SUSCEPTIBILITY OF ORIENTAL FRUIT MOTH, (*GRAPHOLITA MOLESTA* (BUSCK)) TO SELECTED INSECTICIDES AND MIXTURES

BY

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DISSERTATION

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ABSTRACT

A series of experiments assessed the susceptibility of Oriental fruit moth, *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae), to selected insecticides and mixtures. Two populations – a laboratory colony from Rutgers University and a colony established in 2007 from orchards in Calhoun County, Illinois – were tested. Both colonies were reared concurrently on lima bean diet and 'Gala' apples to reduce the likelihood that either colony would be lost to diseases or other factors. Bioassays were analyzed separately for each colony and for progeny of parents reared on each food source.

To determine the baseline susceptibility of *G. molesta* to chlorantraniliprole, spinetoram, spinosad, acetamiprid, thiamethoxam, esfenvalerate, and lambda-cyhalothrin, neonates were placed on wheat germ diet containing a range of concentrations of each insecticide. Overall, the two colonies responded similarly to these insecticides, regardless of parental food source. Results of these bioassays provide baseline data for future monitoring of of resistance.

To develop and test a diagnostic dose for estimation of pyrethroid resistance in the field, the dose-mortality relationship was described for esfenvalerate applied topically to adult males. A range of concentrations was applied in 1 µl of acetone to male moths from the Rutgers colony, and the LD_{99} was estimated to be 0.022 µg per moth. Application of 0.022 µg esfenvalerate per moth to *ca.* 600 male moths from two putatively susceptible populations resulted in mean survivorship approximately equal to the expected level of 1.0%. Application of this dose to *ca.* 375 moths captured in two Calhoun County orchards with histories of pyrethroid use resulted in mean survivorship of 9.4% and 82%. It is proposed that 0.022 µg of esfenvalerate in 1 µl of acetone be used as a diagnostic dose for monitoring pyrethroid resistance.

The toxicities of three mixtures of insecticides to neonates were estimated. Chlorantraniliprole was mixed with acetamiprid, esfenvalerate, or thiamethoxam. These insecticides may be mixed or rotated to provide broad spectrum control of orchard pests. Mixtures of chlorantraniliprole with acetamiprid or thiamethoxam did not exhibit consistent synergism or antagonism. For chlorantraniliprole plus esfenvalerate, mortality was less than expected at nearly all concentrations for both colonies, suggesting antagonism despite different modes of action for the two compounds. The effectiveness of one or both insecticides to Oriental fruit moth might be reduced if they are combined in field applications.

To estimate the toxicity of novaluron, an insect growth regulator, to eggs of the two colonies, eggs on waxed paper were dipped into a range of concentrations. Eggs from the Calhoun colony were more tolerant to novaluron than eggs from the Rutgers colony. Differences in the responses of these colonies may represent natural variation among populations or may be the result of selection by other insecticides used in orchards in Calhoun County before larvae were collected to establish this colony.

Key Words: bioassays, insecticide resistance, chlorantraniliprole, spinetoram, spinosad, acetamiprid, thiamethoxam, esfenvalerate, lambda-cyhalothrin, novaluron, resistance monitoring

To my Mother, Constance M. Jones, The 12th Rose

and my Guardian Angel

Rest in Peace

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

The research presented in this dissertation focuses on the toxicity of several insecticides to the Oriental fruit moth, *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae). Chapters 2-5 are presented with only minor revisions from manuscripts prepared for submission to specific journals under the authorship of Moneen Jones, Jacqueline Robertson, and Richard Weinzierl. As a result, there is some repetition in the introductory paragraphs of each chapter. The introductions to each chapter do not, however, provide a broader review of relevant background information. This *Introduction and Literature Review* provides an overview of the systematics and taxonomy of the Oriental fruit moth and a review of its host plants and distribution. It also describes this insect's life history and phenology, as well as modes of action of key insecticides, insecticide resistance, and resistance management.

Systematics and Taxonomy

The Oriental fruit moth is a tortricid (Lepidoptera: Tortricidae) in the subfamily Tortricinae. The Tortricidae, the only family currently recognized in the superfamily Tortricoidea (Horak and Brown 1991), contains more than 5,000 species (Horak 1984). Distinguishing characteristics of the family include porrect labial palpi (extended forward) and an unscaled proboscis (Horak and Brown 1991). Wingspans range from 8 to 40 mm, and moths are usually dull in color. The forewings of females appear either bell-shaped or rectangular. Many species bear tufts of scales on their thorax and forewings (Scoble 1992).

Lepidopterists currently recognize three subfamilies in the Tortricidae: Tortricinae, Chlidanotinae, and the Olethreutinae (Horak and Brown 1991). Male genitalia are least specialized in the Tortricinae. In the three tribes of Chlidanotinae, the valvae are characterized by deep, dorso-longitudinal invaginations (Tuck 1981, Horak and Brown 1991). Fusion of the aedeagus with the anellus and the juxta characterize the six tribes of the Olethreutinae (Horak and Brown 1991). In addition to the Oriental fruit moth, perennial pests in the family Tortricidae include borers, such as citrus fruit-borer, *Ecdytolopha aurantiana* Lima, leafrollers such as obliquebanded leafroller, *Choristoneura rosaceana* (Harris), the spruce budworms *Choristoneura occidentalis* (Freeman) and *Choristoneura fumiferana* (Clemens), and codling moth, *Cydia pomonella* (L.).

The scientific and common names of the Oriental fruit moth have been revised several times. The genus *Grapholita* was originally proposed by Treitschke (1829) and later as *Grapholitha* (Treitschke 1830). Most early authors were aware of the earlier usage but not the latter, and the 1830 spelling is considered an "unjustified emendation" of the original spelling (J. W. Brown, ARS USDA, Wash. DC 2010, personal communication). The Oriental fruit moth was first described by August Busck of the U. S. Bureau of Entomology as *Laspeyresia molesta* (Quaintance and Wood 1916). Heinrich (1926) included a long list in his synonymy under the name *Laspeyresia molesta* Busck, and was the first person to cite the use of *Grapholitha molesta* (Busck).

Within the tribe Grapholitini, three genus groups – *Dichrorampha*, *Cydia*, and *Grapholita* (Komai 1999) – are recognized. *Laspeyresia* is recorded in the Natural History Museum (London) index as a junior homonym of *Cydia* (Giusti 2004). Bradley (1972) listed *Laspeyresia* as a junior homonym of *Cydia*, and as a synonym to *Grapholita*. Brown (1979) treated the two

as separate genera. Leraut (1980) recorded *Laspeyresia* under *Cydia* (Hübner), and Razowski (1989) documented *molesta* under *Grapholita* (Treitschke). Razowski later treated *Grapholita* as a synonym for *Cydia* and recorded the Oriental Fruit moth as *Cydia molesta* in the tribe Grapholitini (Razowski 1996). Powell (1983) and Powell et al. (1995) placed the species authoritatively into *Grapholita*. Komai (1999) amended the genus with the inclusion of a subgenus *Aspila.*

Through the 1930's the Oriental fruit moth was commonly known in North America as the Oriental peach moth (Wood and Selkregg 1918, Peterson and Haeussler 1930, Schoene et al. 1937), and its recognized scientific name was *Laspeyresia molesta* Busck. In the 1940's, the species was referred to as the Oriental fruit moth with the Latin name, *Grapholitha molesta* (Busck) (note the *h* in the genus name) (Allen and Plasket 1958, Reichart and Bodor 1972). Meanwhile in Australia, it was called *Cydia molesta* Busck (Bailey 1979). In the late 20th and early 21st centuries, European literature continued to use the epithet *Cydia molesta* (Rothschild and Vickers 1991, Natale et al. 2004). The most recent name change in economic literature occurred in the early 2000's, where the Latin name (without the *h*) was recorded as *Grapholita molesta* (Myers et al. 2005).

Distribution and Host Plants

The Oriental fruit moth is thought to have originated in northwestern China (Rothschild and Vickers 1991). The species is a pest in peaches and apples in South Africa (Blomefield and Geertsema, 1990), Hungary (Reichart and Bodor 1972), Slovakia (Hrdy et al. 1993), Australia (Bailey 1980), and Canada (Dustan 1967), as well as the United States. It has been known as a pest of peaches and other fruit crops since its accidental introduction into North America in 1916

(Quaintance and Wood 1916). Thought to have been introduced from Japan in shipments of flowering cherries, peaches, and other fruits, the species was first detected in the District of Columbia (Wood and Selkregg 1918). In Asia, larvae were known to consume the fruits and twigs of cotoneaster (*Cotoneaster* sp.), cherry (*Prunus cerasus* (L.)), and plum (*Prunus domestica* [L.]) (Rothschild and Vickers 1991). In the United States, larvae have been reported to consume twigs of peach (*Amygdalus persica* [L.]), plum (*Prunus* spp.), and cherry (*Prunus* spp.) (Quaintance and Wood 1916). Additional host plants include quince (*Cydonia vulgaris* [Persoon]), almond (*Prunus amygdalaus* [Stokes]), apple (*Malus silvestris* [Miller]), apricot (*Prunus armeniaca* [L.]), Japanese plum (*Prunus japonica* [Thunberg]), and Chinese malus (*Cormus tschonoskii* [Koidz.]) (Schoene et al. 1937, Haeussler 1940, Reichart and Bodor 1972, Sziraki 1979, Rothschild and Vickers 1991).

Following introduction into the eastern United States, the Oriental fruit moth spread across the country in *ca.* 25 years. Snapp and Swingle (1929) state that it was first observed in the southern United States in the fall of 1923, most likely as a result of transportation of fruit in commerce. The species was detected in southern Illinois (Pulaski County) in early winter of 1927 and was thought to have arrived in peaches and young nursery stock (Flint and Chandler 1929). By 1942, Oriental fruit moth had spread to California, where the species caused major economic losses in peach orchards (Summers 1966).

Unmanaged (untreated) orchards sometimes exist near managed orchards and allow development and dispersal of adults throughout the season. Flint and Chandler (1929) reported that apples were infested only if they were interspersed among peach trees. Unsprayed apples have been implicated as important food sources for larvae in August and September after peaches are harvested (Allen and Plasket 1958). The presence of apples and peaches in close

proximity to each other, providing early- and late-season hosts, may favor successful establishment of Oriental fruit moth populations, but this trend does not always hold true (Steiner and Yetter 1933, Allen and Brunson 1943, Sziraki 1979).

Life History and Phenology

In temperate climates, the Oriental fruit moth overwinters in the pupal stage (Flint and Chandler 1929). Factors that induce diapause are temperature and photoperiod, and these abiotic variables are influential during larval development (Dickson 1949). When reared in constant darkness or constant light, very few Oriental fruit moth larvae enter diapause. In addition, few larvae enter diapause when they are raised at low temperatures; this may be an ecological advantage in areas where larvae feed throughout the winter (Dickson 1949).

Voltinism varies according to latitude. There are three to four generations of moths per year in Hungary (Reichart and Bodor 1972), and four to five generations in Japan (Haeussler 1940). In southeastern Canada, three to four generations are apparent per year (Dustan 1967). Four generations develop annually in New Jersey (Stearns and Peterson 1928) and Virginia (Stearns 1921). Five to six generations occur annually in Missouri (Sarai 1970) and South Carolina (Eddy et al. 1930), and six to seven generations develop each year in Georgia (Rothschild and Vickers 1991). Five or more generations develop annually in southern Illinois (Flint and Chandler 1929).

Moths emerge in the spring when peaches bloom. Adults from overwintered pupae emerge as early as mid-March in southern Illinois and begin to deposit eggs on peach buds and leaves. After hatching, larvae tunnel through green shoots until the fourth or fifth instar. Injury

to peach trees in several successive years can stunt growth (Peterson and Haeussler 1926). Larvae migrate to the tree trunk to spin cocoons. Subsequent generations of larvae tunnel into peach fruit, either from the side of the fruit or from the stem. Because stem entries are difficult for peach growers to detect, infested fruit may be sold to processors or consumers (Summers 1966).

Individual moths lay eggs either singularly or in small clutches for a period of 7 to 10 days (Peterson and Haeussler 1930, Smith and Summers 1948). Depending on temperature, developmental times are: eggs 4-8 days; larvae 12-22 days; and pupae 10-16 days. The developmental period from egg to adult averages 30-49 days (Peterson and Haeussler 1926, Peterson and Haeussler 1930, Summers 1966, Reichart and Bodor 1972). There are four or five instars depending on temperature. Roberts et al. (1978) noted that when reared in controlled temperatures, the fifth-instar developed only at 30ºC; they suggested that only four instars develop when larvae feed at temperatures between 15º and 24ºC. Fully grown larvae are 13-15 mm long and are whitish to pink. The head capsule is light brown with dark markings (Quaintance and Wood 1916). An anal fork is present below the anal plate and behind the anal prolegs (Wood and Selkregg 1918).

Peterson (1930) and Dustan and Armstrong (1932) first described the influence of temperature on the rate of development of *G. molesta*. Subsequent researchers estimated the lower developmental threshold to range from 4ºC (Chaudhry 1956) to 11ºC (Tanaka and Yabuki 1978), with an upper developmental threshold of 30ºC to 35ºC (Peterson and Haeussler 1930, Chaudhry 1956). Models estimate the total number of degree-days (dd) required for development (egg-to-egg) to range from 383 dd with a lower threshold of 11ºC (Tanaka and Yabuki 1978) to 535 dd with a lower threshold of 7.2Cº (Rothschild and Vickers 1991). Croft et

al. (1980) estimated dd requirements (converted to ºC) to be 79 for eggs, 213 for larvae, 211 for pupae, and 28 for adults before oviposition. The lower and upper thresholds used in this model were 7.2º (45ºF) and 32.2ºC (90ºF), respectively. Degree-day accumulations after the initial detection of moths in pheromone traps (i.e., the biofix) have been used since the mid-1980s to predict optimal timing for application of insecticides (Rice 1984).

Economic Impacts, Management, and Monitoring

Globally, the economic value of apples and peaches exceeds $730 million (FAS USDA 2008a, 2008b). In 2006-07, apple production reached 46.1 million tons, with 15% produced in the United States; 40,000 tons of apples valued at $39 million were exported from the United States (FAS USDA 2007). Production levels vary annually; the Central region of the United States produced 9 % of the national crop in 2008 and a greater portion in 2009 (Perez and Pollack 2009). In 2008, total apple production in the Central states was 910 million pounds and resulted in *ca*. $260 million in sales; Illinois produced 46 million pounds for *ca.* $21 million in sales (Perez and Pollack 2009). Globally, peach and nectarine production reached 1.4 million tons in 2006; peach production for California, Georgia, and South Carolina was 481,000 tons in 2008. In 2007, the reported value of peach exports by the United States was *ca.* $130 million (FAS USDA 2008b). Illinois produced *ca*. 7,500 tons of peaches in 2009 and ranked 10th in the United States (NASS USDA 2009).

Fruit infestations lead to reduction in grade as well as increased costs for sorting (Summers 1966). Fruit that is infested by Oriental fruit moth must be discarded. The Mid-Atlantic region developed an Apple Pest Management Strategic Plan that lists *G. molesta* as one

of the major pests of apple (Baniecki and Dabaan 2003), and New England's Apple Pest Management Strategic Plan estimated that their entire apple acreage was vulnerable to infestation by *G. molesta* (Gotlieb and Kingsley-Richards 2003).

Various methods have been used to control *G. molesta* and reduce its economic effect on the fruit industry. After Stearns (1920) noted that the first and second generation attacked terminal shoots, he did a series of twig experiments. Early clipping and destruction of infested twigs reduced infestation from 28 percent to < 1%. Field and laboratory experiments with lead arsenate, nicotine sulphate, and lime arsenate at different strengths were also tested (Stearns 1920, Eddy et al. 1930). In these experiments (Stearns 1920) determined nicotine sulphate was the best ovicide, and nicotine-arsenical combinations were most effective against early instars. In the 1930's, bait traps that contained a sugary substance (often molasses) in combination with essential oils such as anise were used to reduce fruit injury up to 80% (Yetter and Steiner 1931, 1932, Steiner and Yetter 1933).

