOWOH, in short) binds to our "molecular target" suggesting a potential role in olfaction. As demonstrated in competitive binding assays, in which AtraPBP1 was exposed to the three ligands at the same time, AtraPBP1 showed slightly higher preference for the behavioral antagonist (NOWOAc), whereas both ZZNOW and NOWOH showed apparently the same binding affinity (Figure 6). Indeed, this compound is also produced in the pheromone gland, as indicated by the analysis of a crude hexane extract of virgin female glands excised at the time of the peak of

pheromone production (Figure 7).

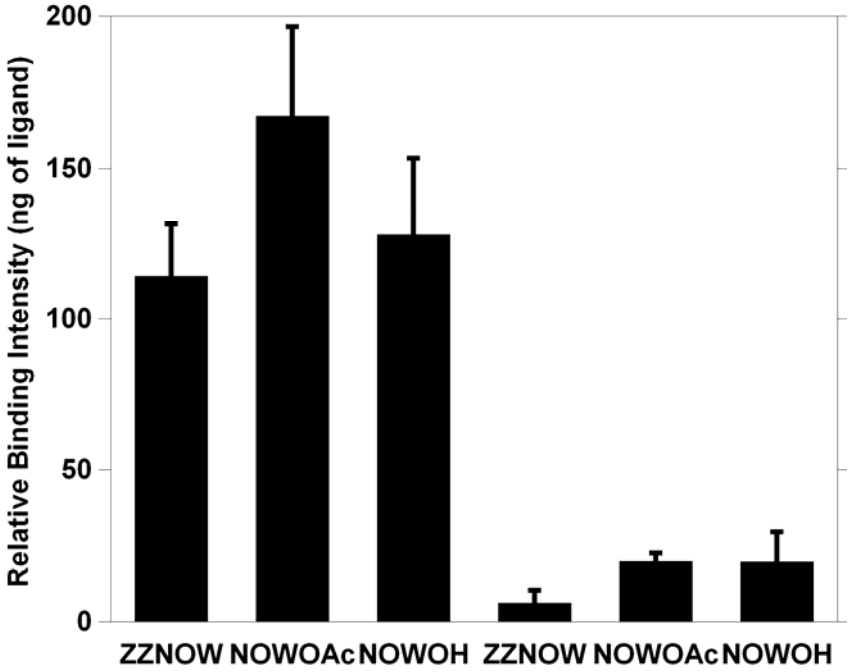

Figure 6. Competitive binding data. The right side of the panel shows the tests with buffer only (controls).

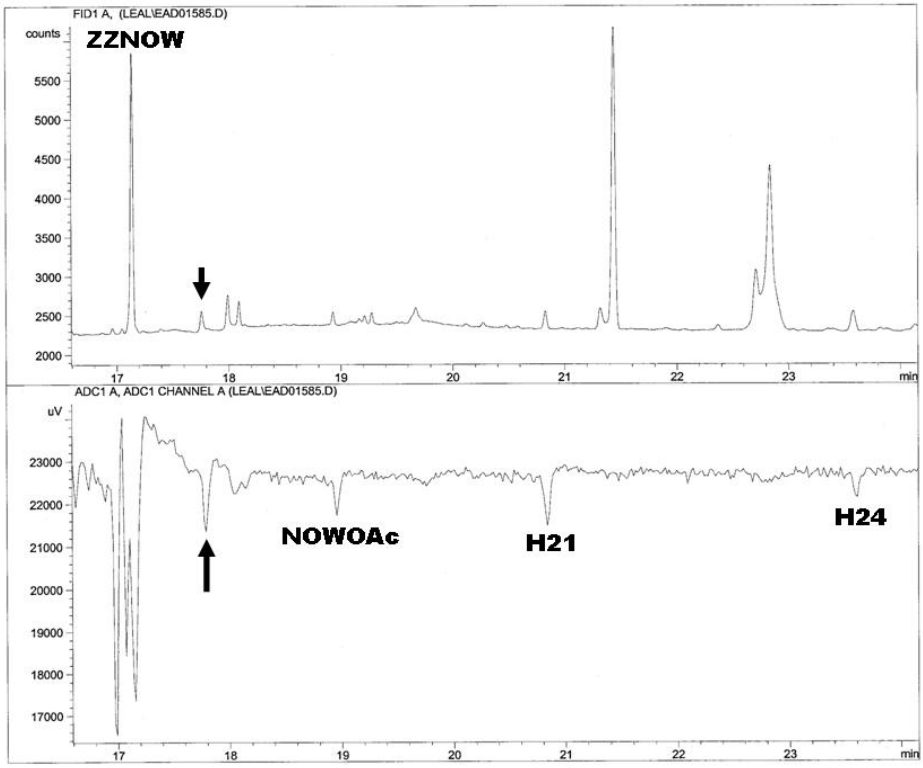

Figure 7. GC-EAD recording from a crude gland extract. In addition to ZZNOW, four other EAD active peaks are highlighted: NOWOAc, two novel pentaenes, H21 and H24,

and (Z,Z)-11,13-hexadecadienol [NOWOH] indicated by an arrow.

