

INSECTICIDE DETOXIFICATION IN THE NAVEL ORANGEWORM *AMYELOIS*
TRANSITELLA (LEPIDOPTERA: PYRALIDAE)

BY

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THESIS

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ABSTRACT

The polyphagous navel orangeworm (*Amyelois transitella*) is considered the most destructive pest of introduced nut crops, including almonds and pistachios, in California orchards. Management of this pest has typically been a combination of cultural controls (including removal of unharvested fruits) and the use of insecticides; insecticide use has increased substantially along with the value of these commodities. In a series of dietary studies the effects of the cytochrome P450 monooxygenase (P450) inhibitor piperonyl butoxide (PBO) and the glutathione-S-transferase (GST) inhibitor diethyl maleate (DEM) were assessed on the toxicity of the insecticides azinphos-methyl, chlorpyrifos, chlorantraniliprole, β -cyfluthrin, and bifenthrin to first instar *A. transitella* larvae from a laboratory strain. Piperonyl butoxide interacted antagonistically with the organophosphate insecticides azinphos-methyl and chlorpyrifos, indicating that they likely are bioactivated by P450s. Piperonyl butoxide synergized the toxicity of the pyrethroids β -cyfluthrin and bifenthrin, which suggests that P450s are involved in detoxification of pyrethroid insecticides. Only azinphos-methyl was a substrate for GSTs in *A. transitella*, as evidenced by diethyl maleate synergism assays. Neither PBO nor DEM influenced the toxicity of the anthranilic diamide chlorantraniliprole. Results suggest that if *A. transitella* detoxify other classes of insecticides used in management through enhanced P450 activity, and resistance begins to develop by this route, then incorporating organophosphate insecticides into rotations may provide an effective means of prolonging efficacy of chemical control because their speed of activation will increase.

In a related series of studies to characterize a putatively pyrethroid-resistant strain of navel orangeworm, eggs from adults originating from almond orchards in which pesticide failures were reported in Kern County, California were shipped to the University of Illinois at

Urbana-Champaign. Their susceptibility to bifenthrin and β -cyfluthrin was compared to that of an established colony of navel orangeworms. Administration of piperonyl butoxide and S,S,S-tributyl phosphorotrithioate (DEF) in bioassays with the pyrethroids bifenthrin and β -cyfluthrin produced synergistic effects and demonstrated that P450s and carboxylesterases (COEs) contribute to resistance in this navel orangeworm population. Resistance is therefore primarily metabolic and likely the result of overexpression of specific P450 and COE genes. Bioassays involving pesticides routinely used in tank mixtures indicate that chlorantraniliprole, which is not detoxified by P450s, may be more effective than methoxyfenozide in overcoming existing pyrethroid resistance because chlorantraniliprole enhanced pyrethroid toxicity and methoxyfenozide had no effect.

Results from median-lethal concentration (LC_{50}) assays revealed that resistance was maintained across eight generations in the laboratory. Life history trait comparisons between the resistant strain and susceptible strain revealed significantly lower pupal weights in resistant males and females reared on the same wheat bran-based artificial diet across three generations. The number of days until the first molt was significantly greater in the resistant strain than the susceptible strain, although overall development time was not significantly different between strains. These experiments indicate that resistance is heritable and may have an associated fitness cost, which could influence the dispersal and expansion of resistant populations.

Acknowledgments

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CHAPTER I: NAVEL ORANGEWORM DETOXIFICATION MECHANISMS FOR DIFFERENT CLASSES OF INSECTICIDES CURRENTLY USED IN MANAGEMENT

Introduction

The navel orangeworm, *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae), is considered the most destructive pest of introduced nut crops in California orchards (Connell 2001; Bentley et al. 2008; Zalom et al. 2012). The geographic range of the navel orangeworm extends from the southern tier of the United States, through Mexico and Central America, and into South America (Heinrich 1956). Larval hosts within its native range belong to diverse plant families, including Asparagaceae, Fabaceae, Rubiaceae and Sapindaceae (Heinrich, 1956). Although this insect pest was initially described feeding on fallen fruits of *Citrus sinensis* (Rutaceae), the introduction of additional nonindigenous fruit crops allowed the navel orangeworm to expand its range and establish itself as a pest in California orchards by the 1940s (Wade 1961). The navel orangeworm causes economic damage to almonds (Rosaceae), pistachios (Anacardiaceae), figs (Moraceae), pomegranates (Lythraceae), and walnuts (Juglandaceae) in the Central Valley of California, demonstrating its capacity to consume host plants with distinct chemistries (Bentley et al. 2008).

Navel orangeworm is an internal feeder with a preference for fallen and mummy (unharvested) fruit or injured and diseased fruits (Heinrich 1956) Neonates tunnel into nuts, resulting in consumption of the nutmeat and the production of large quantities of frass and webbing. Moreover, infestation by navel orangeworm results in an increased susceptibility to infection by *Aspergillus* species, many of which produce aflatoxins, a crop contaminant that causes millions of dollars in losses each year (Campbell et al. 2003; Molyneux et al. 2007; van

Egmond et al. 2007). Management of this insect pest has typically been a combination of cultural control (removal of unharvested fruits) combined with insecticides, but the use of insecticides has increased substantially along with the value of these commodities.

The ability to feed on chemically distinct host plants may have preadapted the navel orangeworm for the different types of xenobiotics it encounters in the Central Valley of California, including synthetic and organic insecticides, mycotoxins, and phytochemicals produced by host plants (Niu et al. 2012). Compared to other insects, navel orangeworm can tolerate unusually high concentrations of aflatoxin ($>100\text{ }\mu\text{g/g}$) administered in artificial diet (Niu et al. 2009) as a result of an active cytochrome P450 monooxygenase (P450) system that can convert aflatoxin B1 into less toxic metabolites (Lee and Campbell 2000). CYP6AB11 has been characterized in the navel orangeworm as a P450 enzyme that can metabolize imperatorin, a furanocoumarin with similar chemistry to other phytochemicals present in several navel orangeworm host plants such as *Citrus* spp. and fig (*Ficus carica*) (Niu et al. 2011). The existence of specialized P450s in the navel orangeworm may have facilitated the transition to non-native crop plants because larvae could detoxify compounds produced by host species with structurally similar phytochemicals.

P450s, GSTs, and carboxylesterases (COE) are three major detoxification enzyme systems utilized in metabolism of pesticides by insects (Feyereisen 2005; Oakeshott et al. 2005; Ranson and Hemingway 2005). Insecticides currently registered for use in California orchards include members of the pyrethroid, organophosphate, anthranilic diamide, diacyl hydrazine, neonicotinoid, and spinosyn classes of insecticides. Insecticides sprayed to control navel orangeworms in heavily infested orchards are usually applied in rotation when the hulls split and the kernel is exposed to larval feeding and infection by *Aspergillus* (Niu et al. 2012). Although

applicators may assume that the rotation of different insecticides with different modes of action, applied either individually or in combination, will delay resistance acquisition, rotational patterns may also raise the risk of acquisition of multiple resistance if rotated insecticides share a common route of detoxification.

In this study, I examined the effects of two synergists, piperonyl butoxide (PBO) and diethyl maleate (DEM), which inhibit detoxification of xenobiotics by P450s and GSTs, respectively, on the toxicity of the insecticides azinphos-methyl, chlorpyrifos, chlorantraniliprole, β -cyfluthrin, and bifenthrin. These insecticides represent member(s) of the organophosphate, anthranilic diamide, and pyrethroid insecticide classes used to control the navel orangeworm (Table 1.1). Synergism (enhancement of mortality beyond additive effects) in the presence of one of these specific inhibitors implicates the contribution of the enzyme system to metabolism of the insecticides assayed.

Materials and Methods

Chemicals:

Piperonyl butoxide was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Azinphos-methyl, bifenthrin, β -cyfluthrin, chlorantraniliprole, chlorpyrifos, and diethyl maleate were purchased from Sigma (St. Louis, MO). Bifenthrin was purchased from Chem Service, Inc. (West Chester, PA). Bifenthrin and chlorpyrifos were dissolved in methanol. Azinphos-methyl, β -cyfluthrin, and chlorantraniliprole were dissolved in acetone. All stock solutions were stored at -20°C.

Navel orangeworm colony:

A laboratory colony of *A. transitella* designated as SPIRL-1966 (Siegel et al. 2010) was kept in the insectary at University of Illinois at Urbana-Champaign and maintained at conditions

of $27 \pm 4^{\circ}\text{C}$ with a photoperiod of 16:8 (L:D) hours. Larvae were mass-reared until pupation in 500-ml glass jars containing 300 g of a wheat bran diet (Finney and Brinkmann 1967). Adults were transferred to additional 500-ml glass jars with tissue paper on the inside and covering the top to serve as oviposition substrate. Eggs were collected every 48 hours from these jars. First instar larvae were selected within 24 hours of hatching. Four larvae were gently transferred into a 28-ml (1-oz) plastic cup containing 5 g of unaugmented (control) diet or diet mixed with different concentrations of insecticide in the bioassays. A total of 20 larvae (5 cups) were prepared for each treatment with or without insecticide application.

Insecticide Preparation:

Insecticides were incorporated into standard diet at specific concentrations and fed to neonate larvae. Insecticides were mixed into the standard diet in its liquid phase and then poured into separate 1-oz cups to harden. Solutions of each insecticide in methanol or acetone were prepared at a range of concentrations (azinphos-methyl: 0.2, 0.5, 1, 2, 3, and 5 $\mu\text{g/g}$; β -cyfluthrin: 50 ng/g, 100 ng/g, 150 ng/g, 500 ng/g, and 1 $\mu\text{g/g}$; bifenthrin: 50 ng/g, 200 ng/g, 300 ng/g, and 500 ng/g; chlorantraniliprole 0.4, 4, 6, 10, and 16 $\mu\text{g/g}$; chlorpyrifos: 0.05, 0.2, 0.3, 0.5, 1, and 2 $\mu\text{g/g}$). In these bioassays, 20 neonates were exposed to each concentration of insecticide. Mortality levels were assessed and recorded after 48 hours with at least three replicates at each concentration. Larvae that did not move when touched with a soft brush were scored as dead.

Synergist Preparation:

A concentration of 200 $\mu\text{g/g}$ for piperonyl butoxide was previously established by Niu et al. (2012) as the maximum concentration determined to be nonlethal for first instar larvae. For diethyl maleate, a concentration of 200 $\mu\text{g/g}$ produced <15% mortality after 48 hours, and this concentration was used in all assays.

Bioassays:

Insecticide assays were conducted using median-lethal concentrations for each insecticide (1.6 µg/g azinphos-methyl; 40 ng/g β-cyfluthrin; 200 ng/g bifenthrin; 4 µg/g chlorantraniliprole; 400 ng/g chlorpyrifos) mixed with standard insect diet in the presence or absence of piperonyl butoxide (200 µg/g) or diethyl maleate (200 µg/g). Controls present in each bioassay consisted of 200 µl insecticide solvent mixed into diet in the absence of insecticide and 200 µg/g of either PBO or DEM. Each bioassay exposed 20 first instar larvae to a single concentration of insecticide. All bioassays were repeated four times. Mortality was recorded daily until at least 80% of the treated insects were dead. On the rare occasions that cannibalism or larval scavenging of already-dead individuals occurred, larvae were removed from the sample size.

Statistical Analyses:

SPSS version 22 software (SPSS Inc., Chicago IL) was used to run the Probit analysis and generate an estimated calculation of the median lethal concentration that would kill 50% of the sample population at 48 hours (LC₅₀) for the five insecticides. Separate analyses using JMP Pro version 9 (SAS Institute, Cary NC) were calculated for each insecticide and insecticide with synergist using multiple regression with orthogonal polynomial contrasts in order to differentiate among treatments.

Results

Synergistic or antagonistic interactions were observed when the mortality of the insecticide with synergist was significantly higher ($P<0.05$) than the combined mortality of the insecticide and synergist. Antagonistic interactions were observed when the mortality of the

insecticide with synergist was significantly lower ($P<0.05$) than the combined mortality of the insecticide and synergist.

The goodness-of-fit test indicated that insecticide concentration mortality data fit the Probit model ($P>0.05$) for azinphos-methyl, chlorpyrifos, chlorantraniliprole, β -cyfluthrin and bifenthrin (Table 1.2). Each class of insecticide displayed differential toxicity toward the navel orangeworm after 48 hours. The pyrethroids β -cyfluthrin and bifenthrin, with LC_{50} s at 40 ng/g and 200 ng/g, respectively, were toxic to neonates at the lowest concentration of all insecticides tested. The organophosphates azinphos-methyl and chlorpyrifos were lethal to neonates at higher concentrations, with LC_{50} s at 1.6 μ g/g and 410 ng/g, respectively, and the anthranilic diamide chlorantraniliprole was least toxic, with an LC_{50} of 4 μ g/g.

Mortality levels were significantly different ($P<0.001$) between insecticide treatments and the controls across all time points examined for the organophosphates azinphos-methyl and chlorpyrifos. The synergist and insecticide solvent controls were not significantly different from each other ($P>0.05$) across each time point examined in these insecticides. Piperonyl butoxide exhibited antagonistic interactions with both insecticides and significantly reduced the toxicity at 36 hours ($P=0.014$) through 144 hours ($P<0.001$) for azinphos-methyl (Fig. 1.1) and at 36 hours ($P=0.016$) through 96 hours ($P<0.001$) for chlorpyrifos (Fig. 1.3) Diethyl maleate synergized the toxicity of azinphos-methyl at 24 hours ($P=0.043$) through 96 hours ($P=0.027$) (Fig. 1.2) but had no significant effect on chlorpyrifos toxicity from 24 hours ($P=0.410$) to 96 hours ($P=0.057$) (Fig. 1.4).

Mortality levels were significantly different ($P<0.001$) between insecticide treatments and the controls from 24 hours to 120 hours in chlorantraniliprole assays. The synergist and insecticide solvent controls were not significantly different from each other ($P>0.05$) from 24

hours to 120 hours in assays with PBO and DEM. Piperonyl butoxide had no significant effect on the toxicity of chlorantraniliprole from 24 hours ($P=0.816$) to 120 hours ($P=0.660$) (Fig. 1.5). Diethyl maleate had no significant effect on the toxicity of chlorantraniliprole from 24 hours ($P=0.629$) to 120 hours ($P=0.659$) (Fig. 1.6).

A significant difference in mortality levels ($P<0.001$) was observed between insecticide treatments and the controls across each time point examined in the pyrethroids β -cyfluthrin and bifenthrin. The synergist and insecticide solvent controls were not significantly different from each other ($P>0.05$) across each time point examined in these pyrethroids. Piperonyl butoxide synergized the toxicity of β -cyfluthrin at 24 hours ($P<0.001$) through 144 hours ($P<0.001$) (Fig. 1.7) and the toxicity of bifenthrin at 24 hours ($P<0.001$) through 144 hours ($P<0.001$) (Fig. 1.9). Diethyl maleate had no significant effect on the toxicity of β -cyfluthrin from 24 hours ($P=0.396$) to 144 hours ($P=0.060$) (Fig. 1.8) or the toxicity of bifenthrin from 24 hours ($P=0.320$) to 144 hours ($P=0.356$) (Fig. 1.10).

Discussion

There are currently numerous insecticides registered for use to control lepidopteran and hemipteran pests in California orchards (Niu et al. 2012). In pistachio orchards, buprofenzin (Applaud) and neonicotinoid insecticides are used to control the mealybug (*Ferrisia gilli*) (Hemiptera: Pseudococcidae) and are applied when navel orangeworms are present (Haviland et al. 2012). Peach twig borers (*Anarsia lineatella*) (Lepidoptera: Gelechiidae) feed on new crop nuts following hull split, and infestations in almond orchards can result in navel orangeworm exposure to additional insecticides because navel orangeworms prefer damaged nuts and are likely to invade any that were previously damaged by peach twig borers (Zalom et al. 2002; Hamby and Zalom 2013). This variation in chemical control strategies for other insect pests

means that navel orangeworms may be exposed to as many as four classes of insecticide in a single season (Niu et al. 2012). The rapid expansion of almond and pistachio orchards over the past seven years into orchards adjacent to cotton, grapes, and stone fruits has increased the probability of nontarget exposure of navel orangeworms to insecticides as a result of drift, which may increase selection pressure on the navel orangeworm for resistance to multiple classes of insecticide (Higbee and Siegel 2009).

Although P450s generally metabolize xenobiotics into less toxic metabolites, they may bioactivate certain compounds instead, resulting in a metabolite more toxic than the original compound (Scott 1999; Wegorek et al. 2011). The results from assays involving chlorpyrifos and azinphos-methyl indicate that both insecticides are bioactivated by PBO in the navel orangeworm because mortality decreased in the presence of the P450 inhibitor. Ahmad and Hollingsworth (2004) observed an increased tolerance to azinphos-methyl and chlorpyrifos when PBO was applied in bioassays involving second instar obliquebanded leafrollers (*Choristoneura rosaceana*) (Lepidoptera: Tortricidae), indicating that P450-mediated bioactivation of these organophosphates occurs in this lepidopteran pest. An increase in survivorship was observed in navel orangeworm bioassays because inhibition of P450s likely prevented the conversion of the organophosphate to the toxic oxon form and subsequent binding to acetylcholinesterase.

