TOXICOLOGICAL STUDIES OF NOVEL REDUCED-RISK INSECTICIDES IN OBLIQUEBANDED LEAFROLLER, CHORISTONEURA ROSACEANA (HARRIS) (LEPIDOPTERA: TORTRICIDAE)

By

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A dissertation submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

WASHINGTON STATE UNIVERSITY
Department of Entomology

AUGUST 2010
To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of

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ACKNOWLEDGMENTS

I would like to thank my wife, Sadaf, for providing such incredible support throughout this whole process—I share this degree with her. To my kids, Haris and Sarah, thanks for loaning me out each morning and giving me a hero’s welcome each night. I have the honor to express my heartiest gratitude and sincere appreciation to my advisor, Jay Brunner, for providing me the opportunity, independence, guidance, and incessant encouragement to undertake my PhD. Current members of the Brunner lab—Mike Doerr, Kathy Pierre, and Keith Granger—have contributed significantly in various ways to my work here, and I thank them all. I offer my earnest thanks to my committee members, John Brown, John Dunley, Steve Garczynski, and Steve Sheppard for their inspiring guidance during my research and constructive criticism to improve my dissertation. I would also like to thank Carol Anelli for providing me opportunities to gain teaching experience, and Laura Lavine for allowing me to conduct special research projects in her laboratory to gain firsthand experience in molecular techniques. I highly acknowledge the cooperation and love of the faculty, students and staff of the WSU Tree Fruit Research and Extension Center. Finally, I greatly appreciate the friendship and advice of faculty, staff, and students in the Department of Entomology.

I also acknowledge the Washington Tree Fruit Research Commission, the Washington State Commission on Pesticide Registration, and Western Sustainable Agriculture and Research (Project No.: GW10-003) for providing funding for this research.
Historically, broad-spectrum insecticides have been the primary tools to manage insect pests in tree fruits. Use of broad-spectrum insecticides such as organophosphates (OPs) for decades has resulted in the development of OP resistance and cross-resistance to other classes of insecticides in major pests including obliquebanded leafroller (OBLR), Choristoneura rosaceana (Harris) (Lepidoptera: Tortricidae). Additionally, regulatory actions such as Food Quality Protection Act of 1996 (FQPA) have put restrictions on the use of broad-spectrum insecticides leading to OP phase-out. The development of insecticide resistance and implementation of FQPA along with restrictions in international markets have led to the development of new chemicals such as chlorantraniliprole and spinetoram which are more environmentally friendly. With the availability of these products, it was critical for tree fruit growers to incorporate the novel reduced-risk insecticides into IPM programs. However, very little information was available to growers on how to utilize these reduced-risk products in IPM programs in a sustainable manner.
With that in mind, studies were initiated to investigate lethal and sublethal effects, stage-specificity, and residual efficacy of these chemicals using OBLR as a model species. Field populations were tested to establish the baseline susceptibility, and a laboratory population was selected for resistance to assess the risk of resistance evolution and determine possible resistance mechanisms. These investigations revealed that chlorantraniliprole and spinetoram have strong lethal as well as sublethal effects. They were highly effective against OBLR larvae regardless of larval age and have very long residual activity. However, evidence of resistance and cross-resistance in the field as well as development of resistance in response to laboratory selection indicates that the risk of resistance evolution against these chemicals exists. Studies also showed that in the absence of selection populations reverted to being susceptible and that different detoxification systems were involved with each chemical. This latter fact indicates that chlorantraniliprole and spinetoram could be effectively incorporated into resistance management programs through strategies of rotation. Implementation of such strategies at this point would be a proactive approach and would lead to management of OBLR and other major pests of tree fruits on a sustainable basis.
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DEDICATION

This dissertation is dedicated to my affectionate parents whose toil and sweat as moral support enshrined and grafted in me an untiring zeal to get on to higher ideals of life.
CHAPTER ONE

BASELINE TOXICITY AND STAGE SPECIFICITY OF RECENTLY DEVELOPED REDUCED-RISK INSECTICIDES CHLORANTRANILIPROLE AND SPINETORAM TO OBLIQUEBANDED LEAFROLLER, CHORISTONEURA ROSACEANA (HARRIS) (LEPIDOPTERA: TORMICIDAE)

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Abstract

BACKGROUND: Studies were designed to assess baseline toxicity of the two recently developed reduced-risk insecticides chlorantraniliprole and spinetoram to obliquebanded leafroller (OBLR), Choristoneura rosaceana (Harris), and determine stage specific effects of these products on OBLR, so that these new chemicals could be strategically incorporated into IPM programs by targeting only the most susceptible stages of OBLR.

RESULTS: Both chlorantraniliprole and spinetoram were highly effective against neonate OBLR larvae at much lower doses than conventional standard azinphosmethyl. Most of the mortality caused by spinetoram occurred during the 4 d exposure period while significant delayed mortality was observed as a result of chlorantraniliprole treatment during the 7 d
recovery period indicating that chlorantraniliprole is a slower acting compound as compared to spinetoram. Unlike broad-spectrum predecessors, the toxicity of chlorantraniliprole significantly increased with larval age. Similarly, the toxicity of spinetoram increased as larvae grew from neonates to third instars, but it slightly decreased in the fifth instars.

CONCLUSION: Chlorantraniliprole and spinetoram are highly effective against OBLR larvae regardless of larval age. The strategic incorporation of these reduced-risk chemistries into IPM programs would contribute to the sustainable management of OBLR in tree fruit systems.

Keywords: obliquebanded leafroller; chlorantraniliprole; spinetoram; baseline toxicity; stage specificity

Introduction

Obliquebanded leafroller (OBLR), *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae) is native to and widely distributed in temperate North America\(^1,2\). Larvae are polyphagous with a wide host range but preferred hosts are predominantly Rosaceae\(^3,4\). This species was not considered an economic pest until initial outbreaks occurred in late 1970’s in apple growing regions of the eastern United States\(^1,5\). Subsequent studies have shown that it has become one of the most serious pests of deciduous tree fruit growing regions of the United States and Canada\(^6,8\). The OBLR larval injury in apple is characterized by the larvae rolling leaves and feeding on the surrounding foliage, flower buds, and developing fruits\(^9,10\). The damage from larval feeding on fruit can occur around bloom, during mid-summer, and
just prior to harvest. Fruit damage caused by the summer brood OBLR is usually more serious than spring feeding by over-wintered larvae because more of the fruit injured later in the season remains on the tree at harvest\textsuperscript{11}.

The OBLR and a wide spectrum of other apple pests have been controlled using organophosphate insecticides (OPs) for over four decades. Even though the OPs are considered highly toxic to biological control agents due to their broad spectrum of activity\textsuperscript{12}, the OP-based apple integrated pest management (IPM) system was relatively stable for many years. The repeated applications of OPs for over four decades have resulted in the development of resistance to OPs, and cross- and multiple-resistance to other classes of insecticides in OBLR\textsuperscript{13-21}. In addition, implementation of the Food Quality Protection Act of 1996 brought pressure for change in the fundamental approach to apple IPM leading to the development of environmentally benign and highly selective insecticides.

Consequently, several insecticides such as spinosad, emamectin benzoate, and insect growth regulators including methoxyfenozide, pyriproxyfen, and novaluron were registered as alternatives to OPs over the last decade. Recently, two reduced-risk novel chemistries chlorantraniliprole and spinetoram were registered for use against major pests of tree fruits including OBLR. Chlorantraniliprole is a member of a new class of insecticides, the anthranilic diamides, which selectively binds to the ryanodine receptors (RyR) in insect muscles resulting in an uncontrolled release of internal calcium stores from the sarcoplasmic reticulum\textsuperscript{22, 23}, causing impaired regulation of muscle contraction leading to feeding cessation, lethargy, paralysis, and death in target organisms. Anthranilic diamides have very low vertebrate toxicity due to a >500-fold differential selectivity toward insect over mammalian RyR\textsuperscript{23}. Spinetoram is a recently developed member of the spinosyns class of insecticides
which primarily activate the nicotinic acetylcholine receptors by binding to a unique and yet unknown site.

Most of the new insecticides are slower acting than conventional nerve poisons because they have little contact activity and must be ingested. Several studies have shown that conventional neurotoxic insecticides such as OPs and carbamates are most effective on the neonate larvae and that the larvae become more tolerant to these compounds as they develop to later instars. Adult moths were almost as susceptible to these insecticides as neonates, but that the pupae were only slightly more susceptible than the last instars. Unlike their broad-spectrum predecessors, most of the new insecticides are relatively selective to specific groups of insects and even to a specific stage of the target pest leading to preserving more of the natural enemies of pests. For instance, tebufenozide had very little ovicidal activity and no toxicity to adults, and fenoxycarb was ineffective on neonates and 200 times more effective on fifth than on third instar larvae of another tortricid pest, tufted apple bud moth. However, the stage specific effects of the two new insecticides chlorantraniliprole and spinetoram have not yet been reported. As our understanding of the dynamics of toxicity of these novel reduced-risk compounds increases, the management of orchard pests should become easier without the use of broad-spectrum insecticides.

The current study was designed to assess baseline toxicity of the two recently developed insecticides chlorantraniliprole and spinetoram relative to the chemicals that have already been registered for use in tree fruit orchards, and determine stage specific effects of these two reduced-risk alternatives to OPs. This information will be useful in incorporating these new insecticides judiciously into IPM programs by targeting only the most susceptible stages of OBLR and other pests of tree fruit systems.
Materials and Methods

**Insects.** The *C. rosaceana* larvae were obtained from a laboratory colony which was established in 1990 from larvae collected from apple orchards in Mattawa, WA. This colony has been reared continuously since their collection on a pinto bean diet following the method of Shorey and Hale\(^36\) under constant conditions of temperature (23 ± 2°C), relative humidity (RH, 70%), and photoperiod (16:8, L:D) and without exposure to insecticides.

**Chemicals.** The insecticides used were chlorantraniliprole (Rynaxypyr\(^{TM}\)/Altacor\(^{®}\) 35WG, E.I. du Pont de Nemours & Co., Wilmington, DE), spinetoram (Delegate\(^{®}\) 25WG, Dow AgroSciences, Indianapolis, IN), spinosad (Delegate\(^{®}\) 25WG, Dow AgroSciences, Indianapolis, IN), emamectin benzoate (Proclaim\(^{®}\) 5SC, Syngenta Crop Protection Inc., Greensboro, NC), novaluron (Rimon\(^{®}\) 0.83EC, Chemtura Corporation, Middlebury, CT), pyriproxyfen (Esteem\(^{®}\) 0.86EC, Valent USA Corporation, Walnut Creek, CA), and azinphosmethyl (Guthion\(^{®}\) 50WP, Bayer, Kansas City, MO).

**Bioassays.** Toxicity of all insecticides except pyriproxyfen to neonate OBLR larvae was determined using a diet incorporation bioassay as described by Sial et al.\(^37\). The insecticide incorporated diet was prepared by mixing different insecticide dilutions (insecticide + water), water, vinegar, and dry diet premix (Stonefly Heliothis Diet, Ward’s Natural Science, Rochester, NY) at a ratio of 10:61:4:25, while an untreated control was prepared by mixing water, vinegar, and dry diet premix at a ratio of 71:4:25. A small portion of insecticide incorporated diet (≈8.0 cm\(^3\)) was added to a plastic Petri dish (Falcon 1006, 50x9 mm, Becton Dickinson Labware, Becton Dickinson and Company, Franklin Lakes, NJ). Depending on availability, 50-150 neonate larvae (>24 h) of OBLR were treated at each concentration by putting five larvae/dish. The dishes were
placed in growth chambers at constant conditions of temperature (23 ± 2°C), relative humidity (RH, 70%), and photoperiod (16:8, L:D). Larval mortality in each bioassay was evaluated after 7 d. Larvae were recorded as dead if they did not move when probed with camel’s hair brush.

In order to determine delayed toxicity of various insecticides to neonate larvae of OBLR, mortality of the treated larvae was assessed after 4 d or 7 d of exposure to an insecticide. The larvae were then transferred to untreated diet and mortality data were recorded again after 7 d of recovery period on untreated diet.

The stage specific effects of chlorantraniliprole and spinetoram were determined by exposing neonate, third instar, and fifth instar OBLR larvae using a diet incorporation bioassay. Survivorship was assessed after 7 d of exposure, and then at adult eclosion.

The toxicity of pyriproxyfen to fifth instar OBLR was assessed by using a leaf-disk bioassay as described by Sial and Brunner\(^38\). Briefly, a series of dilutions (0-30 µL/L) of pyriproxyfen was prepared from the stock solution. Fifth instar OBLR larvae were exposed to a range of pyriproxyfen concentrations (0-30 ppm). The circular leaf-disks of 12 mm diameter were cut out of the leaves using a core sampler (leaf-disk cutter) and 10 µL droplets of pyriproxyfen dilutions were applied to each of the leaf-disks. The control leaf-disks were treated with the same volume of water. Once dried, the leaf-disks were offered to a 5\(^{th}\) instar OBLR in a small petri dish (Falcon 1006, 50x9 mm, Becton Dickinson Labware, Becton Dickinson and Company, Franklin Lakes, NJ). The petri dishes were placed in a growth chamber held at constant conditions of temperature (23 ± 2°C), relative humidity (RH, 70%), and photoperiod (16:8, L:D). After 24 h of exposure, the larvae that consumed the entire leaf-disk were transferred to untreated pinto bean diet and reared in the laboratory, and the survivorship was assessed at adult emergence.
**Data Analysis.** Lethal concentration values (LC_{50} and LC_{90}) and their corresponding 95% fiducial limits (FL) were estimated using POLO^{39}. The instar toxicity ratios at LC_{50} values and their corresponding 95% confidence limits (CL) were calculated using lethal concentration ratio significance test^{40}. Probit model did not fit the mortality data from the bioassays where fifth instars were treated with chlorantraniliprole. Therefore, treatment mortalities of the fifth instar males and females were adjusted for control mortalities according to Abbott^{41} and presented as a bar chart.

**Results**

The results of baseline bioassays showed that all of the chemicals tested were highly toxic to OBLR larvae (Table 1). The LC_{50} values of the new chemicals except insect growth regulators novaluron and pyriproxyfen were much lower than that of the conventional standard azinphosmethyl indicating that the novel reduced-risk insecticides are effective against OBLR at much lower doses than their broad-spectrum predecessor azinphosmethyl.

The bioassays performed to determine delayed effects of various insecticides on neonate OBLR larvae indicated that mortality of the neonates exposed to spinetoram did not increase significantly at the LC_{50} level after 4 d mortality readings (Table 2). In contrast, chlorantraniliprole, emamectin benzoate, and insect growth regulator novaluron continued to cause additional mortality in a week following the 4 d or 7 d exposure. The LC_{50} value of emamectin benzoate after a week of recovery period was 10-fold less than that at 4 d of exposure. Similarly, a 3.5- and 3.2-fold decrease in LC_{50} of chlorantraniliprole and novaluron, respectively, was observed in a week of recovery period following the exposure to insecticides.
In order to determine stage specific effects of chlorantraniliprole and spinetoram on OBLR, diet incorporation bioassays were performed at neonate, and third and fifth instar OBLRs. Based on LC$_{50}$ values, the toxicity of chlorantraniliprole to OBLR significantly increased with the larval age (Table 3). The LC$_{50}$ of chlorantraniliprole for neonates at 7 d of exposure was 2.8-fold higher than that for third instars. Similarly, LC$_{50}$ of chlorantraniliprole for neonates at adult eclosion was 3.6-fold higher than that for third instars (Table 5). The LC$_{50}$ values of chlorantraniliprole for the fifth instars could not be computed because probit model did not fit mortality data. Because both male and female fifth instars treated with chlorantraniliprole at very low concentrations (0.001 and 0.003 mg/L) did not die as larvae. They rather developed into abnormal pupae and then died. The appearances of the abnormal pupae in this case were somewhat similar to those observed as a result of pyriproxyfen treatment (a juvenile hormone mimic) especially the green color of the cuticle. The toxicity of chlorantraniliprole to fifth instars, therefore, could not be compared with that to neonates and third instars. The results of bioassays of chlorantraniliprole with fifth instars are presented as percent corrected mortality at each of the test concentrations (Fig. 1).

The toxicity of spinetoram was also increased with larval age as they developed from neonates to third instars, but it slightly decreased in the fifth instars (Table 4). The LC$_{50}$ values of spinetoram for third instars at 7 d of exposure and adult eclosion were 1.4- and 1.3-fold, respectively, lower than those for neonates (Table 5). The LC$_{50}$ of spinetoram for the fifth instar males was 1.3- and 1.7-fold higher than that for neonates and third instars, respectively. Similarly, an increase of 2.1-fold in LC$_{50}$ of spinetoram for fifth instar females was observed as compared to neonates, and the same of 2.7-fold as compared to third instars.
Discussion

The implementation of Food Quality Protection Act of 1996 has increased pressure for fundamental change in IPM strategies leading to restrictions on the use of OPs and development of reduced-risk alternatives. In order to replace OPs, a number of reduced-risk insecticides including chlorantraniliprole and spinetoram have recently become available for use against OBLR and other pests of tree fruit systems. These OP replacement compounds exhibit a high degree of selectivity, worker safety and are environmentally friendly, thereby providing the potential for more sustainable management of orchard pests. Further understanding of the dynamics of toxicity of these chemicals would be useful in making judicious use of these products in tree fruit orchards.

In the current study, all of the tested insecticides were effective against neonate OBLR larvae. However, the LC$_{50}$ values of chlorantraniliprole and spinetoram were much lower than that of the azinphosmethyl. Although spinetoram belongs to the same class as spinosad, the LC$_{50}$ of spinetoram was much lower than that of spinosad. Our results are in agreement with those of the field trials where chlorantraniliprole and spinetoram were found to be highly effective against OBLR$^{42}$, Brunner unpublished data. The higher LC$_{50}$ values of novaluron and pyriproxyfen observed in this study make sense because both of these compounds are IGRs which do not have contact activity and need to be digested before showing their activity. Moreover, the effectiveness of IGRs cannot be assessed based solely on LC$_{50}$ values because they cause strong sublethal effects on development and reproduction of OBLR$^{38}$ which must be taken into account in order to determine total impact of IGRs.

Spinetoram showed highest effectiveness against neonate OBLR larvae among the tested chemicals both in terms of LC$_{50}$ value and the speed of action. Most of the mortality caused
by spinetoram occurred in the first 4 d of exposure which might be due to the fact that spinetoram is a neurotoxic compound\textsuperscript{27}. The fast activity of spinetoram is also evident from the results of another study where 100\% mortality of neonate OBLR larvae was observed only after one day of exposure to spinetoram\textsuperscript{43}. Spinosad, the first spinoxy molecule, has been shown to have a relatively high speed of action in gypsy moth\textsuperscript{44}. Delayed mortality to a varying degree was observed for all other insecticides tested in this study. An increase of 10-fold was observed in LC\textsubscript{50} of emamectin benzoate after a week following the exposure indicates high potential for delayed mortality. Similarly, delayed mortality has been reported in other tortricid larvae including western spruce budworm and tufted apple bud moth as a result of treatment with abamectin which belongs to the same class as emamectin benzoate\textsuperscript{28, 45}. Novaluron is an IGR that disrupts chitin synthesis process in insects, delayed mortality was observed in OBLR larvae as a result of exposure to novaluron. A similar study has documented delayed mortality in tufted apple bud moth larvae as a result of treatment with hexaflumuron which is also a chitin synthesis inhibitor like novaluron. Chlorantraniliprole belongs to a novel class of insecticides, anthranilic diamides, and primarily activates ryanodine receptors in insect muscle cells. Being a novel chemistry, toxicity profile of chlorantraniliprole is not well understood yet; however, the delayed mortality observed in this study as a result of chlorantraniliprole treatment could be attributed at least in part to the fact that it needs to be ingested to initiate its activity.

The bioassays performed to determine stage specific effects of chlorantraniliprole and spinetoram showed that unlike broad-spectrum predecessors\textsuperscript{28, 29-31}, the toxicity of spinetoram to OBLR larvae also increased as they developed from neonates to 3\textsuperscript{rd} instars, but their susceptibility was slightly decreased as they developed from third to fifth instar. There are no
reports indicating stage specific effects of spinetoram for us to compare our findings with, however, our results are in agreement with those of an earlier study in which the spinosad was more effective on gypsy moth until the third larval instar and then its effectiveness was decreased\textsuperscript{44}.

Likewise, the toxicity of chlorantraniliprole to OBLR larvae significantly increased with larval age as the larvae developed from neonates to third instars. Although LC\textsubscript{50} values of chlorantraniliprole for fifth instars were not available for comparison, the percent corrected mortality readings were indicative of that fact that chlorantraniliprole was more toxic to fifth instars than neonates or third instars. Although stage specific effects of chlorantraniliprole have not been documented in any species, an earlier study reported that the toxicity of abamectin to another tortricid western spruce budworm was increased with larval age as they developed from third instars to sixth instars\textsuperscript{45}. Furthermore, it was surprising to note that the fifth instars treated with chlorantraniliprole developed into abnormal pupae with appearances somewhat similar to those observed as a result of treatment with juvenile hormone mimics. These results point out that effect of chlorantraniliprole may not be limited to the activation of ryanodine receptors. Further studies should be conducted to understand the physiological pathways that are directly or indirectly affected by chlorantraniliprole treatment within the body of target insects.

Our findings demonstrate that the recently developed reduced-risk insecticides chlorantraniliprole and spinetoram are highly effective against OBLR larvae regardless of larval age at much lower doses than previously used broad-spectrum insecticides such as azinphosmethyl. These findings are encouraging especially at a time when OP insecticides are being restricted or even phased-out, and indicate that chlorantraniliprole and spinetoram
have strong potential to control OBLR. Incorporation of these novel reduced-risk chemistries into IPM programs for OBLR and other orchard pests would therefore lead to eco-friendly and sustainable management of tree fruit pests. But concerns regarding negative effects of these products on natural enemies shown by recent studies raise additional questions on how best to integrate their use into such programs. However, further studies investigating sublethal effects of these products on pests as well as natural enemies would be useful in determining the long-term impact of these products on population dynamics of insect pests in orchards.

Acknowledgments

This study was sponsored by the Washington Tree Fruit Research Commission and the Washington State Commission on Pesticide Registration. We thank Michael D. Doerr for his help in initiating this study and standardizing various bioassays performed in this study, and Kathleen Pierre for her assistance in rearing the field-collected populations and maintaining a colony of OBLR in the laboratory.

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Tables and Figures

**Table 1.** Baseline toxicity of various compounds to obliquebanded leafroller neonate larvae (5\textsuperscript{th} instar in case of pyriproxyfen) from a susceptible laboratory colony.

<table>
<thead>
<tr>
<th>Compound</th>
<th>n</th>
<th>Slope (± SE)</th>
<th>LC\textsubscript{50} (ppm) (95% FL)</th>
<th>LC\textsubscript{90} (ppm) (95% FL)</th>
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<tbody>
<tr>
<td>Chlorantraniliprole</td>
<td>180</td>
<td>1.51 (0.18)</td>
<td>0.12 (0.08-0.18)</td>
<td>0.87 (0.53-1.80)</td>
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<tr>
<td>Spinetoram</td>
<td>300</td>
<td>1.86 (0.18)</td>
<td>0.06 (0.03-0.14)</td>
<td>0.32 (0.12-2.00)</td>
</tr>
<tr>
<td>Spinosad</td>
<td>180</td>
<td>1.78 (0.24)</td>
<td>0.16 (0.11-0.24)</td>
<td>0.84 (0.50-1.96)</td>
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<tr>
<td>Emamectin benzoate</td>
<td>1000</td>
<td>3.25 (0.32)</td>
<td>0.06 (0.05-0.07)</td>
<td>0.15 (0.12-0.18)</td>
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<tr>
<td>Novaluron</td>
<td>1350</td>
<td>1.00 (0.11)</td>
<td>14.90 (4.72-27.51)</td>
<td>284.80 (165.61-738.25)</td>
</tr>
<tr>
<td>Pyriproxyfen (5\textsuperscript{th} instar males)</td>
<td>600</td>
<td>1.28 (0.15)</td>
<td>2.35 (1.03-3.98)</td>
<td>23.50 (12.90-70.41)</td>
</tr>
<tr>
<td>Pyriproxyfen (5\textsuperscript{th} instar females)</td>
<td>600</td>
<td>1.53 (0.22)</td>
<td>4.81 (1.70-8.42)</td>
<td>33.06 (17.28-104.92)</td>
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<td>Azinphosmethyl</td>
<td>210</td>
<td>5.10 (1.14)</td>
<td>1.96 (1.44-2.43)</td>
<td>3.49 (2.77-5.31)</td>
</tr>
</tbody>
</table>

n = number of larvae assayed

1 Mortality was assessed at 7 d of exposure (Except for pyriproxyfen in which case data presented here represent mortality assessments made at adult eclosion)

2 95% fiducial limits estimated using POLO (LeOra Software 1987)
Table 2. Delayed toxicity of various compounds to obliquebanded leafroller neonate larvae from a susceptible laboratory colony.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Time of mortality assessment</th>
<th>n</th>
<th>Slope (± SE)</th>
<th>LC₅₀ (ppm) (95% FL)</th>
<th>LC₉₀ (ppm) (95% FL)²</th>
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<tbody>
<tr>
<td>Chlorantraniliprole</td>
<td>4 d</td>
<td>1800</td>
<td>0.78 (0.04)</td>
<td>0.28 (0.14-0.50)</td>
<td>12.07 (5.77-33.39)</td>
</tr>
<tr>
<td></td>
<td>11 d</td>
<td>1800</td>
<td>1.13 (0.06)</td>
<td>0.08 (0.04-0.13)</td>
<td>1.08 (0.59-2.56)</td>
</tr>
<tr>
<td>Spinetoram</td>
<td>4 d</td>
<td>1350</td>
<td>1.22 (0.61)</td>
<td>0.07 (0.02-0.19)</td>
<td>0.84 (0.31-5.47)</td>
</tr>
<tr>
<td></td>
<td>11 d</td>
<td>1350</td>
<td>3.51 (0.37)</td>
<td>0.05 (0.04-0.06)</td>
<td>0.12 (0.10-0.15)</td>
</tr>
<tr>
<td>Emamectin benzoate</td>
<td>7 d</td>
<td>1000</td>
<td>3.25 (0.32)</td>
<td>0.06 (0.05-0.07)</td>
<td>0.16 (0.12-0.18)</td>
</tr>
<tr>
<td></td>
<td>14 d</td>
<td>1000</td>
<td>1.26 (0.11)</td>
<td>0.006 (0.004-0.009)</td>
<td>0.07 (0.05-0.10)</td>
</tr>
<tr>
<td>Novaluron</td>
<td>7 d</td>
<td>1350</td>
<td>1.00 (0.12)</td>
<td>14.91 (4.71-27.51)</td>
<td>284.80 (165.61-738.25)</td>
</tr>
<tr>
<td></td>
<td>14 d</td>
<td>1350</td>
<td>0.98 (0.09)</td>
<td>4.73 (1.07-10.30)</td>
<td>95.86 (49.74-275.81)</td>
</tr>
</tbody>
</table>

n = number of larvae assayed

¹ Mortality was assessed at 4 d of exposure, then again at 11 d including 7 d of recovery period (Except for emamectin benzoate and novaluron where mortality was assessed at 7 d of exposure, then again at 14 d including 7 d of recovery period)

² 95% fiducial limits estimated using POLO (LeOra Software 1987)
Table 3. Toxicity of chlorantraniliprole to neonate and third instar obliquebanded leafrollers from a susceptible laboratory colony.