Between 1928 and 1945, researchers and growers experimented with the importation and release of parasitoids to control Oriental fruit moth (Brunson and Allen 1944, Allen 1958). In the late 1950's, monitoring predators (field mice and lacewing larvae) and parasitoids (*Macrocentrus spp.* (Hymenoptera: Braconidae) and *Trichogramma minutum* Riley (Hymenoptera: Trichogrammatidae)) continued, but the use of inundative releases of parasitoids became almost obsolete (Allen 1958). DDT was approved for agricultural use in 1946, and beginning in the 1950's DDT, parathion, and EPN (O-ethyl O-4-nitrophenyl phenylphosphonothioate) effectively controlled Oriental fruit moth and their use was continued in conjunction with increased removal of debris (Allen 1958). Sprays of DDT or organophosphorous chemicals were timed to coincide with seasonal sprays to control other

orchard pests such as plum curculio, *Conotrachelus nenuphar* (Herbst) (Coleoptera: Curculionidae), and plant bugs (Heteroptera: Miridae) (Allen 1958). Beginning in the 1950's, organophosphates such as azinphosmethyl and phosmet were used widely for Oriental fruit moth control in North America and elsewhere (Allen 1958, Kanga et al. 2003). The use of pyrethroid insecticides for Oriental fruit moth control began in the 1980's and continues to the present (US EPA 2009). Several reduced-risk insecticides are now labeled for Oriental fruit moth control (Midwest Fruit Workers Group 2010). Mating disruption has been used successfully in North Carolina and Pennsylvania apple orchards (Hull et al. 2001, Kovanci et al. 2004), where sprayable pheromones as well as hand-applied dispensers have been more effective than conventional insecticide sprays.

Early approaches to monitoring Oriental fruit moth population levels focused on estimating numbers of larvae in immature fruits and twigs, but this approach did not provide information before control measures were needed (Allen and Plaskett 1958). Pheromone traps improved monitoring. In general, pheromone traps are baited with lures that attract males by using synthetic compounds that mimic the female sex pheromone. The primary component of the mating pheromone of the Oriental fruit moth was identified as cis-8-dodecenyl acetate by Roelofs et al. (1969); further analysis by Cardé et al. (1979) revealed four components – (Z)-8-dodecenyl acetate, (E)-8-dodecenyl acetate, (Z)-8-dodecen-1-ol, and dodecanol – in distinct ratios. Pheromone traps have been used successfully for timing insecticide applications (Phillips 1973), determining distribution (Hrdy et al. 1993), and monitoring *G. molesta* within apple and peach orchards (Baker et al. 1980, Rothschild et al. 1984, Kovanci and Walgenbach 2005).

Insecticides and Modes of Action

Synthetic insecticides are widely used to control Oriental fruit moth in apples and peaches. Insecticides interfere with a variety of processes, including the function of acetylcholine receptors, gamma-aminobutyric acid (GABA)-gated chloride channels, sodium channels, mitochondrial respiration, and chitin synthesis (Yu 2008). The Insecticide Resistance Action Committee currently categorizes available insecticides into over 25 distinct modes of action (IRAC 2010). Modes of action of key groups of insecticides that are or have been used for Oriental fruit moth control are summarized below.

Organochlorines were the first class of modern (synthetic) insecticides. This group includes dichlorodiphenyltrichloroethane (DDT). The use of DDT in the United States was banned in the early 1970's because of its persistence in the environment, but derivatives of DDT such as methoxychlor and dicofol remained in use until recently (Yu 2008). Most organochlorines bind to sodium channels, preventing their closure and leading to repetitive discharge of action potentials (Dresden 1949). Excessive neuroexcitation results in hyperactivity, tremors, and death (Matsumura 1985).

Pyrethrins and pyrethroids also are sodium channel modulators (Matsumura 1985, Khambay and Jewess 2005). Pyrethrins are the insecticidal compounds produced in the flowers of the pyrethrum daisy, *Chrysanthemum cinerariaefolium* (Trevisano); their active ingredients are four esters (pyrethrins I and II and cinerins I and II). Pyrethroids – synthetic pyrethrin-like compounds – generally contain chlorine or other halogens substituted for isobutenyl methyl groups or at other sites to create additional compounds (Coats 1990). Whereas natural pyrethrins are highly unstable in sunlight, currently available pyrethroids are much more photostable and

more effective as agricultural insecticides (Corbett et al. 1984, Matsumura 1985, Scharf 2003, Khambay and Jewess 2005).

Organophosphates and carbamates bind to acetylcholinesterase and prevent the enzyme from hydrolyzing the neurotransmitter acetylcholine, thus causing excessive neuroexcitation (Eldefrawi 1985, Matsumura 1985). Organophosphates are derived from phosphoric acid (Yu 2008); carbamates are esters of carbamic acid (Ecobichon 2001). Many organophosphates and carbamates were developed in the 1960's and 1970's, but few remain in widespread use on food crops in the United States after restrictions imposed by the Food Quality Protection Act of 1996.

Neonicotinoids are analogs of nicotine and are relatively low in toxicity to humans (US EPA 2003a, 2003b, 2003c, 2003d). Nicotine, neonicotinoids, and spinosyns mimic acetylcholine (i.e. are agonists) and activate the nicotinic acetylcholine receptor, causing an influx of sodium ions to flood the receptor. Acetylcholinesterase does not hydrolyze these insecticides, and overstimulation of receptors occurs (Eldefrawi and Eldefrawi 1990).

The spinosyns include spinosad and spinetoram. Spinosad is produced in fermentation culture by the soil actinomycete *Saccharopolyspora spinosa* Mertz & Yao (Mertz and Yao 1990), and spinetoram is a synthetic optimization of spinosad (Sparks et al. 2008). They act on nicotinic receptors and GABA-gated chloride ion channels (Sparks et al. 2001).

Benzoylphenylureas (or benzoylureas) are insect growth regulators that inhibit chitin synthesis (van Daalen et al. 1972, Mulder and Gijawijt 1973, Ishaaya and Casida (1974). Most act by ingestion (Ishaaya 1990), but a novel benzoylphenyl urea, novaluron, acts by contact and ingestion (Ishaaya et al. 1996).

Anthranilic diamides, including flubendiamide and chlorantraniliprole, activate a ryanodine receptor, releasing stored calcium and causing impaired muscle contractions (Cordova

et al. 2006, Ebbinghaus-Kintscher et al. 2007). They are effective in controlling Lepidopteran pests on a variety of crops (Yu 2008).

Insecticide Resistance, Resistance Monitoring, and Resistance Management

Insecticide resistance is a genetically based shift in population response to insecticides (Georghiou and Taylor 1977). Resistance may result from a change in frequencies of a single gene or multiple genes. The alternative alleles may be dominant, recessive (i.e. DDT, spinosyns), incompletely dominant, or incompletely recessive (i.e. pyrethroids) (Stone 1968). More than 500 arthropod species are resistant to one or insecticides or acaricides (Whalon et al. 2008).

Identifying and Quantifying Resistance

The responses of populations to insecticides are most often described by dose-mortality regressions derived from binary bioassays that measure mortality (or another key response) at each of a range of doses (Staetz 1985, Robertson et al. 2007). A logarhythmic transformation of doses is usually used for such analyses because it produces a more uniform increase in response (Bliss 1934, Finney 1971). However, plotting mortality as a percentage on the *y*-axis against log-dose on the *x*-axis generally results in a curvilinear relationship that remains difficult to model accurately (Bliss 1934). Dose-mortality models can be improved by transforming observations of mortality using a probit or logit function, where probit models are more accurate for data that are distributed normally, and logit models provide a better fit if the data are

described by a logistic distribution (Finney 1971). Probits are units of cumulative probability derived from a normal probability curve, with the addition of 5 to the standard deviation from the mean to remove negative numbers (Bliss 1934). Probit or logit models can be used to estimate the slope of dose-response lines and the doses that kill specific portions of the test population (for example the LD_{50} and LD_{90} for 50% and 90% mortality) (Finney 1971, Robertson et al. 2007).

Probit and logit models of data from binary bioassays often yield similar LC_{50} estimates (Savin et al. 1977). To choose the more accurate model, goodness of fit can be estimated for each model by a χ^2 test that compares expected and observed values. If the resulting χ^2 exceeds the critical value for χ^2 at the designated level of probability and the relevant number of degrees of freedom, it indicates significant lack of fit of the dose-mortality model (Robertson et al. 2007). In such instances, a plot of the residuals (the differences between observed and expected values) may reveal outliers, observed values that differ from expected values by more than two standard deviations of the mean. If outliers are not the results of errors in data entry, variability may account for lack of fit (Robertson et al. 2007).

To compare the responses of two populations to the same insecticide or to assess changes in responses over time, LD_{50}'s or LD_{90}'s are compared. Initial methods of identifying the significance of observed differences in paired estimates of LD_{50}'s or LD_{90}'s used the 95% confidence limits for these estimates. If the confidence limits overlapped, the differences were not considered to be significant at P=0.05 (Schenker and Gentleman 2001). This methodology actually imposes a much lower probability of a Type I error than 0.05 and fails to detect differences that are significant at P=0.05 (Wheeler et al. 2005). A more accurate method to determine if differences in two LD_{50}'s (or other lethal dose levels) are significant is to calculate

the lethal dose ratio for the pairing (for example, LD_{50} of insecticide A for population 1 ÷ LD_{50} of insecticide A for population 2) and its confidence interval. If the confidence interval for the ratio does not include 1.0, the two LD estimates are significantly different (Wheeler et al. 2005, Robertson et al. 2007). Where LD_{50}'s or LD_{90}'s for a putatively resistant population are significantly greater than the LD_{50}'s or LD_{90}'s for a susceptible population, the difference may result from natural variation among populations or from evolution in response to selection by insecticide use (Robertson et al. 1995). Multiple estimates of baseline responses of susceptible populations before selection by insecticide use aid in understanding the range of natural variation to specific insecticides.

Mechanisms of Insecticide Resistance

In general, four broad mechanisms of insecticide resistance are recognized – altered behavior, reduced penetration, enhanced metabolism, and target site insensitivity (Mallet 1989, Georghiou 1994). Behavioral resistance typically results from a hypersensitivity or hyperirritability to a toxicant (Yu 2008). Examples in Lepidopterans include carbaryl-resistant fall armyworms (*Spodoptera frugiperda* [J. E. Smith] [Lepidoptera: Noctuidae]) that avoid insecticide-treated leaf surfaces (Young and McMillian 1979), and pyrethroid resistance in diamondback moths (*Plutella xylos*tella [L.] [Lepidoptera: Plutellidae]) that detect the insecticide through their tarsi (Moore et al. 1989).

Resistance by reduced penetration of the cuticle is a common mechanism that offers little resistance by itself but which offers enhanced resistance to some insecticides when present in conjunction with other mechanisms (Forgash et al. 1962, Plapp 1986). Patil and Guthrie (1979)

reported decreased uptake of organophosphates, organochlorines, and carbamates by houseflies with higher levels of lipids, fatty acids, sterols, and other organic chemicals in their cuticles. Terriere (1982) attributed this resistance mechanism to degratory enzymes in the cuticle or a thicker, impermeable cuticle. Reduced penetration is a factor in resistance to pyrethroids in *Helicoverpa armigera* (Lepidoptera: Noctuidae) (Ahmad et al. 2006).

The rate of metabolic detoxification within insects influences tolerance to insecticides. Variants of several enzymes can increase rates of detoxification. Primary (Phase 1) processes include oxidation, hydrolysis, and reduction, and metabolites are either excreted or reduced further in secondary reactions. Secondary (Phase 2) processes involve conjugation reactions that attach and remove chemical groups from insecticides before excretion. Oxidation reactions are carried out by cytochrome-P450s that convert toxins such as DDT and cyclodienes (Esaac and Matsumura 1980), pyrethroids (Tomita et al. 1995, Scharf et al. 1998), and organophosphates (Sabourault et al. 2001) into (generally) less toxic and less stable metabolites. Over-expression of cytochrome-P450 monooxygenases may be an adaptation against secondary chemicals produced by plants (i.e., alkaloids, furanocoumarins) (Feyereisen 1999). In many insects including fall armyworm,, monooxygenases also confer resistance to organophosphates, carbamates, pyrethroids, neonicotinoids, and other classes of insecticides (Yu 1991). Insecticides with ester bonds, including organophosphates, carbamates, and pyrethroids, and juvenoids with ester linkages are detoxified by hydrolysis (Matsumura 1985). Hydrolases cleave carboxylester and phosphorotriester bonds and are involved in resistance to organophosphates and pyrethroid insecticides (Dauterman 1983, 1985).

Three types of reduction reactions – nitro reduction, reductive dechlorination, and aldehyde or ketone reduction – are known to metabolize insecticides. In reductive

dechlorination, DDT is metabolized to form TDE, with methoxy groups replacing p-chloro groups, making them more easily demethylated by cytochrome P450 monooxygenases (Peterson and Robison 1964, Matsumura 1985). Aldehydes and ketones reduce to alcohols (Williams 1959). Amplified aldehyde oxidase is thought to be responsible for resistance in *Culex quinquefasciatus* Say (Diptera: Culicidae) (Coleman et al. 2002). Phase 2 processes include but are not limited to glucose, sulfate, phosphate, amino acid, and glutathione conjugation. Sulfotransferase has been found in the guts of larvae of southern armyworm, *Spodoptera eridania* (Cramer) (Lepidoptera: Noctuidae), tobacco hornworm, *Manduca sexta* (L.) (Lepidoptera: Sphingidae), and monarch butterfly, *Danaus plexippus* L. (Lepidoptera: Nymphalidae), after exposure to phenol compounds (Yang and Wilkinson 1973). Glutathione conjugation is performed by glutathione *S*-transferases that make insecticides more polar and soluble in water for excretion (Matsumura 1985).

Three mechanisms are known to reduce the sensitivity of target sites to insecticides. Reduced sensitivity of neurons makes neural excitation more difficult (Matsumura and Hayashi 1969). Altered forms of acetylcholinesterase are not as vulnerable to inhibition by organophosphates (Smissaert 1964) or carbamates (Yamamoto et al. 1983). Reduced binding property at the target site can decrease attachment of toxins (Matsumura and Hayashi 1969). Altered binding sites in the midguts of tobacco budworm, *Heliothis virescens* (F.) (Lepidoptera: Noctuidae) and pink bollworm (*Pectinophora gossypiella* [Saunders] [Lepidoptera: Gelechiidae]), are responsible for resistance to *Bacillus thuringiensis* toxins (Gahan et al. 2001, Morin et al. 2003).

Insecticide Resistance in Oriental Fruit Moth

By crossing field-caught Oriental fruit moths from the Niagara Peninsula of Ontario with moths from established laboratory colonies, Pree et al. (1998) documented resistance to 14 insecticides, 10 of which were organophosphates. Based on bioassays of P and F_1 moths, they surmised that resistance to organophosphates was conferred by a single recessive gene. The major mechanisms of resistance to organophosphorus and carbamate insecticides in *G. molesta* include enhanced detoxification by E_1 esterase, decreased sensitivity of the target site to acetylcholinesterase inhibition (in adults as well as larvae), and reduced cuticular penetration of carbofuran (Kanga et al. 1997).