Bioassays involving the organophosphates and diethyl maleate indicated that azinphos-methyl was metabolized by GSTs and chlorpyrifos was not. Enhanced GST activity has been established as a mechanism of resistance to organophosphates in populations of lepidopteran pests such as the codling moth (*Cydia pomonella*) (Lepidoptera: Tortricidae) and the obliquebanded leaf roller (Smirle et al. 1998; Ahmad and Hollingsworth 2004; Fuentes-Contreras et al. 2007). The primary mode of detoxification of chlorpyrifos in the navel

orangeworm remains to be investigated. Further work is clearly warranted to delineate the effects of the esterases in detoxification of these organophosphate insecticides.

Pyrethroids are the most widely used insecticide in navel orangeworm management during the growing season (Leal et al. 2009). The primary modes of detoxification for pyrethroid insecticides include P450s and esterases (Ishaaya 1993). The toxicity of both β -cyfluthrin and bifenthrin was enhanced in the presence of PBO, indicating that P450s are involved in the metabolism of pyrethroids in this species. Niu et al. (2012) examined neonate mortality in navel orangeworms when piperonyl butoxide was applied to median-lethal concentrations of the pyrethroids α -cypermethrin and τ -fluvalinate and found that both pyrethroids exhibited synergistic effects with PBO. The findings from these experiments emphasize the participation of P450s in the metabolism of pyrethroids and suggest that disruption of P450 enzyme systems could increase the efficacy of this insecticide class; however, pyrethroids used to manage other insect pests may increase selection pressure on navel orangeworms and lead to the development of resistance if applied in close proximity to almond and pistachio orchards (Niu et al. 2012).

Diethyl maleate did not have any significant impact on the metabolism of β -cyfluthrin or bifenthrin in navel orangeworms. This is consistent with findings of previous researchers that GSTs were not linked to the direct metabolism of pyrethroids (Enayati et al. 2005; Khambay and Jewess 2005; Ranson and Hemingway 2005). Although results from this experiment support P450-mediated metabolism as major detoxification pathways in the navel orangeworm, the full extent of GST involvement in metabolism of pyrethroids remains unclear. GST-dependent sequestration of pyrethroids and detoxification of pyrethroid-induced lipid peroxidation products have been documented as potential resistance mechanisms in other insects such as the mealworm

(*Tenebrio molitor*) (Coleoptera: Tenebrionidae) and the brown planthopper (*Nilaparvata lugens*) (Hemiptera: Delphacidae) (Kostaropoulos et al. 2001; Vontas et al. 2001).

A recent addition to the arsenal of chemicals used in navel orangeworm management is chlorantraniliprole, an anthranilic diamide insecticide with broad-spectrum activity against lepidopteran pests because of its activity on the calcium channels of muscle (Temple et al. 2009). There was no evidence of synergism when PBO and DEM were applied to chlorantraniliprole in bioassays with the navel orangeworm. Wang et al. (2010) reported that PBO and DEF displayed minor synergism with a susceptible strain in the diamondback moth (*Plutella xylostella*) (Lepidoptera: Plutellidae), but PBO, DEM, and DEF had no activity in field populations of this insect. It is possible that P450s and GSTs may not be the main detoxification systems for chlorantraniliprole in the navel orangeworm. Tolerance may be attributable to esterase activity or, alternatively, to varying degrees of target-site insensitivity. The contribution of esterases to chlorantraniliprole will be evaluated in future assays.

Disruption of P450 enzymes responsible for the metabolism of many insecticides (Lee and Campbell 2000; Niu et al. 2012) may be a valid strategy for navel orangeworm control. If the navel orangeworm detoxifies other classes of insecticides through enhanced cytochrome P450 enzyme activity and resistance begins to develop by this route, then incorporating organophosphates into insecticide rotation may provide an effective system for delaying resistance to chemical control. This study demonstrated that chlorantraniliprole is not detoxified by P450s or GSTs, which may allow it to be combined with other insecticide classes that are detoxified by these systems, such as pyrethroids, in order to delay resistance acquisition. It is essential to investigate the role of esterase genes in the detoxification of the different insecticide classes that are used in management of navel orangeworms in order to avoid cross-resistance to

insecticides that share the same modes of detoxification. Understanding how detoxification enzymes respond to selective pressures exerted by different insecticides is critical in order to design sustainable control strategies and minimize the evolution of resistance.

Figures and Tables

Figure 1.1. First instar navel orangeworm mortality across multiple time points following dietary exposure to 1.6 µg/g azinphos-methyl, 1.6 µg/g azinphos-methyl + 200 µg/g piperonyl butoxide (PBO), 200 µg/g PBO, and 200 µl acetone. Error bars represent the standard error. Letters A, B, and C represent significantly different groups ($P < 0.05$).

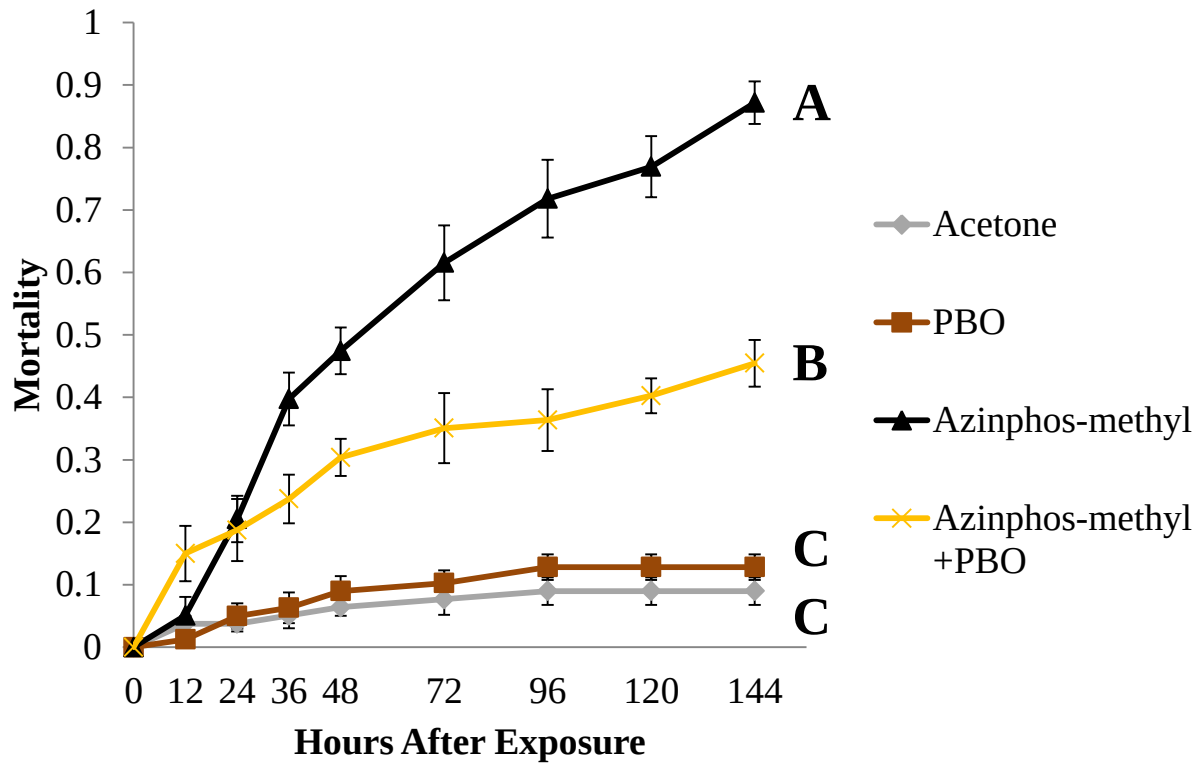


Figure 1.2. First instar navel orangeworm mortality across multiple time points following dietary exposure to 1.6 µg/g azinphos-methyl, 1.6 µg/g azinphos-methyl + 200 µg/g diethyl maleate (DEM), 200 µg/g DEM, and 200 µl acetone. Error bars represent the standard error. Letters A, B, and C represent significantly different groups ($P < 0.05$).

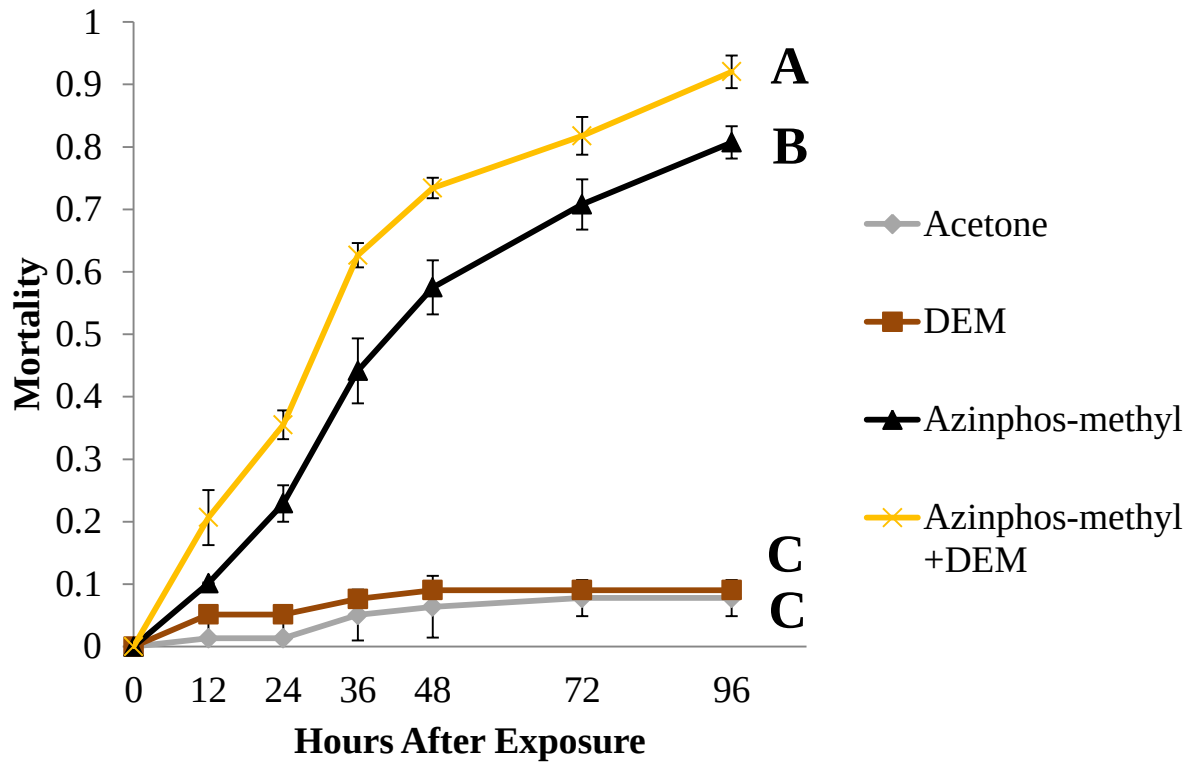


Figure 1.3. First instar navel orangeworm mortality across multiple time points following dietary exposure to 400 ng/g chlorpyrifos, 400 ng/g chlorpyrifos + 200 µg/g piperonyl butoxide (PBO), 200 µg/g PBO, and 200 µl methanol. Error bars represent the standard error. Letters A, B, and C represent significantly different groups ($P < 0.05$).

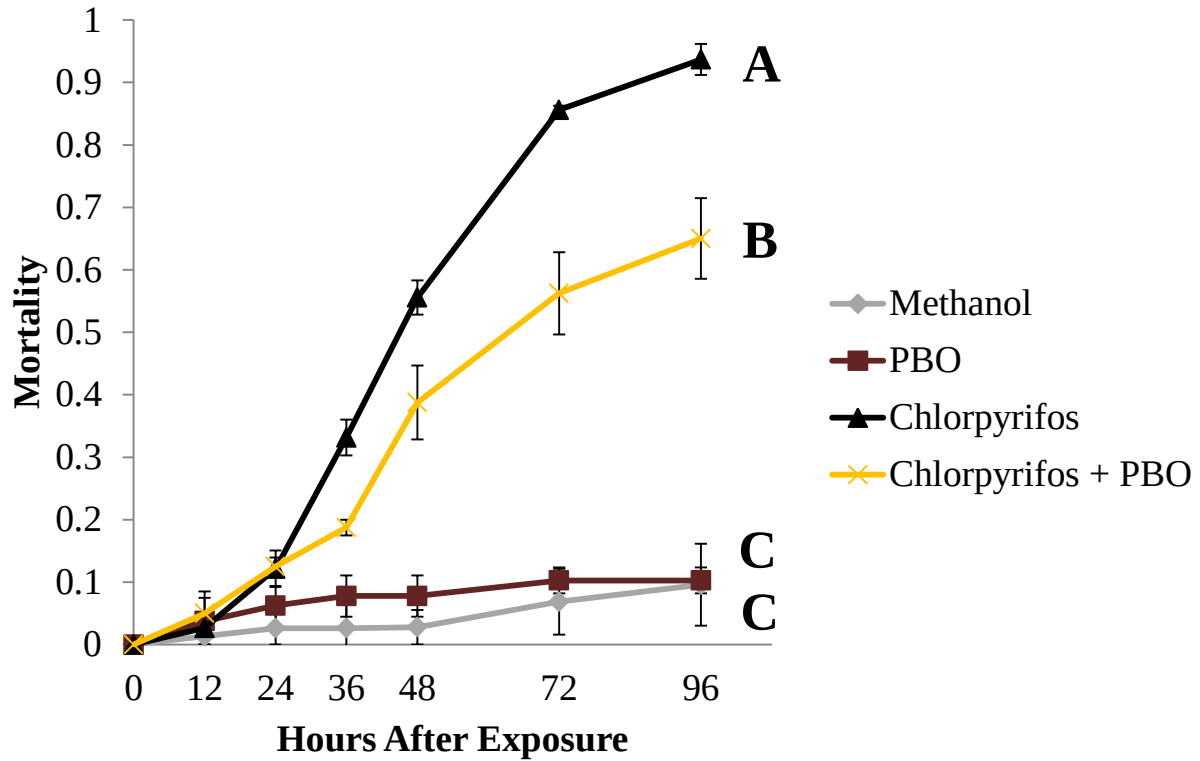


Figure 1.4. First instar navel orangeworm mortality across multiple time points following dietary exposure to 400 ng/g chlorpyrifos, 400 ng/g chlorpyrifos + 200 µg/g diethyl maleate (DEM), 200 µg/g DEM, and 200 µl methanol. Error bars represent the standard error. Letters A and B represent significantly different groups ($P < 0.05$).

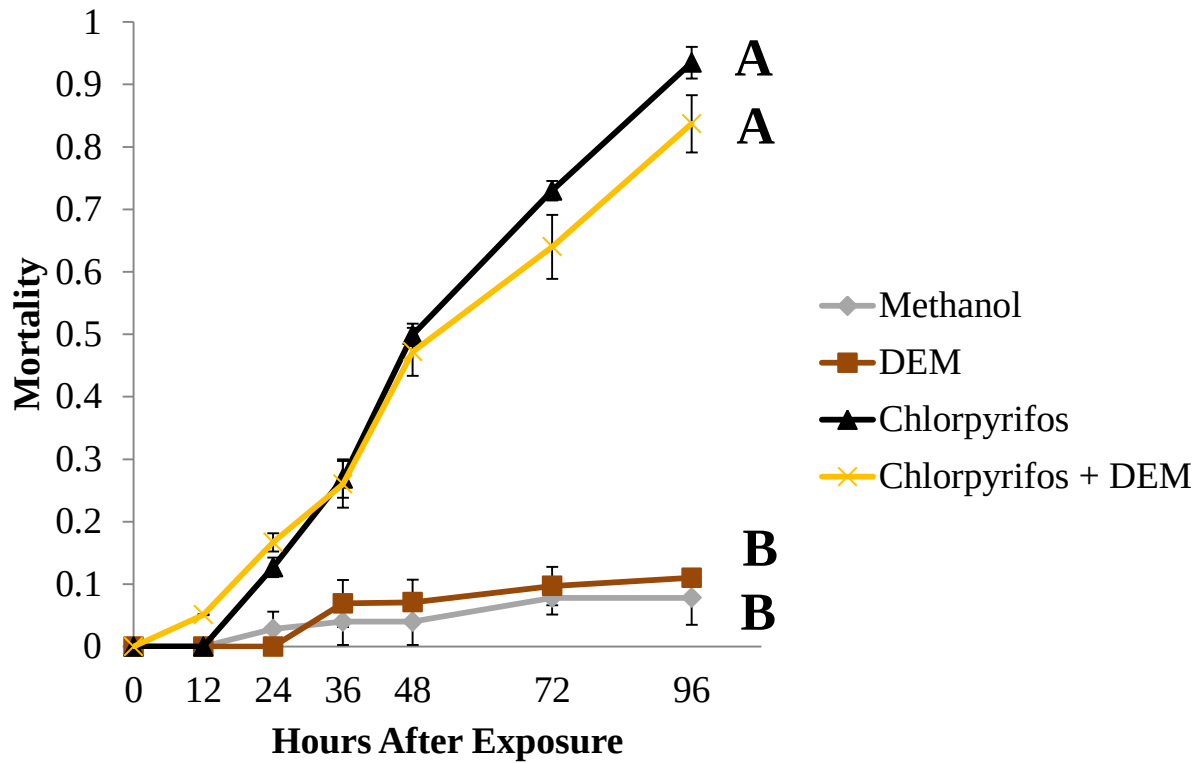


Figure 1.5. First instar navel orangeworm mortality across multiple time points following dietary exposure to 4 $\mu\text{g/g}$ chlorantraniliprole, 4 $\mu\text{g/g}$ chlorantraniliprole + 200 $\mu\text{g/g}$ piperonyl butoxide (PBO), 200 $\mu\text{g/g}$ PBO, and 200 μl acetone. Error bars represent the standard error. Letters A and B represent significantly different groups ($P < 0.05$).