<table>
<thead>
<tr>
<th>Larval age</th>
<th>Time of mortality assessment</th>
<th>n</th>
<th>Slope (± SE)</th>
<th>LC$_{50}$ (ppm) (95% FL)</th>
<th>LC$_{90}$ (ppm) (95% FL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neonate</td>
<td>7 d</td>
<td>1200</td>
<td>2.20 (0.16)</td>
<td>0.12 (0.10-0.15)</td>
<td>0.46 (0.35-0.66)</td>
</tr>
<tr>
<td></td>
<td>AE</td>
<td>1200</td>
<td>2.12 (0.17)</td>
<td>0.05 (0.04-0.07)</td>
<td>0.21 (0.16-0.32)</td>
</tr>
<tr>
<td>Third instar</td>
<td>7 d</td>
<td>1200</td>
<td>1.95 (0.11)</td>
<td>0.04 (0.03-0.05)</td>
<td>0.19 (0.15-0.26)</td>
</tr>
<tr>
<td></td>
<td>AE</td>
<td>1200</td>
<td>1.56 (0.08)</td>
<td>0.02 (0.01-0.03)</td>
<td>0.10 (0.05-0.38)</td>
</tr>
</tbody>
</table>

n = number of larvae assayed

AE = adult eclosion

1 Mortality was assessed at 7 d of exposure, then again at adult eclosion

2 95% fiducial limits estimated using POLO (LeOra Software 1987)
**Table 4.** Toxicity of spinetoram to neonate, third instar, and fifth instar obliquebanded leaf rollers from a susceptible laboratory colony.

<table>
<thead>
<tr>
<th>Larval age</th>
<th>Time of mortality</th>
<th>n</th>
<th>Slope (± SE)</th>
<th>LC₅₀ (ppm) (95% FL)¹</th>
<th>LC₉₀ (ppm) (95% FL)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neonate</td>
<td>7 d</td>
<td>1050</td>
<td>2.72 (0.25)</td>
<td>0.03 (0.02-0.04)</td>
<td>0.08 (0.06-0.12)</td>
</tr>
<tr>
<td></td>
<td>AE</td>
<td>700</td>
<td>3.92 (0.53)</td>
<td>0.01 (0.01-0.02)</td>
<td>0.03 (0.02-0.04)</td>
</tr>
<tr>
<td>Third instar</td>
<td>7 d</td>
<td>1050</td>
<td>2.64 (0.17)</td>
<td>0.02 (0.017-0.024)</td>
<td>0.06 (0.05-0.08)</td>
</tr>
<tr>
<td></td>
<td>AE</td>
<td>750</td>
<td>2.81 (0.24)</td>
<td>0.01 (0.009-0.012)</td>
<td>0.03 (0.025-0.04)</td>
</tr>
<tr>
<td>Fifth instar (♂)</td>
<td>AE</td>
<td>140</td>
<td>1.65 (0.26)</td>
<td>0.02 (0.01-0.03)</td>
<td>0.11 (0.06-0.27)</td>
</tr>
<tr>
<td>Fifth instar (♀)</td>
<td>AE</td>
<td>140</td>
<td>2.32 (0.38)</td>
<td>0.03 (0.01-0.06)</td>
<td>0.10 (0.05-0.54)</td>
</tr>
</tbody>
</table>

n = number of larvae assayed

AE = adult eclosion

¹ Mortality was assessed at 7 d of exposure, then again at adult eclosion

² 95% fiducial limits estimated using POLO (LeOra Software 1987)
Table 5. Instar toxicity ratios of various larval instars of obliquebanded leafroller from a susceptible laboratory colony exposed to chlorantraniliprole and spinetoram.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Time of mortality assessment</th>
<th>Instar toxicity ratio</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3&lt;sup&gt;rd&lt;/sup&gt; / neonate&lt;sup&gt;2&lt;/sup&gt;</td>
<td>5&lt;sup&gt;th&lt;/sup&gt; / neonate&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Chlorantraniliprole</td>
<td>7 d</td>
<td>0.36 (0.29-0.44)*</td>
<td>---</td>
</tr>
<tr>
<td>AE</td>
<td>0.28 (0.22-0.36)*</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Spinetoram</td>
<td>7 d</td>
<td>0.73 (0.60-0.88)*</td>
<td>---</td>
</tr>
<tr>
<td>AE</td>
<td>0.77 (0.62-0.95)*</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>AE (5&lt;sup&gt;th&lt;/sup&gt; instar ♂)</td>
<td>---</td>
<td>1.32 (0.83-2.10)</td>
<td>1.72 (1.08-2.72)*</td>
</tr>
<tr>
<td>AE (5&lt;sup&gt;th&lt;/sup&gt; instar ♀)</td>
<td>---</td>
<td>2.08 (1.41-3.08)*</td>
<td>2.70 (1.84-3.98)*</td>
</tr>
</tbody>
</table>

n = number of larvae assayed

AE = adult eclosion

1 Mortality was assessed at 7 d of exposure, then again at adult eclosion

2 LC<sub>50</sub> of 3<sup>rd</sup> instars / LC<sub>50</sub> of neonate larvae

3 LC<sub>50</sub> of 5<sup>th</sup> instars / LC<sub>50</sub> of neonate larvae

4 LC<sub>50</sub> of 5<sup>th</sup> instars / LC<sub>50</sub> of 3<sup>rd</sup> instars

* Toxicity ratios are significant (α = 0.05) (Robertson et al. 2007)
Fig. 1. Toxicity of chlorantraniliprole to fifth instar obliquebanded leafrollers from a susceptible laboratory colony (Percent treatment mortality adjusted for control mortality according to Abbott (1925) + SEM).
CHAPTER TWO

LETHAL AND SUBLETHAL EFFECTS OF AN INSECT GROWTH REGULATOR, PYRIPROXYFEN, ON OBLIQUEBANDED LEAFROLLER (LEPIDOPTERA: TORTRICIDAE)

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Abstract

The obliquebanded leafroller, *Choristoneura rosaceana* (Harris), is one of the most destructive pests of tree fruits in Washington. The development of insecticide resistance in *C. rosaceana* has led us to explore new management tactics. The use of very low doses of insecticides that have strong sublethal effects represents an environmentally-friendly option to improve existing integrated pest management strategies. We tested the insect growth regulator pyriproxyfen to determine its lethal and sublethal effects on growth and development of *C. rosaceana*. A leaf-disk bioassay was used to test seven concentrations of pyriproxyfen ranging from 0 to 30 ppm on 5th instar *C. rosaceana*. Male and female larvae were assessed separately for mortality as well as other parameters of growth and development. The LC$_{50}$ values for males and females were 2.4 and 4.8 ppm, respectively. The response to
pyriproxyfen was concentration-dependent: only 5-6% of the larvae treated with the highest concentration emerged as morphologically normal adults compared to 86% emergence in the controls. The pupation and adult emergence was significantly delayed at concentrations higher than 1 ppm. The weights of *C. rosaceana* pupae and adults were significantly increased whereas fecundity and fertility were significantly reduced at a sublethal concentration of 0.3 ppm. We conclude that both lethal and sublethal effects might exhibit significant impacts on the population dynamics of *C. rosaceana* in tree fruit orchards treated with low concentrations of pyriproxyfen.

**Key words:** Obliquebanded leafroller, insect growth regulator, pyriproxyfen, lethal and sublethal effects, growth and development

**Introduction**

Obliquebanded leafroller, *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae) is a native species and is widely distributed in North America (Chapman and Lienk 1971, Weires and Riedl 1991). It is a polyphagous insect with a host range of over 50 plant species including members of Rosaceae and Cornaceae (Sanderson and Jackson 1909). Although this species was collected from apples, *Malus domestica* (Borkhausen), in the late 1950’s, it was not considered to be an important pest in commercial apple orchards (Chapman and Lienk 1971). Initial outbreaks in apple orchards occurred in late 1970’s and subsequent studies have shown that it is one of the most destructive lepidopteran pests of deciduous tree fruits in Washington (Brunner 1999). *C. rosaceana* has 1 to 2 generations per year, depending on the locality. It overwinters as half grown larvae (2nd to 3rd instar) in cocoon in sheltered areas on the tree. The *C. rosaceana*
larvae complete their larval development in 5 to 6 instars with 4\textsuperscript{th}, 5\textsuperscript{th}, and 6\textsuperscript{th} instars being the most problematic. In apple, \textit{C. rosaceana} larvae feed on flower buds, leaves, and developing fruits (Howitt 1993, Ohlendorf 1999). Fruit damage from larval feeding can occur in the spring, during mid-summer (the most significant), or just prior to harvest (Beers et al. 1993). For the overwintering larvae, an economic threshold of 3\% of the fruit clusters containing live larvae is used. Economic threshold for summer generation varies from 3\% (for fresh fruit) to 10\% (for processing) infestation rate of live larvae (Beers et al. 1994). Insecticides, effective against large larvae, such as pyriproxyfen, are usually applied at petal fall. If necessary, another insecticide is applied in the summer, when most of the summer brood eggs have hatched.

Broad-spectrum insecticides such as organophosphates have provided stable management of leafrollers and other major pests of pome fruits for decades. Increased public concern over health and environmental effects of broad-spectrum insecticides as well as regulatory actions such as the Food Quality Protection Act of 1996 have led to a greater priority to the development of environmentally benign and highly selective insecticides. In this situation, insect growth regulators (IGRs) have great potential for replacing broad-spectrum insecticides because of their very selective and unique modes of action (Miyamoto et al. 1993). The principal effect of IGRs is to disturb development and metamorphosis, and expression of their activity is, therefore, much slower than that of conventional insecticides. These compounds are effective on a narrow range of sensitive stages of the insect’s life cycle with high potency against the target pests (Casida and Quistad 1998).

Juvenile hormone analogs (JHAs) are one of the major classes of IGRs that mimic the action of juvenile hormone (JH) in insect body (Dhadialla et al. 1998). Pyriproxyfen, 2-[1-
methyl-2-(4-phenoxyphenoxy) ethoxy] pyridine, is an oxime ether with strong JH activity (Hatakoshi et al. 1986). Pyriproxyfen is a synthetic JH mimic that inhibits JH synthesis in the corpora allata thus simulating the activity of endogenous JH (Baker et al. 1986). Pyriproxyfen is a relatively stable compound with very low mammalian toxicity (Yokoyama and Miller 1991). Pyriproxyfen is a highly potent JHA that results in strong suppression of embryogenesis, metamorphosis, and adult formation (Glancey et al. 1990, Koehler and Patterson 1991, Ishaaya and Horowitz 1992, Dhadialla 2005). Pyriproxyfen was first registered for controlling public health pests (Miyamoto et al. 1993), however, later it was found to be highly effective against a variety of agricultural pests including Diptera (Langley et al. 1993), Homoptera (Richardson and Lagos 2007), Dictyoptera (Koehler and Patterson 1991), Lepidoptera (Hatakoshi et al. 1986, Yokoyama and Miller 1991, Oouchi 2005), Hymenoptera (Reimer et al. 1991), Siphonaptera (Palma et al. 1993), Orthoptera (Vennard et al. 1998), and Coleoptera (Abo-Elghar et al. 2004). Pyriproxyfen has negative impacts to a broad spectrum of insects during the embryonic, last larval, or reproductive stages (Dhadialla et al. 1998). Because of its translaminar action, pyriproxyfen provides residual activity in the field (Horowitz and Ishaaya 1994, Ishaaya and Horowitz 1998).

Pyriproxyfen induces supernumerary molts because JH promotes the continued expression of immature characteristics (Hatakoshi et al. 1986, Singh and Sidhu 1992) and causes significant reduction in fecundity (Richardson and Lagos 2007). When larvae are treated with pyriproxyfen it causes imperfect formation of genitalia in the surviving adults (Kuldova et al. 1994), and production of dead and deformed neonates because of a disruption of embryonic development (Liu and Chen 2001). In addition to its strong larvicidal effects, pyriproxyfen showed significant ovicidal effects on codling moth, Cydia pomonella (L.), eggs, whereas
treatment of codling moth adults with pyriproxyfen resulted in significantly reduced egg viability (Yokoyama and Miller 1991).

Pyriproxyfen has been registered for use against codling moth and leafrollers in Washington and provides a safe alternative to OP insecticides for managing leafroller populations, especially the spring brood, in orchards (Brunner, unpublished data). Pyriproxyfen has been reported to suppress the populations of various target pests as a result of its lethal as well as sublethal effects on the target pests (Kerns and Stewart 2000, Liu and Chen 2001, Oouchi 2005, Prabhaker and Tascano 2007, Richardson and Lagos 2007). However, its effects have not been reported against the orchard pest, *C. rosaceana*. The major objective of this study was to assess the lethal as well as sublethal effects of pyriproxyfen on survival, growth and development of *C. rosaceana*. The demonstration of lethal and sublethal effects of pyriproxyfen on *C. rosaceana* could encourage growers to incorporate this reduced-risk compound into the leafroller management programs.

Materials and Methods

Insects. The *C. rosaceana* larvae used in this study were obtained from a laboratory colony, which was established by collecting larvae from apple orchards in Mattawa (Grant County), WA, in 1990. This colony has been reared continuously since their collection on a pinto bean diet following the method of Shorey and Hale (1965) under constant conditions of temperature (23 ± 2°C), relative humidity (RH, 70%), and photoperiod (16:8, L:D).

Insecticides. The material used in this study was pyriproxyfen (Esteem® 0.86EC, EPA Reg. No. 59639-95), procured from Valent USA Corporation, Walnut Creek, CA 94596, USA. This formulation also contains 1-5% naphthalene (91-20-3), and 40-50% total.
hydrocarbons. The rest of the ingredients are inert materials which are maintained as trade secrets by the manufacturer.

**Bioassays.** Pyriproxyfen is mainly effective against older larvae of *C. rosaceana*. The disruptive effects of pyriproxyfen on development appear in terms of morphogenetic abnormalities at the time of larval-pupal molt. Because, by this time, actual JH activity has been ceased in the larval body, and pyriproxyfen promotes continued expression of immature characteristics (Hatakoshi et al. 1986). That is why, 5th instar *C. rosaceana* were used in this study. Effects of pyriproxyfen on 5th instar *C. rosaceana* were determined using leaf-disk bioassay in two separate experiments. In the first experiment, 5th instar *C. rosaceana* were exposed to a range of pyriproxyfen concentrations (0-30 ppm) to assess its effects on the survival, growth and development. The concentrations used in this experiment were determined based on the results of a test bioassay conducted. A stock solution was prepared by diluting pyriproxyfen (Esteem® 0.86EC) with water to the highest concentration (30 ppm) to be used in this bioassay. Serial dilutions were then prepared from the stock solution to get the required concentrations (30, 10, 3, 1, 0.3, and 0.1 ppm). The leaves were collected from apple, *Malus domestica* Borkhausen, ‘Red Delicious’ trees in Block # 5 located at WSU, Tree Fruit Research and Extension Center Wenatchee, WA which had not been treated with any insecticide. The circular leaf-disks of 12 mm diameter were cut out of the leaves using a core sampler (leaf-disk cutter) and 10 µL droplets of pyriproxyfen dilutions were applied to each of the leaf-disks. The control leaf-disks were treated with the same volume of water (plus surfactant). A silicone surfactant (Sylgard® 309, Wilbur-Ellis Company, Fresno, CA 93755, U.S.A.) was used (50 µL/L) to facilitate even distribution of droplets on the surface of leaf-disks and drying. The leaf-disks were air-dried. Once dried, the leaf-disks were offered to a 5th
instar *C. rosaceana* in a small petri dish (Falcon 1006, 50x9 mm, Becton Dickinson Labware, Becton Dickinson and Company, Franklin Lakes, NJ 07417, U.S.A.). The petri dishes, each containing a leaf-disk and a 5th instar *C. rosaceana* larva, were placed in a growth chamber held at constant conditions of temperature (23 ± 2°C), relative humidity (RH, 70%), and photoperiod (16:8, L:D). After 24 h of exposure, the larvae that consumed the entire leaf-disk were transferred to untreated pinto bean diet and reared in the laboratory following the method of Shorey and Hale (1965) under constant conditions of temperature (23 ± 2°C), relative humidity (RH, 70%), and photoperiod (16:8, L:D) until they emerged as adults or died in larval or pupal stage. The 39 male larvae and 19 female larvae were excluded from the study because they were unable to consume the entire leaf disk. The *C. rosaceana* larvae used in this study were selected from the LAB colony not more than 24 hours after molting into 5th instar. The male 5th instar *C. rosaceana* were separated from the females based on the presence of gonads visible from the dorsal side of the abdomen. Twenty male and twenty female larvae were treated at each concentration separately, and all treatments were replicated five times. New pyriproxyfen dilutions were prepared for each replication. For each larva, observations were recorded daily for mortality or growth and development parameters of normal larvae, abnormal larvae, normal pupae, abnormal pupae, normal adults, abnormal adults, and longevity.

In the second experiment, 5th instar *C. rosaceana* were exposed to a sublethal concentration of pyriproxyfen (0.3 ppm) to assess its effects on the development and reproduction. A leaf-disk bioassay was set up exactly the same way as in the first experiment, but only one concentration of pyriproxyfen was used in addition to the untreated control. The concentration of pyriproxyfen used in the second experiment was selected based on results of
the first experiment, and it was the highest concentration at which no significant lethal effects were observed in the first experiment. One hundred male and one hundred female larvae were treated with the selected sublethal concentration of pyriproxyfen and the same numbers of both male and female larvae were treated with water plus surfactant to serve as untreated control. All treatments were replicated five times. For each larva, observations were recorded for pupal weight upon pupation and adult weight upon adult emergence. After recording adult weights, twenty five pairs of adult males and females were set up for mating in each of the following four treatment combinations: Untreated male X Untreated female, Untreated male X Treated female, Treated male X Untreated female, and Treated male X Treated female. For each mating pair, observations were then recorded for fecundity, fertility, percent egg hatch, and number of egg masses.

**Data Analysis.** Lethal concentration values ($LC_{50}$ and $LC_{90}$) and their corresponding 95% fiducial limits (FL) were estimated using POLO (LeOra Software 1987). The significance of difference in $LC_{50}$ values among male and female *C. rosaceana* was calculated using lethal concentration ratio (LCR) significance test (Robertson et al. 2007). Data recorded from both experiments on parameters with three or more treatments, such as time to pupation, time to adult emergence, normal pupation, normal adult emergence, fecundity, fertility, percent egg hatch, and egg masses, were analyzed using analysis of variance (ANOVA) (Proc Mixed, SAS Institute 1999). Data recorded from the second experiment on parameters with only two treatments, such as pupal weight and adult weight, were analyzed using *t* test assuming equal variances. Means for each parameter were separated using Tuckey’s honestly significant difference (HSD) test. Significance was accepted at $\alpha = 0.05$ in all statistical tests used in this study.
Results

Pyriproxyfen treatment significantly affected the survival of 5th instar *C. rosaceana* of both sexes. The data on mortality of both males and females indicated a good fit to the probit model (Pearson’s chi-square test; *P* > 0.05) with the LC$_{50}$ values of 2.35 and 4.81 ppm for males and females, respectively (Table 1). Based on LCR significance test, the female 5th instar *C. rosaceana* were significantly less susceptible to pyriproxyfen than the males with 2.04-fold higher LC$_{50}$ values. The slopes of the probit lines for both males and females were similar suggesting that both males and females had relatively similar variation in their susceptibility to pyriproxyfen.

The 5th instar *C. rosaceana* treated with pyriproxyfen initially developed certain morphological abnormalities in a dose dependent manner and then died. The major morphogenetic effects of pyriproxyfen treatment observed in *C. rosaceana* larvae in this study include abortive molting, larval-pupal intermediates with precocious evagination of wing disks, deformed pupae, and larval-adult intermediates that were unable to give rise to normal adults. The morphological abnormalities observed in *C. rosaceana* adults include partial eclosion, incomplete development of wings and genitalia, and wing twisting. The percentage of treated 5th instar *C. rosaceana* that were able molt into normal pupae was significantly reduced in a concentration-dependent manner in males (F = 64.2; df = 6, 28; *P* < 0.001) as well as females (F = 88.3; df = 6, 28; *P* < 0.001) (Table 2). In males, only 14% showed normal pupation at the highest concentration (30 ppm) as compared 97% in the untreated control, while in females only 7% showed normal pupation as compared to 96% in the
untreated. There was no significant difference in normal pupation between males and females (F = 0.00; df = 1, 68; P = 0.973).

Normal adult emergence was significantly reduced in males (F = 23.4; df = 6, 28; P < 0.001) and females (F = 16.5; df = 6, 28; P < 0.001) in a concentration-dependent manner (Table 2). The percentage of 5th instar *C. rosaceana* treated with pyriproxyfen that emerged as normal adults was 6% at the highest concentration compared to 86% in the untreated control while in females only 5% emerged as normal adults compared to 86% in the untreated control. The normal adult emergence was statistically similar in both sexes (F = 0.24; df = 1, 68; P = 0.629).

In addition to its lethal effects, pyriproxyfen significantly affected the longevity of both sexes in a concentration-dependent manner (Table 3). The time to pupation in males was increased from 5 days in the untreated control to 16 days at the highest concentration (F = 11.9; df = 6, 28; P < 0.001) while in females it increased from 6 days in the untreated control to 16 days at the highest concentration (F = 79.2; df = 6, 28; P < 0.001). Similarly, time to adult emergence was significantly increased from 20 days in the untreated control to 35 days at the highest concentration in males (F = 13.3; df = 6, 28; P < 0.001) while in females it increased from 22 days in the control as compared to 38 days. However, no differences were observed in time to pupation (F = 0.47; df = 1, 68; P = 0.496) or to adult emergence (F = 0.98; df = 1, 68; P = 0.325) between sexes.

The treatment of 5th instar *C. rosaceana* with a sublethal concentration of pyriproxyfen (0.03 ppm - ≈LC$_{10}$) significantly increased the pupal weight of both males (t = 14.3; df = 845; P < 0.001) and females (t = 13.1; df = 879; P < 0.001) (Table 4). The average pupal weight of the treated males was 69.8 mg as compared to 56.7 mg in the untreated control while treated
female pupae weighted 103.2 mg as compared to 85.0 mg in the untreated control. Average adult weight was also higher in treated versus untreated males, 22.6 mg to 32.5 mg ($t = -19.6; \text{df} = 873; P < 0.001$) and 49.0 mg to 61.7 mg females ($t = -13.2; \text{df} = 876; P < 0.001$) (Table 4).

The sublethal concentration of pyriproxyfen significantly affected fecundity ($F = 68.5; \text{df} = 3, 465; P < 0.001$), fertility ($F = 204.6; \text{df} = 3, 465; P < 0.001$), percent egg hatch ($F = 186.9; \text{df} = 3, 465; P = 0.001$) and number of egg masses per female ($F = 55.5; \text{df} = 3, 465; P = 0.001$) of $C. \text{rosaceana}$ (Table 5). The average number of eggs laid per female (fecundity) was 210 where both males and females were treated as compared to 601 in the untreated control. Fertility, the average number of eggs hatched per female, also decreased from 511 in the untreated control to 54 where both males and females were treated. The percent egg hatch decreased from 78.0% in the untreated control to 16.4% where both males and females were treated. The average number of egg masses produced per female was reduced from 4.4 in the untreated control to 1.7 where both males and females were treated. The average fecundity, fertility and number of egg masses were statistically similar in treatments where only the male or female parent was treated. These values were intermediate between those for the untreated control and where both parents were treated. However, percent egg hatch was significantly lower where only males were treated as compared to the treatment where only females were treated (Table 5).

**Discussion**

Our results indicate strong lethal effects of pyriproxyfen on $C. \text{rosaceana}$ larvae from a laboratory colony. The treatment of 5\textsuperscript{th} instars with pyriproxyfen caused significant reduction
in survival of *C. rosaceana* with males being > 2-fold more susceptible than females at median lethal concentration. These results are in agreement with those of Rill et al. (2007) where males of California red scale were 12-fold more susceptible to pyriproxyfen than females. The higher male susceptibility could be due to physiological differences between males and females. The higher susceptibility of males might create a problem for growers using pheromone trapping to estimate population density of *C. rosaceana* in orchards and to determine whether insecticide applications are needed. Because of higher susceptibility of males to pyriproxyfen, pheromone trapping in pyriproxyfen treated orchards could underestimate densities of female *C. rosaceana*. On the positive side, higher male mortality will directly decrease the number of mated females which will suppress the *C. rosaceana* populations in the orchards over time.

The morphogenetic effects of pyriproxyfen we observed are consistent with the disruption of physiological processes in holometabolous insects, the absence of JH thus inducing a commitment for larval-pupal molt mediated by 20-hydroxy ecdysone (Riddiford 1996). The persistence of JH or JHA, such as fenoxy carb or pyriproxyfen, during the critical time in life cycle interrupts the normal commitment for the larval-pupal molt and results in larval-pupal or larval-adult intermediates and other morphological abnormalities. Similar morphogenetic effects have been reported in *Epiphyas postvittana* (McGhie and Tomkins 1988), *Adoxophyes orana* (Charmillot 1989, Charmillot et al. 1994), *Helicoverpa virescens* (Mauchamp et al. 1989), and *C. fumiferana* (Mulye and Gordon 1989, Hicks and Gordon 1992), as a result of treatment with fenoxy carb, a JHA similar to pyriproxyfen. The treatment of *Plutella xylostella* (Ouchi 2005) and *Aphis glycines* (Richardson and Lagos 2007) with pyriproxyfen also resulted in morphological abnormalities similar to those observed in this study.
The production of supernumerary larval-larval molts leading to delayed pupation and adult emergence in larvae treated with JHAs is well documented (Hatakoshi et al. 1986, Mauchamp et al. 1989, Gadenne et al. 1990). The significant increase in time to pupation and adult emergence observed in our study could be attributed to the activity of pyriproxyfen as a strong JHA (Hatakoshi et al. 1986) allowing continued expression of larval characteristics (Wigglesworth 1934, Bauerfeind and Chapman 1984). Similarly, the increase in time to pupation was observed in males of tufted apple bud moth, *Platynota idaeusalis* after treatment with fenoxycarb (Biddinger and Hull 1999). However, the opposite effect has been reported in some studies with hemimetabolous insects where reduction in longevity was observed in *Lipaphis erysimi* (Liu and Chen 2001) and *Aphis glycines* (Richardson and Lagos 2007) after treatment with pyriproxyfen.

The prolonged duration of larval stage as a result of exposure to fenoxycarb caused production of oversized larvae and the larval weight was significantly increased due to occurrence of supernumerary molts (Mauchamp et al. 1989). In our study, larval weight was not recorded, but the pupal as well as adult weight was significantly increased in both sexes of *C. rosaceana* after treatment with a sublethal concentration of pyriproxyfen. Biddinger and Hull (1999) also observed significant increase in pupal weight of female *P. idaeusalis* as a result of exposure to a sublethal concentration of fenoxycarb. Although the larger larvae were produced as a result of pyriproxyfen treatment, their diet consumption was not higher than that of the untreated normal larvae (AAS and JFB, unpublished data), which could be attributed to the internal physiological abnormalities caused by pyriproxyfen treatment. However, the major advantage of pyriproxyfen treatment would be evident in the terms of
highly suppressed populations of *C. rosaceana* in the following generation as a result of its lethal and sublethal effects.

The treatment with JHAs has been known to affect the reproductive potential of insects (Dhadialla et al. 1998). In our study, pyriproxyfen treatment significantly reduced the fecundity, fertility, percent egg hatch and number egg masses per female in *C. rosaceana* regardless of whether the male or female parent had been treated. Similar effects were observed in *C. occidentalis* (Robertson and Kimball 1979), *Trialeurodes vaporarium* (Natskova 1988), *Blattella germanica* (King and Bennet 1990), *C. fumiferana* (Hicks and Gordon 1992), *Spodoptera litura* (Hatakoshi 1992, Nomura and Miyata 2000), and *A. glycines* (Richardson and Lagos 2007) as a result of treatment with JHAs. Our data suggest that when both parents were treated there may have been an additive effect on reproductive parameters, which is in agreement with the observations made by Biddinger and Hull (1999) in *P. idaeusalis* as a result of exposure to fenoxycarb. However, the observation of lower percent egg hatch in mating pairs where only males were treated contrasts with the findings of Biddinger and Hull (1999) where effects were stronger in females.

