University of Tennessee, Knoxville
Trace: Tennessee Research and Creative Exchange

Masters Theses | Graduate School

8-2019

Mechanisms of resistance against insecticidal dsRNA in Leptinotarsa decemlineata (Colorado potato beetle)

Swati Mishra
University of Tennessee, smishra8@vols.utk.edu

Follow this and additional works at: https://trace.tennessee.edu/utk_gradthes

Recommended Citation

Mishra, Swati, "Mechanisms of resistance against insecticidal dsRNA in Leptinotarsa decemlineata (Colorado potato beetle). " Master's Thesis, University of Tennessee, 2019.
https://trace.tennessee.edu/utk_gradthes/5548

This Thesis is brought to you for free and open access by the Graduate School at Trace: Tennessee Research and Creative Exchange. It has been accepted for inclusion in Masters Theses by an authorized administrator of Trace: Tennessee Research and Creative Exchange. For more information, please contact trace@utk.edu.

Mechanism of resistance against insecticidal double stranded RNA (dsRNA) in *Leptinotarsa decemlineata* (Colorado potato beetle)

A Thesis Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Swati Mishra

August 2019

Copyright © 2019 by Swati Mishra and Juan Luis Jurat-Fuentes. “Mechanism of resistance against insecticidal double stranded RNA (dsRNA) in *Leptinotarsa decemlineata* (Colorado potato beetle)”

All rights reserved.

Dedication

To Mom and Dad

Acknowledgements

I would like to express the deepest appreciation to my major advisor, Dr. Juan Luis Jurat-Fuentes, who allowed me to choose this project and work on it independently, while still guiding me whenever I needed it. His guidance not only helped me with my scientific growth, but also my personal growth. His constant support and motivation allowed me to experience incomparable opportunities and I am excited to keep growing and learning from him during my PhD. I would also like to thank my committee members, Dr. William Moar and Dr. Margaret Staton for their valuable feedback on the topics of their expertise. Special thanks to Dr. Moar for giving me the opportunity to interact with professionals in the industrial setting at Monsanto (Bayer).

I want to thank Monsanto (now Bayer) for funding this project and providing all the dsRNA material needed during the project.

Next I would like to thank my lab members, James Dee and Nayma Dias for their assistance with certain experiments. Also, thanks to my other past and present fellow lab members Dr. Heba Abdelgaffar, Dr. Caroline De Bortoli, Dr. Rafael Ferreira Dos Santos and Tejas Rao, for making the lab feel like a family. Special thanks to my office mates who walked the same journey of getting a masters with me and made it a little easier with an uninterrupted supply of coffee.

Last, I want to thank my parents, my brother and my sister-in-law for their constant encouragement, love and support. I wouldn't be here without any of them. Most importantly, thanks to my friends Tvisha, Sahira, Ishita, Aanchal and Kirtan, for always making me believe in myself.

Abstract

In the last decade, silencing of vital genes by RNA interference (RNAi) through dsRNA ingestion has been identified as a revolutionary bioinsecticide technology. As commercialization of insecticidal dsRNA technology approaches, it becomes crucial to develop resistance management tools for the sustainability of this technology. Using chronic exposure through larval development, we developed a population (CEAS) of *Leptinotarsa decemlineata* (Colorado potato beetle, CPB) that is >5,000-fold resistant to insecticidal dsRNA targeting the V-ATPase subunit A gene. The current Thesis was focused on identification of candidate resistance mechanisms and cross-resistance to Cry3Aa, one of the most active insecticidal protein from *Bacillus thuringiensis* against CPB. Here we provide molecular evidence for lack of gene silencing in dsRNA resistant population CEAS and cross-resistance to alternative dsRNA target. Up-regulation of V-ATPase subunit A transcript upon treatment with V-ATPase subunit A dsRNA was observed and may represent a resistance mechanism specific to this target. Comparison of dsRNA stability in digestive fluids from susceptible and resistant CPB support and that degradation of dsRNA by nucleases is not involved in resistance. Monitoring uptake of fluorescently labeled dsRNA by midgut cells using confocal microscopy supports reduced uptake of dsRNA in midgut cells of CEAS compared to susceptible larvae. This is partly supported by results from small RNA (sRNA) sequencing, which also suggests the existence of an additional mechanism of resistance involving up-regulation of target gene. Additionally, CEAS appears to be >3-fold less susceptible to Cry3Aa toxin when compared to GC. Results from this project will guide development of Insect Resistance Management (IRM) strategies for insecticidal RNAi and its combined used with insecticidal proteins from *B. thuringiensis* against CPB and will allow the optimization of insecticidal RNAi technology.

Table of Contents

List of Figures

List of Tables

Chapter One

RNA interference (RNAi): Applications and Resistance

RNA interference (RNAi): Description and Applications

RNA-interference (RNAi) is a gene silencing mechanism triggered by the presence of double stranded RNA (dsRNA) complimentary to a target gene. This suppression in gene expression is achieved by degradation of target mRNA. Though the term RNA-interference was initially coined by Fire et. al through their work in *Caenorhabditis elegans* (Fire, Albertson et al. 1991), the mechanism was first observed in plants (Wingard 1928, Napoli, Lemieux et al. 1990), and has been described in several eukaryotic organisms since. In animals, this process is termed as RNA-interference (RNAi), whereas in plants it has also been referred to as post transcriptional gene silencing (Baum, Bogaert et al. 2007). RNAi is a highly conserved process believed to have evolved as a defense mechanism against foreign nucleic acids like viruses and transposable elements (Mello and Conte 2004, Dykxhoorn and Lieberman 2005). Other than this cellular defense role, RNAi also aids in regulation of endogenous developmental genes and chromatin (Mello and Conte 2004).

Though an endogenous process, RNAi can be exploited to silence genes of interest by delivering complementary dsRNA into the organism. In insects, this can be achieved by the delivery of dsRNA to the target tissue, usually by feeding, soaking or injection, followed by uptake in the target cells. Two different types of RNAi response, cell-autonomous and non-cell autonomous, have been described based on where the silencing effect is observed (Whangbo and Hunter 2008). In cell-autonomous RNAi, reduction in gene expression is observed only in the cells in which dsRNA is introduced or expressed. This type of RNAi response has been observed in the model organism *Drosophila melanogaster,* which has allowed for tissue-specific functional genomic studies. In non-cell autonomous RNAi, the silencing effect is observed in cells capable of up taking the dsRNA or tissues beyond the site of dsRNA application. For example, injection of dsRNA into the head or tail of *C. elegans* produced silencing of the target gene throughout the individual, and was even passed on to its progeny (Fire, Xu et al. 1998). Two types of non-cell autonomous RNAi, environmental and systemic, are described. In environmental RNAi, cells take up dsRNA from their environment and exhibit the silencing effect, while in systemic RNAi the silencing effect is transferred from cells exposed to dsRNA to other cells or tissues (Whangbo and Hunter 2008, Huvenne and Smagghe 2010). In plants and nematodes exhibiting systemic RNAi, the mechanism of spread is explained by the presence of an RNA-dependent RNA polymerase

(RdRP) producing secondary small interfering RNAs (siRNAs) using the target mRNA as template, also known as transitive RNAi (Vélez and Fishilevich 2018).

Insects belonging to different orders have been shown to display systemic RNAi (Aronstein and Saldivar 2005, Zhou, Oi et al. 2006, Zhang, Zhang et al. 2010, Bolognesi, Ramaseshadri et al. 2012). In Western corn rootworm (WCR, *Diabritoca virgifera virgifera*), quantitative real-time PCR (qRT-PCR) studies measuring transcript levels of widely-expressed genes detected >80% knockdown using the whole body when dsRNA was fed via artificial diet (Bolognesi, Ramaseshadri et al. 2012, Baum and Roberts 2014, Vélez, Khajuria et al. 2016). In contrast, for a non-systemic system one would expect to observe partial knockdown for widely expressed transcripts, localized in the area of dsRNA exposure. Additional experimental evidence for systemic RNAi in insects comes from RNA *in-situ* hybridization studies in WCR (*D. v. virgifera*), where feeding of dsRNA resulted in knockdown of target transcript levels in distal fat bodies not connected to the midgut (Hu, Richtman et al. 2016, Li, Bowling et al. 2018). Further evidence for systemic RNAi comes from knockdown observed in eggs of dsRNA-treated females, a process called parental RNAi (Bucher, Scholten et al. 2002, He, Cao et al. 2006, Khajuria, Vélez et al. 2015, Shukla, Kalsi et al. 2016). However, unlike plants and nematodes, insect genomes lack RdRP homologs (Gordon and Waterhouse 2007), and there is no evidence for the presence of secondary siRNAs (Li, Bowling et al. 2018). This indicates that the systemic RNAi response in insects is not transitive and may involve different mechanisms for uptake and spread.

Whether it is systemic or not, the first step in any RNAi response is the entry of dsRNA into the cells. Though the complete process of uptake is not very well understood, some insights have been provided in *C. elegans*. In this nematode, systemic RNA interference-deficiency (SID) proteins (SID-1, SID-2, SID-3 and SID-5) are involved in uptake of dsRNA (Rocheleau 2012). In multiple insects, SID-1-like proteins (SILA, SILB and SILC) have been hypothesized to be involved in uptake of dsRNA (Miyata, Ramaseshadri et al. 2014, Cappelle, De Oliveira et al. 2016). In *Drosophila* dsRNA uptake is followed by clathrin-mediated endocytosis (Saleh, van Rij et al. 2006), which has also been identified in other insects (Wynant, Santos et al. 2014, Xiao, Gao et al. 2015, Cappelle, De Oliveira et al. 2016). Multiple dsRNA uptake pathways, including SID-1-like transmembrane proteins and clathrin-mediated endocytosis, have been described in the Colorado potato beetle (*Leptinotarsa decemlineata*) (Cappelle, De Oliveira et al. 2016), highlighting the complexity of the dsRNA uptake mechanism and systemic response in insects.

Upon uptake into the cytoplasm, the dsRNA is cleaved into 21-25 bp long small interfering RNAs (siRNAs) by action of a multidomain ribonuclease (RNase) type III enzyme called Dicer (Carthew and Sontheimer 2009). These siRNAs are loaded into an enzymatic complex termed the RNA induced silencing complex (RISC). The RISC loading complex (RLC), composed of dicer-2 (Dcr2) and an RNA binding protein, R2D2, is responsible for incorporation of siRNAs into RISC (Preall and Sontheimer 2005). Since the siRNAs are double stranded at uptake, they are unwound during RISC formation to yield a guide strand complementary to the target mRNA and a passenger strand that gets degraded. The guide strand then directs the RISC complex to its complimentary mRNA, where it binds. An important component of RISC is an enzyme containing an RNAase H-like domain termed Argonaute, which is responsible for degradation of the target mRNA, thus inhibiting gene expression. Given their relevance to the process, dicer, Argonaute and siRNAs are commonly considered essential parts, and evidence of a functional RNAi machinery for silencing of genes (Carthew and Sontheimer 2009).

RNAi can be exploited for specific inhibition of expression of virtually any gene, and its sequence-specific nature makes it a highly potent tool with applications in various fields including functional genomics (Travella, Klimm et al. 2006, Liu, Ge et al. 2016, Inwood, Betenbaugh et al. 2018), therapeutics (Dykxhoorn and Lieberman 2005, Egli and Manoharan 2019, Manisit, Sara et al. 2019, Yoo, Jordan et al. 2019) and agriculture (described below in detail).

Uses of RNAi against insects of agricultural importance

Different applications of RNAi related to agriculture have been proposed, including protection of beneficial insects from pathogens (Hunter, Ellis et al. 2010, Zotti and Smagghe 2015), control of insect pests (Baum, Bogaert et al. 2007, Gordon and Waterhouse 2007, Price and Gatehouse 2008, Zhou, Wheeler et al. 2008, Andrade and Hunter 2016, San Miguel and Scott 2016), plant pathogens (Tenllado, Martínez-García et al. 2003, Koch, Kumar et al. 2013, Åsman, Dixelius et al. 2015, Koch, Biedenkopf et al. 2016) and nematodes (Walawage, Britton et al. 2013, Youssef, Kim et al. 2013), or managing herbicide resistance in weeds (Zhang, Zhang et al. 2011, Guo, Zhang et al. 2012). For example, protection of honeybees by RNAi against the Israeli acute paralysis virus resulted in lower disease incidence associated with colony collapse disorder (Maori, Paldi et al. 2009).

The first experimental evidence of insecticidal activity of dsRNA was provided by Baum et. al. (Baum, Bogaert et al. 2007), laying the foundation for the potential of RNAi technology in control of insect pests belonging to different orders. In that study, genetically modified plants expressing dsRNAs targeting vital genes were shown to cause lethality in *D. v. virgifera* and demonstrated significant protection against this insect. However, diverse studies demonstrate great differences in sensitivity to RNAi among insect orders with coleopterans being the most sensitive and lepidopterans and hemipterans being largely recalcitrant to RNAi (Baum, Bogaert et al. 2007, Terenius, Papanicolaou et al. 2011, Baum and Roberts 2014). These differences in RNAi sensitivity are based on physiological differences among these insect orders as well as differences in the RNAi mechanism and machinery. The relative insensitivity of lepidopteran insects to dsRNA, compared to coleopterans, is due to differences in dsRNA stability in the insect gut and subsequent lack of uptake and processing of dsRNAs. Initial evidence of gut nucleases degrading dsRNA was provided in larvae of *Bombyx mori* (Arimatsu, Furuno et al. 2007*)*. These nucleases that degrade dsRNA and inhibit the RNAi response were also shown to be present in the hemolymph of *Manduca sexta* (Garbutt, Bellés et al. 2013). Comparison of dsRNA stability in hemolymph and gut fluids from a lepidopteran (*Heliothis virescens*) and a coleopteran (*L. decemlineata*) insect demonstrated that dsRNA is degraded faster in *H. virescens* (Shukla, Kalsi et al. 2016) than in *L. decemlineata*. However, the role of nucleases in dsRNA degradation in insect gut fluids and its effect on RNAi efficiency has also been documented for coleopterans such as *Anthonomus grandis* (Almeida Garcia, Lima Pepino Macedo et al. 2017), the hemipteran *Lygus lineolaris* (Allen and Walker 2012) and the homopteran *Acyrthosiphon pisum* (Christiaens, Swevers et al. 2014).

