Detoxification of Insecticides Individually and in Combinations in Navel Orangeworm (NOW) (*Amyelois transitella*) Populations Resistant to Pyrethroid Insecticides

Project No.: **15-ENTO1-Berenbaum**

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Objectives:

1) Compare toxicity of chlorantraniliprole, methoxyfenozide, flubendiamide, and spinosad between the bifenthrin-resistant and susceptible colonies through median-lethal concentration assays
2) Determine if P450s and/or other detoxification enzymes (glutathione-S-transferases, esterases) are involved in the detoxification of chlorantraniliprole, methoxyfenozide, flubendiamide, and spinosad by using synergists in neonate feeding assays for both colonies
3) Examine contact toxicity through insecticide sprays of field doses for chlorantraniliprole, methoxyfenozide, flubendiamide, and spinosad in resistant and susceptible colonies
4) Use RNA-Seq next-generation sequencing techniques to identify specific genes and allelic variants associated with pyrethroid resistance
5) Continued exploratory research: Identify survivorship differences in resistant and susceptible adults in response to consumption of hostplant phytochemicals and compare with survivorship in a hostplant-adapted population
6) Exploratory research: Examine impacts of mutualistic association with Aspergillus flavus on development, survivorship, and toxin tolerance

Interpretive Summary:

The polyphagous navel orangeworm (NOW) (*Amyelois transitella*) (Walker) (Lepidoptera:Pyralidae) continues to be the primary insect pest of almonds and other introduced tree nut crops in California orchards (Zalom et al. 2012). Management strategies for this insect typically combine effective sanitation with insecticides use, but the demand for these commodities has resulted in an increase in insecticide applications to limit damage by NOW. In addition to causing direct losses through feeding, infestation by NOW makes nuts more susceptible to infection by *Aspergillus* species that produce aflatoxins. In 2012, navel orangeworm resistance to the pyrethroid insecticide bifenthrin (Brigade, Bifenture, Fanfare) (IRAC Group 3) was first reported in almond orchards in Kern County (B. Higbee: Wonderful Orchards). Our experiments with this bifenthrin-resistant strain (R347) of NOW started in 2013

when we received eggs collected from wild-caught moths in order to establish a colony at the the University of Illinois at Urbana-Champaign. Feeding assays that tested effects of specific inhibitors of major detoxification pathways in NOW in first instar larvae of the R347 strain demonstrated that the mechanism of resistance in this population was likely metabolic and the result of overexpression of cytochrome P450 and esterase genes (Demkovich et al. 2015a).

Our most recent work with the R347 strain investigated the potential for cross-resistance with other insecticides used in management of navel orangeworm. Using our established feeding assay protocols with first instar larvae, we identified median-lethal concentrations (LC_{50}s) for the spinosyn insecticide spinosad (IRAC Group 5) and the anthranilic diamide insecticide chlorantraniliprole (Altacor®) (IRAC Group 28) in both the R347 strain and the susceptible strain (CPQ) of NOW. Using the spray tower located at the USDA-ARS in Parlier, CA, we applied a range of chlorantraniliprole concentrations to filter papers and then placed CPQ and R347 eggs on sprayed papers to simulate contact toxicity. Results from LC_{50} and contact toxicity assays suggest that there is potential cross-resistance to chlorantraniliprole in the pyrethroid-resistant R347 population. We also identified the LC_{50} for a phytochemical present in almonds, chlorogenic acid, in R347, CPQ, and a population collected from fig orchards (FIG) in order to evaluate more broadly the detoxification capabilities of these different strains.

We continued to use synthetic inhibitors to identify major detoxification pathways in NOW. Through the use of the synergist piperonyl butoxide (PBO), we determined that cytochrome P450 monooxygenases are not involved in the detoxification of chlorantraniliprole in the CPQ strain of NOW (Demkovich et al. 2015b). In an effort to identify the main detoxification pathway of chlorantraniliprole in NOW and assess any potential for cross-resistance to chlorantraniliprole, we applied PBO and the esterase inhibitor S,S,S-tributyl phosphorotrithioate (DEF) in first instar feeding assays with the R347 strain. The P450 inhibitor had no effect on chlorantraniliprole toxicity, but DEF in the diet enhanced the toxicity of chlorantraniliprole, which implicates esterases in the metabolism of this diamide insecticide.

This year we also investigated the impact of adjuvants applied to enhance spray deposition, adhesion, and penetration of insecticides used in NOW managment. Using the USDA-ARS spray tower, we examined toxicity of the methylated seed oils (MSOs) Dyne-Amic™ and FastStrike™, the wetter-spreader Induce™, and the spreader-stickers Cohere™ and Latron B-1956™ toward NOW when combined with the anthranilic diamide chlorantraniliprole and the phthalic acid diamide flubendiamide (Belt®) (IRAC Group 28). Adjuvants were applied in the presence and absence of these insecticdes in a 60% methanol carrier and sprayed on adult NOW and eggs. Results indicate that MSOs may be effective when combined with diamide insetcicides against both NOW adults and eggs and spreader-stickers may be effective when combined with diamide insecticides against NOW eggs.

Based on previous findings in the literature on interactions between other insect species and fungi, we undertook a line of investigation aimed at characterizing the nature of the association between NOW and *Aspergillus* and its impact on detoxification of hostplant phytochemicals. The navel orangeworm is frequently associated with phytopathogenic fungi, and there is mounting evidence that the association between the navel orangeworm and *Aspergillus flavus* is a facultative mutualism (Palumbo et al. 2014, Ampt et al. 2015). Some species of *Aspergillus* fungi are known to detoxify both phytochemicals and synthetic insecticides (Mukherjee and

Mittal 2005, Myung et al. 2008), and we suspect that there may be a detoxification aspect to this association. To determine whether the presence of the fungus affects NOW tolerance of toxic phytochemicals characteristic of a subset of hostplants, we conducted a series of laboratory bioassays in which NOW growth rates, survivorship, and pupal weight were measured on an artificial potato dextrose agar (PDA) diet containing almond meal, in the presence and absence of the furanocoumarins xanthotoxin and bergapten, which occur in some fruit hostplant species. Xanthotoxin and bergapten in the diet reduce the growth rate of navel orangeworm larvae, but only when the fungus is absent. Larvae grow considerably more quickly in the presence of *A. flavus* and in fact, the mold seems to almost eliminate most inhibitory effects of xanthotoxin on the development time of navel orangeworm. However, even in the presence of *A. flavus*, pupal weights are diminished in the presence of xanthotoxin; this indicates that the larvae suffer some developmental penalty owing to xanthotoxin even when development time is unaffected.

Materials and Methods:

Colonies of *A. transitella* were reared under conditions of 27 ± 4ºC with a 16:8 (L:D) hour photoperiod.

Objectives 1 and 5: For median-lethal concentration assays, insecticides were mixed in at a range of different concentrations with a standard diet in its liquid phase and then poured into seprate 28-ml (1-oz) cups to harden. Twenty neonates were placed on diet containing insecticide at each concentration, and mortality was assessed after 48 hours. Following three replicates of each concentration, LC_{50}s were generated through Probit analysis using SPSS version 22 software (SPSS Inc., Chicago IL). Results from these experiments were considered significant when there was no overlap in the 95% confidence intervals produced in the Probit analysis.

Objective 2: In chlorantraniliprole feeding assays that incorporated detoxification inhibitors, the median-lethal concentration of chlorantraniliprole was applied for the CPQ and R347 strains. PBO and DEF were incorporated at the concentration of 200 µg/g (200 micrograms of synergist per gram of standard diet) based on previous research (Niu et al. 2012; Demkovich et al. 2015a) because this concentration produced <15% mortality toward neonates within the first 48 hours and allowed us to observe any synergism of chlorantraniliprole in the form of enhanced toxicity. Synergistic interactions were designated when the mortality of chlorantraniliprole with synergist was significantly higher (P<0.05) than the combined mortality of chlorantraniliprole and synergist. Separate analyses using JMP Pro version 12 (SAS Institute, Cary NC) were calculated for chlorantraniliprole and chlorantraniliprole with synergists using multiple regression with orthogonal polynomial contrasts in order to differentiate among treatments.

Objective 3: Assays examining contact toxicity with chlorantraniliprole were conducted using the USDA-ARS spray tower in Parlier, California. Filter papers were sprayed with 10 ml chlorantraniliprole in a 60% methanol carrier at 2.5, 25, and 250 ppm. Filter papers sprayed with the carrier were used as control. Sprayed filter papers were then placed in Petri dishes containing wheat bran diet used for NOW rearing. Sheets of R347 and CPQ eggs were cut into strips of 20 eggs each and placed individually on each sprayed filter paper. All controls

and concentrations used in chlorantraniliprole contact toxicity assays were tested against 160-240 eggs. Larval survivorship was assessed after two weeks. Differences in R347 and CPQ larvae were determined using Chi-Square analysis.

