Effects of altered lipid metabolism in bifenthrin-resistant navel orangeworm Amyelois transitella on cuticle permeability and potential associated cross-resistance to diacylhydrazine and diamide pesticides

Project No.: ENTO1-Berenbaum

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A. Summary

Cuticular hydrocarbons (CHCs) are the main components of the epicuticular wax layer in many insects. Functioning primarily as a barrier against desiccation, CHCs play other roles including serving as sex pheromones and colony-, caste-, species- and sex-recognition signals in social insects. More recently, they have been implicated in resistance to insecticides in many insects, including in the navel orangeworm (NOW). Numerous studies have also reported that the content and composition of CHCs can vary with age, diet, development stage, environment, and sex. We investigated the potential sources of variation in CHC profiles in NOW, specifically the effect of age, sex and strain (i.e., pyrethroid-resistant vs. susceptible), and discovered that all three of these factors are significant. Our work suggests that pyrethroid resistance in NOW is associated with differences in the quantity and/or composition of CHCs, which may reduce insecticide penetration of the cuticle.

In addition, we screened the genome of NOW for evidence of differences between pyrethroid-resistant and susceptible strains that might account for differential CHC profiles or other resistance mechanisms. Bifenthrin resistance of NOW in Kern County may be a greater threat to the almond industry than previously realized, because it appears to involve both enzyme-mediated detoxification and reduced cuticle permeability. There does appear to be a region in the genome of resistant NOW that has experienced intense selection and has undergone a selective sweep, and this region includes genes that are candidates for involvement in pyrethroid resistance.

If NOW populations have developed resistance to pyrethroids in part due to alterations in CHC profiles, we would expect cross-resistance with other chemistries, because many pesticides depend on penetration of the cuticle. In order to establish a method for contact exposure LC_{50} assays, we developed a bioassay to assess insecticide toxicity through direct exposure to eggs and contact exposure across a treated surface. These preliminary assays were performed using bifenthrin.

Another method of insect control that targets the cuticle is topical application of kaolin, an inert naturally occurring clay currently registered for use in several crops as Surround®. We performed laboratory and field experiments to determine whether kaolin could be used as an

effective insecticide against NOW or as a synergist with chemical insecticides. The laboratory assays used both pyrethroid-resistant and susceptible strains to determine whether their differing CHC profiles might make the resistant individuals more resistant to kaolin applications. The field trials, performed in pistachio orchards, used Surround in conjunction with commonly used adjuvants and Altacor. Both our laboratory assays and our field contact toxicity trials provide evidence that kaolin is effective in reducing NOW infestations. Kaolin may act as a viable insecticide on its own in almonds, as it inhibited infestation by NOW larvae in the laboratory. As well, it synergized the chemical insecticides used in both experiments. Both results are encouraging from the perspective of broader NOW control strategies. However, we uncovered evidence of cross-resistance in a pyrethroid-resistant NOW population.

B. Objectives

Specific Objective 1 (Ngumbi, Calla). Confirm overexpression of hydrocarbon-synthesizing P450s in cuticle and impacts on epicuticular wax layer content and composition of all life stages of resistant NOW.

Milestone: Confirmation of multiple resistance mechanisms in NOW, identifying life stage differences in CHC profiles, allowing for strategic management to mitigate resistance and reduce selection pressures to avoid resistance development.

Specific Objective 2 (Demkovich). Determine LC_{50} contact toxicity of chlorantraniliprole and methoxyfenozide to adult and larval NOW, with pyrethroid as positive control for resistance.

Milestone: Development of viable assay using bifenthrin.

Specific Objective 3 (Bush, Demkovich, Siegel). Determine whether kaolin clay can prevent egg hatch and/or cause neonate mortality.

Milestone: Confirmation of kaolin as an effective insecticide; confirmation of kaolin as a synergist of chlorantraniliprole; evidence of cross-resistance to chlorantraniliprole and kaolin of pyrethroid-susceptible NOW.