The molecular characteristics of the receptor proteins by which JH or JHAs manifest their activity and the mechanisms by which JHAs reduce fecundity and fertility are still poorly understood, especially the mechanisms by which the application of JHAs to males affects the fecundity or fertility of their female partners. Oviposition in *S. litura* was inhibited as a result of pyriproxyfen treatment due to the lack of release of an oviposition stimulating factor that was present in the hemolymph of mated untreated females (Hatakoshi 1992). The JHAs could block embryonic development if transferred directly to the eggs (Retnakaran et al. 1985). A chitin synthesis inhibitor, diflubenzuron, was transferred to the eggs of house fly from the
sperms of treated males (Ivie and Wright 1978, Chang and Barkovec 1990). Likewise, the effects of male treatment on the reproduction of untreated females observed in the current study may indicate transfer of pyriproxyfen to the eggs via sperm.

Our findings demonstrate that pyriproxyfen was highly effective against *C. rosaceana* in the laboratory by causing not only direct mortality but also disrupting normal growth and development, and reducing their reproductive potential. These results indicate that pyriproxyfen has a strong potential to suppress *C. rosaceana* populations through its lethal as well as sublethal effects, and thus provides a powerful tool to manage *C. rosaceana* in the orchards, possibly at concentrations that minimize impacts on non-target beneficial insects. Because pyriproxyfen has been shown to have adverse effects on some natural enemies (Mendel et al. 1994, Hattingh and Tate 1995, Liu and Chen 2002), but not the others (McMullen 1990, Liu and Stansly 1997, Ellsworth and Martinez-Carillo 2001, Naranjo 2001, Naranjo et al. 2004, Cloyd and Dickinson 2006), it should be incorporated into a management program that minimizes its impact on local natural enemies. Further studies are needed in the laboratory to reveal the mechanisms by which pyriproxyfen causes its lethal as well as sublethal effects, and in the field to further understand the impact of cumulative effects of pyriproxyfen on population dynamics of *C. rosaceana*.

**Acknowledgments**

This study was sponsored by the Washington Tree Fruit Research Commission and the Washington State Commission on Pesticide Registration. We thank Michael D. Doerr for his help in initiating this study and Dr. John E. Dunley for his comments on the manuscript, and
Kathleen Pierre for her assistance in rearing the field-collected populations and maintaining a colony of OBLR in the laboratory.

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fenoxycarb after treatment of the fourth and the fifth instars of the tobacco budworm,
Heliothis virescens F. Pestic. Sci. 26: 283-301.

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**Tables and Figures**

**Table 1:** Results of probit analyses for pyriproxyfen using leaf disk bioassay on 5\textsuperscript{th} instar *C. rosaceana* from a laboratory population

<table>
<thead>
<tr>
<th>Population</th>
<th>( n )</th>
<th>Slope (± SE)</th>
<th>( \chi^2 )</th>
<th>( LC_{50} ) (ppm)</th>
<th>( LC_{90} ) (ppm)</th>
<th>LCR(^2) (95% CL)(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>561</td>
<td>1.28 (0.15)</td>
<td>2.14</td>
<td>2.35 (1.53-3.27)</td>
<td>23.50 (15.94-40.72)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>581</td>
<td>1.53 (0.22)</td>
<td>4.51</td>
<td>4.81 (1.70-8.42)</td>
<td>33.06 (17.28-65.78)</td>
<td>2.04 (1.22-3.42)*</td>
</tr>
</tbody>
</table>

\( n = \) number of larvae assayed

\(^1\) 95\% fiducial limits estimated using POLO (LeOra Software 1987)

\(^2\) LCR, lethal concentration ratio = \( LC_{50} \) (Females)/\( LC_{50} \) (Males)

\(^3\) 95\% confidence limits estimated using lethal concentration ratio significance test (Robertson et al. 2007)

* \( LC_{50} \) of females significantly different (\( \alpha = 0.05 \)) from that of the male population (Robertson et al. 2007)
Table 2: Percent normal pupation and adult emergence of *C. rosaceana* treated as 5\textsuperscript{th} instar with pyriproxyfen using leaf disk bioassay

<table>
<thead>
<tr>
<th>Concentration (ppm)</th>
<th>Normal pupation\textsuperscript{a} (%)</th>
<th>Normal adult emergence\textsuperscript{b} (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Mean ± SEM)</td>
<td>(Mean ± SEM)</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>0</td>
<td>96.9 ± 1.28a</td>
<td>95.9 ± 1.89a</td>
</tr>
<tr>
<td>0.1</td>
<td>98.0 ± 1.22a</td>
<td>90.8 ± 1.08b</td>
</tr>
<tr>
<td>0.3</td>
<td>96.7 ± 1.39a</td>
<td>93.0 ± 3.74ab</td>
</tr>
<tr>
<td>1</td>
<td>76.2 ± 5.22b</td>
<td>83.0 ± 3.00b</td>
</tr>
<tr>
<td>3</td>
<td>47.8 ± 6.18c</td>
<td>60.9 ± 4.29c</td>
</tr>
<tr>
<td>10</td>
<td>19.6 ± 5.17d</td>
<td>20.6 ± 6.55d</td>
</tr>
<tr>
<td>30</td>
<td>13.7 ± 7.22d</td>
<td>6.6 ± 4.27c</td>
</tr>
</tbody>
</table>

Means within columns not followed by the same letter are significantly different from each other (\(\alpha = 0.05\); Tukey’s HSD test)

\textsuperscript{a} Normal pupation, percentage of 5\textsuperscript{th} instar *C. rosaceana* treated with pyriproxyfen that managed to transform into normal pupae

\textsuperscript{b} Normal adult emergence, percentage of 5\textsuperscript{th} instar *C. rosaceana* treated with pyriproxyfen that managed to emerge as normal adults
Table 3: Effects of pyriproxyfen on longevity of *C. rosaceana* treated as 5\textsuperscript{th} instar using leaf disk bioassay

<table>
<thead>
<tr>
<th>Concentration (ppm)</th>
<th>Time to pupation(^a) (days)</th>
<th>Time to adult emergence(^b) (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male (Mean ± SEM)</td>
<td>Female (Mean ± SEM)</td>
</tr>
<tr>
<td>0</td>
<td>5 ± 0.2a</td>
<td>6 ± 0.2a</td>
</tr>
<tr>
<td>0.1</td>
<td>6 ± 0.2a</td>
<td>7 ± 0.2ab</td>
</tr>
<tr>
<td>0.3</td>
<td>6 ± 0.4a</td>
<td>7 ± 0.3ab</td>
</tr>
<tr>
<td>1</td>
<td>7 ± 0.4a</td>
<td>8 ± 0.6b</td>
</tr>
<tr>
<td>3</td>
<td>8 ± 0.8a</td>
<td>9 ± 0.5c</td>
</tr>
<tr>
<td>10</td>
<td>13 ± 1.8b</td>
<td>11 ± 0.4d</td>
</tr>
<tr>
<td>30</td>
<td>16 ± 2.3b</td>
<td>16 ± 0.2e</td>
</tr>
</tbody>
</table>

Means within columns not followed by the same letter are significantly different from each other (\(\alpha = 0.05\); Tukey’s HSD test)

\(^a\) Time to pupation, number of days from treatment to pupation

\(^b\) Time to adult emergence, number of days from treatment to adult emergence
**Table 4:** Pupal weight and adult weight of *C. rosaceana* treated with a sublethal concentration (~LC₁₀) of pyriproxyfen as 5<sup>th</sup> instar using leaf disk bioassay

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pupal weight&lt;sup&gt;a&lt;/sup&gt; (mg)</th>
<th>Adult weight&lt;sup&gt;b&lt;/sup&gt; (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Mean ± SEM)</td>
<td>(Mean ± SEM)</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Untreated</td>
<td>56.7 ± 0.59&lt;sup&gt;a&lt;/sup&gt;</td>
<td>85.0 ± 0.87&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Treated</td>
<td>69.8 ± 0.70&lt;sup&gt;b&lt;/sup&gt;</td>
<td>103.2 ± 1.10&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Means within columns not followed by the same letter are significantly different from each other (α = 0.05; *t*-Test)

<sup>a</sup>Pupal weight, weight of *C. rosaceana* pupae recorded within 24 h of pupation

<sup>b</sup>Adult weight, weight of *C. rosaceana* adults recorded within 24 h of adult emergence
**Table 5:** Fecundity, fertility, percent egg hatch, and number of egg masses per female of *C. rosaceana* treated with a sublethal concentration (≈LC$_{10}$) of pyriproxyfen s 5th instar using leaf disk bioassay

<table>
<thead>
<tr>
<th>Treatment Combination</th>
<th>Fecundity$^a$ (Mean ± SEM)</th>
<th>Fertility$^b$ (Mean ± SEM)</th>
<th>Egg hatch$^c$ (%) (Mean ± SEM)</th>
<th>Egg masses$^d$ (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$U_{Male} \times U_{Female}$</td>
<td>601 ± 22.6a</td>
<td>511 ± 21.5a</td>
<td>78.0 ± 2.29a</td>
<td>4.4 ± 0.15a</td>
</tr>
<tr>
<td>$U_{Male} \times T_{Female}$</td>
<td>345 ± 21.6b</td>
<td>148 ± 14.4b</td>
<td>31.7 ± 2.26b</td>
<td>2.3 ± 0.16b</td>
</tr>
<tr>
<td>$T_{Male} \times U_{Female}$</td>
<td>330 ± 20.1b</td>
<td>105 ± 10.2bc</td>
<td>22.5 ± 1.91c</td>
<td>2.4 ± 0.18b</td>
</tr>
<tr>
<td>$T_{Male} \times T_{Female}$</td>
<td>210 ± 15.0c</td>
<td>54 ± 7.0c</td>
<td>16.4 ± 1.74c</td>
<td>1.7 ± 0.14c</td>
</tr>
</tbody>
</table>

U, untreated; T, treated with pyriproxyfen

Means within columns not followed by the same letter are significantly different from each other (α = 0.05; Tukey’s HSD test)

$a$ Fecundity, number of eggs laid per adult female

$b$ Fertility, number of eggs hatched per adult females

$c$ Egg hatch, percent egg hatch

$d$ Egg masses, number of egg masses produced per adult females
CHAPTER THREE

TOXICITY AND RESIDUAL EFFICACY OF CHLORANTRANILIPROLE, SPINETORAM AND EMAMECTIN BENZOATE TO OBLIQUEBANDED LEAFROLLER (LEPIDOPTERA: TORTRICIDAE)

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Abstract

Studies were conducted to determine the residual toxicity of spinetoram, chlorantraniliprole and emamectin benzoate to obliquebanded leafroller, Choristoneura rosaceana (Harris). Larvae were exposed to apple foliage collected at different intervals after an airblast sprayer application at the manufacturer recommended field rate and half the field rate. A mortality of 100% was recorded at field rate applications of spinetoram, chlorantraniliprole, and emamectin benzoate through 59, 38, and 10 days after treatment (DAT), respectively. Significantly less foliage was consumed by C. rosaceana larvae surviving in the emamectin benzoate, chlorantraniliprole, and spinetoram treatments compared to those exposed to untreated foliage ($P < 0.05$). Third instar C. rosaceana exposed to fresh residues on terminal foliage showed 100% mortality after 5 d exposure to spinetoram residues and after 10 d
exposure to chlorantraniliprole and emamectin benzoate. The effect of larval movement from foliage with fresh residues was examined by transferring neonate larvae from foliage treated with spinetoram, chlorantraniliprole, or emamectin benzoate to untreated foliage after various exposure intervals. An exposure of 1, 3, and 6 d was required for spinetoram, chlorantraniliprole, and emamectin benzoate to cause 100% mortality at field rate, respectively. The higher the concentration of chlorantraniliprole and emamectin benzoate, the less exposure time was necessary to cause high levels of mortality in *C. rosaceana* neonates. Our results indicate that these novel insecticides are highly toxic to *C. rosaceana* larvae. Implications of these results for *C. rosaceana* management programs are discussed.

**Key words:** *Choristoneura rosaceana*, chlorantraniliprole, spinetoram, residues, exposure

**Introduction**

Obliquebanded leafroller (OBLR), *Choristoneura rosaceana* (Harris), is a polyphagous insect with a broad host range of plant species (Chapman and Lienk 1971) including members of family Rosaceae and Cornaceae (Sanderson and Jackson 1909). It is one of the major pests of deciduous pome fruits in the United States and Canada (Brunner 1999) and can cause economic damage in apple *Malus domestica* (Borkhausen) (Madsen and Madsen 1980). In apple orchards, *C. rosaceana* larvae feed on flower buds, leaves, and developing fruits (Howitt 1993, Ohlendorf 1999). Fruit damage from larval feeding can occur in the spring (after bloom), during mid-summer (when the most significant fruit injury occurs), or just prior to harvest (Beers et al. 1993). Leafroller control in commercial orchards in Washington has been achieved primarily through the use of broad-spectrum insecticides such as
organophosphates, including azinphosmethyl, chlorpyrifos, parathion, and methyl parathion for over four decades (Beers et al. 1996, Brunner 1999). However, reliance on these insecticides has resulted in the development of resistance to azinphosmethyl and other insecticides in some populations of *C. rosaceana* (Reissig et al. 1986, Brunner 1996, Lawson et al. 1997, Waldstein et al. 1999, Ahmad et al. 2002, Smirle et al. 2002, Dunley et al. 2006). In addition, the occurrence of cross-resistance between azinphosmethyl and insecticides from other classes such as tebufenozide and methoxyfenozide (Dunley et al. 2006) has made it difficult for growers to manage *C. rosaceana* in orchards.

The development of insecticide resistance as well as restrictions on the use of organophosphate insecticides as a result of implementation of the Food Quality Protection Act of 1996 (FQPA) (USEPA 1996) have spurred interest of apple growers in incorporating other insecticides into *C. rosaceana* management programs. A number of insecticides including insect growth regulators, spinosad and emamectin benzoate are available for leafroller control. In addition, chlorantraniliprole and spinetoram are two reduced-risk insecticides (USEPA 1997) with novel chemistries that were recently registered for *C. rosaceana* control in apple orchards.

Emamectin benzoate (Proclaim®) is an effective broad-spectrum lepidopteran insecticide (Dybas et al. 1989, Trumble et al. 1987, Loriatti et al. 2009a) derived from the avermectin family, which belongs to insecticide resistance action committee (IRAC) mode of action class 6 (IRAC 2009). Avermectins are naturally occurring macrocyclic lactones isolated from fermentation products of the soil micro-organism *Streptomyces avermitilis* M S & D MA-4680 strain (Ishaaya et al. 2002). They bind with high affinity to sites in the head and muscle neuronal membranes of various insect species (Deng et al. 1992, Rohrer et al. 1995) thereby
acting as agonists for GABA-gated chloride channels (Mellin et al. 1983, Albrecht et al. 1987). Emamectin benzoate causes irreversible activation of chloride channels in the nervous system of insects, resulting in prevention of muscle contraction, cessation of feeding and death. Because of its rapid decomposition (Crouch and Feely 1995), contact activity of emamectin benzoate against beneficial arthropods on the leaf surfaces is limited to very short period of time, allowing selective control of some lepidopteran pests (Chukwudebe et al. 1997) including codling moth and leafrollers (Loriatti et al. 2009a).

Chlorantraniliprole (Rynaxypyr™/Altacor®) is a novel anthranilic diamide insecticide which belongs to insecticide resistance action committee (IRAC) mode of action class 28 (IRAC 2009). Insecticides in this class selectively bind to the ryanodine receptors (RyR) in muscle cells resulting in activation of RyR causing an uncontrolled release of \( \text{Ca}^{2+} \) from internal stores in the sarcoplasmic reticulum (Lahm et al. 2005, Cordova et al. 2006, Lahm et al. 2007), leading to depletion of calcium, muscle paralysis, and ultimate death (Cordova et al. 2007). The symptoms of chlorantraniliprole intoxication in treated insects include impaired regulation of muscle contraction leading to feeding cessation, lethargy, paralysis, and death of target organisms. Anthranilic diamides have very low vertebrate toxicity due to a >500-fold differential selectivity toward insect over mammalian RyR (Cordova et al. 2006, Lahm et al 2007).

Spinetoram (Delegate™ WG) is a recently developed spinosyn which belongs to IRAC mode of action class 5 (IRAC 2009). Insecticides in this class primarily activate the nicotinic acetylcholine receptors by acting on a unique site (Salgado 1998, Salgado et al. 1998). Both chlorantraniliprole and spinetoram were highly effective against \textit{C. rosaceana} in laboratory and field trials (Hull et al. 2009).
Laboratory bioassays provide information on the inherent and relative toxicity of an insecticide, but it is the longevity of residues that informs growers about retreatment intervals. Therefore, in order for growers to effectively incorporate new insecticide technologies into *C. rosaceana* management programs, information on their residual toxicity under field conditions is needed. Because some of the newly registered insecticides such as chlorantraniliprole have been reported to cause feeding cessation in the target pest (Cordova et al. 2006), the assessment of the effect of these insecticides on foliage consumption by *C. rosaceana* would also be valuable in determining the total impact of these chemicals on this pest. As our knowledge about the field performance of these chemicals increases, their utility in the *C. rosaceana* management programs should improve.

This study was designed to investigate the influence of *C. rosaceana* larval biology and toxicological properties of emamectin benzoate, chlorantraniliprole, and spinetoram on the effectiveness of these compounds under field conditions. The objectives of this study were to determine the toxicity of field-aged foliar residues of spinetoram, chlorantraniliprole and emamectin benzoate to *C. rosaceana* neonates, to assess the consumption of treated foliage by live *C. rosaceana* larvae, and to evaluate the effect of length of exposure of *C. rosaceana* neonates to the treated foliage. Because it is known that *C. rosaceana* larvae do not remain in the same feeding shelter for long periods (Waldstein 2000), we also evaluated the mortality of third instar *C. rosaceana* confined on foliage in the absence of terminal growth or movement of larvae to foliage with sublethal residues, and examined the effects of emamectin benzoate, chlorantraniliprole, and spinetoram on *C. rosaceana* larvae moving from treated foliage to untreated diet after different exposure intervals.
Materials and Methods

**Insects.** A laboratory colony (LAB) of *C. rosaceana* was established in 1990 from larvae collected from apple orchards in Mattawa, WA. This colony has been reared continuously since their collection on a pinto bean diet following the method of Shorey and Hale (1965) under constant conditions of temperature (23 ± 2°C), relative humidity (RH, 70%), and photoperiod (16:8, L:D) and without exposure to insecticides.

**Insecticides.** Materials used in these experiments were emamectin benzoate (Proclaim® 5SC), EPA Reg. No. 100-904, Syngenta Crop Protection Inc., Greensboro, NC 27149, U.S.A.; chlorantraniliprole [DPX E2Y45-410] (Rynaxypyr™/Altacor® 35WG), EPA Est. No. 352-DE-002, E.I. du Pont de Nemours & Co., Wilmington, DE 19898, U.S.A.; and spinetoram [XDE-175] (Delegate® 25WG), EPA Est. No. 62719-IN-1, Dow AgroSciences LLC, Indianapolis IN 46268, U.S.A.

**Field-Aged Leaf Residue Bioassay.** This study was conducted at Washington State University, Tree Fruit Research and Extension Center (WSU-TFREC), Wenatchee WA. Spinetoram, chlorantraniliprole and emamectin benzoate were applied to apple cultivar ‘Red Delicious’. Trees were ≈4.0 m high and planted 3.0 by 6.0 m apart. The three insecticides were tested at manufacturer recommended field rate 105 g(AI)/acre of spinetoram, 74 g(AI)/acre of chlorantraniliprole and 14 g(AI)/acre of emamectin benzoate (field rate) and at half of these rates (½ field rate). An untreated control (water only) was also included as a treatment. All treatments were replicated three times and arranged in a randomized complete block design. Each treatment was applied to 4-6 trees in the same row. A treatment was separated by 2-3 trees to avoid insecticide drift from other treatments. The treatment blocks were separated by 1-2 untreated rows to avoid possible insecticide drift from adjacent blocks.
An airblast sprayer calibrated to deliver 757 liters/acre was used to apply treatments. Treatments were applied on 5 August 2008 to minimize dilution effects of active growth of apple foliage. Twenty to thirty leaves were collected from the central trees in each treatment and placed in labeled zip-lock plastic bags. Care was taken to sample only mature foliage that was present when the insecticide treatments were applied. Leaf samples were collected 1, 3, 6, and 10 d after the insecticide application and then weekly thereafter until significant survival was observed in larvae exposed to the insecticide treated foliage. Leaves were transported to the laboratory and two 20-mm-diameter discs were cut from each with a cork borer. These discs were placed in a plastic Petri dish (Falcon 1006, 50x9 mm, Becton Dickinson Labware, Becton Dickinson and Company, Franklin Lakes, NJ 07417, U.S.A.) with one *C. rosaceana* neonate (<48 h old). Twenty-five Petri dishes were prepared for each treatment including controls. Petri dishes were maintained in growth chambers under constant conditions of temperature (23 ± 2°C), relative humidity (RH, 70%), and photoperiod (16:8 h, L:D). Mortality of neonates was assessed after 7 d. Larvae were scored as dead if they failed to respond to a probe with a camel’s hair brush. Treatment mortality was adjusted for control mortality according to Abbott (1925).

**Feeding Comparison.** Leaf discs from the Field-Aged Leaf Residue Bioassays were visually examined to determine the percentage consumed by larvae. Feeding was only assessed on leaf discs where live larvae were detected at the 7-d evaluation, therefore mortality did not influence the results. An ANOVA was conducted separately for each insecticide and collection period. Means were separated using Tuckey’s honestly significant difference (HSD) test at $\alpha = 0.05$. Because of the potential for rounding error, means were
carried out to the hundredth place even though percentage consumption was estimated using whole numbers.

**Mortality of Larvae Confined on Field Foliage.** This portion of the study was conducted using the treatment blocks as described above (Field-Aged Leaf Residue Bioassay). Small shoots along with terminal foliage were collected from a height of 1 to 2 m on the exterior canopy from all treatments 24-30 h after insecticide application. Each terminal was cut to a length of 20 cm and placed in a cage. The cage consisted of an empty one-gallon plastic container (Crystal Geyser®, Alpine Spring Water, 55 Francisco Street Suite 410, San Francisco, CA 94133, U.S.A.) with dimensions, 14 cm X 14 cm X 25 cm. The one-gallon plastic container was placed on the top of a 16 oz translucent plastic disposable food container (www.acemart.com, item # SOLMN16X-0100) containing 250 ml of water. The container was enclosed with a lid to cover the water. A hole of 8-10 mm diameter was made through bottom of the one-gallon plastic container and the lid of the disposable food container using cork borer. The bottom of the terminal was put in the water through the hole to prevent the terminal foliage from drying. Five third instar *C. rosaceana* were placed on the terminal foliage in each cage. A 25 cm X 25 cm piece of muslin cloth was tied with a rubber band on the top of the cage as a cover. Three replicates of five cages were set up for each treatment and the control. The cages were maintained in growth chambers under constant conditions of temperature (23 ± 2°C), relative humidity (RH, 70%), and photoperiod (16:8 h, L:D). Mortality was assessed at 5, 10, and 15 d after the terminals had been collected. Treatment mortality was adjusted for control mortality according to Abbott (1925).

**Exposure to the Field Treated Foliage.** This portion of the study was conducted using the treatments and treatment blocks as described above (Field-Aged Leaf Residue Bioassay).
Samples were collected 24-30 h after insecticide application and handled as described above in the Field-Aged Leaf Residue Bioassay section. Three replications of 100 dishes were prepared for each treatment including controls (300 larvae per treatment for a total of 900 larvae tested with each of the three insecticides). Larvae in twenty-five dishes for each treatment received different exposure durations of 1, 3, 6, and 10 d. After an exposure period, larvae were transferred to untreated apple leaf discs for 9, 7, 4, and 0 d, respectively, so that all larvae were exposed for the same duration (10 d) to either treated or untreated foliage. Petri dishes were maintained in growth chambers under constant conditions of temperature (23 ± 2°C), relative humidity (RH, 70%), and photoperiod (16:8 h, L:D). Percentage mortality was assessed at 10 d. Treatment mortality was adjusted for control mortality according to Abbott (1925).

Exposure to the Laboratory Treated Foliage. This study was conducted in the laboratory using a leaf-dip bioassay. Apple leaves were collected from an unsprayed ‘Red Delicious’ apple orchard located at WSU-TFREC. The apple leaves were treated by dipping them in aqueous solutions of emamectin benzoate, chlorantraniliprole or spinetoram for 5 s. The leaves were allowed to air dry and then 20-mm-diameter leaf discs were cut out of the leaves with a cork borer. Two leaf discs were placed into a Petri dish (Falcon 1006, 50x9 mm, Becton Dickinson Labware, Becton Dickinson and Company, Franklin Lakes, NJ 07417, U.S.A.) and then one C. rosaceana neonate from the LAB colony was placed onto the leaf discs. The insecticides in this study were tested at 56.2, 28.1, 5.6 and 0.6 mg(AI)/L of spinetoram, 39.3, 19.7, 3.9 and 0.4 mg(AI)/L of chlorantraniliprole, and 7.5, 3.8, 0.8, and 0.08 mg(AI)/L of emamectin benzoate. These concentrations were chosen based on label rates for these products, and are equivalent to the field rate, \( \frac{1}{2} \) field rate, \( \frac{1}{10} \)th field rate, and \( \frac{1}{100} \)th
field rate, respectively. A control consisted of leaves dipped in distilled water. Five replications of 20 larvae each were tested at each concentration (100 larvae per concentration for a total of 500 larvae tested with each of the three insecticides). One-fourth of the total for each concentration (25 Petri dishes) was divided into four different exposure durations including 1, 3, 6, and 10 d. After exposure to treated discs, larvae were transferred to untreated apple leaf discs for 9, 7, 4, and 0 d, respectively, for total exposure duration to treated and untreated foliage of 10 d. The larvae put on apple leaf discs were maintained in growth chambers under constant conditions of temperature (23 ± 2°C), relative humidity (RH, 70%), and photoperiod (16:8 h, L:D). Percentage mortality was assessed at 10 d. Treatment mortality was adjusted for control mortality according to Abbott (1925).

Results

Field-Aged Leaf Residue Bioassay. Spinetoram had the longest residual activity in the field followed by chlorantraniliprole and then emamectin benzoate. Mortality of C. rosaceana neonates did not decline until 59 days after treatment (DAT) when exposed to spinetoram treated foliage using the field rate (Fig. 1a). Mortality declined from 100% to 84.4% at 66 DAT and then dropped below 50% at 73 and 80 DAT. Residual activity of spinetoram at ½ field rate was similar to that of the field rate causing 100% mortality of C. rosaceana neonates consistently until 38 DAT. Mortality then gradually declined to 68.8% at 66 DAT. Mortality on the last two sample days was below 50% and similar to that observed for the field rate.

Mortality of C. rosaceana neonates exposed to chlorantraniliprole treated foliage at the field rate did not decline significantly from 100% until 38 DAT (Fig. 1a). In the following four weeks there was a gradual decline in mortality and it dropped below 50% at 73 DAT.
Although mortality of *C. rosaceana* neonates exposed to the ½ field rate of chlorantraniliprole declined slightly between 24 and 38 DAT, it remained consistently above 90% until 38 DAT. The residues of chlorantraniliprole then declined steadily and the mortality of *C. rosaceana* neonates dropped below 50% at 66 DAT, and was only 9.9% at 80 DAT.

There was 100% mortality of *C. rosaceana* neonates exposed to emamectin benzoate residues at the field rate through 10 DAT and the ½ field rate through 6 DAT (Fig. 1c) after which there was a continuous gradual decline in percent mortality at both rates during the following weeks. The mortality of *C. rosaceana* neonates was below 50% at both rates of emamectin benzoate by 45 DAT.

