Chapter 5

Volatile Natural Products for Monitoring the California Tree Nut Insect Pest *Amyelois transitella*

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The navel orangeworm (*Amyelois transitella*) is a major insect pest that inflicts serious economic damage to the California tree nut industry. Feeding by navel orangeworm larvae causes physical damage resulting in lower kernel quality; more importantly larvae are purported to vector the aflatoxigenic fungi. Aflatoxins are toxic metabolites produced by aspergilli and represent a major food safety concern. Over the years volatile natural products have played a large role in efforts to control or monitor navel orangeworm moths. The two most important sources of relevant natural products have been female navel orangeworm, which produce a complex sex pheromone blend; and, the almond host plant, which has recently been described as the source of a blend of volatiles that attract both male and female navel orangeworm. Provided herein is an overview of natural products and their role in efforts to control or monitor navel orangeworm moths in California almonds, pistachios, and walnuts.

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Introduction

Natural products have long been considered important bioactive chemical compounds with a wide variety of practical uses, including: medicinal, toxic agents, pesticides, and fungicides, among others (*1*). Volatile natural products also play a large role in the chemical cues of insects; examples include location of a food source, safe ovipositional sites, and avoidance of non-host plants (*2–8*). One example is an agricultural insect pest, the navel orangeworm (*Amyelois transitella*) (Figure 1), a major insect pest of California tree nuts (*9*) dating back to the 1960s (*10*). A blend of natural product host plant volatiles has recently been reported as an attractant for both male and female navel orangeworm moths (*11*) and the chemical components of the female sex pheromone are known (*12*). In addition to the physical damage to the tree nut kernels caused by larval feeding, navel orangeworm larvae are purported to vector aflatoxigenic fungi (*13*), thus contaminating the product and raising significant concerns regarding the safety of tree nut consumption.

Figure 1. The navel orangeworm (Amyelois transitella) moth, shown next to a whole almond, is an insect pest of California almonds, pistachios, and walnuts.

Aflatoxins

Navel orangeworm moths have been associated with aflatoxins, which are a group of compounds produced by certain mycotoxigenic aspergilli ubiquitous in California tree nut orchards (*9*, *14*). Specifically, the fungus *Aspergillus flavus* produces the aflatoxins B_1 and B_2 and *A. parasiticus* produces B_1, B_2, G_1, and

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G_2 (Figure 2), compounds **1**-**4**, respectively (*15*). Aflatoxins are considered carcinogenic and teratogenic. In addition to their food safety threat, tree nuts contaminated with aflatoxins constitute an international trade issue when exported (*9*, *15*).

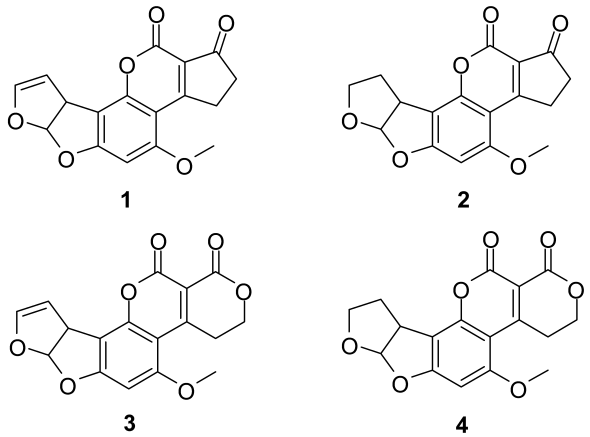

*Figure 2. Chemical structures of aflatoxins B_1, B_2, G_1, and G_2, compounds **1**-**4**, respectively.*

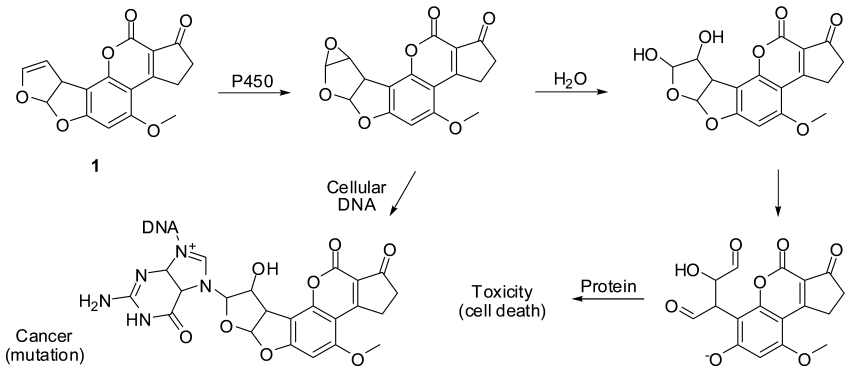

Figure 3. Mechanism of aflatoxin B_1 carcinogenicity and toxicity. (Reproduced with permission from references (17) and (18). Copyright 1998 and 2006 Elsevier.)