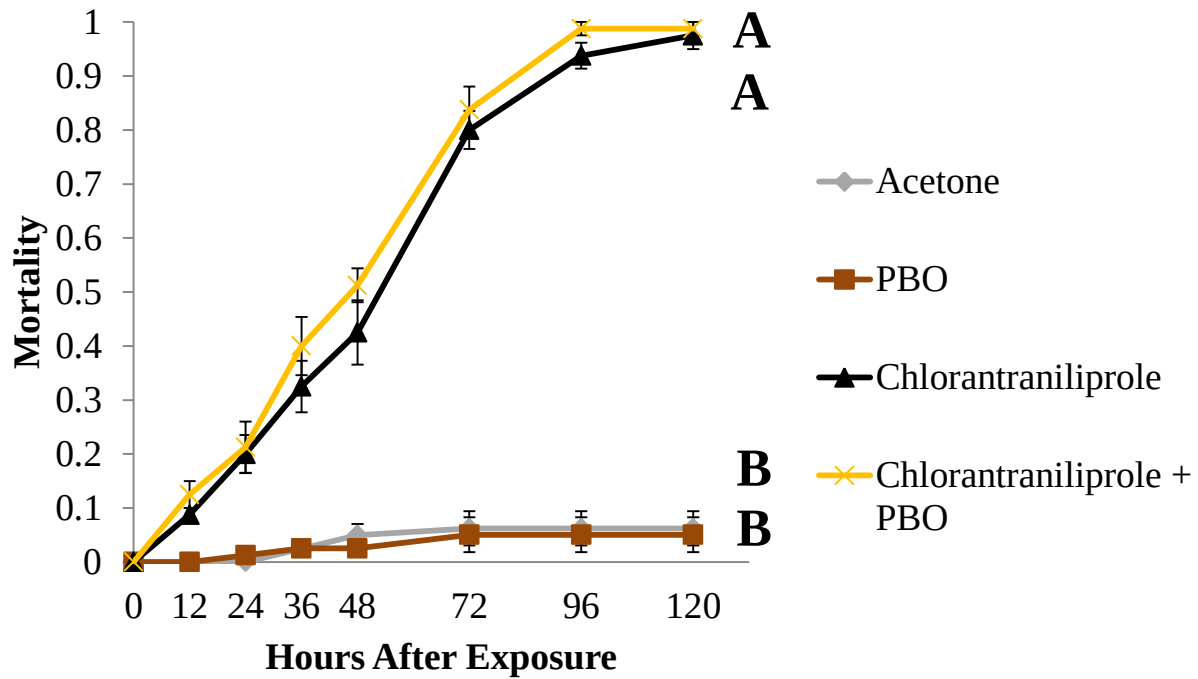


Figure 1.6. First instar navel orangeworm mortality across multiple time points following dietary exposure to 4 $\mu\text{g/g}$ chlorantraniliprole, 4 $\mu\text{g/g}$ chlorantraniliprole + 200 $\mu\text{g/g}$ diethyl maleate (DEM), 200 $\mu\text{g/g}$ DEM, and 200 μl acetone. Error bars represent the standard error. Letters A and B represent significantly different groups ($P < 0.05$).

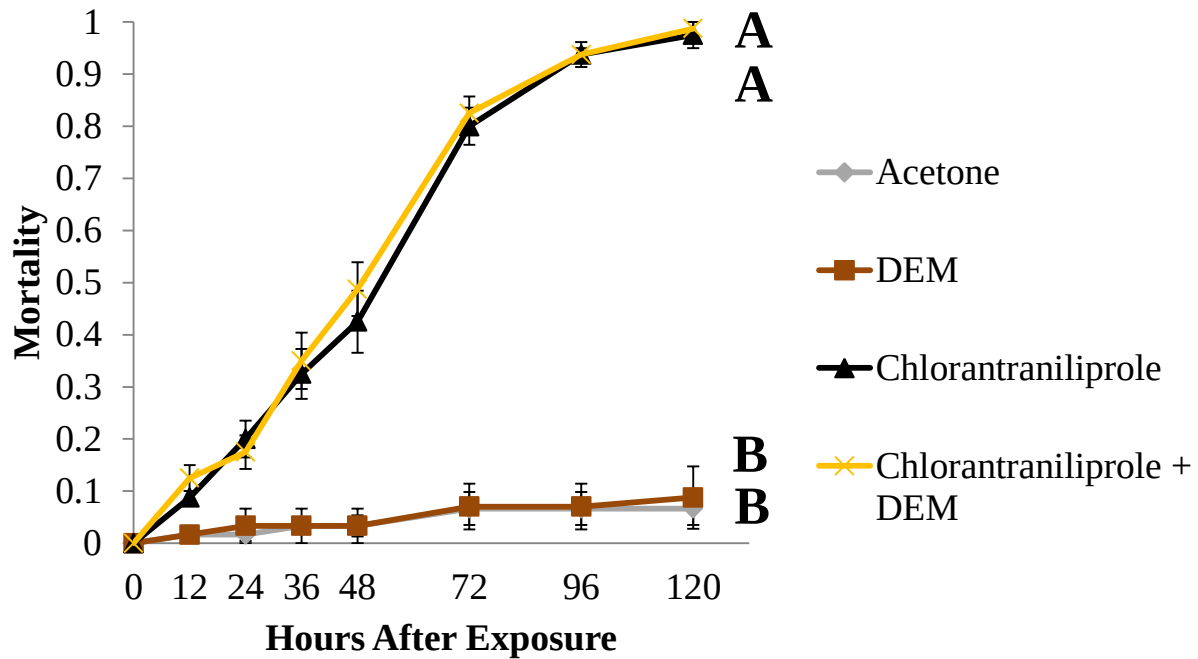


Figure 1.7. First instar navel orangeworm mortality across multiple time points following dietary exposure to 40 ng/g β -cyfluthrin, 40 ng/g β -cyfluthrin + 200 μ g/g piperonyl butoxide (PBO), 200 μ g/g PBO, and 200 μ l acetone. Error bars represent the standard error. Letters A, B, and C represent significantly different groups ($P < 0.05$).

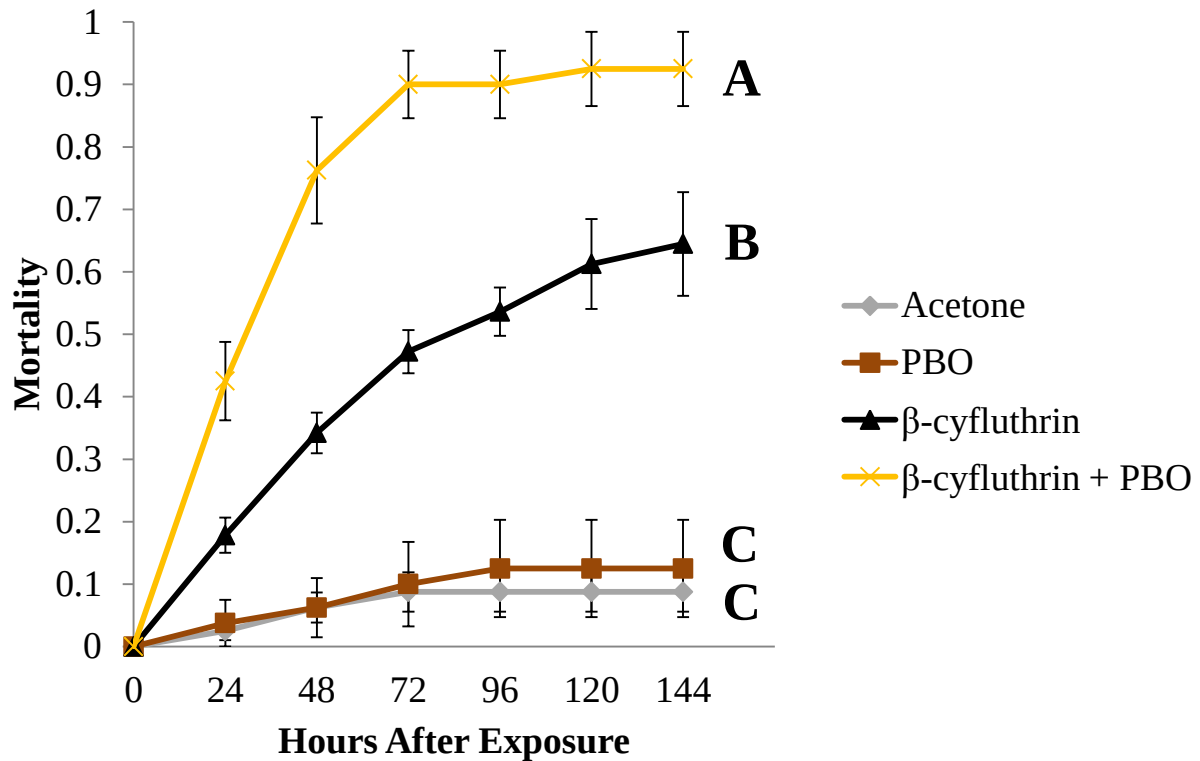


Figure 1.8. First instar navel orangeworm mortality across multiple time points following dietary exposure to 40 ng/g β -cyfluthrin, 40 ng/g β -cyfluthrin + 200 μ g/g diethyl maleate (DEM), 200 μ g/g DEM, and 200 μ l acetone. Error bars represent the standard error. Letters A and B represent significantly different groups ($P < 0.05$).

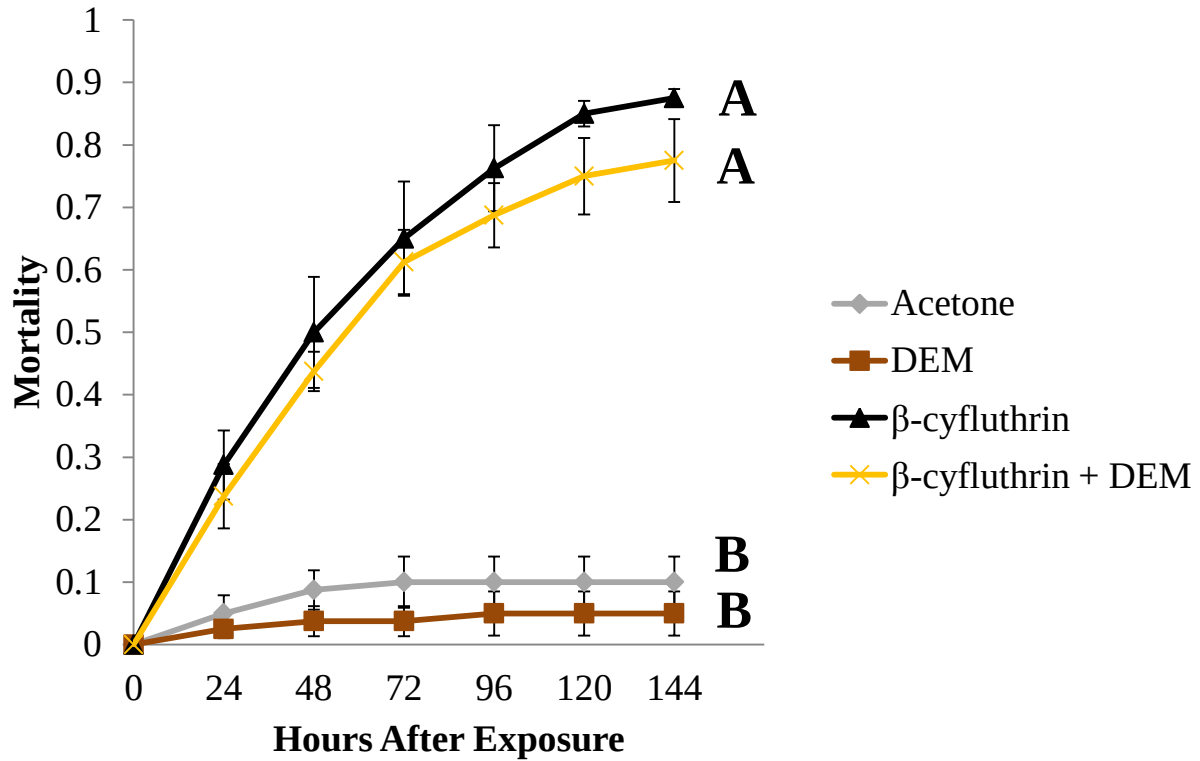


Figure 1.9. First instar navel orangeworm mortality across multiple time points following dietary exposure to 200 ng/g bifenthrin, 200 ng/g bifenthrin + 200 µg/g piperonyl butoxide (PBO), 200 µg/g PBO, and 200 µl methanol. Error bars represent the standard error. Letters A, B, and C represent significantly different groups ($P < 0.05$).

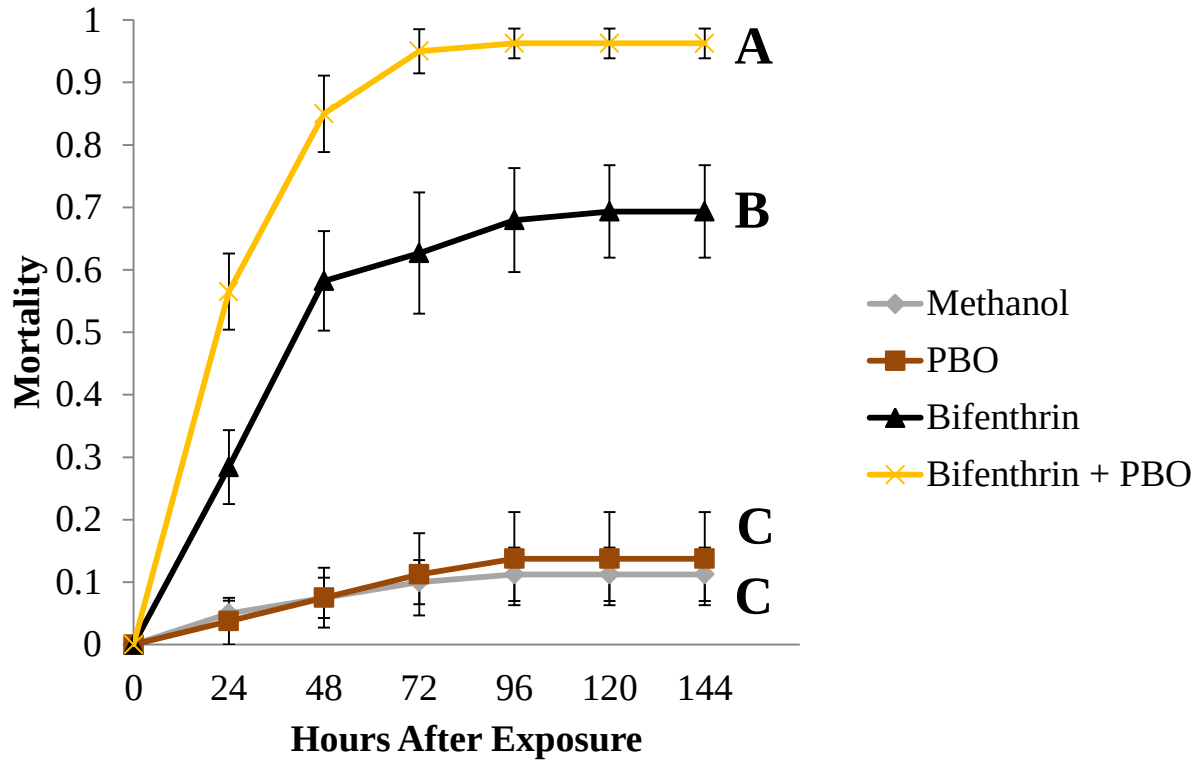


Figure 1.10. First instar navel orangeworm mortality across multiple time points following dietary exposure to 200 ng/g bifenthrin, 200 ng/g bifenthrin + 200 µg/g diethyl maleate (DEM), 200 µg/g DEM, and 200 µl methanol. Error bars represent the standard error. Letters A and B represent significantly different groups ($P < 0.05$).