C. Annual Results and Discussion

Overall, we identified 47 cuticular hydrocarbons, ranging from C_{17} to C_{43} in the cuticular extracts of the navel orangeworm including straight-chain alkanes and a variety of mono-, di-, and trimethylalkanes. Six hydrocarbons dominated NOW CHC profiles (Figure 3), together comprising 59% of all hydrocarbons. We found significant differences between the pesticide-resistant (R347) and susceptible (ALMOND) strains, with first two principal components explaining 59% of the total variance (Figure 1). In addition, means of 23 out of the 47 identified compounds were significantly different.

There were significant age-based differences between pyrethroid-resistant and susceptible NOW strains, differences that were reflected in the PCAs (Figure 2). Post-eclosion, total CHCs increased with adult age. In general, Day 1 adults had significantly lower amounts of hydrocarbons compared to Day 3, Day 5 and Day 7 in the most abundant compounds (Figure 2 and 3). In addition, within each age class, adults from resistant populations had greater quantities of CHCs in total than those from susceptible strains (Figure 3).

Figure 1. Principal component analysis of cuticular hydrocarbons from pesticide-resistant (R347) and susceptible (ALMOND) adult navel orangeworm strains

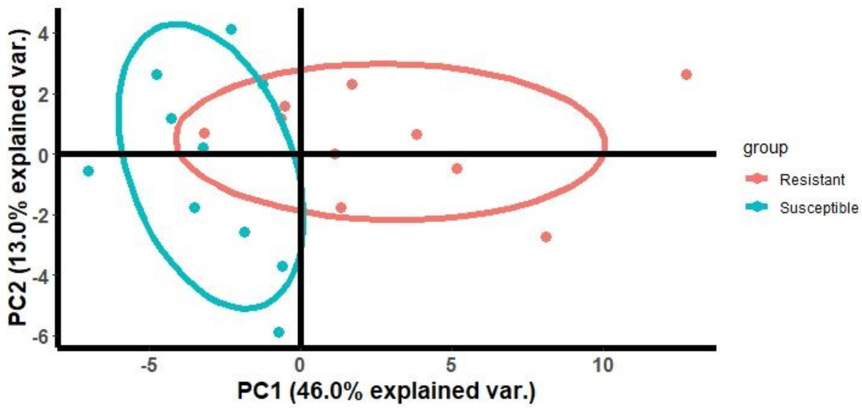
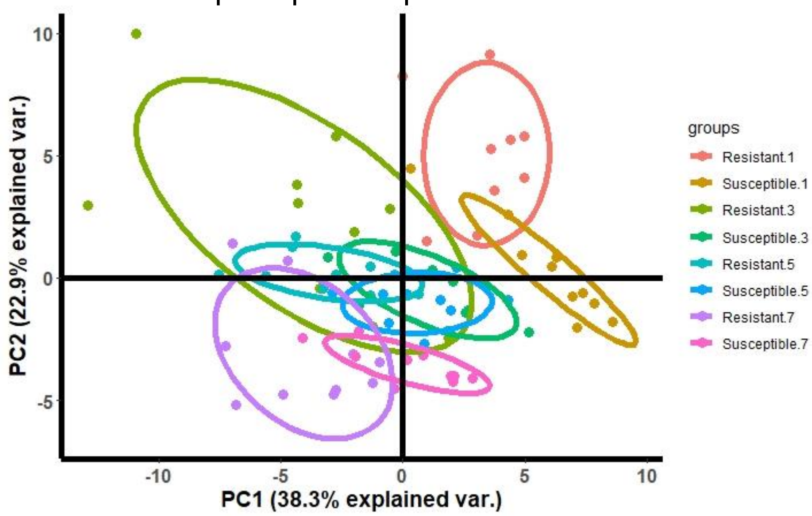


Figure 2. Principal component analysis of cuticular hydrocarbons from pesticide-resistant (R347) and susceptible (ALMOND) adult navel orangeworm strains aged 1, 3, 5, and 7 days. The PCA shows first and second principal components.