A mechanism for toxicity of aflatoxin has been shown to occur at the 8,9-alkene located in the furan ring of aflatoxins B_1 and G_1 (compounds **1** and **3**). Figure 3 illustrates the oxidation of this double bond by cytochrome P450 to the corresponding oxirane. The anomeric-like carbon is now highly activated toward nucleophilic attack by DNA or water. Ring opening by DNA results in the N^7-guanyl adduct which leads to mutation (*16*). An alternative pathway is oxirane

ring opening by water to form the diol, which opens further to form a dialdehyde. This dialdehyde is thought to bind with protein to form an imine adduct leading to cell death (*16*, *17*).

For several years aflatoxin contamination in almonds has been associated with feeding damage by navel orangeworm larvae (*19*, *20*). Indeed, a recent report demonstrated that navel orangeworm larvae transport the spores of *Aspergillus flavus*, thus acting as a vector for the mycotoxigenic fungus (*13*). Studies have shown the same association between navel orangeworm damage and aflatoxin contamination in pistachios (*21*). For walnuts, insect damage is assumed to be a factor for aflatoxin contamination (*9*).

Navel Orangeworm

As its name implies the navel orangeworm was originally found on navel oranges, although its geographic origin appears to be uncertain. For instance, one report from Arizona in 1922 (*22–24*) reported a new pest to oranges; however, a 1965 State of Florida Department of Agriculture document shows *Paramyelois transitella* (synonymous with *Amyelois transitella*) was first found in 1863 in the "United States, probably Florida…" (*25*). Interest in navel orangeworm in California walnuts (*26*) and almonds (*27*, *28*) appears in the literature in the late 1950s and early 1960s, respectively. These were followed by two investigations that comment on the difficulty in controlling navel orangeworm infestations (*10*, *29*). In his 1961 paper, Wade (*27*) provided nice detail of the biology of the navel orangeworm as well as its movement from southern California citrus and walnut storage areas to important fruit and nut crops in the upper Central Valley of northern California. The food safety issues, economic costs, and physical damage caused by navel orangeworm has led to numerous reports and control efforts over the years by tree nut industry, academic, and USDA-ARS researchers (*9*, *11*, *20*, *30–32*).

Host Plant Volatile Natural Products Associated with Monitoring Navel Orangeworm

Various efforts involving non-pheromonal tactics have been either investigated or implemented for control or monitoring of navel orangeworm in tree nuts – each with varying results. These efforts include either the exploration or implementation of the following: diamalt bait and terpinyl acetate in various media (*27*); pathogens of navel orangeworm (*29*, *33*, *34*); stringent orchard sanitation (*20*, *35–37*); navel orangeworm frass extracts (*38*); use of natural enemies of navel orangeworm (*39*, *40*); black light (*41*); ovipositional baits (*42*) or disruption (*43*); almond by-products (*44*); almond oil fatty acids (*45*); or, the use of the nonhost compound, phenyl propionate (*32*, *46*).

Negative results or poor performance from many of these studies prompted investigators to continue to explore other options. It was the use of almond press cake (*47*) in the early 1980s that started the more enduring utilization of almond

parts for the monitoring of navel orangeworm (*43*, *44*, *48*). Almond press cake is "the solid…residue that remains after almond oil has been mechanically pressed or removed…" (*47*). More recently, the use of almond meal, or almond meal with small percentages of crude almond oil mixed in has been the standard tool for monitoring navel orangeworm in almond orchards (*49*). Press cake is ground to produce the almond meal (personal communication, Liberty Vegetable Oil).