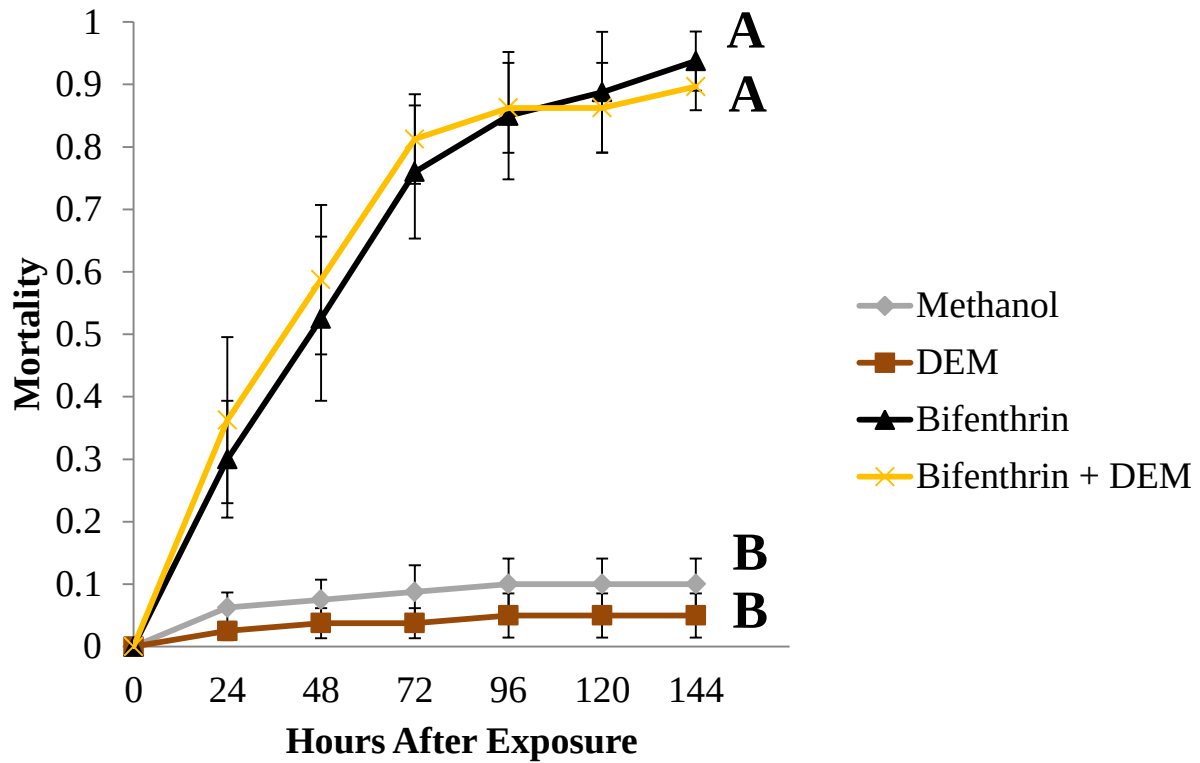


Table 1.1. Insecticides and synergists tested against first instar larvae from a laboratory strain (SPIRL-1966) of *Amyelois transitella*.

Active Ingredient	Commercial Name(s)	Chemical Family	Mode of Action	Dosage used ($\mu\text{g/g}$)
Azinphos-methyl	Guthion	Organophosphate	Acetylcholinesterase inhibitor	1.60
β -cyfluthrin	Baythroid	Pyrethroid	Sodium channel modulators	0.04
Bifenthrin	Brigade Bifenture	Pyrethroid	Sodium channel modulators	0.20
Chlorantraniliprole	Altacor Coragen	Anthranilic diamide	Ryanodine receptor modulators	4.00
Chlorpyrifos	Lorsban	Organophosphate	Acetylcholinesterase inhibitor	0.40
Diethyl Maleate		Synergist	Glutathione-S-transferase inhibitor	200
Piperonyl Butoxide	Butacide	Synergist	Cytochrome P450 monooxygenase inhibitor	200

Table 1.2. Probit analysis data for azinphos-methyl, chlorpyrifos, chlorantraniliprole, β -cyfluthrin, and bifenthrin in neonate *A. transitella* from a laboratory strain (SPIRL-1966).

Insecticide	<i>n</i>	Slope (SE)	LC ₅₀ $\pm$ 95% CL ($\mu\text{g/g}$)	χ^2	<i>P</i>
Azinphos-methyl	636	2.73 (0.19)	1.50 (1.35-1.65)	6.22	0.40
Chlorpyrifos	555	4.20 (0.33)	0.41 (0.38-0.45)	7.49	0.19
Chlorantraniliprole	320	1.10 (0.33)	3.20 (0.92-4.83)	0.76	0.68
β -cyfluthrin	355	1.33 (0.22)	0.03 (0.01-0.05)	5.65	0.23
Bifenthrin	240	2.82 (0.35)	0.21 (0.18-0.25)	0.31	0.86

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CHAPTER II: MECHANISM OF RESISTANCE ACQUISITION IN NAVEL ORANGEWORMS EXPOSED TO PYRETHROID INSECTICIDES

Introduction

The ability of insects to develop resistance to synthetic insecticides presents a challenge for sustainable pest management programs. Selection pressure from repeated exposure to insecticides favors individuals with biochemical mechanisms that confer resistance by increasing the rate at which detoxification occurs or that decrease overall sensitivity toward insecticides as a result of changes in target site structure (Li et al. 2007). If resistance is heritable, then genes encoding these mechanisms will be passed on to successive generations, resulting in populations that cannot be controlled effectively through insecticide use (Khambay and Jewess 2005).

The navel orangeworm, *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae), is a polyphagous insect pest that is able to feed on a diversity of chemically distinct and economically important host plants, including almonds, pistachios, figs, walnuts, pomegranates, and citrus (Connell 2001; Bentley et al. 2008; Zalom et al. 2012). The ability of the navel orangeworm to cope with toxins produced from such divergent plant families suggests a versatile detoxification system that may be preadapted for the acquisition of insecticide resistance (Li et al. 2007).

Navel orangeworm resistance to standard applications of the pyrethroid insecticide bifenthrin has recently been reported in almond orchards in Kern County, California (B. Higbee, Paramount Farming Company, personal communication). Many factors, such as the type of crop and history of insecticide use, can affect selection pressure and lead to resistance within an insect species (Gunning et al. 1991). Kern County had the largest acreage in production of almond and pistachio crops from 2011-2012, with 20.1% of almond acres (148,913) in 2012 and 41.8% of

pistachio acres (62,831) in 2011 (ACP 2011; NASS 2012). Row crops grown adjacent to almond and pistachio orchards, such as pomegranates and figs, may provide sources of immigrants when infested by navel orangeworm (Burks et al. 2008). Chemical control practices that target navel orangeworms and other insect pests within almond and pistachio orchards as well as neighboring crops may increase selection pressure and promote resistance as a result of both movement between hosts and drift exposure. Consequently, the navel orangeworm may be exposed to as many as four insecticide classes within a single growing season and multiple insecticides within each class (Niu et al. 2012).

The primary mechanisms involved in the generation of insecticide resistance to pyrethroids involve target-site insensitivity, increased metabolism, or a combination of both (Khambay and Jewess 2005; Li et al. 2007; Feyereisen 2011). Target-site resistance to pyrethroids occurs through the inheritance of point mutations in the *para*-type sodium channels, which are the binding sites for this insecticide class (Casida et al. 1983; Davies et al. 2007). Metabolic resistance often occurs through contributions of cytochrome P450 monooxygenases (P450s), glutathione-S-transferase (GSTs), and carboxylesterase (COEs) enzymes (Feyereisen 2005; Oakeshott et al. 2005; Ranson and Hemingway 2005; Liu 2012) and results in a decrease in the effective dose of insecticide available at the target site.

Pyrethroids registered for use in almond and pistachio orchards include β cyfluthrin, bifenthrin, esfenvalerate, fenpropathrin, lambda-cyhalothrin, and permethrin (CPRB 2012). Pyrethroids mixed with diamides (*e.g.*, chlorantraniliprole-Altacor; flubendiamide-Belt) or diacyl hydrazines (*e.g.*, methoxyfenozide-Intrepid) are also applied as tank mixes or as a premix (Volium Xpress) in almonds and pistachios. Applicators assume that the rotation of different insecticides with different modes of action, applied either individually or in combination, will

delay resistance acquisition but applying mixtures may also raise the risk of acquisition of cross-resistance if the insecticides applied in mixtures share a common route of detoxification.

A compelling form of evidence for the contribution of an enzyme system in resistance is enhancement of toxicity in the presence of an insecticide synergist that compromises the enzyme in question (Feyereisen 2011). Suppression of resistance or reduction in levels of resistance by the synergist, as measured by increased mortality, indicates involvement of the particular enzyme system inhibited by the synergist in insecticide metabolism. In this experiment, the synergists piperonyl butoxide (PBO) and S,S,S-tributyl phosphorotrithioate (DEF) were assessed in bioassays with the pyrethroids bifenthrin and β -cyfluthrin in order to determine if P450s or COEs contribute to pyrethroid resistance in a field-derived population of navel orangeworm from Kern County, California. A susceptible laboratory colony of navel orangeworm was used to compare the effects of the synergists and to calculate resistance level differences. Finally, chlorantraniliprole and methoxyfenozide, in combination with bifenthrin and β -cyfluthrin, were added to artificial diets to determine if insecticide tank mixtures could be effective in overcoming resistance in this species.

Materials and Methods

Chemicals:

Piperonyl butoxide was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). β -cyfluthrin, chlorantraniliprole, and methoxyfenozide were purchased from Sigma (St. Louis, MO). Bifenthrin and S,S,S-tributyl phosphorotrithioate were purchased from Chem Service, Inc. (West Chester, PA). Bifenthrin, and methoxyfenozide were dissolved in methanol and β -cyfluthrin and chlorantraniliprole were dissolved in acetone. All stock solutions were stored at -20°C.

Navel orangeworm colonies:

A susceptible colony of *A. transitella* designated as CPQ (J. Siegel, USDA-ARS, Parlier, CA) and a resistant colony designated as R347 (B. Higbee, Paramount Farming Company, Bakersfield, CA) were kept in an incubator at University of Illinois at Urbana-Champaign and maintained at conditions of $27 \pm 4^{\circ}\text{C}$ in the absence of a light cycle. Larvae were mass-reared until pupation in 500-ml glass jars containing 300 g of a wheat bran-based artificial diet (Finney and Brinkmann 1967). Adults were transferred to additional 500-ml glass jars with tissue paper on the inside and covering the top to serve as an oviposition substrate. Eggs were collected every 48 hours from these jars. First instar larvae were selected within 24 hours of egg hatch. Four larvae were gently transferred into each 28-ml (1-oz) plastic cups containing 5 g of unaugmented (control) diet or diet mixed with different concentrations of insecticide in the bioassays. A total of 20 larvae (5 cups) were prepared for each treatment with or without insecticide application.

Insecticide Preparation:

Insecticides were incorporated into the artificial diet at specific concentrations and fed to neonate larvae. Insecticides were mixed in with the diet in its liquid phase and then poured into separate 28-ml (1-oz) cups to harden. Solutions of each pyrethroid in methanol or acetone were prepared at a range of concentrations for each strain. The concentrations tested in the susceptible strain were 50 ng/g, 200 ng/g, 300 ng/g, and 500 ng/g for bifenthrin and 50 ng/g, 100 ng/g, 150 ng/g, 500 ng/g, and 1 $\mu\text{g/g}$ for β cyfluthrin. The concentrations tested in the resistant strain were 500 ng/g, 1 $\mu\text{g/g}$, 1.5 $\mu\text{g/g}$, 2 $\mu\text{g/g}$, and 4 $\mu\text{g/g}$ for bifenthrin and 50 ng/g, 100 ng/g, 500 ng/g, 1 $\mu\text{g/g}$, and 2 $\mu\text{g/g}$ for β cyfluthrin. In each bioassay, 20 neonates were exposed to each concentration of insecticide. Mortality levels were assessed and recorded at each concentration

after 48 hours. Bioassays were replicated three times. Larvae that did not move when touched with a soft brush were scored as dead.

Insecticides selected for use in tank mix assays with pyrethroids were prepared along a range of concentrations and fed to neonate larvae (methoxyfenozide: 20 ng/g, 76 ng/g, 130 ng/g, and 250 ng/g; chlorantraniliprole: 1 µg/g, 2 µg/g, 4 µg/g, and 8 µg/g). A 130 ng/g dose of methoxyfenozide and 4 µg/g dose of chlorantraniliprole were selected based on mortality after 48 hours (~20%) for use in bioassays with the LC₅₀ doses of bifenthrin and β-cyfluthrin.

Synergist Preparation:

A concentration of 200 µg/g for piperonyl butoxide was previously established by Niu et al. (2012) as the maximum concentration determined to be nonlethal for first instar larvae. For S,S,S-tributyl phosphorotrithioate, a concentration of 100 µg/g produced <15% mortality after 48 hours, and this concentration was used in all assays.

Bioassays:

Median-lethal concentrations determined for β-cyfluthrin and bifenthrin within each strain of navel orangeworms were mixed into the artificial diet in the presence or absence of piperonyl butoxide or S,S,S-tributyl phosphorotrithioate. Controls in each bioassay included a diet treatment in which the synergists PBO and DEF were mixed in with diet in the absence of insecticide for the susceptible strain. The synergist DEF was selected as the only control for pyrethroid assays with the resistant strain. Each bioassay in the susceptible strain involved 20 first instar larvae exposed to a single concentration of insecticide with or without synergist and was replicated four times. Lower egg production from the resistant strain resulted in fewer replicates, fewer individuals per replicate, and the absence of a PBO control. Bioassays in the resistant strain involved 15 first instar larvae exposed to a single concentration of insecticide

with or without synergist and were replicated four times. Bioassays involving insecticide mixes were administered with 15 first instar larvae per treatment and replicated three times. Controls in the insecticide mix assays consisted of 200 µl insecticide solvent mixed into diet in the absence of insecticide. On the rare occasions that cannibalistic behavior or scavenging on already-dead caterpillars occurred, larvae were removed from the sample size.

Statistical Analyses:

SPSS version 22 software (SPSS Inc., Chicago IL) was used to run the Probit analysis and generate an estimated calculation of the median lethal concentration that would kill 50% of the sample population at 48 hours (LC_{50}) for the pyrethroids. The median lethal concentration of each insecticide was set as a baseline, and synergistic were observed when the mortality of the insecticide with synergist was significantly higher ($P < 0.05$) than the mortality of the insecticide alone. Separate analyses using JMP Pro version 9 (SAS Institute, Cary NC) were calculated for each insecticide and insecticide with synergist using multiple regression with orthogonal polynomial contrasts in order to differentiate among treatments.

Results

The LC_{50} values for bifenthrin and β -cyfluthrin for the resistant and susceptible strains are significantly different as shown by their non-overlapping 95% confidence intervals (Fig. 2.1). The LC_{50} was on average 8.7 -fold greater (ranging from 6.2 to 11.7-fold) in the resistant strain than the susceptible strain for bifenthrin, and the LC_{50} was 11-fold greater (ranging from 4.7 to 17.8-fold) in the resistant strain than the susceptible strain for β -cyfluthrin.

In the susceptible strain, mortality levels were significantly different ($P < 0.001$) across all time points examined between insecticide treatments and the control in bifenthrin assays. There was no significant difference observed between PBO and DEF ($P > 0.05$) at any time point

examined. Both PBO and DEF synergized the toxicity of bifenthrin at 72 hours ($P<0.001$) through 144 hours ($P<0.001$) (Fig. 2.2). The treatments including either PBO or DEF with bifenthrin were not significantly different from each other after 144 hours ($P=0.154$). Mortality levels were significantly different ($P<0.001$) across all time points between insecticide treatments and the control in β -cyfluthrin assays. PBO and DEF synergized the toxicity of β -cyfluthrin at 72 hours ($P<0.001$) through 144 hours ($P<0.001$) (Fig. 2.3). Treatments including either PBO or DEF with β -cyfluthrin were significantly different from each other at 72 hours ($P=0.005$) through 144 hours ($P=0.035$).

In the resistant strain, mortality levels were significantly different ($P<0.001$) across all time points between insecticide treatments and the control in bifenthrin assays. Both PBO and DEF synergized the toxicity of bifenthrin at 72 hours ($P<0.001$) through 144 hours ($P<0.001$) (Fig. 2.4). Treatments including either PBO or DEF with bifenthrin were not significantly different from each other after 144 hours ($P=0.136$). Mortality levels were significantly different ($P<0.001$) across all time points between insecticide treatments and the control in β -cyfluthrin assays. Both PBO and DEF synergized the toxicity of β -cyfluthrin at 48 hours ($P<0.001$) through 144 hours ($P<0.001$) (Fig. 2.5). Treatments including either PBO or DEF with β -cyfluthrin were not significantly different from each other after 144 hours ($P=0.486$).

In bioassays aimed at determining the effect of methoxyfenozide on toxicity of pyrethroids, mortality levels were significantly different ($P<0.001$) across all time points between insecticide treatments and the control for bioassays that combined bifenthrin and β -cyfluthrin with methoxyfenozide. Methoxyfenozide had no significant effect on the toxicity of either bifenthrin in mixtures (Fig. 2.6) from 48 hours ($P=0.106$) to 72 hours ($P=0.459$) or β -cyfluthrin in mixtures (Fig. 2.7) from 48 hours ($P=0.831$) to 72 hours ($P=0.355$). The toxicity of

methoxyfenozide was significantly different from the toxicity of bifenthrin at 48 hours ($P<0.001$) but not 72 hours ($P=0.222$). The toxicity of methoxyfenozide was significantly different from the toxicity of β -cyfluthrin at 48 hours ($P<0.001$) but not 72 hours ($P=0.182$).