**Feeding Comparison.** Because there was no survival of *C. rosaceana* neonates in the early samples for all treatments, first observation for foliage consumption was not started until 17, 45, and 66 DAT for emamectin benzoate, chlorantraniliprole, and spinetoram, respectively. The percentage of leaf disc consumed by *C. rosaceana* neonates was significantly lower at both the field rate and ½ field rate of spinetoram relative to the untreated control collected from field until 80 DAT (*F* = 99.69; df = 2, 2, 151; *P* < 0.001) (Table 1). However, foliage consumption at the ½ field rate (5.13%) was significantly higher than that at the field rate (4.15%).

The percentage of leaf disc consumed by *C. rosaceana* neonates was significantly lower for both field rate (2.17%) and ½ field rate (3.19%) of chlorantraniliprole compared to the untreated control (8.92%) at 45 DAT (*F* = 57.64; df = 2, 2, 83; *P* < 0.001) and was still lower at 80 DAT (*F* = 82.64; df = 2, 2, 177; *P* < 0.001) (Table 2). However, percentage leaf disc consumption at the ½ field rate was significantly higher than at the field rate on 66, 73 and 80 DAT.
The percentage of leaf disc consumed by a *C. rosaceana* neonate was significantly lower with emamectin benzoate at both field rate (1.99%) and ½ field rate (3.36%) compared to the untreated control (7.71%) at 17 DAT (F = 27.98; df = 2, 2, 84; *P* < 0.001) (Table 3). Foliage consumption increased evidently because foliar residues declined. The percentage leaf disc consumption at the ½ field rate (8.29%) was statistically similar to that at untreated control (8.81%) at 45 DAT and beyond, and by 59 DAT there was no significant difference in the percentage foliage consumption at field rate (8.31%) and ½ field rate (8.41%) rate compared to the untreated control (8.40%) (F = 0.01; df = 2, 2, 194; *P* = 0.992).

**Mortality of Larvae Confined on Field Foliage.** Mortality of third instar *C. rosaceana* confined on terminal foliage treated with spinetoram at a field rate or ½ field rate was 100% after 5 d exposure, therefore no further evaluation was conducted. Mortality of third instar *C. rosaceana* exposed to field rate residues of emamectin benzoate and chlorantraniliprole for 5 d was 95.5 and 88.6%, respectively (Table 4). Mortality increased to 100% for both emamectin benzoate and chlorantraniliprole after 10 d exposure to the field rate treatment but did not reach 100% in the ½ field rate until after 15 d of exposure (Table 4).

**Exposure to the Field Treated Foliage.** Mortality of *C. rosaceana* neonates was 100% at the end of 10 d period, when they were exposed to foliar residues of either field rate or ½ field rate of spinetoram for any period before transfer to untreated foliage (Table 5). Mortality of *C. rosaceana* neonates exposed for 1 d to foliage with fresh residues of chlorantraniliprole at field rate and ½ field rate treatments was less than 100%, but when exposed for 3 d mortality in the field rate treatment was 100% and less than 90% in the ½ field rate treatment. However, larval mortality was 100% in both the field rate and ½ field rate treatments after 6 or 9 d exposure (Table 5). Mortality of *C. rosaceana* neonates exposed for 1 or 3 d to foliage
with fresh residues of emamectin benzoate at field rate or ½ field rate treatments before transfer to untreated foliage was less than 100% (Table. 5). After 6 or 10 d exposure, larval mortality was 100% in both the field rate and ½ field rate treatments.

**Exposure to the Laboratory Treated Foliage.** When *C. rosaceana* neonates were exposed to different residue levels (field rate to 1/100 field rate) of spinetoram, chlorantraniliprole and emamectin benzoate for different periods of time larval mortality was variable. There was 100% mortality in all rates and durations of exposure, when *C. rosaceana* neonates were exposed to residues of spinetoram (Fig 2a). Mortality of *C. rosaceana* neonates as a result of exposure to chlorantraniliprole residues was dependent on the rate and duration of exposure. With 1 d exposure to chlorantraniliprole residues mortality was 79.89, 67.09, 47.16, and 24.34% at the concentrations tested (Fig. 2a). Mortality increased to 100% after 3 d exposure to the field rate, and after 6 and 10 d of exposure to the ½ field rate treatment. However, mortality at 1/10 and 1/100 rate treatments never reached 100%. When *C. rosaceana* neonates were exposed to residues of emamectin benzoate, mortality was dependent on the rate and duration of exposure. With 1 d exposure to emamectin benzoate residues, larval mortality was 89.59, 80.90, 64.62, and 66.14% at the field rate, ½ field rate, 1/10 field rate, and 1/100 field rate, respectively (Fig. 2c). Mortality increased to 100% after 3 d exposure to the field rate and ½ field rate treatments, after 6 d exposure to the 1/10 field rate treatment, and after 10 d exposure to the 1/100 field rate treatment (Fig 2c).
Discussion

The residual efficacy of chlorantraniliprole and spinetoram at the field rate reported in this study was much longer than any other insecticide that has been reported to date (Brunner et al. 1995a, Brunner et al. 1995b, Waldstein and Reissig 2001, Borchert et al. 2004). Loriatti et al. (2009b) reported that the mortality of *Lobesia botrana* (Lepidoptera: Tortricidae) declined at 14 DAT as a result of exposure to field applied residues of chlorantraniliprole whereas we did not observe a decline in mortality until after 38 DAT. These differences could be related to the nature of plant materials used in bioassays, i.e., mature apple foliage versus actively growing grapes. More than 50 mm of rainfall received in the other study (Loriatti et al. 2009b) might have also contributed to the observed differences. However, the residual efficacy of chlorantraniliprole at the ½ field rate declined quickly compared to that of spinetoram. These data emphasize the need to obtain good spray coverage in the field so that *C. rosaceana* larvae are exposed to high concentrations of chlorantraniliprole therefore achieving acceptable control. The residual efficacy of emamectin benzoate declined quickly compared to the other two insecticides in our study. Short residual activity of emamectin benzoate was also observed against codling moth (Loriatti et al. 2009b), which could be attributed to the rapid decomposition of this chemical on the foliage (Crouch and Feely 1995, Chukwudebe et al. 1997). Despite a quick initial decline in residual efficacy, the rate of residue decline in emamectin benzoate was slower than that in chlorantraniliprole and spinetoram, which could be due to its translaminar activity (Wright et al. 1985).

While the long residual activity of chlorantraniliprole and spinetoram makes these reduced-risk insecticides useful tools for management of *C. rosaceana*, it also raises concerns about the resistance management. The low levels of resistance against chlorantraniliprole, and
cross-resistance between spinosad and spinetoram has already been observed in *C. rosaceana* populations collected from tree fruit orchards in Washington (Sial et al. 2009). Resistance management strategies should be implemented to reduce multiple-generation exposure to lethal or sublethal residues of these insecticides. The advantage of the shorter residue of emamectin benzoate may be an advantage in preventing or slowing the development of resistance in pests like *C. rosaceana*.

In the absence of terminal growth and movement of *C. rosaceana* to untreated foliage or foliage with sublethal residues, mortality of third instar *C. rosaceana* increased with increasing exposure time for emamectin benzoate and chlorantraniliprole at field rate and ½ field rate treatments. Similarly, the mortality of the *C. rosaceana* neonates also increased as the length of larval exposure to treated foliage increased prior to the larvae being moved to untreated foliage. The similar results have been demonstrated for tebufenozide in laboratory bioassays on tufted apple bud moth (Biddinger et al. 1996) and in semi-field studies on *C. rosaceana* (Waldstein and Reissig 2001). In case of spinetoram, the mortality of *C. rosaceana* neonates was similar for all exposure periods, which is in agreement with the observations made by Biddinger et al. (1996) in bioassays of azinphosmethyl on tufted apple bud moth, and Waldstein and Reissig (2001) in studies of chlorpyrifos and esfenvalerate on *C. rosaceana*. The *C. rosaceana* larvae do not remain in the same feeding shelter for long periods (Waldstein 2000). This behavior may have greater impact on the efficacy of emamectin benzoate and chlorantraniliprole than on spinetoram, because the larvae moving to foliage with lower residue levels would be expected to have a higher survival. For the three insecticides we tested, 100% mortality of neonate and third instars of *C. rosaceana* was achieved at both the field rate and ½ field rate. These results indicate that emamectin
benzoate, chlorantraniliprole, and spinetoram could be effective against older larvae in the field situations.

Both field-aged residue and laboratory leaf-dip bioassays indicated that the higher the concentration of emamectin benzoate and chlorantraniliprole, the lower exposure time required to cause high levels of mortality of neonates and third instar *C. rosaceana*. These results emphasize the need to obtain good coverage in the field to ensure that the larvae are exposed to lethal concentrations of these insecticides. Spray coverage depends on various factors such as tree size, spray volume, sprayer type and condition, and tractor speed. It can be difficult to achieve adequate spray coverage when using reduced spray volumes, especially in the orchards with large trees and therefore it is more difficult for growers to control *C. rosaceana* in these kinds of orchards (Reissig et al. 1998, 1999). Spray coverage could be improved by increasing spray volume and decreasing tractor speed. Spray coverage in larger trees could also be improved by following pruning practices, which would allow for better spray coverage and therefore effectively reduce *C. rosaceana* damage in orchards with larger apple trees (Lawson et al. 1998).

In addition to mortality, foliage consumption by *C. rosaceana* larvae that survived 7 d exposure to residues of emamectin benzoate, chlorantraniliprole, and spinetoram was significantly decreased compared to consumption on untreated foliage. A decrease in foliage consumption was also reported by Waldstein and Reissig (2001) when *C. rosaceana* larvae were exposed to residues of tebufenozide and chlorpyrifos. Foliage consumption was somewhat higher in our study when *C. rosaceana* larvae were exposed to emamectin benzoate residues compared to chlorantraniliprole and spinetoram. These results could most likely be
attributed to the rapid decomposition of emamectin benzoate (Crouch and Feely 1995, Chukwudebe et al. 1997).

Although significantly less than the untreated control, noticeable feeding of the leaf discs was observed by the *C. rosaceana* neonates that died from exposure to emamectin benzoate as compared to no obvious feeding in case of chlorantraniliprole and spinetoram treatments. This could be because it takes 4-7 times longer for lepidopteran larvae to stop feeding on emamectin benzoate treated foliage than that on chlorantraniliprole treated foliage (Hannig et al. 2009). The low level of foliage consumption with chlorantraniliprole and spinetoram observed in our study indicates that field applications would result in very low levels of foliar damage in the apple orchards. While it is difficult to know for sure, it is very likely that the reduction in foliage consumption would translate into reduce fruit injury. Further studies are needed to thoroughly investigate the effects of sublethal concentrations of chlorantraniliprole and spinetoram on the biology of *C. rosaceana* in order to assess cumulative impact of these novel insecticides on *C. rosaceana* populations in orchards.

**Acknowledgments**

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Tables and Figures

Table 1. Percentage of leaf disc consumed (mean ± SEM) by *C. rosaceana* neonates feeding on untreated apple foliage and foliage treated with spinetoram applied at manufacturer recommended field rate (Field Rate) and half of the manufacturer recommended field rate (½ Field Rate) using airblast sprayer

<table>
<thead>
<tr>
<th>Time after application (d)</th>
<th>Treatment</th>
<th>% Consumption ± SEM</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>66</td>
<td>Control</td>
<td>8.87 ± 0.14a</td>
<td>104</td>
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<tr>
<td></td>
<td>½ Field Rate</td>
<td>5.37 ± 0.32b</td>
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<td></td>
<td>Field Rate</td>
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<td>73</td>
<td>Control</td>
<td>7.57 ± 0.14a</td>
<td>143</td>
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<td></td>
<td>½ Field Rate</td>
<td>4.91 ± 0.19b</td>
<td></td>
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<tr>
<td></td>
<td>Field Rate</td>
<td>3.99 ± 0.22c</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>Control</td>
<td>7.66 ± 0.18a</td>
<td>156</td>
</tr>
<tr>
<td></td>
<td>½ Field Rate</td>
<td>5.13 ± 0.21b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Field Rate</td>
<td>4.15 ± 0.23c</td>
<td></td>
</tr>
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Table 2. Percentage of leaf disc consumed (mean ± SEM) by *C. rosaceana* neonates feeding on untreated apple foliage and foliage treated with chlorantraniliprole applied at manufacturer recommended field rate (Field Rate) and half of the manufacturer recommended field rate (½ Field Rate) using airblast sprayer.

<table>
<thead>
<tr>
<th>Time after application (d)</th>
<th>Treatment</th>
<th>% Consumption ± SEM</th>
<th>N</th>
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<tbody>
<tr>
<td>45</td>
<td>Control</td>
<td>8.92 ± 0.31a</td>
<td>88</td>
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<td>½ Field Rate</td>
<td>3.19 ± 0.35b</td>
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<td></td>
<td>Field Rate</td>
<td>2.17 ± 0.36b</td>
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<tr>
<td>52</td>
<td>Control</td>
<td>8.67 ± 0.16a</td>
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<td>½ Field Rate</td>
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<td></td>
<td>Field Rate</td>
<td>2.50 ± 0.37b</td>
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<tr>
<td>59</td>
<td>Control</td>
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<td>½ Field Rate</td>
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<td></td>
<td>Field Rate</td>
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<td>½ Field Rate</td>
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<td></td>
<td>½ Field Rate</td>
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<td>80</td>
<td>Control</td>
<td>7.71 ± 0.23a</td>
<td>182</td>
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<td>½ Field Rate</td>
<td>5.36 ± 0.24b</td>
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<td></td>
<td>Field Rate</td>
<td>4.03 ± 0.25c</td>
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Table 3. Percentage of leaf disc consumed (mean ± SEM) by *C. rosaceana* neonates feeding on untreated apple foliage and foliage treated with emamectin benzoate applied at manufacturer recommended field rate (Field Rate) and half of the manufacturer recommended field rate (½ Field Rate) using airblast sprayer.

<table>
<thead>
<tr>
<th>Time after application (d)</th>
<th>Treatment</th>
<th>% Consumption ± SEM</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>Control</td>
<td>7.77 ± 0.30a</td>
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<tr>
<td>½ Field Rate</td>
<td>3.36 ± 0.65b</td>
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</tr>
<tr>
<td>Field Rate</td>
<td>1.99 ± 1.21b</td>
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<tr>
<td>24</td>
<td>Control</td>
<td>7.47 ± 0.23a</td>
<td>109</td>
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<td>½ Field Rate</td>
<td>4.62 ± 0.36b</td>
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<td>Field Rate</td>
<td>3.29 ± 0.48b</td>
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<tr>
<td>31</td>
<td>Control</td>
<td>8.96 ± 0.17a</td>
<td>123</td>
</tr>
<tr>
<td>½ Field Rate</td>
<td>5.24 ± 0.34b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Field Rate</td>
<td>3.87 ± 0.37c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>Control</td>
<td>8.87 ± 0.30a</td>
<td>139</td>
</tr>
<tr>
<td>½ Field Rate</td>
<td>8.00 ± 0.36b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Field Rate</td>
<td>6.59 ± 0.40c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>Control</td>
<td>8.81 ± 0.18a</td>
<td>182</td>
</tr>
<tr>
<td>½ Field Rate</td>
<td>8.29 ± 0.19ab</td>
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<tr>
<td>Field Rate</td>
<td>7.86 ± 0.22b</td>
<td></td>
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</tr>
<tr>
<td>52</td>
<td>Control</td>
<td>8.87 ± 0.18a</td>
<td>183</td>
</tr>
<tr>
<td>½ Field Rate</td>
<td>8.31 ± 0.20ab</td>
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<td></td>
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<tr>
<td>Field Rate</td>
<td>8.03 ± 0.23b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>59</td>
<td>Control</td>
<td>8.4 ± 0.17a</td>
<td>199</td>
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<tr>
<td>½ Field Rate</td>
<td>8.41 ± 0.18a</td>
<td></td>
<td></td>
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<tr>
<td>Field Rate</td>
<td>8.37 ± 0.18a</td>
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</table>
Table 4. Mortality (mean ± SEM) of 3rd instar *C. rosaceana* confined on terminal foliage collected from apple trees 24 h after application of spinetoram, chlorantraniliprole, and emamectin benzoate at manufacturer recommended field rate (Field Rate) and half of the manufacturer recommended field rate (½ Field Rate) using airblast sprayer

<table>
<thead>
<tr>
<th>Exposure duration (d)</th>
<th>% corrected mortality ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spinetoram</td>
</tr>
<tr>
<td></td>
<td>½ Field Rate</td>
</tr>
<tr>
<td>5</td>
<td>100 ± 0.0</td>
</tr>
<tr>
<td>10</td>
<td>100 ± 0.0</td>
</tr>
<tr>
<td>15</td>
<td>100 ± 0.0</td>
</tr>
</tbody>
</table>
Table 5. Effect of exposure time on mortality of *C. rosacea* neonates on spur foliage collected from apple trees 24 h after application of emamectin benzoate, chlorantraniliprole, and spinetoram at manufacturer recommended field rate (Field Rate) and half of the manufacturer recommended field rate (½ Field Rate) using airblast sprayer

<table>
<thead>
<tr>
<th>Exposure duration (d)</th>
<th>% corrected mortality ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spinetoram</td>
</tr>
<tr>
<td></td>
<td>½ Field Rate</td>
</tr>
<tr>
<td>1</td>
<td>100 ± 0.0</td>
</tr>
<tr>
<td>3</td>
<td>100 ± 0.0</td>
</tr>
<tr>
<td>6</td>
<td>100 ± 0.0</td>
</tr>
<tr>
<td>10</td>
<td>100 ± 0.0</td>
</tr>
</tbody>
</table>
Fig. 1. Mortality of *C. rosaceana* after 7 d exposure to foliage collected at various times from apple trees treated with spinetoram (a), chlorantraniliprole (b), and emamectin benzoate (c) at manufacturer recommended field rate (Field Rate) and half of the manufacturer recommended field rate (½ Field Rate) using airblast sprayer.
Fig. 2. Effect of exposure time on mortality of *C. rosaceana* neonates on apple foliage treated with different dilutions of spinetoram (a), chlorantraniliprole (b), and emamectin benzoate (c) equivalent to manufacturer recommended field rate (Field Rate), half of the field rate (½ Field Rate), 1/10 Field Rate, and 1/100 Field Rate using leaf dip bioassay.
CHAPTER FOUR

SUSCEPTIBILITY OF OBLIQUEBANDED LEAFROLLER (LEPIDOPTERA: TORTRICIDAE) TO TWO NEW REDUCED-RISK INSECTICIDES

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Abstract

The response of field-collected populations of the obliquebanded leafroller, Choristoneura rosaceana (Harris) (Lepidoptera: Tortricidae), to chlorantraniliprole, spinetoram, spinosad, and azinphosmethyl was assessed using a diet incorporation bioassay. Populations of OBLR were collected from nine orchards in Chelan, Douglas, Grant, and Okanogan counties of Washington. The neonates of the F₁ or F₂ generation were used in all assays. The parameters of probit regression lines were estimated and lethal concentration ratios (LCRs) were calculated for all populations compared with a susceptible laboratory population. Significant variation was detected in response to all four insecticides including chlorantraniliprole and spinetoram, which had never been used in the field. LCRs were 3.9-39.7 for azinphosmethyl, 0.5-3.6 for spinosad, 1.2-5.3 for chlorantraniliprole, and 0.5-4.1 for spinetoram. Correlation analysis indicated possibility of cross-resistance between spinosad and spinetoram, which are
both members of spinosyn class. The occurrence of low but significant levels of resistance against chlorantraniliprole and spinetoram in field-collected populations of *C. rosaceana* before their first field application indicates that the risk of resistance evolution against these two new reduced-risk insecticides exists. However, it is likely that these low levels of resistance can be managed if the insecticides are used judiciously in conjunction with sound resistance management programs. Implications of these results for developing and implementing resistance management strategies are discussed.

**Key words:** Obliquebanded leafroller, field-collected populations, chlorantraniliprole, spinetoram, insecticide resistance

**Introduction**

The obliquebanded leafroller (OBLR), *Choristoneura rosaceana* (Harris), is a tortricid moth native to North America (Weires and Riedl 1991). It is a major pest of pome fruits, second only to codling moth, *Cydia pomonella* (L), in Washington (Brunner 1999). In apple, OBLR larvae feed on flower buds, leaves, and developing fruits (Howitt 1993, Ohlendorf 1999). Fruit damage from larval feeding can occur in the spring, during mid-summer (the most significant), or just prior to harvest (Beers et al. 1993). Leafrollers, and the key apple pest, *Cydia pomonella* (L.), have been controlled using the organophosphate (OP) insecticides for over four decades. However, reports of decreasing efficacy of OP insecticides against leafrollers have been attributed to the development of insecticide resistance (Brunner 1996). Insecticide resistance to OPs and cross-resistance to other groups of chemicals have been documented in OBLR (Reissig et al. 1986, Lawson et al. 1997, Waldstein et al. 1999, Ahmad
et al. 2002, Smirle et al. 2002, Dunley et al. 2006), in some cases even before the new insecticides had been used in the field (Sauphanor et al. 1998, Dunley and Welter 2000). In addition, the implementation of Food Quality Protection Act of 1996 (FQPA) (USEPA 1996) has increased restrictions on the use of broad-spectrum insecticides; especially the OPs.

The development of insecticide resistance as well as regulatory actions such as FQPA have led to a greater priority in the development of reduced-risk insecticides as OP alternatives (USEPA 1997). Chlorantraniliprole and spinetoram are the reduced-risk insecticides recently registered as OP alternatives. Chlorantraniliprole is an anthranilic diamide, which belongs to insecticide resistance action committee (IRAC) mode of action class 28 (IRAC 2009). Anthranilic diamides selectively bind to the ryanodine receptors (RyR) in insect muscles resulting in an uncontrolled release of calcium from internal stores in the sarcoplasmic reticulum (Lahm et al. 2005, Cordova et al. 2006), causing impaired regulation of muscle contraction leading to feeding cessation, lethargy, paralysis, and death of target organisms. Anthranilic diamides have very low vertebrate toxicity due to a >500-fold differential selectivity toward insect over mammalian RyR (Cordova et al. 2006). Spinetoram is a recently developed spinosyn, which belongs to IRAC mode of action class 5 (IRAC 2009). Spinosyns primarily activate the nicotinic acetylcholine receptors by acting on a unique site (Salgado 1998, Salgado et al. 1998). Both chlorantraniliprole and spinetoram have a high degree of efficacy against OBLR in laboratory and field trials (Hull et al. 2009; Brunner, unpublished data).

With the availability of effective OP alternatives, it is critical for growers to incorporate the reduced-risk insecticides into C. rosaceana management programs for successful production of tree fruits on a sustainable basis. However, the development of resistance is a
continual threat, especially to the novel chemistries such as chlorantraniliprole and spinetoram. The total cost associated with resistance is difficult to assess, but the loss of insecticide effectiveness almost invariably entails the use of increased concentrations and application frequency. Eventually replacement compounds (National Research Council 1986) are needed that are more expensive due to increased costs in discovery, development, registration, and manufacturing (Metcalf 1980). Characterizing susceptibility to new insecticides in field populations would be valuable for *C. rosaceana* management programs by providing an early detection of potential problems of resistance and cross-resistance. This would allow growers to change their *C. rosaceana* control strategies and potentially slow the spread of resistance. It could also provide evidence that resistance is not a problem associated with control failures, and encourage growers to address operational factors, e.g. sprayer calibration or target coverage, contributing to the lack of control. The objective of this study was to survey current levels of susceptibility of field-collected populations of *C. rosaceana* to the two new reduced-risk insecticides chlorantraniliprole and spinetoram before their introduction into *C. rosaceana* management programs, and to assess the potential for the occurrence of cross-resistance to the currently used insecticides such as azinphosmethyl and spinosad.

**Materials and Methods**

**Insects.** *Laboratory Population.* A *C. rosaceana* laboratory colony (LAB) was established by collecting larvae from apple orchards in Mattawa, WA, in 1990. This colony has been reared continuously since their collection on a pinto bean diet following the method
of Shorey and Hale (1965) under constant conditions of temperature (23 ± 2°C), relative humidity (RH, 70%), and photoperiod (16:8 h, L:D).

**Field Populations.** In 2007, field populations were collected from the overwintering (spring brood) larvae at three locations JON (Quincy, Grant County, WA), PTH (Mattawa, Grant County, WA), and JAR (Brewster, Okanogan County, WA). In 2008, field populations were collected from the overwintering brood at four locations, STM (Stemilt Hill, Wenatchee, Chelan County, WA), KMP and CLK (Chelan, Chelan County, WA), and WEB (Quincy, Grant County, WA); and from summer brood larvae at two locations, GRF (Crane, Douglas County, WA) and ROB (Brewster, Okanogan County, WA). The field populations were collected as third to fifth instars, returned to the laboratory, transferred to 96 ml plastic portion cups (#S-300, Prairie Packaging Inc., Bedford Park, IL 60638, U.S.A.) containing artificial pinto bean diet (Shorey and Hale 1965), and reared to the adult stage. The neonate larvae of the first laboratory generation, and in some cases the second laboratory generation, were used in bioassays.


**Bioassays.** Toxicity of each insecticide to neonate larvae of *C. rosaceana* was determined using a diet incorporation bioassay. A dry premix of a Heliothis diet (Stonefly Heliothis Diet
[Item # 38 V 0600], Ward’s Natural Science, Rochester, NY 14692, U.S.A.) was used in the bioassay. A stock solution was prepared by diluting the test insecticide at 10X the highest concentration to be used in the bioassay. Then serial dilutions were prepared from the stock solution, each at 10X the target concentration to be used in the bioassay. For each concentration, a treatment solution was prepared by weighing 61 g of water adding 4 g of vinegar and then 10 g of the 10X insecticide dilution. This treatment solution was then added to 25 g of dry diet premix to complete the insecticide incorporated diet. An untreated control was prepared by using water and vinegar plus the dry diet premix. For chlorantraniliprole, five concentrations (0.1-10 ppm) were used in 2007 whereas six concentrations (0.01-3 ppm) were used in 2008. For bioassays performed in 2008, six concentrations were used for spinetoram (0.003-1 ppm) and spinosad (0.3-10 ppm), and seven concentrations (1-1000 ppm) were used for azinphosmethyl. Enough insecticide incorporated diet was prepared prior to the start of the bioassays, so that all tests were run on the same diet mixtures.

A small portion of insecticide incorporated diet (approximately 8.0 cm\(^3\)) was added to a plastic Petri dish (Falcon 1006, 50x9 mm, Becton Dickinson Labware, Becton Dickinson and Company, Franklin Lakes, NJ 07417, U.S.A.). The diet was pushed firmly along the edges of the dish, and scored with a pin so that \(C.\) \textit{rosaceana} neonates could readily colonize the diet. Petri dishes were chosen randomly, and five 1-day-old \(C.\) \textit{rosaceana} larvae were transferred into each dish using a camel’s hair brush. Six to ten dishes were prepared for each treatment (30-50 larvae/treatment) depending on the availability of neonate larvae. The dishes were placed in growth chambers at constant conditions of temperature (23 ± 2°C), relative humidity (RH, 70%), and photoperiod (16:8 h, L:D).
In 2007, larval mortality was evaluated after 4 d. Larvae were recorded as dead if they did not move when probed with a camel’s hair brush. In bioassays associated with other studies we found that a high percentage of the larvae treated with chlorantraniliprole that were recorded alive in 2007 bioassays using the criteria described above for assessing larval mortality, were actually moribund and died after a few days. In order to incorporate this new knowledge and improve the quality of our bioassay techniques for chlorantraniliprole, we revised the criteria of assessing larval mortality in 2008 bioassays. In these bioassays larval mortality in all populations, including the LAB colony, was evaluated after 7 d, and moribund larvae were recorded as dead. To ensure that offspring of many females were assayed, larvae emerging from any given egg mass were systematically distributed among various concentrations so that a maximum of 5-10 larvae per egg mass were treated at any one concentration.