Sex-based differences in CHC profiles between pyrethroid-resistant and susceptible NOW strains were recorded, as reflected in PCAs (Figure 4). The first two principal factors accounted for 54% of the variance.

Our study revealed that navel ornageworm CHC profiles are complex, with variations attributable to strain, age, and sex. Overall, the pesticide-resistant strains had greater amounts of CHCs compared to the susceptible strain. Our results suggest that CHCs and CHC content may be useful as a biomarker to differentiate between pesticide-resistant and susceptible NOW populations. Furthermore, our results demonstrate that sex is one of the factors associated with CHC variations in NOW, suggesting that CHCs could conceivably be combined with sex pheromones to improve the performance of lures that are currently used for monitoring and controlling NOW. Knowledge of the factors associated with variations in CHC profiles in NOW is an important step toward enhancing existing monitoring and management strategies.

Figure 3. Effect of age on the composition of cuticular hydrocarbons in pesticide-resistant (R347) and susceptible navel ornageworm strains. Figure shows the six dominant hydrocarbons.

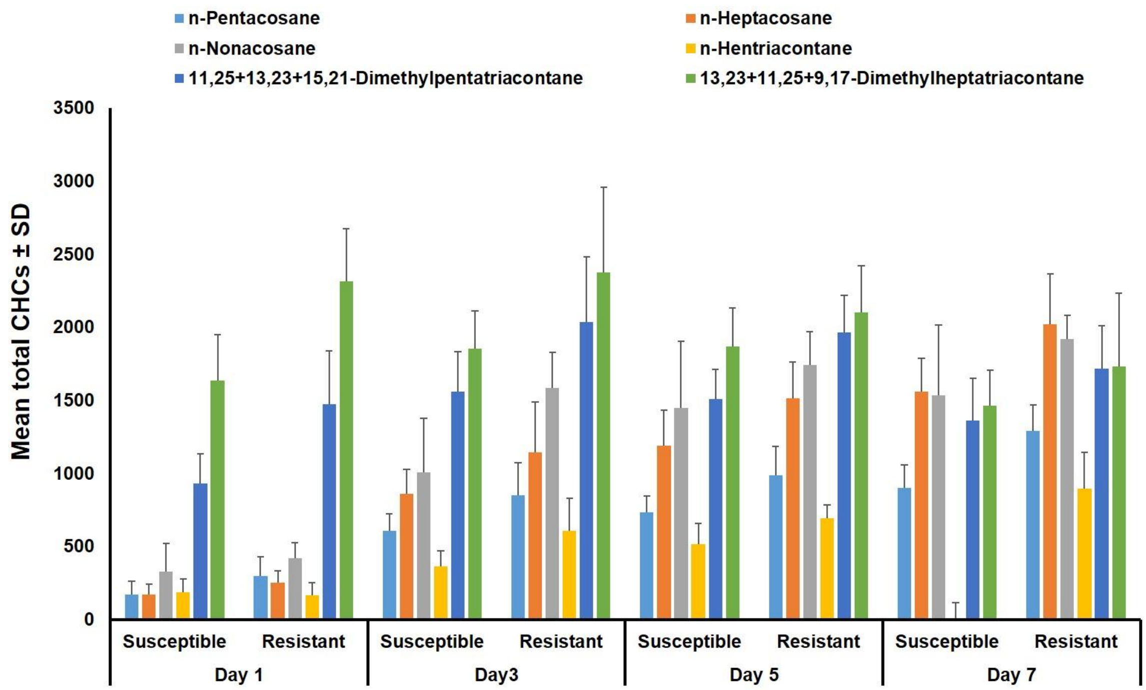
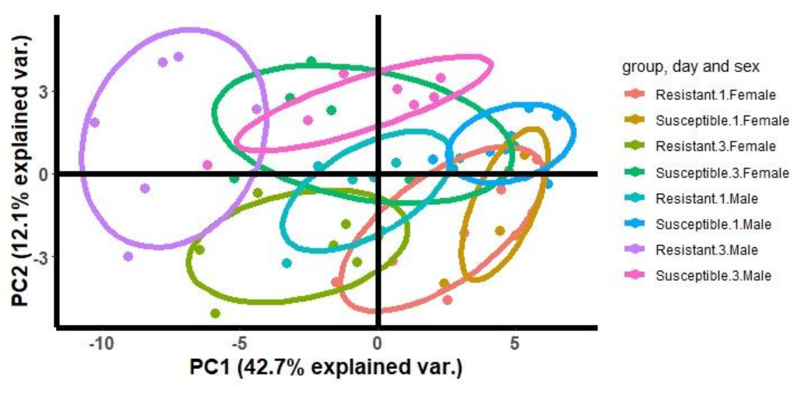
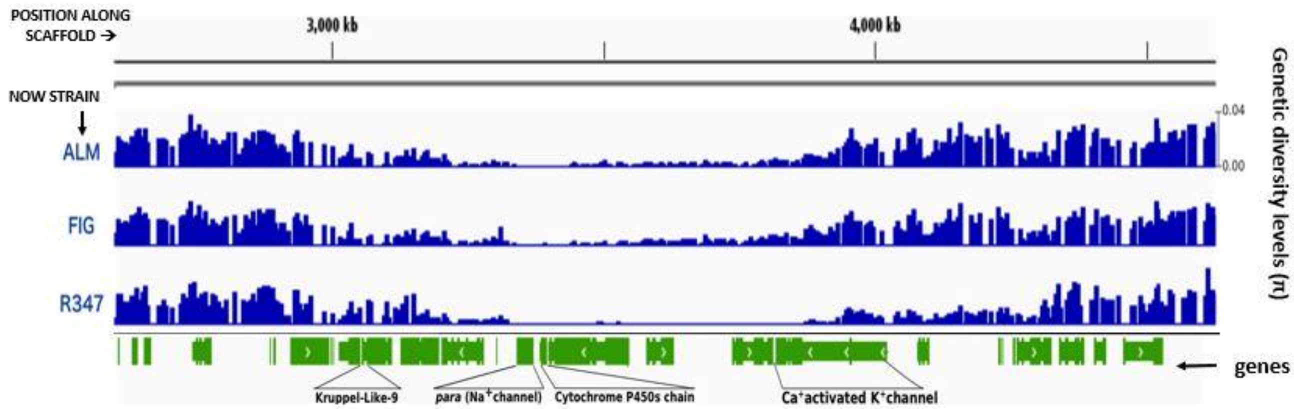