Unexpectedly, even after efficient dsRNA uptake, siRNAs corresponding to dsRNA administered to cell lines were detected in coleopteran but not in lepidopteran cells (Yoon, Shukla et al. 2016). This observation could be explained by accumulation of dsRNA in early and late endosomes resulting in inefficient RNAi, as reported in *Spodoptera frugiperda* (Yoon, Gurusamy et al. 2017). The availability of complete genomes for certain insect species have shown that the number of core RNAi machinery genes, such as Argonaute (Ago1 and Ago2), Dicer (Dcr2) and RNA binding protein (R2D2), varies among different insect groups (Dowling, Pauli et al. 2016), providing another explanation for the observed differences in RNAi efficacy among different insect species. For example, the insect model species *Tribolium castaneum* shown to be highly

sensitive to dsRNA has two paralogs of both Ago2 and R2D2, while most insects have one (Tomoyasu, Miller et al. 2008). Another species sensitive to dsRNA, *L. decemlineata*, has duplicated core components of the siRNA pathway, including Dcr2 and Ago2 (Schoville, Chen et al. 2018). Understanding these differences in insect response to dsRNA is crucial for successful development and use of RNAi-based insecticidal technology.

The application of dsRNA as an insecticidal approach can be of two types depending on the delivery technology. Transformative RNAi comprises transgenic plants expressing dsRNA targeting a vital gene in a particular insect pest. This approach, also known as host-induced gene silencing (HIGS), has been demonstrated as a successful tool in protection of crops against specific insects (Baum, Bogaert et al. 2007, Mao, Cai et al. 2007, Head, Carroll et al. 2017, Zotti, dos Santos et al. 2018). In fact, SmartStax Pro®, a maize transformation event expressing three insecticidal crystal (Cry) genes from *Bacillus thuringiensis* and a RNAi based trait which expresses hairpin loop RNA containing a 240-bp dsRNA fragment of the *D. v. virgifera* sucrose non-fermenting 7 (*DvSnf7*) gene has already been registered by the US Environmental Protection Agency (Head, Carroll et al. 2017). This approach combines two diverse modes of action as RNAi-based traits cause lethality in insects by disrupting target cellular function while Cry toxins act by disrupting midgut function through ion channels and pores (Vachon, Laprade et al. 2012, Moar, Khajuria et al. 2017, Khajuria, Ivashuta et al. 2018). Hence, pyramiding of Cry toxins and RNAi-based traits provides increased protection and is expected to delay the onset of resistance. However, transgenic crops are not always a realistic option for controlling insect pests due to many factors including political, legislative, economic issues, or difficulty in transforming certain crops. Therefore, alternative non-transformative methods of dsRNA delivery have also been proposed.

The non-transformative methods of dsRNA delivery include topical sprays, root drenches and trunk injections, that require less cost to develop compared to transformative technologies and lesser number of experiments than required for regulatory approvals for transgenic crops. However, one drawback of non-transformative RNAi strategies that may add extra labor and cost for growers, is the need for repeated treatments to ensure protection, while a transgenic crop can continuously produce dsRNA. Effective use of foliar dsRNA sprays to induce RNAi, also known as spray induced gene silencing (SIGS), has been demonstrated for target pests such as the Colorado potato beetle (*Leptinotarsa decemlineata*) (Zhu, Xu et al. 2011, San Miguel and Scott 2016). Experiments show that a foliar dsRNA spray is not only highly effective in controlling *L.*

decemlineata populations under greenhouse conditions, but also that the dsRNA is sufficiently stable on leaves and not removed by water once dried on leaves (San Miguel and Scott 2016). Irrigating rice and maize with a solution containing dsRNA targeting the actin gene showed increased mortality in planthoppers and Asian corn borers (Li, Guan et al. 2015). These results demonstrate efficient absorption of dsRNA by plant roots and induction of RNAi in insects feeding on such plants, providing effective control of piercing-sucking and stem-borer insects by root drenching, a non-transformative strategy (Li, Guan et al. 2015). In another study, full-sized citrus and grapevine trees were treated with dsRNA using foliar sprays, root drenching, or trunk injections and two hemipteran insects, a xylem- and a phloem-feeder, and a coleopteran chewing insect, were fed on treated trees. While dsRNA was still detected at least 57 days in the plants post treatment, it was detected in insects 5-8 days after ingestion of treated plants (Hunter, Glick et al. 2012). Persistence of dsRNA on plants treated using different non-transformative methods of dsRNA delivery offer potent pest control approaches in wide areas like crop fields. Such encouraging results have paved the way for RNAi-based commercial products for pest control with certain products expected to reach market by the end of 2019 (Zotti, dos Santos et al. 2018).

Resistance to RNAi

There are only a handful of studies addressing the potential of resistance evolution to insecticidal dsRNA. Reports in mammalian cells demonstrate development of resistance to siRNA-mediated RNAi and the role of RNA editing by adenosine deaminases acting on RNA (ADARs) in the resistance mechanism (Zheng, Tang et al. 2005). Viruses are known to develop resistance to siRNA inhibition either by mutating their target region or by producing suppressors of RNAi (Zheng, Tang et al. 2005). Resistance to dsRNA induced by conditional expression siRNAs derived from short hairpin RNA (shRNA) has also been reported in mouse liver and mammary gland, although the mechanism of resistance was not discussed (Ajiro, Jia et al. 2015).

Several possible mechanisms of resistance to dsRNA in insects have been proposed. Stability of dsRNA in the insect gut is an important factor for a successful RNAi response (Garbutt et al. 2013; Garcia et al. 2017; Spit et al. 2017), and increased nuclease expression can lead to degradation of dsRNA and subsequent RNAi failure. Artificial reduction of Dcr-2 and Ago2 expression has been reported to confer complete protection to *D. v. virgifera* adults against an insecticidal dsRNA, with no phenotypic effects in adults or larvae (Vélez et al. 2016). These

observations suggest that mutations in RNAi machinery genes is another possible resistance mechanism. However, it is important to consider that these RNAi machinery genes encode proteins essential for normal processing of endogenous dsRNAs and microRNAs (Carthew and Sontheimer 2009), and thus reduces the likelihood of resistance evolution (Wu et al. 2017). Another plausible mechanism of resistance is mutations in the target gene sequence such that siRNAs cannot identify the target mRNA (Auer and Frederick 2009). However, because each long insecticidal dsRNA molecule can produce numerous siRNAs with different sequences and a single 21 bp match to the target sequence is efficacious (Bolognesi, Ramaseshadri et al. 2012), this possibility appears difficult.

As most of these possible resistance mechanisms act independently of the dsRNA sequence, it is reasonable to assume the same initial mode of action for all naked dsRNA molecules. Thus, diverse dsRNAs should have similar cellular uptake mechanisms and stability in the gut lumen environment of the insect, even if the dsRNA molecules target completely different intracellular pathways. This is a valid concern and will have to be carefully considered when developing novel pest control strategies based on the RNAi technology and when estimating the associated resistance risks (Spit et al. 2017).

The sole experimental evidence on resistance to RNAi in an insect comes from a recent study by Khajuria et al (Khajuria, Ivashuta et al. 2018). In this study, a population of field-collected *D. v. virgifera* was used for screening and selection for resistance against maize expressing dsRNA targeting the *DvSnf7* gene. After seven episodes of selection, resistant *D. v. virgifera* showed significantly lower mortality compared to susceptible insects while feeding on maize expressing dsRNA. These insects were also cross-resistant to other insecticidal dsRNAs, suggesting that the resistance mechanism is not sequence-specific. Importantly, no cross-resistance to the Cry3Bb1 toxin was observed, thus supporting combined use of the two technologies to delay the onset of resistance (Moar, Khajuria et al. 2017). *In vitro* incubation of dsRNA with digestive fluids from resistant and susceptible *D. v. virgifera* showed no difference in nuclease activity between the two populations, thus indicating that nucleases are not associated with the resistance mechanism. In contrast, reduced uptake of fluorescently labeled dsRNA in midgut cells from resistant compared to susceptible rootworms, and the lack of siRNAs corresponding to the DvSnf7 dsRNA in resistant insects supports impaired uptake of dsRNA as candidate resistance mechanism in *D. v. virgifera*.

These results have high significance as they provide the first insight into resistance mechanisms against insecticidal dsRNAs.

Selection experiments with dsRNA targeting the inhibitor of the *apoptosis 1* gene in the Lepd-SL1 cell line from *L. decemlineata* led to moderate levels of resistance (Yoon, Mogilicherla et al. 2018). In these resistance cells, the levels of expression of StaufenC, a dsRNA binding protein required for processing of dsRNA were reduced when compared with susceptible cells. The same study showed that coleopteran-specific StaufenC is required for RNAi in *T. castaneum*, further supporting that its reduced expression can result in RNAi resistance.

As with every novel pest control strategy, RNAi is not exempt from the concern of resistance development in insect pests. Combining technologies with different modes of action (such as Cry toxins and dsRNAs) in a single product, along with implementation of Integrated Pest Management (IPM), should delay the onset of resistance (Roush 1998). However, understanding potential resistance mechanisms to insecticidal dsRNA is important to compose effective Insect Resistance Management (IRM) strategies to ensure durability and optimization of the technology. Consequently, a more comprehensive picture, obtained by studies conducted from different insects and using different selection regimes is required. This thesis was aimed at understanding how *L. decemlineata* as a model pest targeted by dsRNA sprays might develop resistance to insecticidal dsRNA targeting the V-ATPase subunit A gene topically applied to host plant material.

Colorado potato beetle: a model for resistance evolution

The Colorado potato beetle (CPB, *Leptinotarsa decemlineata*) is the most significant pest of potato crop, *Solanum tuberosum* L., though its host range also includes other solanaceous plants such as buffalobur (*S. rostratum*), eggplant (*S. melongena*), silverleaf nightshade (*S. elaeagnifolium*), horsenettle (*S. carolinense*), bittersweet nightshade (*S. dulcamara*), tomato (*S. lycopersicum*), and tobacco (*Nicotiana tabacum*). CPB was first observed by Thomas Nuttall in 1811 and then formally described by Thomas Say in 1824 (Jacques 1988), yet it did not gain pest status until ~1859, when it began destroying potato crops in Omaha (Nebraska) (Jacques 1988). After that, CPB quickly spread in the 20th century across an area of 16 million km^2 including parts of North America, Europe and Asia.

Eggs of *L. decemlineata* are yellow to orange in color and laid on the underside of leaves in batches of about 30. The females are highly prolific, capable of laying over 500 eggs in 4-5

weeks and can undergo multiple overlapping generations each season. After hatching, in about 4-15 days the larvae go through four instars, with first through third instar being 2-3 days long and the fourth one lasting from 4-7 days. The fourth instar larvae spend a few days as non-feeding prepupae and dig several inches into the soil to pupate. In about 5-10 days, adult beetles emerge from the soil and start feeding and mating. The whole cycle from eggs to adult usually lasts 21 days if the temperature is favorable. On the other hand, in case of unfavorable temperature, light duration or host availability conditions, adults can enter diapause and delay their emergence. This ability to correctly time diapause has been demonstrated to aid the expansion and adaptation of this pest to various climate ranges (Walsh 1865, Hare 1990, Piiroinen, Ketola et al. 2011, Lehmann, Lyytinen et al. 2014).

A remarkable ability of *L. decemlineata* is its rapid development of resistance to a wide range of insecticides, making it one of the most adaptable and challenging insect pests to control. In fact, this ability is believed to have driven the development of the modern pesticide industry, starting with the application of Paris Green (cuprous acetoarsenite) to control it (Gauthier 1981). As of today, different populations of *L. decemlineata* have been described as resistant to 56 different compounds (Whalon 2019) belonging to all major insecticide classes and with different modes of action (Table 1.1). Studies to elucidate resistance in *L. decemlineata* revealed a variety of mechanisms, including target site insensitivity, increased metabolism, reduced insecticide penetration, increased excretion and behavioral resistance (Alyokhin, Baker et al. 2008). Increased oxidative metabolism by cytochrome P450-dependent monooxygenases is the most common resistance mechanism in *L. decemlineata*. Thus, pre-treatment of resistant *L. decemlineata* larvae or adults with piperonyl butoxide (PBO), an oxygenase inhibitor, has been shown to decrease resistance against azinphosmethyl (Ahammadsahib, Hollingworth et al. 1994), carbofuran and carbaryl (Rose and Brindley 1985), fenvalerate (M. Soderlund, W. Hessney et al. 1987) (Forgash, Silcox et al. 1985, Harris and Turnbull 2012), permethrin (Forgash, Silcox et al. 1985) and abamectin (Yoon, Nelson et al. 2002). In the case of imidacloprid-resistant *L. decemlineata*, treatment with PBO reduced resistance from 300-fold to 108-fold, again demonstrating the association between monooxygenase activity and pesticide resistance (Mota-Sanchez, Hollingworth et al. 2006). Though the metabolism of imidacloprid to produce a less toxic olefin metabolite is observed in both susceptible and resistant *L. decemlineata* strains, it is observed at a greater extent in resistant insects.

Table 1.1- List of insecticides against which *L. decemlineata* has developed resistance in response to field exposure or laboratory selection (Whalon 2019).

IRAC classification	**Common names**
Acetylcholinesterase (AChE) inhibitors	Aldicarb, carbaryl, carbofuran, cloethocarb, dioxacarb, oxamyl, propoxur, carbosulfan, azinphosethyl, chlorpyrifos, azinphosmethyl, chlorfenvinphos, malathion, methamidophos, methidathion, monocrotophos, parathion, parathion-methyl, phorate, phosmet, phoxim, phosalone quinalphos, tetrachlorvinphos, trichlorfon
Sodium channel modulators	DDT, methoxychlor
GABA-gated chloride channel blockers	Aldrin, chlordane, dieldrin, endosulfan, endrin, lindane, toxaphene
Mitochondrial complex IV electron transport inhibitors	Hydrogen cyanide
Sodium channel modulators	Cypermethrin, cypermethrin-alpha, cyhalothrin, deltamethrin, esfenvalerate, fenvalerate, permethrin
Mitochondrial complex I electron transport inhibitor	Rotenone
Nicotinic acetylcholine receptor (nAChR) competitive modulators	thiamethoxam, acetamiprid, clothianidin, dinotefuran, imidacloprid, N-desmethylthiamethoxam, nitenpyram, thiacloprid
Nicotinic acetylcholine receptor (nAChR) channel blockers	Cartap, Bensultap
Nicotinic acetylcholine receptor (nAChR) allosteric modulators - Site I	Spinosad
Microbial disruptors of insect midgut membranes	Bt

In addition to monooxygenase activity, increased metabolism due to arylesterase (Ferro, Argentine et al. 1989), carboxylesterase (Argentine, Lee et al. 1995, Lee and Clark 1996) and gluthione-S-transferase (Ahammadsahib, Hollingworth et al. 1994) activity have also bene shown to contribute to resistance against different pesticides in resistant *L. decemlineata* populations from diverse geographical areas.