**Data Analysis.** Lethal concentration values (LC\(_{50}\) and LC\(_{90}\)) and their corresponding 95% fiducial limits (FL) were estimated using POLO (LeOra Software 1987) and lethal concentration ratios (LCR) at LC\(_{50}\) and LC\(_{90}\) values and their corresponding 95% confidence limits (CL) were calculated using lethal concentration ratio significance test (Robertson et al. 2007). The laboratory colony (LAB) served as the reference susceptible population for comparison purposes and was assigned a ratio of 1.0. The lethal concentration (LC\(_{50}\) and LC\(_{90}\)) values of the field-collected populations were considered significantly different from those of the LAB population if the 95% CL of their corresponding LCR values did not include the value of 1.0 (\(\alpha = 0.05\)). Pearson’s Product Moment Correlation (Pearson’s correlation) was used to detect the occurrence of cross-resistance between the chemicals tested in this study.
Results

Larvae of *C. rosaceana* collected from different orchards in Washington State in 2007 showed varying susceptibility to chlorantraniliprole (Table 1). The mortality for populations from PTH, JAR and JON indicated a good fit to a probit model (Pearson’s chi-square test; \( P > 0.05 \)). Larvae from PTH were the most susceptible of populations tested in 2007, with \( \text{LC}_{50} \) and \( \text{LC}_{90} \) of 0.39 and 1.03 ppm, respectively. Based on the LCR significance test, both JAR and JON populations were less susceptible to chlorantraniliprole than the LAB colony with 3.07- and 4.18-fold higher \( \text{LC}_{50} \) values, respectively. The slope values suggested that the JAR population had relatively less variation in susceptibility to chlorantraniliprole as compared to the JON population.

In 2008, *C. rosaceana* field-collected larvae showed varying degree of susceptibility to chlorantraniliprole (Table 2). Populations from STM, KMP, WEB and CLK (spring brood) and from GRF and ROB (summer brood) indicated good fit to a probit model (Pearson’s chi-square test; \( P > 0.05 \)). The STM and CLK populations were the most susceptible to chlorantraniliprole. Based on the LCR significance test, all field-collected populations evaluated in 2008 were significantly less susceptible than the LAB colony with 1.70- to 5.26-fold higher \( \text{LC}_{50} \) values. However, \( \text{LC}_{90} \) of the STM and CLK populations were not significantly different from the LAB colony. The WEB and CLK populations had relatively less variation in their susceptibility to chlorantraniliprole, as indicated by the high slope values. The \( \text{LC}_{50} \) values of the LAB colony observed in 2008 were less than those recorded in 2007. This was due primarily to the change in the criteria for assessment of larval mortality as explained in materials and methods section, and not to an inherent change in the susceptibility of the LAB colony.
In 2008, the larvae of field-collected populations of *C. rosaceana* showed significant variation in their susceptibility to spinetoram (Table 3). Populations from STM, WEB and CLK (spring brood), and GRF and ROB (summer brood) indicated good fit to a probit model (Pearson’s chi-square test; $P > 0.05$). Larvae from STM and WEB were the most susceptible to spinetoram. Based on the LCR significance test the KMP, CLK, GRF and ROB populations were significantly less susceptible than the LAB colony with 1.70- to 4.05-fold higher LC$_{50}$ values while the STM and WEB populations were significantly more susceptible than the LAB colony with LCRs of 0.50 and 0.40 at LC$_{50}$, respectively. However, LC$_{90}$ values of the STM and WEB populations were not significantly different from that of the LAB colony. The KMP population had relatively more variation in its susceptibility to spinetoram, as indicated by low slope value, whereas the CLK and ROB populations had relatively less variation as indicated by high slope values.

The larvae of *C. rosaceana* populations collected in 2008 showed significant variation in their susceptibility to spinosad (Table 4). Populations from STM and KMP (spring brood), and GRF (summer brood) indicated good fit to a probit model (Pearson’s chi-square test; $P > 0.05$). Based on the LCR significance test, LCRs at LC$_{50}$ for four out of five populations were significant ($\alpha = 0.05$). The KMP, GRF and ROB populations were significantly less susceptible than the LAB colony with 2.4- to 3.6-fold higher LC$_{50}$ values while the STM population was significantly more susceptible than the LAB colony with an LCR of 0.50. The susceptibility of larvae from the WEB population to spinosad was statistically similar to that of the LAB colony. However, LC$_{90}$ values of the KMP and GRF populations were significantly higher than that of the LAB colony. The STM and KMP populations had relatively more variation in their susceptibility to spinosad, as indicated by low slope values,
whereas the GRF and ROB populations had relatively less variation in their response to spinosad, as indicated by high slope values.

The larvae of the field-collected populations (2008) of *C. rosaceana* showed significant variation in their susceptibility to azinphosmethyl (Table 5). Populations from KMP and CLK (spring brood), and GRF and ROB (summer brood) indicated good fit to a probit model (Pearson’s chi-square test; *P* > 0.05). Based on LCR significance test, the neonate larvae from all of the five field-collected populations were significantly less susceptible (*α* = 0.05) than those from the LAB colony with 3.9- to 39.7-fold higher LC$_{50}$ values. The WEB, CLK, and KMP populations were more heterogeneous in their response to azinphosmethyl, as indicated by low slope values.

There was significant positive correlation between the tolerances of field-collected populations of *C. rosaceana* to spinosad and spinetoram at LC$_{50}$ with Pearson’s correlation coefficient (*r*) = 0.92 (df = 3; *P* = 0.026) indicating the possibility of cross-resistance between the two chemicals (Fig. 1). There was no correlation among tolerances of *C. rosaceana* to any of the other insecticides tested. The correlation coefficient (Pearson’s *r*) for chlorantraniliprole and azinphosmethyl was 0.54 (df = 3; *P* = 0.35), for chlorantraniliprole and spinosad was 0.45 (df = 3; *P* = 0.44), for chlorantraniliprole and spinetoram was 0.20 (df = 4; *P* = 0.71), for azinphosmethyl and spinosad was −0.69 (df = 2; *P* = 0.31), and for azinphosmethyl and spinetoram was −0.81 (df = 3; *P* = 0.09) across the field-collected populations.

**Discussion**

The occurrence of insecticide resistance is a major risk to the sustainability of integrated pest management (IPM) programs for *C. rosaceana*. Effective resistance management
strategies could slow the development of resistance only if implemented in a timely manner. The information on existing levels of resistance and cross-resistance to different classes of insecticides is an important factor in developing a successful resistance management program for *C. rosaceana*. All of the populations of *C. rosaceana* tested in this study were resistant to azinphosmethyl, which is consistent with the results of Waldstein et al. (1999), Pree et al. (2001), Ahmad et al. (2002), Smirle et al. (2002), and Dunley et al. (2006). The prevalence of azinphosmethyl resistance can be attributed to the decades of its use in *C. pomonella* management programs, which incidentally exposed *C. rosaceana* to azinphosmethyl.

Implementation of FQPA has increased pressure for fundamental change in IPM strategies leading to the development of reduced-risk OP alternatives including chlorantraniliprole, spinosad and spinetoram. Chlorantraniliprole, a member of a novel class of insecticides, the anthranilic diamides, was registered for use on tree fruit in 2008. The field-collected populations tested in this study had never been exposed to chlorantraniliprole. The significant variation in the susceptibility of field-collected *C. rosaceana* populations represents the first documentation of pre-existing resistance to chlorantraniliprole and suggests that higher levels of resistance could occur rapidly after its use in the field. That there was no significant correlation between chlorantraniliprole and azinphosmethyl resistance in *C. rosaceana* field populations argues against cross-resistance to OPs. The presence of low level resistance in *C. rosaceana* to an insecticide before its first introduction has been reported before (Waldstein and Reissig 2000, Ahmad et al. 2002, Smirle et al. 2002, Dunley et al. 2006). The *C. rosaceana* is a polyphagous insect with a host range of over 50 plant species including members of family Rosaceae and Cornaceae (Sanderson and Jackson 1909). Its adaptive evolutionary response to insecticides, even novel chemistries, could be attributed to the
breadth of compounds that *C. rosaceana* has been exposed to through its diet. All of the *C. rosaceana* populations tested in 2007 and 2008 were collected from conventionally managed orchards except the STM population that came from an organically managed orchard. This population was the most susceptible of all populations tested in 2008. These differences reflect the usage pattern of insecticides under organic and conventional management. IPM strategies can impact the susceptibility of pests to insecticides, regardless of which class they belong to, which has been documented elsewhere (Smirle et al. 2003).

Spinosad was the first spinosyn insecticide registered for use against *C. rosaceana* in 1998. Resistance of *C. rosaceana* populations to spinosad was documented by Dunley et al. (2006) and can be attributed to its extensive use over six years when no other effective control existed. The recent introduction of spinetoram, a chemical in the same class, raised questions about cross-resistance between these two chemicals. Our results showed significant variation in tolerance of the field-collected populations of *C. rosaceana* to spinosad and spinetoram. Moreover, based on LCRs at LC$_{50}$ spinetoram resistance was highly correlated with spinosad resistance, suggesting the possibility of cross-resistance. This was the first study to document the evidence of correlated cross-resistance between spinosad and spinetoram.

Genetic variation provides the basis for evolutionary change. The significant variation in susceptibility of *C. rosaceana* populations to chlorantraniliprole and spinetoram indicates a high risk of resistance evolution in this pest. Our findings establish baseline susceptibility of the field-collected *C. rosaceana* populations to chlorantraniliprole and spinetoram and serve as an early warning for the growers and pest managers, pointing out that implementing a sound resistance management program is essential to the preservation of these reduced-risk insecticides for *C. rosaceana* control on sustainable basis.
At a time when OP insecticide use is being restricted or even phased-out, a number of alternative insecticides with different modes of action have become available for *C. rosaceana* control including chlorantraniliprole and spinetoram. These OP alternative insecticides are highly effective against *C. rosaceana* (Hull et al. 2009; Brunner, unpublished data), exhibit a high degree of worker safety and are environmentally friendly, thereby providing the potential for more sustainable management of *C. rosaceana*. However, these insecticides must be used wisely in the framework of a well-informed resistance management program that reduces selection pressure on *C. rosaceana*, taking into account the potential for cross-resistance between different classes of insecticides (Georghiou 1983, ffrench-Constant and Roush 1990). Further studies are needed to determine the biochemical and molecular basis of mechanisms conferring *C. rosaceana* resistance to chlorantraniliprole and spinetoram, so that insecticides not affected by the same mechanism could be incorporated into IPM programs.

**Acknowledgments**

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Tables and Figures

Table 1. Results of probit analyses for chlorantraniliprole using diet incorporation bioassays on *C. rosaceana* neonate larvae from a laboratory as well as field-collected populations in 2007

<table>
<thead>
<tr>
<th>Population</th>
<th>n</th>
<th>Slope (± SE)</th>
<th>$\chi^2$</th>
<th>P</th>
<th>LC$_{50}$ (ppm) (95% FL)$^1$</th>
<th>LC$_{90}$ (ppm) (95% FL)$^1$</th>
<th>LCR-LC$_{50}$$^2$ (95% CL)$^4$</th>
<th>LCR-LC$_{90}$$^3$ (95% CL)$^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAB</td>
<td>250</td>
<td>2.61 (0.39)</td>
<td>1.08</td>
<td>0.78</td>
<td>0.33 (0.24-0.77)</td>
<td>1.03 (0.77-1.60)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTH</td>
<td>250</td>
<td>3.06 (0.70)</td>
<td>0.18</td>
<td>0.98</td>
<td>0.39 (0.19-0.57)</td>
<td>1.03 (0.71-2.10)</td>
<td>1.18 (0.88-1.59)</td>
<td>1.00 (0.59-1.68)</td>
</tr>
<tr>
<td>JAR</td>
<td>250</td>
<td>5.12 (0.99)</td>
<td>0.75</td>
<td>0.86</td>
<td>1.21 (1.01-1.44)</td>
<td>2.16 (1.76-3.19)</td>
<td>3.07 (2.19-4.29)*</td>
<td>2.09 (1.35-3.25)*</td>
</tr>
<tr>
<td>JON</td>
<td>250</td>
<td>2.91 (0.37)</td>
<td>4.97</td>
<td>0.17</td>
<td>1.39 (1.02-1.85)</td>
<td>3.83 (2.74-6.58)</td>
<td>4.18 (2.91-5.99)*</td>
<td>3.71 (2.30-5.99)*</td>
</tr>
</tbody>
</table>

n = number of larvae assayed.

1 95% fiducial limits estimated using POLO (LeOra Software 1987).

2 LCR-LC$_{50}$, lethal concentration ratio at LC$_{50}$ = LC$_{50}$ (field population)/LC$_{50}$ (LAB population).

3 LCR-LC$_{90}$, lethal concentration ratio at LC$_{90}$ = LC$_{90}$ (field population)/LC$_{90}$ (LAB population).

4 95% confidence limits estimated using lethal concentration ratio significance test (Robertson et al. 2007).
* LC$_{50}$ or LC$_{90}$ of field collected population significantly different ($\alpha = 0.05$) from that of the LAB population (Robertson et al. 2007).
Table 2. Results of probit analyses for chlorantraniliprole using diet incorporation bioassays on *C. rosaceana* neonate larvae from a laboratory as well as field-collected populations in 2008

<table>
<thead>
<tr>
<th>Population</th>
<th>n</th>
<th>Slope (± SE)</th>
<th>χ²</th>
<th>P</th>
<th>LC₅₀ (ppm) (95% FL)</th>
<th>LC₉₀ (ppm) (95% FL)¹</th>
<th>LCR-LC₅₀ (95% CL)⁴</th>
<th>LCR-LC₉₀ (95% CL)⁴</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
<td><strong>Spring Brood:</strong></td>
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</tr>
<tr>
<td>LAB</td>
<td>180</td>
<td>2.65 (0.57)</td>
<td>0.25</td>
<td>0.99</td>
<td>0.11 (0.07-0.16)</td>
<td>0.35 (0.24-0.69)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STM</td>
<td>180</td>
<td>2.55 (0.45)</td>
<td>1.34</td>
<td>0.85</td>
<td>0.19 (0.13-0.26)</td>
<td>0.61 (0.42-1.13)</td>
<td>1.70 (1.02-2.79)*</td>
<td>1.76 (0.92-3.38)</td>
</tr>
<tr>
<td>KMP</td>
<td>180</td>
<td>1.46 (0.31)</td>
<td>4.68</td>
<td>0.32</td>
<td>0.27 (0.10-0.47)</td>
<td>2.00 (1.05-8.72)</td>
<td>2.40 (1.19-4.64)*</td>
<td>5.79 (2.44-13.77)*</td>
</tr>
<tr>
<td>WEB</td>
<td>180</td>
<td>3.84 (0.84)</td>
<td>0.84</td>
<td>0.93</td>
<td>0.57 (0.40-0.75)</td>
<td>1.23 (0.92-2.12)</td>
<td>5.03 (3.11-8.13)*</td>
<td>3.56 (1.97-6.44)*</td>
</tr>
<tr>
<td>CLK</td>
<td>180</td>
<td>3.52 (0.92)</td>
<td>1.60</td>
<td>0.81</td>
<td>0.19 (0.12-0.26)</td>
<td>0.44 (0.32-0.91)</td>
<td>1.70 (1.02-2.78)*</td>
<td>1.27 (0.68-2.39)</td>
</tr>
<tr>
<td><strong>First Summer Brood:</strong></td>
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</tr>
<tr>
<td>LAB</td>
<td>180</td>
<td>2.20 (0.44)</td>
<td>3.44</td>
<td>0.49</td>
<td>0.15 (0.08-0.22)</td>
<td>0.57 (0.33-1.30)</td>
<td></td>
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</tr>
<tr>
<td>GRF</td>
<td>180</td>
<td>1.94 (0.47)</td>
<td>0.43</td>
<td>0.98</td>
<td>0.79 (0.43-1.20)</td>
<td>3.60 (2.14-11.97)</td>
<td>5.26 (2.91-9.50)*</td>
<td>6.29 (2.47-16.04)*</td>
</tr>
<tr>
<td>ROB</td>
<td>180</td>
<td>2.54 (0.39)</td>
<td>6.76</td>
<td>0.15</td>
<td>0.71 (0.52-0.96)</td>
<td>2.28 (1.58-4.10)</td>
<td>4.76 (2.88-7.87)*</td>
<td>3.98 (2.02-7.84)*</td>
</tr>
</tbody>
</table>

n = number of larvae assayed.

¹ 95% fiducial limits estimated using POLO (LeOra Software 1987).
2 LCR-LC\textsubscript{50}, lethal concentration ratio at LC\textsubscript{50} = LC\textsubscript{50} (field population)/LC\textsubscript{50} (LAB population).

3 LCR-LC\textsubscript{90}, lethal concentration ratio at LC\textsubscript{90} = LC\textsubscript{90} (field population)/LC\textsubscript{90} (LAB population).

4 95\% confidence limits estimated using lethal concentration ratio significance test (Robertson et al. 2007).

* LC\textsubscript{50} or LC\textsubscript{90} of field collected population significantly different (\(\alpha = 0.05\)) from that of the LAB population (Robertson et al. 2007).
Table 3. Results of probit analyses for spinetoram using diet incorporation bioassays on *C. rosaceana* neonate larvae from a laboratory as well as field-collected populations in 2008

<table>
<thead>
<tr>
<th>Population</th>
<th>n</th>
<th>Slope (± SE)</th>
<th>χ²</th>
<th>P</th>
<th>LC₅₀ (ppm) (95% FL)¹</th>
<th>LC₉₀ (ppm) (95% FL)¹</th>
<th>LCR-LC₅₀²</th>
<th>LCR-LC₉₀³</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Spring Brood:</strong></td>
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<td></td>
</tr>
<tr>
<td>LAB</td>
<td>180</td>
<td>3.82 (0.82)</td>
<td>1.56</td>
<td>0.82</td>
<td>0.09 (0.07-0.12)</td>
<td>0.20 (0.15-0.34)</td>
<td></td>
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</tr>
<tr>
<td>STM</td>
<td>180</td>
<td>2.25 (0.55)</td>
<td>2.73</td>
<td>0.60</td>
<td>0.04 (0.02-0.07)</td>
<td>0.16 (0.11-0.37)</td>
<td>0.50 (0.27-0.82)*</td>
<td>0.80 (0.43-1.52)*</td>
</tr>
<tr>
<td>KMP</td>
<td>180</td>
<td>1.38 (0.19)</td>
<td>15.04</td>
<td>0.01</td>
<td>0.23 (0.11-0.61)</td>
<td>1.92 (0.68-2.50)</td>
<td>2.41</td>
<td>9.49</td>
</tr>
<tr>
<td>WEB</td>
<td>180</td>
<td>1.97 (0.50)</td>
<td>4.40</td>
<td>0.36</td>
<td>0.04 (0.01-0.06)</td>
<td>0.16 (0.12-0.40)</td>
<td>0.40</td>
<td>0.78</td>
</tr>
<tr>
<td>CLK</td>
<td>180</td>
<td>3.23 (0.88)</td>
<td>2.86</td>
<td>0.58</td>
<td>0.16 (0.09-0.25)</td>
<td>0.39 (0.28-0.90)</td>
<td>1.70</td>
<td>1.95</td>
</tr>
<tr>
<td><strong>First Summer Brood:</strong></td>
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</tr>
<tr>
<td>LAB</td>
<td>180</td>
<td>3.05 (0.64)</td>
<td>1.14</td>
<td>0.89</td>
<td>0.02 (0.01-0.03)</td>
<td>0.05 (0.04-0.10)</td>
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</tr>
<tr>
<td>GRF</td>
<td>180</td>
<td>2.97 (0.59)</td>
<td>2.28</td>
<td>0.68</td>
<td>0.06 (0.04-0.09)</td>
<td>0.16 (0.11-0.33)</td>
<td>3.00</td>
<td>3.08</td>
</tr>
<tr>
<td>ROB</td>
<td>180</td>
<td>3.27 (0.39)</td>
<td>0.97</td>
<td>0.91</td>
<td>0.08 (0.06-0.11)</td>
<td>0.20 (0.15-0.37)</td>
<td>4.05</td>
<td>3.80</td>
</tr>
</tbody>
</table>

n = number of larvae assayed.

¹ 95% fiducial limits estimated using POLO (LeOra Software 1987).

² LCR-LC₅₀, lethal concentration ratio at LC₅₀ = LC₅₀ (field population)/LC₅₀ (LAB population).
3 LCR-LC$_{90}$, lethal concentration ratio at LC$_{90}$ = LC$_{90}$ (field population)/LC$_{90}$ (LAB population).

4 95% confidence limits estimated using lethal concentration ratio significance test (Robertson et al. 2007).

* LC$_{50}$ or LC$_{90}$ of field collected population significantly different ($\alpha = 0.05$) from that of the LAB population (Robertson et al. 2007).
Table 4. Results of probit analyses for spinosad using diet incorporation bioassays on *C. rosaceana* neonate larvae from a laboratory as well as field-collected populations in 2008

<table>
<thead>
<tr>
<th>Population</th>
<th>n</th>
<th>Slope (± SE)</th>
<th>$\chi^2$</th>
<th>$P$</th>
<th>LC$_{50}$ (ppm) (95% FL)$^1$</th>
<th>LC$_{90}$ (ppm) (95% FL)$^1$</th>
<th>LCR-LC$_{50}$$^2$ (95% CL)$^4$</th>
<th>LCR-LC$_{90}$$^3$ (95% CL)$^5$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Spring Brood:</strong></td>
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</tr>
<tr>
<td>LAB</td>
<td>180</td>
<td>2.43 (0.33)</td>
<td>13.45</td>
<td>0.01</td>
<td>0.26 (0.16-0.43)</td>
<td>0.87 (0.50-2.63)</td>
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</tr>
<tr>
<td>STM</td>
<td>180</td>
<td>1.78 (0.25)</td>
<td>5.59</td>
<td>0.23</td>
<td>0.13 (0.08-0.19)</td>
<td>0.68 (0.41-1.66)</td>
<td>0.50 (0.32-0.78)*</td>
<td>0.78 (0.38-1.60)</td>
</tr>
<tr>
<td>KMP</td>
<td>180</td>
<td>1.86 (0.22)</td>
<td>5.90</td>
<td>0.21</td>
<td>0.61 (0.28-1.45)</td>
<td>2.96 (1.28-21.60)</td>
<td>2.36 (1.52-3.66)*</td>
<td>3.41 (1.71-6.83)*</td>
</tr>
<tr>
<td>WEB</td>
<td>180</td>
<td>2.09 (0.27)</td>
<td>14.07</td>
<td>0.01</td>
<td>0.25 (0.17-0.36)</td>
<td>1.01 (0.63-2.17)</td>
<td>0.95 (0.62-1.45)</td>
<td>1.16 (0.59-2.28)</td>
</tr>
<tr>
<td><strong>First Summer Brood:</strong></td>
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<td></td>
</tr>
<tr>
<td>LAB</td>
<td>180</td>
<td>1.78 (0.24)</td>
<td>15.02</td>
<td>0.01</td>
<td>0.16 (0.11-0.24)</td>
<td>0.84 (0.50-1.96)</td>
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</tr>
<tr>
<td>GRF</td>
<td>180</td>
<td>3.75 (1.02)</td>
<td>5.08</td>
<td>0.28</td>
<td>0.58 (0.33-0.78)</td>
<td>1.27 (0.93-2.49)</td>
<td>3.61 (2.22-5.89)*</td>
<td>1.52 (1.08-2.77)*</td>
</tr>
<tr>
<td>ROB</td>
<td>180</td>
<td>3.46 (0.89)</td>
<td>1.45</td>
<td>0.84</td>
<td>0.49 (0.30-0.66)</td>
<td>1.16 (0.84-2.31)</td>
<td>3.09 (1.91-5.00)*</td>
<td>1.39 (0.70-2.75)</td>
</tr>
</tbody>
</table>

n = number of larvae assayed.

1 95% fiducial limits estimated using POLO (LeOra Software 1987).

2 LCR-LC$_{50}$, lethal concentration ratio at LC$_{50} = $LC$_{50}$ (field population)/LC$_{50}$ (LAB population).

3 LCR-LC$_{90}$, lethal concentration ratio at LC$_{90} = $LC$_{90}$ (field population)/LC$_{90}$ (LAB population).
4 95% confidence limits estimated using lethal concentration ratio significance test (Robertson et al. 2007).

* LC$_{50}$ or LC$_{90}$ of field collected population significantly different (α = 0.05) from that of the LAB population (Robertson et al. 2007).
Table 5. Results of probit analyses for azinphosmethyl using diet incorporation bioassays on *C. rosaceana* neonate larvae from a laboratory as well as field-collected populations in 2008

<table>
<thead>
<tr>
<th>Population</th>
<th>n</th>
<th>Slope (± SE)</th>
<th>$\chi^2$</th>
<th>P</th>
<th>$\text{LC}_{50}$ (ppm) (95% FL)</th>
<th>$\text{LC}_{90}$ (ppm) (95% FL)</th>
<th>$\text{LCR-LC}_{50}^1$ (95% CL)</th>
<th>$\text{LCR-LC}_{90}^1$ (95% CL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Spring Brood:</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>LAB</td>
<td>210</td>
<td>4.25 (0.88)</td>
<td>8.20</td>
<td>0.15</td>
<td>1.33</td>
<td>2.66</td>
<td>(1.04-1.66)</td>
<td>(2.05-4.42)</td>
</tr>
<tr>
<td>KMP</td>
<td>210</td>
<td>2.11 (0.27)</td>
<td>1.17</td>
<td>0.95</td>
<td>9.92</td>
<td>40.04</td>
<td>(7.29-13.52)</td>
<td>(26.99-72.11)</td>
</tr>
<tr>
<td>WEB</td>
<td>180</td>
<td>1.52 (0.18)</td>
<td>17.32</td>
<td>0.004</td>
<td>52.69</td>
<td>368.33</td>
<td>(33.85-83.81)</td>
<td>(202.03-963.10)</td>
</tr>
<tr>
<td>CLK</td>
<td>210</td>
<td>1.64 (0.19)</td>
<td>10.81</td>
<td>0.06</td>
<td>15.56</td>
<td>94.32</td>
<td>(10.52-23.24)</td>
<td>(56.26-206.07)</td>
</tr>
<tr>
<td><strong>First Summer Brood:</strong></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAB</td>
<td>210</td>
<td>5.10 (1.14)</td>
<td>0.004</td>
<td>1.00</td>
<td>1.96</td>
<td>3.49</td>
<td>(1.44-2.43)</td>
<td>(2.77-5.31)</td>
</tr>
<tr>
<td>GRF</td>
<td>210</td>
<td>2.78 (0.50)</td>
<td>3.02</td>
<td>0.70</td>
<td>50.55</td>
<td>146.28</td>
<td>(32.22-72.61)</td>
<td>(98.60-289.39)</td>
</tr>
<tr>
<td>ROB</td>
<td>210</td>
<td>1.48 (0.25)</td>
<td>4.48</td>
<td>0.48</td>
<td>7.56</td>
<td>55.66</td>
<td>(3.46-12.79)</td>
<td>(31.85-138.84)</td>
</tr>
</tbody>
</table>

n = number of larvae assayed.

1 95% fiducial limits estimated using POLO (LeOra Software 1987).

2 $\text{LCR-LC}_{50}$, lethal concentration ratio at $\text{LC}_{50} = \text{LC}_{50}$ (field population)/$\text{LC}_{50}$ (LAB population).

3 $\text{LCR-LC}_{90}$, lethal concentration ratio at $\text{LC}_{90} = \text{LC}_{90}$ (field population)/$\text{LC}_{90}$ (LAB population).
95% confidence limits estimated using lethal concentration ratio significance test (Robertson et al. 2007).

