Developmental and environmental impacts on pesticide detoxification in navel orangeworm (NOW) (*Amyelois transitella*)

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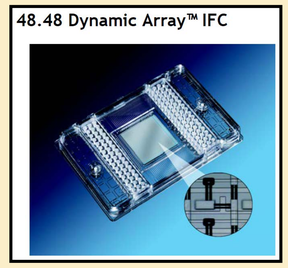
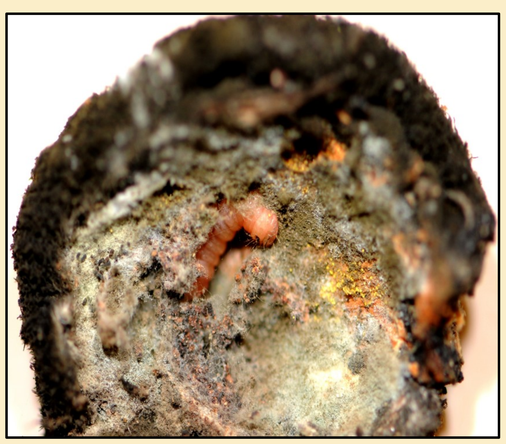
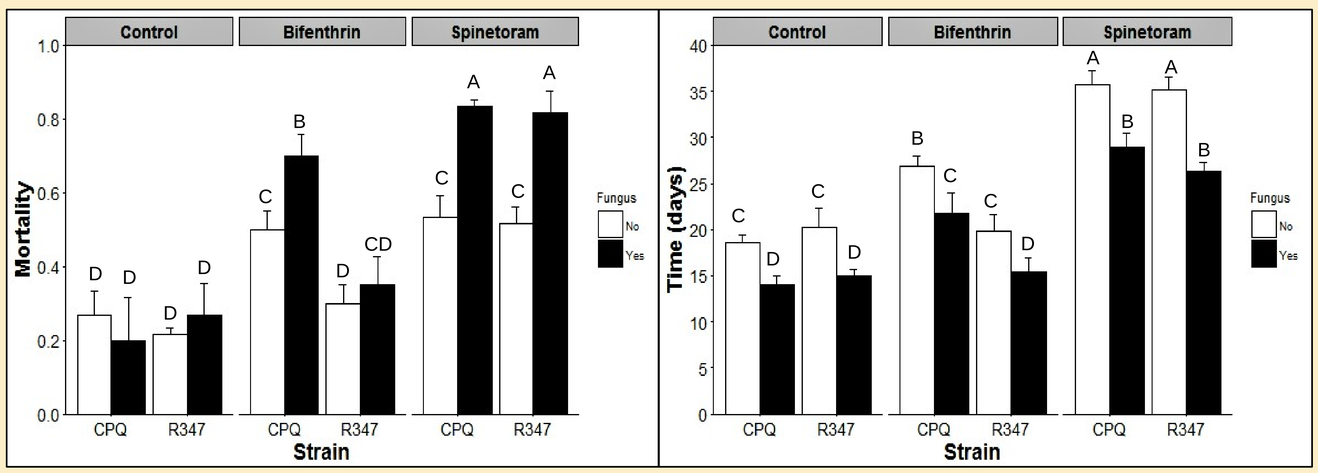


Detoxification Mutualism Projects: Assess the effects of *Aspergillus flavus* presence on toxicity of synthetic insecticides to NOW larvae

Methods & Discussion: Ascomycete fungi can metabolize some chemical insecticides.[1, 2] In this study, we investigated the effects of *A. flavus* presence on insecticide toxicity to the navel orangeworm (NOW). We conducted feeding assays with third instars on almond agar diet into which the insecticides bifenthrin and spinetoram were incorporated, and half of the treatments were inoculated with *A. flavus*. Two strains of NOW were used—a susceptible strain ("CPQ") and a pyrethroid-resistant strain ("R347"). **Both strains of larvae grew faster and survived better on bifenthrin-treated diet than on diet containing spinetoram** (Figs. 1 and 2). As expected, **R347 grew faster and survived better than susceptible larvae on bifenthrin treatments**. ***A. flavus* presence generally increased mortality on pesticide diets** (Fig. 1). This outcome may result from sublethal effects of insecticides on the ability of larvae to prevent opportunistic fungal infection. If pyrethroid resistance ameliorates the negative effects of *A. flavus.* Pyrethroid resistance mat be a greater concern to growers if it ameliorates the negative effects of *A. flavus* under stressful conditions..





Figures 1 and 2. Mortality and development time of two strains of navel orangeworm (susceptible strain "CPQ" and pyrethroid-resistant strain "R347") in the presence of the fungus *Aspergillus flavus* (AF36) and the insecticides bifenthrin (a pyrethroid, IRAC Group 3A) and spinetoram (a spinosyn, IRAC Group 5): **(1)** Differential mortality assessed with a three-way ANOVA and a Tukey's mean separation procedure; results with the same letter are not significantly different; **(2)** Differential time to pupation.