Figure 4. Principal component analysis of cuticular hydrocarbons from pesticide-resistant (R347) and susceptible (ALMOND) adult male and female navel orangeworm strains aged 1 and 3 days. The PCA shows first and second principal components.



In the process of screening genomic signatures of insecticide selection in three populations of the navel orangeworm with varying levels of resistance, we identified a 1.1 Mb selective sweep. The target of selection could be in the ***para*** gene with the mutation "*kdr*" (knock-down-resistance), which confers resistance to pyrethroids, and/or in a chain of cytochrome P450s (***CYP6AB54*, *CYP6AB55*,** and ***CYP6AB66***) situated in the center of the sweep. CYP6AB enzymes have been previously implicated in pyrethroid resistance in other lepidopterans (e.g., cotton bollworm, *Helicoverpa armigera*).

Figure 5. Genomic diversity levels for three strains of navel orangeworm (NOW): ALM (pyrethroid-susceptible NOW from almond orchards), FIG (NOW from figs with some resistance to pyrethroids), and R347 (pyrethroid-resistant NOW from Kern County)



In our preliminary LC_{50} assays using bifenthrin, we observed significant reductions in egg mortality in the resistant strain relative to the susceptible strain. Additionally, more larvae survived the contact exposure in the resistant strain when total survivorship counts were assessed three weeks after the sprays. With a successful method using bifenthrin as a positive control, we intend to examine chlorantraniliprole and methoxyfenozide under similar conditions.