Another mechanism of resistance observed in insecticide-resistant *L. decemlineata* populations is target site insensitivity. Mutations in the acetylcholine esterase (*AChE*) gene targeted by organophosphates and carbamates are related with resistance (Ioannidis, Grafius et al. 1992, Wierenga and Hollingworth 1993, Stanković, Zabel et al. 2004). For example, two mutations in AChE led to decreased enzyme sensitivity against azinphosmethyl and carbofuran (Kim, Dunn et al. 2006). Similarly, a single mutation in AChE provided high resistance against carbofuran and lower level resistance to azinphosmethyl (Kim, Yoon et al. 2007). Target site mutations have also been observed in permethrin-resistant *L. decemlineata* strains. In these beetles, an amino acid change (leucine to phenylalanine) from a single base-pair mutation in an α-subunit of the sodium channel, was responsible for nerve insensitivity and resistance to permethrin (Lee, Dunn et al. 1999, Kim, Hawthorne et al. 2005)

Reduced penetration and increased excretion are other mechanisms of resistance observed in various *L. decemlineata* populations. Even though these mechanisms may not confer high resistance on their own, they still have an important role to play in pest control. For instance, they were found to contribute to reduced toxicity of azinphosmethyl by acting along with enhanced metabolism and target site insensitivity (Argentine, Zhu et al. 1994). They have also been shown to play an important role in carbaryl resistance as well (Rose and Brindley 1985). In addition, increased excretion to remove toxic compounds like imidacloprid and glycoalkaloids (Krishnan, Kodrík et al. 2007) has also been identified.

Changes in insect behavior, such as increased flight activity in resistant compared to susceptible beetles, was studied in *L. decemlineata* resistant to endotoxins produced by *Bacillus thuringiensis* (Ferro 1993, Alyokhin and Ferro 1999). Such a response allowed beetles to escape toxic environments leading to physiological resistance. Behavioral resistance was also reported by Hoy and Head (Hoy and Head 1995), who observed that larvae of *L. decemlineata* resistant to *B. thuringiensis* were also more responsive and moved away from treated foliage.

Heavy dependence on chemical insecticides and high selection pressure are likely contributors to high resistance incidence, yet other factors may have played a major role in facilitating *L. decemlineata* resistance. For instance, these beetles evolved to feed on solanaceous plants, which are otherwise highly toxic to other herbivore insects as they contain steroidal alkaloids and glycoalkaloids (Milner, Brunton et al. 2011, Cárdenas, Sonawane et al. 2015). Additionally, in response to beetle feeding, potato plants upregulate pathways related to terpenoid, alkaloid and phenylpropanoid biosynthesis, along with a range of protease inhibitors (Lawrence, Novak et al. 2008). A complex of digestive cysteine proteases helps *L. decemlineata* to respond to such plant-induced defenses (Novillo, Castañera et al. 1997, Petek, Turnšek et al. 2012). Moreover, *L. decemlineata* larvae adapted to excrete (Armer 2004) and sequester plant derived toxic compounds in the hemolymph (Hsiao and Fraenkel 1969). The physiological mechanisms involved in detoxification of toxic plant compounds and other xenobiotics are believed to contribute to the evolution of pesticide resistance (Alyokhin and Chen 2017). Another characteristic of *L. decemlineata* that may aid in resistance development is its high fecundity, which ensures quick selection and spread of favorable resistance-conferring alleles in the population. An insight into the genome of *L. decemlineata* showed increased presence of transposable elements (17% of the whole genome) and high nucleotide diversity (1 in every 22 base pairs is different) (Schoville, Chen et al. 2018). These features may allow for rapid evolutionary change and increased incidence of pesticide resistance in this insect.

Currently, tens of millions of dollars are spent annually to manage *L. decemlineata* (Grafius 1997) and the costs are expected to reach billions of dollars if left unmanaged (Skryabin 2010), which makes it important to develop new efficient control strategies. As mentioned above, studies have shown that *L. decemlineata* is able to uptake dsRNA from the gut lumen and subsequently induce a potent systemic RNAi response (Zhu, Xu et al. 2011), suggesting that RNAi could be a feasible control method for this insect pest. In fact, the presence of duplications in core RNAi machinery genes, including Dicer-2 and Argonaute-2, in the genome of *L. decemlineata* helps explain its high sensitivity to insecticidal dsRNA (Schoville, Chen et al. 2018). The history of insecticide resistance in *L. decemlineata* along with its susceptibility to dsRNA, makes it an ideal insect model to study development of resistance to insecticidal RNAi.

Selection for resistance to dsRNA in *L. decemlineata*

Colorado potato beetle colonies from ten different locations across the United States were collected with the help of collaborators (Fig. 1.1). All of these colonies were pooled together, and the resulting colony, named General Colony (GC), was selected for resistance using dsRNA targeting the Vacuolar-type H+-ATPase subunit A (V-ATPase subunit A) via a 20-day bioassay. In this bioassay, eggs from the previous pooled population were collected from the greenhouse and kept in an incubator at 25 ºC. As the eggs hatched, neonates were fed leaves treated with V-ATPase subunit A dsRNA. Leaves were first dipped in a dsRNA solution made with 0.1% Tween-20. Tween-20 is a surfactant used to provide a homogenous spread of dsRNA on the leaf surface. Treated leaves were dried completely before feeding larvae. This process was repeated for about 9 days until larvae reached the late fourth instar and stopped feeding as a prepupa. At this time, they were transferred to soil from which they emerged as adults (after 20 days from the initiation of the bioassay). Progeny of adults surviving the previous bioassay were exposed to increasing concentrations of dsRNA. These selections resulted in the development of a population of *L. decemlineata* resistant to V-ATPase subunit A dsRNA (Table 1.2). This population was named CEAS for "chronically exposed adult survivors". To estimate the level of resistance in CEAS, it was tested at 2,000 µg/ml dsRNA concentration and demonstrated no significant mortality compared to the control or treated only with 0.1% Tween-20. When compared to the LC_{50} for GC (0.38 µg/ml) the resistance level was estimated at >5,000-fold. Hence, GC was used as a susceptible control to perform further experiments characterizing and elucidating the mechanism of resistance to V-ATPase subunit A dsRNA in CEAS.

Figure 1.1- Map showing the regions (red circles) from which *L. decemlineata* colonies were collected.

Table 1.2- Selection for resistance to dsRNA targeting the V-ATPase subunit A transcript in *L. decemlineata*. Shown are the dsRNA concentration and mortality observed (adult emergence endpoint) for the unselected parental (GC) and selected (CEAS) colonies. NT= not tested.

Selection episode	dsRNA concentration used (µg/ml)	Percentage mortality	
		GC	CEAS
1	0.38	100	96
2	0.38	NT	44.4
3	1.11	NT	33.2
4	1.88	NT	41.7
5	5.68	96.7	6.7
6	9.41	100	26.7
7	30	97.7	17.7
8	300	100	5
9	2,000	100	0

Chapter Two

Role of nucleases in resistance against insecticidal dsRNA in Colorado potato beetle (*Leptinotarsa decemlineata*)

Abstract

Gene silencing using double-stranded RNA (dsRNA) via the RNA-interference (RNAi) pathway presents an is a novel mode of action that may be leveraged in developing insecticides. As with every insecticidal technology, targeted insects will be expected to develop resistance to insecticidal dsRNAs. Consequently, understanding dsRNA resistance mechanisms is important to ensure durability and effective resistance management strategies for dsRNA technology. Here we explore the role of nucleases present in digestive fluids in conferring resistance against dsRNA in a V-ATPase dsRNA-resistant population of *L. decemlineata,* a coleopteran insect otherwise highly susceptible to gene silencing induced by dsRNA. Degradation of dsRNA in the insect gut due to increased nuclease activity is observed in insects insensitive to dsRNA and hence suggests a potential mechanism of resistance. Comparison of dsRNA stability in gut fluids from susceptible and V-ATPase dsRNA-resistant *L. decemlineata* populations demonstrate no difference in nuclease activity between the two. We conclude that nucleases are not involved in the resistance mechanism against dsRNA in resistant *L. decemlineata* population, and further steps involved in the RNAi pathway need to be examined for their potential involvement in resistance.

Introduction

Gene silencing by RNA-interference (RNAi) is a mechanism triggered by the presence of double stranded RNA (dsRNA) complimentary to a target transcript that is degraded, hence suppression in gene expression. Due to its dependence on sequence complementarity, RNAi has been proposed as a highly specific pesticide representing a new mode of action and with exciting applications for pest control in agriculture and for protection of beneficial insects (Zotti, dos Santos et al. 2018). Since the first description of the insecticidal use of RNAi through production of dsRNA by transgenic maize plants against *Diabrotica virgifera virgifera* larvae (Baum, Bogaert et al. 2007), several researchers have explored the use of insecticidal dsRNA delivered through transgenics, foliar sprays, root drenches and trunk injections (Hunter, Glick et al. 2012, Li, Guan et al. 2015, San Miguel and Scott 2016). RNAi technology is expected to complement other modes of action, such as insecticidal crystal (Cry) proteins from *Bacillus thuringiensis*, in pyramided transgenic crops for increased efficacy and delay of resistance evolution (Head, Carroll et al. 2017).

As with any novel pest control strategy, it is expected that insect pests will develop resistance to RNAi, that can evolve from any alterations in its mode of action. In insects, the process of RNAi is initiated by the delivery of dsRNA to the target tissue by feeding, soaking or injection, followed by uptake in the target cells. Upon uptake into the cell cytoplasm, the dsRNA is cleaved into 21-25 bp long small interfering RNAs (siRNAs) by action of a multidomain ribonuclease (RNase) type III enzyme called Dicer. These siRNAs are loaded into an enzymatic complex termed the RNA induced silencing complex (RISC). The RISC loading complex (RLC), composed of the enzyme Dicer-2 (Dcr2) and the RNA binding protein R2D2, is responsible for incorporating siRNAs into RISC. Since the siRNAs are double stranded, they are unwound during RISC formation to form a guide strand, complementary to the target mRNA, and a passenger strand which gets degraded. The guide strand then directs the RISC complex to its complimentary mRNA, followed by mRNA degradation by the enzyme Argonaute. Given their relevance to the process, Dicer, Argonaute and siRNAs, are commonly considered essential parts and evidence of a functional RNAi machinery for silencing of genes. In theory, any alteration in these steps or RNAi machinery genes could result in resistance, and several possible mechanisms of resistance to RNAi in insects have been proposed (Baum and Roberts 2014). However, the sole experimental evidence on resistance to RNAi in an insect suggests reduced uptake of dsRNA as the mechanism of resistance to maize producing dsRNA targeting the *DvSnf7* gene in a resistant population of *D. v. virgifera* (Khajuria, Ivashuta et al. 2018).

Stability of dsRNA in the insect gut is an important factor for a successful RNAi response (Garbutt et al. 2013; Garcia et al. 2017; Spit et al. 2017), and nucleases degrading dsRNA in insect gut fluids hinder RNAi efficiency in coleopterans such as *Anthonomus grandis* (Almeida Garcia, Lima Pepino Macedo et al. 2017), the hemipteran *Lygus lineolaris* (Allen and Walker 2012) and the homopteran *Acyrthosiphon pisum* (Christiaens, Swevers et al. 2014). Several studies investigating the relative insensitivity of lepidopterans to RNAi compared to other insect groups (such as most coleopterans), reveal a central role for differences in dsRNA stability in the insect gut. Initial evidence of gut nucleases degrading dsRNA was provided in larvae of *Bombyx mori* (Arimatsu, Kotani et al. 2007). These nucleases degrade dsRNA and inhibit the RNAi response, and were also shown to be present in the hemolymph of *Manduca sexta* (Garbutt, Bellés et al. 2013). Comparison of dsRNA stability in hemolymph of a lepidopteran (*Heliothis virescens*) and a coleopteran (*L. decemlineata*) demonstrated that dsRNA is degraded relatively faster in *H.*

virescens (Shukla, Kalsi et al. 2016). Based on this information, increased nuclease activity and reduced stability of dsRNA in digestive fluids, is a possible mechanism to develop resistance against insecticidal dsRNA in insects.

In our group we developed a strain of Colorado potato beetle (*Leptinotarsa decemlineata*) with >5,000-fold resistance to dsRNA targeting the V-ATPase subunit A gene, delivered via feeding (Dee *et al*, in preparation). The goal of this work was to test the role of the first potential step affecting the RNAi mode of action in these beetles; the stability of dsRNA in the digestive fluids in the resistance phenotype. Understanding resistance mechanisms to insecticidal RNAi is important to compose effective Insect Resistance Management (IRM) strategies to ensure durability and optimization of the technology.

Materials and Methods

Insects

Susceptible (GC) and resistant (CEAS) Colorado potato beetle adult populations are kept in a greenhouse bay at 25 ºC and 85% relative humidity. The CEAS colony is kept under continuous selection during the larval stage with 400 µg/ml of dsRNA targeting the V-ATPase A subunit gene. Eggs are collected from the greenhouse and as they hatch, neonates were fed leaves treated with V-ATPase subunit A dsRNA. Leaves are first dipped in a dsRNA solution made with 0.1% Tween-20. Tween-20 is a surfactant used to provide a homogenous spread of dsRNA on the leaf surface. Treated leaves are dried completely before feeding larvae. This process is repeated for about 9 days until larvae reach the late fourth instar and stop feeding as a prepupa. They are then transferred to soil for pupation, from which they emerge as adults. The adults are then reared on untreated potato plants in the greenhouse. These plants are changed every 2-3 days. Eggs of similar age are collected to perform various experiments. Unused eggs are removed from potato plants and frozen to prevent competition between larval and adult populations.

Collection of gut fluids

Eggs from the GC and CEAS populations were collected and hatched larvae were reared on untreated potato leaves until 3rd instar. Digestive fluids were obtained from five 3rd instar larvae by dissecting the entire gut, while regurgitate was obtained by gently pressing the sides of the abdomen using blunt forceps. Dissected guts were homogenized in 20 µl of Ringer's solution (150

mM NaCl, 1 mM $CaCl_2$, 10 mM KCl, 40 mM $MgCl_2$, 40 mM $NaHCO_3$, 90 mM sucrose, pH 7.2). Gut debris was separated from fluid content by centrifugation at 15,000 rpm for 5 min at room temperature. Digestive fluid samples were quantified using the Qubit fluorometer (Invitrogen) and diluted to a 1 µg/µl stock in Ringer's solution (150 mM NaCl, 1 mM $CaCl_2$, 10 mM KCl, 40 mM $MgCl_2$, 40 mM $NaHCO_3$, 90 mM sucrose, pH 7.2). The stock was either used immediately or stored at -20 ºC until further use.

Nuclease assay

The 1 µg/µl stock of digestive fluids or regurgitate was used to prepare different dilutions (2X, 5X, 50X, 100X and 500X) in Ringer's solution. Digestive fluid and regurgitate samples were incubated with 500 ng of V-ATPase A dsRNA for 10 mins or 1h at room temperature (25ºC). The samples were then resolved in a 1% agarose gel to monitor stability of dsRNA.