Resistance Monitoring

Monitoring the presence and prevalence of resistant individuals in a population is a key step in insecticide resistance management (Roush and Miller 1986). Roush and Miller (1986) indicated that the goals for resistance monitoring programs include detecting resistance before control failures, estimating the frequency of resistant individuals, making field-level choices of insecticides, and monitoring changes in resistance frequency. They noted that, when frequencies of resistant individuals are low, typical binary bioassays do not detect small changes in slopes or lethal concentrations efficiently. An alternative to using a full binary bioassay to detect resistance is the use of a single discriminating or diagnostic dose (Brown and Pal 1971, Roush and Miller 1986, Halliday and Burnham 1990, Usmani and Shearer 2001). Halliday and Burnham (1990) noted that the term discriminating dose is used when genetic and toxicological

tests reveal differences in response by genotypes, whereas the term diagnostic dose is used to monitor changes in phenotypic response. When a diagnostic dose is administered to a sufficiently large sample of insects, survivorship that significantly exceeds the expected level (often 1 percent, but determined by the selection of the diagnostic dose) is considered possible evidence of resistance (WHO 1976). Even though the use of a diagnostic dose increases the efficiency of monitoring efforts designed to detect resistance at an early stage, thousands of insects must be tested to detect resistance at phenotypic frequencies of 0.001 or less (Roush and Miller 1986).

The life stages tested in monitoring programs and the methods of admistering insecticides in bioassays vary. Glass vials coated with insecticide may be used to assess the toxicity of residual contact insecticides to adult moths (Kanga and Plapp 1995, Kanga et al. 1995, 1997, 2003). For bioassays of larvae, insecticides may be applied to the surface of artificial diets (Mascarenhas et al. 1998, Bouvier et al. 2002, Shearer et al. 2007) or incorporated into the diet (Borchert et al. 2005). Topical bioassays with adults or larvae can be done with Potter-spray towers (Pree et al. 1998) or with a repeating micropipette or syringe (Staetz 1985, Dunley and Welter, 2000, Usmani and Shearer 2001). Where ovicidal activity is the focus of bioassays, eggs laid on plant tissue or artificial surfaces may be dipped into water containing an insecticide or miticide (Brunner et al. 2005). Leaf disks or leaf dip bioassays are most often used for testing contact pesticides for mites (Bergh et al. 1999) and insect larvae (Zhao et al. 2006).

Resistance monitoring efforts have focused on Oriental fruit moth adults and larvae to develop probit models of dose-response relationships (Kanga et al. 1997, Usmani and Shearer 2001). Diagnostic doses have been used to quantify resistance in field populations of Oriental

fruit moth and related Lepidopteran pests (Bush et al. 1993, Varela et al. 1993, Kanga et al. 1995, 2003, Pasquier and Charmillot 2003, Soleno et al. 2008).

Resistance Management

Georghiou (1994) proposed three broad categories of practices for managing (preventing or delaying) the evolution of resistance in arthropods – moderation, saturation, and multiple attack. **Moderation** emphasizes the importance of the continued presence of susceptible individuals in a population and includes the use of low insecticide rates, infrequent application, and the use of refugia. Killing all susceptible homozygotes and heterozygotes by use of high rates and frequent applications of insecticides is the goal of **saturation**. A **multiple attack** approach uses a combination of stressors on the insect and includes the use of mixtures and insecticides used in rotation.

Tabashnik and Croft (1985) found that, if dose and frequency of application are reduced, the development of resistance can be delayed in European red mites (*Panonychus ulmi* (Koch). Acari: Tetranychidae). Selection of pesticides that are less persistent slows resistance by reducing the duration of selection pressure (Georghiou 1980).

The use of mixtures and rotations of insecticides with different modes of action to delay the evolution of resistance depends on several factors including the independent and uncorrelated action of the components. Mixtures and rotations are most likely to slow the development of resistance if the modes of action of the chemicals differ and they are not subject to detoxification by the same enzymes and metabolic pathways (Georghiou 1994). If the toxicity of two components in a mixture is independent, their combined action is described by the equation:

$$p = p_0 + (1 - p_0)p_1 + (1 - p_0)(1 - p_1)p_2$$

where p = the probability of mortality at a given dose or concentration of a 2-component mixture; p_0 = natural mortality; p_1 = the probability of mortality caused by component 1; and p_2 = the probability of mortality caused by component 2 (Robertson et al. 2007). For p_1 and p_2, the probability of mortality is a function of dose or concentration. The joint action of chemicals in mixtures can be assessed by analyzing binary bioassays of the individual components and their mixtures (Robertson et al. 2007).

Rationale and Objectives

Insecticides are used widely in the management of Oriental fruit moth and other arthropod pests of apples and peaches. Pyrethroids and several newer insecticides are now used instead of organophosphates. Examples of newer insecticides tested in my research include reduced-risk insecticides (*e.g.*, acetamiprid, chlorantraniliprole, and spinetoram), an insecticide approved for use in organic production (*e.g.*, spinosad), and a chitin inhibitor (*e.g.*, novaluron). These insecticides have diverse modes of action for use in rotations for insecticide resistance management. Even so, multiple applications of insecticides each season in apples and peaches may result in high selection pressure. Ongoing resistance management practices will be necessary to maintain their effectiveness. To support such efforts, the research presented in this dissertation was designed to meet the following objectives:

1. Describe the susceptibility of *G. molesta* larvae to acetamiprid, chlorantraniliprole, spinetoram, and spinosad. Such baseline information can be used for comparisons in future resistance monitoring efforts.
2. Describe the susceptibility of *G. molesta* larvae to the pyrethroids esfenvalerate and lambda-cyhalothrin and develop a diagnostic dose of esfenvalerate for field detection of resistance using adults.
3. Describe the joint action of mixtures of chlorantraniliprole with acetamiprid, esfenvalerate, or thiamethoxam. Understanding whether interactions of these mixture components are antagonistic, additive, or synergistic will contribute to their optimal use in rotations for resistance management and in mixtures for broad-spectrum pest management.
4. Describe the susceptibility of *G. molesta* eggs to novaluron. As in Objective 1, such baseline information can be used for comparisons in future resistance monitoring efforts.

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CHAPTER TWO: SUSCEPTIBILITY OF ORIENTAL FRUIT MOTH (LEPIDOPTERA: TORTRICIDAE) LARVAE TO SELECTED REDUCED-RISK INSECTICIDES

ABSTRACT: To determine their baseline susceptibility to chlorantraniliprole, spinetoram, spinosad, and acetamiprid, Oriental fruit moth (*Grapholita molesta* [Busck]) neonates were placed on diet containing a range of concentrations of each insecticide. Mortality was assessed after 96 h. Two populations – a long-term laboratory colony from Rutgers University and a colony established in 2007 from a southwestern Illinois (Calhoun County) field population – were tested. We compared the responses of Calhoun colony neonates from parents reared on 'Gala' apples with those of neonates from parents reared on lima bean diet. We also compared the responses of Calhoun colony neonates with those of Rutgers colony neonates (all from parents reared on apples). LC_{50}'s (ppm in diet) for Calhoun colony progeny of adults reared on apples were 0.08, 0.06, 0.41, and 0.30 for chlorantraniliprole, spinetoram, acetamiprid, and spinosad, respectively. Parental food source did not consistently influence the concentration-mortality relationships. Based on LC_{50}'s and toxicity ratio tests, Calhoun colony neonates were slightly but significantly less susceptible to spinetoram and acetamiprid than were Rutgers colony neonates. Similarly, LC_{90}'s and toxicity ratio tests indicated that Calhoun colony neonates were slightly but significantly less susceptible to chlorantraniliprole. However, toxicity ratios (Calhoun/Rutgers) were low in all instances, and the highest ratio was 1.73 at LC_{90} for chlorantraniliprole. Overall, the two colonies responded similarly to these insecticides. Results reported here provide baseline data for future monitoring of resistance development.

Key Words: bioassays, insecticide resistance, spinetoram, chlorantraniliprole, spinosad, acetamiprid, *Grapholita molesta*

ORIENTAL FRUIT MOTH, *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae), has been a serious pest of peaches, apples, and other fruit crops since its introduction into North America in the early 1900's (Quaintance and Wood 1916, Wood and Selkregg 1918). In the late 1990's, infestations in apples generally increased in the eastern US (Baniecki and Dabaan 2003, Shearer et al. 2007); severe infestations were observed in Illinois apples in 2005 (R.A.W., unpublished data).

Increased prevalence of Oriental fruit moth in apple orchards since the late 1990's may have resulted from changes in management practices for codling moth, *Cydia pomonella* L. (Lepidoptera: Tortricidae), or from insecticide resistance. Use of the organophosphorous insecticides methyl parathion and chlorpyrifos, which was common until 2000, has been prohibited by the United States Environmental Protection Agency. Also, use of the organophosphates azinphosmethyl and phosmet has declined because of their ineffectiveness against resistant populations of codling moth. Timing of applications of newer insecticides to control codling moth in apples may not provide coincidental control of Oriental fruit moth. In addition, Oriental fruit moth populations resistant to one or more organophosphates, carbamates, or pyrethroids have been detected in apple orchards in New Jersey and Ontario (Kanga et al. 1997, Usmani and Shearer 2001, Kanga et al. 2003). Widespread failures of pyrethroids, primarily permethrin, to control Oriental fruit moth in peach orchards were observed in Calhoun County, IL, in 2004 and 2005 (R. A.W., unpublished data). Where control in peach orchards has been compromised by insecticide resistance, increased populations in those orchards are likely to

spread to nearby apple orchards in late season (Allen and Brunson 1943). In some instances, dispersal of resistant individuals may explain the greater prevalence of Oriental fruit moth in apple orchards during the last 10-15 years.

Reduced-risk insecticides are now recommended for use in fruit orchards (Midwest Fruit Workers Group 2009). These insecticides are generally more selective and cause less harm to non-target organisms. Although the effectiveness of some reduced-risk chemicals against Oriental fruit moth has been confirmed in field trials, dose- or concentration-response relationships have not been studied in detail in the laboratory except in a preliminary investigation by Shearer et al. (2007). We chose to estimate concentration-mortality relationships for four of these insecticides – an anthranilic diamide (chlorantraniliprole), two spinosyns (spinetoram and spinosad), and a neonicotinoid (acetamiprid). Anthranilic diamides activate a ryanodine receptor that releases stored calcium and causes impaired muscle contractions (Cordova et al. 2006). Spinosyns target the nicotinic receptor and GABA-gated chloride ion channels (Sparks et al. 2001). Spinosad is produced in fermentation culture by the soil actinomycete *Saccharopolyspora spinosa* Mertz & Yao (Mertz and Yao 1990), and spinetoram is a synthetic optimization of spinosad (Sparks et al. 2008). Neonicotinoids such as acetamiprid mimic acetylcholine; they activate the nicotinic acetylcholine receptor, causing an influx of sodium ions to flood the receptor (Tomizawa and Casida 2005). Acetylcholinesterase does not hydrolyze the neonicotinoids, and overstimulation of receptors results (Eldefrawi and Eldefrawi 1990).

Baseline data from susceptible populations are a prerequisite for understanding the development of resistance to insecticides in the field. Because resistance is a genetically-based shift in population response, resistance monitoring is aided by the initial quantification of

responses to toxins by susceptible populations (Robertson et al. 2007). To determine the baseline susceptibility of *G. molesta* to these reduced-risk insecticides, we estimated the statistical parameters of concentration-response relationships observed in binary bioassays (Robertson et al. 2007). We tested neonates because Oriental fruit moth larvae damage shoots and fruits and are the primary targets of the insecticides that we selected (Midwest Fruit Workers Group 2009). We examined responses of two Oriental fruit moth colonies, one that had been in long-term laboratory culture and another established from a field population in Calhoun County, IL in 2007. For each insecticide, we tested the null hypothesis that the responses of the two colonies would be the same. For the Calhoun colony, we also compared responses of insects from parents reared on 'Gala' apples to insects from parents reared on lima bean diet.

Materials and Methods

Laboratory Colonies. Two laboratory colonies, designated "Rutgers" and "Calhoun," were maintained. The Rutgers colony, acquired from Rutgers University, New Brunswick, NJ, in 2007 was originally established in a USDA laboratory near Fresno, CA, *ca.* 40 years ago and has been reared since 1995 at Rutgers. The Calhoun colony was established with larvae from untreated peach fruits and shoots from orchards in Calhoun County in southwestern Illinois from May through August, 2007. After a late frost in April 2007 that resulted in a severe crop loss, orchards used as collection sites were left untreated through most of the season, allowing heavy infestation of shoots and remaining fruit. At the time the colony was established, peach orchards in this area had never been treated with any of the insecticides tested in our bioassays. Only acetamiprid, which was used in 2006 in apple orchards *ca.* 10 meters from one of the 2007

collection sites, had been used by apple growers before the laboratory colony was established. Infested peach shoots and fruit were brought to the laboratory and placed into plastic trays with corrugated cardboard strips that served as pupation sites. Trays were covered with ventilated lids and held under constant light (to prevent larvae from entering diapause) and ambient temperature.

Rearing methods similar to those of Yokoyama et al. (1987), Vetter et al. (1989), and Pree (1985) were used to maintain the laboratory colonies. Each colony was split for rearing concurrently on 'Gala' apples and on lima bean diet (Yokoyama et al. 1987) to reduce the likelihood of colony loss if one of the rearing methods failed. We did not permanently separate populations reared on apples from those reared on diet for either colony. Instead, when numbers of insects reared on the lima bean diet decreased, eggs from moths reared on apples were placed on diet to maintain colony vigor. Likewise, eggs from moths reared on diet were placed on apples as needed. Consequently, larvae used in bioassays were identified by colony (Rutgers versus Calhoun) and by parental food source (apple versus lima bean diet).

Moths were held for oviposition in cages (30.5 x 30.5 x 30.5 cm and lined on five sides with black cardstock) and provisioned with de-ionized water and 10% sucrose in 110 ml jars packed with cotton balls. One 'Gala' apple was added to each cage to provide volatiles to promote oviposition (Peter Shearer, Rutgers University, personal communication). Eggs were collected twice weekly on a continuously fed roll of wax paper (30.5 cm wide) stretched across the inside of the lighted face of the cage. For rearing on apples, wax paper sheets with eggs were placed into upright plastic containers (*ca.* 9.5 x 16.5 x 24 cm; 3785 ml) with ventilated lids. Five to six apples were placed into each container to feed larvae. Cardboard strips were provided for pupation. These containers were held in constant light at 26-27°C. After larvae had pupated

inside the cardboard strips (*ca.* 3 weeks), strips containing pupae were removed and placed into emergence cages (30.5 x 30.5 x 30.5 cm screened cage) and held at 26.8±2 ºC, 60% RH, and a photoperiod of 16:8 (L:D). Emergence cages also contained a 10% sucrose solution in a small jar with a cotton wick. After eclosion, adults were collected in aspirators and transferred to oviposition cages.

For rearing larvae on lima bean diet, the hot diet mixture was poured to a depth of *ca*. 2.5 cm in 23 x 33 cm glass baking dishes with ventilated lids where it cooled and solidified. Sheets of eggs from oviposition cages were cut into 3 strips (each *ca.*10 x 30 cm) and placed above the surface of the lima bean diet; the sheets were supported by paper clips to provide an air space between the wax paper and the surface of the diet. After 10 d, the lima bean diet with larvae was transferred to upright plastic jars (*ca.* 14 x 14 x 19.5 cm, 3785 ml) with ventilated lids. These jars were wrapped with black cardstock so that larvae would crawl to the top (toward light) to pupate. Pupation sites were provided by coiling 2.5-cm wide strips of corrugated cardboard to produce a 7.5-cm diam. plug that was suspended in the mouth of the jars beneath the ventilated lid. These cardboard plugs were replaced twice weekly and placed into oviposition cages. Egg-to-egg generation time was *ca*. 30 days on lima bean diet or apples.