Experiments examining the impact of adjuvants also involved the use of the USDA-ARS spray tower. Solutions of chlorantraniliprole and flubendiamide were prepared at 125 ppm, which falls in the range of current field applications. All insecticide, adjuvant, and insecticide-adjuvant combinations were sprayed at 10 ml in a 60% methanol carrier solution. Adjuvant applications were scaled down to 10 ml sprays and applied at the following rates: Dyne-Amic 8 oz/100 gal; FastStrike 64 oz/100 gal; Induce 8 oz/100 gal; Cohere 8 oz/100 gal; Latron B-1956 3.5 oz/100 gal. With the exception of Dyne-Amic, all of these applications for adjuvants fall within the range of ground application rates per 100 gallons. Dyne-Amic was applied at a rate below the label recommendations because it was more reflective of the amount that growers are currently using in field applications (J. Siegel, personal observation).

All sprays conducted with NOW involved the use of the susceptible CPQ strain. Adults were selected within 48 hr of emergence, sealed in mesh bags and sprayed. Forty adults per control and treatment were sprayed at an equal sex ratio. Mortality was recorded at 24 hr, 48 hr, and 72 hr following each spray, and all adult sprays were replicated three times. A separate series of sprays was also conducted using CPQ eggs obtained weekly from the insectary at the USDA-ARS. Sheets of NOW eggs were cut into strips of 50 eggs, placed into a Petri dish, and sprayed with 5 strips per plate, for a total of 250 eggs per control and treatment. All egg sprays were replicated three times. Sprayed egg strips were then placed in new Petri dishes containing a rearing wheat-bran diet for NOW. Egg and larval mortality were assessed after two weeks. Data for all adjuvant trials were assembled recently using a series of chi-squares as a preliminary analysis. Significant differences were observed when mortality was greater ($P<0.05$) in all chi-square comparisons with adult and egg data. We intend to use a different statistical approach for these data in a manuscript in preparation and are in the current stages of analysis using multiple regression with dummy coding and orthogonal polynomial contrasts in order to differentiate among treatments.

Objective 6: For bioassays conducted with *A. flavus*, newly emerged first instar larvae were placed on a semi-defined standard lepidopteran diet and then transferred to the experimental medium upon reaching the third instar. An almond PDA medium was prepared using 400 mL of water, 15.6 g of PDA (Sigma-Aldrich, St. Louis, MO), 21.88 g of Bob's Red Mill almond meal (Milwaukee, OR), and 0.057 g of streptomycin (Sigma-Aldrich) for every ~10 (8.5 cm diameter) plate. For treatments excluding fungus, 1 mL of 10% formaldehyde (diluted from 36.5-38.0% stock (Macron, Center Valley, PA)) was also added to prevent fungal growth. A laboratory culture of atoxigenic *A. flavus* (AF36) was started from infected wheat seeds provided by Themis Michailides (University of California, Kearney Ag Research and Extension Center and Davis). In the bioassays, almond PDA plates were inoculated with an agar plug (5 mm diameter) taken from the margins of a sporulating culture (10-15 d old).

Five larvae (reared through first and second instar on a semidefined artificial diet formulated for lepidopterans) were transferred to each of eight plates of almond PDA. Half of the plates were then inoculated with *A. flavus* plugs. There were four replicates per treatment, for a total of twenty individuals per treatment. Larval growth and survival were monitored at 24 h intervals

until pupation, and pupal weights were obtained for all survivors. The plates were maintained under conditions of 28 ± 4^{o} C with a 16:8 (L:D) h photoperiod. The same experimental design was used to test furanocoumarin toxicity, with sixteen almond PDA plates, but half of the plates contained diet mixed with xanthotoxin (Sigma-Aldrich, 1.99 g per 10 plates), and half contained diet mixed with bergapten (Sigma-Aldrich, 2.88 g per 10 plates); furanocoumarins were incorporated into the diet by grinding the crystals with mortar and pestle and stirring them into the diet after autoclaving. After diet was poured into the plates, half of the plates for each furanocoumarin treatment were inoculated with *A. flavus* as before. Larval survival and development time were monitored as before under the same rearing conditions.

We created stage-specific life tables tracking the time to develop to the next instar for each larva, and we also compared pupal weights for all individuals that reached pupation, using SPSS version 22 (SPSS Inc., Chicago IL). Analysis of variance (ANOVA) was used to assess differences among all treatments in development time to the fourth instar. ANOVA was also used to test for significant differences in time to pupation and mortality rate among almond PDA treatments. Paired *t*-tests or Fisher's Least Significant Difference (LSD) test (post hoc) were used to identify significant differences between pairs of treatments.

Results and Discussion:

Objectives 1 and 2: Median-lethal concentrations for neonates were different between the resistant strain for chlorantraniliprole but not spinosad (**Figure 1**). Chlorantraniliprole feeding assays that incorporated the use of inhibitors revealed that PBO did not increase toxicity of chlorantraniliprole in the CPQ ($F \leq 699.73$; df = 2, 237; $P \geq 0.6598$) strain and the R347 strain ($F \leq 7.62$; df = 2, 237; $P \geq 0.24$) (**Figure 2 - 3**). The esterase inibitor DEF, however, increased the toxicity of chlorantraniliprole from 24 hr through 72 hr ($F \leq 7.62$; df = 2, 237; $P \leq 0.0006$) (**Figure 3**).

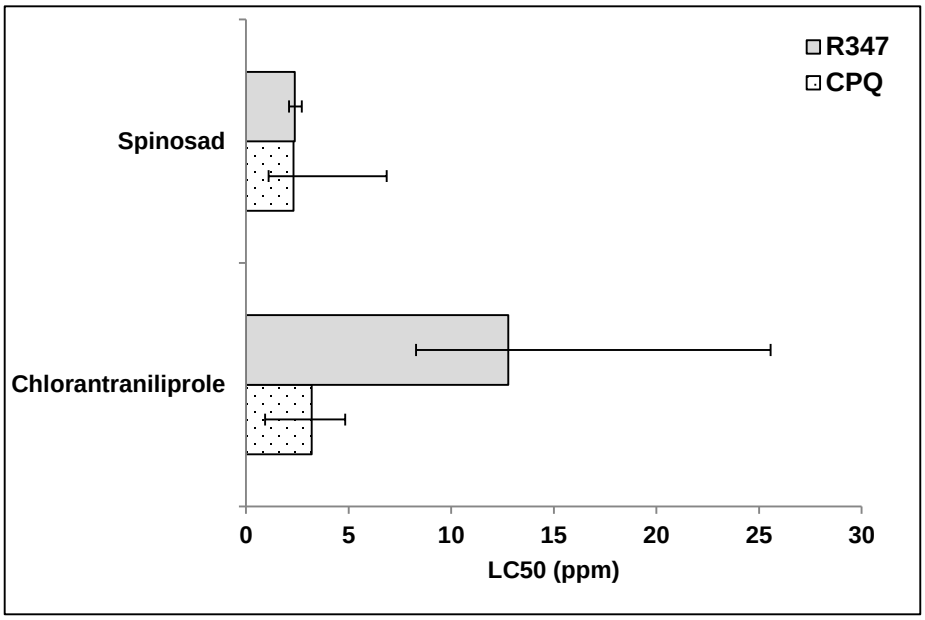

Figure 1. Median-lethal concentrations for chlorantraniliprole (Altacor, diamide, IRAC Group 28) and spinosad (spinosyn, IRAC Group 5) in a bifenthrin-resistant strain (R347) and a susceptible strain (CPQ). Error bars represent the 95% confidence interval of the LC_{50}.