Other EAD-active compounds produced by pheromone gland, in addition to ZZNOW, NOWOAc, and (Z,Z)-11,13-hexadecadienal (Figure 7), are two novel pentaenes, H21 and H24. These compounds have been fully identified as (Z,Z,Z,Z,Z)-3,6,9,12,15-tricosapentaene and (Z,Z,Z,Z,Z)-3,6,9,12,15-pentacosapentaene (Leal et al., 2005). Other minor constituents identified by GC-EAD are (Z)-11-hexadecenal and (Z)-13-hexadecenal.

All compounds identified as sex pheromone attractants and the behavioral antagonist were synthesized by Bedoukian Research Inc (Leal et al., 2005). Due to possible chemical instability of some of the pheromone constituents, synthetic lures were formulated and evaluated in Davis where populations of the navel orangeworm are very low. Clearly, the new synthetic lure is active (Figure 8), but long-lasting formulations are still under development. When the synthetic pheromone was formulated in rubber septa, trap captures during the first night that the traps were deployed were high (given the low population), but the captures decreased dramatically on the second night in the field (Figure 8). This was observed not only with NOW, but also with *P. farinalis*, which has a higher population in the location where the tested were conducted.

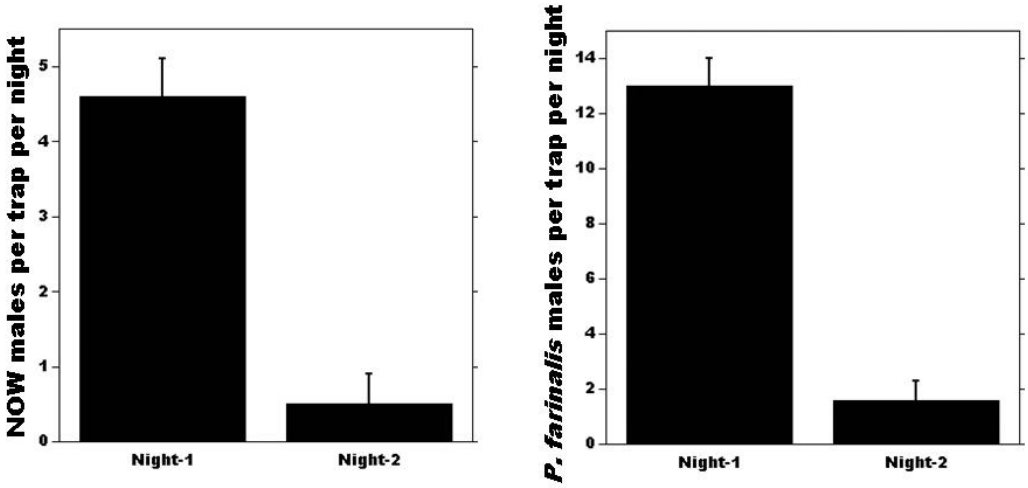

Figure 8. Capture data for the navel orangeworm (left) and *P. farinalis* (right) indicating a dramatic decrease in activity after one day exposure in the field. Control (empty) traps caught no males and were omitted in the figure.

In general, pheromones formulated in rubber septa do not last long in the field, but the lures are active for at least 3-5 days. The rapid decrease in activity of the NOW pheromone formulated in rubber septa suggests additional problem(s), which may be derived at least in part from exposure of the pheromone to sun light. Thus, for monitoring populations of the navel orangeworm a long-lasting formulation is still needed. However, this may not be the case with applications of the new synthetic pheromone when used for mating disruption and applied using a 'puffer'. Here, the pheromone mixture is canned and thus protected from sun light. A puffer may be programmed to release intermittent sprays of pheromone mixture (in a precise ratio) in the dark during the time of flight and mating activity of the navel orangeworm.

In an attempt to develop additional attractants, we have screened a large number of

plant volatiles, floral compounds, and other semiochemicals by both electroantennogram (EAG) and gas chromatography with electroantennographic detection (GC-EAD) using male and female antennae. We identified two compounds, which generate electroantennographic detection by male and female antennae, a potential unisex attractant. In addition, we have identified a number of compounds, which generate strong EAD activity in female, but not male antennae. These compounds are potential ovipostion attractants.

Note: The year-3 of this project was funded by the Almond Board of California and the California Pistachio Commission.

Conclusions and practical applications. With a better understanding of the molecular basis of olfaction in the navel orangeworm, we obtained "molecular hints" that ultimately led to the characterization of eight additional constituents of the sex pheromone system of the navel orangeworm. One of the new compounds is a behavioral antagonist produced by females of the navel orangeworm to repel males of *P. farinalis*. The other constituents are sex attractants. Traps baited with the new sex pheromone system, formulated in rubber septa, caught significant numbers of males of the navel orangeworm, even in areas with low populations. Long-lasting formulations for commercial applications in monitoring are under development. The new synthetic pheromone has potential application in a mating disruption strategy for controlling populations of the navel orangeworm. We are exploring the development of additional attractants, particularly those targeting gravid females.

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