In bioassays aimed at determining the effect of chlorantraniliprole on toxicity of pyrethroids, mortality levels were significantly different ($P<0.001$) across all time points between insecticide treatments and the control for bioassays that combined bifenthrin and β -cyfluthrin with chlorantraniliprole. Chlorantraniliprole significantly increased the toxicity of bifenthrin in mixtures (Fig. 2.8) at 72 hours ($P=0.003$) but not 48 hours ($P=0.196$). Chlorantraniliprole increased the toxicity of β -cyfluthrin in mixtures (Fig. 2.9) at 48 hours ($P=0.015$) and ($P<0.001$) at 72 hours. The toxicity of chlorantraniliprole was significantly different from the toxicity of bifenthrin after 48 hours ($P=0.045$) but not 72 hours. ($P=0.179$). The toxicity of chlorantraniliprole was significantly different from the toxicity of β -cyfluthrin after 48 hours ($P=0.010$) but not 72 hours ($P=0.592$).

Discussion

Suppression of resistance *in vivo* in bioassays that incorporate inhibitors such as PBO or DEF accentuates the contributions of P450s and COEs in the detoxification of pyrethroids (Oakeshott et al. 2005), as opposed to target-site resistance that is usually inferred when insects display no change in pesticide sensitivity in the presence of such synergists (Khambay and Jewess 2005). As expected, applications of PBO and DEF synergized the toxicity of bifenthrin and β -cyfluthrin in the susceptible strain of navel orangeworms, confirming that P450s and COEs are involved in detoxification in this strain as well. Results from our bioassays show that the same synergistic concentrations of PBO and DEF in the susceptible strain decreased the LC_{50}

for bifenthrin and β -cyfluthrin in the resistant strain. Resistance is therefore at least partly metabolic and likely occurs through overexpression of specific P450 and COE genes.

Recent studies involving highly polyphagous herbivorous pests such as the corn earworm (*Helicoverpa zea*) (Lepidoptera: Noctuidae) suggest that P450s responsible for insecticide detoxification may have evolved from P450s that detoxify host plant allelochemicals (Li et al. 2007). In *H. zea*, CYP321A1 and CYP6B8 are involved in the metabolism of the pyrethroid cypermethrin as well as many phytochemicals (Li et al. 2004; Sasabe et al. 2004). The use of pyrethroid insecticides to control the Old World bollworm (*Helicoverpa amigera*) (Lepidoptera: Noctuidae) has resulted in populations that owe their resistance to overexpression of detoxificative P450s (Martin et al. 2002). In navel orangeworm, CYP6AB11 metabolizes imperatorin, a furanocoumarin related to those found in figs and citrus species (Niu et al. 2011). Whether P450s that are specialized for metabolizing a narrow range of alleochemicals in the broadly polyphagous navel orangeworm contribute to development of resistance to pyrethroids is an open question, but the fact that synthetic pyrethroids are structurally derived from plant alleochemicals makes such a scenario plausible (Li et al. 2007).

β -cyfluthrin is classified as a type II pyrethroid because it contains an α -cyano group at its ester linkage (Davies et al. 2007). The presence of the α -cyano group makes pyrethroids less susceptible to hydrolytic detoxification by carboxylesterases (Casida et al. 1983; Oakeshott et al. 2005). β -cyfluthrin detoxification differed between the resistant and the susceptible strains because the magnitude of the effect of the COE inhibitor was significantly less than the effect of the P450 inhibitor in the susceptible strain. In the resistant strain, the inhibitors had the same effect on the LC_{50} , which may implicate equal contributions from P450s and COEs in the detoxification of β -cyfluthrin, but results may be confounded by the fact that PBO is also a

moderate inhibitor of esterases in some insects, and DEF has some capacity to inhibit P450 genes in some insects (Ahmad and Hollingsworth 2004).

Cross-resistance is a major problem in designing management strategies once an insect pest has acquired resistance to a particular insecticide (Liu et al. 2012). If resistance to one insecticide class can emerge as the result of enhanced P450 and COE activity, then cross-resistance may arise at an increasing rate if resistant navel orangeworms are exposed to other insecticides that share similar modes of detoxification. I evaluated the efficacy of methoxyfenozide and chlorantraniliprole, which are combined in tank mixes with pyrethroids that are registered for use in almond and pistachio orchards.

Niu et al. (2012) determined that methoxyfenozide is not detoxified by P450s in a susceptible strain of navel orangeworms. Methoxyfenozide did not synergize or inhibit the toxicity of bifenthrin or β -cyfluthrin in the resistant strain of navel orangeworms. Pyrethroids and methoxyfenozide in mixes may be detoxified through different pathways in the navel orangeworm, but, based on the results of our bioassays, methoxyfenozide would not be expected to effectively restore susceptibility in resistant strains through synergistic effects.

In contrast, chlorantraniliprole synergized the toxicity of bifenthrin and β -cyfluthrin. Although its main detoxification pathway has yet to be determined in the navel orangeworm, our results suggest that chlorantraniliprole can be used in combination with pyrethroids to circumvent resistance because of its ability to synergize combined with an independent mode of detoxification. Future assays that involve combinations of pesticides already registered for use against navel orangeworm can provide insight into whether mixtures containing chlorantraniliprole can be effective in overcoming existing pyrethroid resistance.

The proximity between almond and pistachio orchards in California's Central Valley could have a major impact on the dispersal and establishment of resistant populations. Higbee and Siegel (2009) assessed navel orangeworm damage patterns in almonds and found significantly more damage in nuts harvested within 3 miles (4.8 km) of pistachio orchards compared to those farther away. Studies in 2003 and 2004 (Burks et al. 2008) showed that pistachio orchards in Kern County contained denser populations of navel orangeworms than in almond orchards. Sanitation is more difficult to accomplish in pistachio orchards than in almond orchards because pistachios cannot be readily shredded by tillage, which results in a greater abundance of mummies and thus overwintering sites for the navel orangeworm (Siegel et al 2008). The proximity of these other hosts and the different timing of insecticide use in these crops also increase the possibility of nontarget exposure of navel orangeworms to sublethal insecticide concentrations, which could accelerate resistance acquisition and threaten integrated management plans.

Figures

Figure 2.1. Median-lethal concentration (LC_{50}) values after 48 hours for the pyrethroid insecticides bifenthrin and β -cyfluthrin. Error bars represent the 95% confidence limits in the LC_{50} for each insecticide

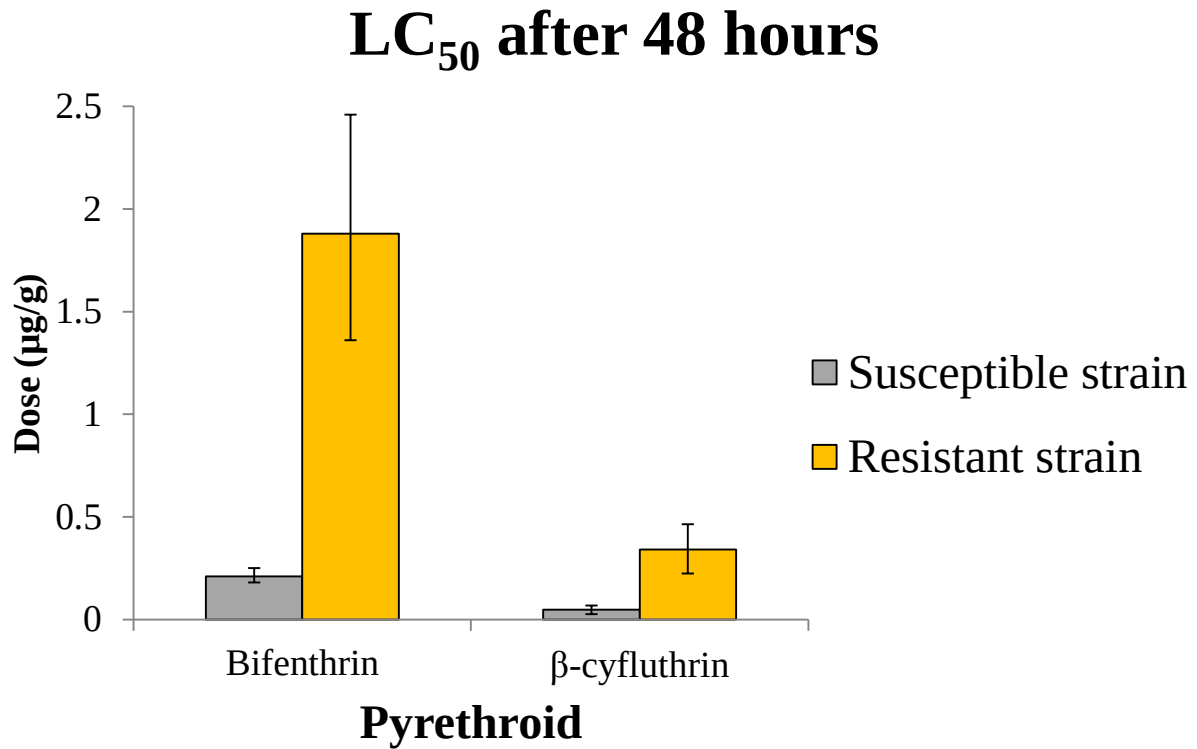


Figure 2.2. First instar navel orangeworm (CPQ) mortality across multiple time points following dietary exposure to 200 ng/g bifenthrin, 200 ng/g bifenthrin +200 µg/g piperonyl butoxide (PBO), 200 ng/g bifenthrin + 100 µg/g S,S,S-tributyl phosphorotrithioate (DEF), 200 µg/g PBO, and 100 µg/g DEF. Error bars represent the standard error. Letters A, B, and C represent significantly different groups ($P<0.05$).

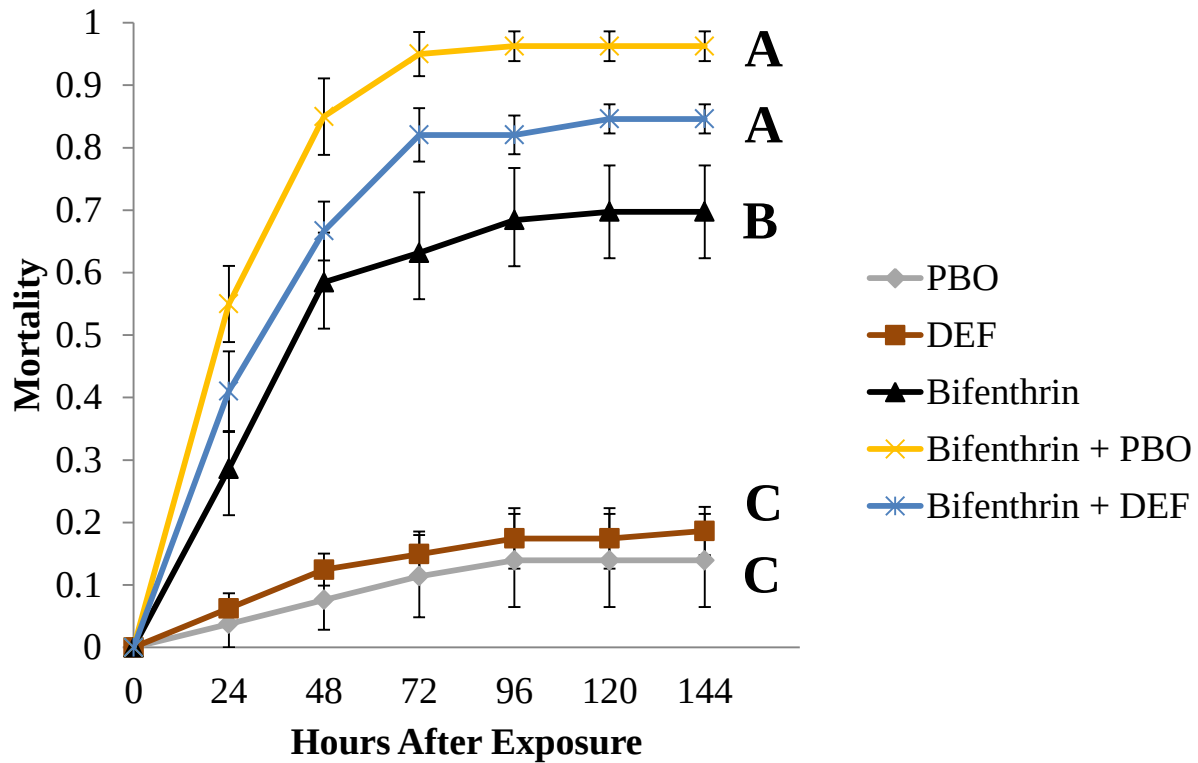


Figure 2.3. First instar navel orangeworm (CPQ) mortality across multiple time points following dietary exposure to 40 ng/g β -cyfluthrin, 40 ng/g β -cyfluthrin + 200 μ g/g piperonyl butoxide (PBO), 40 ng/g β -cyfluthrin + 100 μ g/g S,S,S-tributyl phosphorotrithioate, 200 μ g/g PBO, and 100 μ g/g DEF. Error bars represent the standard error. Letters A, B, C, and D represent significantly different groups ($P < 0.05$).

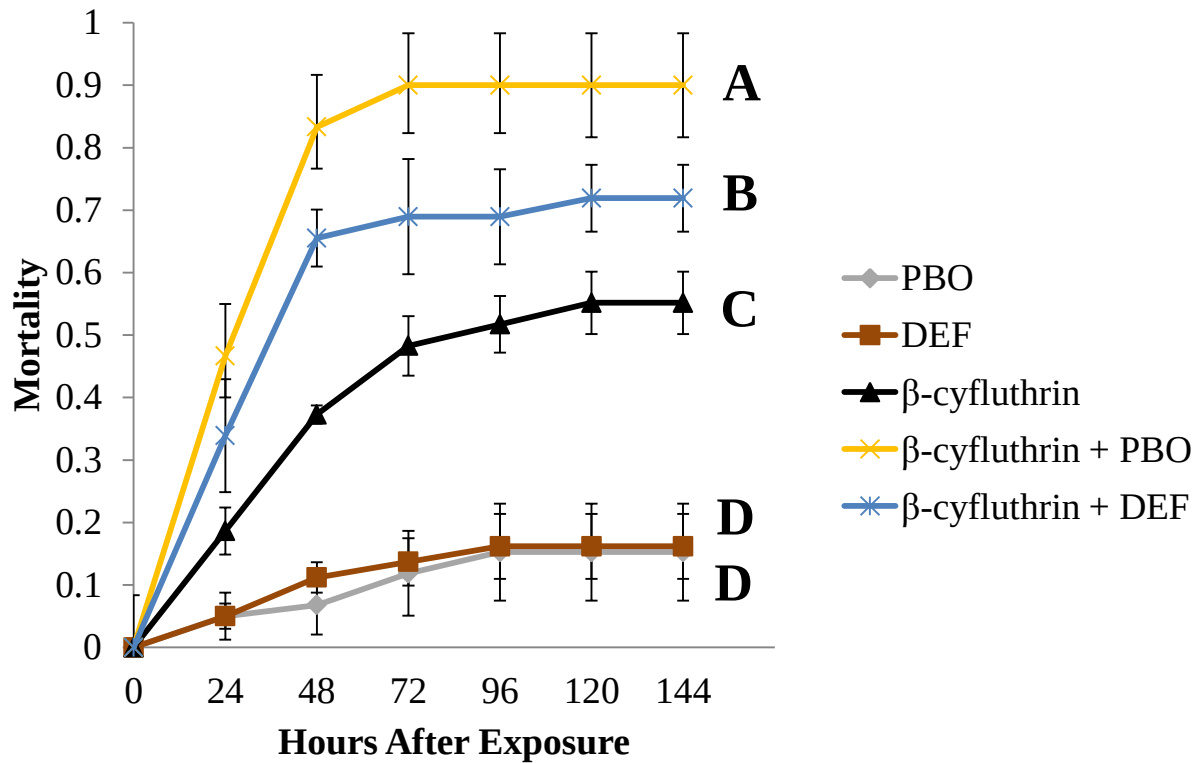


Figure 2.4. First instar navel orangeworm (R347) mortality across multiple time points following dietary exposure to 1.6 $\mu\text{g/g}$ bifenthrin, 1.6 $\mu\text{g/g}$ bifenthrin + 200 $\mu\text{g/g}$ piperonyl butoxide (PBO), 1.6 $\mu\text{g/g}$ bifenthrin + 100 $\mu\text{g/g}$ S,S,S-tributyl phosphorotrithioate (DEF), and 100 $\mu\text{g/g}$ DEF. Error bars represent the standard error. Letters A, B, and C represent significantly different groups ($P < 0.05$).