Insecticide Bioassays. Commercial formulations of chlorantraniliprole (Altacor 35WG, DuPont Agricultural Products, Wilmington, DE), spinetoram (Delegate 25WG, Dow AgroSciences, Indianapolis, IN), acetamiprid (Assail 30SG, United Phosphorus, King of Prussia, PA), and spinosad (Entrust 80WP, Dow AgroSciences, Indianapolis, IN) were diluted in de-ionized water for incorporation into diet using methods similar to those of Sial et al. (2010). For each concentration of each product tested, we prepared 150 g of a wheat germ-based diet (*Heliothis* premix, Item # 38 V 0600, Ward's Natural Science, Rochester, NY) by mixing 1 part

diet per 3 parts water-plus-insecticide to yield the final concentrations listed in Table 1. Diet was mixed without heating in 500-ml beakers, transferred into 150-ml diam. Petri dishes, and scored to produce diet blocks *ca.*1 cm^3 in size. Diet cubes were placed into 30-ml plastic diet cups (Bio-Serv, Frenchtown, NJ), and a single larva that had hatched within the previous 24 hours was added to each diet cup. These cups were held in environmental chambers at 26.8±2 ºC, 60% RH, and a photoperiod of 16:8 (L:D). Mortality was evaluated after 96 h. Larvae were recorded as dead if they were unresponsive to prodding with a blunt probe.

Calhoun colony larvae used in bioassays had been in laboratory culture for 2 to 20 generations during the time bioassays were done. Bioassays of chlorantraniliprole were done with generations 2 to 10; bioassays of spinetoram, acetamiprid, and spinosad were done with generations 10 to 11, 12 to 16, and 18 to 20, respectively. Bioassays of each insecticide were completed over roughly the same time period for both colonies (Oct. 2007-June 2008 for chlorantraniliprole, June-July 2008 for spinetoram, August-December 2008 for acetamiprid, and February-April 2009 for spinosad).

Statistical Analyses. Although neonates used in bioassays had not fed before they were placed on the treated diet cubes, their responses to insecticides may have differed according to parental food source. Consequently, although we did not expect parental food to influence the results of bioassays, we separated the Calhoun colony trials and compared responses of progeny of insects reared on apples with those of progeny of insects reared on lima bean diet. We also compared responses of neonates from the Calhoun and Rutgers colonies (all from parents reared on apples) (Tables 2 and 3).

Concentration-response (mortality) relationships were estimated assuming the probit or logit models with PoloPlus (LeOra Software, Petaluma, CA) described by Robertson et al.

(2007). If the probit model did not fit, logit analysis was used. Plots of standardized residuals were examined for outliers (Robertson et al. 2007). Slopes, LC_{50}'s, and LC_{90}'s were estimated for each bioassay. To determine if paired LC_{50}'s or LC_{90}'s differed from each other, PoloPlus was used to calculate lethal concentration ratios (progeny of parents reared on apples/progeny of parents reared on lima bean diet and Calhoun colony/Rutgers colony) and 95% confidence intervals for those ratios. The LC's were considered to be significantly different if the 95% confidence interval for the lethal concentration ratio did not include 1.0 (Robertson et al. 2007). We chose this method of hypothesis testing because the lethal concentration ratio test is more powerful for distinguishing differences between populations than using the confidence limits around each LC and concluding that differences are significant if there is no overlap (Wheeler et al. 2005).

Results and Discussion

For all insecticides tested, the probit model fit the concentration-response data for Calhoun colony neonates from parents reared on apples or lima bean diet (Table 2). Confidence intervals for all the LC_{50} ratios in Table 2 included 1.0, indicating that the corresponding LC_{50}'s did not differ significantly based on parental food source. For chlorantraniliprole, the confidence interval for the LC_{90} ratio (1.83) did not include 1.0; this indicated that the LC_{90}'s differed significantly ($P = 0.013$) based on parental diet. Overall, however, parental food source did not appear to influence the responsesof neonates to these insecticides.

For the Rutgers colony response to chlorantraniliprole and spinetoram, probit models did not fit the data and a logit model was used (Table 3). To allow comparison of the Rutgers and

Calhoun colony responses to these two insecticides, the logit model also was used to describe the response of the Calhoun colony. LC_{50} and LC_{90} estimates for the Calhoun colony based on the logit model (Table 3) differed little from those based on the probit model (Table 2). The probit model adequately described larval responses of both colonies to acetamiprid and spinosad (Table 3).

The LC_{50} ratio tests (Calhoun/Rutgers) for spinetoram and acetamiprid indicated that the Calhoun colony was slightly and significantly more tolerant to these insecticides than the Rutgers colony, but the greatest ratio was 1.41 for spinetoram. The LC_{90} ratio tests indicated that larvae from the Calhoun County were significantly more tolerant to chlorantraniliprole, spinetoram, and acetamiprid. Again, the highest ratio was relatively low at 1.73 for chlorantraniliprole. Overall, we observed little difference in the responses of the Calhoun colony versus the Rutgers colony to these four insecticides. The minor differences that we observed most likely resulted from natural variation among populations (Robertson et al. 1995). It is possible, however, that the slightly greater LC_{50}'s and LC_{90}'s recorded for the Calhoun colony resulted from selection exerted on the population in the field by other insecticides before we established the colony. For example, organophosphate-selected cross-resistance to spinosad has been shown in two leafrollers (Dunley et al. 2006). Conversely, Mota-Sanchez et al. (2008) found no evidence of organophosphate-selected cross-resistance to acetamiprid or spinosad in populations of the codling moth. If any of the insecticides (organophosphates, carbamates, and pyrethroids) used in Calhoun County orchards selected for traits that also increased Oriental fruit moth survival in the presence of the reduced-risk insecticides that we tested, there was little evidence of that selection at the time we completed these bioassays.

Shearer et al. (2007) estimated concentration-mortality relationships for acetamiprid and spinosad (and other insecticides) by using surface-treated diet and assessing Oriental fruit moth survival from neonate placement on diet to adult emergence. They found that spinosad was *ca.* 4 times more toxic than acetamiprid at LC_{50} and LC_{90}. Although these assessments used the same Rutgers colony from which we established ours, we did not see the same relationship in our 96-h bioassays of neonates. LC_{50}'s for our Rutgers neonates (from parents reared on apples) were nearly identical at 0.30 and 0.28 ppm for acetamiprid and spinosad, respectively (Table 3). Other comparisons with the work of Shearer et al. (2007) are difficult because their bioassays used surface applications of insecticides to diet instead of incorporation and they assessed mortality after 7 days or through adult emergence.

These results provide baseline data on the susceptibility of Oriental fruit moth to chlorantraniliprole, spinetoram, acetamiprid, and spinosad and will allow comparisons with future estimates of concentration-response relationships. Results from both colonies can be considered to represent the responses of susceptible populations. Continued monitoring of Oriental fruit moth response to these reduced-risk insecticides will be essential to detect and manage resistance.

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Tables

Table 1. Concentrations of insecticides in wheat germ diet for neonate Oriental fruit moth bioassays.

Insecticide	Concentrations in final diet (ppm)
chlorantraniliprole	0.0, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 10.0
spinetoram	0.0, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 10.0
acetamiprid	0.0, 0.1, 0.2, 0.3, 0.6, 1.0, 3.0
spinosad	0.0, 0.1, 0.2, 0.3, 0.6, 1.0, 3.0

Table 2. Concentration-mortality relationships for Calhoun colony Oriental fruit moth neonates on wheat germ diet containing reduced-risk insecticides (probit analysis). Progeny of insects reared on 'Gala' apples are compared with progeny of insects reared on lima bean diet.

Insecticide and colony[a]	n	Slope ± SE	χ^{2} [b]	LC_{50}[c]	95% CL Lower-Upper	LCR[d]	95% CI [e] Lower-Upper	LC_{90}[c]	95% CL Lower-Upper	LCR[d]	95% CI [e] Lower-Upper
chlorantraniliprole											
A	1082	1.55±0.11	12.92*	0.08	0.04-0.13	1.00	0.68-1.47	0.52	0.31-1.09	1.83	1.19-2.80
D	532	2.27±0.30	1.89	0.08	0.06-0.10			0.28	0.22-0.41		
spinetoram											
A	840	2.29±0.24	3.89	0.06	0.04-0.07	0.95	0.69-1.32	0.20	0.16-0.27	0.85	0.57-1.28
D	616	2.12±0.24	0.57	0.06	0.05-0.07			0.24	0.18-0.35		
acetamiprid											
A	810	6.40±0.63	10.67*	0.41	0.32-0.50	1.07	0.91-1.26	0.66	0.54-0.92	0.96	0.81-1.15
D	434	5.22±0.66	1.79	0.39	0.33-0.44			0.68	0.44-0.60		
spinosad											
A	509	3.25±0.32	6.19	0.30	0.23-0.39	1.18	0.99-1.43	0.75	0.57-1.22	1.08	0.84-1.39
D	1025	2.93±0.22	1.83	0.26	0.23-0.29			0.70	0.61-0.82		

[a] A = Calhoun colony, parents reared on 'Gala' apples. D = Calhoun colony, parents reared on lima bean diet.

[b] * indicates significant lack of fit at $P = 0.05$. PoloPlus uses a heterogeneity factor to calculate confidence limits of estimates of slopes, LC_{50}'s, and LC_{90}'s to compensate for lack of fit.

[c] Insecticide concentration in diet, ppm.

[d] Lethal concentration ratios; A LC_{50}/D LC_{50} and A LC_{90}/D LC_{90} (Robertson et al. 2007).

[e] Confidence intervals for lethal concentration ratios. Where intervals include 1.0, the corresponding LC_{50}'s or LC_{90}'s were not significantly different at $P = 0.05$.

Table 3. Logit and probit regressions[a] of concentration-mortality responses of Oriental fruit moth neonates on wheat germ diet containing reduced-risk insecticides. Calhoun colony neonates (C) are compared with Rutgers colony neonates (R).

Insecticide and colony[b]	n	Slope ± SE	χ^{2} [c]	LC_{50} [d]	95% CL Lower-Upper	LCR[e]	95% CI [f] Lower-Upper	LC_{90} [d]	95% CL Lower-Upper	LCR[e]	95% CI [f] Lower-Upper
chlorantraniliprole											
C	1082	2.91±0.25	2.87	0.08	0.06-0.10	1.01	0.74-1.37	0.45	0.34-0.62	1.73	1.20-2.50
R	1624	4.23±0.36	7.26	0.08	0.05-0.11			0.26	0.17-0.56		
spinetoram											
C	840	4.03±0.45	6.65	0.06	0.03-0.08	1.41	1.03-1.93	0.20	0.13-0.38	1.46	1.05-2.02
R	1491	4.15±0.41	24.32*	0.04	0.01-0.07			0.14	0.08-0.73		
acetamiprid											
C	810	6.40±0.63	10.67*	0.41	0.27-0.53	1.37	1.23-1.53	0.66	0.51-1.19	1.38	1.20-1.59
R	1160	6.46±059	7.12	0.30	0.25-0.35			0.48	0.40-0.69		
spinosad											
C	509	3.25±0.32	6.19	0.30	0.23-0.39	1.08	0.90-1.31	0.75	0.57-1.22	1.05	0.81-1.35
R	816	3.13±0.27	7.37	0.28	0.21-0.35			0.72	0.56-1.06		

[a] Logit models were used for chlorantraniliprole and spinetoram. Probit models were used for acetamiprid and spinosad.

[b] C = Calhoun colony, parents reared on 'Gala' apples. R = Rutgers colony, parents reared on 'Gala' apples..

[c] * indicates significant lack of fit at $P = 0.05$. PoloPlus uses a heterogeneity factor to calculate confidence limits of estimates of slopes, LC_{50}'s, and LC_{90}'s to compensate for lack of fit.

[d] Insecticide concentration in diet, ppm.

[e] Lethal concentration ratios; Calhoun LC_{50}/Rutgers LC_{50} and Calhoun LC_{90}/Rutgers LC_{90} (Robertson et al. 2007).

[f] Confidence intervals for lethal concentration ratios. Where intervals include 1.0, the corresponding LC_{50}s or LC_{90}s were not significantly different at P=0.05.

CHAPTER THREE: SUSCEPTIBILITY OF ORIENTAL FRUIT MOTH (LEPIDOPTERA: TORTRICIDAE) TO TWO PYRETHROIDS AND A PROPOSED DIAGNOSTIC DOSE FOR FIELD DETECTION OF RESISTANCE

ABSTRACT: Laboratory colonies of Oriental fruit moth, *Grapholita molesta* (Busck), were reared on 'Gala' apples and lima bean diet. Neonates from these colonies were placed on wheat germ diet containing a range of concentrations of esfenvalerate or lambda-cyhalothrin; mortality was assessed after 96 h. For a long-term laboratory colony, LC_{50}'s of esfenvalerate and lambda-cyhalothrin were 0.35 and 0.12 ppm, respectively, for progeny of insects reared on apples. For a colony established from Calhoun County, IL in 2007, LC_{50}'s of esfenvalerate and lambda-cyhalothrin were 0.37 and 0.10 ppm, respectively, for progeny of insects reared on apples. The LC_{50}'s of these insecticides did not differ significantly from these values for either colony when progeny of insects reared on lima bean diet were tested. We observed no consistent evidence of pyrethroid resistance in the Calhoun colony after laboratory culture for 21-23 generations. We described the dose-response relationship for esfenvalerate applied topically in 1 µl of acetone to male moths and estimated the LD_{99} to be 0.022 µg per moth. Application of 0.022 µg esfenvalerate per moth to *ca.* 600 male moths from two putatively susceptible populations resulted in mean survivorship approximately equal to the expected level of 1.0%. Application of the same dose to *ca.* 375 field-captured moths from two Calhoun County orchards with histories of pyrethroid use resulted in mean survivorship of 9.4% and 82%. We propose that 0.022 µg of esfenvalerate in 1 µl of

acetone can be used as a diagnostic dose for monitoring pyrethroid resistance in Oriental fruit moth in the field.

Key words: bioassays, resistance monitoring, *Grapholita molesta*

ORIENTAL FRUIT MOTH, *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae), has been a severe pest of apples and peaches in North America since its introduction in the early 1900's (Quaintance and Wood 1916, Wood and Selkregg 1918). To reduce its economic impact, various insecticides including botanicals, inorganic compounds (Stearns 1920), organophosphates (Rothschild and Vickers 1991, Kovanci and Walgenbach 2005), carbamates (Rothschild and Vickers 1991), pyrethroids (Kanga et al. 2003), neonicotinoids (Elbert et al. 2008), and more recently registered insecticides with novel modes of action (Midwest Fruit Workers Group 2009) have been applied in apple and peach orchards. Pyrethroids have been used in peach orchards in the Midwest for >20 years to control Oriental fruit moth, plant bugs, and stink bugs (US EPA 2009). Kanga et al. (2003) documented pyrethroid resistance in *G. molesta* in Ontario. Resistance to pyrethroids has been suspected in Calhoun County in southwestern Illinois after growers experienced control failures in 2004 and 2005 despite their application of permethrin, esfenvalerate, and lambda-cyhalothrin at rates, volumes, and intervals that had been effective in previous years (R.A.W., unpublished data). In response, growers in Calhoun County altered their management programs to reduce (but not eliminate) reliance on pyrethroids for control of Oriental fruit moth, but the importance of resistance in earlier control failures remained unclear when research presented here started in 2007.

Detection and quantification of insecticide resistance requires an understanding of the relationship between dose or concentration and mortality for at least one susceptible population. We use the word **dose** to indicate a known amount of one or more insecticides administered to a test animal. For insects, the toxicant is usually administered topically. We use the word **concentration** to describe the amount of one or more insecticides applied to an insect's habitat or food. In these applications, the exact amount of insecticide deposited on the cuticle or ingested is not measured. Basic binary bioassays that use toxins incorporated into diet or deposited on the inner surface of glass vials or Petri plates that hold test insects are accurately called concentration-mortality bioassays.

