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# 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

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## A. Summary

Cuticular hydrocarbons (CHCs) are the main components of the epicuticular wax layer in many insects. Typically, CHCs prevent desiccation while also serving as sex pheromones and colony-, caste-, species- and sex-recognition signals in social insects. In addition, CHCs have been implicated in resistance to insecticides, including resistance to pyrethroids in the navel orangeworm (NOW) (*Amyelois transitella*). Numerous studies have also demonstrated that the content and composition of CHCs can vary with age, diet, development stage, environment, and sex. We investigated potential sources of variation in CHC profiles in NOW, particularly the effects of age, sex and NOW 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.

If altered CHC profiles in NOW populations are responsible in part for resistance to pyrethroids due to reduced penetration, cross-resistance with other chemistries can be expected because toxicity of many pesticides depends on penetration of the cuticle. To establish methods for contact exposure LC<sub>50</sub> assays, we developed three bioassays to assess insecticide toxicity through direct exposure to eggs and contact exposure across a treated surface. These preliminary assays were performed with bifenthrin and chlorantraniliprole, two key insecticides used in NOW management.

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 to improve the performance of chemical insecticides. The laboratory assays tested both pyrethroid-resistant and susceptible strains to determine whether their differing CHC profiles make insecticide-resistant individuals more resistant to kaolin applications as well. The field trials, performed in pistachio orchards, used Surround in conjunction with the adjuvant Kinetic and with Altacor (chlorantraniliprole). Both our laboratory assays and our field contact toxicity trials provide evidence that kaolin is effective in reducing

the intensity of 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 improved the efficacy of the chemical insecticides tested in both experiments. Both of these findings are encouraging from the perspective of broader NOW control strategies. However, we uncovered evidence of cross-resistance to kaolin in a pyrethroid-resistant NOW population.

## B. Objectives

**Specific Objective 1:** Determine and compare cuticular hydrocarbon profiles between insecticide-resistant and susceptible strains of navel orangeworm (NOW)

**Milestone:** Published our findings that strain, age, and sex have an effect on CHC composition, which may be a factor in insecticide resistance (Ngumbi et al. 2020)

**Specific Objective 2:** Determine LC<sub>50</sub> contact toxicity of bifenthrin, methoxyfenozide, and chlorantraniliprole to larval NOW from a range of populations

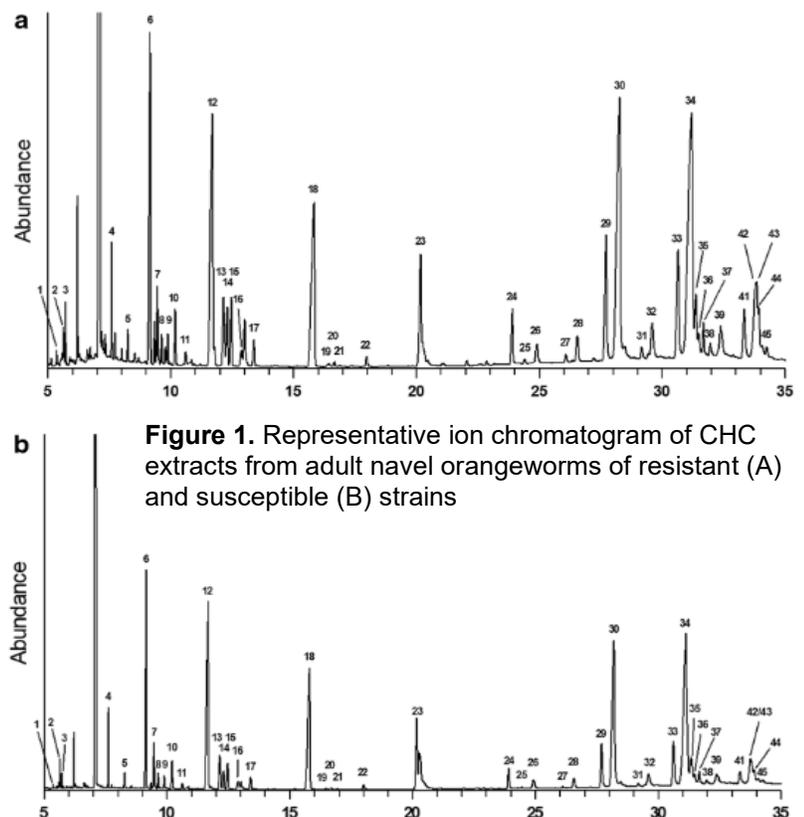
**Milestone:** Developed both laboratory and field assays to assess contact toxicity of bifenthrin and chlorantraniliprole