There exists a lack of information regarding the chemical composition of both almond meal or press cake. Work performed in 2009 by Beck and co-workers (unpublished material) showed the majority of the headspace volatile composition of almond meal (no crude almond oil added) to be made up of several pyrazine analogues. Some of the volatiles detected during the survey of almond meal via solid phase microextraction analysis (tentative identifications for pyrazines) included limonene, methyl pyrazine (unknown isomer), 2,5-dimethyl pyrazine, 2-ethyl-5-methyl pyrazine, 3-ethyl-2,5-dimethyl pyrazine, among other alkyl pyrazines. Other compounds tentatively identified included two methyl butanol isomers, small chain alkanals, and benzaldehyde. Subsequent electroantennographic (EAG) analysis and limited field studies of a few available isomeric components (similar to related work ref. (*11*)) did not provide reason for the pyrazine compounds to be considered further as possible attractant candidates by these researchers.

Other studies have explored various host plant materials to determine the chemical composition and possible association to navel orangeworm. For instance, Buttery and co-workers (*50*) studied the chemical composition of steamed almond hulls and postulated association of similar compounds from navel oranges as having possible relation to navel orangeworm. A large number of compounds detected included alkyl aldehydes typical of fatty acid oxidation/breakdown, among others (*51*).

Another volatile investigated for its ability to attract navel orangeworm was phenyl propionate (*32*, *46*, *52*). In field trapping studies, this compound attracted navel orangeworm moths and held the interest of researchers for a number of years. However, its origin was not divulged (*52*) and thus its classification as a natural product related to navel orangeworm hosts is unsubstantiated.

In 2009 a study (*31*) using EAG analysis was used to screen a large number of volatile natural products for potential attractiveness to the navel orangeworm. The volatiles were detected *in situ* from whole almonds and were studied under the hypothesis that female navel orangeworm use the background volatiles as a way to help distinguish a site for oviposition (*53*). Based on their antennal responses during EAG bioassay a number of compounds were identified as potential candidates, however none have been demarcated as having significant navel orangeworm behavioral activity.

In 2012 a blend of volatiles based on damaged almond hulls and almonds undergoing hull split was reported to attract both male and female navel orangeworm during field trapping studies (*11*). The navel orangeworm attractant blend comprised the structurally simple natural products (±)-1-octen-3-ol (**5**), (±)-(*E*)-conophthorin (**6**), acetophenone (**7**), ethyl benzoate (**8**), and methyl salicylate (**9**) (Figure 4).

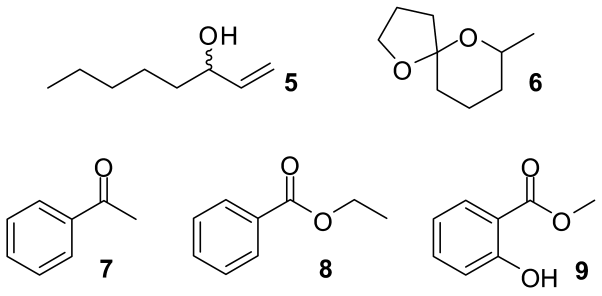

Figure 4. Chemical components of the host plant volatile blend that attracts adult navel orangeworm.

In the report, the blend of almond host plant volatiles underwent field-trapping studies over the course of a growing season (*11*). Table I shows the total number of male and female navel orangeworm moths captured in California almond and pistachio orchards in 2011 and compared to the standard for monitoring, almond meal. See also Beck et al. 2012 (*11*) for full statistical analysis of almond trap captures; Table I also provides unreported trap capture data for pistachio orchards in 2011.

Table I. Navel orangeworm moths captured in delta-sticky traps baited with a host-plant volatile blend (compounds 5-9), almond meal, and blanks in California almond and pistachio orchards in 2011

		Navel Orangeworm Moths Captured		
Orchard	***Treatment***	***Total***	***Female***	***Male***
Almond	Blend	155	59	96
	Meal	20	19	1
	Blank	2	1	1
Pistachio	Blend	32	20	12
	Meal	2	2	0
	Blank	0	0	0

A surprising result for moth captures in both almond and pistachio orchards was the relatively large number of males captured by the host plant blend. Almond meal is known for its ability to attract gravid female navel orangeworm, but is not an attractant for males. The host plant blend attracted numerically greater number of males in almonds, yet fewer males than females in pistachios. This phenomenon of male/female capture ratios is being examined further in trapping studies in both

orchards. Correspondingly, almond meal in pistachio orchards is known to become less effective in the month of June and by July the almond meal-baited egg traps are not attractive (personal observation, Bradley Higbee) to female navel orangeworm.