By definition, resistance is characterized by changes in dose- or concentration-mortality relationships in comparison with susceptible populations (Roush and Miller 1986, Denholm 1990, Robertson et al. 2007). However, typical binary bioassays are not sensitive enough to detect small changes in slopes or lethal concentrations efficiently when resistance frequencies are low (Roush and Miller 1986). An alternative to using basic binary bioassays to detect resistance is to use a single discriminating or diagnostic dose (Brown and Pal 1971, Roush and Miller 1986, Halliday and Burnham 1990). Standardized bioassays that used a diagnostic dose were developed decades ago for monitoring and detecting resistance in agricultural pests (ESA 1968) and disease vectors (WHO 1976). Diagnostic doses have been used to detect resistance in field populations of codling moth, *Cydia pomonella* L. (Lepidoptera: Tortricidae) (Bush et al. 1993, Varela et al. 1993, Pasquier and Charmillot 2003, Soleno et al. 2008), and Kanga et al. (1999,

2003) used a diagnostic concentration of cypermethrin to monitor resistance in *G. molesta.*

Despite decades of use of diagnostic doses in bioassays to monitor resistance, criteria for establishing diagnostic doses are not well-defined. They often are based on or derived from LD_{95}'s or LD_{99}'s (Roush and Miller 1986, Halliday and Burnham 1990, Robertson et al. 2007). Robertson et al. (2007) stressed that precise estimation of LD_{95}'s and LD_{99}'s requires that bioassays use a large number of doses in the upper (>95% mortality) response level and extremely large sample sizes at these doses. Mass-rearing and large-scale collecting may not provide enough insects to do such bioassays in all species of interest, particularly Lepidoptera. Halliday and Burnham (1990) developed a simulation model to assess the statistical power of experiments that use diagnostic doses to monitor insecticide resistance when dose-response lines for susceptible and heterozygous strains overlap. They found that slope, resistance factor, frequency of resistance, inheritance of resistance, dose, and numbers of insects tested (susceptible strain and putatively resistant strain) contributed to the power of bioassays. They cautioned against using extremely high doses (multiples of the LD_{99} or $LD_{99.9}$) as diagnostic doses because such an approach would likely fail to detect resistance in some instances.

If a diagnostic dose is to be valuable in detecting resistance, it must: (1) cause the expected level of mortality in susceptible field populations, and (2) allow significantly greater survivorship in populations with low frequencies of resistance. Interpretation of survivorship at a diagnostic dose may be complicated by natural variation among populations (Robertson et al. 2007). Where a diagnostic dose is based on studies of one

or only a few susceptible populations, its application to other populations with slightly greater natural tolerance may result in unexpected levels of survivorship that should not be interpreted as evidence of resistance.

To characterize Oriental fruit moth response to two pyrethroids and develop a diagnostic dose for practical use in the field, we: (1) used bioassays to describe and compare the responses of neonates from two laboratory colonies to esfenvalerate and lambda-cyhalothrin incorporated into wheat germ diet; (2) compared responses of neonates from parents reared on 'Gala' apples and neonates from parents reared on lima bean diet (3) used a topical bioassay to describe the responses of adult males of a susceptible laboratory colony to esfenvalerate and estimate an LD_{99}; (4) tested *ca.* 600 male moths from two susceptible populations with the LD_{99} from the topical bioassay to determine if observed survivorship differed from the expected level of 1%; and (5) tested the LD_{99} from the topical bioassay as a possible diagnostic dose on adult males from two populations with histories of selection by pyrethroids to determine if observed survivorship differed from 1%.

Materials and Methods

Laboratory Colonies. Two laboratory colonies, designated "Rutgers" and "Calhoun" were maintained. The Rutgers colony was established in a USDA laboratory near Fresno, CA, *ca.* 40 years ago and had been reared since 1995 at Rutgers University, New Brunswick, NJ. The Calhoun colony was established with field-collected larvae from untreated peach fruit and shoots from orchards in Calhoun County in southwestern

Illinois from May through August, 2007. Orchards where collections were made had been treated multiple times per season with pyrethroids, primarily permethrin, esfenvalerate, and lambda-cyhalothrin, for several years before 2007. We used rearing methods similar to those of Pree (1985), Yokoyama et al. (1987), and Vetter et al. (1989) to maintain these colonies. Each colony was split and reared concurrently on 'Gala' apples and on lima bean diet to reduce the likelihood of colony loss if one of the rearing methods failed. A detailed summary of rearing methods was described in Chapter 2. At the time bioassays were done from May through July, 2009, the Calhoun colony had been in laboratory culture for 21 to 23 generations.

Larval bioassays. Commercial formulations of esfenvalerate (Asana XL, DuPont Agricultural Products, Wilmington, DE) and lambda-cyhalothrin (Warrior CS, Syngenta Crop Protection, Greensboro, NC) were diluted in deionized water for incorporation into a wheat germ diet (Stonefly Industries, Item #38 V 0600, Ward's Natural Science, Rochester, NY) for larval bioassays using methods similar to those of Sial et al. (2010)(Chapter 2). Diet containing seven concentrations of each insecticide (0.03, 0.1, 0.3, 1.0, 2.0, 3.0, and 10.0 ppm esfenvalerate; 0.03, 0.06, 0.1, 0.3, 1.0, 3.0, and 10.0 ppm lambda-cyhalothrin) was prepared, along with untreated wheat germ diet. Diet cubes (*ca.*1 cm^3) were placed into 30-ml plastic diet cups (Bio-Serv, Frenchtown, NJ), and a single larva that had hatched within the previous 24 hours was added to each diet cup. These cups were held in environmental chambers at 26.8±2ºC, 60% RH, and a photoperiod of 16:8 (L:D). Mortality was evaluated after 96 h. Larvae were counted as dead if they were unresponsive to prodding with a blunt probe.

Adult Topical Bioassays. To determine the dose-response relationship of adult males from the Rutgers Colony to esfenvalerate, we applied a range of doses of technical grade esfenvalerate (Du Pont Agricultural Products, Wilmington, DE) in acetone to the dorsal surface of the abdomen and thorax of 0- to 4-d old males collected from emergence cages with an aspirator. Only males were used in bioassays; male pupae were separated from female pupae using methods described by George (1965). All moths used in this bioassay were reared on lima bean diet. Bioassays were done from April to August, 2009. Moths were anesthetized for 5 s with CO_2 and placed ventral-side down on a sticky liner from a Pherocon VI pheromone trap (Trecé Inc., Adair, OK). One µl of acetone or acetone plus esfenvalerate was applied to the dorsal surface of the abdomen and thorax with a 50-µl micro-syringe mounted on a repeating dispenser (#PB600-1, Hamilton, Reno, NV). After preliminary experiments established the range of doses that influenced mortality, six doses of esfenvalerate (0.001, 0.005, 0.01, 0.015, 0.02, 0.025 µg/moth) were administered, along with an acetone-only control. Treated moths were held at 26.8±2ºC, 60% RH, and a photoperiod of 16:8 (L:D), and mortality was assessed after 24 h by prodding the moths with a blunt probe and observing their movement. A moth was considered to be alive if it was able to move its antennae, legs, wings, or head when prodded. Moths that exhibited only rapid fluttering of the wings or twitching of the wings, abdomen, or antennae were considered to be moribund and combined with dead insects (no movement) for analysis.

To examine the possible use of the LD_{99} from the Rutgers Colony (0.022 µg per male moth) as a diagnostic dose, we administered it to *ca.* 600 additional male moths from two populations that had not been exposed to pyrethroids for many generations –

the Rutgers colony itself and moths captured in an abandoned 2-ha block of apples near Urbana, IL that had not been treated with insecticides for >10 years – to determine if mortality would be *ca.* 99% as expected. For the Rutgers colony, male moths that had been reared on lima bean diet were collected from emergence cages within 4 d after eclosion, anesthetized, and treated as described above. A total of 310 male moths from the Rutgers colony were treated with the diagnostic dose on a single date in July 2009, and 25 moths were treated with acetone only. To obtain moths from the untreated Urbana location, Pherocon VI traps baited with Oriental fruit moth lures (Trecé Inc., Adair, OK) were hung before dusk. Trap liners were collected and returned to the laboratory the next morning on multiple dates in 2009. Bioassays began as soon as moths were brought to the laboratory, and male moths on the trap liners were treated as described above. We used moths only if they were stuck ventral-side down and moved vigorously when prodded. Others were removed from sticky liners and discarded. For each date we treated *ca.* 30 percent of the available moths with acetone only and recorded their survival after 24 h as well.

We also administered the putative diagnostic dose to larger numbers of moths from three populations previously exposed to selection by pyrethroids – (1) our Calhoun colony, (2) moths captured in 2009 in commercial blocks of apples and peaches (designated CHA) in Calhoun County that were treated with pyrethroids and other insecticides with limited success that season, and (3) moths captured in the spring of 2010 in a commercial peach orchard (designated CEI) in Calhoun County where severe infestations were observed late in the 2009 season despite repeated uses of pyrethroids. On each bioassay date we treated *ca.* 30 percent of the available moths with acetone only.

For the Calhoun colony, male moths that had been reared on lima bean diet were collected from emergence cages within 4 d after eclosion, anesthetized, and treated as described above. To obtain moths from the Calhoun field populations, Pherocon VI traps baited with Oriental fruit moth lures were hung before dusk, and trap liners were collected and returned to the laboratory the next morning on six dates in July and August, 2009, and on four dates in April, 2010. Bioassays began as soon as moths were returned to the laboratory, and male moths on trap liners were treated as described above.

Statistical Analyses. Although neonates used in bioassays had not fed before they were placed on the treated diet cubes, their responses to insecticides may have differed according to parental food source. Consequently, although we did not expect parental food to influence the results of bioassays, we separated all trials and compared responses of progeny of insects reared on apples with those of progeny of insects reared on lima bean diet. We also compared responses of neonates from the Calhoun and Rutgers colonies.

Concentration-mortality relationships were estimated with PoloPlus (LeOra Software 2005) assuming the probit or logit model as described by Robertson et al. (2007). Lethal concentrations were considered to be significantly different from each other if the 95% confidence interval for the lethal concentration ratio did not include 1.0 (Robertson et al. 2007). As noted in Chapter 2, the lethal concentration ratio test is more powerful for detecting differences in the responses of two populations than using overlapping confidence limits for the LC_{50}'s or LC_{90}'s (Wheeler et al. 2005). The dose-response relationship for adult males from the Rutgers colony treated with esfenvalerate also was analyzed using PoloPlus.

To determine whether or not the LD_{99} from the Rutgers colony (0.022 µg/moth) also caused 99 percent mortality when applied to larger numbers of moths from susceptible populations, we compared observed survivorship with expected survivorship. For the 310 moths from the Rutgers colony, our single observation of survivorship was informative but not suitable for hypothesis testing. For samples from the Urbana population from an abandoned and untreated site, mortality for each sampling date was corrected with Abbott's (1925) formula. Means of survivorship from multiple sampling dates were compared with the expected level of 1% using a 1-sample *t*-test and a 2-tailed probability distribution (to determine whether observed means were greater or less than 1.0) (Little et al. 1996, PROC TTEST, SAS Institute 2003).

To determine if survivorship of moths from the Calhoun County CHA and CEI populations (suspected to be resistant) was > 1.0 % after treatment with the proposed diagnostic dose, observations of survivorship from multiple sampling dates were corrected with Abbott's (1925) formula and means were compared with the expected level of 1.0% using a 1-sample *t*-test and a 1-tailed probability distribution (Little et al. 1996; PROC TTEST, SAS Institute 2003).

Results and Discussion

Larval Bioassays. The probit model adequately fit the concentration-response data for all bioassays of esfenvalerate (Table 4). For assessment of the concentration-response relationship for lambda-cyhalothrin and Calhoun colony neonates from parents reared on diet, the probit model did not fit the data, and the logit model was used. To

allow comparisons between separate bioassays, the logit model was used to describe all bioassays of lambda-cyhalothrin (Table 4).

In comparisons of the concentration-response relationships for larvae from parents reared on 'Gala' apples versus larvae from parents reared on lima bean diet, confidence intervals for all the LC_{50} ratios in Table 4 included 1.0, indicating that the corresponding LC_{50}'s did not differ significantly based on parental food source. Similarly, LC_{90}'s of esfenvalerate did not differ significantly based on parental food source for either colony. For lambda-cyhalothrin, LC_{90} ratios for progeny of parents reared on apples/progeny of parents reared on lima bean diet were 0.55 and 1.58 for the Calhoun and Rutgers colonies, respectively. These ratios indicated significant differences ($P \leq 0.05$) in the LC_{90}'s according to parental food source. Similarly, regression models of concentration-mortality relationships for progeny of apple-reared versus diet-reared parents for the Calhoun and Rutgers colonies differed significantly from each other ($P=0.001$ for the Calhoun colony; $P=0.036$ for the Rutgers colony). However, the differences in LC_{90}'s were inconsistent (greater for progeny of diet-reared than apple-reared parents for the Calhoun colony but less for progeny of diet-reared than apple-reared parents for the Rutgers colony), and the LC_{90} ratios deviated only slightly from 1.0. We concluded that the two parental diets that we used had little impact on responses of neonates to these insecticides.

To compare the concentration-response relationships for larvae from the Calhoun colony versus the Rutgers colony, we used PoloPlus to calculate lethal concentration ratios based on the parameters of the regression models presented in Table 5. Confidence intervals for all the LC_{50} ratios included 1.0 (Table 5), indicating no significant

differences in the LC_{50}'s of these insecticides between the two colonies, regardless of parental diet. Confidence intervals for the LC_{90} ratios (Calhoun/Rutgers) for lambda-cyhalothrin did not include 1.0 and indicated that the LC_{90}'s of lambda-cyhalothrin listed in Table 4 for the Calhoun and Rutgers colonies differed significantly from each other ($P<0.05$) for progeny of apple-reared and diet-reared parents. Regression models of the concentration-mortality relationship for lambda-cyhalothrin also differed significantly between the Calhoun and Rutgers colonies ($P = 0.005$ for progeny of insects reared on apples; $P = 0.008$ for progeny of insects reared on lima bean diet). Overall, however, the two colonies responded similarly in these bioassays. Differences in the LC_{90}'s were small (Calhoun/Rutgers ratios were between 0.5 and 1.6) and inconsistent (Calhoun/Rutgers ratio was <1.0 for progeny of apple-reared adults and > 1.0 for progeny of diet-reared adults). Together, the analyses summarized in Tables 4 and 5 provide baseline data for future bioassays to investigate resistance with neonates.

Although we suspected some level of pyrethroid resistance in the Calhoun population at the time of field collection in 2007, it is not surprising that the larval bioassays reported here did not support that suspicion. Kanga et al. (2003) concluded that pyrethroid resistance in Oriental fruit moth was not stable in the absence of selection, and Smirle et al. (1998) and Djihinto et al. (2009) also documented reversions to susceptibility in other Lepidopterans. The Calhoun colony had been in culture for 20-22 generations when these bioassays were done, and its response to esfenvalerate and lambda-cyhalothrin may have reflected either an absence of resistant individuals at the time of collection or a decline of resistance frequencies over time. To better assess the

status of pyrethroid resistance in the field in Calhoun County and elsewhere we focused on bioassays of adults and development of a diagnostic dose for field use.