**Specific Objective 3:** Determine whether kaolin clay can prevent egg hatch and/or cause neonate mortality

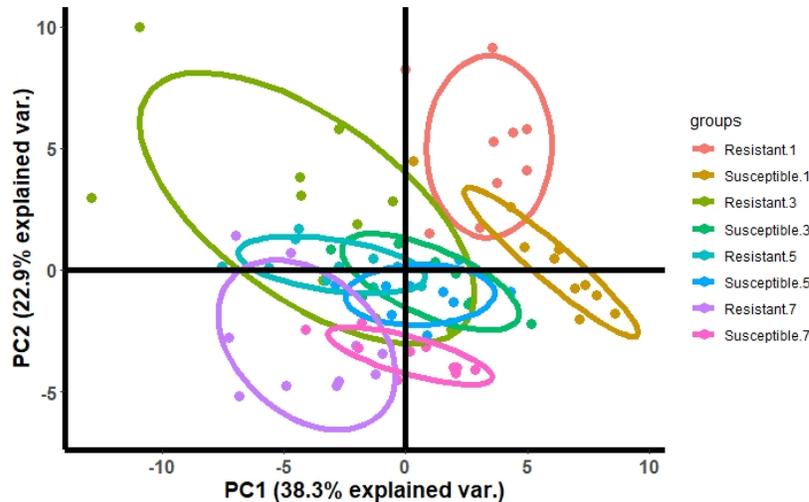
**Milestone:** Preparing manuscript based on study demonstrating that kaolin can reduce infestation intensity and increase neonate mortality, independently or as an addition to chemical pesticides

## C. Results and Discussion

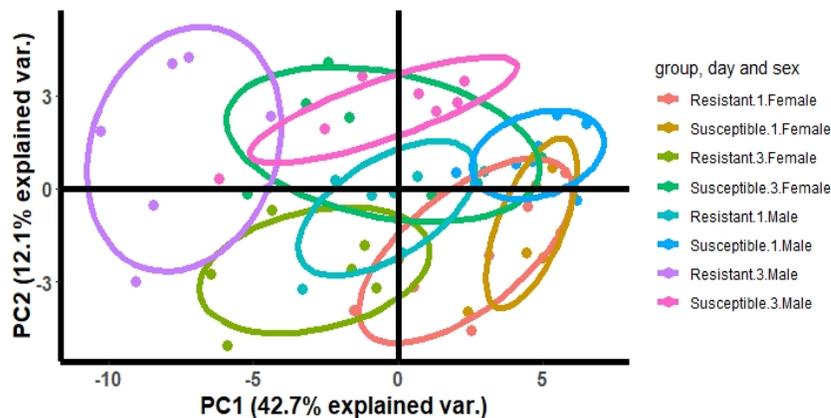
**Objective 1:** The insect wax layer comprises cuticular hydrocarbons (CHCs) that both prevent desiccation and provide intraspecific communication signals. CHCs can also contribute to insecticide resistance via reduced penetrance. We assessed the role of CHCs in NOW insecticide resistance. We found differences in cuticular hydrocarbon profiles between adults of pyrethroid-resistant (R347) and susceptible (ALMOND) strains (Figure 1). Hydrocarbon profiles consisted of 47 compounds of lengths ranging from C<sub>17</sub> to C<sub>43</sub>. Generally, R347 adults had greater total CHC quantities, but relative quantities of individual components were similar. We also found that CHC quantities increased with age (Figure 2) and differed by sex



(Figure 3). Our results indicate that CHCs vary by all three factors and could potentially be useful biomarkers to differentiate between insecticide-resistant and susceptible strains. A manuscript from this work has been published in *Journal of Chemical Ecology* (Ngumbi et al. 2020).



**Figure 2.** Principal components analysis of first and second principal components for CHCs of adult navel orangeworms from pesticide-resistant and susceptible strains aged 1, 3, 5, and 7 d.



**Figure 3.** Principal components analysis of first and second principal components for CHCs of adult navel orangeworms, male and female, from pesticide-resistant and susceptible strains.