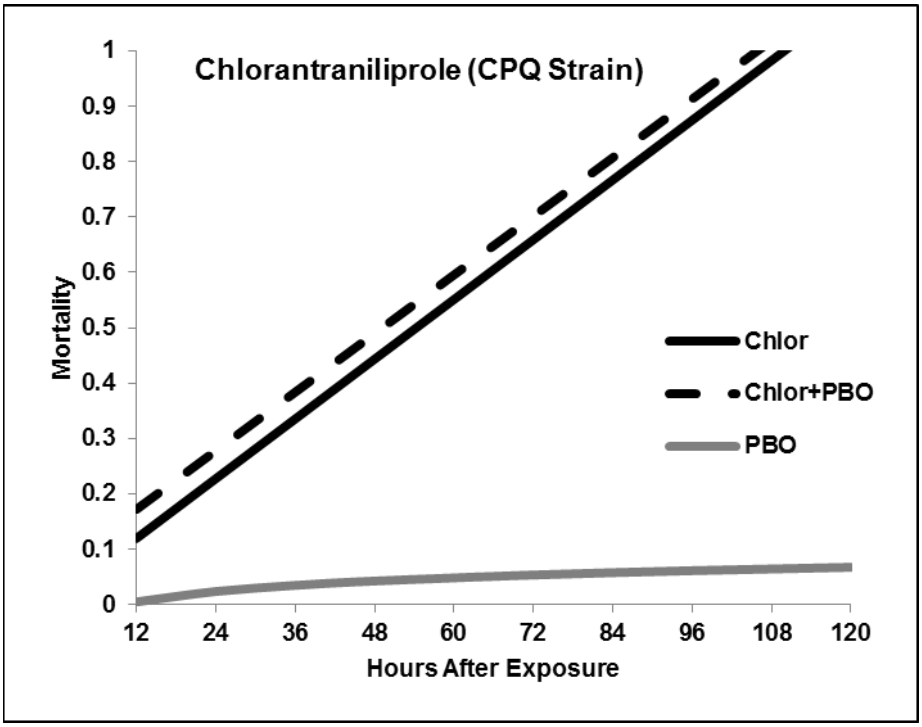

Figure 2. Predicted NOW mortality during the first 120 h in feeding assays with the CPQ strain and 4 µg/g chlorantraniliprole in the presence and absence of 200 µg/g PBO. Figure taken from Demkovich et al. (2015b).

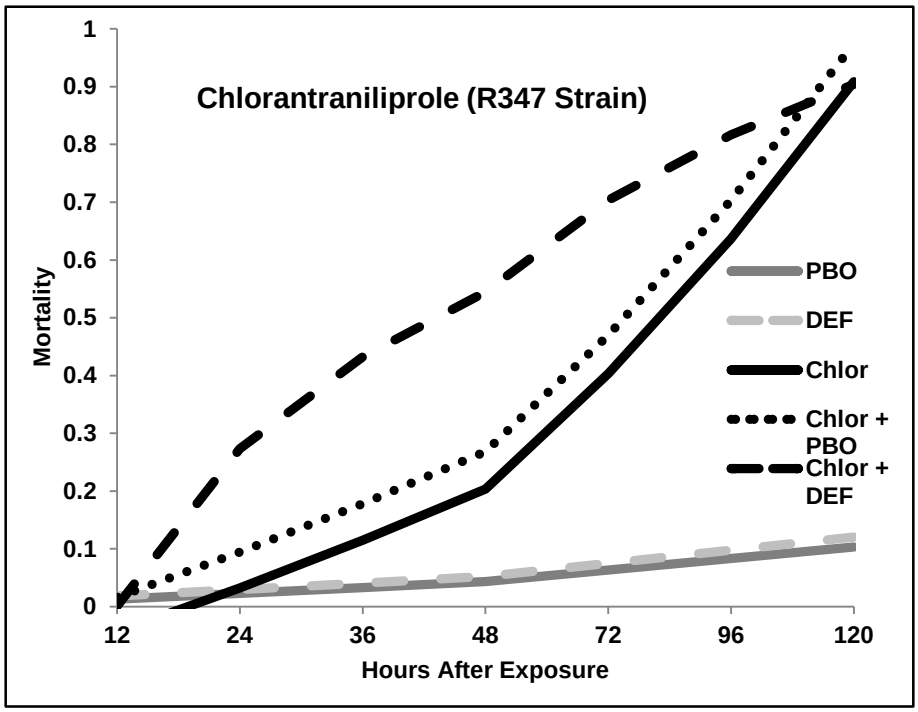

Figure 3. Predicted NOW mortality during the first 120 h in feeding assays with the R347 strain and 8 µg/g chlorantraniliprole in the presence and absence of 200 µg/g PBO (cytochrome P450 inhibitor) and 200 µg/g DEF (esterase inhibitor).

Insecticide feeding assays with the resistant and susceptible strain revealed an approximate 4-fold difference between the median-lethal concentration for chlorantraniliprole and no difference for spinosad. These results suggest an overlapping mechanism in the detoxification of pyrethroids and the diamide insecticide chlorantraniliprole in the resistant strain of navel orangeworm. Applicators assume that the rotation of insecticides based on different modes of action will delay resistance but individual applications ot mixtures may also raise the risk of cross or multiple resistance if the different pesticides share a common route of detoxification. The resistance mechanism of R347 was identified in Demkovich et al. (2015a) as attributable to P450 and esterase activity. If P450s and/or esterases are involved in chlorantraniliprole detoxification in a strain of navel orangeworm with enhanced metabolic capabilities, then cross-resistance may arise with diamide exposure.

Objective 5: The median-lethal concentrations were greater for the R347 and FIG strains over the CPQ strain for the phytochemicals chlorogenic acid, bergapten, and xanthotoxin (**Figure 4**). There was no difference between the R347 and FIG strain for any of the phytochemicals examined.

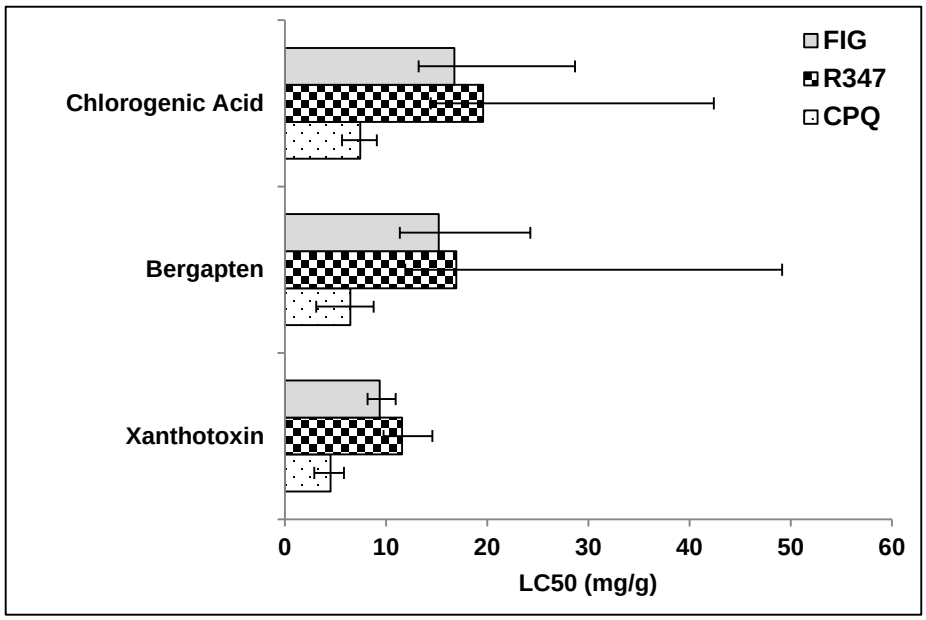

Figure 4. Median-lethal concentrations for phytochemicals present in NOW hostplants (chlorogenic acid-almond, bergapten-fig, xanthotoxin-citrus) in a resistant strain (R347), fig population (FIG), and susceptible strain (CPQ). The LC_{50} is measured in milligrams of phytochemical per gram of standard diet prepared. Error bars represent the 95% confidence interval of the LC_{50}.

When challenged in feeding assays with phytochemicals present in almonds and figs, the resistant line and population collected from fig orchards exhibited a greater tolerance than the susceptible (laboratory colony) line. These results suggest that the FIG colony may have a robust detoxification system comparable to that of the R347 strain. Median-lethal concentration results in the R347 strain indicate that it is capable of detoxifying a wide array of chemistries from synthetic insecticides to phytochemicals present in hostplants.

Objective 3: In filter paper assays with chlorantraniliprole, the resistant R347 strain also displayed a greater tolerance to this diamide insecticide relative to the laboratory susceptible CPQ strain (**Figure 5**). Survivorship in the R347 strain was greater at 25 ppm ($x^2 = 9.56$; df = 1; $P < 0.01$) and 250 ppm ($x^2 = 4.31$; df = 1; $P < 0.05$).