* LC$_{50}$ or LC$_{90}$ of field collected population significantly different ($\alpha = 0.05$) from that of the LAB population (Robertson et al. 2007).
Fig. 1. Regression between spinosad and spinetoram lethal concentration ratios at LC$_{50}$ in populations of *C. rosaceana* collected from pome fruit orchards in Washington.
CHAPTER FIVE

ASSESSMENT OF RESISTANCE RISK IN OBLIQUEBANDED LEAFROLLER (LEPIDOPTERA: TORTRICIDAE) TO THE REDUCED-RISK INSECTICIDES CHLORANTRANILIPROLE AND SPINETORAM

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Department of Entomology, Washington State University, Tree Fruit Research and Extension Center, 1100 N. Western Avenue, Wenatchee, WA 98801

Abstract

Obliquebanded leafroller, Choristoneura rosaceana (Harris), is a major pest of pome fruits in Washington. The use of broad-spectrum insecticides for decades has led to the development of insecticide resistance in C. rosaceana. Recently registered insecticides with novel modes of action such as chlorantraniliprole and spinetoram have provided effective C. rosaceana control, but resistance remains a threat. The risk of insecticide resistance development in a pest can be assessed by artificial selection in the laboratory. Subsequently, this information can be valuable in developing strategies to retain susceptibility in the field. A laboratory population of C. rosaceana was selected following repeated exposure to chlorantraniliprole and spinetoram to determine the risk of resistance evolution. After six generations of selection, 6.58- and 3.64-fold increases in LC50 were recorded for chlorantraniliprole and spinetoram, respectively. The realized heritability (h²) of resistance was estimated as 0.17 for
chlorantraniliprole and 0.18 for spinetoram using threshold trait analysis. The rates of resistance development were compared using the response quotient ($Q$), which was estimated as 0.11 and 0.07 for chlorantraniliprole and spinetoram, respectively. Projected rates of resistance evolution indicated that if $h^2 = 0.2$ and 80% of the population was killed at each generation, then a 10-fold increase in LC$_{50}$ would be expected in less than six generations for chlorantraniliprole and ten generations for spinetoram. These results indicate that the risk of resistance development in *C. rosaceana* exists to both of these insecticides, but resistance development in *C. rosaceana* would be slower against spinetoram than chlorantraniliprole.

**Key words:** Obliquebanded leafroller, chlorantraniliprole, spinetoram, resistance, heritability

**Introduction**

The obliquebanded leafroller, *Choristoneura rosaceana* (Harris), is one of the most destructive lepidopteran pests of pome fruits in Washington (Brunner 1999). The use of broad spectrum organophosphorus insecticides (OPs) against *C. rosaceana* and a wide spectrum of other tree fruit pests for over four decades has led to the development of insecticide resistance in *C. rosaceana* (Brunner 1996). Insecticide resistance against OPs and cross-resistance to other groups of chemicals have been documented in *C. rosaceana* (Reissig et al. 1986, Lawson et al. 1997, Waldstein et al. 1999, Ahmad et al. 2002, Smirle et al. 2002, Dunley et al. 2006), in some cases to newly developed insecticides even before they had been used in the field (Sauphanor et al. 1998, Dunley and Welter 2000). The evolution of insecticide resistance, and increased public concern over health and environmental effects of broad-spectrum insecticides has led to a greater priority in the development of reduced-risk
insecticides (USEPA 1997). Some reduced-risk insecticides with novel chemistries such as chlorantraniliprole and spinetoram have recently been registered as OP alternatives for use in tree fruit production.

Chlorantraniliprole is a member of a new class of insecticides, the anthranilic diamides. Anthranilic diamides selectively bind to the ryanodine receptors (RyR) in insect muscles resulting in an uncontrolled release of internal calcium stores from the sarcoplasmic reticulum (Lahm et al. 2005, Cordova et al. 2006), causing impaired regulation of muscle contraction leading to feeding cessation, lethargy, paralysis, and death in target organisms. Anthranilic diamides have very low vertebrate toxicity due to a >500-fold differential selectivity toward insect over mammalian RyR (Cordova et al. 2006). Spinetoram is a recently developed member of the spinosyns class of insecticides. They primarily activate the nicotinic acetylcholine receptors by acting on a unique site (Salgado 1998, Salgado et al. 1998). Both chlorantraniliprole and spinetoram have shown high efficacy against C. rosaceana (Hull et al. 2009, Brunner unpublished data).

The integration of these highly selective insecticides into pest management programs is critical for successful production of tree fruits on a sustainable basis. However, the development of pest resistance is a continual threat, especially to recently introduced insecticides. Resistance management strategies are often implemented after resistance has been detected in field populations, reducing their value in managing resistance. Therefore, the assessment of resistance risk in a pest like C. rosaceana before resistance occurs in the field would provide valuable information supporting a proactive implementation of strategies to manage and maintain susceptibility in field populations and thus to delay the development of resistance.
There are several techniques available to assess resistance risk for an insecticide, including selecting for resistance in the laboratory (National Research Council 1986, Brown and Payne 1988). Data from selection experiments can be analyzed using quantitative genetic models that consider resistance as a continuous variable and estimate heritability of resistance (Firko and Hayes 1990). Estimation of heritability (narrow-sense), the proportion of phenotypic variation accounted for by additive genetic variation (Falconer and Mackay 1996), provides a standardized way to quantify and summarize results from selection experiments (Tabashnik 1992), and can therefore be used to predict rate and direction of the genetic change associated with resistance (Firko and Hayes 1990).

Assessing risk of insecticide resistance development in *C. rosaceana* to newly introduced insecticides is an important factor to managing resistance against these chemicals and thus sustaining the efficacy of these products for as long as possible. In this study we assessed the risk of resistance development in *C. rosaceana* to chlorantraniliprole and spinetoram using truncated selection whereby successive generations of a susceptible population are selected and maintained in the laboratory.

**Materials and Methods**

**Insects.** A laboratory colony (LAB) of *C. rosaceana* was established in 1990 from larvae collected from apple orchards in Mattawa, WA. This colony has been reared continuously since their collection on a pinto bean diet following the method of Shorey and Hale (1965) under constant conditions of temperature (23 ± 2°C), relative humidity (RH 70%), and photoperiod (16:8 L:D) and without exposure to insecticides.
**Insecticides.** The insecticides tested were chlorantraniliprole (Rynaxypyr™/Altacor® 35WG [active ingredient: 35%]), E.I. du Pont Co., Wilmington, DE; and spinetoram (Delegate® 25WG [active ingredient: 25%]), Dow AgroSciences, Indianapolis, IN.

**Bioassays.** Toxicity of chlorantraniliprole and spinetoram to neonate *C. rosaceana* larvae was determined using a diet incorporation bioassay. Diet used in the bioassay was a dry premix of a Heliothis diet (Stonefly Heliothis Diet [Item # 38 V 0600], Ward’s Natural Science, Rochester, NY). Insecticide incorporated diet was prepared by mixing insecticide dilution (insecticide + water), water, vinegar, and dry diet premix at a ratio of 10:61:4:25 to produce 100 g of final product. Vinegar was used in the diet as recommended by the manufacturer. A stock solution of each insecticide was prepared by diluting it at 10X the highest concentration to be used in the bioassay. Serial dilutions were then prepared from the stock solution at 10X each of the target concentrations to be used in the bioassay. A treatment solution was prepared by combining 61 g of water to 4 g of vinegar and then with 10 g of the appropriate 10X insecticide dilution. The treatment solution was then added to 25 g of dry diet premix to complete an insecticide incorporated diet of known concentration. An untreated control was prepared by combining water only (75 g) with the dry diet premix. Enough insecticide incorporated diet was prepared prior at the start of the bioassays, so that all tests were conducted using the same diet mixtures, and new diet was prepared for each replication.

A small portion of insecticide incorporated diet (approximately 8.0 cm$^3$) was added to a plastic 50x9 mm Petri dish (Becton Dickinson and Company, Franklin Lakes, NJ). The diet was pushed firmly along the edges of the dish, and scored with a pin so that *C. rosaceana* neonates could readily colonize the diet. Petri dishes were chosen randomly, and five 1-day-old *C. rosaceana* larvae were transferred into each dish using a camel’s hair brush. Six to ten
dishes were prepared for each treatment (30-50 larvae/treatment) depending on the availability of neonate larvae. The dishes were placed in growth chambers at constant conditions of temperature (23 ± 2°C), RH 70%, and 16:8 L:D photoperiod. Larval mortality in each bioassay was evaluated at 7 d. Larvae were recorded as dead if they did not move when probed with camel’s hair brush. To ensure that offspring of many females were assayed, larvae emerging from any given egg mass were systematically distributed among various concentrations so that a maximum of 5-10 larvae per egg mass were exposed to any one concentration.

**Selection for Resistance.** Based on results from initial bioassays, cohorts of larvae from the laboratory colony were selected with chlorantraniliprole (RYN) or spinetoram (SPIN) for six consecutive generations while another cohort treated in the same way but without exposure to insecticides served as a control (LAB). In the first selection, neonate larvae were exposed to LC$_{70}$ of the baseline established for the LAB population. After 4 d of exposure, surviving larvae were transferred to untreated pinto bean diet, and reared in the laboratory under conditions described above. The concentration of chlorantraniliprole and spinetoram used to select each subsequent generation was the ≈LC$_{70}$ based on the results of bioassays from the previous generation. The number of neonate larvae used for each generation varied (1000-2000) depending on availability. A total of six rounds of selection (generations) were conducted with each insecticide. Based on availability, a subset of the progeny of the *C. rosaceana* surviving each selection was exposed to a range of concentrations using diet incorporation bioassay to determine the effect of selection on the susceptibility of the selected populations. Each time a bioassay was conducted on larvae from the selected colonies, a bioassay was also conducted on larvae from the LAB colony. There were larvae available for
bioassays only at generations 1, 3, 5, and 6 for the RYN colony and at generations 1, 2, 4, and 6 for the SPIN colony to conduct the concentration response bioassays.

**Data Analysis.** Lethal concentration values (LC$_{50}$ and LC$_{90}$) and their corresponding 95% fiducial limits (FL) were estimated using POLO (LeOra Software 1987) and lethal concentration ratios (LCR) at LC$_{50}$ values and their corresponding 95% confidence limits (CL) were calculated using lethal concentration ratio significance test (Robertson et al. 2007). A laboratory population that was not selected with any of the insecticides but otherwise treated the same way served as the reference susceptible population for comparison purposes and was assigned a ratio of 1.0. Lethal concentrations of the LAB population before and after selection were considered significantly different if the 95% CL of their corresponding LCR did not include the value of 1.0.

**Estimation of Realized Heritability.** Realized heritability ($h^2$) of resistance to chlorantraniliprole and spinetoram in *C. rosaceana* was estimated using a threshold trait analysis method (Tabashnik 1992, Tabashnik and McGaughey 1994), where resistance was considered a threshold trait with an underlying continuous variable called tolerance (Falconer and Mackay 1996):

$$ h^2 = \frac{R}{S} $$  \hspace{1cm} (1)

; where $R$ is the response to selection and $S$ is the selection differential (Falconer and Mackay 1996).

Response to selection ($R$), the difference in mean phenotype between the offspring of the selected parents and the whole parental generation before selection was estimated as:

$$ R = \frac{\log \text{(final LC}_{50}) - \log \text{(initial LC}_{50})}{n} $$  \hspace{1cm} (2)
; where final LC\textsubscript{50} is the LC\textsubscript{50} of offspring of the \emph{C. rosaceana} surviving after n generations of selection, initial LC\textsubscript{50} is the LC\textsubscript{50} of LAB population after n generation without selection, and \( R \) is the average response to selection per generation.

The selection differential (\( S \)), the difference in mean phenotype between the selected parents and the entire parental generation (Hartl 1988) was estimated as:

\[
S = i \sigma_p
\]  \hspace{1cm} (3)

; where \( i \) is the intensity of selection and \( \sigma_p \) is the phenotypic standard deviation. The intensity of selection (\( i \)) was estimated from \( p \), the percentage of population with values above the selection threshold (i.e., the percentage of treated larvae surviving selection), using Appendix-A of Falconer and Mackay (1996), which is based on the properties of normal distribution.

The phenotypic standard deviation was estimated as the reciprocal of mean of the estimated slopes of the probit regression lines from the LAB population after n generations without selection (initial slope) and the offspring of the RYN or SPIN populations after \( n \) generations of selection (final slope):

\[
\sigma_p = \left[ \frac{1}{2} \left( \text{initial slope} + \text{final slope} \right) \right]^{-1}
\]  \hspace{1cm} (4)

We also used the regression method proposed by Tanaka and Noppun (1989) for estimating narrow-sense heritability (\( h^2 \)), where \( h^2 \) is the regression of breeding values (\( R \)) on phenotypic values (\( S \)), and is equivalent to the ratio of additive genetic variance (\( V_a \)) to total phenotypic variance (\( V_P \)): \( h^2 = V_a/V_P \) (Falconer and Mackay 1996). The values of parameters \( R \) and \( S \) were determined at every generation of selection and \( h^2 \) was estimated as the regression coefficient of cumulative response to selection on cumulative selection differential.
To determine if the genetic parameters such as $R$, $S$, or $h^2$ changed during the course selection, we calculated each of the parameters for the first and second halves of each experiment separately. The split between the two parts was as close to half as allowed by the data.

The response to selection ($R$) can be estimated as the product of heritability ($h^2$) and selection differential ($S$) (Falconer and Mackay 1996):

$$R = h^2 S. \quad (5)$$

Based on the response of *C. rosaceana* to selection in laboratory, predictions about the risk of resistance development were made under varying conditions of heritability and slope at different selection intensities in terms of number of generations required for a 10-fold increase in $\text{LC}_{50}$ ($G$), which is the reciprocal of $R$ (Tabashnik 1992):

$$G = R^{-1}. \quad (6)$$

For any particular value of $S$, the rate of resistance development will be directly proportional to $h^2$ (equation 5) and inversely proportional to slope (equation 4). $S$ can be constant across insecticides for a particular intensity of selection only if the slope of the probit regression lines (and thus $\sigma_p$) is constant across insecticides (equations 3 and 4), but slope is not necessarily constant across insecticides. Thus, response quotient ($Q$) was used to compare the rates of resistance development against chlorantraniliprole and spinetoram, which can be defined as $R$ divided by $i$ (Tabashnik and McGaughey 1994):

$$Q = R/i. \quad (7)$$

The value of $Q$ enables comparing the rates of resistance evolution among different insecticides without reference to slope, and thus evaluate the durability of an insecticide against a particular pest population.
Results

Susceptibility of RYN population to chlorantraniliprole significantly decreased as a result of selection with chlorantraniliprole (Table 1). The first round of selection resulted in 2.2-fold increase in $\text{LC}_{50}$ of the RYN population as compared to that of the unselected LAB population. $\text{LC}_{50}$ of the RYN population continued to increase as a result of each of the subsequent rounds of selection leading to LCR of 6.6 at $F_5$. However, there was no additional increase in $\text{LC}_{50}$ as a result of sixth round of selection.

Similarly, a significant decrease in the susceptibility of SPIN population was observed as a result of selection with spinetoram (Table 2). There was a 1.26-fold increase in $\text{LC}_{50}$ of the SPIN population as a result of first round of selection as compared to the unselected LAB population. After six rounds of selection, $\text{LC}_{50}$ of the SPIN population was increased to 3.64-fold as compared to the unselected LAB population.

The overall mean estimate of $h^2$ of chlorantraniliprole resistance using threshold trait analysis in *C. rosaceana* was 0.17 with mean response to $R$ of 0.19 and an overall mean $S$ of 1.12 (Table 3). The $h^2$ of spinetoram resistance was 0.18 with an $R$ of 0.13 and an $S$ of 0.70 (Table 4). However, the values of heritability of chlorantraniliprole and spinetoram resistance using the regression method were estimated as 0.13 ($R^2 = 0.96$) (Fig. 2) and 0.18 ($R^2 = 0.96$) (Fig. 3), respectively.

The estimates of $h^2$ of chlorantraniliprole resistance were higher for the first half of the selection experiment (mean = 0.28) than the second half (mean = 0.11) (Fig. 1a). Although the $S$ was higher in the second half of the selection experiment (mean = 1.41) than in the first half (mean = 0.82), $R$ was higher in the first half of the selection experiment (mean = 0.24) than in the second half (mean = 0.15) (Table 3).
Likewise, the estimates of $h^2$ of spinetoram resistance were higher for the first half of the selection experiment (mean = 0.22) than those for the second half (mean = 0.15) (Fig. 1b). Although $S$ was higher in the second half of the selection experiment (mean = 0.75) than that in the first half (mean = 0.65), $R$ was higher in the first half of the selection experiment (mean = 0.14) than that in the second half (mean = 0.11) (Table 4). These results indicate that the proportion of total phenotypic variation for resistance against chlorantraniliprole attributable to additive genetic variation in resistance declined during the selection experiment. This also was the case for spinetoram.

The mean values of $Q$ for resistance against chlorantraniliprole and spinetoram were 0.11 and 0.07, respectively. These results indicate that resistance evolution would be slower against spinetoram than that against chlorantraniliprole, and thus spinetoram would be more durable than chlorantraniliprole against this particular population of *C. rosaceana*.

**Discussion**

Insect populations maintained in the laboratory for several years without being exposed to any insecticides are likely to have less genetic variation than field populations (Keiding 1986, Tanaka and Noppun 1989, Firku and Hayes 1990). It took only six generations of selection of a susceptible laboratory strain of *C. rosaceana* with chlorantraniliprole and spinetoram to produce a 6.6- and 3.6-fold increase in LC$_{50}$, respectively. The increase in levels of tolerance indicates that resistance could result in the field situations where selection pressures can be much higher than in the laboratory and populations are likely to be more heterogeneous.

Estimation of $h^2$ based on the method proposed by Tabashnik (1992) provides a standardized means to quantify the results of selection experiments by incorporating estimates of the
strength of selection as well as the rate of resistance development. It also places the results of selection experiments in the broader context of the empirical and theoretical literature of evolutionary biology (Mousseau and Roff 1987, Falconer and Mackay 1996). In this study, $R$ declined for both chlorantraniliprole and spinetoram as the selection progressed whereas $S$ did not, leading to significantly higher $h^2$ in the first half of the selection experiment compared to the second half. These results are in agreement with those of Tabashnik (1992) where substantial additive genetic variation was present initially (i.e., alleles for resistance were not rare) and then declined as selection proceeded.

There was no change observed in the LCR as selection for chlorantraniliprole resistance proceeded from $F_5$ to $F_6$ and only a slight increase in the LCR as selection for spinetoram resistance proceeded from generations $F_4$ to $F_6$. These results point out that most of the variation in susceptibility of the LAB population to chlorantraniliprole and spinetoram had been exhausted. Likewise, a decrease in $h^2$ of chlorantraniliprole after three generations and spinetoram after four generations of selection indicates that most of the additive genetic variation in susceptibility of that particular LAB population to chlorantraniliprole and spinetoram had been exhausted. In addition, high mortalities observed after the fifth round of selection for both chlorantraniliprole and spinetoram resistances indicate the occurrence of bottlenecks which might have contributed to the reduced variation in the selected populations (Falconer and Mackay 1996, Saccheri et al. 2001).

The estimated values of $h^2$ of chlorantraniliprole resistance from our selection experiment using two different methods, 0.17 (Tabashnik 1992) and 0.13 (Tanaka and Noppun 1989) are in close agreement, and those of spinetoram resistance, 0.18 (Tabashnik 1992) and 0.18 (Tanaka and Noppun 1989) are the same. These results indicate that $\approx 13-17\%$ of the total
variation in chlorantraniliprole susceptibility and \( \approx 18\% \) of that in spinetoram susceptibility of the LAB population was caused by additive genetic variation. In a summary of thirteen previously reported studies, estimates of \( h^2 \) to various insecticides for other insect pests ranged from 0.05 to 0.85, with a mean of 0.29 (Omer et al. 1993). Thus, our estimates of \( h^2 \) of resistance to chlorantraniliprole (0.13 and 0.17) and spinetoram (0.18) in \textit{C. rosaceana} were lower than the mean for other reported cases.

Like any laboratory studies, the estimation of \( h^2 \) based on selections (Tanaka and Noppun 1989, Tabashnik 1992) is not free from limitations including technical problems in estimating parameters and uncertainty about extrapolation of experimental results to the field populations. Falconer and Mackay (1996) discussed technical difficulties generally encountered in estimating \( h^2 \) from selection experiments, whereas Tanaka and Noppun (1989) and Tabashnik (1992) examined the specific problems in the context of estimating \( h^2 \) of insecticide resistance. A detailed analysis of the factors that may introduce bias into the estimates of \( S \) (selection differential) was provided by Rosenheim (1991). One of these factors, the unequal selection of males and females, was minimized by selecting neonates without regard to sex. Efforts were made in bioassays to minimize the individual differences in exposure, but we do not know the extent of bias in estimation of \( S \) introduced by unequal treatment of individual neonates (which can overestimate \( S \)), and sublethal effects (which can underestimate \( S \)). Chlorantraniliprole caused mating disruption in codling moth when adult moths were exposed to its residues (Knight and Flexner 2007) but sublethal effects of larval exposure are largely unknown for chlorantraniliprole and spinetoram because these are novel insecticides and were only recently registered (2008) for use in orchards.
Despite recognized difficulties in extrapolating laboratory results to the field, we used estimates of realized $h^2$ and slope of probit lines in conjunction with varying selection intensities to project the rates of resistance development (Figs. 4 and 5). The projected rate of resistance evolution is directly proportional to $h^2$ and selection intensity [equation 5] (Fig. 4). For instance: assuming a slope of 1.6 [the average slope observed for chlorantraniliprole in this study] ($\sigma_p = 0.625$), $h^2$ of 0.17, and a selection mortality of 80% at each generation, then $R = 0.12$ and LC$_{50}$ increases 10-fold in eight generations. However, in the same would happen in 12 generations if selection mortality is only 50%. Moreover, in a population with $h^2$ of 0.07 and 50% of the population selected at each generation ($R = 0.04$), a 10-fold increase in LC$_{50}$ is expected in 29 generations. If $h^2 = 0.27$ and 90% of the population is killed in each generation ($R = 0.30$), only three generations are needed to increase LC$_{50}$ by a factor of 10. Similarly, assuming a slope of 2.8 [the average slope recorded for spinetoram in this study] ($\sigma_p = 0.357$), $h^2$ of 0.18, and selection mortality of 80% at each generation, then $R = 0.09$ and LC$_{50}$ increases 10-fold in 11 generations. However, the same would happen in 19 generations if selection mortality is only 50% ($R = 0.05$). Moreover, if the population had an $h^2$ of 0.08 and 50% mortality at each generation, then $R = 0.02$ and a 10-fold increase in LC$_{50}$ would take more than 43 generations. If $h^2 = 0.28$ and 90% of the population is killed in each generation ($R = 0.18$), only less than six generations would be needed to increase LC$_{50}$ by a factor of 10.

On the other hand, the projected rate of resistance evolution is inversely proportional the slope of the probit line (Fig. 5). For example: assuming that $h^2 = 0.17$ [the heritability of chlorantraniliprole resistance observed in this study] and selection mortality = 70%, a 10-fold increase in LC$_{50}$ would occur in only eight generations at a slope of 1.6 whereas it would take
more than 18 generations for the same to happen at a slope of 3.6. Similarly, assuming that $h^2 = 0.18$ [the heritability of spinetoram resistance observed in this study] and selection mortality = 80%, it would take seven generations for a 10-fold increase in LC$_{50}$ at a slope of 1.8 whereas the same would happen in more than 15 generations at a slope of 3.8.

However, predictions must be interpreted cautiously because they are based on estimates of $h^2$ of a laboratory reared population. The $h^2$ of resistance to a particular insecticide can vary between conspecific populations as well as within a population through time because of changes in allele frequencies, environmental variation, or both. Nonetheless, the predictions based on the equation ($G = R^{-1}$) from quantitative genetic theory using a laboratory population provide information that could be valuable in developing strategies to manage resistance (Via 1986, Firku and Hayes 1990, Tabashnik 1992) even before the occurrence of resistance in the field populations.

Relatively quick response of a laboratory population selected with chlorantraniliprole and spinetoram suggests that a risk for resistance development in *C. rosaceana* to both insecticides exists. The higher value of response quotient ($Q$) for chlorantraniliprole (0.11) compared to that for spinetoram (0.07) suggests that resistance to chlorantraniliprole could evolve faster than to spinetoram in *C. rosaceana*. Our findings serve as an early warning for the growers and pest managers, and point out that implementation of resistance management strategies should occur when these chemistries are registered for use.

Although insecticide resistance management in *C. rosaceana* in tree fruit orchards is a challenge for growers and pest managers, especially at the time when broad-spectrum insecticides such as OPs are being phased out, a wide range of newer insecticides with different modes of action is available to control this pest. Significant variation in response of
the field-collected populations to the two novel insecticides, chlorantraniliprole and spinetoram, has already been documented before their first field application (Sial et al. 2010). These insecticides must be used wisely in the framework of a well thought-out resistance management program. However, resistance management strategies can only be successful if no cross-resistance occurs between different insecticides used in a resistance management program (Georghiou 1983). Therefore, further studies are required to explore the biochemical and molecular basis of mechanisms conferring resistance to chlorantraniliprole and spinetoram so that the insecticides that would not be affected by the same detoxification mechanisms could be incorporated into a pest management program in a manner that would minimize selection for resistance.

Acknowledgments

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Tables and Figures

**Table 1.** The effect of chlorantraniliprole contaminated diet on *Choristoneura rosaceana* neonate larvae from chlorantraniliprole selected population (RYN) and unselected susceptible laboratory population (LAB)

<table>
<thead>
<tr>
<th>Selected Generation</th>
<th>Population</th>
<th>n</th>
<th>Slope (± SE)</th>
<th>( \chi^2 )</th>
<th>( LC_{50} ) (ppm) (95% FL)</th>
<th>( LC_{90} ) (ppm) (95% FL)</th>
<th>LCR-LC(_{50}) (95% CL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>RYN</td>
<td>450</td>
<td>1.02 (0.39)</td>
<td>20.74</td>
<td>0.16 (0.07-0.32)</td>
<td>2.94 (1.41-8.37)</td>
<td>2.2 (1.02-4.65)*</td>
</tr>
<tr>
<td>LAB</td>
<td></td>
<td>450</td>
<td>1.08 (0.10)</td>
<td>25.07</td>
<td>0.08 (0.03-0.15)</td>
<td>1.17 (0.56-3.42)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>RYN</td>
<td>350</td>
<td>1.72 (0.17)</td>
<td>17.10</td>
<td>0.26 (0.20-0.34)</td>
<td>1.46 (1.00-2.43)</td>
<td>3.1 (2.12-4.43)*</td>
</tr>
<tr>
<td>LAB</td>
<td></td>
<td>350</td>
<td>2.24 (0.28)</td>
<td>6.62</td>
<td>0.08 (0.06-0.11)</td>
<td>0.32 (0.23-0.49)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>RYN</td>
<td>210</td>
<td>1.19 (0.17)</td>
<td>10.31</td>
<td>0.77 (0.31-1.48)</td>
<td>9.26 (4.40-33.02)</td>
<td>6.6 (3.27-13.24)*</td>
</tr>
<tr>
<td>LAB</td>
<td></td>
<td>210</td>
<td>1.90 (0.28)</td>
<td>3.01</td>
<td>0.12 (0.08-0.16)</td>
<td>0.55 (0.36-1.09)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>RYN</td>
<td>180</td>
<td>1.88 (0.36)</td>
<td>7.71</td>
<td>1.03 (0.50-1.66)</td>
<td>4.93 (2.88-14.19)</td>
<td>6.6 (3.68-11.79)*</td>
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<td></td>
<td>180</td>
<td>1.59 (0.19)</td>
<td>11.83</td>
<td>0.16 (0.11-0.23)</td>
<td>1.00 (0.59-2.17)</td>
<td></td>
</tr>
</tbody>
</table>

n = number of larvae assayed.