**Objective 2:** We developed a laboratory bioassay to assess insecticide toxicity through direct exposure to eggs and contact exposure across a treated surface. Bifenthrin, chlorantraniliprole, and methoxyfenozide were sprayed on eggs resting on filter paper placed on the surface of artificial diet in Petri dishes. Neonates thus experienced contact exposure to treated filter paper before reaching their food underneath the treated surface. The resistant strain experienced significantly lower egg mortality relative to the susceptible strain. More larvae survived the contact exposure in the resistant strain up to three weeks after the sprays. Also with this method using bifenthrin as a positive control, we have preliminary data on contact toxicity of chlorantraniliprole and methoxyfenozide.

In addition, we developed two bioassays to measure the effects of contact toxicity beginning at the time of oviposition. See the Methods and Results for Objective 3 for full details; in short, paper towels (in the laboratory) or filter papers (in the experimental orchards) were impregnated with mixtures of bifenthrin or chlorantraniliprole, alone or together with kaolin and adjuvants. These methods proved successful, and they provide

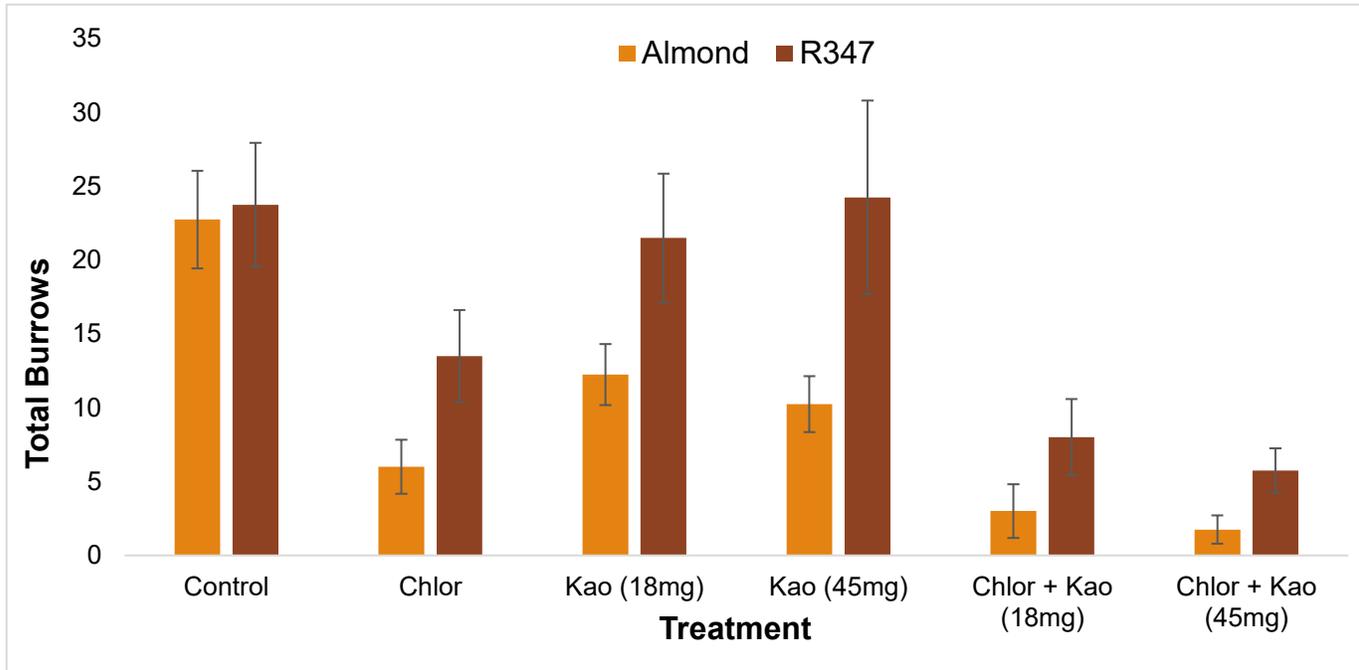
alternative methods of determining contact toxicity of bifenthrin, chlorantraniliprole, and methoxyfenozide for NOW eggs and neonates.