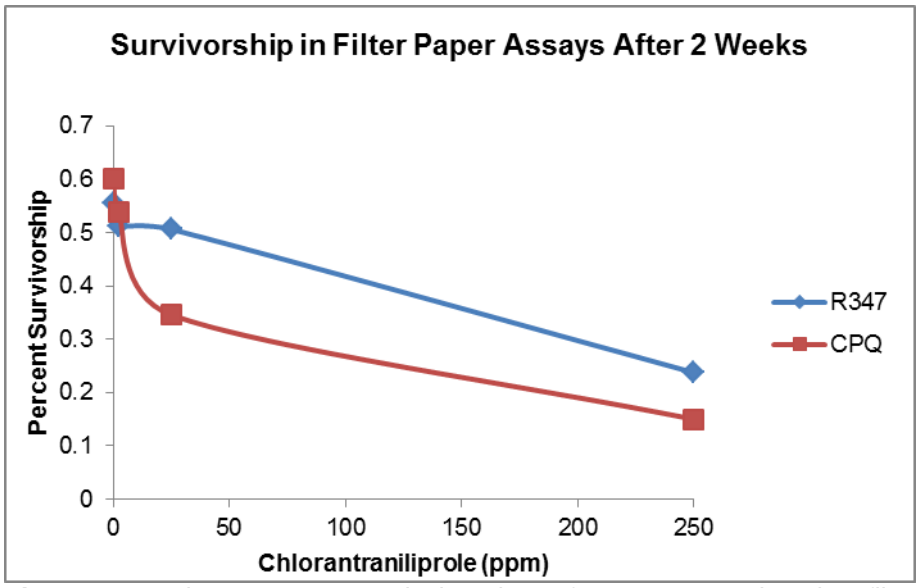

Figure 5. Navel orangeworm survival 14 days after eggs were placed on filter papers sprayed with chlorantraniliprole at 0 ppm, 2.5 ppm, 25 ppm, and 250 ppm in a resistant colony (R347) and a susceptible colony (CPQ).

Our series of experiments involving the resistant strain of NOW exposed first instar larvae to chlorantraniliprole though both ingestion and contact. Results from filter paper assays, combined with the experiments involving feeding assays (**Figures 1 - 3**), suggest that the enhanced enzymatic capabilites of the R347 strain (Demkovich et al. 2015a) may confer resistance to additional classes of inseciticides beyond pyrethroids. This year we demonstrated cross-resistance in bifenthrin-resistant NOW to other classes of pesticide. Identifying the potential extent of cross-resistance with a pyrethroid-resistant strain of NOW and additional insecticide classes will be invaluable to growers when they consider options for both individual insecticide applications and tank mixes in order to prevent resistance or manage existing resistant populations.

The 48 hr time point was selected for analysis in all adjuvant experiments with chlorantraniliprole and flubendiamide (**Figures 6-9**). Analysis of the controls in adult sprays with both chlorantraniliprole revealed that FastStrike was the only adjuvant that increased adult mortality over the 60% aqueous methanol carrier (**Figure 6**). None of the adjuvants had any impact when sprayed without insecticide (**Figure 7**). FastStrike, Induce, Dyne-Amic, and Cohere all increased adult mortality when added to chlorantraniliprole compared to chlorantraniliprole sprayed without adjuvants (**Figure 6**). Overall adult mortality was lower in flubendiamide sprays compared to chlorantraniliprole sprays. In flubendiamide adult sprays, mortality in response to flubendiamide sprayed without adjuvants was not different from mortality in the 60% methanol solutions (**Figure 7**). The only adjuvant that increased adult mortality when added to flubendiamide was FastStrike.

Adjuvant applications without insecticide had more impact in egg sprays. In chlorantraniliprole trials, Cohere and FastStrike were more toxic to eggs than the 60% methanol carrier (**Figure 8**). In flubendiamide trials, Cohere, FastStrike, and Latron B-1956 were more toxic than the carrier solution (**Figure 9**). When chlorantraniliprole was sprayed with adjuvants on NOW eggs, FastStrike, Cohere, Dyne-Amic, and Latron B-1956 all enhanced egg mortality when

added to sprays with chlorantraniliprole compared to chlorantraniliprole alone (**Figure 8**). The additions of Cohere and FastStrike to chlorantraniliprole were the most toxic insecticide-adjuvant ccombinations to eggs in chlorantraniliprole sprays. The wetter-spreader Induce was the only adjuvant that did not increase egg mortality when added to chlorantraniliprole. In flubendiamide egg sprays, Dyne-Amic, FastStrike, Cohere, and Latron B-1956 all increased egg mortality when sprayed with flubendiamide relative to flubendiamide sprays without adjuvants (**Figure 9**). There was no difference between these four flubendiamide-adjuvant combinations. Egg mortality was not affected by the addition of Induce to flubendiamide compared to flubendiamide alone.

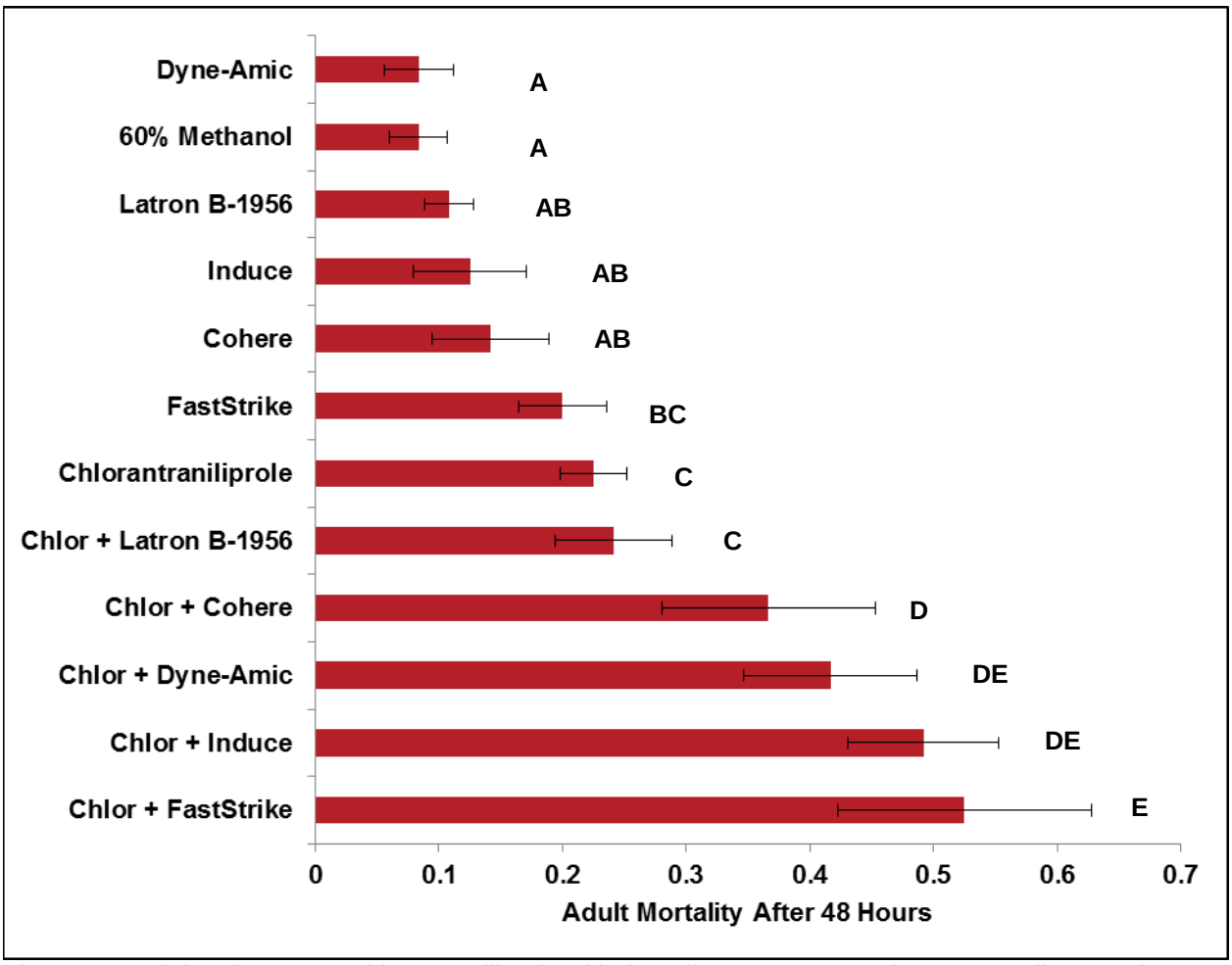

Figure 6. Toxicity of 125 ppm chlorantraniliprole with the adjuvants Dyne-Amic™, FastStrike™, Induce™, Cohere™, and Latron B-1956™ against NOW adults. Adjuvant application rates scaled down to 10 ml sprays were Dyne-Amic 8 oz/100 gal; FastStrike 64 oz/100 gal; Induce 8 oz/100 gal; Cohere 8 oz/100 gal; Latron B-1956 3.5 oz/100 gal. Adult mortality (n=120 per control/treatment) was assessed after 48 hours when sprayed with 10 ml chlorantraniliprole at 125 ppm with and without adjuvants. Error bars represent the standard error of mortality within 3 replicates of each control/treatment.