Results

An initial experiment was performed using regurgitate and a 10-minute incubation with dsRNA (Fig. 2.1). Degradation of the dsRNA was observed as lack of a detectable band in the highest (stock) concentration of regurgitate (1µg/µl) tested in both GC and CEAS samples, when compared to the dsRNA stock. Dilution of this stock (100x or 500x) resulted in reduced nuclease activity and increased stability of dsRNA (Fig. 2.1). Importantly, no obvious differences in dsRNA stability were observed between the samples from GC and CEAS populations for the same digestive fluid dilution.

A second independent experiment was performed with samples both from regurgitate and whole gut fluids collected by dissection, one day prior to the nuclease assay. We only tested the stock concentration (1 µg/µl), based on the results observed in the previous experiment. To test for effects of length of incubation period, we incubated the digestive fluid and regurgitate samples with dsRNA for 10 minutes and 1 hour (Fig. 2.2). For both incubation periods, degradation was observed in GC and CEAS samples obtained via regurgitation, while dsRNA appeared to be stable in gut fluids collected by dissection. This may be due to the loss of enzyme activity in gut fluids due to storage. Interestingly, no differences in degradation were observed when comparing the two time points for any sample tested. Notably, the results agreed with the lack of differences in the

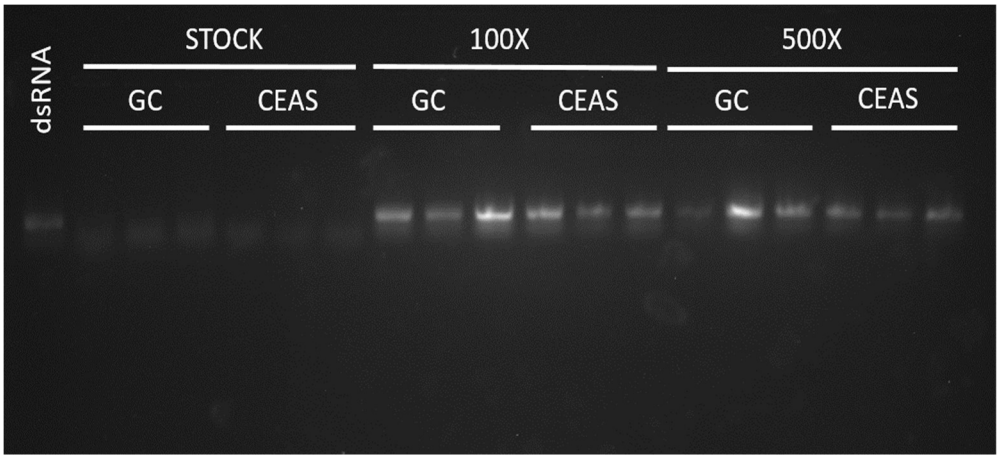


Figure 2.1- Agarose gel showing relative stability of dsRNA when incubated with 1µg/µl of GC and CEAS regurgitate (stock) or with 100X and 500X dilutions for 10 minutes at room temperature.

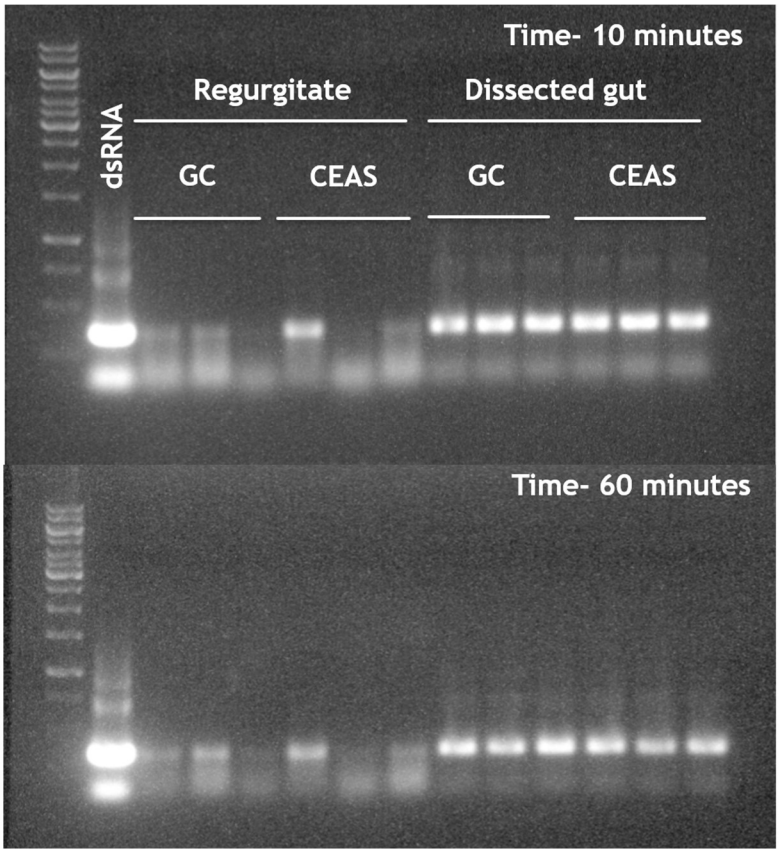


Figure 2.2- Agarose gel showing relative stability of dsRNA when incubated with 1µg/µl of GC and CEAS gut fluids collected as regurgitate or by dissection, for 10 minutes and 1 hour at room temperature.

nuclease activity and dsRNA stability between the GC and CEAS populations, as observed in the previous experiment.

Based on these results, a third experiment was conducted in which GC and CEAS regurgitate and gut fluids obtained by dissection were tested as a stock (1µg/µl) concentration and as 5X, 50X and 100X dilutions. All samples were collected on the same day as the assay to avoid loss of enzyme activity upon storage. Only the 1-hour incubation period was tested, and products were resolved on a 1% agarose gel (Fig. 2.3). dsRNA degradation was observed for the stock concentrations in both regurgitate and gut fluids for both GC and CEAS. In contrast, dsRNA was stable at higher dilutions of both regurgitate and digestive fluids. Most importantly, no difference was observed in the nuclease activity between GC and CEAS, in consistency with the previous results.

Discussion

Sensitivity to RNAi is highly variable among insects belonging to different orders. Most coleopteran insects, including *L. decemlineata*, have been shown to be highly sensitive to RNAi (Zhu, Xu et al. 2011) while others like lepidopterans are recalcitrant (Terenius, Papanicolaou et al. 2011). These differences in RNAi efficiency are based on physiological differences among these insect orders as well as differences in the RNAi mechanism and machinery. Increased nuclease activity leading to dsRNA degradation has frequently been associated with poor response or insensitivity to RNAi in some of these recalcitrant insects (Wang, Peng et al. 2016). Interestingly, nuclease activity was observed in the gut of *L. decemlineata* adults and larvae, despite their high sensitivity to RNAi, although dsRNA degradation was lower than in *Schistocerca gregaria,* a species insensitive to RNAi (Spit, Philips et al. 2017). A role for nucleases in RNAi efficacy in *L. decemlineata* was demonstrated by knockdown of two nucleases specific to the *L. decemlineata* gut leading to increased susceptibility towards orally delivered dsRNA (Spit, Philips et al. 2017). Consequently, increased nuclease activity could hypothetically represent a potential mechanism of resistance to insecticidal RNAi in *L. decemlineata*.

In this work, we test this hypothesis by comparing dsRNA-specific nuclease activity between susceptible (GC) and dsRNA-resistant (CEAS) populations of *L. decemlineata*. Based on the results from three independent experiments performed with three biological replicates, we conclude that there is no significant difference in nuclease activity and dsRNA stability in the

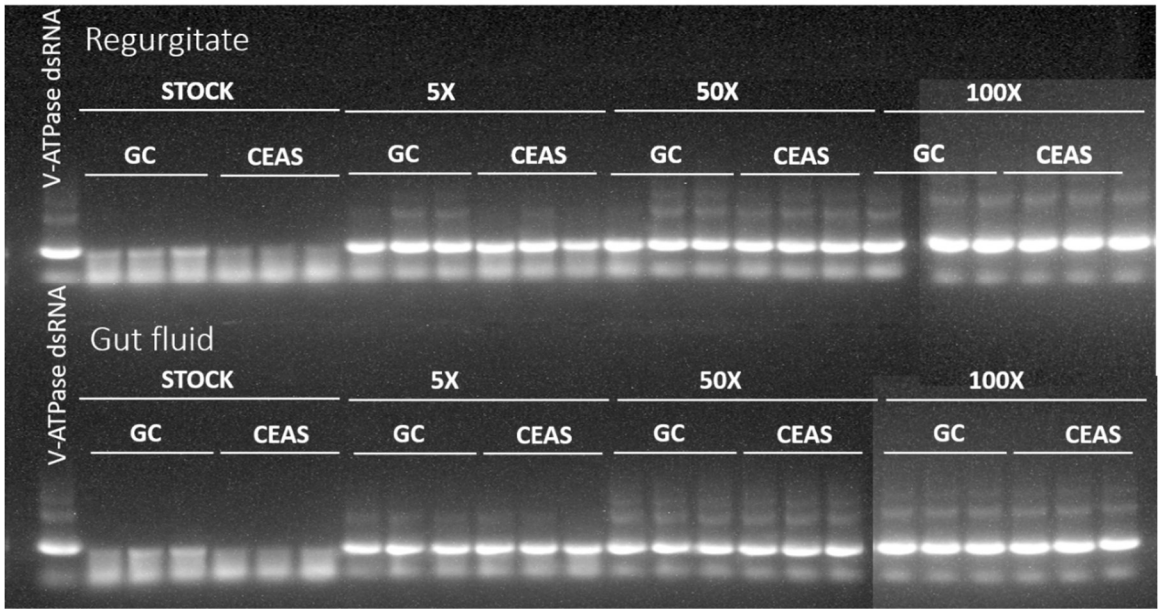


Figure 2.3- Agarose gel showing relative stability of dsRNA when incubated with stock (1µg/µl), 5X, 50X and 100X dilutions of GC and CEAS gut fluids collected as regurgitate or by dissection, for 1 hour at room temperature.

digestive fluids or regurgitate of GC and CEAS larvae. Therefore, degradation of dsRNA by nucleases present in the gut does not explain the >5,000-fold resistance against insecticidal RNAi in the CEAS *L. decemlineata* population. Our results are also supported by similar observations made for a dsRNA-resistant population of *D. v. virgifera,* in which impaired dsRNA uptake and not dsRNA degradation was associated with resistance (Khajuria, Ivashuta et al. 2018). In the third chapter of this Thesis, we demonstrate that CEAS is cross-resistant to other dsRNAs in addition to dsRNA targeting the V-ATPase subunit A gene used for selection, thus concluding that target site mutations cannot be the resistance mechanism in CEAS (Auer and Frederick 2009). In our future research we plan to test other plausible mechanisms such as reduced or impaired uptake or alterations in the RNAi machinery genes to elucidate the mechanism of resistance to dsRNA in the CEAS population.

Chapter Three

Cross-resistance to insecticidal dsRNAs and Cry3Aa protoxin from *Bacillus thuringiensis* in a dsRNA resistant Colorado potato beetle population

Abstract

Larvae and adults of Colorado potato beetle (*Leptinotarsa decemlineata,* CPB) are voracious defoliators of potato and solanaceous crops in North America, Europe and Asia. Its remarkable ability to develop resistance against pesticides and high susceptibility to gene silencing through RNA-interference (RNAi) makes it a model insect to study insect resistance mechanisms against insecticidal dsRNA technology. Here we provide molecular evidence for lack of gene silencing in a dsRNA resistant Colorado potato beetle population, previously selected using dsRNA targeting the V-ATPase subunit A gene. Instead, up-regulation of V-ATPase subunit A transcript upon treatment with V-ATPase subunit A dsRNA was observed and may represent a resistance mechanism specific to this target. Cross-resistance to an alternative dsRNA indicate that resistance is not sequence specific and that target site mutations are not associated with resistance. This also supports the presence of at least two different mechanisms of resistance, one general mechanism conferring cross-resistance to other dsRNAs and another mechanism specific to V-ATPase subunit A dsRNA. Additionally, CEAS showed increased tolerance to a Bt Cry toxin, Cry3Aa. These results provide useful insights into the development of resistance against dsRNA in insects and calls for careful risk assessment while developing insecticidal products based on dsRNA technology.

Introduction

The mechanism of gene silencing by RNA-interference (RNAi) is initiated by the presence of double stranded RNA (dsRNA) complimentary to a target gene. The RNAi mechanism is highly conserved among eukaryotic organisms and can be exploited for specific inhibition of expression of virtually any gene. Importantly, its sequence specific nature makes it a highly potent tool with applications in various fields, including agriculture where it can be used as a control technique for harmful pests, resistance management and protection of beneficial insects (Zotti, dos Santos et al. 2018).

During RNAi, silencing is achieved by degradation of the targeted messenger RNA (mRNA). The complete process of RNAi commences with uptake into the target cells of dsRNA complementary to the target mRNA, where it is cleaved into small interfering RNAs (siRNAs) by the action of a multidomain ribonuclease (RNase) type III enzyme called Dicer. These siRNAs are loaded into an enzymatic complex termed the RNA induced silencing complex (RISC). Since the

siRNAs are double stranded, they are unwound during RISC formation to form a guide strand complementary to the target mRNA, and a passenger strand which gets degraded. The guide strand then directs the RISC complex to its complimentary mRNA, where it binds Argonaute, an enzyme containing an RNAase H-like domain, which degrades the complementary mRNA and inhibiting gene expression.

The first example of RNAi application for pest control was the use of dsRNA targeting vital genes that cause lethality when silenced in the chrysomelid beetle *Diabrotica virgifera virgifera* (western corn rootworm, WCR) (Baum, Bogaert et al. 2007). In that report, the authors used genetically modified plants to deliver dsRNAs to the insects by feeding, and since then several researchers have explored the use of insecticidal dsRNA delivered in the form of not only transgenics but also foliar sprays, root drench and trunk injections (Hunter, Glick et al. 2012, Li, Guan et al. 2015, San Miguel and Scott 2016). Given the high sequence-driven specificity of RNAi, insecticidal RNAi is considered a highly specific insecticide (Bachman, Bolognesi et al. 2013) with a unique mode of action (Moar, Khajuria et al. 2017, Khajuria, Ivashuta et al. 2018), amenable to pyramiding with other insecticidal technologies (Roush 1998). As a practical example, a maize event expressing three insecticidal crystal (Cry) genes from *Bacillus thuringiensis* (Bt) and a dsRNA targeting the *D. v. virgifera* sucrose non-fermenting 7 (*DvSnf7*) gene is the first approved RNAi crop by the US Environmental Protection Agency for commercialization (Head, Carroll et al. 2017).