Adult Topical Bioassays. The logit model provided the best fit for the data from the bioassay of esfenvalerate that used adult males from the Rutgers colony (Table 6). The LD_{99} from this analysis (0.022 µg per moth) was selected as a possible diagnostic dose for detecting resistance in the field. Multiplying the LD_{95} or LD_{99} by an adjustment factor (2X or 3X) or using the upper value of the 95% CL for the estimate of the LD_{99} to establish a putative diagnostic dose has been suggested as a way to reduce the likelihood that random variation among susceptible populations might result in survivorship that is interpreted incorrectly as resistance (Roush and Miller 1986, Suckling et al. 1987, Subramanyam et al, 1989, Robertson et al. 2007). We chose not to use a dose greater than the estimated LD_{99} because the slope of the dose-mortality regression was very steep (Table 6), and there was only a 2-fold difference between the LD_{50} and LD_{99}. We suspected that using a dose greater than the estimated LD_{99} might kill moths that exhibit resistance to field rates of insecticide application and result in a failure to identify resistant populations (Halliday and Burnham 1990). We chose to determine if the 0.022-µg dose provided the expected 99 % mortality in susceptible populations and then determine whether or not that dose could be used to detect resistance where it was suspected in field populations.

Application of 0.022 µg of esfenvalerate to Rutgers colony males resulted in 1.3% survivorship. Although we did not split this bioassay into separate samples to allow calculation of measures of variability or confidence limits, it provided evidence that the proposed LD_{99} caused roughly the expected level of mortality in the Rutgers colony.

Application of 0.022 μg of esfenvalerate to field-collected males from the Urbana field site (considered to be a susceptible population) in 2009 resulted in a mean of 0.70% survivorship (Table 7). A two-tailed *t*-test indicated that this mean did not differ significantly from 1.0 percent (t = -0.67; df = 5; P = 0.534). We concluded that a dose of 0.022 μg per male moth adequately represented the LD_{99} for at least two susceptible populations.

Application of 0.022 μg of esfenvalerate to adult males from the Calhoun colony, the 2009 collection of male moths from Calhoun County CHA, and the 2010 collection of male moths from Calhoun County CEI resulted in 3.1 %, 9.4 %, and 82 % survivorship, respectively. Mean survivorship in the Calhoun colony did not differ significantly from 1 percent (t = 1.51; df = 5; P = 0.096). This result was expected after our bioassays with neonates indicated no difference between the Calhoun and Rutgers colonies' responses to esfenvalerate. As noted earlier, this may reflect the absence of resistant moths in the Calhoun colony at the time of collection in 2007 or the instability of resistance in laboratory culture. Mean survivorship in the moths collected from CHA in 2009 and CEI in 2010 differed significantly from 1.0% (t = 2.90; df = 4; P = 0.022 for CHA; t = 11.18; df = 3; P = 0.000). Survivorship observed in the two bioassays of field-collected moths from sites where resistance was suspected, coupled with the expected level of *ca.* 1% survivorship of moths from two susceptible populations, indicates that the proposed diagnostic dose of 0.022 μg of esfenvalerate can be used to detect pyrethroid resistance.

This bioassay uses the most common pheromone traps used by growers to monitor Oriental fruit moth in the Midwestern US and can be done readily by researchers or extension entomologists in the region. Although pyrethroids have been used widely

since the 1980s because of their low mammalian toxicity and high toxicity to insects (Ecobichon 2001), our work provides evidence of one more instance of resistance in a key crop pest. We propose that topical bioassays using the diagnostic dose reported in this paper can be used to monitor pyrethroid resistance in the Oriental fruit moth and guide future management programs.

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Tables

Table 4. Concentration-mortality relationships for Oriental fruit moth neonates on wheat germ diet containing esfenvalerate or lambda-cyhalothrin[a]. Progeny of insects reared on 'Gala' apples are compared with progeny of insects reared on lima bean diet.

Insecticide and colony[b]	n	Slope ± SE	χ^{2c}	LC_{50}[d]	95% CL Lower-Upper	LCR[e]	95% CI[f] Lower-Upper	LC_{90}[d]	95% CL Lower-Upper	LCR[e]	95% CI[f] Lower-Upper
esfenvalerate											
CA	706	2.06±0.17	8.50	0.37	0.25-0.51	0.90	0.70-1.17	1.56	1.10-2.58	0.96	0.71-1.31
CD	818	2.16±0.14	4.26	0.41	0.33-0.51			1.62	1.27-2.22		
RA	878	2.29±0.22	6.23	0.35	0.24-0.46	1.03	0.72-1.47	1.26	0.96-1.84	0.87	0.63-1.20
RD	635	2.03±0.20	11.05	0.34	0.16-0.53			1.45	0.96-2.56		
lambda-cyhalothrin											
CA	729	5.62±0.74	4.13	0.10	0.09-0.12	1.01	0.80-1.27	0.25	0.20-0.34	0.55	0.37-0.81
CD	817	3.35±0.28	11.53*	0.10	0.07-0.14			0.46	0.29-1.00		
RA	839	3.60±0.32	12.75*	0.11	0.08-0.17	1.06	0.85-1.32	0.46	0.28-1.15	1.58	1.08-2.33
RD	689	5.04±0.51	6.29	0.11	0.07-0.15			0.29	0.19-0.73		

[a] Probit model used for esfenvalerate; logit model used for lambda-cyhalothrin.

[b] C = Calhoun colony; R = Rutgers colony; A = parents reared on 'Gala' apples; D = parents reared on lima bean diet.

[c] * indicates significant lack of fit at $P = 0.05$. PoloPlus uses a heterogeneity factor to calculate confidence limits of estimates of slopes, LC_{50}'s, and LC_{90}'s to compensate for lack of fit.

[d] Insecticide concentration in diet, ppm.

[e] Lethal concentration ratios; RA LC_{50}/RD LC_{50} and RA LC_{90}/RD LC_{90}; CA LC_{50}/CD LC_{50} and CA LC_{90}/CD LC_{90} (Robertson et al. 2007).

[f] Confidence intervals for lethal concentration ratios. Where intervals include 1.0, the corresponding LC_{50}s or LC_{90}s were not significantly different at P=0.05.

Table 5. Lethal concentration ratios for esfenvalerate and lambda-cyhalothrin in wheat germ diet fed to Oriental fruit moth neonates from two laboratory colonies.

Insecticide	Colonies[a]	LC_{50} Ratio and 95% CI[b]		LC_{90} Ratio and 95% CI[b]	
esfenvalerate	CA/RA	1.07	0.79-1.45	1.24	0.90-1.71
	CD/RD	1.22	0.88-1.68	1.12	0.83-1.51
lambda-cyhalothrin	CA/RA	0.91	0.72-1.15	0.55	0.37-0.82
	CD/RD	0.95	0.76-1.19	1.58	1.09-2.30

[a] C = Calhoun colony; R = Rutgers colony; A = parents reared on 'Gala' apples; D = parents reared on lima bean diet.

[b] Lethal concentration ratios; where confidence intervals include 1.0, the corresponding LC_{50}'s or LC_{90}'s (see Table 4) were not significantly different at P=0.05.

Table 6. Dose mortality relationship for esfenvalerate applied topically to Rutgers colony adult male Oriental fruit moths.

n	Slope ± SE	χ^2	LD_{50}[a] (95% CL)	LD_{90}[a] (95% CL)	LD_{99}[a] (95% CL)
212	14.82±2.08	1.48	0.011 (0.008-0.012)	0.015 (0.014-0.019)	0.022 (0.018-0.040)

[a] Dose expressed as µg/moth.

Table 7. Mean 24-h survival of adult male Oriental fruit moths treated topically with a proposed diagnostic dose of 0.022 µg esfenvalerate in 1 µl acetone .

Population	Year	Sampling Dates	N	Mean Percent Survivorship (SEM)
Rutgers colony	2009	29 July	310	1.29
Urbana Field	2009	25, 26, 27, and 28 Apr., 03 and 04 July	286	0.70 (0.45)
Calhoun colony	2009	29 June, 03, 07, 11, and 21 July, 12 Aug.	240	3.13 (1.41)
Calhoun County CHA	2009	17 July, 13, 14, 15, and 28 Aug.	156	9.40 (2.90)
Calhoun County CEI	2010	6, 7, 13, 14 April	218	81.92 (7.24)

CHAPTER FOUR – TOXICITY OF THIAMETHOXAM AND MIXTURES OF CHLORANTRANILIPROLE PLUS ACETAMIPRID, ESFENVALERATE, OR THIAMETHOXAM TO NEONATES OF ORIENTAL FRUIT MOTH (LEPIDOPTERA: TORTRICIDAE)

ABSTRACT: To assess the toxicity of thiamethoxam and three mixtures of insecticides to Oriental fruit moth, *Grapholita molesta* (Busck), we added the insecticides to diet and fed it to neonates of two laboratory colonies; mortality was assessed after 96 h. Thiamethoxam was much less toxic than insecticides previously tested. Five of six analyses of the joint action of chlorantraniliprole plus acetamiprid, esfenvalerate, or thiamethoxam indicated that toxicity was not independent and uncorrelated. For chlorantraniliprole plus acetamiprid, mortality was slightly lower than expected at low concentrations and greater than expected at high concentrations. For chlorantraniliprole plus esfenvalerate, mortality was less than expected at nearly all concentrations, suggesting antagonism despite the two compounds' different modes of action. For chlorantraniliprole plus thiamethoxam, observed mortality exceeded expected mortality at low concentrations, but this trend did not continue at higher concentrations. Although the null hypothesis of independent and uncorrelated toxicity was rejected for chlorantraniliprole plus acetamiprid and chlorantraniliprole plus thiamethoxam in three of four analyses, differences between observed and expected mortality were minor and inconsistent over the range of concentrations tested. We do not expect these mixtures to exhibit significant synergism or antagonism in the field. Apparent antagonism between chlorantraniliprole and esfenvalerate is particularly relevant because these insecticides (or chlorantraniliprole plus a different pyrethroid) may be used together in apples or peaches for control of Oriental fruit moth

and Hemipteran pests. The effectiveness of each insecticide against Oriental fruit moth might be reduced in such applications.

Key Words: bioassays, insecticide resistance, joint action, *Grapholita molesta*

MIXTURES OF PESTICIDES are used for at least two reasons – to control a broad spectrum of pest species and to manage pesticide resistance (Georghiou 1980, LeBaron 1986, Kataria and Gisi 1990, Mavroeidi and Shaw 2006). Compounds may be sold as pre-mixed formulated products, or they may be tank-mixed by applicators (Hammock and Soderlund 1986, Ahmad 2004). Using tank-mixes of insecticides and fungicides is common in integrated pest management in tree fruits where multiple insects and plant pathogens are targeted (Midwest Fruit Workers Group 2010). Combining insecticides is most common where one is fairly selective to one or a few species, but other species not susceptible to that ingredient can be controlled by another insecticide. When populations of insect species are resistant to a commonly used insecticide, an additional ingredient may be used to target the resistant individuals. The value of mixtures is greatest if the components are synergistic and can be used effectively at reduced rates and costs (Turner 1951, El-Sebae et al. 1964, Wolfenbarger and Cantu 1975, Marking 1985). Organophosphates and carbamates have been shown to synergize pyrethroids applied to populations of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) and *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) that have increased levels of mixed-function oxidases (Martin et al. 2003, Bielza et al. 2007).

When used for insecticide resistance management, mixtures or rotations are most likely to slow the evolution of resistance if the modes of action of the components differ and they are

not subject to detoxification by the same enzymes and metabolic pathways (Georghiou 1980). The independent action of two insecticides can be assessed with bioassays of each component and of a combination of the two (Robertson et al. 2007).

Chlorantraniliprole controls Oriental fruit moth – including populations thought to be resistant to pyrethroids – in apples and peaches, but it does not control other insect pests such as aphids (Hemiptera: Aphididae), stink bugs (Hemiptera: Pentatomidae), plant bugs (Hemiptera: Miridae), plum curculio, *Conotrachelus nenuphar* (Herbst) (Coleoptera: Curculionidae), and Japanese beetle, *Popillia japonica* Newman (Coleoptera: Scarabeidae) (Midwest Fruit Workers Group 2010). Insecticides that might be mixed with chlorantraniliprole include acetamiprid for control of plum curculio and Japanese beetle, esfenvalerate for control of stink bugs, plant bugs, or Japanese beetle, and thiamethoxam for control of aphids, plum curculio, and Japanese beetle (Midwest Fruit Workers Group 2010). Acetamiprid might also be used in rotations with chlorantraniliprole for managing insecticide resistance in Oriental fruit moth and codling moth, *Cydia pomonella* L. (Lepidoptera: Tortricidae). We previously assessed the toxicity of chlorantraniliprole, acetamiprid, and esfenvalerate to two laboratory colonies of Oriental fruit moth (Chapters 2 and 3). Here we report the baseline susceptibility of Oriental fruit moth neonates to thiamethoxam and to 1:1 mixtures of chlorantraniliprole with acetamiprid, esfenvalerate, and thiamethoxam. We also test the hypothesis that the toxicity of chlorantraniliprole is independent of the toxicity of each of the other insecticides.

Materials and Methods

Laboratory Colonies. Two laboratory colonies, designated "Rutgers" and "Calhoun" were maintained. The Rutgers colony was established in a USDA laboratory near Fresno, CA, *ca.* 40 years ago and had been reared since 1995 at Rutgers University, New Brunswick, NJ. The Calhoun colony was established with larvae collected from untreated peach fruit and shoots from orchards in Calhoun County in southwestern Illinois from May through August, 2007 (Chapter 2). Orchards from which collections were made had been treated multiple times per season with pyrethroids, primarily permethrin, esfenvalerate, and lambda-cyhalothrin, and organophosphates such as chlorpyrifos and phosmet for >15 years before 2007 (R. A. W., unpublished data). As a result, the Calhoun County population had experienced some selection pressure from these insecticides before the colony was established. We used rearing methods similar to those of Pree (1985), Yokoyama et al. (1987), and Vetter et al. (1989) to maintain these colonies. Each colony was split and reared concurrently on 'Gala' apples and lima bean diet to allow maintenance of stable colonies. A detailed summary of rearing methods was described in Chapter 2. The Calhoun colony had been in laboratory culture for 23-29 generations when bioassays reported here were done from July 2009 through January 2010.

Larval bioassays. Commercial formulations of chlorantraniliprole (Altacor 35WG, DuPont Agricultural Products, Wilmington, DE), acetamiprid (Assail 30SG, United Phosphorus, King of Prussia, PA), thiamethoxam (Actara 25WG, Syngenta Crop Protection, Greensboro, NC), and esfenvalerate (Asana XL, DuPont Agricultural Products, Wilmington, DE) and a 1:1 commercial premix of chlorantraniliprole:thiamethoxam (Voliam Flexi 40WG, Syngenta Crop Protection, Greensboro, NC) were used. These insecticides were diluted in deionized water for

incorporation into wheat germ diet (Stonefly Industries, Item #38 V 0600, Ward's Natural Science, Rochester, NY) for bioassays of larvae using methods described in Chapter 2. We prepared 5 to 7 concentrations of thiamethoxam and each mixture in diet (and diet with no insecticide) for each bioassay. The range of concentrations in diet were 1.0-15 ppm thiamethoxam, 0.12-0.6 ppm chlorantraniliprole plus acetamiprid, 0.03-0.6 ppm chlorantraniliprole plus esfenvalerate, and 0.06-1.0 ppm chlorantraniliprole plus thiamethoxam. For mixtures, concentrations were expressed as the sum of the concentrations of each component (for example, 0.1 ppm chlorantraniliprole combined with 0.1 ppm acetamiprid = 0.2 ppm A.I.). Diet cubes (*ca.*1 cm^3) were placed into 30-ml plastic cups (Bio-Serv, Frenchtown, NJ), and a single larva that had hatched within the previous 24 hours was added to each cup. All larvae were progeny of adults reared on apple. Cups were held in environmental chambers at 26.8±2ºC, 60% RH, and a photoperiod of 16:8 (L:D). Mortality was evaluated after 96 h. Larvae were counted as dead if they were unresponsive to prodding with a blunt probe.