In laboratory trials, application of kaolin and chlorantraniliprole reduced penetration of NOW larvae into almonds as well as the percentage of kernels infested after one week. Kaolin and chlorantraniliprole interacted synergistically. The pyrethroid-resistant strain R347 was more resistant to all treatments but was still significantly adversely affected (Figure 6). The addition of Surround in pistachio field trials not only increased larval mortality over Altacor alone (pooled across days 1, 7, and 12 after application), but Altacor + Kinetic + Surround also significantly outperformed Altacor + Vintre (resulting in a 6.7% decrease in mortality (Table 1)).

Although we had hypothesized a role for cuticular hydrocarbons in resistance to pyrethroids and possibly kaolin, we did not foresee increased resistance to chlorantraniliprole, because is structurally and mechanistically unrelated to pyrethroids. The CHC profile in the resistant strain may reduce penetration of chemical insecticides in general and may even confer resistance to the disruption of the cuticle by kaolin. The potential spread of insecticide resistance in the Central Valley presents a major challenge for NOW control; although our results with kaolin are generally encouraging, Surround may be less effective against such populations. In addition, our field trials were performed on pistachio, not almond. In 2020, we hope to repeat the experiment in almond orchards. We are in the process of checking populations from counties north of the Central Valley to determine if pyrethroid resistance has evolved elsewhere.

Figure 3. Effects of kaolin and chlorantranilprole on NOW larvae from pyrethroid-susceptible populations from almond orchards (ALM) and resistant populations from Kern County (R347).

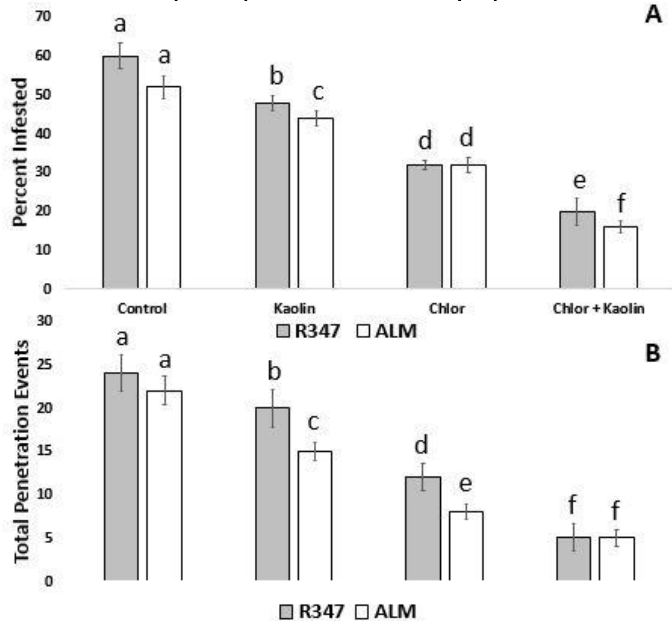


Table 1. Pooled navel orangeworm mortality data 1, 7, and 12 days after applications of Altacor in pistachio with adjuvants Kinetic, Vintre, and kaolin (Surround). Application rates:
Insecticide: Altacor, at 4.5 oz/acre
Adjuvant 1: Vintre at 16 oz/100 gal (0.125%)
Adjuvant 2: Kinetic at 4 oz/100 gal (0.031%)
Adjuvant 3: Kinetic at 4 oz/100 gal (0.031%) + Surround at 15 lbs/acre (1.875%)

Treatment	Sig	Mortality Effect
Altacor Mortality ± SD Kinetic Alone		
83.25% ± 0.086. (N=59) (2,456/2,950)	A	
Altacor Mortality ± SD Vintre Alone		
86.80% ± 0.083. (N=60) (2,604/3,000)	B	4.3% Increase over Kinetic
Altacor Mortality ± SD Kinetic + **Surround**		
88.80% ± 0.066. (N=60) (2,664/3,000)	**C**	**6.7% Increase over Kinetic**