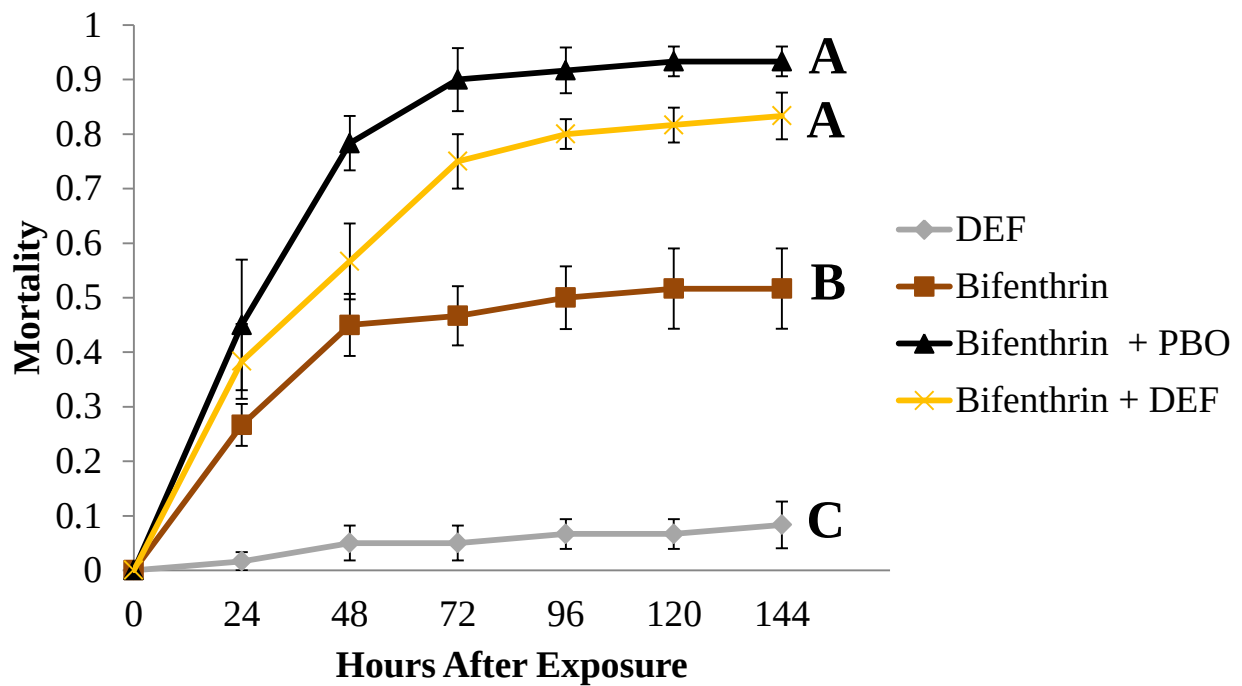


Figure 2.5. First instar navel orangeworm (R347) mortality across multiple time points following dietary exposure to 350 ng/g β -cyfluthrin, 350 ng/g β -cyfluthrin + 200 μ g/g piperonyl butoxide (PBO), 350 ng/g β -cyfluthrin + 100 μ g/g S,S,S-tributyl phosphorotrithioate (DEF), and 100 μ g/g DEF. Error bars represent the standard error. Letters A, B, and C represent significantly different groups ($P < 0.05$).

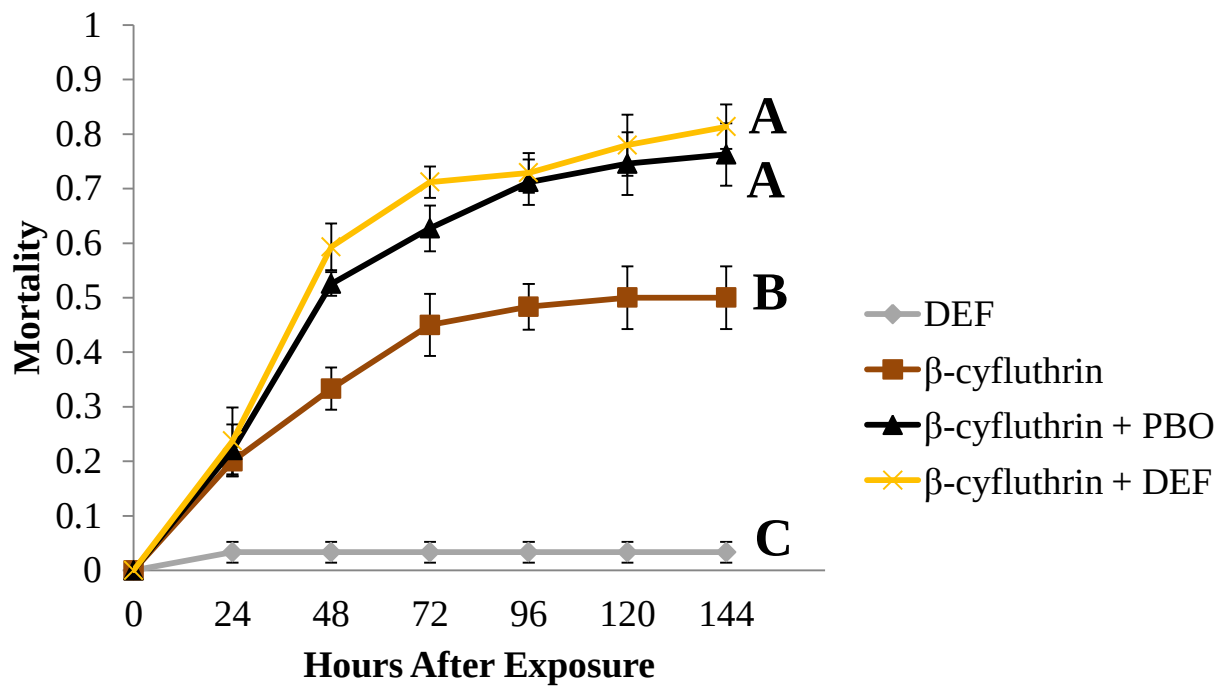


Figure 2.6. First instar navel orangeworm (R347) mortality across multiple time points following dietary exposure to 1.6 $\mu\text{g/g}$ bifenthrin, 130 ng/g methoxyfenozide, 1.6 $\mu\text{g/g}$ bifenthrin + 130 ng/g methoxyfenozide, and a control (200 μl methanol). Error bars represent the standard error. Letters represent significantly different groups ($P < 0.05$). Letters A and B represent significantly different groups ($P < 0.05$).

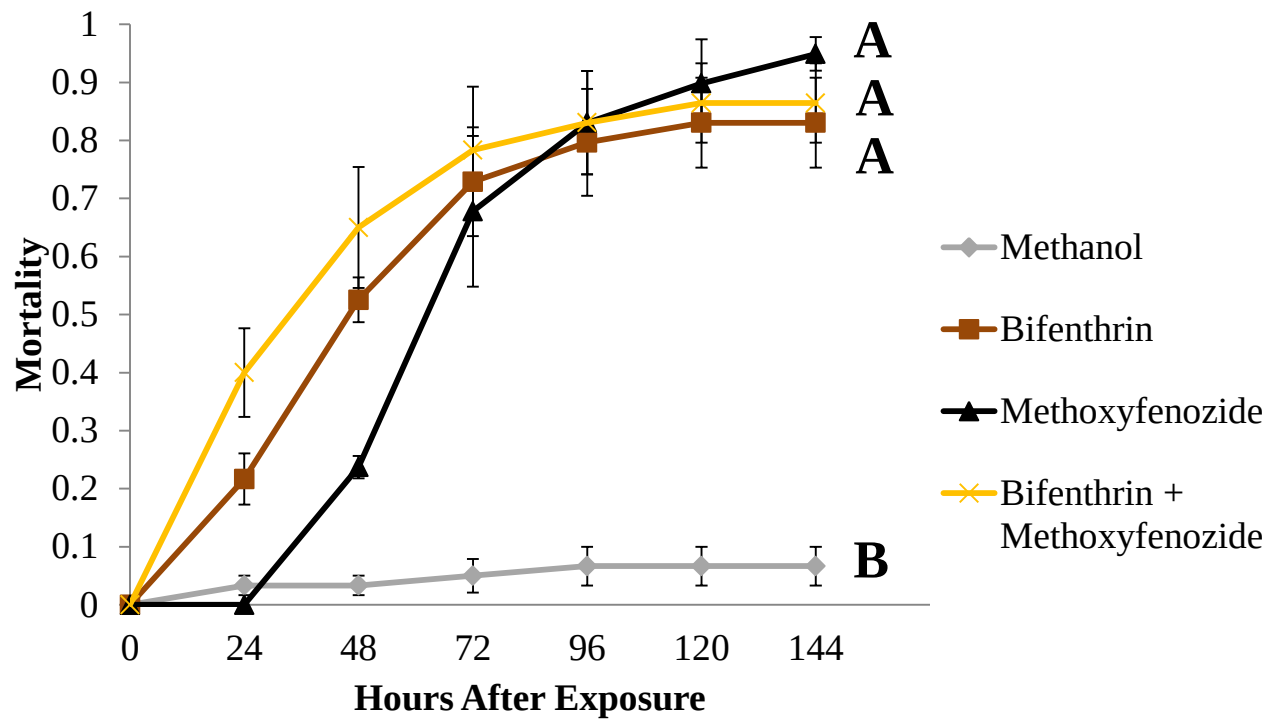


Figure 2.7. First instar navel orangeworm (R347) mortality across multiple time points following dietary exposure to 350 ng/g β -cyfluthrin, 130 ng/g methoxyfenozide, 350 ng/g β -cyfluthrin + 130 ng/g methoxyfenozide, and a control (200 μ l methanol). Error bars represent the standard error. Letters A and B represent significantly different groups ($P < 0.05$).

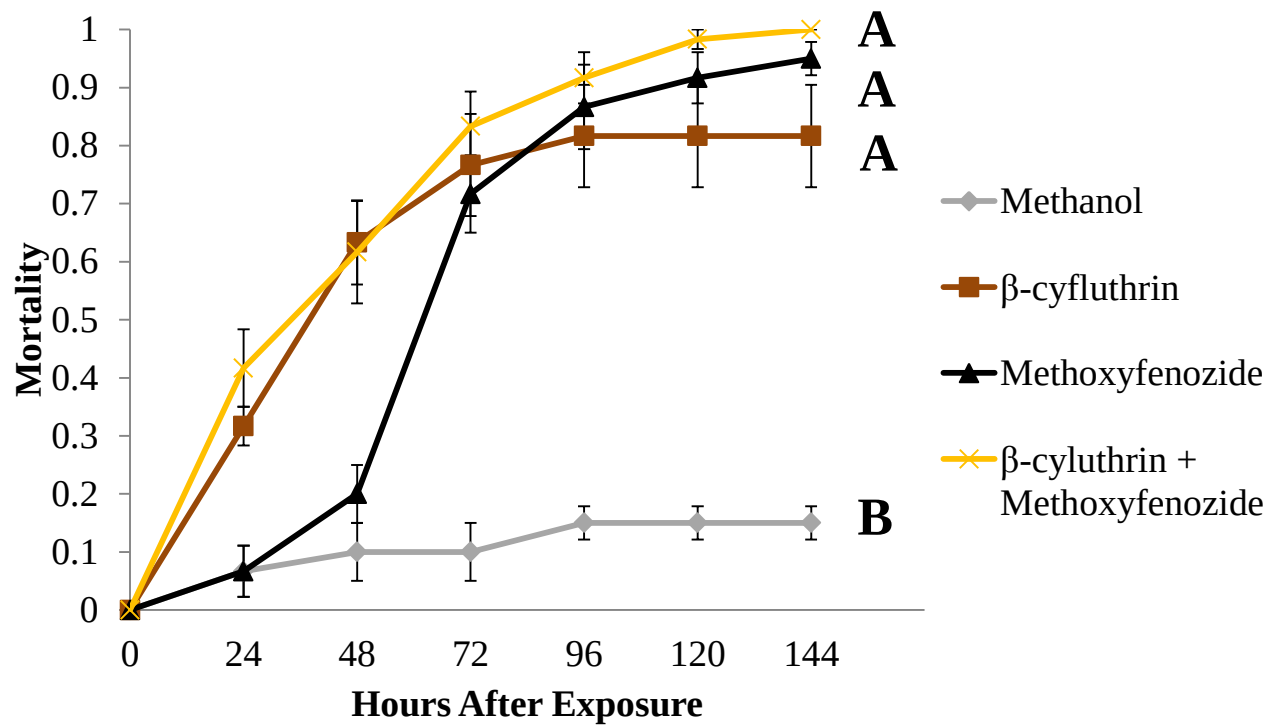


Figure 2.8. First instar navel orangeworm (R347) mortality across multiple time points following dietary exposure to 1.6 µg/g bifenthrin, 4 µg/g chlorantraniliprole, 1.6 µg/g bifenthrin + 4 µg/g chlorantraniliprole, and a control (200 µl acetone). Error bars represent the standard error. Letters A, B, and C represent significantly different groups ($P < 0.05$).

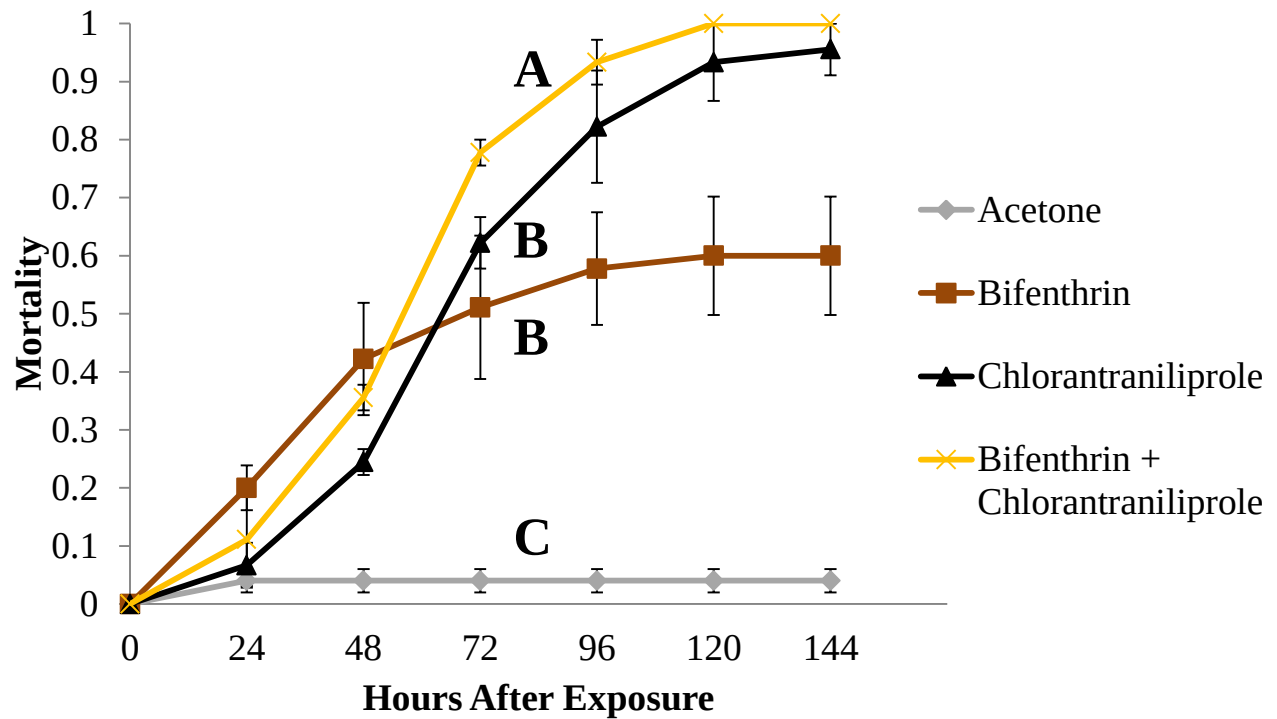
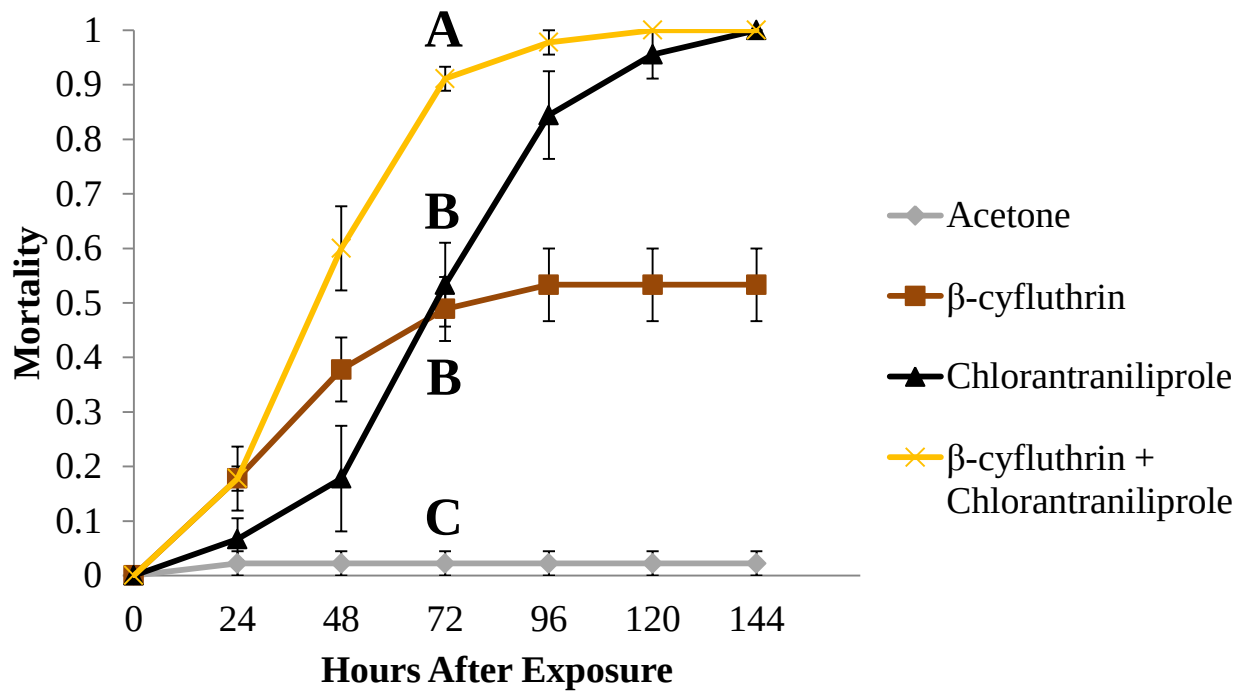


Figure 2.9. First instar navel orangeworm (R347) mortality across multiple time points following dietary exposure to 350 ng/g β -cyfluthrin, 4 μ g/g chlorantraniliprole, 350 ng/g β -cyfluthrin + 4 μ g/g chlorantraniliprole, and a control (200 μ l acetone). Error bars represent the standard error. Letters A, B, and C represent significantly different groups ($P < 0.05$).