1 95% fiducial limits estimated using POLO (LeOra Software 1987).

2 LCR-LC\(_{50}\), lethal concentration ratio at \( LC_{50} = LC_{50} \) (field population)/\( LC_{50} \) (LAB population).

3 95% confidence limits estimated using lethal concentration ratio significance test (Robertson et al. 2007).

* Indicates that Lethal Concentration Ratio was significant (\( \alpha = 0.05 \)) (Robertson et al. 2007).
Table 2. The effect of spinetoram contaminated diet on *Choristoneura rosaceana* neonate larvae from chlorantraniliprole selected population (RYN) and unselected susceptible laboratory population (LAB)

<table>
<thead>
<tr>
<th>Selected Generation</th>
<th>Population</th>
<th>n</th>
<th>Slope (± SE)</th>
<th>$\chi^2$</th>
<th>LC$_{50}$ (ppm) (95% FL)$^1$</th>
<th>LC$_{50}$ (ppm) (95% FL)$^1$</th>
<th>LCR-LC$_{50}$$^2$ (95% CL)$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SPIN</td>
<td>450</td>
<td>2.56 (0.37)</td>
<td>4.18</td>
<td>0.10 (0.07-0.12)</td>
<td>0.31 (0.23-0.48)</td>
<td>1.26 (0.86-1.85)</td>
</tr>
<tr>
<td>LAB</td>
<td></td>
<td>450</td>
<td>4.00 (0.90)</td>
<td>3.62</td>
<td>0.08 (0.06-0.10)</td>
<td>0.16 (0.13-0.26)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>SPIN</td>
<td>350</td>
<td>2.53 (0.33)</td>
<td>3.96</td>
<td>0.12 (0.09-0.15)</td>
<td>0.39 (0.29-0.59)</td>
<td>2.3 (1.59-3.26)$^*$</td>
</tr>
<tr>
<td>LAB</td>
<td></td>
<td>350</td>
<td>1.75 (0.16)</td>
<td>7.61</td>
<td>0.05 (0.02-0.19)</td>
<td>0.29 (0.12-2.10)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>SPIN</td>
<td>350</td>
<td>3.63 (0.58)</td>
<td>2.98</td>
<td>0.17 (0.14-0.20)</td>
<td>0.38 (0.30-0.56)</td>
<td>3.5 (2.37-5.09)$^*$</td>
</tr>
<tr>
<td>LAB</td>
<td></td>
<td>350</td>
<td>2.72 (0.55)</td>
<td>6.22</td>
<td>0.05 (0.02-0.07)</td>
<td>0.14 (0.10-0.39)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>SPIN</td>
<td>210</td>
<td>3.01 (0.48)</td>
<td>2.52</td>
<td>0.22 (0.17-0.29)</td>
<td>0.59 (0.43-1.02)</td>
<td>3.64 (2.42-5.46)$^*$</td>
</tr>
<tr>
<td>LAB</td>
<td></td>
<td>210</td>
<td>1.97 (0.24)</td>
<td>7.59</td>
<td>0.06 (0.04-0.11)</td>
<td>0.27 (0.15-0.87)</td>
<td></td>
</tr>
</tbody>
</table>

n = number of larvae assayed.

$^1$ 95% fiducial limits estimated using POLO (LeOra Software 1987).

$^2$ LCR-LC$_{50}$, lethal concentration ratio at LC$_{50}$ = LC$_{50}$ (field population)/LC$_{50}$ (LAB population).

$^3$ 95% confidence limits estimated using lethal concentration ratio significance test (Robertson et al. 2007).

* Indicates that Lethal Concentration Ratio was significant ($\alpha = 0.05$) (Robertson et al. 2007).
Table 3. Estimation of response to selection ($R$) and selection differential ($S$) of chlorantraniliprole selected population of *Choristoneura rosaceana*

<table>
<thead>
<tr>
<th>Selected Generation</th>
<th>Estimation of response to selection</th>
<th>Estimation of selection differential</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial $\text{LC}_{50}$ (95% FL)</td>
<td>Final $\text{LC}_{50}$ (95% FL)</td>
</tr>
<tr>
<td>1</td>
<td>0.08 (0.03-0.15)</td>
<td>0.16 (0.07-0.32)</td>
</tr>
<tr>
<td>3</td>
<td>0.08 (0.06-0.11)</td>
<td>0.26 (0.20-0.34)</td>
</tr>
<tr>
<td>5</td>
<td>0.12 (0.08-0.16)</td>
<td>0.77 (0.31-1.48)</td>
</tr>
<tr>
<td>6</td>
<td>0.16 (0.11-0.23)</td>
<td>1.03 (0.50-1.66)</td>
</tr>
</tbody>
</table>
Table 4. Estimation of response to selection ($R$) and selection differential ($S$) of spinetoram selected population of *C. rosaceana*

<table>
<thead>
<tr>
<th>Selected Generation</th>
<th>Initial LC$_{50}$ (95% FL)</th>
<th>Final LC$_{50}$ (95% FL)</th>
<th>Estimation of response to selection</th>
<th>Estimation of selection differential</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>$R$</td>
<td>$i$ Initial slope Final slope $\sigma_p$ $S$</td>
</tr>
<tr>
<td>1</td>
<td>0.08 (0.06-0.10)</td>
<td>0.10 (0.07-0.12)</td>
<td>0.10</td>
<td>1.400 4.00 (0.90) 2.56 (0.37) 0.305 0.43</td>
</tr>
<tr>
<td>2</td>
<td>0.05 (0.02-0.19)</td>
<td>0.12 (0.09-0.15)</td>
<td>0.19</td>
<td>1.858 1.75 (0.16) 2.53 (0.33) 0.467 0.87</td>
</tr>
<tr>
<td>4</td>
<td>0.05 (0.02-0.07)</td>
<td>0.17 (0.14-0.20)</td>
<td>0.13</td>
<td>1.627 2.72 (0.55) 3.63 (0.58) 0.315 0.51</td>
</tr>
<tr>
<td>6</td>
<td>0.06 (0.04-0.11)</td>
<td>0.22 (0.17-0.29)</td>
<td>0.09</td>
<td>2.459 1.97 (0.24) 3.01 (0.48) 0.402 0.99</td>
</tr>
</tbody>
</table>
Fig. 1. Heritability in the narrow-sense ($h^2$) of resistance to chlorantraniliprole (a) and spinetoram (b) in a laboratory population of *Choristoneura rosaceana* selected for resistance in the laboratory.
Fig. 2. Regression of cumulative response to chlorantraniliprole selection on cumulative selection differential in Choristoneura rosaceana.
Fig. 3. Regression of cumulative response to spinetoram selection on cumulative selection differential in *Choristoneura rosaceana.*
Fig. 4. Effect of heritability ($h^2$) on number of generations of *Choristoneura rosaceana* required for a 10-fold increase in LC$_{50}$ of chlorantraniliprole (Slope = 1.6) and spinetoram (Slope = 2.8) at different selection intensities ($i$).
**Fig. 5.** Effect of slope on number of generations of *Choristoneura rosaceana* required for a 10-fold increase in LC$_{50}$ of chlorantraniliprole ($h^2 = 0.17$) and spinetoram ($h^2 = 0.18$) at different selection intensities ($i$).
CHAPTER SIX

BIOCHEMICAL CHARACTERIZATION OF CHLORANTRANILIPROLE AND SPINETORAM RESISTANCE IN OBLIQUEBANDED LEAFROLLER, CHORISTONEURA ROSACEANA (HARRIS) (LEPIDOPTERA: TORTRICIDAE)

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Abstract

Neonate larvae of obliquebanded leafroller, Choristoneura rosaceana, from a laboratory colony were exposed to two reduced-risk insecticides, chlorantraniliprole and spinetoram. After nine generations of selection, significant levels of resistance to each insecticide were observed. Biochemical assays were performed on third instar larvae to determine potential resistance mechanisms. Enzyme assays indicated that esterase activity was significantly increased in chlorantraniliprole-selected colony, whereas mixed-function oxidase levels were elevated in spinetoram-selected colony. No difference in glutathione-S-transferase activity was seen in either of the insecticide selected colonies. These results indicate the potential involvement of esterases and mixed-function oxidases as detoxification mechanisms responsible for resistance to chlorantraniliprole and spinetoram, respectively. Furthermore,
the results of this study suggest that chlorantraniliprole and spinetoram do not share detoxification mechanisms and could therefore be incorporated into resistance management programs in tree fruit leading to sustainable management of *C. rosaceana*.

**Key words:** Obliquebanded leafroller, chlorantraniliprole, spinetoram, resistance mechanisms, esterases, mixed-function oxidases, glutathione-S-transferase

**Introduction**

The obliquebanded leafroller (OBLR), *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae), is a major pest of pome fruits, second only to codling moth, *Cydia pomonella* (L), in the state of Washington [1]. Broad-spectrum insecticides, such as organophosphates (OPs), have been used to control OBLR for over four decades, leading to the development of resistance to OPs, and cross-resistance to other classes of insecticides [2-8]. OBLR has been reported to develop resistance to new insecticides, such as spinosad, only six years after its introduction into the field [8]; and in some cases high levels of resistance were documented for OBLR to chemicals that have never been used for insect control in tree fruits (i.e. indoxacarb) [6, 8, 9].

The development of insecticide resistance as well as regulatory actions such as Food Quality Protection Act of 1996 have prioritized the development of reduced-risk insecticides [10]. Chlorantraniliprole and spinetoram are reduced-risk insecticides which were recently registered for use in tree fruits as alternatives to OPs. Chlorantraniliprole is an anthranilic diamide which belongs to insecticide resistance action committee (IRAC) mode of action class 28 [11]. Anthranilic diamides selectively bind to ryanodine receptors (RyR) in insect
muscles resulting in an uncontrolled release of calcium from internal stores in the sarcoplasmic reticulum [12, 13], causing impaired regulation of muscle contraction leading to feeding cessation, lethargy, paralysis, and death of target organisms. Anthranilic diamides have very low vertebrate toxicity due to a >500-fold differential selectivity toward insect over mammalian RyR [13]. Spinetoram is a recently developed spinosyn belonging to IRAC mode of action class 5 [11]. Spinosyns primarily activate the nicotinic acetylcholine receptors by acting on a unique and yet unknown binding site [14-16]. Both chlorantraniliprole and spinetoram were highly effective against OBLR in both laboratory and field trials [17, Brunner unpublished data].

For successful production of tree fruits on a sustainable basis, it is critical for growers to incorporate reduced-risk insecticides into OBLR management programs. However, the development of resistance, and the possibility of cross-resistance, to previously used insecticides are major concerns with all new insecticides. The rational development of resistance management strategies for newly developed insecticides may not be possible without identification of mechanisms conferring resistance to those particular insecticides. Mechanisms responsible for resistance to broad-spectrum insecticides including azinphosmethyl, chloropyrifos, cypermethrin, chlorfenapyr, indoxacarb, and methoxyfenozide (an insect growth regulator) have been investigated in OBLR [18-20]. Biochemical mechanisms responsible for resistance to spinosad have been reported in other species of insects [21-26], but not for OBLR. Although resistance to chlorantraniliprole and spinetoram in some field-collected populations of OBLR has been reported [27], mechanisms conferring resistance to these chemicals have not been investigated.
In this paper, we report the results of our studies designed to characterize potential biochemical mechanisms of resistance to chlorantraniliprole and spinetoram in OBLR. This information will enable growers to incorporate these reduced-risk insecticides into more scientific knowledge based resistance management programs, leading to successful control of OBLR on a sustainable basis.

Materials and Methods

**Insects.** *C. rosaceana* were obtained from a laboratory colony that was established in 1990 from larvae collected from apple orchards in Mattawa, WA, and maintained at WSU Tree Fruit Research and Extension Center (Wenatchee, WA). This colony has been reared continuously since their collection on a pinto bean diet following the method of Shorey and Hale [28] under constant conditions of temperature (23 ± 2°C), relative humidity (RH, 70%), photoperiod (16:8, L:D), and without exposure to insecticides.

**Chemicals.** The insecticides used were chlorantraniliprole (Rynaxypyr™/Altacor® 35WG) obtained from E.I. du Pont de Nemours & Co., Wilmington, DE 19898, USA; and spinetoram (Delegate® 25WG) obtained from Dow AgroSciences (Indianapolis, IN). Bovine serum albumin (BSA), Cytochrome c from bovine heart (≥95%), 3,3',5,5'-tetramethyl benzidine dihydrochloride [TMBZ] (≥98%), α-naphthyl acetate [αNA] (≥98%), Fast Garnet GBC sulfate salt, L-glutathione reduced [GSH] (≥98%), 1-chloro-2,4-dinitrobenzene [CDNB] (97%), and 30% hydrogen peroxide were obtained from Sigma-Aldrich® (St. Louis, MO).

**Bioassays.** Toxicity of chlorantraniliprole and spinetoram to neonate *C. rosaceana* larvae was estimated by using a diet incorporation bioassay as described by Sial et al. [27] where a stock solution of each insecticide was prepared by diluting it at 10X the highest concentration
to be used in the bioassay. Serial dilutions were then prepared from the stock solution at 10X of each of the target concentrations to be used in the bioassay. Insecticide incorporated diet was prepared by mixing insecticide dilution (insecticide + water), water, vinegar, and dry diet premix (Stonefly *Heliothis* Diet Ward’s Natural Science, Rochester, NY) at a ratio of 10:61:4:25. An untreated control was prepared by mixing water, vinegar, and dry diet premix at a ratio of 71:4:25. Enough insecticide incorporated diet was prepared prior to the start of the bioassays, so that all tests were run on the same diet mixtures, and new diet was prepared for each replication.

A small portion of insecticide incorporated diet (approximately 8.0 cm³) was added to a plastic 50x9 mm Petri dish (Becton Dickinson and Company, Franklin Lakes, NJ). The diet was pushed firmly along the edges of the dish, and scored with a pin so that *C. rosaceana* neonates could readily colonize the diet. Petri dishes were chosen randomly, and five 1-day-old *C. rosaceana* larvae were transferred into each dish using a camel’s hair brush. Six to ten dishes were prepared for each treatment (30-50 larvae/treatment) depending on the availability of neonate larvae. These dishes were placed in growth chambers at constant conditions of temperature (23 ± 2°C), relative humidity (RH, 70%), and photoperiod (16:8, L:D). Larval mortality in each bioassay was evaluated after 7 d. Larvae were recorded as dead if they did not move when probed with camel’s hair brush. To ensure that offspring of different females were assayed, larvae emerging from any given egg mass were systematically distributed among various concentrations so that a maximum of 5-10 larvae per egg mass were exposed to any one concentration.

**Selection for Resistance.** Based on results from initial bioassays, cohorts of larvae from the laboratory colony were selected with chlorantraniliprole (RYN) or spinetoram (SPIN)
using diet incorporation bioassays, while the unselected control (LAB) was treated identically but without exposure to insecticides. In the first selection, neonate larvae were exposed to insecticides at concentrations corresponding to the LC$_{70}$ of the baseline established for the LAB colony. After 4 d of exposure, surviving larvae were transferred to untreated pinto bean diet, and reared in the laboratory under conditions described above. The concentration of chlorantraniliprole and spinetoram used to select each subsequent generation was LC$_{70}$ based on the results of bioassays from the previous generation. The number of neonate larvae used for each generation varied (1,000-2,000) depending on availability.

**Enzyme Assays.** Esterase, oxidase, and glutathione-S-transferase activities were determined according to the methods of Martin et al. [29] with modification as described below. For enzyme assays, 30 third-instar *C. rosaceana* larvae (10-15 mg each) from the susceptible colony (LAB) and each of the selected resistant colonies (RYN and SPIN) were used. Individual insects were homogenized in 200 µl ice cold potassium phosphate buffer (0.1M, pH 7.2), and then spun in a microfuge at ~21,000 g for 2 min. All reactions were carried out in disposable 96-well microplates (Greiner Bio-One, VWR International, West Chester, PA), as detailed below.

**Esterase Assay.** Esterase activity was measured using αNA as substrate. Briefly, hydrolysis of αNA was performed by incubating 10 µl homogenate with 90 µl of phosphate-buffered saline plus 1% Triton X-100 [PBS] (pH 6.5) for 10 min at room temperature. One hundred µl solution containing αNA (60 mM) in PBS was added and the mixture was incubated for 30 min at room temperature. The reaction was stopped by adding 100 µl Fast Garnet salt (2.4 mM) and the mixture was incubated for 10 min at room temperature. Absorbance was read at $\lambda = 550$ nm in a PowerWave 340 microplate spectrophotometer.
(BioTek, Winooski, VT) against blanks and converted to esterase activity (nmol/min/µg protein) based on the standard curve. The standard curve of α-Naphthol product was linear with an $r^2$ value of 0.9997.

**Oxidase Assay.** Total oxidase activity was measured using TMBZ as substrate. The reaction mixture consisted of 50 µl of 100 mM potassium phosphate buffer (pH 7.2), 50 µl of larval homogenate, 200 µl of 1.3 mM TMBZ 25% methanol, 250 mM sodium acetate buffer (pH 5.0). Then 25 µl of hydrogen peroxide (3.0%) was added, giving a final volume of 325 µl. Absorbance was read at $\lambda = 630$ nm against blanks (wells containing all reaction components except larval homogenate) in a PowerWave 340 microplate reader after 30 min incubation at room temperature. Total oxidase activity was expressed as pmol equivalent cyt-P450/mg protein. The standard curve of cytochrome c was linear with an $r^2$ value of 0.9947.

**Glutathione-S-transferase Assay.** Glutathione-S-Transferase (GSH) activity was measured using CDNB as a model substrate. The reaction mixture contained 100 µl of 10 mM GSH in 100 mM sodium phosphate buffer (pH 6.5) and 100 µl of larval homogenate. The reaction was initiated by the adding 100 µl CDNB (6 mM in methanol), resulting in a final volume of 300 µl. The plates were immediately transferred to a microplate reader. The reactions were allowed to continue for 5 min and absorbance readings were taken at $\lambda = 340$ nm automatically once per min against blanks (wells containing all reaction components except larval homogenate). The increase in absorbance was linear throughout the 5-min reading interval. An extinction coefficient of 9.6 mM$^{-1}$ cm$^{-1}$ was used to calculate the amount of CDNB conjugated.

**Protein Assay.** Protein contents were determined by the method of Bradford (1976) [30] using Bio-Rad dye reagent (Bio-Rad Laboratories, Hercules, CA) with BSA as a
standard. Three replicates of 10 µl were assayed for each larval homogenate. Absorbance was read at \( \lambda = 595 \) nm against blanks and converted to protein concentration based on the standard curve.

**Data Analysis.** Median lethal concentration (LC\(_{50}\)) values and their corresponding 95% fiducial limits (FL) were estimated using POLO [31]. Lethal concentration ratios (LCR) at LC\(_{50}\) and their corresponding 95% confidence limits (CL) were calculated using lethal concentration ratio significance test [32]. A laboratory colony (LAB) that was not selected with any of the insecticides but otherwise treated the same way served as the reference susceptible population for comparison purposes and was assigned a ratio of 1.0. Lethal concentrations of the RYN and SPIN selected colonies, and the unselected LAB colony were considered significantly different if the 95% CL of their corresponding LCR-LC\(_{50}\) did not include the value of 1.0. Mean enzyme activities recorded in larvae from the RYN and SPIN selected colonies were compared with those from larvae in the unselected LAB colony using \( t \)-test. Significance was accepted at \( \alpha = 0.05 \) in all statistical tests used in this study.

**Results**

Bioassays show that both chlorantraniliprole and spinetoram are toxic to the OBLR LAB colony, with LC\(_{50}\) values of 0.12 ppm and 0.06 ppm, respectively (Table 1 and Table 2). After nine generations of selection for resistance, susceptibility of RYN and SPIN selected colonies significantly decreased in diet incorporation bioassays with both chlorantraniliprole and spinetoram, with LC\(_{50}\) values of 0.96 ppm and 0.28 ppm, respectively (Table 1 and Table 2). Diet incorporation bioassays showed an 8-fold increase in the LC\(_{50}\) value of the RYN colony after nine consecutive generations of selection as compared to the unselected LAB
colony (Table 1). Similarly, a 5-fold increase was observed in the LC50 value of the SPIN colony after selection for the same number of generations as compared to the unselected LAB colony (Table 2).

To determine the potential role of detoxification enzymes in resistance to chlorantraniliprole and spinetoram in the selected OBLR colonies, enzyme assays to measure the levels of esterases, oxidases, and glutathione-S-transferases were performed. The activity of non-specific esterases was significantly higher in third-instar OBLR larvae from the RYN colony compared to the LAB colony ($t = -3.00$; df = 55; $p = 0.004$) when determined using αNA as substrate (Fig. 1), indicating the possible involvement of esterases in conferring resistance to chlorantraniliprole. For spinetoram selected OBLR however, there was no significant difference in the esterase activity in OBLR larvae from the SPIN colony compared to the LAB colony ($t = -0.81$; df = 54; $p = 0.420$) (Fig. 1).

In contrast to the results above, the activity of oxidases was significantly higher in third-instar OBLR larvae from the SPIN colony compared to the LAB colony ($t = -2.11$; df = 54; $p = 0.039$) when determined using TMBZ as substrate (Fig. 2), suggesting a possible role of oxidases in conferring resistance to spinetoram. For chlorantraniliprole selected OBLR, there was no significant differences in oxidase activity in OBLR larvae from the RYN colony when compared to the LAB colony ($t = 0.06$; df = 47; $p = 0.950$) (Fig. 2).

The laboratory colonies of OBLR selected for resistance to chlorantraniliprole and spinetoram were also assessed for glutathione-S-transferase activity. In each case, there was no significant differences in the activity of glutathione-S-transferases in third-instar OBLR larvae from either the RYN colony ($t = 0.78$; df = 55; $p = 0.441$) or the SPIN colony ($t = -1.97$; df = 54; $p = 0.054$), when compared with the LAB colony (Fig. 3). These results
indicate that glutathione-S-transferases probably have no role in detoxification of chlorantraniliprole or spinetoram, at least in these laboratory-selected colonies of *C. rosaceana*.

**Discussion**

Insecticide resistance presents a major risk to the sustainability of integrated pest management (IPM) programs for *C. rosaceana*. Resistance management strategies could slow the development of resistance only if implemented in a timely manner. However, the effectiveness of resistance management strategies may be reduced without the knowledge of biochemical mechanisms conferring resistance to insecticides used in IPM programs. In this study, we selected *C. rosaceana* neonate larvae from a laboratory colony for resistance to two recently registered insecticides, chlorantraniliprole and spinetoram.

Our results show that artificial selection for resistance in the laboratory resulted in development of significant levels of resistance to both chlorantraniliprole and spinetoram in OBLR. Moreover, the level of resistance observed after nine generations of selection was higher for to chlorantraniliprole (8-fold) than that for spinetoram (5-fold). These results suggest that resistance to chlorantraniliprole could develop at a faster rate than resistance to spinetoram in field populations of *C. rosaceana* [33].

This study represents the first report on the mechanisms involved in resistance to chlorantraniliprole and spinetoram in *C. rosaceana*. Chlorantraniliprole is the first anthranilic diamide registered for use in *C. rosaceana* control programs. Chlorantraniliprole or other diamides have never been used for insect control in tree fruit in the past, but we have reported significant levels of resistance to chlorantraniliprole in some of the field populations of *C.
*rosaceana* even before its first field application [27]. However, specific mechanisms responsible for chlorantraniliprole resistance have not been reported in any other insects. The higher activities of esterases in *C. rosaceana* larvae from the RYN colony are indicative of the possible involvement of esterases in conferring resistance to chlorantraniliprole. The esterase enzymes have previously been reported to be involved in resistance to azinphosmethyl in *C. rosaceana* [18, 20] and other tortricid moths, such as light brown apple moth, *Epiphyas postvittana* [34]. The azinphosmethyl-resistance in *C. rosaceana* mediated by general esterases usually extends to several types of organophosphates, carbamates, and other classes of insecticides [20], and has been associated with cross-resistance to pyrethroids [35-37].

Spinetoram is a second generation spinosyn which was recently registered for *C. rosaceana* control in tree fruit. Spinosad, the first spinosyn registered, has been used in tree fruit since 1998 and *C. rosaceana* populations developed resistance to this compound only six years after its introduction [8]. In a recent study, we reported low levels of resistance to spinetoram in some field populations of *C. rosaceana*, which were highly correlated with resistance to spinosad, even before its registration [27]. Resistance to spinosad has been characterized in several species of insects; however, mechanisms responsible for spinetoram-resistance have not yet been reported. Significant elevation in the level of oxidases in *C. rosaceana* larvae from the SPIN colony suggests that resistance to spinetoram in this laboratory-selected colony was mediated by oxidases. Our findings are in agreement with the previous studies reporting the involvement of oxidases as a mechanism for resistance to spinosad in *Musca domestica* [21], *Spodoptera exigua* [22], and *Helicoverpa armigera* [25], an anticipated result since spinosad and spinetoram are both spinosyns.
Insecticide resistance management in *C. rosaceana* in tree fruit orchards is a challenge for growers especially at a time when broad-spectrum insecticides such as OPs are being phased out and a wide range of newer insecticides with different modes of action are available to control this pest. Both chlorantraniliprole and spinetoram are highly effective against *C. rosaceana* in preliminary studies in laboratory and field situations [17, Brunner unpublished data], and this study indicates that they do not share detoxification mechanisms. These findings support our contention that chlorantraniliprole and spinetoram could be incorporated into a resistance management program leading to sustainable management of *C. rosaceana* in tree fruit. Unfortunately the evidence of pre-existing resistance to chlorantraniliprole and cross-resistance between spinosad and spinetoram in some field populations of *C. rosaceana* [27] coupled with a high potential for resistance evolution witnessed in selection experiments may make resistance management more difficult. Therefore, chlorantraniliprole and spinetoram must be used wisely in the framework of a well thought-out resistance management program taking into account all other insecticide options and avoiding the use of those detoxified by the similar enzyme systems against consecutive generations of *C. rosaceana*.

The mechanisms responsible for resistance to the same chemical may vary from one population to another [18]. Therefore, detoxification mechanisms responsible for chlorantraniliprole and spinetoram resistance observed in the laboratory-selected populations in the current study may not be the same as those present in the field populations. Further studies are required to establish the mechanisms of resistance in field-collected populations.
Acknowledgments

This study was sponsored by the Washington Tree Fruit Research Commission, the Washington State Commission on Pesticide Registration, and Western Sustainable Agriculture Research and Education (Project No.: GW10-003). We thank Dr. John J. Brown (Washington State University) for his comments on the manuscript; Dr. John E. Dunley (Washington State University, Tree Fruit Research and Extension Center Wenatchee) for his guidance and advice on the selection experiments, Mike Doerr (WSU) for his assistance in the standardizing diet incorporation bioassays, Kathy Pierre (WSU) for her help in maintaining colonies of *C. rosaceana* in the laboratory, and Robert McCurdy and Laura Willett (USDA-ARS) for their assistance in enzyme assays.

References


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[33] A. A. Sial, J. F. Brunner, Assessment of Resistance Risk in Obliquebanded Leafroller (Lepidoptera: Tortricidae) to the Reduced-Risk Insecticides Chlorantraniliprole and Spinetoram, J. Econ. Entomol. 103 (2010), In press.