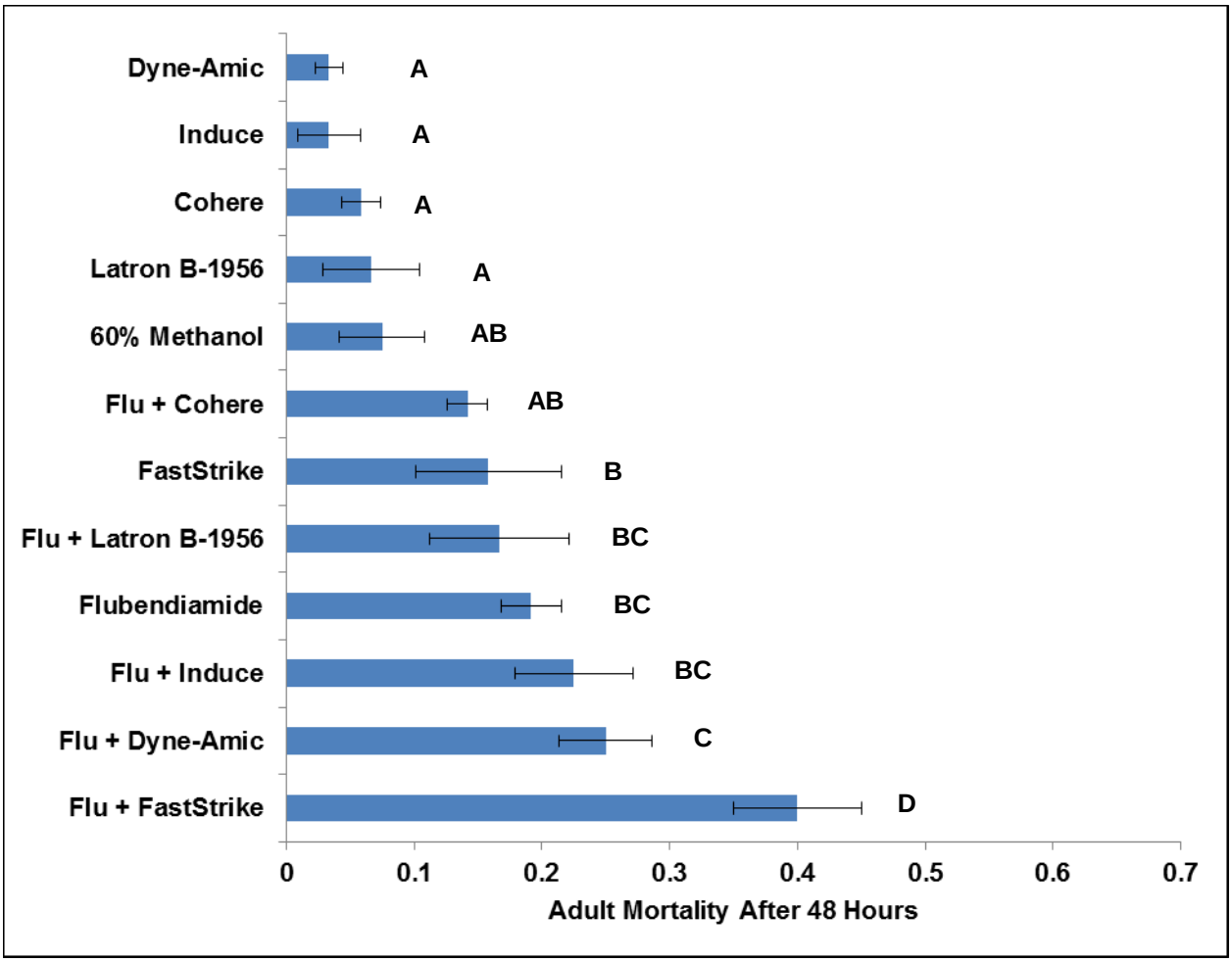

Figure 7. Toxicity of 125 ppm flubendiamide with the adjuvants Dyne-Amic™, FastStrike™, Induce™, Cohere™, and Latron B-1956™ against NOW adults. Adjuvant application rates scaled down to 10 ml sprays were Dyne-Amic 8 oz/100 gal; FastStrike 64 oz/100 gal; Induce 8 oz/100 gal; Cohere 8 oz/100 gal; Latron B-1956 3.5 oz/100 gal. Adult mortality (n=120 per control/treatment) was assessed after 48 hours when sprayed with 10 ml flubendiamide at 125 ppm with and without adjuvants. Error bars represent the standard error of mortality within 3 replicates of each control/treatment.

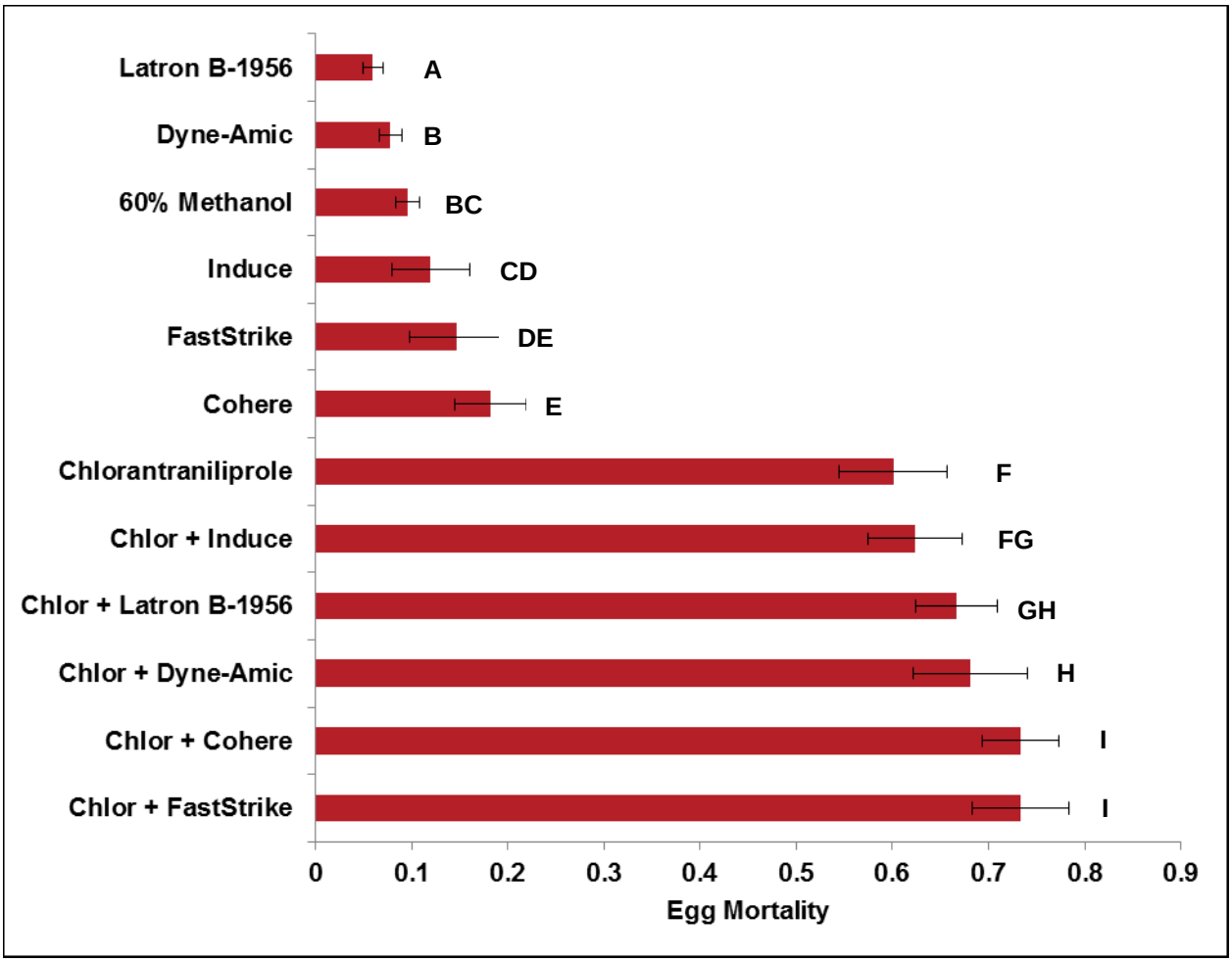

Figure 8. Toxicity of 125 ppm chlorantraniliprole with the adjuvants Dyne-Amic™, FastStrike™, Induce™, Cohere™, and Latron B-1956™ against NOW eggs. Adjuvant application rates scaled down to 10 ml sprays were Dyne-Amic 8 oz/100 gal; FastStrike 64 oz/100 gal; Induce 8 oz/100 gal; Cohere 8 oz/100 gal; Latron B-1956 3.5 oz/100 gal. Egg mortality (n=750 per control/treatment) assessed when sprayed with 10 ml chlorantraniliprole at 125 ppm with and without adjuvants. Error bars represent the standard error of mortality within the 15 sets of egg strips per control/treatment (50 eggs/strip).