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CHAPTER III: LIFE HISTORY DIFFERENCES BETWEEN A RESISTANT AND SUSCEPTIBLE STRAIN OF NAVEL ORANGEWORMS

Introduction

The navel orangeworm, *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae), is the primary pest of almonds and pistachios in California orchards (Siegel et al. 2010). The navel orangeworm is managed primarily through winter removal and destruction of mummies along with insecticide use. Navel orangeworm populations undergo two generations on mummies before adults emerge and oviposit on new crop nuts during hull split from late July to August (Sanderson et al. 1989; Bentley et al. 2008). Insecticides that target the most damaging third generation may be timed based on oviposition activity or onset of crop susceptibility (Sanderson et al 1989). Pyrethroid insecticides and insect growth regulators are the insecticides most widely used during the growing season (Leal et al. 2009).

Resistance to the pyrethroid insecticide bifenthrin has recently been reported in almond orchards located in Kern County. Insect pests can acquire resistance to pyrethroid insecticides through multiple mechanisms, including as target-site insensitivity, overexpression of essential detoxification enzymes, increased sequestration, and reduced cuticular penetration (Khambay and Jewess 2005; Li et al. 2007; Liu 2012). Our previous study (Chapter 2) demonstrated that the mechanism of resistance in this resistant population involves elevated levels of cytochrome P450 monooxygenase (P450) and carboxylesterase (COE) activity.

The acquisition of resistance to synthetic insecticides often carries an associated fitness cost. A reduction in fitness occurs in resistant populations when resources directed toward fitness-enhancing traits are diverted in favor of the mechanisms involved in the production and maintenance of resistance (Carriere et al. 1994). Costs associated with insecticide resistance such

as increased development time of larval and pupal stages have been observed in many lepidopteran pests, including the tobacco budworm (*Heliothis virescens*) (Lepidoptera: Noctuidae) (Sayyed et al. 2008), the cotton bollworm (*Helicoverpa armigera*) (Lepidoptera: Noctuidae) (Wang et al. 2010), and the obliquebanded leafroller (*Choristoneura rosaceana*) (Harris) (Carriere et al. 1994).

In this study, I compared life history traits in a resistant strain of navel orangeworms and a susceptible strain in an effort to identify fitness costs associated with the initial development of resistance to pyrethroid insecticides. Life tables offer an effective means of summarizing the survival and mortality rates in a population and allow for statistical inferences about the population under study (Chiang 1984). I constructed life tables from first instar through pupation for resistant and susceptible strains on a semi-synthetic artificial diet (Niu et al. 2012). Overall survivorship, development time, and pupal weights were compared between strains. In addition, pupal weights were compared directly across three generations from resistant and susceptible strains reared on wheat bran diet. Median-lethal concentrations (LC₅₀) for bifenthrin were also recorded through eight generations to determine if resistance was maintained in the absence of bifenthrin selection pressure.

Materials and Methods

Insects:

A laboratory colony of *A. transitella* designated as CPQ (J. Siegel: USDA-ARS, Parlier, CA) and a resistant colony designated as R347 (B. Higbee, Paramount Farming Company, Bakersfield, CA) were kept in an incubator at University of Illinois at Urbana-Champaign and maintained at conditions of $27 \pm 4^{\circ}\text{C}$ in the absence of a light cycle. Larvae were mass-reared until pupation in 500 ml glass jars containing 300 g of a wheat bran diet (Finney and Brinkmann

1967). Adults were transferred to additional 500-ml glass jars with tissue paper on the inside and covering the top to serve as oviposition substrate. Eggs were collected every 48 hours from these jars.

Life Table:

Eggs were collected on the same day for both the susceptible and the resistant colonies. After egg hatch, 50 neonates from each strain were transferred individually onto separate 28-ml (1-oz) plastic cups containing 5 g of artificial diet. Larvae were checked every day for the occurrence of a molt and for survivorship through the first five larval instars prior to pupation. The presence of a shed head capsule indicated a molt and a transition to the next stadium. Days when molting occurred were recorded until the larvae pupated. Individuals that survived to pupation were removed, separated by sex, and weighed for comparison between strains. Life tables were generated based on the percentage survivorship at the beginning of each stage (l_x), the number dying during each stage (d_x), and the mortality rate between instars (q_x) (Table 3.1). Boxplots were constructed to examine the distribution of days until pupation and the first molt from individuals in the susceptible and resistant strains. The diet was replaced once each week in order to avoid any effects of spoilage throughout the experiment.

Pupal Weights:

Pupal weights of at least 200 individuals were randomly sampled in each generation from the susceptible and resistant colonies reared on the wheat bran diet. Pupae from each strain were separated by sex within each generation and the number of males and females was recorded. The sex of a pupa was determined by the location of the gonopore, which occurs on the eighth segment in females and ninth segment in males (Husseiny and Madsen 1964).

Median-lethal Concentration Assays:

Bifenthrin was mixed into the artificial diet during the liquid phase at specific concentrations and poured into separate 1 oz (28 ml) cups to harden. Solutions of bifenthrin in methanol were prepared at a range of concentrations (500 ng/g, 1 µg/g, 2 µg/g, 3 µg/g, and 8 µg/g) and fed to neonate larvae. For each bioassay, 20 neonates were exposed to each concentration of bifenthrin. Mortality levels were assessed and recorded at each concentration after 48 hours. Larvae that did not move when touched with a soft brush were scored as dead. Bioassays were repeated twice in the second and third generations and three times in generations three through eight.

Statistical Analyses:

SPSS version 22 software (SPSS Inc., Chicago IL) was used to construct Kaplan-Meier (1958) curves and test for a significant difference in survivorship across five larval instars and through pupation in the resistant and susceptible strains. A two-tailed t-test was used in SPSS to check for significant differences between pupal weights and development times of both strains from life table experiments. A two-way analysis of variance (ANOVA) was run in SPSS to test for significant differences in pupal weights across multiple generations for females and males of each strain that were reared on wheat bran diet. Strain and generation were the categorical variables in the ANOVA. Finally, the Probit analysis was used to calculate median-lethal concentrations that would kill 50% of resistant neonates at 48 hours for the insecticide bifenthrin.

Results

Kaplan-Meier survivorship curves were constructed to determine if the difference in survivorship between the susceptible strain and the resistant strain was significant from first instar through pupation (Fig. 3.1). Individuals that survived to pupation were marked as censored

and removed from the analysis. One individual present from each strain in the fifth instar was also censored after 60 days (indicative of developmental abnormalities) since the start of the experiment. The Log-Rank Test indicated that survivorship was not significantly different between the susceptible strain and the resistant strain ($P=0.146$).

Results from the t-test confirmed there was no significant difference in time to pupation between strains ($P=0.732$) (Fig. 3.2). The boxplot of time to the first molt displayed two outliers at 9 days and 14 days in the distribution from the resistant strain. These outliers were removed in the two-tailed t-test, which concluded that the difference in the number of days until the molt into second instar between strains was significant ($P<0.001$) (Fig. 3.3).

The t-test confirmed there was no significant difference in weight of female pupae from the susceptible and resistant strains, although there was a trend toward larger weights in the susceptible strain ($P=0.062$) (Fig. 3.4). There was no significant difference in weight of male pupae from the susceptible and resistant strains ($P=0.333$). The effects of strain ($P<0.001$) and generation ($P<0.001$) were significant in the two-way ANOVA for females, while the interaction was not significant ($P=0.081$) (Fig. 3.5). In males, the effects of strain ($P<0.001$) and generation ($P<0.001$) were significant in the two-way ANOVA as was the interaction ($P<0.001$) (Fig. 3.6). Separate one-way ANOVAs were conducted between males from the susceptible and resistant strains in generations 4, 5, and 6. Results from the one-way ANOVAs were significant between strains for each generation of males ($P<0.001$).

The goodness-of-fit test indicated that bifenthrin concentration mortality data fit the Probit model ($P>0.05$) in each of the eight generations (Table 3.2). The LC_{50} values for bifenthrin in the resistant strain across eight generations were not considered significantly different as evidenced by their non-overlapping 95% confidence intervals.

Discussion

Although genetically determined resistance may provide a selective advantage to individuals in the presence of an insecticide, resistance genes are rarely fixed in natural populations and can be lost in the absence of selection pressure exerted by insecticides (Coustau and Chevillon 2000). If insecticide resistance carries a strong associated fitness cost, then a return to an insecticide-free environment could result in a strong phenotypic change with deleterious effects (Coustau and Chevillon 2000; Klot and Ghanim 2012). Boivin et al. (2003) reported a decline in laboratory-reared codling moths (*Cydia pomonella*) (Lepidoptera: Tortricidae) resistant to the benzamide insecticide diflubenzuron after 10 generations in the absence of insecticide pressure. Because navel orangeworm resistance to bifenthrin developed recently, it is possible that alleles underlying enhanced detoxification have not become fixed within the population and may be lost in the absence of bifenthrin selection pressure. Median-lethal concentration assays for bifenthrin over first 8 generations under laboratory conditions indicate resistance is heritable thus far and that contributing alleles are maintained in the resistant colony.

Initial differences in development of the resistant strain compared with the susceptible strain could have an impact on management practices for navel orangeworm. The increased mortality displayed in the initial stages of growth from the life table experiment and the significant difference in the number of days to the first molt could influence efficacy of insecticide sprays. If resistant strains develop more slowly during the initial stages of growth in almond orchards, then populations may experience greater exposure and impact from allelochemicals present in the shells and hulls of almonds in addition to prolonged insecticide treatments before they can tunnel into the nut (Siegel et al. 2010).

The recorded survival and development across all larval stages and through pupation, however, did not support the existence of fitness costs associated with development in the resistant strain. Control failure in navel orangeworm populations may result when variation in development times produces faster-growing individuals, potentially resulting in an extra generation or generations that substantially increases the standing population; alternatively, individuals that develop more slowly may emerge and oviposit on new-crop nuts after insecticide treatment has degraded (Siegel et al. 2010). If there is no effect on the development of navel orangeworm populations resistant to pyrethroids, then the timing of insecticide sprays that target adult oviposition during the spring and at hull split may not result in deviations from standard management practices.

The positive relationship between female pupal weight and fecundity has been documented in many species of Lepidoptera (Haukioja and Neuvonen 1985; Calvo and Molina 2005; Konopka et al. 2012). Comparisons of pupal weights across three generations revealed that females were larger in the susceptible strain than the resistant strain. If these observed differences are reflected in resistant field populations, then the dispersal and establishment of additional resistant populations could be severely hindered by reduced fecundity associated with smaller adult size.

Pupal weight comparisons across three generations in males also indicated a potential fitness cost in the resistant strain through significant reductions in size. Male size can have a direct impact on ejaculate size and nutritional content in spermatophores (Wiklund and Kaitala 1995). In the male European corn borer (*Ostrinia nubilalis*) (Lepidoptera: Crambidae), the volume of the first spermatophore produced is correlated with pupal weight, and investments in the production of sperm and accessory gland secretions may limit reproductive outputs (Royer

and McNeil 1993). Pyrethroid resistance in navel orangeworm populations may limit the number of offspring males can produce if smaller size has an impact on the quality of spermatophores in field populations.

Identifying fitness costs resulting from pyrethroids can be extremely valuable in designing pest management programs that can limit the spread of the resistant population. Results from this study suggest a fitness cost associated with pyrethroid resistance, but such assessments conducted under optimal laboratory conditions may not accurately reflect the conditions experienced by field populations of navel orangeworms (Kliot and Ghanim 2012). Pupal weight differences may simply reflect heterogeneity in the resistant strain or initial adaptation to the laboratory conditions as opposed to fitness costs. It is possible that differences noted in the resistant strain may have resulted from suites of alleles maintained or lost through founder effects or drift. Differences in weights between susceptible and resistant lines are confounded by a separate origin for the two lines, combined with a large difference in the number of generations in laboratory culture. Examining additional navel orangeworm populations in the orchards of Kern and its neighboring counties will be invaluable in determining the distribution of resistant individuals and its association with different histories of pyrethroid use.

Figures and Tables

Figure 3.1. Kaplan-Meier Survivorship Curves for a resistant (R347) and susceptible strain (CPQ) of *Amyelois transitella*. The sixth instar on the X-axis represents the pupal stage.

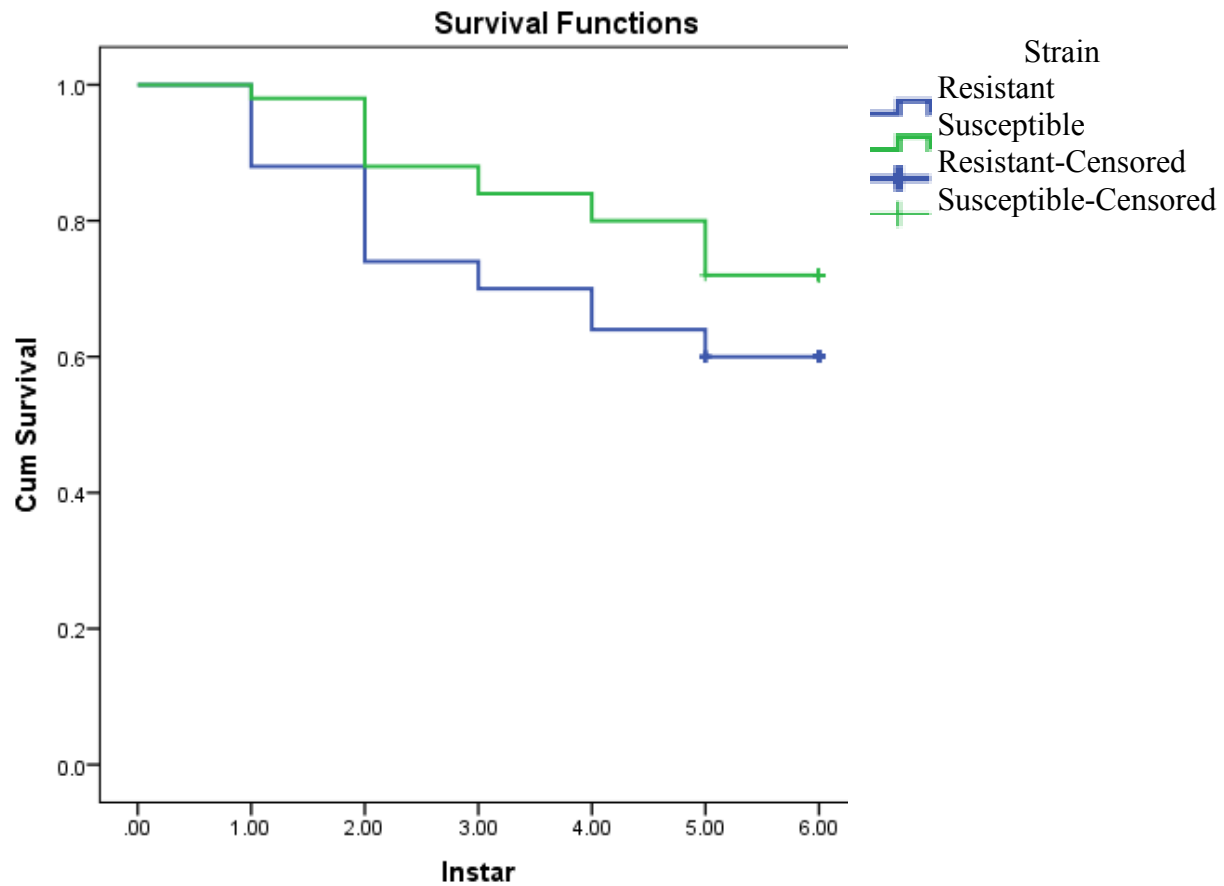


Figure 3.2. Distribution of days to pupation for a resistant (R347) and susceptible strain (CPQ) of *A. transitella* from a life table experiment. Error bars (whiskers) represent the highest and lowest values below 1.5 and 3 times the interquartile range. Circles represent outliers.