**Tables and Figures**

**Table 1.** Results of probit analyses for diet incorporation bioassays of chlorantraniliprole with *C. rosaceana* neonate larvae from chlorantraniliprole-selected (RYN) colony after nine generations of selection for resistance and the unselected laboratory (LAB) colony

<table>
<thead>
<tr>
<th>Colony</th>
<th>n</th>
<th>Slope (± SE)</th>
<th>χ²</th>
<th>LC₅₀ (ppm)</th>
<th>LCR² (95% FL)¹</th>
<th>(95% CL)³</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAB</td>
<td>180</td>
<td>1.59 (0.19)</td>
<td>11.83</td>
<td>0.12 (0.06-0.20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RYN</td>
<td>180</td>
<td>1.65 (0.29)</td>
<td>5.20</td>
<td>0.96 (0.32-1.95)</td>
<td>7.95 (3.81-16.59)*</td>
<td></td>
</tr>
</tbody>
</table>

n = number of larvae assayed.

¹ 95% fiducial limits estimated using POLO (LeOra Software 1987).

² LCR, lethal concentration ratio = LC₅₀ (RYN colony)/LC₅₀ (LAB colony).

³ 95% confidence limits estimated using lethal concentration ratio significance test (Robertson et al. 2007).

* Indicates that Lethal Concentration Ratio was significant (α = 0.05) (Robertson et al. 2007).
Table 2. Results of probit analyses for diet incorporation bioassays of spinetoram with *C. rosaceana* neonate larvae from spinetoram-selected (SPIN) colony after nine generations of selection for resistance and the unselected laboratory (LAB) colony

<table>
<thead>
<tr>
<th>Colony</th>
<th>N</th>
<th>Slope (± SE)</th>
<th>$\chi^2$</th>
<th>LC$_{50}$ (ppm)</th>
<th>LCR$^2$ (95% FL)$^1$</th>
<th>(95% CL)$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAB</td>
<td>300</td>
<td>2.07 (0.20)</td>
<td>9.34</td>
<td>0.06 (0.03-0.10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPIN</td>
<td>300</td>
<td>2.60 (0.29)</td>
<td>4.76</td>
<td>0.28 (0.17-0.29)</td>
<td>4.99 (3.61-6.90)*</td>
<td></td>
</tr>
</tbody>
</table>

n = number of larvae assayed.

1 95% fiducial limits estimated using POLO (LeOra Software 1987).

2 LCR, lethal concentration ratio = LC$_{50}$ (SPIN colony)/LC$_{50}$ (LAB colony).

3 95% confidence limits estimated using lethal concentration ratio significance test (Robertson et al. 2007).

* Indicates that Lethal Concentration Ratio was significant ($\alpha = 0.05$) (Robertson et al. 2007).
Fig. 1. Activity of esterases (Mean + SEM) in chlorantraniliprole-selected (RYN) and spinetoram-selected (SPIN) colonies after nine generations of selection for resistance in laboratory, and the unselected laboratory (LAB) colony of *C. rosaceana*. Graph bars containing similar letters on the top are not significantly different ($\alpha = 0.05$, *t*-test).
Fig. 2. Level of oxidases (pmol equivalent cytochrome-P450 U) (Mean + SEM) in chlorantraniliprole-selected (RYN) and spinetoram-selected (SPIN) colonies after nine generations of selection for resistance in laboratory, and the unselected laboratory (LAB) colony of *C. rosaceana*. Graph bars containing similar letters on the top are not significantly different (α = 0.05, *t*-test).
Fig. 3. Activity of glutathione-S-transferases (Mean + SEM) in chlorantraniliprole-selected (RYN) and spinetoram-selected (SPIN) colonies after nine generations of selection for resistance in laboratory, and the unselected laboratory (LAB) colony of *C. rosaceana*. Graph bars containing similar letters on the top are not significantly different ($\alpha = 0.05$, *t*-test).
CHAPTER SEVEN

SELECTION FOR RESISTANCE, REVERSION TOWARD SUSCEPTIBILITY, AND SYNERGISM OF CHLORANTRANILIPROLE AND SPINETORAM IN OBLIQUEBANDED LEAFROLLER, *CHORISTONEURA ROSACEANA* (LEPIDOPTERA: TORTRICIDAE)

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Abstract

Larvae of the obliquebanded leafroller, *Choristoneura rosaceana*, derived from a laboratory colony, were selected for resistance to two reduced-risk insecticides chlorantraniliprole and spinetoram. Significant levels of resistance to each insecticide were observed after twelve generations of selection. In the absence of selection pressure, susceptibility of a subset of larvae from both chlorantraniliprole- and spinetoram-selected populations reverted to pre-selection levels after five and six generations, respectively, indicating that resistance to both chlorantraniliprole and spinetoram was unstable in *C. rosaceana*. In synergist bioassays performed after twelve generations of selection, S,S,S-tributylphosphorothioate (DEF) and piperonyl butoxide (PBO) synergized the toxicity of chlorantraniliprole and spinetoram,
respectively; suggesting the involvement of esterases in chlorantraniliprole resistance and that of mixed-function oxidases in spinetoram resistance. These findings suggest that chlorantraniliprole and spinetoram could be incorporated into C. rosaceana resistance management programs by using rotational strategies.

**Key words:** Obliquebanded leafroller, chlorantraniliprole, spinetoram, resistance, selection, reversion, synergism

**Introduction**

Obliquebanded leafroller, *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae) is a polyphagous insect with a broad host range including members of Rosaceae and Cornaceae [1]. It is native to North America [2, 3], and is an economic pest of deciduous tree fruits throughout major tree fruit growing regions in the United States and Canada [2, 4-6]. Broad-spectrum insecticides such as organophosphates (OPs) have been widely used to control major pests of tree fruits including *C. rosaceana*. The reliance on repeated applications of conventional neurotoxic insecticides has resulted in the development of resistance to those insecticides and cross-resistance to other insecticides in *C. rosaceana* [7-15], and in some cases to the new classes of insecticides prior to them being applied in the field [13-17].

The occurrence of resistance, cross- and multiple-resistance in *C. rosaceana* necessitates the introduction of resistance management strategies in order to ensure adequate control and conserve chemical control options [18, 19] which are scarce resources, indeed. Additionally, the implementation of Food Quality Protection Act of 1996 has put restrictions on the use of
conventional broad-spectrum insecticides, and expedited the registration of reduced-risk insecticides [20]. Chlorantraniliprole and spinetoram are two of the reduced-risk insecticides recently registered as OP alternatives for use in tree fruit production. Chlorantraniliprole is a member of a new class of insecticides, the anthranilic diamides, which selectively binds to the ryanodine receptors in insect muscles resulting in an uncontrolled release of internal calcium stores from the sarcoplasmic reticulum, causing impaired regulation of muscle contraction leading to feeding cessation, lethargy, paralysis, and death of the target insect [21, 22]. Spinetoram is a recently developed spinosyn which primarily activates the nicotinic acetylcholine receptors by acting on a unique and yet unknown site [23-26]. Both chlorantraniliprole and spinetoram have shown high efficacy against *C. rosaceana* [27, Brunner unpublished data].

Resistance management strategies aimed at prolonging the efficacy of newly introduced insecticides are critical components of sustainable pest management. Once resistance has developed, tactics such as rotation of insecticides across generations are practiced to maintain the efficacy of the new insecticides [19, 28, 29]. However, the success of rotation as a strategy to delay the onset of resistance may strongly depend on the magnitude of the fitness costs associated with resistance, and the pattern of cross-resistance among the alternated insecticides [28-33]. Although chlorantraniliprole and spinetoram are new chemistries, the evidence of pre-existing resistance to chlorantraniliprole and positively correlated cross-resistance between spinetoram and spinozad in *C. rosaceana* [17] may reduce the effectiveness of resistance management programs for *C. rosaceana*. Further understanding of mechanisms conferring resistance to these new insecticides and whether the resistance of *C. rosaceana* to these chemicals would be stable in the absence of selection pressure seems
necessary to develop effective resistance management strategies and to determine if rotation of these chemicals would be an appropriate tactic.

In this paper we describe results of experiments investigating the effect of selection of *C. rosaceana* for resistance in the laboratory to chlorantraniliprole and spinetoram, the stability of resistance after removal of selection pressure, and the effect of metabolic synergists on toxicity of chlorantraniliprole and spinetoram. This information would enable growers and pest management consultants to develop rational resistance management strategies by incorporating these novel reduced-risk insecticides into tree fruit pest management programs leading to sustainable management of *C. rosaceana*. It would also be useful in suggesting resistance mechanisms and in improving effectiveness of chlorantraniliprole and spinetoram spray programs in commercial orchards.

**Materials and Methods**

**Insects.** The *C. rosaceana* larvae were obtained from a laboratory colony which was established in 1990 from larvae collected from apple orchards in Mattawa, WA. This colony has been reared continuously since their collection on a pinto bean diet following the method of Shorey and Hale [34] under constant conditions of temperature (23 ± 2°C), relative humidity (RH, 70%), and photoperiod (16:8, L:D) and without exposure to insecticides.

**Chemicals.** The insecticides used were chlorantraniliprole (Rynaxypyr™/Altacor® 35WG, E.I. du Pont de Nemours & Co., Wilmington, DE) and spinetoram (Delegate® 25WG, Dow AgroSciences, Indianapolis, IN). The synergists tested with chlorantraniliprole and spinetoram were DEF (S,S,S-Tributylphosphorothioate, Chem Service, West Chester, PA),
DEM (Diethyl maleate, 97% technical, Aldrich, Milwaukee, WI), and PBO (Piperonyl butoxide, 90% technical, Aldrich, Milwaukee, WI).

**Selection for Resistance.** Selection for resistance to chlorantraniliprole and spinetoram was performed in the laboratory as described in Sial and Brunner [35]. Briefly, cohorts of larvae from a susceptible laboratory colony were selected with chlorantraniliprole (RYN) or spinetoram (SPIN) while another cohort treated in the same way, but without exposure to insecticides, served as unselected control (LAB). In the first selection, neonate larvae were exposed to LC$_{70}$ of the baseline established for the LAB colony. After 4 d of exposure, surviving larvae were transferred to untreated pinto bean diet, and reared in the laboratory under conditions described above. The concentration of chlorantraniliprole and spinetoram used to select each subsequent generation was the ≈LC$_{70}$ based on the results of bioassays from the previous generation. The number of neonate larvae used for each generation varied (1,000-2,000) depending on availability. Selection of some generations was deferred because sufficient numbers of progeny were not available.

**Reversion toward Susceptibility:** After six generations of selection and after resistance had been documented, a subset of *C. rosaceana* larvae from each of the selected RYN and SPIN populations was removed from selection to establish two new populations, RYN-Rev and SPIN-Rev, respectively. The main objective of establishing these colonies was to determine whether or not the resistance in the selected populations was stable. The *C. rosaceana* larvae in RYN-Rev and SPIN-Rev populations were reared in the laboratory without any further exposure to insecticides. Susceptibility of neonate larvae from the RYN-Rev and SPIN-Rev populations was assessed and compared with that of the neonate larvae from the unselected LAB populations at each generation using a diet incorporation bioassay.
Bioassays. Depending on availability of larvae, diet incorporation bioassays were performed as described by Sial et al. [17] on the RYN and SPIN populations after each selected generation, and on the RYN-Rev and SPIN-Rev populations at each generation without selection to determine their susceptibility to chlorantraniliprole or spinetoram. Every time a bioassay was performed on RYN, SPIN, RYN-Rev, or SPIN-Rev, *C. rosaceana* larvae from the unselected LAB colony were tested using the same experimental materials in order to minimize the experimental error. Bioassays were not performed on G2 and G4 of the RYN population, and G3 and G5 of the SPIN population because sufficient larvae were not available. The *C. rosaceana* larvae of the RYN population at G10 were not tested because selection had not been performed on their parental generation due to unavailability of enough neonate larvae.

Synergist Bioassays. A diet overlay bioassay as described by Ahmad and Hollingworth [36] was used to test the effect of synergists DEF, DEM and PBO on toxicity of chlorantraniliprole and spinetoram to RYN and SPIN (resistant), and the LAB (susceptible) populations of *C. rosaceana*. Diet was prepared from dry diet premix (Stonefly Heliothis Diet, Ward’s Natural Science, Rochester, NY) as described by Sial et al. [17]. A small portion of the diet (~5-8 cm³) was added to one-ounce plastic cups (Solo Cup Company, Highland Park, IL). Synergists were dissolved in 100% acetone and added to distilled water. The final solutions consisted of six concentrations of insecticides each with 20 µl per liter synergist in 5% solution of acetone in distilled water for a total volume of 200 ml. A concentration of 20 µl per liter of synergists was used because this was the maximum concentration that could be used without significant (>5%) mortality to *C. rosaceana* neonates [16, 37]. For each synergist and colony, 5 larvae were tested for each of the 6-10 replications.
of six concentrations for a total of 180-300 larvae tested. Mortality of neonates was assessed after 7 d.

**Data Analysis.** Median lethal concentration (LC$_{50}$) values and their corresponding 95% fiducial limits (FL) were estimated using POLO [38]. Lethal concentration ratios (LCR) at LC$_{50}$ and their corresponding 95% confidence limits (CL) were calculated using lethal concentration ratio significance test [39]. A laboratory colony (LAB) that was not selected with any of the insecticides but otherwise treated the same way served as the reference susceptible population for comparison purposes and was assigned a ratio of 1.0. LCRs were considered significant ($\alpha = 0.05$) if their 95% CL did not include the value of 1.0. Similarly, synergism ratios (SR) at LC$_{50}$ (LC$_{50}$ of insecticide/LC$_{50}$ of insecticide + synergist) and their corresponding 95% CL were calculated using lethal concentration ratio significance test [39]. SRs were considered significant ($\alpha = 0.05$) if their 95% CL did not include the value of 1.0.

**Results**

Our results indicate that the toxicity of both chlorantraniliprole (Fig. 1) and spinetoram (Fig. 2) to *C. rosaceana* neonate larvae was significantly decreased as a result of selection for resistance in the laboratory. However, the level of resistance against chlorantraniliprole was higher than spinetoram after the same number of selected generations. After 12 generations of selection, the LC$_{50}$ values of chlorantraniliprole and spinetoram to *C. rosaceana* were increased to 8.5- and 5.3-fold, respectively, compared to the unselected LAB colony (Fig. 1 and Fig. 2).

After six generations of selection to chlorantraniliprole and spinetoram resistance levels of 7-fold and 4-fold had been documented, and a subset of larvae from the RYN and SPIN
populations were used to establish two new colonies RYN-Rev and SPIN-Rev, respectively. The RYN-Rev and SPIN-Rev populations were reared in laboratory without exposure to insecticides. In the absence of insecticide selection, both the RYN-Rev and SPIN-Rev populations reverted to susceptibility after five and six generations, respectively (Fig. 3 and Fig. 4). Although level of chlorantraniliprole resistance in the RYN-Rev population at the beginning of the reversion experiment was higher than that of spinetoram resistance in the SPIN-Rev population, it took five generations for the RYN-Rev population to return to susceptibility statistically similar to the unselected LAB population (Fig. 3) whereas a similar return to susceptibility took six generations for the SPIN-Rev population (Fig. 4).

To determine the potential effects of metabolic synergists on toxicity of chlorantraniliprole and spinetoram on *C. rosaceana* neonate larvae from the RYN, SPIN, and susceptible LAB populations, diet overlay bioassays, with and without synergists (DEF, DEM, and PBO), were performed. The toxicity of chlorantraniliprole to both the LAB (susceptible) and the RYN (resistant) population was increased by the addition of DEF (Table 1) suggesting the involvement of esterases as a potential mechanism for chlorantraniliprole resistance. The LC$_{50}$ of chlorantraniliprole to the RYN population was significantly decreased (2.54-fold) whereas there was only a slight decrease (1.44-fold) in LC$_{50}$ of the chlorantraniliprole to the LAB population. The addition of DEM or PBO did not result in any significant change in LC$_{50}$ of chlorantraniliprole to the LAB or RYN populations (Table 1).

In contrast to the results above, the toxicity of spinetoram to both the LAB (1.8-fold) and the SPIN (3.6-fold) populations was significantly increased by the addition of PBO (Table 2) indicating the involvement of oxidases as a potential mechanism for spinetoram resistance. There was also a decrease, though not significant, in LC$_{50}$ of spinetoram to the SPIN
population (1.66-fold) by the addition of DEM, but not in the LAB population, pointing toward a possible role of glutathione-S-transferases in spinetoram detoxification as a secondary mechanism. The susceptibility of the LAB or the SPIN populations to spinetoram was not affected by DEF (Table 1).

Discussion

Chlorantraniliprole and spinetoram are two recently registered insecticides that are highly effective against *C. rosaceana* [27, Brunner unpublished data]. However, reports of pre-existing resistance to these insecticides in the field populations of *C. rosaceana* and cross-resistance between spinosad and spinetoram [17], present a major risk to the effective life of these insecticides in the field. The effective life of new insecticides can be prolonged by the implementation of a resistance management program [40]. The success of such programs depends on better understanding of different characteristics of resistance such as the relative risk of resistance evolution, stability of resistance, and resistance mechanisms. In an attempt to further our understanding of chlorantraniliprole and spinetoram resistance, we selected *C. rosaceana* neonate larvae for resistance in the laboratory. Significant levels of resistance to both chlorantraniliprole and spinetoram were observed soon after selection was initiated but selection continued through 12 generations. These results indicate that high levels of resistance against these new chemistries could occur in relatively shorter period of time in the field where selection pressures are likely to be much higher than those imposed in our laboratory selection and where populations are likely to be more heterogeneous [35].

For the first time we demonstrate two new characteristics of chlorantraniliprole and spinetoram resistance in *C. rosaceana*: the instability of resistance in the absence of selection
pressure, and the synergism of toxicity of chlorantraniliprole and spinetoram by DEF and PBO, respectively. It is evident from the results of reversion experiments where, in the absence of selection pressure, both of the selected populations reverted to being susceptible that the chlorantraniliprole and spinetoram resistance in *C. rosaceana* was unstable. These findings are encouraging for resistance management programs aimed at slowing the process of resistance evolution against chlorantraniliprole and spinetoram, and prolonging the useful life of these new insecticides against *C. rosaceana* in the field. Our results suggest that chlorantraniliprole and spinetoram resistance could revert in *C. rosaceana* in the field when selection pressure was relaxed, however, it could take several generations for this to occur. One of the operational strategies that can be used to reduce selection pressure is rotation of chlorantraniliprole and spinetoram treatments with other chemicals that do not have cross-resistance to these insecticides. Reversion of resistance to pre-selection levels has been demonstrated in *C. rosaceana* [41] and other species [42, 43], and is sometimes cited as a prerequisite for the success of rotational strategies for resistance management in the field [44].

The reasons for reversion toward susceptibility are unclear, but it likely reflects fitness costs associated with resistance development. Fitness costs associated with resistance have been demonstrated in *C. rosaceana* [33] and other species [45-49]. Azinphosmethyl resistance mediated by elevated activity of esterases resulted in lower larval weights, reduced pupal weights, and longer development times in *C. rosaceana* [33]. Fitness disadvantages associated with resistance are particularly relevant to insecticide resistance management. Once resistance has evolved, the use of substitute chemicals that do not have cross-resistance will halt or even revert the evolution of resistance [28, 30] as witnessed in the current study.
The fitness cost associated with resistance increases with the degree of resistance [33, 50] which was evident in the current study where the RYN population had higher level of resistance than SPIN population in the beginning of reversion experiment, but reverted to being susceptible faster than the SPIN population. Based on the faster occurrence of reversion, we hypothesize that the fitness costs associated with chlorantraniliprole resistance would be higher than those of spinetoram resistance in *C. rosaceana*. This hypothesis was further strengthened by the results of synergism studies where chlorantraniliprole resistance was mediated by esterases, which have been shown to be costlier than oxidases [19], which were responsible for spinetoram resistance.

The synergism of the toxicity of chlorantraniliprole and spinetoram primarily by DEF and PBO, respectively, suggests that chlorantraniliprole resistance was mediated by esterases whereas oxidases were the primary mechanism responsible for spinetoram resistance in *C. rosaceana*. In other species, PBO has been previously reported to synergize the toxicity of spinosad [51-53] indicating possible involvement of oxidases in resistance to spinosad, which is a spinosyn just like spinetoram. Additionally, a small degree of synergism of spinetoram toxicity in the SPIN population by DEM is indicative of a possible role of glutathione-S-tranferases as a secondary mechanism in spinetortam resistance. In contrast, the involvement of esterases has been reported as a secondary mechanism involved in spinosad detoxification in other species [51-53]. These differences might be due to species difference because earlier studies have characterized spinosad resistance in other species and not in *C. rosaceana*.

Our findings that the resistance to chlorantraniliprole and spinetoram in *C. rosaceana* is unstable and that these two new insecticides appear to be detoxified by different enzyme systems suggest that chlorantraniliprole and spinetoram could be incorporated into *C.
management programs, and management of resistance may be possible with rotational strategies. The information that chlorantraniliprole and spinetoram was esterase- and oxidase-based will also be helpful in making sound choices regarding the best alternation of materials to be used in such management programs. Furthermore, synergism of chlorantraniliprole and spinetoram by DEF and PBO, respectively, also indicates that DEF and PBO could be useful in improving the efficacy of these compounds. However, prior to such use, further studies should be conducted to determine the effects of metabolic synergists on toxicity of these insecticides to field populations of *C. rosaceana*.

**Acknowledgments**

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**References**


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### Tables and Figures

**Table 1.** Toxicity of chlorantraniliprole to *C. rosaceana* neonate larvae from a colony (RYN) subjected to selection for resistance to chlorantraniliprole for 12 generations and the unselected laboratory (LAB) colony after synergism.

<table>
<thead>
<tr>
<th>Colony</th>
<th>Compound</th>
<th>N</th>
<th>Slope (± SE)</th>
<th>LC$_{50}$ (ppm)</th>
<th>SR$^2$ (95% FL)</th>
<th>(95% CL)$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAB</td>
<td>Chlorantraniliprole</td>
<td>180</td>
<td>1.51 (0.18)</td>
<td>0.12 (0.08-0.18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chlorantraniliprole + DEF</td>
<td>180</td>
<td>1.59 (0.20)</td>
<td>0.09 (0.06-0.12)</td>
<td>1.44 (0.86-2.39)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chlorantraniliprole + DEM</td>
<td>180</td>
<td>1.44 (0.18)</td>
<td>0.13 (0.09-0.19)</td>
<td>0.95 (0.56-1.61)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chlorantraniliprole + PBO</td>
<td>180</td>
<td>1.71 (0.26)</td>
<td>0.13 (0.08-0.20)</td>
<td>0.94 (0.54-1.64)</td>
<td></td>
</tr>
<tr>
<td>RYN</td>
<td>Chlorantraniliprole</td>
<td>180</td>
<td>2.60 (0.24)</td>
<td>1.05 (0.31-1.91)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chlorantraniliprole + DEF</td>
<td>180</td>
<td>1.65 (0.25)</td>
<td>0.41 (0.26-0.62)</td>
<td>2.54 (1.41-4.6)*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chlorantraniliprole + DEM</td>
<td>180</td>
<td>1.63 (0.29)</td>
<td>1.08 (0.51-1.90)</td>
<td>0.97 (0.53-1.78)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chlorantraniliprole + PBO</td>
<td>180</td>
<td>1.63 (0.25)</td>
<td>0.99 (0.41-2.02)</td>
<td>1.07 (0.59-1.92)</td>
<td></td>
</tr>
</tbody>
</table>

n = number of larvae assayed.

$^1$ 95% fiducial limits estimated using POLO (LeOra Software 1987).

$^2$ SR, synergistic ratio = LC$_{50}$ (without synergist)/LC$_{50}$ (with synergist).

$^3$ 95% confidence limits estimated using lethal concentration ratio significance test (Robertson et al. 2007).

* Indicates that synergistic ration was significant ($\alpha = 0.05$) (Robertson et al. 2007).
Table 2. Toxicity of spinetoram to *C. rosaceana* neonate larvae from a colony (SPIN) subjected to selection for resistance to spinetoram for 12 generations and the unselected laboratory (LAB) colony after synergism.

<table>
<thead>
<tr>
<th>Colony</th>
<th>Compound</th>
<th>n</th>
<th>Slope (± SE)</th>
<th>LC$_{50}$ (ppm) (95% FL)$^1$</th>
<th>SR$^2$ (95% CL)$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAB</td>
<td>Spinetoram</td>
<td>300</td>
<td>1.86 (0.18)</td>
<td>0.06 (0.03-0.14)</td>
<td></td>
</tr>
<tr>
<td>LAB</td>
<td>Spinetoram + DEF</td>
<td>300</td>
<td>2.56 (0.44)</td>
<td>0.07 (0.05-0.09)</td>
<td>0.95 (0.65-1.40)</td>
</tr>
<tr>
<td>LAB</td>
<td>Spinetoram + DEM</td>
<td>300</td>
<td>2.11 (0.29)</td>
<td>0.06 (0.04-0.08)</td>
<td>1.11 (0.75-1.64)</td>
</tr>
<tr>
<td>LAB</td>
<td>Spinetoram + PBO</td>
<td>300</td>
<td>1.71 (0.16)</td>
<td>0.04 (0.02-0.06)</td>
<td>1.83 (1.26-2.64)*</td>
</tr>
<tr>
<td>SPIN</td>
<td>Spinetoram</td>
<td>300</td>
<td>2.71 (0.34)</td>
<td>0.34 (0.27-0.42)</td>
<td></td>
</tr>
<tr>
<td>SPIN</td>
<td>Spinetoram + DEF</td>
<td>300</td>
<td>2.58 (0.32)</td>
<td>0.33 (0.26-0.41)</td>
<td>1.05 (0.76-1.44)</td>
</tr>
<tr>
<td>SPIN</td>
<td>Spinetoram + DEM</td>
<td>300</td>
<td>1.99 (0.19)</td>
<td>0.21 (0.12-0.38)</td>
<td>1.66 (0.68-2.31)</td>
</tr>
<tr>
<td>SPIN</td>
<td>Spinetoram + PBO</td>
<td>300</td>
<td>1.76 (0.27)</td>
<td>0.10 (0.04-0.16)</td>
<td>3.58 (2.28-5.61)*</td>
</tr>
</tbody>
</table>

n = number of larvae assayed.

$^1$ 95% fiducial limits estimated using POLO (LeOra Software 1987).

$^2$ SR, synergistic ratio = LC$_{50}$ (without synergist)/LC$_{50}$ (with synergist).

$^3$ 95% confidence limits estimated using lethal concentration ratio significance test (Robertson et al. 2007).

* Indicates that synergistic ration was significant ($\alpha = 0.05$) (Robertson et al. 2007)
Fig. 1. Toxicity of chlorantraniliprole (LC$_{50}$ + 95% CL) to *C. rosaceana* neonate larvae from a colony (RYN) subjected to artificial selection for resistance to chlorantraniliprole in laboratory for 12 generations. Numbers on top of the graph bars represent resistance ratios (RR); and *Indicates that the RR is significant ($\alpha = 0.05$).
Fig. 2. Toxicity of spinetoram (LC$_{50} + 95\%$ CL) to *C. rosaceana* neonate larvae from a colony (SPIN) subjected to artificial selection for resistance to spinetoram in laboratory for 12 generations. Numbers on top of the graph bars represent resistance ratios (RR); and *Indicates that the RR is significant ($\alpha = 0.05$).
Fig. 3. Toxicity of chlorantraniliprole (LC$_{50}$ + 95% CL) to $C.\;rosaceana$ neonate larvae from chlorantraniliprole-selected colony when reared in the absence of selection pressure (RYN-Rev). Numbers on top of the graph bars represent resistance ratios (RR); and *Indicates that the RR is significant ($\alpha = 0.05$).
Fig. 4. Toxicity of spinetoram (LC\textsubscript{50} + 95% CL) to \textit{C. rosaceana} neonate larvae from spinetoram-selected colony when reared in the absence of selection pressure (SPIN-Rev). Numbers on top of the graph bars represent resistance ratios (RR); and *Indicates that the RR is significant (\(\alpha = 0.05\)).