Four of the five components of the attractive host plant blend are derived from the volatile emissions of *in situ* almonds undergoing hull split (*11*) – the three benzenoids and the spiroketal conophthorin. The fifth component, 1-octen-3-ol, is generally considered to be a volatile associated with fungal contamination (*54*). All of the components have a history of semiochemical activity with other insects, yet none of the individual compounds elicited strong behavioral responses from navel orangeworm in field trapping studies (*11*).

Since the time of the study that reported on the host plant volatile blend's ability to attract navel orangeworm, there have been two other reports of conophthorin detected from sources other than hull split almonds (*11*) or from non-host angiosperms in relation to scolytid bark beetles (*55*). Conophthorin was recently detected from fungal spores on fatty acids (*56*) and from bacteria on varying laboratory media (*57*). These studies broaden the complexity of the origin of this particular spiroketal and add to the rich history of this natural product as a semiochemical.

Sex Pheromone Volatile Natural Products for Navel Orangeworm

Sex pheromones are important tools for monitoring and potentially controlling insect pests and need to be accurately identified and synthesized before their use as a tool to monitor or control insect populations. Sex pheromones are particularly valuable for techniques such as mating disruption, lure and kill, or mass trapping (*58*). In 1979, the major component of the sex pheromone emitted by female navel orangeworm moths was identified as (11*Z*,13*Z*)-hexadecadienal (compound **10** in Figure 5) by Coffelt and co-workers (*12*). Using their results from laboratory-based male behavioral bioassays, which demonstrated wing-fanning, orientation, and some upwind movement, Coffelt and co-workers (*59*) believed that this aldehyde would be sufficient as a monitoring lure. This supposition was supported by numerous examples in the literature that major components of lepidopteran sex pheromones were sufficiently attractive for use as a monitoring tool in various trapping schemes (*60–62*). However, efforts to develop a monitoring lure for navel orangeworm using only (11*Z*,13*Z*)-hexadecadienal were unsuccessful (*63*, *64*). It was demonstrated that relative to traps baited with virgin female moths, very few male navel orangeworm moths were captured in traps baited with synthetic (11*Z*,13*Z*)-hexadecadienal (*63*). This result suggested that additional components may be present in the natural pheromone blend produced by female moths. Additional studies that focused on purity, dosage, formulations on various substrates (e.g. rubber septa), and stabilizers confirmed that the synthetic form of (11*Z*,13*Z*)-hexadecadienal alone was so much less attractive than virgin females that ultimately its use as a field lure was not feasible (*65*).

As previously mentioned, a number of species of lepidopteran pests have been successfully managed using synthetically derived sex pheromones as mating disruptants (*65*). Since the discovery of the major sex pheromone component for navel orangeworm, the possibility of developing a management strategy based

on pheromone-mediated mating disruption has been of great interest (*66*). This reflects the importance of navel orangeworm as a pest to the almond and pistachio industries and also the shortcomings of the conventional insecticidal approach. Groups of studies over several years demonstrated that (11*Z*,13*Z*)-hexadecadienal had biological activity on males in the field; more specifically, interference of the orientation of male moths to unmated female moths used as bait (interpreted as trap shutdown) and damage reduction effects in small (1-8 ha) almond plots (*67–69*). Methods of dispensing pheromone into orchard systems can be divided into three broad groups based on the number of dispensing units and amount of pheromone emitted by each unit. In 2006, Sarfraz and co-workers (*70*) categorized formulations as microencapsulated, hand-applied and high-emission dispensers. Results were mixed for initial almond trials, which used a variety of hand-applied dispensers. Although complete trap shutdown was achieved, damage levels were unacceptable in some trials due to high levels of egg deposition by mated females within the plots, likely due to immigration of mated females from the surrounding area which was not permeated with (11*Z*,13*Z*)-hexadecadienal (*69*). Technical problems with the pheromone chemistry and release of the pheromone were also suspected to contribute to the inconsistent reduction in damage (*69*).