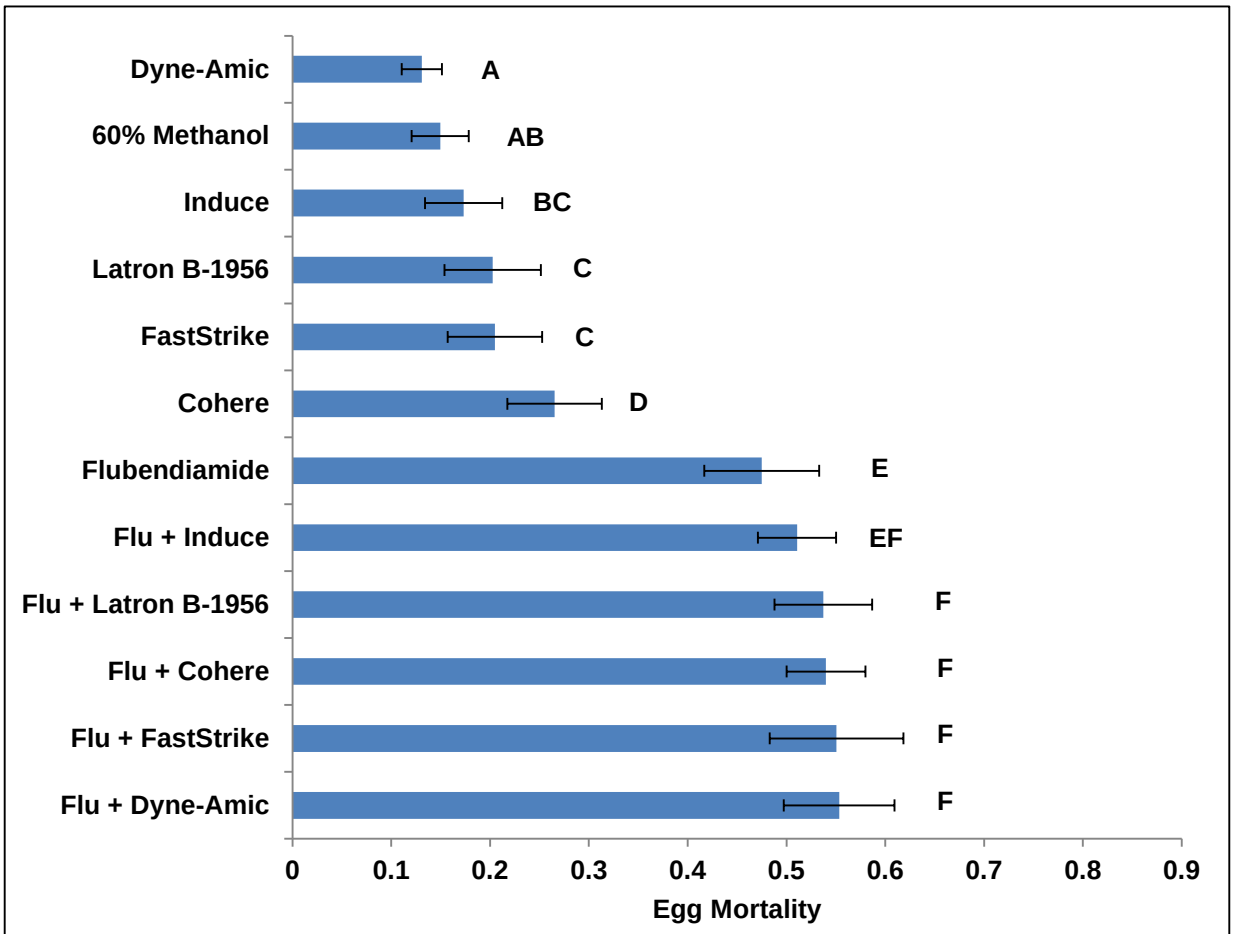

Figure 9. Toxicity of 125 ppm flubendiamide with the adjuvants Dyne-Amic™, FastStrike™, Induce™, Cohere™, and Latron B-1956™ against NOW eggs. Adjuvant application rates scaled down to 10 ml sprays were Dyne-Amic 8 oz/100 gal; FastStrike 64 oz/100 gal; Induce 8 oz/100 gal; Cohere 8 oz/100 gal; Latron B-1956 3.5 oz/100 gal. Egg mortality (n=750 per control/treatment) assessed when sprayed with 10 ml flubendiamide at 125 ppm with and without adjuvants. Error bars represent the standard error of mortality within the 15 sets of egg strips per control/treatment (50 eggs/strip).

Our research on adjuvants toxicity with the diamide insecticides indicates that these chemical formulations may have a significant impact on the toxicity of insecticides applied to control navel orangeworm. The MSO FastStrike was the only adjuvant that enhanced mortality for both chlorantraniliprole and flubendiamide when applied to adults and eggs. Egg mortality was enhanced by each class of adjuvant except for the wetter-spreader Induce for both chlorantraniliprole and flubendiamide. Results from these sets of assays indicate that navel orangeworm may be more vulnerable to certain insecticide-adjuvant combinations at different stages in its life cycle. The spreader-stickers Cohere and Latron B-1956 may have a greater impact on the eggs through adhesion of the insecticide to the chorion. The MSOs FastStrike and Dyne-Amic are penetrators and their mode of action may increase toxicity of insecticides by allowing the insecticides to move through the chorion and the cuticle of the adults. Differential impact of adjuvants on the toxicity of current insecticides used to control navel orangeworm may allow for new chemical management strategies that incorporate effective insecticide-adjuvant combinations in field sprays. In these experiments, we examined only the diamides, but there may also be adjuvants that are more effective with some of the other

insecticide classes used to control NOW such as the pyrethroids, organophosphates, spinosyns, and the diacylhydrazines.

Objective 6: Larvae on almond PDA reached adulthood 14 d faster in the presence of *A. flavus* than on uninoculated plates (t = 16.06; df = 3; P < 0.001). In addition, male and female pupal weights were higher on average for individuals reared on *Aspergillus*-inoculated diet than for those on uninoculated diet (t = 5.56; df = 3; P = 0.011 and t = 7.16; df = 3; P = 0.006, respectively). Larvae raised on diets containing furanocoumarins experienced considerably longer (56 d for xanthotoxin, and 48 d for bergapten) development times (F = 1013.73; df = 3; P < 0.001). However, on plates inoculated with *A. flavus*, development time was much shorter (19 d for xanthotoxin and 25 d for bergapten), less than in the control treatment (diet alone) and almost as short as in the *A. flavus* treatment without furanocoumarins (**Figure 10**). In contrast, pupal weights were significantly depressed by the presence of furanocoumarins in the diet (14 g for males and 24 g for females with xanthotoxin; 14 g for males and 28 g for females with bergapten), irrespective of whether *A. flavus* was present, although fungal growth did allow significantly greater pupal weights in bergapten treatments (F = 328.09; df = 5; P < 0.001 for males, F = 83.00; df = 5; P < 0.001 for females) (**Figures 11 and 12**). Pre-pupal mortality rates were much higher for larvae raised on diet containing furanocoumarins and no *A. flavus* (80% with xanthotoxin and 75% with bergapten), but not on a diet inoculated with *A. flavus* (19% with xanthotoxin and 25% with bergapten) (F = 9.61; df = 5; P < 0.001) (**Figure 13**).