As is the case with any pest control strategy, insect pests are expected to develop resistance to RNAi. Consequently, it is important to understand resistance mechanisms to insecticidal dsRNAs in order to compose effective Insect Resistance Management (IRM) strategies that ensure durability and optimization of the technology. Several possible mechanisms of resistance to RNAi in insects have been proposed, including increased nuclease activity in the insect gut leading to reduced stability of dsRNA, reduced or impaired uptake of dsRNA into target cells, mutation(s) in RNAi machinery genes or mutation(s) in the target gene (Auer and Frederick 2009, Baum and Roberts 2014). Currently, the sole experimental evidence on resistance to dsRNA in an insect comes from a recent study by Khajuria et al (Khajuria, Ivashuta et al. 2018). In that study, a population of field-collected *D. v. virgifera* was used for screening and selection for resistance against maize expressing dsRNA targeting the *DvSnf7* gene. After seven episodes of selection, resistant *D. v. virgifera* exhibited significantly lower mortality compared to susceptible insects

while feeding on maize expressing dsRNA. These insects were also cross-resistant to other insecticidal dsRNAs, but not to the Cry3Bb1 toxin from Bt. This observation supports the combined use of the Bt and RNAi-based technologies to delay the onset of resistance in *D. v. virgifera*. Further experiments demonstrated no difference in nuclease activity, but impaired uptake of dsRNA into the target gut cells of resistant compared to susceptible larvae as a plausible mechanism of resistance.

The goal of this Thesis was to understand how another chrysomelid beetle (*Leptinotarsa decemlineata*) would develop resistance to dsRNA upon selection with dsRNA targeting the V-ATPase subunit A gene topically applied to host plant material. This selection resulted in a population of *L. decemlineata* (CEAS; chronically exposed adult survivors), that when compared to the susceptible population (GC; general colony) displayed >5,000-fold resistance (Dee et al, unpublished).

One possibility for this high-level resistance in CEAS is the selection of mutated target genes so that the target mRNA could not be identified by the RISC complex carrying the corresponding guide siRNA strand (Auer and Frederick 2009). However, because each long insecticidal dsRNA molecule can produce numerous siRNAs with different sequences and a single 21 bp match to the target sequence can have at least some toxicity (Bolognesi, Ramaseshadri et al. 2012), this possibility appears negligible. Nevertheless, this candidate mechanism easily could be tested by studying cross-resistance to insecticidal dsRNAs targeting alternative genes, as resistance due to mutations in the target gene should not confer cross-resistance to alternative dsRNA targets. While given the unique mode of action of RNAi, cross-resistance to Bt toxins is not expected. Therefore, we were interested in testing potential cross-resistance to Cry3Aa, one of the most active Bt toxins against *L. decemlineata* (Ferro 1989) and the Bt trait found in New Leaf potato to control *L. decemlineata* that was primarily commercialized in the 1990's.

The objectives of the current chapter were to determine if gene silencing occurs in CEAS and GC beetles upon feeding on dsRNA targeting the V-ATPase subunit A gene, determine cross-resistance by assessing silencing induced by dsRNAs targeting alternative genes, and testing cross-resistance to Cry3Aa protoxin in CEAS. We used quantitative PCR to determine relative transcript levels for the targeted genes at increasing time intervals after feeding on dsRNA. Bioassays using adult emergence as the endpoint were performed to determine the susceptibility of larvae from the

GC and CEAS populations to Cry3Aa protoxin. Results from these experiments demonstrate that resistance in CEAS is not sequence specific, and that CEAS is >3-fold less-susceptible to Cry3Aa.

Materials and Methods

Insects

Susceptible (GC) and resistant (CEAS) *L. decemlineata* populations are kept in a greenhouse at 25ºC and 85% relative humidity. The CEAS colony is kept under continuous selection during the larval stage by feeding on potato leaves coated in a solution of 400 µg/ml of dsRNA targeting the V-ATPase subunit A gene (Dee et. al., in preparation). Adults are then reared on untreated potato plants in the greenhouse. These plants are changed every 2-3 days. Eggs of similar age are collected to perform various experiments. Unused eggs are removed from potato plants and frozen to prevent competition for resources between the larval and adult population. In addition to GC and CEAS, another population of *L. decemlineata,* ME (provided by Aaron Buzza and Andrei Alyokhin, University of Maine), also was used as an additional susceptible control in some experiments.

Treatments

Larvae from susceptible (GC or ME) and resistant (CEAS) *L. decemlineata* populations were reared on untreated potato leaves until treatment with dsRNA. Early fourth instar larvae were starved for 24 hours and then fed a 5 µl droplet containing 25 µg of dsRNA targeting the V-ATPase subunit A gene and 0.5 mg of sucrose to promote ingestion. Larvae were closely monitored until they completely consumed the droplet. After ingestion, the larvae were transferred to untreated potato leaves until gut dissection at 0, 6, 12, 24, 48 and 72 hours post drop-feeding.

To test for cross-resistance, a different dsRNA target, Mon337 (provided by Monsanto Company), was used following the methodology described above but only testing after 0, 48, and 72 hours post drop-feeding. To assess the effect of feeding on dsRNA-treated leaves versus drop feeding on target transcript abundance, early fourth instar larvae were fed potato leaves coated in a solution of 400 µg/ml of dsRNA targeting the V-ATPase subunit A gene for 48 hours, and then their guts were dissected. In all the set-ups, dissected guts were used for extracting RNA for quantitative PCR (qPCR).

Quantification of transcripts for targeted genes by real time quantitative PCR (RTqPCR)

Total RNA was isolated from dissected guts using the RNeasy Mini kit (Qiagen), according to manufacturer's specifications. Purified total RNA (2 µg) was used to synthesize cDNA with random hexamer primers using the PrimeScript 1st strand cDNA Synthesis Kit (Takara), according to manufacturer's instructions. Quantitative PCR assays included 3 µl of cDNA diluted 10X, 10 µl of mastermix (PerfeCTa® qPCR FastMix® II, Low ROX™, QuantaBio), 1 µl at 10 µM of each primer, 0.5 µl at 10 µM probe and 4.5 µl of nuclease-free water, for a total volume of 20 µl. The thermocycler conditions were one cycle at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 10 s and annealing/extension at 60°C for 30s. Transcript levels for target genes were estimated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) using the *RP18* housekeeping gene for normalization (Shi, Guo et al. 2013). Mean transcript and corresponding standard errors were calculated from three independent biological replicates (3 guts per replicate) tested in triplicate for each target gene. Transcript levels at time zero or in untreated samples were considered as a relative level of "1" and all other expressed relative to levels at time zero.

Purification of and bioassays with Cry3Aa protoxin

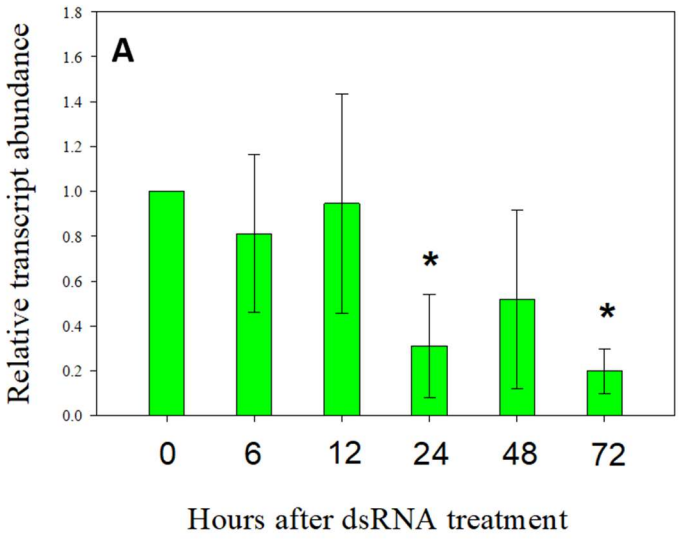
Bacillus thuringiensis var. *tenebrionis* was used to produce Cry3Aa protoxin. An isolated colony from a 1/3 TSB agar plate was suspended in 1 ml of autoclaved water and heated for 45 min at 70ºC, and then 500 µl was used to inoculate 1 L of 1/3 TSB medium. The culture was incubated for 3 days at 28ºC and 160 rpm agitation until sporulation was confirmed by microscopic observation. Cultures were centrifuged and pellets washed three times with 1M NaCl containing 0.1% triton X-100, followed by three washes with distilled water. The final pellet was resuspended in solubilizing solution (50 mM Na_2CO_3, 0.1% β-mercaptoethanol, 0.1 M NaCl) and incubated overnight at 30ºC and 200 rpm. The solubilized solution was centrifuged at 14,500 rpm for 30 min to pellet spores. After centrifugation, the supernatant was loaded on a HiTrap_Q_HP column pre-equilbrated in 50 mM Na_2CO_3, pH 9 (buffer A) for purification by anion exchange. Elution was performed using a linear gradient of Buffer A containing 1M NaCl. One major peak was detected during the elution and the fractions contained in this peak were analyzed by SDS-10%PAGE. The fractions were then pooled together and kept at -80ºC until used.

Bioassays were performed by exposing susceptible (GC) and dsRNA-resistant (CEAS) *L. decemlineata* larvae to potato leaves coated with solutions of 0.1% Tween-20 containing Cry3Aa protoxin at concentrations 0.5 µg/ml, 2 µg/ml, 5 µg/ml, 20 µg/ml and 50 µg/ml. Larvae fed on leaves treated with 0.1% Tween-20 solution were used as a control. Three biological replicates with 15 larvae each were performed for each concentration. One replicate was conducted at a different time than other two. Eggs were collected from different egg batches for all replicates, to ensure genetic variability. After 48h of exposure, larvae were transferred to containers with toxin-free potato foliage and put on soil once they stopped eating as prepupae. Successful adult emergence after approximately 20 days since initiation was considered to be the end point of the bioassay.

Results and Discussion

Lack of gene silencing in CEAS

In samples from susceptible the *L. decemlineata* population (GC) we detected a significant reduction in V-ATPase A transcript levels after 24 h and up to 72 h after treatment, when compared to the 0 h time point (p-value<0.001, Dunnett's multiple comparison) (Fig. 3.1A). The highest reduction in relative transcript amounts was observed at 72 h post treatment, with a 4.6-fold decrease compared to 0 h (p-value<0.05, Dunnett's multiple comparison). At 24 h post treatment, 3.7-fold reduction was detected (p-value<0.05, Dunnett's multiple comparison) while at 48h from treatment, a 2-fold reduction in expression level was detected, however it was not significantly different from 0 h (p-value<0.05, Dunnett's multiple comparison). For another susceptible population tested (ME), a similar pattern of transcript level reduction was observed, though the extent of level reduction was much higher. Significant reduction in V-ATPase A transcript level was observed starting at 24h post treatment until 72h when compared to 0 h (p-value<0.001, Dunnett's multiple comparison) (Fig. 3.1B). However, the highest reduction (16.4-fold), was detected after 24h post treatment (p-value<0.05, Dunnett's multiple comparison), which was not significantly different from 48h and 72h post treatment (11-fold and 11.8-fold reduction respectively, Dunnett's multiple comparison). This difference may be due to the distinct genetic background of the two strains.



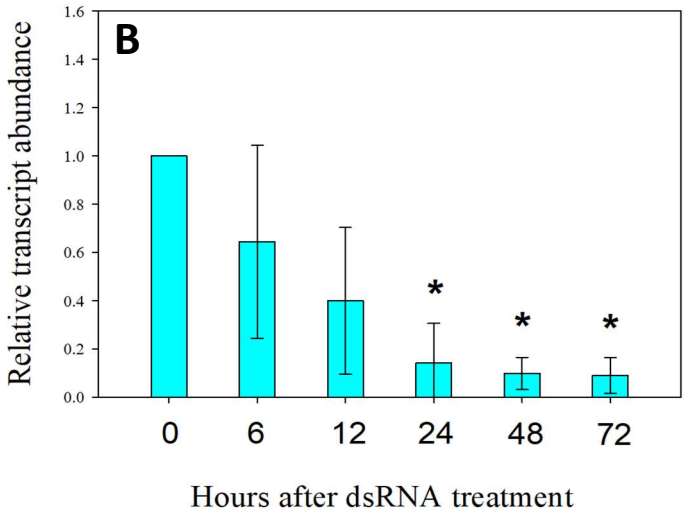


Figure 3.1- Change in V-ATPase A transcript level in the susceptible L. decemlineata population (A) GC, (B) ME and (C) resistant population CEAS after treatment with potato leaves containing dsRNA targeting the V-ATPase subunit A gene. Asterisks represents significant difference from time 0 (Dunnett's multiple comparison test, $p<0.05$).

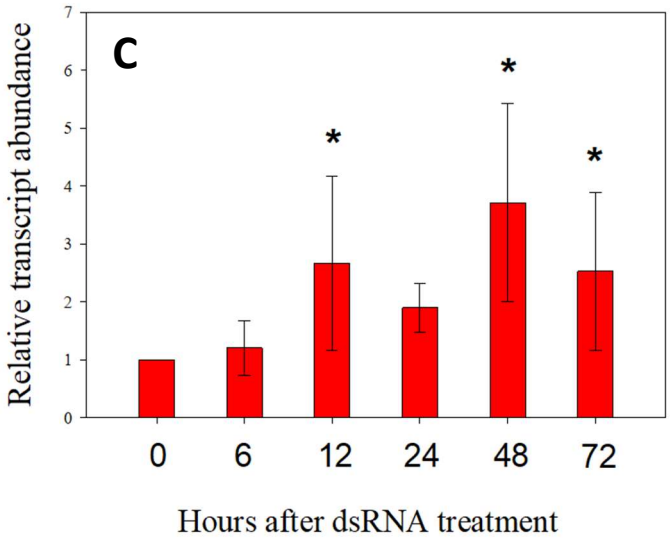


Figure 3.1 continued.

In contrast to results from susceptible strains, in samples from the resistant (CEAS) population, a significant increase in abundance of V-ATPase subunit A transcript was observed beginning at 12 h post treatment (p-value<0.001, Dunnett's multiple comparison) (Fig. 3.1C). The highest transcript level was recorded at 12 h and 48h post treatment, with a 3.1 and 3.2-fold increase respectively, when compared to 0 h (p-value<0.05, Dunnett's multiple comparison). A 2-fold increase was also detected at 24 h and 72 h post treatment (p-value<0.05, Dunnett's multiple comparison).