D. Outreach Activities

In the Insect Ecology course IB444 taught by May Berenbaum at the University of Illinois at Urbana-Champaign, lab students are required to conduct six new experiments. In the most recent term, Professor Berenbaum and teaching assistant Daniel Bush designed a set of assays testing the effectiveness of kaolin and diatomaceous earth (DE, another inert dust used to abrade insect cuticle) in navel orangeworm control. The students monitored the effects of kaolin and DE on adult, egg, and neonate mortality in rearing containers. This lab was focused on the subject of autecology—the study of interactions between individuals and their environment—and taught students how naturally-occurring substrates may have insecticidal or deterrant properties.

In addition, both Dr. Mark Demkovich and Dr. Esther Ngumbi have delivered departmental talks within the last year on work funded by this project. These talks were part of the colloquium series for the Department of Entomology, intended to share new discoveries with members of the School of Integrative Biology. Some of the findings in this report have also been shared at other conferences by various project cooperators and personnel.

E. Materials and Methods

Cuticular hydrocarbons were extracted from resistant and susceptible adults of different ages (1, 3, 5 and 7 days post-eclosion) and both sexes following methods by Nelson and Buckner with modifications. Individual adults were submerged for 10 min in 200 µl hexane containing 1-bromooctadecane as the internal standard (25 ng per µl). Extracts were transferred to clean glass vials. The adults were rinsed with an additional 200 µl of hexane containing the internal standard, which was combined with the initial extract. We analyzed extracts using gas chromatography-mass spectrophotometry (GC-MS). Ten replicates were done. Data from the three comparative analyses (strain, age, sex) were separately subjected to statistical analysis. We used Principal Components Analyses (PCA) to visualize overall treatment (strain, age, and sex) effects on hydrocarbon profile, and treatment effects on the compounds that represented the top two principal components were tested with one-, two-, and three-way analyses of variance (ANOVA) models in R (RStudio v.0.98.1083, R Foundation, Vienna, Austria). Statistical differences were considered significant if $P < 0.05$. (Ngumbi et al. 2020)

For the preliminary LC_{50} assays, bifenthrin concentrations of 5 ppm, 10 ppm, 20 ppm, and 40 ppm were sprayed on eggs resting on filter paper placed on top of rearing diet in Petri dishes. Hatching larvae had to receive contact exposure on treated filter paper before reaching rearing diet underneath the treated surface.

In our lab trials for the kaolin experiment, we treated almond kernels with kaolin, chlorantraniliprole, or both together. Kaolin concentrations were based on field applications of 15 lb/100 gal, and chlorantraniliprole concentrations were based on a 0.67% field dose. We measured % almonds (n = 25) infested by larvae (n = 50) after sprays containing kaolin, chlorantraniliprole, or both, as well as the total number of inhabited burrows created. A three-way ANOVA was conducted on the results, followed by a Tukey's mean separation procedure (RStudio v.0.98.1083, R Foundation, Vienna, Austria). In our first field trials with pistachios, we used ~15 lb/acre kaolin sprays to avoid clogging or other sprayer problems. In this experiment, there were four treatments: Surround with Altacor and the adjuvant Kinetic, Altacor alone, Altacor with Kinetic alone, and Altacor with the adjuvant Vintre alone. The spray rig was an AiroFan PTO with multinozzles (GB36) (Speed: 2 mph; Volume: 100 gal/acre). There were 6 rows per treatment, separated by 4 row buffers. The middle two rows were used for each treatment to hang hooks.

F. Publications that emerged from this work

Ngumbi E.N., Hanks L.M., Suarez A.V., Millar J.G. and Berenbaum M.R. (2020). Factors associated with variation in cuticular hydrocarbon profiles in the navel orangeworm, *Amyelois transitella* (Lepidoptera: Pyralidae). *Journal of Chemical Ecology*. 46: 40-47.
(acknowledgments were inadvertently omitted from this publication; we will ask the journal to publish an erratum to correct the omission).