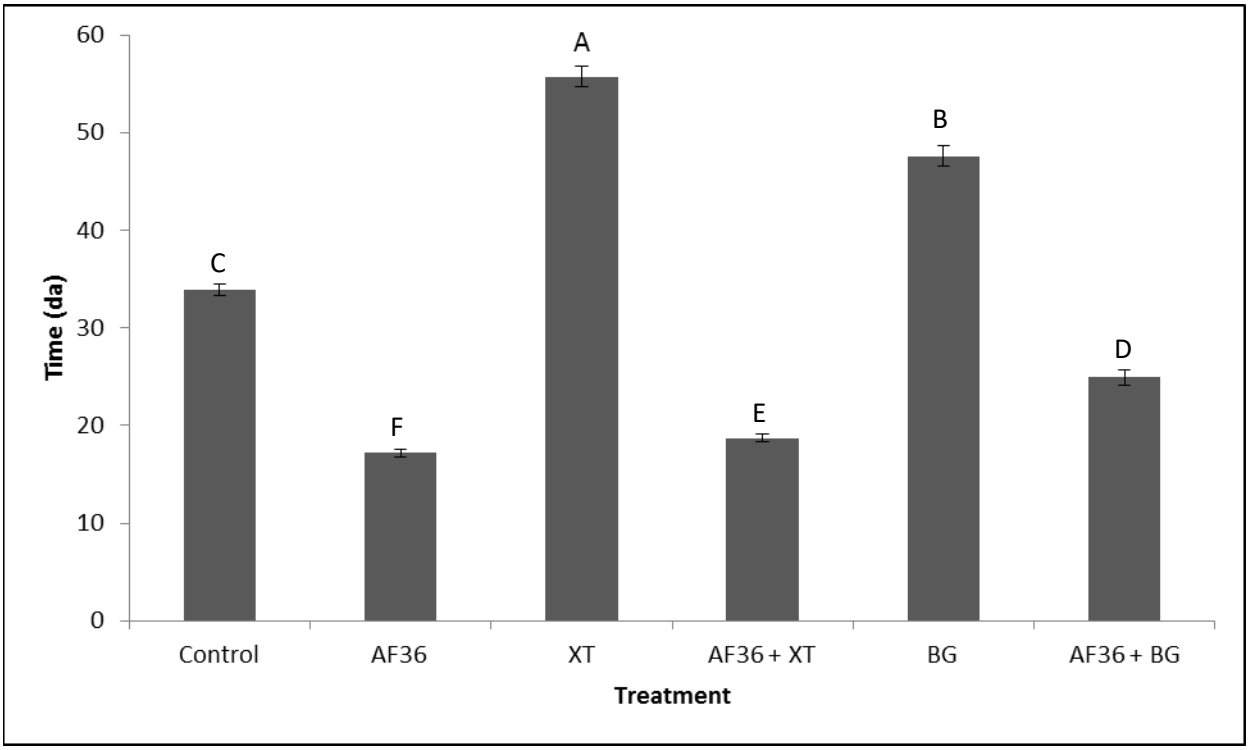

Figure 10. Mean time (± 1 SE) to pupation for third-instar navel orangeworm (*Amyelois transitella*) larvae on almond PDA (Control), with *Aspergillus flavus* (AF36), on diets containing furanocoumarins (xanthotoxin, XT and bergapten, BG), and with both *A. flavus* and furanocoumarins (AF36 + XT and BG).

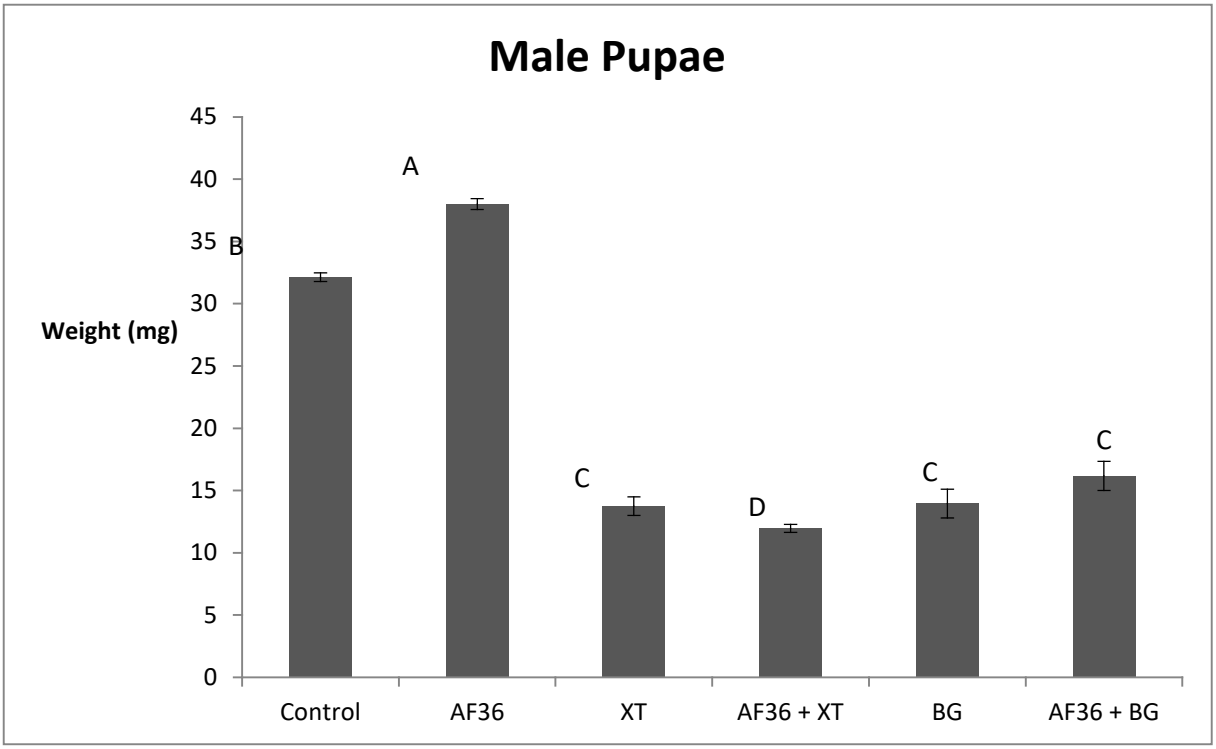

Figure 11. Average pupal weights (± 1 SE) for male navel orangeworm (*Amyelois transitella*) surviving to pupation on almond PDA (Control), with *Aspergillus flavus* (AF36), on diets containing furanocoumarins (XT and BG), and with both *A. flavus* and furanocoumarins (AF36 + XT and BG).

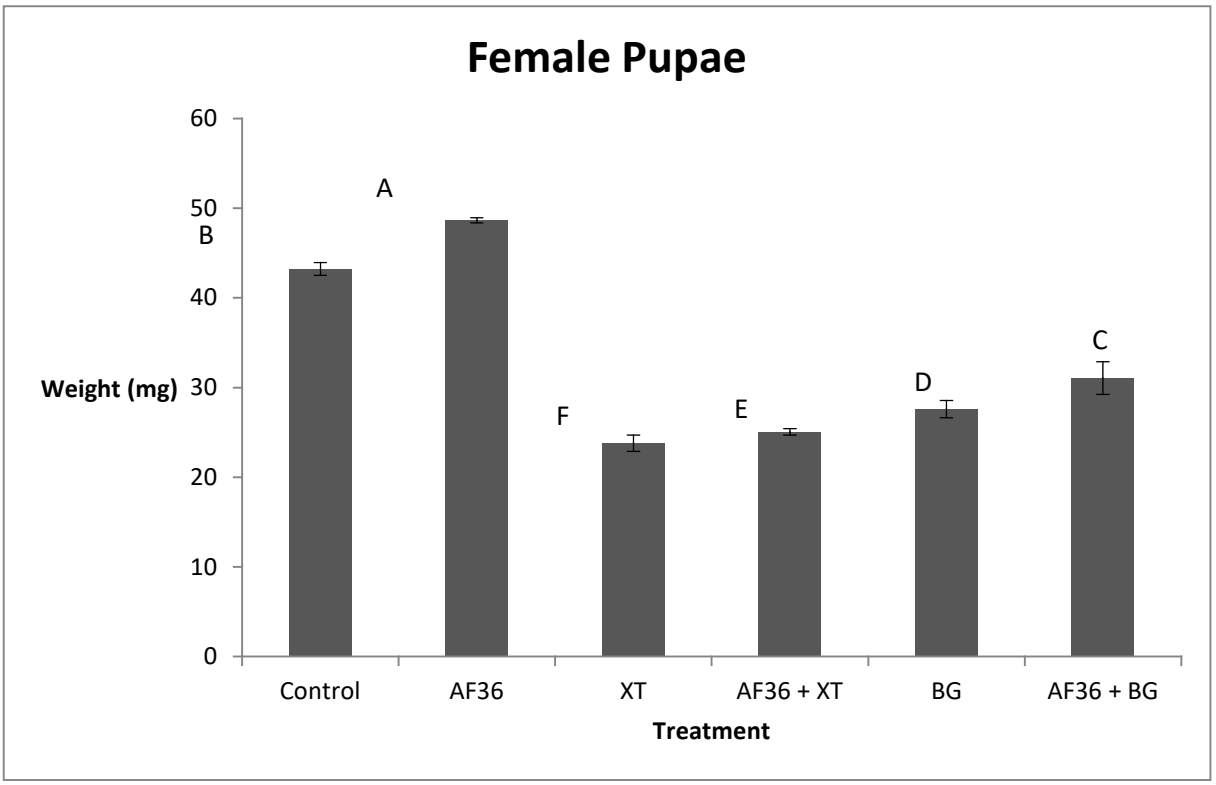

Figure 12. Average pupal weights (± 1 SE) for female navel orangeworm (*Amyelois transitella*) surviving to pupation on almond PDA (Control), with *Aspergillus flavus* (AF36), on diets containing furanocoumarins (XT and

Ber), and with both *A. flavus* and furanocoumarins (AF36 + XT and Ber).