Up-regulation of genes in response to dsRNA and subsequent failure of RNAi has been reported before in mice, where application of a high concentration of siRNAs led to increased expression of RNAi inhibitors, ADAR-1 and ERI-1 (Sledz, Holko et al. 2003, Hirai, Terenius et al. 2004, Hong, Qian et al. 2005). Increasing levels of target mRNA after treatment with dsRNA were also reported in the nematode *Meloidogyne incognita* (Dalzell, Warnock et al. 2010, Chi, Wang et al. 2016) and in the oriental fruit fly *Bactrocera dorsalis* (Li, Zhang et al. 2011), though the mechanism behind this up-regulation is not yet understood. This up-regulation has been previously proposed as a potential mechanism explaining refractoriness of some gene targets to dsRNA (Bellés and Piulachs 2007). In fact, in *B. dorsalis* this up-regulation after dsRNA treatment appeared to be specific to one out of 4 target genes tested (Li, Zhang et al. 2011). Based on these reports, we tested whether the increased V-ATPase subunit A transcript levels observed in CEAS

was a general response or a specific response only observed after treatment with dsRNA targeting the V-ATPase subunit A gene. As shown in Fig. 3.2 no significant change (p-value<0.05, Dunnett's multiple comparison) in V-ATPase subunit A transcript levels was detected after treatment of CEAS larvae with dsRNA targeting Mon337. These results support that the up-regulation of V-ATPase subunit A in CEAS is a specialized response to treatment with dsRNA targeting this gene, and not a generalized phenomenon. This specialized response may be related to the different efficacy of RNAi based on the target gene, as shown before (Terenius, Papanicolaou et al. 2011). Another possibility is that given that the CEAS population was developed through selection with V-ATPase subunit A dsRNA, it may have developed multiple mechanisms against dsRNA. Thus, one general mechanism would confer resistance and cross-resistance to diverse dsRNAs, and another mechanism specifically counteracting silencing of V-ATPase subunit A. As up-regulation is observed only in response to dsRNA treatment, it suggests the presence of some type of feedback mechanism regulating the levels of V-ATPase subunit A transcript. An example of this type of feedback regulatory mechanism was reported for *Helicoverpa armigera*, where higher concentrations of dsRNA targeting juvenile hormone acid methyl transferase (*jhamt*) resulted in a reduced silencing effect due to the over-expression of an upstream gene in the juvenile hormone biosynthetic pathway (Asokan, Chandra et al. 2013).

Alternatively, it is possible that the V-ATPase subunit A is involved in an immune response.Sledz et. al. reported the activation of the interferon system and global up-regulation of interferon-stimulated genes in response to siRNAs (Sledz, Holko et al. 2003). Similarly, in Chinese oak silk moth, *Antheraea pernyi*, targeting of *Hemolin* gene using dsRNA resulted in its over-expression (Hirai, Terenius et al. 2004). The authors concluded that *Hemolin* is involved in immune response against viral infections, that led to its enhanced expression upon dsRNA treatment, as dsRNA is recognized as a virus-like molecule. It is possible that a similar immune response against virsus and dsRNAs, involving V-ATPase, is associated with the increased expression of V-ATPase subunit A in CEAS. If this specific up-regulation is involved in resistance, one would expect lower levels of resistance to alternate dsRNAs, like Mon337, compared to the >5,000-fold resistance observed in CEAS against V-ATPase subunit A dsRNA. Performing comparative bioassays with dsRNA against Mon337 and V-ATPase subunit A would help test this hypothesis.

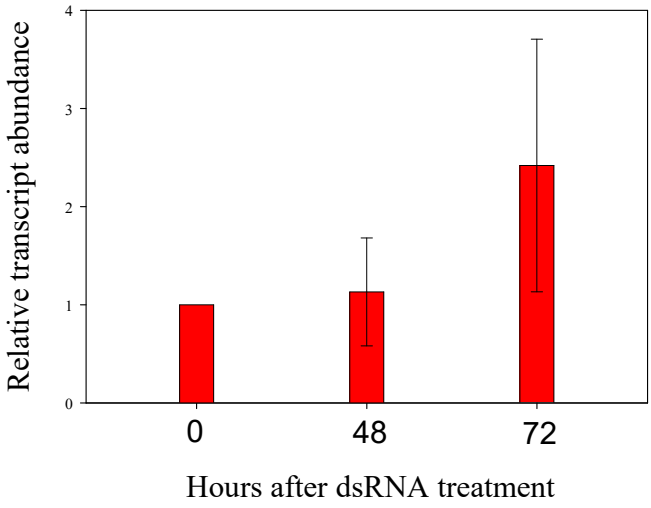


Figure 3.2- Change in V-ATPase A transcript level in *L. decemlineata* resistant population CEAS, after treatment with Mon337 dsRNA. No significant difference was observed at 48 hours or 72 hours when compared to time 0 (Dunnett's multiple comparison test, $p<0.05$).

Results from experiments using dsRNA delivery via potato leaf feeding were in agreement with drop feeding exposure (Fig. 3.3). A 3-fold reduction in V-ATPase subunit A transcript levels was observed in the GC population when compared with untreated samples (p-value< 0.001, t-test). Conversely, no significant difference in the transcript level was observed between treated and untreated samples in CEAS larvae (p-value<0.05, t-test).

Taken together, these results provide molecular evidence for lack of gene silencing associated with resistance to V-ATPase subunit A dsRNA in the CEAS population. In contrast up-regulation of the target genes was observed in CEAS samples. Multiple studies have reported up-regulation of target gene transcripts in response to treatment with dsRNA. However, the fact that up-regulation of V-ATPase subunit A transcripts after dsRNA treatment is observed only in the CEAS population and not in GC and ME, suggests its potential involvement in resistance to insecticidal dsRNA.

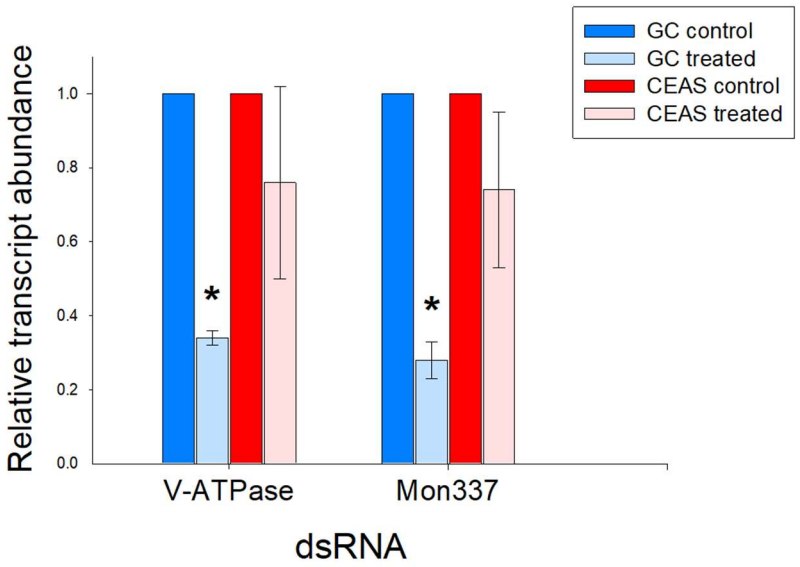


Figure 3.3- Change in transcript levels in larvae from *L. decemlineata* populations GC (susceptible) and CEAS (resistant) after a drop feeding treatment with V-ATPase subunit A and Mon337 dsRNAs. Asterisks represents significant difference from untreated samples (t-test, $p<0.05$).

Cross-resistance to alternative dsRNA targets in CEAS

To determine if the CEAS population is cross-resistant to other dsRNA targets, we measured transcript levels after treatment with an alternative dsRNA target (Mon337). In the samples treated by the drop feeding method, a significant 1.6-fold reduction (p-value<0.05, t-test) in transcript abundance for the gene targeted by the Mon337 dsRNA was observed in samples from the GC population after 48 h (Fig. 3.4A). However, Mon337 transcript levels at 72h were not significantly different from 0 h (p-value<0.05, t-test).

In the ME population a 7.4-fold reduction in Mon337 transcript levels was observed after 48 h when compared with initial levels (p-value<0.001, t-test) (Fig. 3.4B). The reduction in Mon337 transcript levels in the susceptible populations supports that Mon337 dsRNA can induce silencing and hence was used to test for cross-resistance in CEAS. No significant differences in Mon337 transcript levels were observed in samples from CEAS at 48 h or 72 h post treatment (p-value<0.05, t-test) (Fig. 3.4C).

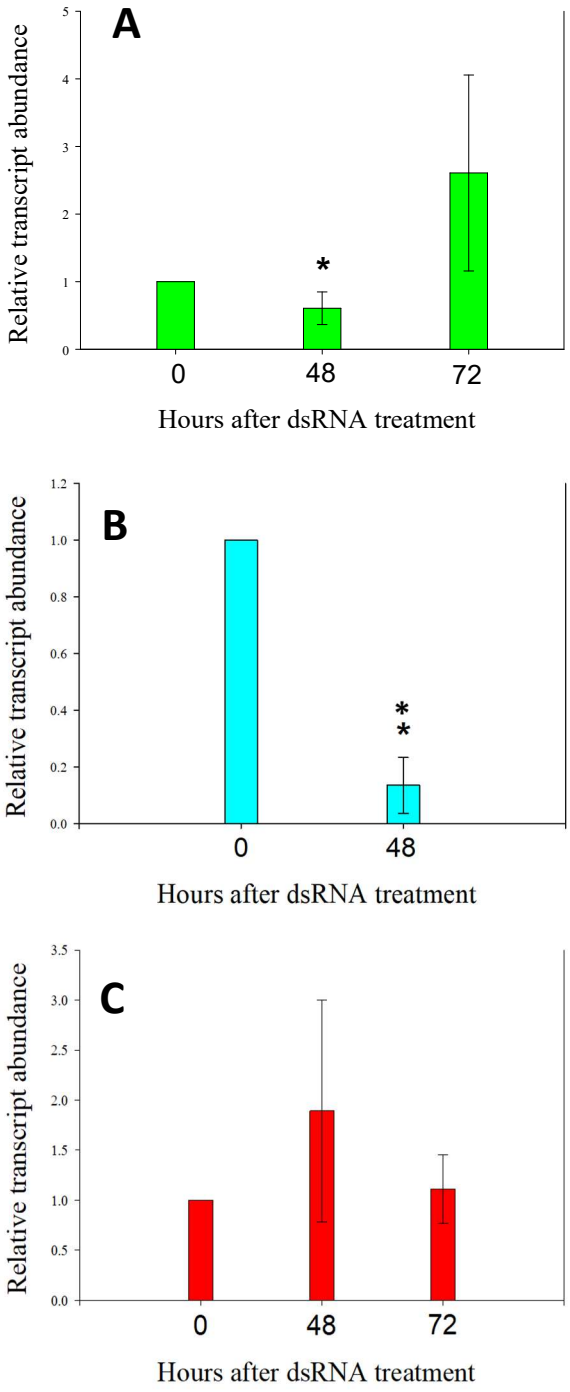


Figure 3.4- Change in Mon 337 transcript levels in larvae from *L. decemlineata* populations (A) GC (susceptible), (B) ME (susceptible) and (C) CEAS (resistant) after treatment with Mon337 dsRNA. * represents significant difference from time zero ((A and B) Dunnett's multiple comparison and (C) t-test). One asterisk $p<0.05$, two asterisks $p<0.01$.

Similar to the tests with drop feeding, in case of dsRNA treatment via leaf feeding the GC population displayed a 3.5-fold reduction in Mon337 transcript levels in comparison to untreated samples (p-value<0.05, t-test) (Fig. 3.3). In contrast, no significant differences in Mon337 transcript levels were detected between the treated and untreated CEAS samples (p-value<0.05, t-test) (Fig. 3.3).

Cross-resistance to alternative targets supports that resistance in CEAS is not sequence specific and that target site mutations are not involved in the resistance mechanism (Auer and Frederick 2009). This is in accordance with the findings of Khajuria et. al. (Khajuria, Ivashuta et al. 2018) and previous statements proposing limited possibility of resistance development through target site mutations (Bolognesi, Ramaseshadri et al. 2012). These results also suggest that the fate of all dsRNA molecules would be similar in an insect resistant to dsRNA, irrespective of the gene they target, calling for careful assessment of resistance risks (Spit, Philips et al. 2017).

Reduced susceptibility to Cry3Aa protoxin in CEAS

To assess if dsRNA-resistant CEAS larvae are also cross-resistant to a Bt Cry toxin active against *L. decemlineata*, we performed bioassays comparing susceptibility to the Cry3Aa protoxin in larvae from the GC and CEAS populations. An LC_{50}, defined as the Cry3Aa protoxin concentration resulting in 50% of the adults not emerging when treated as larvae, was calculated for each population. The LC_{50} for GC was 4.5 µg/ml, while the LC_{50} for CEAS was 14.13 µg/ml, indicating a >3-fold significant (based on non-overlapping 95% confidence intervals) cross-resistance in CEAS (Table 3.1).

Table 3.1- Comparison of LC_{50} for GC and CEAS from Cry3Aa bioassay.

Population	LC_{50}	95% Confidence Interval	Slope
GC (Susceptible)	4.519 µg/ml	1.99 – 9.03 µg/ml	1.31 ± 0.19
CEAS (Resistant)	14.129 µg/ml	9.59 – 21.83 µg/ml	1.45 ± 0.27

In the work done by Khajuria et. al., two high doses of Cry3Bb1 toxin (117 µg/cm^2 and 235 µg/cm^2) were used to test for cross-resistance in *D. v. virgifera* dsRNA resistant population (Khajuria, Ivashuta et al. 2018). They observed 100% mortality in both susceptible and resistant population for both the concentration. It is possible that no significant difference would be observed in mortality between GC and CEAS if a high dose of Cry3Aa is used. However, results from bioassay using lower concentrations of Cry3Aa toxin suggest that under continuous treatment with Cry3Aa (especially at non "high dose" levels), a greater difference in susceptibility to Cry3Aa might be observed between GC and CEAS, with GC being more susceptible.

Alternatively, the >3-fold difference in Cry3Aa susceptibility between GC and CEAS can be due to the generally increased vigor of a population selected continuously for every generation (CEAS) and a population which is not selected (GC). As suggested by Moar et. al. it is important to consider and compare these attributes, in addition to mortality, when evaluating resistance and cross-resistance between different populations (Moar, Khajuria et al. 2017).