Statistical Analysis. Concentration-mortality relationships for thiamethoxam and the three mixtures were estimated with PoloPlus (LeOra Software 2005) as described by Robertson et al. (2007). Data were analyzed assuming the probit model. To assess the joint action of components in mixtures, we tested the null hypothesis that the toxicity of the two insecticides was independent and uncorrelated (i.e., the toxicity of each component is unaffected by the toxicity of the other, and susceptibility of the insect to one component is not affected by susceptibility to the other component). We used PoloMix (LeOra Software 2005) to determine if observed mortality caused by insecticide mixtures deviated from levels predicted by the null hypothesis (Robertson et al. 2007). Parameters from models of concentration-mortality relationships for chlorantraniliprole, acetamiprid, and esfenvalerate (chapters 2 and 3) were

combined with the results of bioassays reported here. For each of the three mixtures analyzed with PoloMix (chlorantraniliprole plus acetamiprid, esfenvalerate, or thiamethoxam), we entered observations of mortality for at least 5 concentrations. Where possible, we limited data entry to concentrations that resulted in >10 percent and <90 percent mortality for a more precise estimates of expected values (Robertson and Smith 1989). We examined mortality (observed versus expected) at each concentration and the χ^2 values for each analysis. If the total calculated χ^2 value for a data set exceeded the critical value for χ^2 based on degrees of freedom (number of concentrations, including the control, minus 1), we rejected the null hypothesis of independent and uncorrelated toxicity.

Results and Discussion

For both colonies, thiamethoxam alone was much less toxic to Oriental fruit moth neonates (much higher LC_{50}'s and LC_{90}'s) than the other individual insecticides (Table 8) (as reported in chapters 2 and 3). Because thiamethoxam (Actara) is not labeled for use against Oriental fruit moth (Syngenta Crop Protection 2010) and field observations have not shown it to be effective against this insect (R. A. W., unpublished data), this result was expected. The Rutgers and Calhoun colonies responded similarly (though not identically) in all bioassays. This also was true in previous bioassays of acetamiprid, chlorantraniliprole, spinosad, spinetoram, esfenvalerate, and lambda-cyhalothrin (chapters 2 and 3).

Analysis of the joint action of chlorantraniliprole plus acetamiprid resulted in the rejection of the null hypothesis of independent and uncorrelated toxicity for data from the Calhoun colony but not the Rutgers colony (Figure 1, A and B). For both colonies, observed

mortality tended to be slightly lower than expected at low concentrations and greater than expected at high concentrations. For chlorantraniliprole plus esfenvalerate, we rejected the null hypothesis for both colonies (Figure 1, C and D). Mortality was less than expected for nearly all concentrations examined. This result suggests antagonism between the two compounds despite their different modes of action. We also rejected the null hypothesis for both colonies' responses to chlorantraniliprole plus thiamethoxam (Figure 1, E and F). At low concentrations, observed mortality consistently exceeded expected mortality in both colonies, but this trend did not hold true at higher concentrations.

Although analyses led to rejection of the null hypothesis of independent and uncorrelated toxicity for mixtures of chlorantraniliprole plus acetamiprid for the Calhoun colony and chlorantraniliprole plus thiamethoxam for both colonies, the differences between observed and expected mortality illustrated in Figure 1 (A, B, E, and F) were generally minor. For these mixtures, the relationships between observed and expected mortality were inconsistent over the range of concentrations tested. Consequently, we conclude that these mixtures are not likely to result in significant synergism or antagonism in field use against Oriental fruit moth.

For chlorantraniliprole plus esfenvalerate, observed mortality was significantly ($P<0.05$) and consistently less than expected in the Rutgers colony. This relationship was less evident but significant in analysis of bioassays with the Calhoun colony as well. This apparent antagonism between the toxic action of chlorantraniliprole and esfenvalerate is particularly relevant because these insecticides (or mixtures of chlorantraniliprole and a different pyrethroid) may be used together in apples or peaches for control of Oriental fruit moth and Hemipteran pests that cause cat-facing injury or other distortions of fruit (Midwest Fruit Workers 2010). Our results suggest

that the effectiveness of each insecticide against Oriental fruit moth might be reduced when they are applied in combination.

The reasons for antagonism between chlorantraniliprole and esfenvalerate remain undetermined. In studies of the combined effects of various pyrethroids and organophosphates on *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), Ahmad (2004) concluded that synergism was likely the result of organophosphates binding to the active site of pyrethroid-hydrolyzing esterases but was unable to explain observations of antagonism. Similarly, El-Guindy et al. (1983) found that combinations of pyrethroids and insect growth regulators resulted in antagonism, dependent on the mixture. They attributed antagonism to possible interference by one component with enzymes responsible for activation of the other; they based this explanation primarily upon DuBois' (1961) work with various organophosphates. Metabolism of pyrethroids is catalyzed by pyrethroid-hydrolyzing esterases (i.e. carboxylesterases/B-esterases) (Gunning et al. 1998) and cytochrome P450 monooxygenases (Brown and Bryson 1995). Metabolism of chlorantraniliprole in mammals is catalyzed by cytochrome P450s and hydroxylases (US EPA 2008, PMRA 2008). Based on findings of Sial et al. (2010), esterases also catalyze metabolism of chlorantraniliprole in a related fruit pest, *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae). How chlorantraniliprole and esfenvalerate might alter the metabolism of each other (and therefore make the combination less effective than expected) or affect its action at target sites is unclear.

Given that pyrethroids are widely used in peaches against Hemipteran pests and that chlorantraniliprole is one of the reduced-risk insecticides used at the same time to control pyrethroid-resistant Oriental fruit moths, a greater understanding of chlorantraniliprole-pyrethroid interactions is needed. Investigations of combinations of chlorantraniliprole and

additional pyrethroids at a range of ratios might reveal the extent of any antagonism between compounds. The use of synergists or direct measures of enzyme activity might provide clues about the nature of specific antagonistic relationships.

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Tables and Figures

Table 8. Concentration mortality relationships for Oriental fruit moth neonates on wheat germ diet containing thiamethoxam and mixtures of chlorantraniliprole with acetamiprid, esfenvalerate, or thiamethoxam[a].

Colony and Insecticide	n	Slope ± SE	χ^{2b}	LC_{50}	95% CL Lower-Upper	LC_{90}[c]	95% CL Lower-Upper
Rutgers							
Thiamethoxam	553	3.11±0.28	2.02	1.94	1.72-2.17	5.02	4.28-6.21
chlorantraniliprole + acetamiprid	818	7.76±0.73	0.94	0.17	0.16-0.17	0.24	0.23-0.27
chlorantraniliprole + esfenvalerate	471	3.58±0.35	0.91	0.20	0.18-0.22	0.45	0.39-0.55
chlorantraniliprole + thiamethoxam	544	3.11±0.35	8.02	0.14	0.11-0.18	0.37	0.28-0.61
Calhoun							
thiamethoxam-p	555	2.76±0.25	8.04	2.41	1.81-3.09	7.01	5.06-12.85
chlorantraniliprole + acetamiprid	615	5.30±0.47	7.09	0.17	0.15-0.20	0.30	0.24-0.48
chlorantraniliprole + esfenvalerate	441	2.64±0.31	10.07*	0.17	0.08-0.28	0.51	0.30-4.86
chlorantraniliprole + thiamethoxam	529	2.34±0.21	9.97	0.12	0.09-0.16	0.44	0.31-0.79

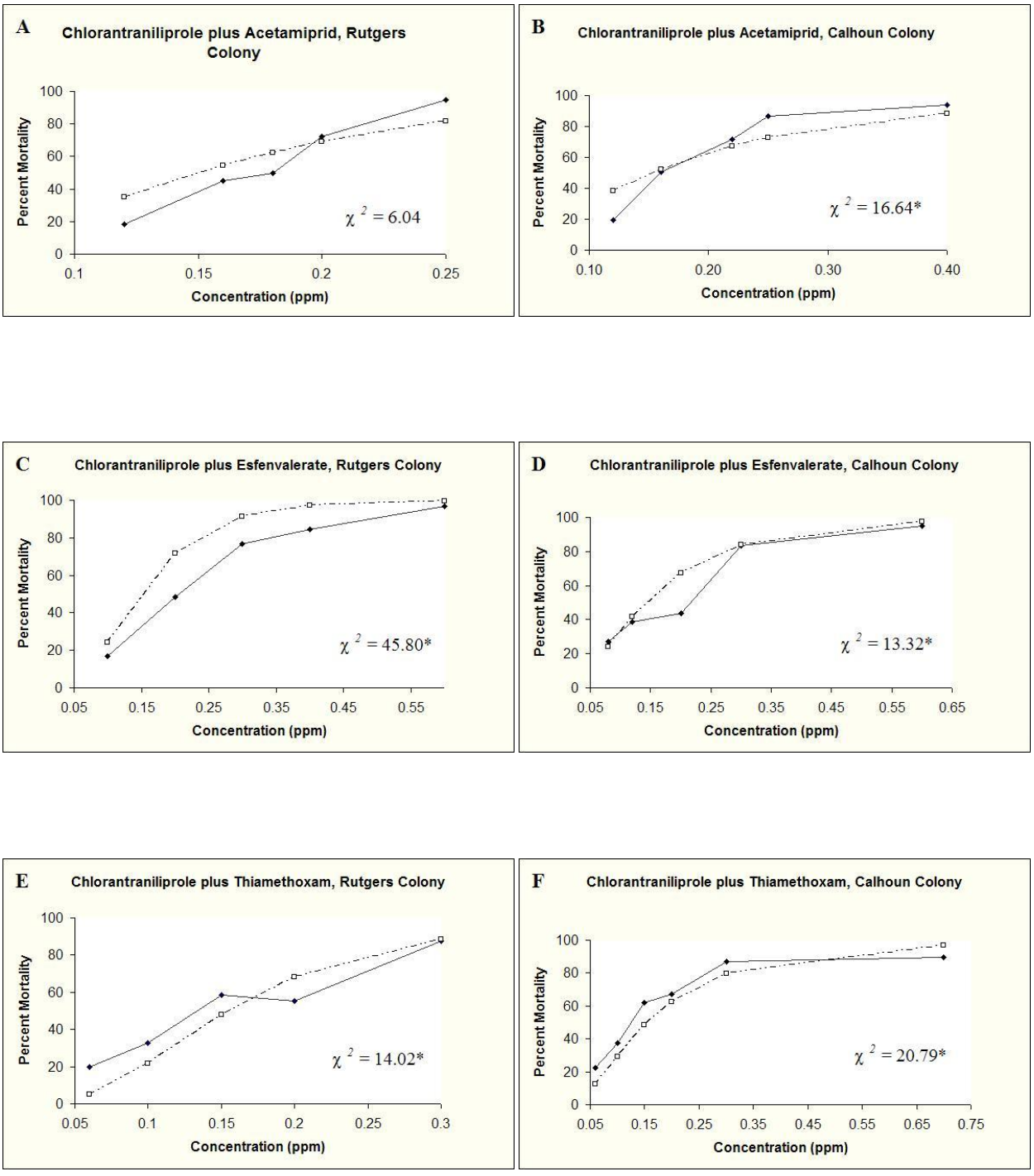
[a] Concentration-mortality models for chlorantraniliprole, acetamiprid, and esfenvalerate were reported by in chapters 2 and 3.

[b] * indicates significant lack of fit at $P = 0.05$.

[c] Insecticide concentration in diet, ppm.

Figure 1. Observed (solid lines) versus expected (dashed lines) mortality of Oriental fruit moth neonates on wheat germ diet containing 1:1 mixtures of chlorantraniliprole with acetamiprid, esfenvalerate, or thiamethoxam. * signifies χ^2 is significant ($P<0.05$), and the null hypothesis of independent and uncorrelated action was rejected (Robertson et al. 2007).

Figure 1 (cont.)



CHAPTER FIVE: SUSCEPTIBILITY OF EGGS FROM TWO LABORATORY COLONIES OF ORIENTAL FRUIT MOTH (LEPIDOPTERA: TORTRICIDAE) TO NOVALURON

ABSTRACT: To estimate the toxicity of novaluron to eggs from two populations of Oriental fruit moth, *Grapholita molesta* (Busck), we dipped eggs on waxed paper into a range of concentrations. Treated eggs were held on wheat germ diet, and mortality was assessed after 10 d. We compared the concentration-mortality relationships of eggs from parents reared on 'Gala' apples with those of eggs from parents reared on lima bean diet. We also compared the responses of a long-term laboratory colony from Rutgers University and a colony established from Calhoun County in southwestern Illinois. LC_{50}'s of novaluron ranged from 0.10 to 0.83 ppm and did not differ significantly based on parental diet. LC_{50}'s differed significantly between colonies; LC_{50}'s estimated for the Calhoun colony were 2.5 and 8 times greater than those for the Rutgers colony in bioassays that used eggs from parents reared on diet and apples, respectively. LC_{90}'s ranged from *ca.* 38 to 1,000 ppm. For the Calhoun colony, the LC_{90} for novaluron applied to eggs from parents reared on apples was *ca.* 10 times greater than the LC_{90} for novaluron applied to eggs from parents reared on lima bean diet. For eggs from parents reared on apples, the LC_{90} for the Calhoun colony was *ca.* 9 times greater than the LC_{90} for the Rutgers colony. Differences in the colonies' responses may represent natural variation among populations or may be the result of selection by other insecticides used in Calhoun County orchards before we collected larvae for our colony.

Key Words: bioassays, insecticide resistance, insect growth regulator, Rimon, *Grapholita molesta*

ORIENTAL FRUIT MOTH, *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae) is a serious pest of peaches, apples, and other fruit crops. The 2010 Midwest Tree Fruit Spray Guide lists >15 insecticides labeled for its control in apples and peaches (Midwest Fruit Workers Group 2010). Among alternatives to organophosphates and pyrethroids are benzoylphenyl ureas that act as chitin inhibitors (Ishaaya and Casida 1974, Hajjar and Casida 1979). Novaluron is a benzoylphenyl urea that is toxic to Lepidopteran eggs and larvae by contact and ingestion (Ishaaya et al. 1996, Hadapad et al. 2001, Ishaaya et al. 2002). Benzoylphenyl ureas are characterized by low mammalian toxicity (US EPA 2010a, 2010b). They are effective against a range of Lepidopteran pests, including key pests of forests (Robertson and Kimball 1979a and 1979b, Thorpe et al. 1997), vegetables (Hadapad et al. 2001, Maxwell and Fadamiro 2006), and tree fruits (Midwest Fruit Workers Group 2010). Novaluron is labeled in the United States for control of Oriental fruit moth and codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) in apples and peaches (US EPA 2010c).

The evolution of resistance to insecticides in target pests threatens the sustained effectiveness of many insecticides, especially those that may be applied multiple times per season to control multivoltine insects (Georghiou and Taylor 1977a, 1977b). Novaluron may be used in this way to control Oriental fruit moth in apples and peaches (Midwest Fruit Workers Group 2010). In studies of insecticide resistance, baseline data on the toxicity of insecticides to susceptible populations is essential. Here we describe

baseline toxicity of novaluron to eggs of two colonies of Oriental fruit moth, one held in culture for many years and the other established in 2007 from a population in southwestern Illinois. We tested the null hypothesis that the responses of the two colonies would be the same. We also compared responses of eggs from parents reared on 'Gala' apples and eggs from parents reared on lima bean diet.

Materials and Methods

Laboratory Colonies. Two laboratory colonies, designated "Rutgers" and "Calhoun" were maintained as described in Chapter 2. The Rutgers colony was established in a USDA laboratory near Fresno, CA *ca.* 40 years ago and had been reared since 1995 at Rutgers University, New Brunswick, NJ. The Calhoun colony was established with larvae collected from untreated peach fruit and shoots from orchards in Calhoun County in southwestern Illinois from May through August, 2007. Novaluron had never been used in peach orchards in the area of our collections, but it had been applied in apple orchards since 2005. Its use in Calhoun County before 2007 was uncommon, however, and limited to one or two applications per season in the few orchards where it was used (R. A.W., unpublished data).