Genomics Projects: Identify specific NOW cytochrome P450 monooxygenase detoxification genes encoding enzymes involved in bifenthrin resistance

CYP-2 Clan	Mitochondrial	CYP3-Clan	CYP4-Clan
CYP18 (1)	CYP301 (2)	**CYP6 (22)**	CYP4 (13)
CYP303 (1)	CYP302 (1)	**CYP9 (6)**	CYP340 (3)
CYP304 (3)	CYP315 (1)	**CYP321 (6)**	CYP341 (11)
CYP305 (1)	CYP333(4)	**CYP324 (2)**	CYP367 (2)
CYP306 (1)	CYP339 (1)	**CYP338 (1)**	
CYP307 (1)	CYP428 (1)	**CYP354 (1)**	
		CYP365 (1)	

Table 1. Cytochrome P450 genes in the navel orangeworm genome listed by Clan and sorted according to family.

Methods:
Cytochrome P450 (P450) monooxygenases in Clan 3 of insects are frequently associated with pesticide metabolism.[3] In these experiments, we conducted quantitative real-time PCR (qRT-PCR) using all known NOW Clan 3 P450 monooxygenase genes. Two NOW strains (**susceptible strain ALMOND** and **pyrethroid-resistant strain R347**[4]) were compared across Clan 3 P450s. **These experiments were conducted to identify differentially expressed P450s in larval midguts between ALMOND and R347 larvae that may contribute to bifenthrin metabolism and resistance.**

Gene Name	Results	tValue	Fold Change	fdr_P-value
CYP6AE54	Elevated expression in R347-CTRL over ALMOND-CTRL (**constitutive expression**)	6.03	11.72	0.001
	Decreased expression in R347-BFN relative to R347-CTRL (**downregulated by bifenthrin**)	2.95	3.34	0.094
CYP321C1v2	Elevated expression in R347-BFN over ALMOND-CTRL	3.61	6.55	0.060
	Elevated expression in ALMOND-BFN over ALMOND-CTRL (***upregulation by bifenthrin**)	3.14	5.12	0.077
	Elevated expression in bifenthrin over controls (**treatment effect**)	3.03	3.05	0.091
CYP321C4	Elevated expression in R347over ALMOND (**strain effect**)	2.86	3.34	0.102
CYP321F6	Elevated expression in R347 over ALMOND (**strain effect**)	2.78	3.70	0.110
CYP365A1	Elevated expression in R347 over ALMOND-CTRL (**constitutive expression**)	2.79	2.98	0.110
CYP6AB110v1	Elevated expression in R347 over ALMOND-CTRL (**constitutive expression**)	3.63	10.93	0.060
CYP6AB39	Elevated expression in R347-CTRL over ALMOND-CTRL (**constitutive expression**)	2.96	3.28	0.094
CYP6AB40	Elevated expression in ALMOND-CTRL over R347-CTRL (**constitutive expression**)	4.07	20.92	0.037
CYP6AN17	Elevated expression in R347-CTRL over ALMOND-CTRL (**constitutive expression**)	3.90	8.83	0.046
	Decreased expression in R347-BFN relative to R347-CTRL (**downregulated by bifenthrin**)	2.69	4.50	0.115
CYP6B44v2	Elevated expression in R347-CTRL over ALMOND-CTRL (**constitutive expression**)	3.21	3.27	0.077
CYP6B54	Elevated expression in R347-CTRL over ALMOND-CTRL (**constitutive expression**)	2.76	2.29	0.114
CYP6B55	Elevated expression in R347-CTRL over ALMOND-CTRL (**constitutive expression**)	2.78	2.37	0.110
CYP9A64	Elevated expression in bifenthrin over controls (**treatment effect**)	2.68	3.00	0.115
CYP9G13	Elevated expression in R347-CTRL over ALMOND-CTRL (**constitutive expression**)	3.23	14.45	0.077

Table 2. Candidate P450s involved in bifenthrin resistance from Clan 3 identified and described from qRT-PCR experiments.

Discussion: We conducted a complete survey of Clan 3 P450s in NOW to identify candidate genes involved in bifenthrin metabolism and resistance and identified one P450 that likely directly metabolizes bifenthrin--CYP321C1v2. Additionally, our analysis revealed differences in expression across strains on control diets, which may reflect differences in constitutive expression for certain P450s. Constitutive expression was greater in the resistant strain for nine P450s than in the susceptible strain. We are currently conducting our analysis on Clan 4 P450s to identify any additional differences between P450s of each strain that may contribute to bifenthrin resistance. Our complete analysis of P450 response to bifenthrin in NOW and our ongoing whole-genome approach with Pool-seq[5] will facilitate the detection of resistance mechanisms in NOW and allow for screening of NOW populations for pyrethroid resistance.