Conclusions

The observed results provide insights into the mechanism of resistance to topically applied V-ATPase subunit A dsRNA in *L. decemlineata*. Comparing these results with a similar study on *D. v. virgifera* selected using a different dsRNA target and method of dsRNA delivery (Khajuria, Ivashuta et al. 2018), allows for a more comprehensive understanding of resistance to insecticidal RNAi technology. Here we provide molecular evidence for the absence of gene silencing in CEAS, in agreement with its resistance to insecticidal dsRNA. We also conclude that resistance in CEAS is not specific to the sequence of dsRNA, supporting that target site mutation is not the mechanism of resistance to dsRNA in CEAS. This calls for careful risk assessment while developing dsRNA based insecticidal products, as resistance developed against one dsRNA target may confer cross-resistance to any other targets. One surprising result from this study is the up-regulation of V-ATPase subunit A transcript observed in CEAS in response to treatment with dsRNA targeting the V-ATPase subunit A gene. This up-regulation is specific to this treatment and may represent an additional, more specific, mechanism contributing to resistance in CEAS, which needs to be further explored. Additionally, the CEAS population demonstrates >3-fold decrease in susceptibility to the Cry3Aa protoxin from Bt. This observation poses a concern on the prospective combined use of Bt and dsRNA technology for control of this pest, as insects maybe quick to

develop resistance against both technologies when put under selection pressure, especially if low, non-lethal dose of Bt toxin is used. However, this difference can also be due the general increased robustness of a constantly selected population and a non-selected population. This makes it important to consider different attributes and not only mortality when evaluating cross-resistance to different toxins. Taken together, these results provide useful insights into the development of resistance to insecticidal dsRNA and will help compose insect resistance management (IRM) strategies to ensure effectiveness and durability of insecticidal RNAi technology.

Chapter Four

Reduced uptake of dsRNA into target cells as a mechanism of resistance to insecticidal dsRNA in *Leptinotarsa decemlineata*

Abstract

In the last decade, silencing of vital genes by RNA-interference (RNAi) through dsRNA ingestion has been identified as a revolutionary bioinsecticide technology. As commercialization of insecticidal dsRNA technology approaches, it becomes crucial to develop resistance management tools for the sustainability of this technology. Here, we share findings from research focused on investigating if reduced uptake of dsRNA inside target cells is associated with resistance in dsRNA resistant *L. decemlineata* population. Monitoring uptake of fluorescently labeled dsRNA by midgut cells supports reduced uptake of dsRNA in midgut cells of resistant population compared to susceptible larvae. This is partly supported by results from small RNA (sRNA) sequencing, which also suggests the existence of an additional mechanism of resistance involving up-regulation of the target gene. The results suggest that insect resistance against dsRNAs can be complicated and may involve more than one mechanism. Thorough understanding of these mechanisms is thus important to compose effective resistance management strategies.

Introduction

Gene silencing induced by the presence of double-stranded RNA (dsRNA) complimentary to a target gene, termed RNA-interference (RNAi), is a highly conserved mechanism among eukaryotic organisms and can be used to specifically inhibit the expression of virtually any gene. Owing to its ubiquitous nature and high sequence specificity, RNAi has allowed the study of gene function in different model organisms and the development of gene based therapeutic tools (Lu, Xie et al. 2005, Liao and Tang 2016). More recently, RNAi has found applications in agriculture where it can be employed as a potent tool for control of harmful pests, resistance management and protection of beneficial insects (Zotti, dos Santos et al. 2018).

The first experimental evidence for the use of RNAi in insect control was provided in 2007 in a study targeting essential genes to control *Diabrotica virgifera virgifera* (Western corn rootworm) through the expression of dsRNA in transgenic plants (Baum, Bogaert et al. 2007). Since then, several researchers have explored the potential of RNAi as insecticidal technology against different pests using diverse dsRNA delivery methods, including transgenic plants expressing dsRNA, dsRNA foliar sprays, root drench and trunk injections (Hunter, Glick et al. 2012, Li, Guan et al. 2015, San Miguel and Scott 2016). The sequence-specific targeting of genes makes insecticidal RNAi a relatively safe approach with limited off-target effects (Bachman,

Bolognesi et al. 2013) and provides a new mode of action, allowing pyramiding with other insecticidal technologies. Thus, the first plant incorporated protectant (PIP) based on RNAi approved for commercialization by the US Environmental Protection Agency (EPA), is a pyramided transgenic maize event expressing three insecticidal crystal (Cry) genes from *Bacillus thuringiensis* and a dsRNA targeting the *D. v. virgifera* sucrose non-fermenting 7 (*DvSnf7*) gene (Head, Carroll et al. 2017).

As with every insect control strategy, it is important to consider evolution of insect resistance to insecticidal RNAi technology. Considering that the RNAi mode of action includes multiple steps, theoretically, alterations in any of them could lead to development of resistance. The RNAi mechanism commences with delivery of dsRNA and its subsequent uptake into the target cells. Upon uptake, the dsRNA is cleaved into small interfering RNAs (siRNAs) by the action of a multidomain ribonuclease (RNase) type III enzyme called Dicer. These siRNAs are loaded into an enzymatic complex termed the RNA induced silencing complex (RISC). Since the siRNAs are double stranded, they are unwound during RISC formation to form a guide strand, complementary to the target mRNA, and a passenger strand which gets degraded. The guide strand then directs the RISC complex to its complimentary mRNA, where it binds Argonaute, an enzyme containing an RNAase H-like domain, which degrades the complementary mRNA thus inhibiting gene expression.

The importance of successful uptake and processing of dsRNA into the target cells for successful insecticidal RNAi has recently been emphasized by the observations made in a dsRNA-resistant *D. v. virgifera* population (Khajuria, Ivashuta et al. 2018). This population displayed impaired dsRNA entry into the midgut cells, when compared to susceptible insects, as the potential mechanism of resistance. These results have tremendous significance as they provide the first insight into resistance mechanisms against insecticidal dsRNAs, yet studies on different insects and using different dsRNA selection procedures are required to gain a more thorough understanding of resistance to insecticidal RNAi. This information is also needed for development of effective Insect Resistance Management (IRM) strategies and optimization of this new insecticidal technology.

This Thesis is aimed at understanding how a coleopteran pest (*Leptinotarsa decemlineata*) would develop resistance to non-transformative RNAi. This type of RNAi involves the use of dsRNA sprays, root drench or trunk injections, while transformative RNAi comprises transgenic

plants expressing dsRNA. Our group selected a strain of *L. decemlineata* via feeding on dsRNA targeting the V-ATPase subunit A gene, resulting in >5,000-fold resistance when compared to the parental strain (Dee *et al*, in preparation). We named this strain CEAS for chronically exposed adult survivors. In previous chapters, we presented evidence suggesting that nucleases and target gene alterations were not involved in resistance, as reported for the *D. v. virgifera* resistant population (Khajuria, Ivashuta et al. 2018). The objective of the current chapter was to test if the reported reduced or impaired uptake of dsRNA inside the gut cells for *D. v. virgifera* was associated with resistance to insecticidal RNAi in the CEAS population. To test this hypothesis, we monitored dsRNA uptake using fluorescence microscopy and determined processing of dsRNA in the gut cells by sequencing small RNAs (sRNAs) and siRNAs generated. Uptake of the fluorescently labeled dsRNA by midgut cells in CEAS larvae appeared reduced compared to uptake in susceptible larvae. Results from sequencing of siRNAs support reduced (but not eliminated) uptake of dsRNA into gut cells of CEAS larvae when compared to susceptible individuals, although high variability was detected among tested samples.

Materials and Methods

Insects

Susceptible (GC) and resistant (CEAS) Colorado potato beetle adult populations were kept in a greenhouse bay at 25ºC and 85% relative humidity. The CEAS colony has been kept under continuous selection during the larval stage with 400 µg/ml of dsRNA targeting the V-ATPase A subunit gene of *L. decemlineata*. This was achieved by collecting eggs from the greenhouse and feeding dsRNA treated leaves as the neonates emerge. Leaves were first dipped in a 400 µg/ml dsRNA solution made with 0.1% Tween-20. Treated leaves were dried completely before feeding to the larvae. The larvae were fed freshly treated leaves everyday till fourth instar. Late fourth instar larvae ware transferred to soil for pupation, from which they emerged as adults. The adults were then reared on untreated potato plants in the greenhouse. These plants were changed every 2-3 days as needed. Eggs of similar age were collected to perform various experiments, and unused eggs were removed from potato plants and frozen to prevent competition between larval and adult populations.

V-ATPase subunit A dsRNA

The dsRNA targeting the V-ATPase subunit A gene of *L. decemlineata* used in this study was provided by Monsanto company (now Bayer). This dsRNA is a 302 bp fragment corresponding to the 270-572 bp region in the V-ATPase subunit A transcript of *L. decemlineata* (2,464 bp, accession number XM_023156517.1).

Histological gut sections to monitor uptake of dsRNA

Labeling of dsRNA targeting the V-ATPase subunit A gene with the Cy3 dye was conducted using the Silencer™ siRNA labeling kit (Ambion), following manufacturer's recommendations. Labeling was confirmed by detecting fluorescence (~550 nm excitation and ~570 nm emission) of the labeled dsRNA resolved in an agarose gel under a GE Typhoon Trio imager (Fig. 4.1).

Larvae from susceptible (GC) and resistant (CEAS) *L. decemlineata* populations were reared on untreated potato leaves until treatment with dsRNA. Early 4th instar larvae were starved for 24 h, and then fed a 5 µl droplet containing 500 ng of Cy3-labeled or un-labelled dsRNA targeting the V-ATPase subunit A. Larvae were closely monitored until they completely consumed the droplet to ensure ingestion. Guts were dissected 30 mins or 1-hour post-feeding and fixed in Carnoy's solution (60% ethanol, 30% chloroform, 10% acetic acid).

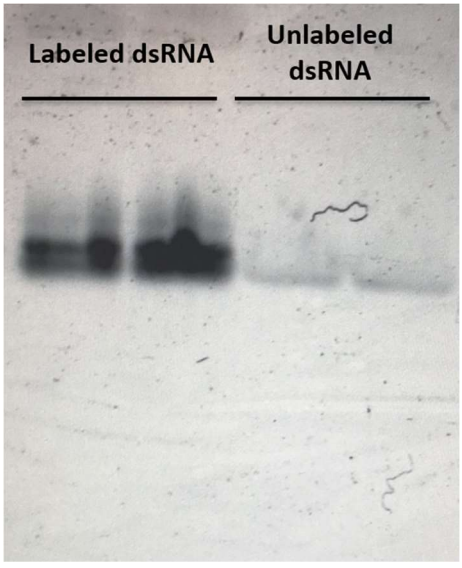


Figure 4.1- Detection of fluorescently labeled dsRNA in an agarose gel in a fluorescence imager.

Fixed tissues were then sent for sectioning to the Histology Department at the University of Tennessee College of Veterinary Medicine (Knoxville, TN). Prepared sections were processed for rehydration (10 min in xylol, 5 min each in 100% ethanol, 70% ethanol and water) to allow staining with DAPI, a water-based nuclear stain. Sections were incubated with DAPI (Molecular probes) for 5 minutes and then washed 3 times with water to remove excess stain. These were then mounted on slides using Clear-Mount with TRIS buffer (Fisher). Sections were observed under a confocal microscope (Leica SP8 White Light Laser Confocal System) at the Advanced Microscopy and Imaging Center (University of Tennessee, Knoxville).

Sample preparation for sRNA sequencing

Larvae from susceptible (GC) and resistant (CEAS) *L. decemlineata* populations were reared on untreated potato leaves until treatment with dsRNA. Potato leaves were dipped in a 400µg/ml V-ATPase A dsRNA solution prepared in 0.1% Tween-20. Leaves were air-dried completely before feeding to the larvae. Third instar larvae were fed the prepared potato leaves for 48 hours, and then their guts were dissected, snap frozen in liquid nitrogen and stored at -80ºC until further use. For each treatment, there were 3 biological replicates and each biological replicate consisted of a pool of 3 guts. Dissected gut samples were sent to the Genomics Core Facility at the University of Texas in San Antonio for further processing and sRNA sequencing.

sRNA sequence analysis

sRNA reads were first trimmed and adapters removed using Cutadapt (Martin 2011). The trimmed files were then analyzed to ensure good quality reads using FastQC (Andrews 2014). These files were filtered to select reads of 21 bp length, based on the size of siRNAs produced typically during RNAi (Zotti, dos Santos et al. 2018). Mapping and visualization of reads was done using CLC Genomics Workbench version 12 (QIAGEN). Reads were either mapped to the V-ATPase subunit A dsRNA fragment used for treatment or to the full-length *L. decemlineata* V-ATPase subunit A transcript (accession number XM_023156517). The percentage of mapped reads was calculated for each sample by dividing the number of mapped reads by the total number of reads in the sample.

Results and Discussion

Reduced uptake of Cy3-labeled dsRNA in midgut cells of CEAS larvae

The Cy-3 labeled dsRNA, indicated by a red signal, was observed accumulating around the gut cells, identifiable by blue signal from cell nuclei due to DAPI staining, in midgut sections from susceptible (GC) larvae 30 mins after ingestion (Fig. 4.2A). Thirty minutes later (1 h post treatment), most of the Cy3-labeled dsRNA signal was localized in the midgut epithelium (Fig. 4.2A). These results support that midgut cells in GC larvae uptake dsRNA, which is in agreement with the potent RNAi response resulting in mortality observed in these larvae (detailed in Chapter Three). In contrast, in gut sections from the resistant (CEAS) population, the Cy3-labeled dsRNA was observed collecting along the sides of the gut lumen after 30 min or 1 h post treatment intervals (Fig. 4.2B).

These observations suggest that CEAS midgut cells may not be able to uptake dsRNA efficiently, which could suggest a potential resistance mechanism. Even more drastic observations were reported for dsRNA-resistant *D. v. virgifera* dsRNA (Khajuria, Ivashuta et al. 2018). In susceptible *D. v. virgifera* cells, localization of the Cy3-labeled dsRNA was observed inside the midgut cells while no localization was observed inside resistant midgut cells, suggesting association of impaired uptake of dsRNA with resistance. In the oriental fruit fly (*Bactrocera dorsalis*), exposure to dsRNA targeting an endogenous gene was shown to induce refractoriness to a second exposure to the same dsRNA (Li, Dong et al. 2015). This lack of silencing during the second exposure was shown to be due to lack of dsRNA uptake. Further experimentation showed that disruption of endocytic pathways involved in the uptake of dsRNA contributed to this refractoriness.

In *L. decemlineata,* dsRNA uptake in midgut cells is mediated by both clathrin-dependent endocytosis and two SID-1-like transmembrane proteins, *SIL-A* and *SIL-C* (Cappelle, De Oliveira et al. 2016). Independent silencing of the genes involved in these two pathways was shown to partially block RNAi in Lepd-SL1cells (Yoon, Shukla et al. 2016). The importance of successful dsRNA uptake in RNAi is also supported by successful dsRNA uptake and increased RNAi efficiency in BmN4 *Bombyx* cells expressing a SID-1 protein from *C. elegans* (Kobayashi, Tsukioka et al. 2012). These observations grant testing expression and alterations in genes involved in dsRNA uptake pathways in *L. decemlineata* in larvae from the GC and CEAS populations.