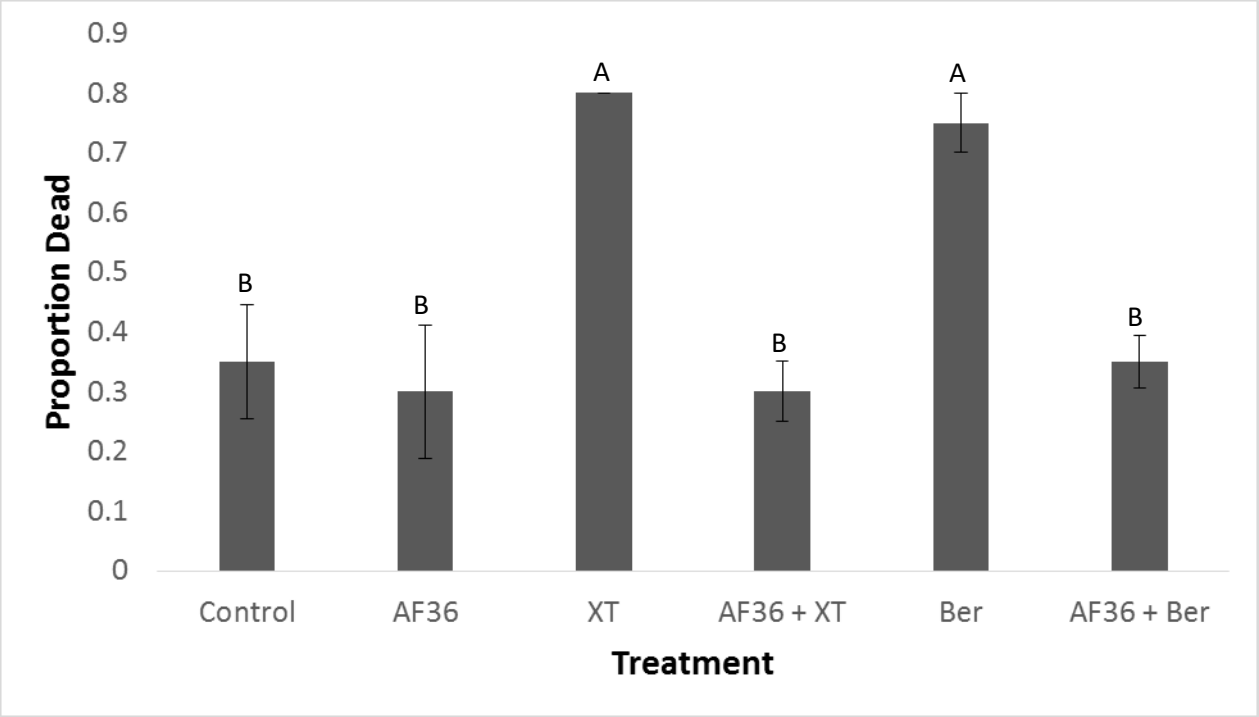

Figure 13. Proportion of navel orangeworm (*Amyelois transitella*) individuals (± 1 SE) to die before reaching pupation on almond PDA (Control), with *Aspergillus flavus* (AF36), on diets containing furanocoumarins (XT and Ber), and with both *A. flavus* and furanocoumarins (AF36 + XT and Ber).

Ingestion of furanocoumarins such as xanthotoxin and bergapten is detrimental to larval performance. However, larval survival and development time are “rescued” to a great degree by the presence of *A. flavus*, so much so that mortality rates in the presence of the fungus are not significantly different from control treatments. There is prior evidence that some ascomycetes (including *Aspergillus* species) are capable of metabolizing furanocoumarins such as xanthotoxin (Myung et al. 2008). Similarly, *Aspergillus* fungi are known to detoxify several classes of insecticides, including organophosphates (Ramadevi et al. 2012) and organochlorines (Mukherjee and Mittal 2005). This experiment is a proof-of-concept that *Aspergillus* species can contribute to the detoxification of natural toxins, and we are planning to perform similar studies in the future using synthetic insecticides.

Objective 4 Update: Instead of using RNA-Seq for the identification of genes involved with pyrethroid resistance, we have decided to alter our approach and use Pool-Seq (Schlötterer et al. 2014) to examine differences in the genomes of NOW collected from almonds, figs, and the R347 population. Pool-Seq is a more cost-effective approach that will allow us to identify regions in the genomes of these different populations of NOW that are under selection. This approach may allow us to identify all genes that distinguish the pyrethroid-resistant R347 population, in addition to genes that allow NOW to adapt to hostplants with more challenging chemistries, such as the fig population. All specimens needed for this analysis have been collected (n=100 adults from each strain) and we are currently in the stage of conducting DNA extractions on these samples and expect sequencing to be underway soon.

Research Effort Recent Publications:

Ampt, E. A., Bush, D. S., Siegel, J. P., and Berenbaum, M. R. 2015. Larval preference and performance of *Amyelois transitella* (navel orangeworm, Lepidoptera: Pyralidae) in relation to the fungus *Aspergillus flavus*. Environ. Entomol. 45(1): 155-162.

Bagchi, V. A., Siegel, J. P., Demkovich, M., Zehr, L. N., and Berenbaum, M. R. 2016. Impact of pesticide resistance on toxicity and tolerance of hostplant phytochemicals in Amyelois transitella (Lepidoptera:Pyralidae). J. Insect Sci. 16(1): 1-7.

Bush, D. S., Lawrance, A., Siegel, J. P., and Berenbaum, M. R. In preparation. Preference of *Amyelois transitella* (Lepidoptera: Pyralidae) for oviposition and feeding sites infected by *Aspergillus flavus* (in progress).

Demkovich, M., Siegel, J. P., Higbee, B. S., and Berenbaum, M. R. 2015a. Mechanism of resistance acquisition and potential associated fitness costs in *Amyelois transitella* (Lepidoptera: Pyralidae) exposed to pyrethroid insecticides. Environ. Entomol. 44(3): 855-863.

Demkovich, M., Dana, C. E., Siegel, J. P., and Berenbaum, M. R. 2015b. Effect of piperonyl butoxide on the toxicity of four classes of insecticides to navel orangeworm (Amyelois transitella) (Lepidoptera: Pyralidae). J. Econ. Entomol. 108(6): 2753-2760.

References Cited:

Ampt, E. A., Bush, D. S., Siegel, J. P., and Berenbaum, M. R. 2015. Larval preference and performance of *Amyelois transitella* (navel orangeworm, Lepidoptera: Pyralidae) in relation to the fungus *Aspergillus flavus*. Environ. Entomol. 45(1): 155-162

Demkovich, M., Siegel, J. P., Higbee, B. S., and Berenbaum, M. R. 2015a. Mechanism of resistance acquisition and potential associated fitness costs in *Amyelois transitella* (Lepidoptera: Pyralidae) exposed to pyrethroid insecticides. Environ. Entomol. 44(3): 855-863.

Demkovich, M., Dana, C. E., Siegel, J. P., and Berenbaum, M. R. 2015b. Effect of piperonyl butoxide on the toxicity of four classes of insecticides to navel orangeworm (Amyelois transitella) (Lepidoptera: Pyralidae). J. Econ. Entomol. 1–8 (2015); DOI: 10.1093/jee/tov237.

Mukherjee, I., and Mittal, A. 2005. Bioremediation of endosulfan using *Aspergillus terreus* and *Cladosporium oxysporum*. Bull. Environ. Contam. Toxicol. 75: 1034-1040.

Myung, K., Manthey, J. A., and Narciso, J. A. 2008. *Aspergillus niger* metabolism of citrus furanocoumarin inhibitors of human cytochrome P450 3A4. Appl. Microbiol. Biotechnol. 78: 343-349.

Niu, G., Pollock, H., Lawrance, A., Siegel, J., and Berenbaum, M. 2012. Effects of a naturally occurring and a synthetic synergist on toxicity of three insecticides and a phytochemical to navel orangeworm (Lepidoptera: Pyralidae). J. Econ. Entomol. 105 (2): 410-417.

Palumbo, J. D., Mahoney, N. E., Light, D. M., Siegel, J., Puckett, R. D., and Michailides, T. J. 2014. Spread of *Aspergillus flavus* by navel orangeworm (*Amyelois transitella*) on almond. Plant Dis. 98: 1194-1199.

Ramadevi, C., Nath, M. M., and Prasad, M. G. 2012. Mycodegradation of malathion by a soil fungal isolate, *Aspergillus niger*. Int. J. Basic and Appl. Chem. Sci. 2: 108-115.

Schlötterer, C., Tobler, R., Kofler, R., and Nolte, V. 2014. Sequencing of individuals—mining genome-wide polymorphism data without big funding. Nature Reviews Genetics 15(11): 749-763.

Zalom, F. G., Pickle, C., Bentley, W. J., Haviland, D. R., Van Steenwyk, R. A., Rice, R. E., Hendricks, L. C., Coviello, R. L., and Freeman, M. W. 2012. UC IPM Pest Management Guidelines: Almond. University of California ANR Publication 3431.