These difficulties remained unsolved until subsequent studies, which used larger plots (16 ha), high emission rates, and metered and timed mechanical devices (puffers) (*71*, *72*). In 1996, Shorey and Gerber (*71*) placed puffers around the perimeter of each plot and demonstrated that trap shutdown could be achieved as effectively as the more numerous hand-applied dispensers (with lower emission rates) applied throughout the smaller plots in previous trials. In the 1996 study, relatively few (5/ha) puffers rather than many (200-400/ha) passive dispensers were tested in almonds. Complete trap shutdown could be achieved in almonds, but not walnuts. The potential problems of dispersal of mated females, air movement impact on pheromone dispersion, pheromone loss through adsorption on foliage, and vertical mixing were identified as potentially interfering with the ability of navel orangeworm males to orient to females used as bait in a sticky trap. In 2008, Higbee and Burks (*72*) compared biological and damage effects in a series of experiments using 8 and 16 ha plots in almonds and pistachios. Puffers deployed peripherally, puffers gridded evenly throughout the plot, and hand-applied membrane dispensers were compared to control plots receiving no treatments in 16 ha plots. The puffers in the gridded deployment were superior to peripherally placed puffers and hand-applied dispensers on both biological (trap shutdown and suppression of mating in sentinel females placed in the center of plots) and damage reduction impacts in almonds and biological impacts in pistachios. In addition, data on estimation of release rates for the puffers and membrane release dispensers indicated that the release rate of the membrane dispensers, which is temperature dependent, was much more variable than the puffers over the season. Whereas the puffer provides a stable and protected environment for the pheromone formulation and emits pheromone only during the hours navel orangeworm are active (*73*). In these later studies, the use of larger plots was able to overcome the problems of immigration of mated females, and puffers solved the problems of pheromone instability and complete release of the pheromone formulation.

More than 25 years after the discovery of (11*Z*,13*Z*)-hexadecadienal and many attempts by chemical ecologists to discover additional navel orangeworm sex pheromone components, a combination of approaches (including molecular biology and sensory physiology) was successful in identifying a number of minor pheromone natural product components (*74*). These natural products include analogs of the major aldehydic component (compound **10**), but in different oxidation states – (11*Z*,13*Z*)-hexadecadien-1-yl acetate and ethyl-(11*Z*,13*Z*)-hexadecadienoate (compounds **18** and **21**, respectively in Figure 5), in addition to two unusual polyunsaturated hydrocarbons – (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-tricosapentaene and (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-pentacosapentaene (compounds **12** and **22**, respectively in Figure 5). Subsequent studies suggested that many of these minor constituents were not important in the attraction of male navel orangeworm, while two- to three of the compounds when mixed with the major component resulted in a highly attractive blend in wind-tunnel assays and field experiments (*64*, *75*). Although this blend of natural products was highly attractive in the field, this attraction was short-lived and it was suspected that degradation products and/or impurities interfered with the response of male moths.

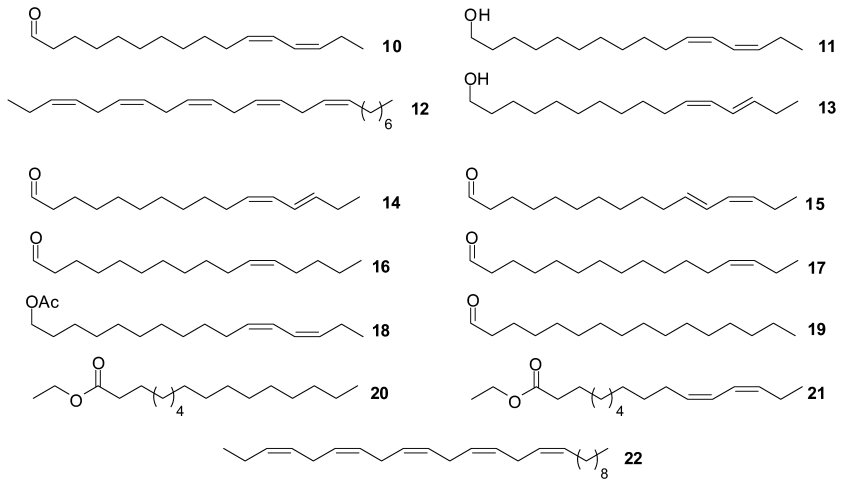

*Figure 5. Components of the current pheromone-based lure (**10-13**) and the identified female navel orangeworm sex pheromone components (**10-12, 14-22**).*

With the discovery and optimization of the complete sex pheromone blend for navel orangeworm, it seemed that an attractive lure that could be used for monitoring this pest would be immediately forthcoming. However, despite the use of stabilizers and various methods of release, such as specially treated plastic vials along with conventional rubber septa, attractiveness of lures decreased rapidly after placement in the field.