**Objective 3:** Kaolin clay hampers feeding and survival in some insect pests; one suggested mechanism is abrasion or disruption of CHCs, resulting in desiccation or vulnerability to toxins and pathogens. Dr. Joel Siegel conducted kaolin field trials to see if kaolin improved contact toxicity of other insecticidal treatments. He compared the effects of kaolin to two adjuvants that are commonly used in California orchards in order to determine whether kaolin would be a good alternative as an addition to spray mixes. We found that the addition of kaolin (Surround) to a mixture of Altacor (chlorantraniliprole) and Kinetic resulted in the the most effective treatment mix tested (Table 1). It improved the performance of the chemical insecticide mix to which it was added and out-performed the frequently-used adjuvant Vintre.

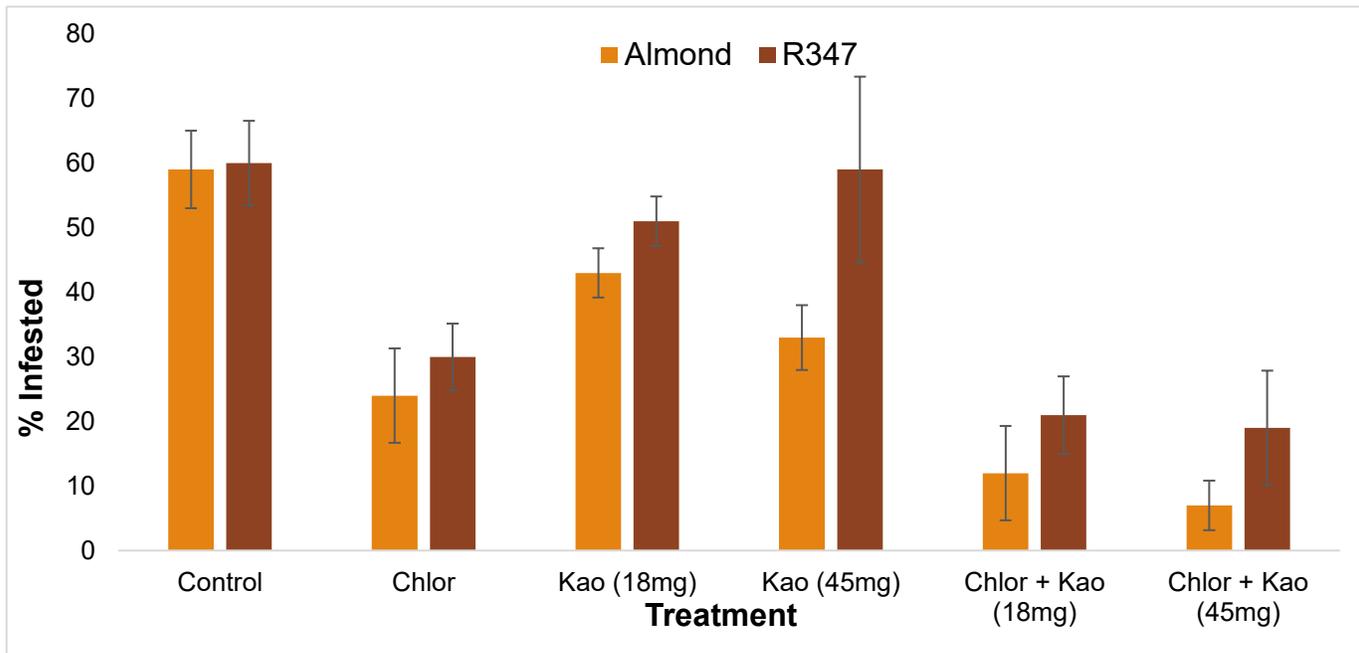
**Table 1.** Contact toxicity mortality rates of navel orangeworm neonate larvae exposed to Altacor tank mixes containing the adjuvants Kinetic and Vintre and the kaolin product Surround

Treatment	Sig	Mortality Effect
Altacor + Kinetic Mortality $\pm$ SD		
83.25% $\pm$ 0.086 (N = 59) (2,456/2,950)	A	
Altacor + Vintre Mortality $\pm$ SD		
86.80% $\pm$ 0.083 (N = 60) (2,604/3,000)	B	4.3% Increase over Kinetic
Altacor + Kinetic + <b>Surround</b> Mortality $\pm$ SD		
88.80% $\pm$ 0.066 (N = 60) (2,664/3,000)	<b>C</b>	<b>6.7% Increase over Kinetic</b>