We used rearing methods similar to those of Pree (1985), Yokoyama et al. (1987), and Vetter et al. (1989) to maintain both colonies. Each colony was split and reared concurrently on 'Gala' apples and lima bean diet. A detailed summary of rearing methods was described in Chapter 2. The Calhoun colony had been in laboratory culture

for 23 to 29 generations, and the Rutgers colony had been in laboratory culture for decades at the time bioassays were done from July 2009 through January 2010.

Bioassays. The toxicity of novaluron to eggs of the Oriental fruit moth was estimated using methods similar to those reported by Brunner et al. (2005). Novaluron (Rimon 0.83EC, Chemtura Corporation, Middlebury, CT) was diluted in deionized water to prepare 10 concentrations ranging from 0.01 to 10,000 ppm. Based on preliminary data for each colony, 6 to 9 concentrations and deionized water as an untreated check were used in each bioassay.

Eggs were collected on waxed paper exposed to moths for 0-72 h on the inside of the lighted face of oviposition cages (Chapter 2). The waxed paper was cut into pieces with 7-9 eggs per piece. Each replicate of the bioassay used 25-45 eggs per concentration. The pieces of waxed paper were dipped into deionized water or a dilution of novaluron for 1 s and allowed to air-dry before placing them individually into 30-ml cups (Bio-Serv, Frenchtown, NJ) with 1 cm^3 of wheat-germ diet (Stonefly Industries, Item #38 V 0600, Ward's Natural Science, Rochester, NY) (Chapter 2). Cups containing eggs were then placed into an environmental chamber at 26.8°C ± 1°C, 60% RH, and a photoperiod of 16:8 (L:D). Egg hatch was recorded after 10 days. As per Brunner et al. (2005), eggs that showed no larval development were recorded as dead; eggs that hatched were considered to have survived (regardless of subsequent larval survival).

Statistical Analyses. Concentration-response (mortality) relationships were estimated with probit analysis (PoloPlus; LeOra Software 2005) as described by Robertson et al. (2007). Although we did not expect parental food source to influence the results of bioassays, we separated all trials and compared responses for eggs from insects

reared on apples with those from insects reared on lima bean diet. We also compared responses of eggs from the Calhoun and Rutgers colonies.

Lethal concentration ratios and 95% confidence intervals for those ratios were calculated to identify differences in responses to novaluron related to parental diet or colony. Lethal concentrations were considered to be significantly different if the 95% confidence interval for the lethal concentration ratio did not include 1.0 (Robertson et al. 2007). We chose this method of hypothesis testing because the lethal concentration ratio test is more powerful for detecting true differences in toxicity than the confidence interval overlap test as noted in Chapter 2 (Wheeler et al. 2005).

Results and Discussion

Probit parameters for the relationships between concentrations of novaluron and mortality of Oriental fruit moth eggs are summarized in Table 9. The very low slopes for all of these models indicate high levels of heterogeneity in response to novaluron in both colonies. Regardless of colony, concentration-mortality relationships showed no significant differences based on parental food source at the LC_{50}. For bioassays of the Calhoun colony, the LC_{90} for novaluron was *ca.* 10 times greater for eggs from parents reared on apples than for eggs from parents reared on diet.

In general, the Calhoun colony was less susceptible to novaluron than the Rutgers colony. Differences in responses between colonies were greater for eggs from parents reared on apples than for eggs from parents reared on diet. For bioassays that used eggs from parents reared on apples, the LC_{50} and LC_{90} ratios (Calhoun/Rutgers) were 8.1 [95%

CI, 3.23-20.08] and 8.6 [95% CI, 2.12-35.04], respectively. For bioassays that used eggs from parents reared on diet, the LC_{50} ratio (Calhoun/Rutgers) for novaluron was 2.5 [95% CI, 1.16-5.36]; LC_{90}'s did not differ significantly between colonies.

Our primary objective for doing these bioassays was to estimate the toxicity of novaluron to eggs of two populations of Oriental fruit moth that had experienced little or no prior selection pressure from this insecticide. The significant differences in response to novaluron that we observed related to parental diet and laboratory colony remain difficult to interpret. Differences in mortality based on parental diet were significant for the Calhoun colony only at the LC_{90} response level, and there were no significant differences in responses based on parental diet for the Rutgers colony. Differences in the models in relation to parental diet were not the result of selection during laboratory culture. We did not consistently separate populations reared on apples from those reared on diet for either the Rutgers or the Calhoun colony. Instead, when numbers of insects reared on lima bean diet decreased, eggs from moths reared on apples were placed onto diet to maintain colony vigor. Likewise, eggs from moths reared on diet were placed on apples as needed (Chapter 2). Although parental diet could influence survival of eggs, this was not evident in observations of colony dynamics or in bioassays of other insecticides that used Oriental fruit moth larvae (chapters 2-4). Studies of additional populations and food sources are needed to determine whether or not our single observation of a difference in response of eggs of Oriental fruit moth to novaluron based on parental diet is consistent and might indicate that host plants could influence the efficacy of this insecticide.

Reasons for the elevated LC_{50}'s and LC_{90} for novaluron applied to eggs from the Calhoun colony versus eggs from the Rutgers colony also remain unclear. Differences in the colonies' responses to novaluron may represent natural variation among populations or may be the result of selection by insecticide use in Calhoun County orchards before we collected larvae for our colony. Organophosphates and pyrethroids had been applied multiple times per season to apples and peaches there for many years. Reuveny and Cohen (2004) found that populations of codling moth that were resistant to azinphosmethyl exhibited tolerance to novaluron without prior application of novaluron in apple orchards. The greater tolerance we observed in the Calhoun colony may have resulted from selection by other insecticides used in orchards surrounding and including the sites where we collected larvae to establish this colony. Future use of novaluron for Oriental fruit moth control and development of resistance management plans will require an expanded understanding of the interaction of novaluron with other insecticides and the interrelationship of any resistance mechanisms.

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Tables

Table 9. Concentration-mortality relationships for Oriental fruit moth eggs dipped into a range of concentrations of novaluron. LC50's and LC90's are compared for eggs of insects reared on 'Gala' apples and for eggs of insects reared on lima bean diet.

Colony[a]	n	Slope ± SE	χ^{2}[b]	LC_{50}[c]	95% CL Lower-Upper	LCR[d]	95% CI[e] Lower-Upper	LC_{90}[c]	95% CL Lower-Upper	LCR[d]	95% CI[e] Lower-Upper
CA	1209	0.41±0.03	9.30	0.83	0.32-1.83	1.88	0.81-4.36	1034.00	289.35-6848.50	10.40	2.45-44.10
CD	833	0.54±0.04	1.37	0.44	0.24-0.75			99.46	44.32-283.08		
RA	1292	0.42±0.03	15.03*	0.10	0.03-0.29	0.58	0.25-1.36	119.85	34.13-782.43	3.19	0.98-10.37
RD	1209	0.55±0.05	11.65	0.18	0.06-0.39			37.62	12.11-235.23		

[a] R = Rutgers colony; C = Calhoun colony; A = parents reared on 'Gala' apples; D = parents reared on lima bean diet.

[b] * indicates significant lack of fit at $P = 0.05$. PoloPlus uses a heterogeneity factor to calculate confidence limits of estimates of slopes, LC_{50}'s, and LC_{90}'s to compensate for lack of fit.

[c] Insecticide concentration in deionized water (ppm).

[d] Lethal concentration ratios (CA LC_{50}/CD $LC_{50;}$ CA LC_{90}/CD LC_{90}; RA LC_{50}/RD LC_{50} and RA LC_{90}/RD LC_{90} (Robertson et al. 2007).

[e] Confidence intervals for lethal concentration ratios. Where intervals include 1.0, the corresponding LC_{50}s or LC_{90}s were not significantly different at P=0.05

CHAPTER 6: SUMMARY AND CONCLUSIONS

The Oriental fruit moth has been a serious pest of apples and peaches since its introduction into North America. It is managed primarily with insecticides and, to a lesser extent, by mating disruption. Resistance to pyrethroid insecticides in populations of *G. molesta* has been confirmed in other areas and was suspected in southwestern Illinois at the time this project began.

The work reported in this dissertation assessed the toxicity of eight insecticides and three mixtures of insecticides to this insect. Bioassays used insects from two laboratory colonies and field-collected moths. An existing laboratory colony (Rutgers) and a new colony established from field-collected larvae (Calhoun) were each maintained on two food sources – lima bean diet and 'Gala' apples. Concurrent rearing on apples and diet allowed consistent maintenance of stable colonies, but where eggs or larvae were used in bioassays, separate analyses were required for offspring of parents reared on diet or apples.

Depending on the characteristics of each insecticide and the objective of each bioassay, eggs, larvae, or adults were tested. Eggs were used in bioassays of novaluron because it is recommended for use primarily as an ovicide. Bioassays of most insecticides – chlorantraniliprole, acetamiprid, spinosad, spinetoram, thiamethoxam, esfenvalerate, and lambda-cyhalothrin – used neonates because they are the primary targets of these insecticides in apples and peaches. Adult males were used in bioassays of esfenvalerate because the objective of these bioassays was to develop a method for monitoring resistance to pyrethroids in the field, and pheromone traps can be used to collect male moths for immediate testing.

Bioassays reported in chapters 2-4 estimated the baseline susceptibility of Oriental fruit moth neonates to five reduced-risk insecticides and two pyrethroids. Chlorantraniliprole, acetamiprid, spinosad, and spinetoram were grouped together in initial bioassays because they are all classified as reduced-risk pesticides by the US EPA and are labeled for use against Oriental fruit moth. Thiamethoxam, another reduced-risk insecticide, is not labeled on its own against Oriental fruit moth and was tested later in conjunction with investigations of mixtures because it is combined with chlorantraniliprole in a formulated pre-mix that is labeled for use against Oriental fruit moth and other insects. The pyrethroids esfenvalerate and lambda-cyhalothrin are not reduced-risk insecticides but are widely used in peaches; resistance to pyrethroids was suspected at the site where larvae were collected to establish the Calhoun colony.

The slopes, LC_{50}'s, and LC_{90}'s generated by probit or logit analyses and reported for these insecticides in tables 2, 3, 4, and 8 characterize the responses of the two colonies. Comparisons of LC_{50}'s or LC_{90}'s among the various insecticides provide some useful observations (for example, spinetoram is roughly 3-7 times more toxic to neonates than spinosad, and thiamethoxam is much less toxic to neonates than all the other compounds tested), but the primary intent of these bioassays was to document the response of susceptible populations to these insecticides. In general, there were few differences in LC_{50}'s or LC_{90}'s between the two colonies, and parental food source did not consistently influence the concentration-mortality relationships. These concentration-mortality models provide a robust representation of baseline susceptibility for comparisons in future bioassays designed to detect the development of resistance.

Larval bioassays of esfenvalerate and lambda-cyhalothrin did not detect any differences in the responses of the Rutgers and Calhoun colonies even though larvae for the Calhoun colony were collected from a site where resistance was suspected. If the population at that site was resistant, resistance was not stable over several generations of laboratory culture.

Identifying a diagnostic dose for bioassays of field-collected moths provides a way to monitor resistance immediately without establishing and building a lab colony over time (and allowing reversion to susceptibility). Bioassays that used topical application of esfenvalerate to adult males were analyzed to develop a logit model that described the dose-response relationship. The estimated LD_{99} from this model, 0.022 µg/moth, was selected for further testing as a diagnostic dose. It was administered to *ca.* 600 additional moths from two susceptible populations to determine if survival would be *ca.* 1% as expected. Survival averaged 1%. The 0.022-µg dose was applied to *ca.* 375 field-collected moths from two orchards in Calhoun County where pyrethroid resistance was suspected. One was a mixed apple and peach orchard where pyrethroids had been used with other insecticides and control had been poor for 2-3 years. The other was a peach orchard where pyrethroids had been used almost exclusively and control failures led to high levels of crop loss in 2009. Survivorship in these bioassays averaged 9% and 82%, respectively, indicating possible resistance in these field populations. These results suggest that the 0.022-µg dose can be used in future monitoring efforts as a diagnostic dose for efficient detection of resistance in the field.

When chlorantraniliprole is used for the control of Lepidopteran pests, including Oriental fruit moth, it is often mixed or rotated with other insecticides to provide broad spectrum control of other orchard pests, including plant bugs, plum curculio, and Japanese beetles. Using mixtures and rotations of insecticides with different modes of action can delay the evolution of

resistance, but such a benefit depends on several factors, including the independent and uncorrelated action of the components.

Mixtures of chlorantraniliprole and acetamiprid, esfenvalerate, or thiamethoxam were tested in bioassays. Analyses tested the null hypothesis that the toxicity of the two insecticides was independent and uncorrelated. If the null hypothesis is not rejected, the toxicity of each component is unaffected by the toxicity of the other. The null hypothesis was rejected for mixtures (1:1) of chlorantraniliprole plus acetamiprid or thiamethoxam, but these mixtures did not exhibit consistent antagonism or synergism. Their use in mixtures is likely to result in additive toxicity. If so, mixtures or rotations of chlorantraniliprole with acetamiprid or thiamethoxam may aid in resistance management.

The 1:1 mixture of chlorantraniliprole plus esfenvalerate was less toxic than expected (based on bioassays of individual components) over a range of doses. The reasons for this apparent antagonism are unclear, but these results suggest that the application of esfenvalerate for Hemipteran control in conjunction with chlorantraniliprole to control pyrethroid-resistant populations of Oriental fruit moth may result in reduced effectiveness.

The toxicity of novaluron, a chitin inhibitor, was assessed using eggs. The very low slopes of the regression lines that described the concentration-mortality relationships indicated a high level of heterogeneity in both colonies. Heterogeneity may favor the development of resistance in response to selection pressure. LC_{50}'s estimated for the Calhoun colony were 2.5 and 8 times greater than those for the Rutgers colony in bioassays that used eggs from parents reared on diet and apples, respectively. For eggs from parents reared on apples, the LC_{90} for the Calhoun colony was *ca.* 9 times greater than the LC_{90} for the Rutgers colony. These differences in responses between colonies may represent natural variation among populations or may be the

result of selection by other insecticides used in Calhoun County orchards before larvae were collected to establish this colony. Within colonies, LC_{50}'s did not differ significantly based on parental diet. However, for the Calhoun colony, the LC_{90} for novaluron applied to eggs from parents reared on apples was ca. 10 times greater than the LC_{90} for novaluron applied to eggs from parents reared on diet. It is unclear whether or not this single observation of a difference in response of eggs of Oriental fruit moth to novaluron based on parental diet might indicate that host plants could influence the efficacy of this insecticide.

The findings reported in this dissertation estimate the baseline susceptibility of Oriental fruit moth to several insecticides, and this information will be useful for comparisons in future studies of the development of insecticide resistance. Findings from these studies also provide key information for immediate actions – methods for a diagnostic bioassay to monitor resistance to pyrethroids and a caution about possible antagonism in mixtures of chlorantraniliprole and esfenvalerate (and possibly other pyrethroids).

Insecticides are and will remain an integral part of integrated pest management. Insecticide resistance and effective resistance management are key determinants of the long-term usefulness of insecticides, especially in intensively managed crops such as apples and peaches. Dose- or concentration-mortality models that describe the responses of susceptible populations to key insecticides and practical methods that allow detection of resistance in the field are essential for monitoring and managing resistance. The findings reported in this dissertation contribute to the information base that will guide efforts to manage resistance in the Oriental fruit moth and to the body of knowledge that supports a broad range of resistance management efforts.