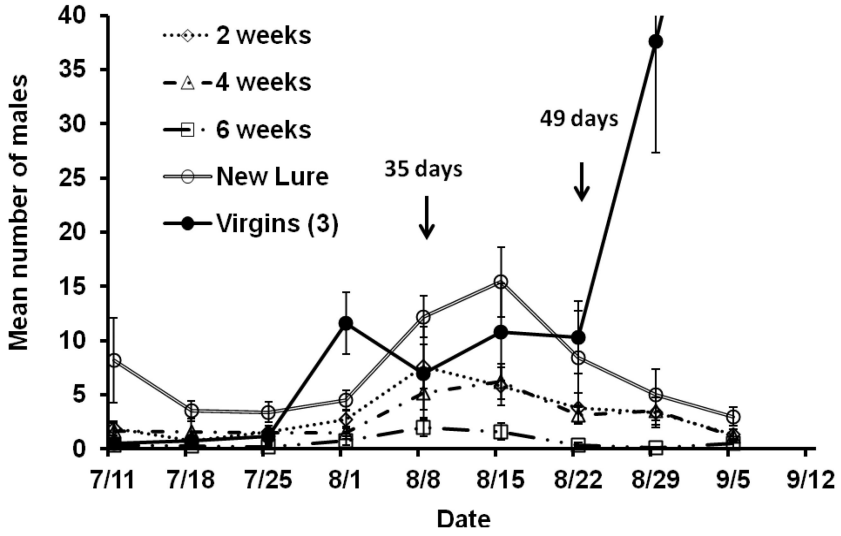

*Figure 6. Male navel orangeworm captures in delta-sticky traps baited with lures aged for 2, 4, and 6 weeks prior to deployment and compared to traps baited with fresh lures in addition to traps baited with three virgin female moths. Lures were formulated with a four-component blend of sex pheromone natural products (compounds **10-13**). Trials conducted by B. Higbee in Kern County, CA, 2012. Error bars represent standard errors.*

A breakthrough occurred when a four-component blend comprised of the sex pheromone natural products, (11*Z*,13*E*)-hexadecadienal, (11*Z*,13*Z*)-hexadecadien-1-ol, (3Z,6Z,9Z,12Z,15Z)-tricosapentaene, and (11Z,13E)-hexadecadien-1-ol (compounds **10**-**13**, respectively in Figure 5) was formulated with *tert*-butylhydroquinone and castor oil in a membrane system (Suterra LLC, Bend, Or). The result was a lure that lasted 4-6 weeks under field conditions and was as attractive to male navel orangeworm as virgin-baited traps in almond orchards (Figure 6; unpublished information).

In addition to the natural products mentioned above, Leal (*74*) and later Kuenen (*64*) and co-workers identified other minor components of the natural sex pheromone blend in 2010. The full suite of compounds is shown in Figure 5. While a number of the identified compounds from the natural sex pheromone mixture do not play a role in attraction of male navel orangeworm, the compound (11**Z**,13**Z**)-hexadecadien-1-yl acetate (*18*) antagonizes attraction of another Pyralidae species, the meal moth, *Pyralis farinalis* (*64*, *74*). Thus, this compound, and possibly other minor components may function as behavioral antagonists, thereby mediating interspecific interactions (*75*).

It should be mentioned that the history of the navel orangeworm and the subsequent control efforts in California orchards is plentiful and complex. Moreover, numerous researchers from industry, academia, and government laboratories have contributed vastly to this history. This current overview and further explanation of the relationship between natural products and the navel orangeworm only touches briefly on the overall history, thus it is not a comprehensive review of the chemical ecology of the navel orangeworm.

This overview of natural products and the California tree nut insect pest, navel orangeworm serves as just one example to highlight the important role volatile natural products play in chemical ecology. Moreover, this example of natural products emphasizes the critical relationship between results from laboratory-based experiments and results generated from field-based experimentation (*76*). Ongoing investigations by scientists from several disciplines continue to contribute important knowledge regarding natural products and their role in the chemical communication of navel orangeworm. Important to the California tree nut industry is what appears to be the forthcoming transfer of positive results to technology applicable to the successful monitoring of navel orangeworm.

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