Determine whether effects on larval performance are caused by metabolism of dietary toxins by *A. flavus*

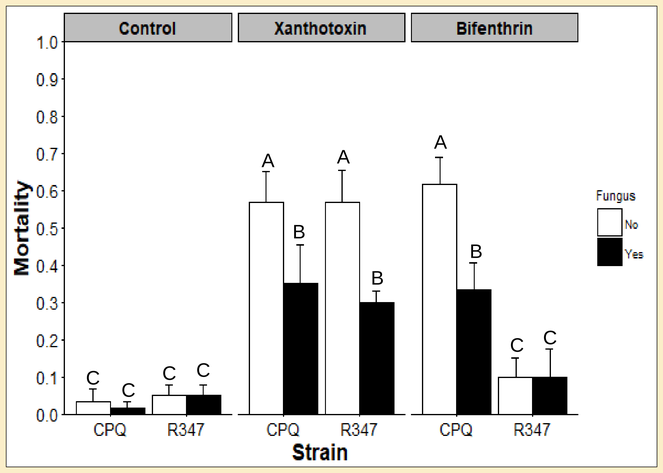


Figure 3. Mortality of navel orangeworm, strains CPQ and R347, in the presence of the furanocoumarin xanthotoxin and insecticide bifenthrin, before and after incubation with *Aspergillus flavus*; differential mortality assessed with a three-way ANOVA and a Tukey's mean separation procedure; results with the same letter are not significantly different.

Methods & Discussion: Previous work suggested *A. flavus* metabolizes toxins to the benefit of associated NOW larvae. We prepared minimal nutrient broth with and without bifenthrin and xanthotoxin (a furanocoumarin found in some host plants), then added *A. flavus* (AF36*)* to half of the flasks for each treatment. We then vacuum-filtered each medium and took an aliquot of the supernatant, which was added to a semi-synthetic artificial diet according to pre-determined LC_{50} concentrations. We then placed NOW neonates (CPQ and R347) onto the diet. **Mortality (48-h) was higher for larvae of both strains consuming diet containing xanthotoxin as compared to control diet** (Fig. 3). **CPQ larvae raised on bifenthrin-treated diet experienced increased mortality; R347 larvae did not**. **Diet from AF36 flasks resulted in lower mortality for CPQ larvae on both gifenthrin and xanthotoxin diets and for R347 larvae on xanthotoxin diet**. Fungal metabolism of dietary toxins may complement genetic resistance mechanisms in allowing larvae to withstand synthetic or natural toxins. We plan to assess the effects of other chemical classes on R347 performance.

Determine the degree to which larval detoxification changes: Neonate mortality using recently collected susceptible populations of NOW with resistant strains collected from Kern County in 2015 and 2016 on artificial diet containing field concentrations of bifenthrin

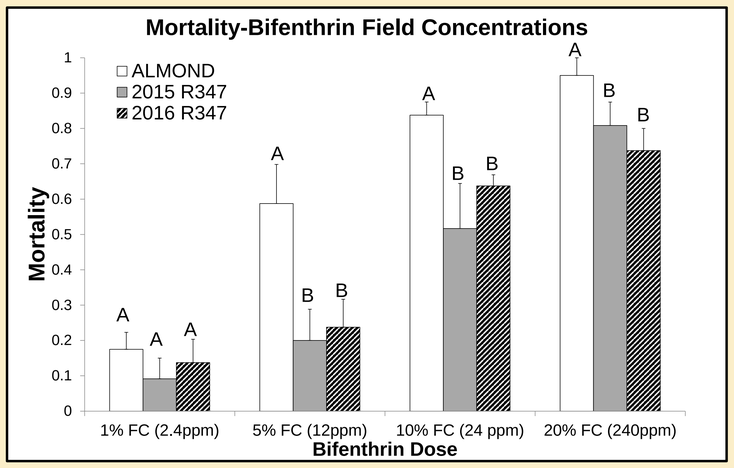


Figure 4. NOW neonate mortality after 48 hours on artificial diets containing bifenthrin field concentrations (FC) for a **12th generation susceptible population (Madera County)**, a **24th generation pyrethroid-resistant (R347) population (Kern County) in 2015**, and a **9th generation pyrethroid-resistant (R347) population (Kern County) in 2016**. Mortality was absent in diet controls without insecticides (data not shown) after 48 hours. Data were analyzed independently at each concentration using Chi-Square with means separated when $P < 0.05$.

Discussion: NOW mortality was greater at a 5%, 10%, and 20% bifenthrin field concentration in the susceptible ALMOND strain compared to resistant populations collected from Kern County in 2015 and 2016 ($X^2 \leq 7.08$, $P < 0.01$). The resistant strains collected from 2015 and 2016 experienced the same mortality across all bifenthrin concentrations in this study. **At a 20% field concentration, the 2015 R347 strain exhibits resistance after 24 generations in a laboratory in the absence of bifenthrin selection relative to a susceptible strain more recently collected from almond orchards**. This finding suggests that resistance may have become stable in more recently collected populations and may no longer have an associated metabolic cost to maintain. Resistance remains a challenge for growers, and developing strategies for managing populations and identifying its genetic basis will continue to be a primary objective in our experiments.

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Acknowledgments
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