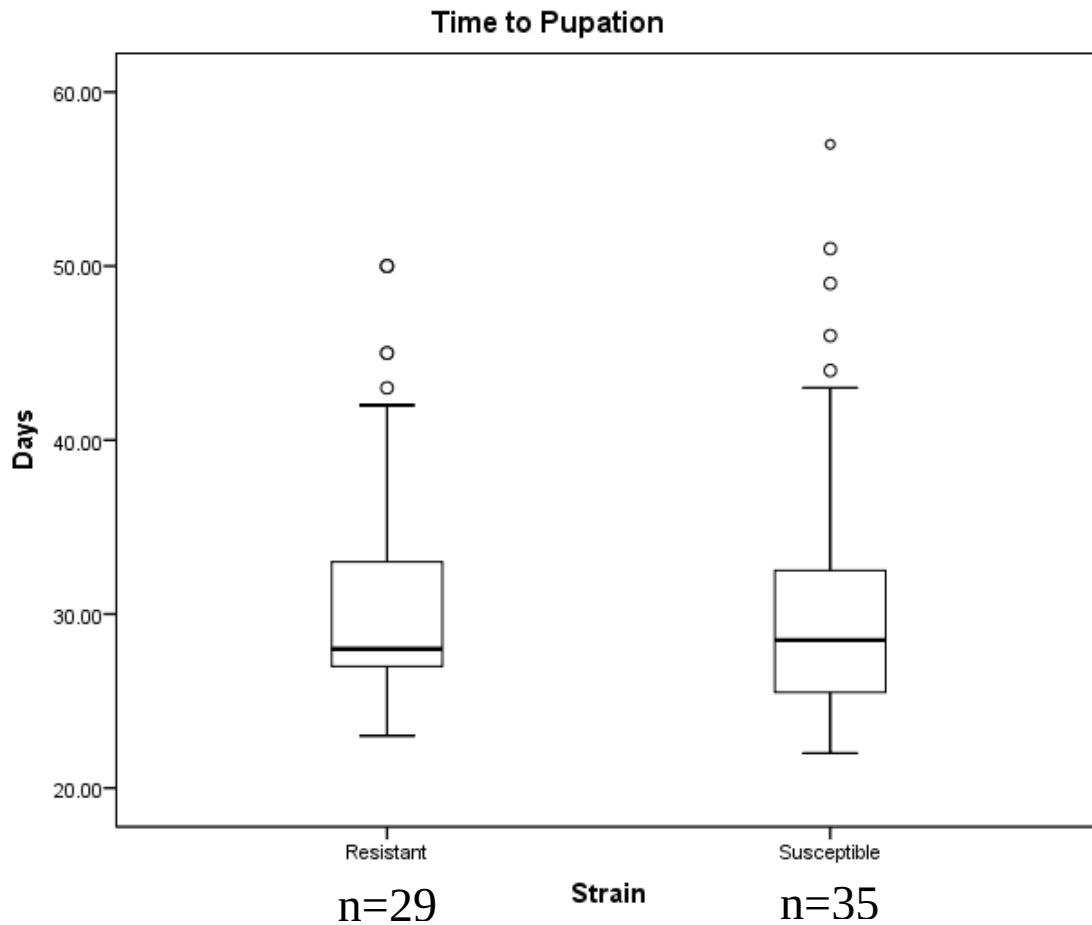


Figure 3.3. Distribution of days until first molt for a resistant (R347) and susceptible strain (CPQ) of *A. transitella* from a life table experiment. Error bars (whiskers) represent the highest and lowest values below 1.5 and 3 times the interquartile range. Circles represent outliers. Outliers at 9 days and 14 days in the resistant strain were removed in the test for significance.

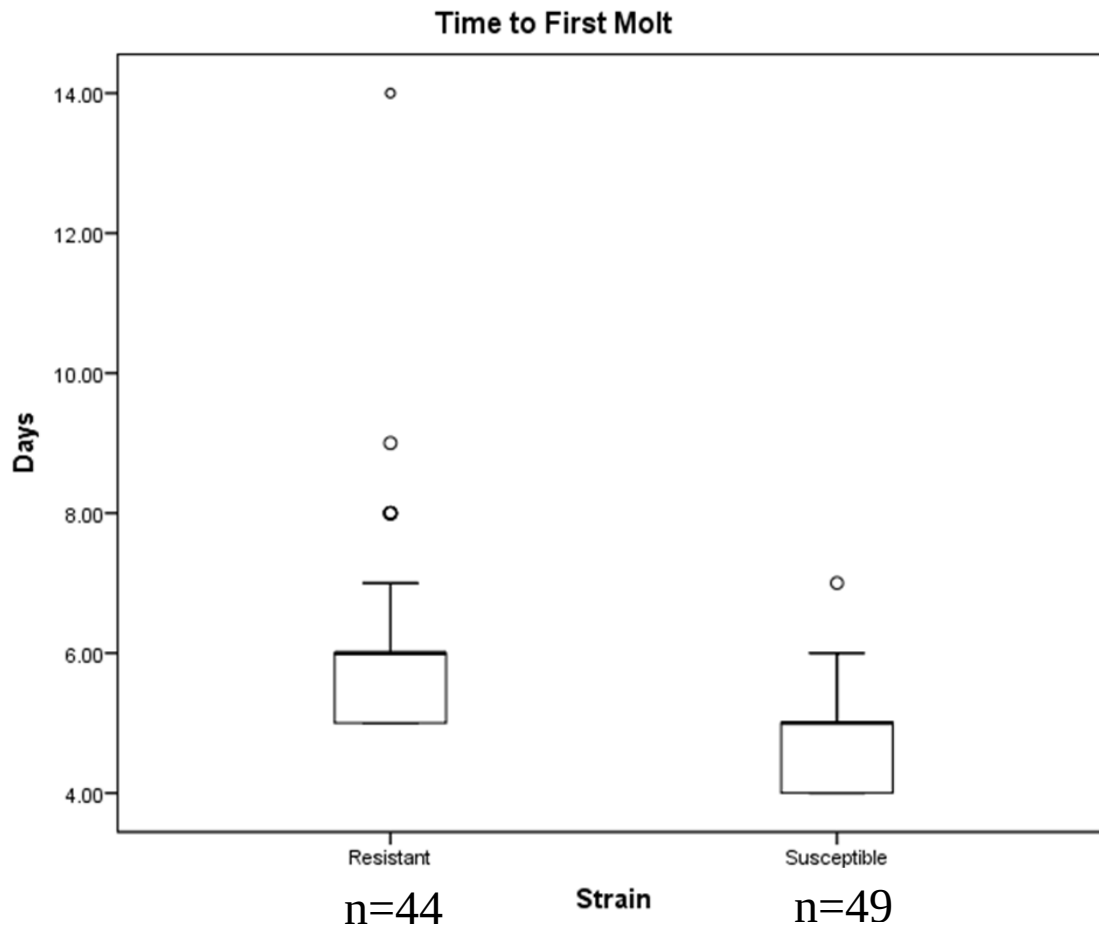


Figure 3.4. Pupal weights for male and female survivors during life table experiments in a resistant (R347) and susceptible strain (CPQ) of *A. transitella*. Error bars represent standard error.

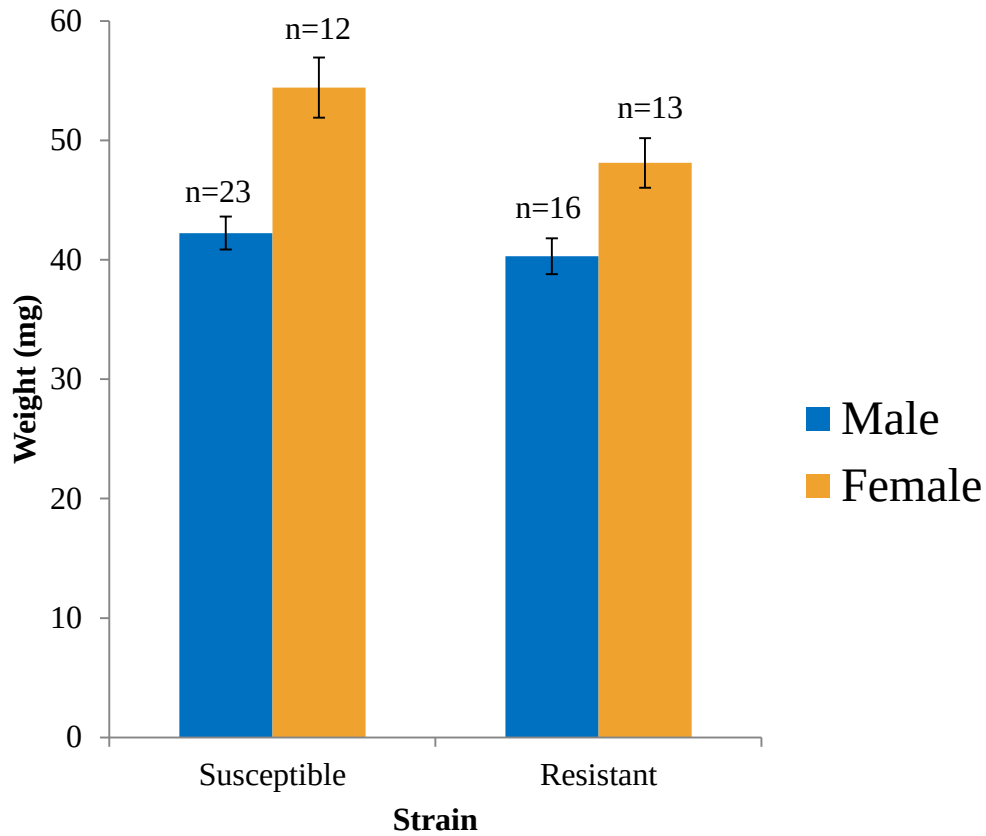


Figure 3.5. Mean pupal weight of females reared on wheat bran diet from a resistant (R347) and susceptible strain (CPQ) of *A. transitella* across multiple generations. Error bars represent the standard error.

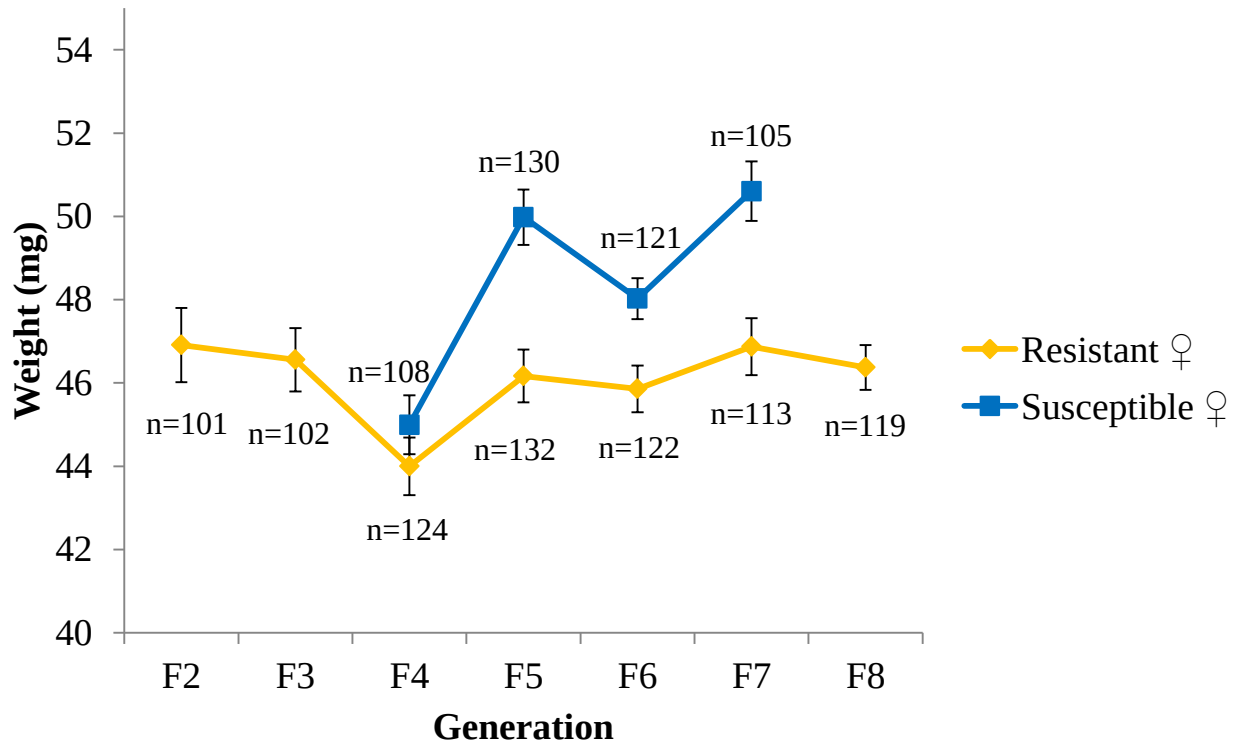


Figure 3.6. Mean pupal weight of males reared on wheat bran diet from a resistant (R347) and susceptible strain (CPQ) of *A. transitella* across multiple generations. Error bars represent the standard error.

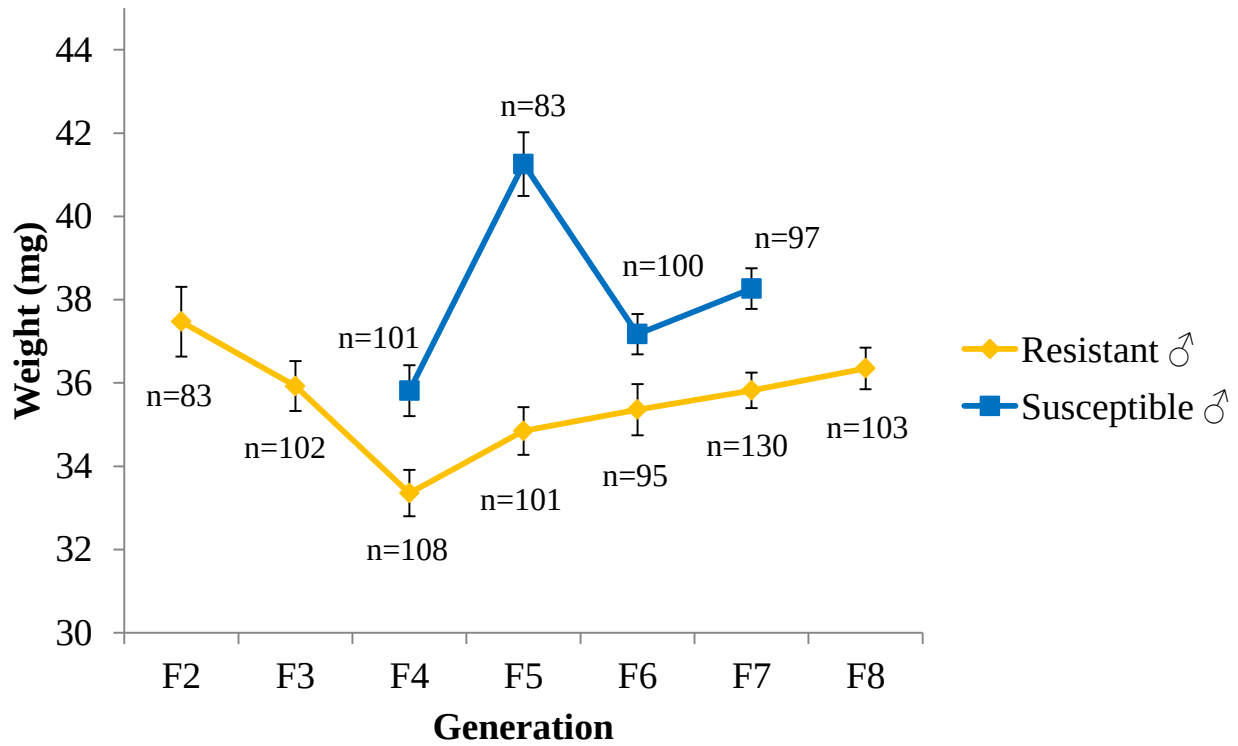


Table 3.1. Life table for a resistant (R347) and susceptible strain (CPQ) of *A. transitella*.

x	Strain	n	lx	dx	qx
Egg	Susceptible	50	1	0	0
	Resistant	50	1	0	0
1st instar	Susceptible	50	1	0	0
	Resistant	50	1	0	0
2nd instar	Susceptible	49	0.98	1	0.02
	Resistant	44	0.88	6	0.12
3rd instar	Susceptible	44	0.88	5	0.102
	Resistant	37	0.74	7	0.159
4th instar	Susceptible	42	0.84	2	0.045
	Resistant	35	0.7	2	0.054
5th instar	Susceptible	40	0.8	2	0.048
	Resistant	32	0.64	3	0.086
Pupae	Susceptible	35	0.7	4	0.1
	Resistant	29	0.58	2	0.063

Table 3.2. Probit analysis data for bifenthrin in resistant neonate *A. transitella* across eight generations maintained under laboratory conditions.

Generation	<i>n</i>	Slope (SE)	LC50 ± 95% CL (ppm)	<i>x</i> ²	<i>P</i>
F2	200	1.93 (0.23)	1.88 (1.36-2.46)	3.58	0.17
F3	200	1.32 (0.27)	1.47 (1.05-1.98)	0.35	0.84
F4	300	1.87 (0.29)	1.89 (1.53-2.38)	0.48	0.79
F5	300	1.69 (0.20)	1.75 (1.40-2.16)	1.82	0.61
F6	300	1.90 (0.31)	1.52 (1.08-1.94)	1.58	0.45
F7	300	2.14 (0.23)	1.52 (1.27-1.81)	1.26	0.74
F8	300	1.88 (0.22)	1.83 (1.50-2.22)	0.56	0.91

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