In laboratory assays, we treated almond kernels with kaolin, chlorantraniliprole, or both together. In two sets of trials, we used kaolin concentrations based on field applications of either ~40 lb or ~15 lb per 100 gallons. In each of these experiments, kaolin and chlorantraniliprole reduced penetration of NOW larvae into the almonds (Figure 4) and the percentage of kernels infested after one week (Figure 5). R347 (the pyrethroid-resistant strain) was generally more resistant to all treatments (including chlorantraniliprole, which was unexpected) but was still significantly affected. However, our treatments were much more effective at reducing the total number of larval burrows than reducing the percentage of affected kernels. Kaolin may be more useful for lowering the intensity of damage, as opposed to its spread (the latter is more important for growers). Nevertheless, these results indicate that kaolin can improve chlorantraniliprole efficacy, possibly by disrupting CHCs. We repeated this experiment with bifenthrin and found similar results (data not shown here), although, as expected, R347 continues to display resistance to pyrethroid treatments. The cross-resistance to chlorantraniliprole we found in the R347 strain is concerning; the modes of action for these insecticides are different, so our results suggest that either R347 is developing multiple resistances or that its resistance to pyrethroids is due partially to adaptation in its CHC composition (or some other unidentified mechanism) that confers resistance to multiple classes of pesticide.



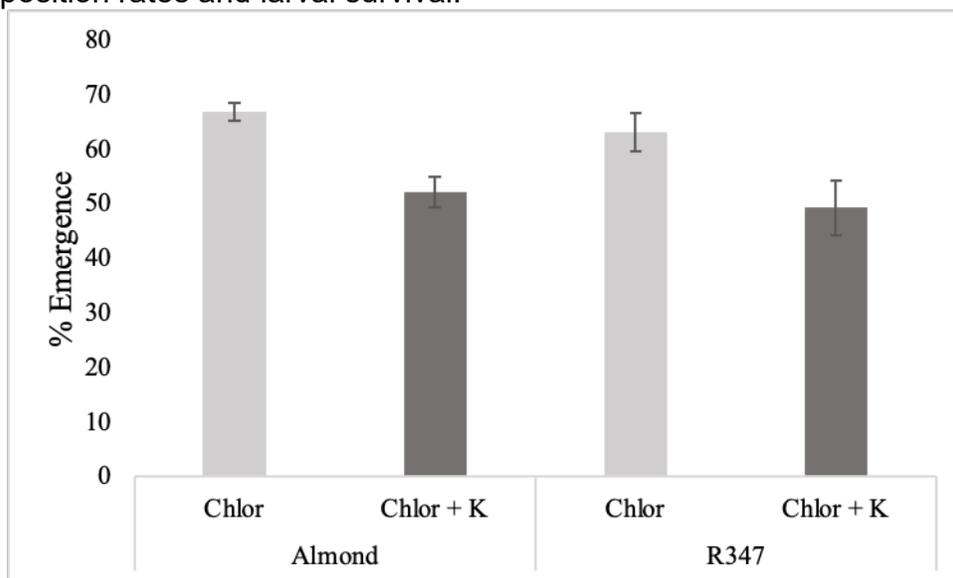
**Figure 4.** Average burrows ( $\pm$  SD) made by navel orangeworm larvae from pyrethroid-susceptible (Almond) and -resistant (R347) strains on almond kernels treated with water (Control), chlorantraniliprole, kaolin, or mixtures of chlorantraniliprole and kaolin



**Figure 5.** Average percentage of almond kernels infested ( $\pm$  SD) by navel orangeworm larvae from pyrethroid-susceptible (Almond) and -resistant (R347) strains on almond kernels treated with water (Control), chlorantraniliprole, kaolin, or mixtures of chlorantraniliprole and kaolin

In order to provide another method for Objective 2 and further evidence for Objective 3, we also performed laboratory assays using oviposition surfaces treated with kaolin and chlorantraniliprole sprays. We maintained the same treatments and concentrations as in the almond kernel assays above. Although there was no effect of kaolin or chlorantraniliprole

sprays on oviposition rates, and kaolin on its own did not have any significant effects, the addition of kaolin to chlorantraniliprole treatments did reduce the emergence and survival of NOW neonate larvae (Figure 6). This may be a result of kaolin abrading the weak cuticle of the larvae and allowing more efficient penetration of the insecticide, or this mixture may have been able to penetrate the eggs more easily. Preliminary data from a similar experiment also indicate that diatomaceous earth (another sorptive dust) negatively affects both NOW oviposition rates and larval survival.



**Figure 6.** Percentage ( $\pm$  SD) of pyrethroid-susceptible (Almond) and -resistant (R347) first instar navel orangeworm larvae to successfully emerge from eggs laid on surfaces treated with chlorantraniliprole or chlorantraniliprole and kaolin.

In all three sets of assays performed for Objective 3 we found evidence for the viability of kaolin as a tool for navel orangeworm control. In particular, it improves the effectiveness of chlorantraniliprole sprays in reducing the intensity of kernel infestations and increasing the mortality of newly-emerged larvae. Kaolin shows promise as a relatively inexpensive addition to the NOW management toolbox that is safe for human contact and consumption.

#### D. Outreach Activities

Dr. Esther Ngumbi has delivered a departmental talk in the last year on work funded by this project as 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; Daniel Bush received an award for his presentation (covering part of this work) at the annual meeting of the Entomological Society of America held online in November 2020.

#### E. Materials and Methods:

In the CHC experiment, CHCs were extracted from adults by submerging freeze-killed individuals in hexane containing 1-bromooctadecane as an internal standard. The adults were rinsed and combined with the initial extract. Extracts were stored at 4 °C, then concentrated under nitrogen and resuspended in hexane. Extracted CHCs were analyzed on a Hewlett-Packard (HP) 6890 GC and HP 5973 mass-selective detector (MSD), with helium carrier gas. Hydrocarbon peaks were identified based on their retention indices relative to a ladder of

straight-chain hydrocarbons and from interpretation of their mass spectra. The abundance of each identified hydrocarbon peak was calculated relative to the internal standard. Trimethylalkanes were identified by attention to retention indices and by analysis of the fragmentation patterns. Hydrocarbons were extracted from females and males of the pyrethroid-resistant and susceptible strains. To examine the influence of age, we extracted hydrocarbons from ten adults each from four age classes: 1-, 3-, 5-, and 7-d post-eclosion. Separately, we extracted another ten male and female moths from both strains at 1-d and 3-d to determine whether CHCs differ between sexes. Results were subjected to principal components analyses (PCA) and analyses of variance (ANOVA) in R to visualize overall treatment (strain, age, and sex) effects on hydrocarbon profile.

For the preliminary LC<sub>50</sub> 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 field trials, we used ~15 lb/acre kaolin sprays to avoid clogging or other sprayer problems. In this experiment, there were three treatments: Surround with Altacor and the adjuvant Kinetic, 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 with filter papers, and the spray-impregnated papers were then used for contact trials. Mortality results were analyzed using ANOVA and orthogonal contrasts.

In our laboratory 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. This procedure was repeated for pyrethroid-resistant and susceptible strains to help probe for resistance mechanisms. A three-way ANOVA was conducted on the results, followed by a Tukey's mean separation procedure. For oviposition/contact toxicity trials, we sprayed the same treatments (and concentrations) on paper towels and then allowed adults to lay eggs on the treated towels. The papers were monitored for 7 d, and the proportions of surviving larvae were compared via T-test.

#### **F. Publications that emerged from this work**

**Published:** Ngumbi EN, LM Hanks, AV Suarez, JG Millar, MR Berenbaum, 2020. Factors associated with variation in cuticular hydrocarbon profiles in the navel orangeworm, *Amyelois transitella* (Lepidoptera: Pyralidae). *Journal of Chemical Ecology* 46: 40-47.

Calla B, M Demkovich, JP Siegel, JPG Viana, KKO Walden, HM Robertson, MR Berenbaum, 2021 Selective sweeps in a nutshell: the genomic footprint of rapid insect resistance evolution in the almond agroecosystem. *Genome Biology and Evolution*

<https://doi.org/10.1093/gbe/evaa234>

**In Preparation:** Bush DS, M Demkovich, V Aldunate, JP Siegel, MR Berenbaum, 2021. Kaolin as a management alternative for insecticide-resistant navel orangeworm, *Amyelois transitella* (Lepidoptera: Pyralidae).