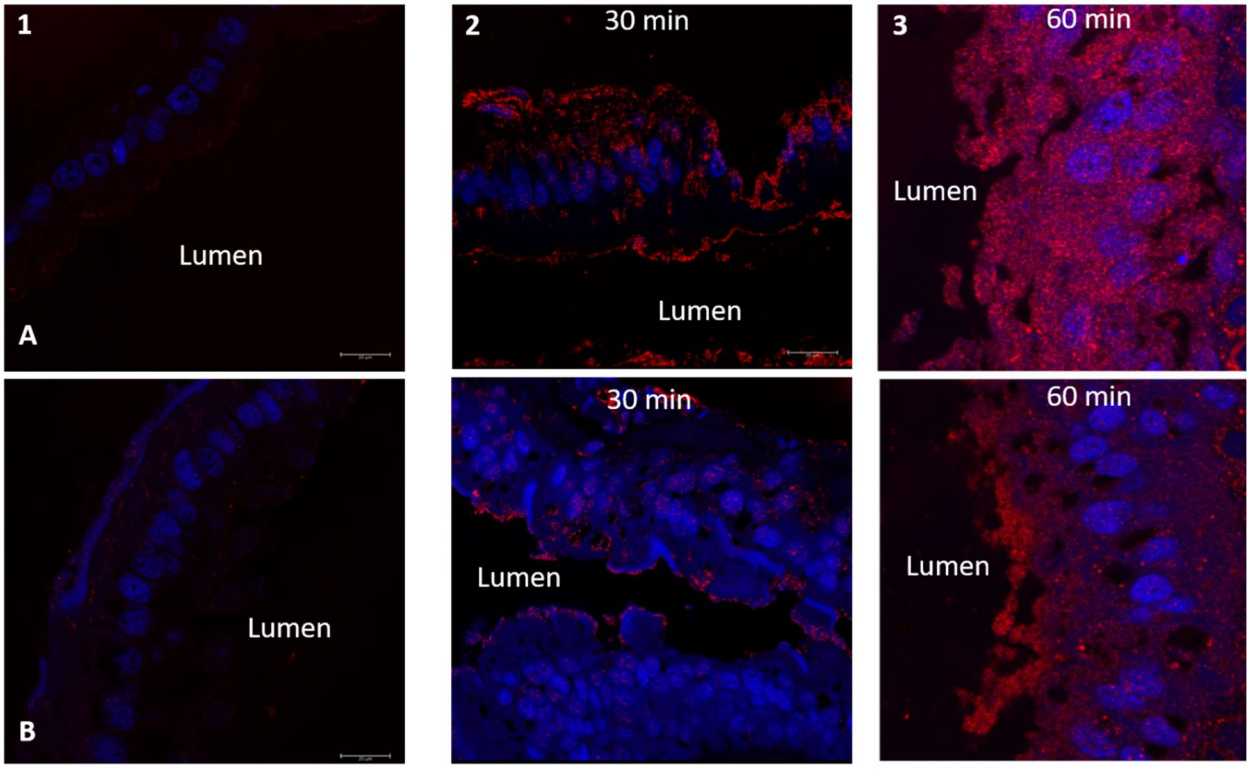


Figure 4.2- Histological sections observed under confocal microscopy to detect Cy3-labeeld dsRNA in gut sections of *L. decemlineata* larvae. Upper row (A): gut sections from the susceptible (GC) population. Lower row (B): gut sections from the dsRNA-resistant population CEAS. (1) Control gut sections from non-treated larvae. (2) Gut sections from larvae treated with Cy3-labeled dsRNA and dissected 30 mins after treatment. (3) Gut sections from larvae treated with Cy3-labeled dsRNA and dissected 1 hour after treatment.

Abundance of sRNAs mapping to the V-ATPase A dsRNA fragment

In further testing to determine if reduced dsRNA uptake was associated with resistance in the CEAS strain, we sequenced small RNAs from the guts of control and dsRNA-treated larvae from both the GC and CEAS strains. In the process of RNAi, dsRNA is processed intracellularly into siRNAs by the enzyme Dicer. Hence, we hypothesized that in untreated larvae we would only detect siRNAs from endogenous RNAi regulatory pathways. As expected, the percentage of reads mapping to the V-ATPase subunit A full length cDNA was lowest in samples from larvae not treated with the dsRNA, independently of the strain (Table 4.1). Unexpectedly, we detected that except for a negligible number of reads, the vast majority of reads in these untreated larvae matched to the transcript region targeted by the dsRNA (Fig. 4.3).

It is possible that the 302 bp region in the V-ATPase subunit A transcript targeted by the V-ATPase subunit A dsRNA is the region involved in endogenous regulation of V-ATPase subunit A expression. It has been reported that endogenous small non-coding RNAs, 20-30 bp long, are involved in the regulation of genes in eukaryotes and are known to function through a process similar to RNAi (Carthew and Sontheimer 2009). This would explain the presence of a smaller number of sRNAs in untreated (compared to treated samples) mapping to the V-ATPase subunit A transcript, particularly in the region targeted by the dsRNA. It is possible that targeting this region in the transcript using an exogenous dsRNA results in an enhanced silencing effect compared to alternative regions in the transcript.

Considering the reduced uptake of dsRNA in CEAS larvae suggested by the fluorescence microscopy data, we hypothesized that after treatment with dsRNA we would detect higher number of siRNAs corresponding to the V-ATPase subunit A dsRNA in GC compared to CEAS larvae. In all the samples, significant variation was observed among the replicates for the same treatment (Table 4.1). Because of this, averages were not used to compare GC and CEAS. This high variability may be explained by the difference in the genetic make-up of individuals in each strain. For example, sample CEAS Treated 1 had a high relative percentage of 21 bp long siRNAs mapping to V-ATPase subunit A dsRNA (5.07%) while sample CEAS Treated 3 only had 0.2%. If the inheritance of resistance is recessive, a heterozygous individual in the CEAS population would still be susceptible and hence accumulate more siRNAs after treatment with dsRNA. A similar observation was reported by Khajuria et. al. (Khajuria, Ivashuta et al. 2018), where the

Table 4.1- Relative percentage from the total number of sRNA reads mapping to the dsRNA sequence or the full-length V-ATPase subunit A transcript.

Sample	% of total reads mapped to V-ATPase A dsRNA	% of 21 bp reads mapped to V-ATPase A dsRNA	% of 21 bp reads mapped to full length V-ATPase A transcript
GC Untreated 1	0.12	7.36	7.19
GC Untreated 2	0.01	1.26	1.24
GC Untreated 3	0.004	0.03	0.03
GC Treated 1	3.45	10.71	10.38
GC Treated 2	1.68	4.73	4.54
GC Treated 3	1.04	4.19	4.06
CEAS Untreated 1	0.01	0.03	0.03
CEAS Untreated 2	0.003	0.007	0.007
CEAS Untreated 3	0.005	0.013	0.016
CEAS Treated 1	2.52	5.07	4.78
CEAS Treated 2	0.39	1.01	0.95
CEAS Treated 3	0.10	0.24	0.22

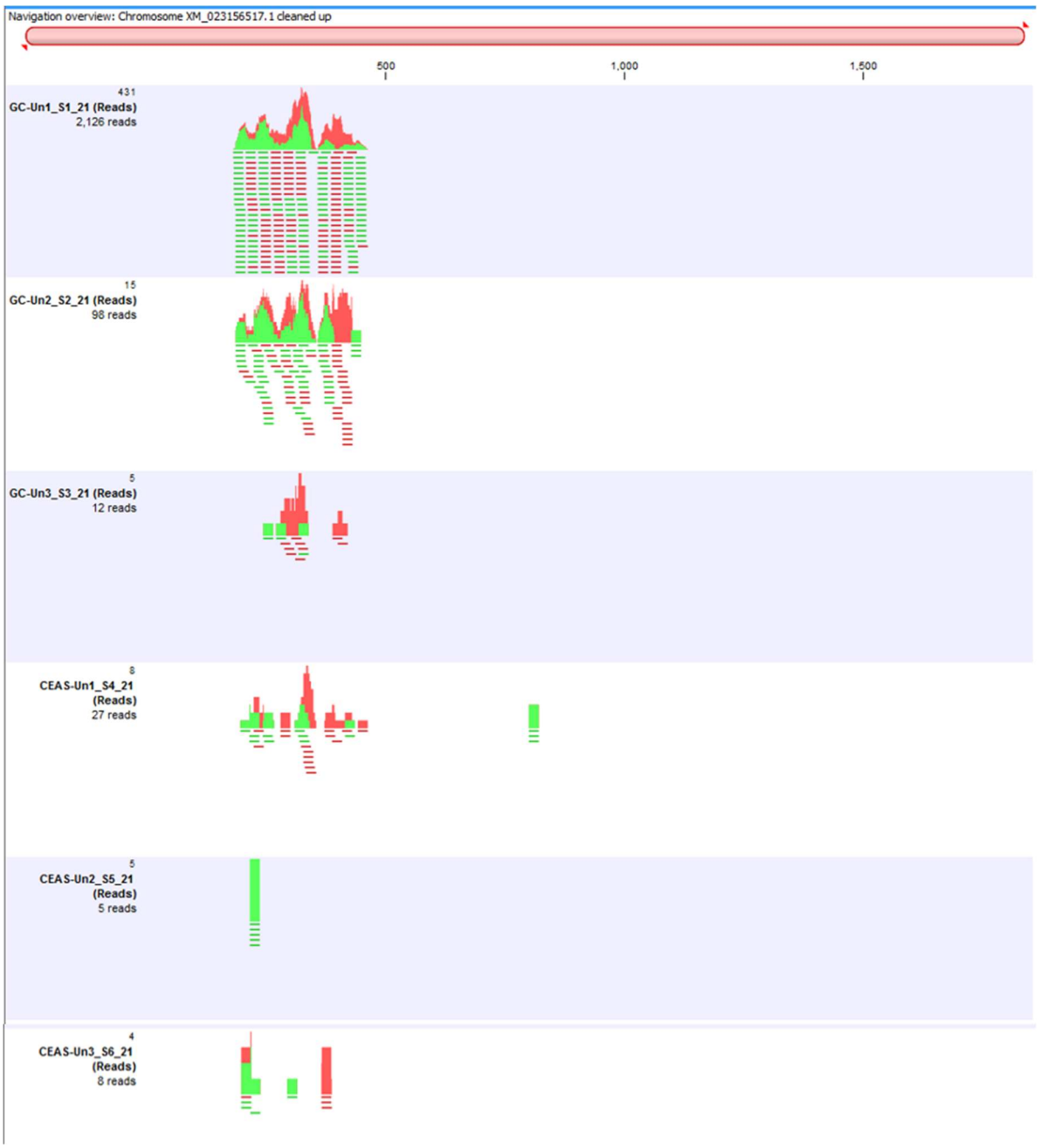


Figure 4.3- Mapping of 21 bp sRNA reads from the three untreated GC and CEAS samples to the complete V-ATPase subunit A transcript.

inheritance of resistance was reported to be recessive and some of some of the larvae in the *D. v. virgifera* dsRNA resistant population did display a susceptible phenotype (reduction in DvSnf7 transcript levels after treatment with dsRNA). In that study they resolved this issue by quantifying target transcripts and detected accumulation of siRNAs corresponding to the DvSnf7 dsRNA in the individual from the resistant colony that displayed a susceptible phenotype, while complete absence of DvSnf7 siRNAs was observed in the other samples from the resistant population. A similar mechanism may explain the high percentage of mapped siRNAs found in the CEAS Treated 1 sample.

To test if the same phenomenon was affecting our analysis, we evaluated V-ATPase subunit A transcript levels in the treated samples used for sRNA sequencing. In the results from quantitative PCR assays (Table 4.2), we detected a relationship between the relative percentage of mapped siRNA reads and V-ATPase subunit A transcript levels, although this relationship was opposite when comparing GC and CEAS. Thus, in the GC samples we observed that a higher relative percentage of siRNA reads mapped to the V-ATPase subunit A transcript was related to reduced transcript levels, suggestive of successful silencing. In contrast, for CEAS samples we detected an inverse relationship between the relative percentage of siRNA reads and transcript levels.

One possible explanation for these observations is the involvement of two mechanisms in resistance to V-ATPase subunit A dsRNA in CEAS, as speculated in Chapter Three. It is possible that the uptake of dsRNA is reduced in some CEAS individuals (as detected for CEAS Treated 2 and 3 samples when compared to GC Treated samples). In addition, for some CEAS individuals in which uptake is not affected (as for CEAS Treated 1 sample), an alternative mechanism involving up-regulation of V-ATPase subunit A transcript levels is activated. This hypothesis is supported by the high V-ATPase subunit A transcript levels detected in the CEAS Treated 1 sample, even though 5.07% of siRNAs mapped to the V-ATPase subunit A dsRNA in that sample. An alternative possibility is that the V-ATPase subunit A up-regulation may reflect the activation of and enhanced immune response in CEAS (as discussed in Chapter Three).

Nonetheless, the opposite trend between percentage of mapped reads and change in V-ATPase subunit A transcript level between GC and CEAS indicate differences in the RNAi response to treatment with dsRNA. Based on the confocal microscopy pictures, we speculate that

Table 4.2- Relative percentage from the total of 21 bp long sRNA reads mapping to the V-ATPase subunit A dsRNA and corresponding V-ATPase subunit A transcript levels in treated samples, relative to untreated samples (assigned transcript level of 1).

Sample	% of 21 bp long sRNAs mapped to V-ATPase A dsRNA	V-ATPase subunit A transcript levels
GC Treated 1	10.71	0.56
GC Treated 2	4.73	0.55
GC Treated 3	4.19	1.48
CEAS Treated 1	5.07	1.27
CEAS Treated 2	1.02	0.43
CEAS Treated 3	0.24	0.70

reduced uptake of dsRNA into the CEAS midgut cells might be playing a role in resistance to insecticidal dsRNA. However, analysis of sRNA sequencing data supports a second mechanism resulting in up-regulation of the targeted gene may be at play in resistance as well. Further experimentation is required to test this hypothesis of two resistance mechanisms in CEAS. One experiment would be to perform sRNA sequencing after treatment with Mon337 dsRNA, as no upregulation of Mon337 transcript was observed in this case for CEAS.

Conclusions

Development and commercialization of RNAi based insecticidal products necessitates careful assessment of risks related to evolution of resistance against this technology. A recent study demonstrated impaired uptake of dsRNA into the midgut cells of dsRNA-resistant *D. v. virgifera* population (Khajuria, Ivashuta et al. 2018). That study provided the first evidence for development and mechanisms of resistance to dsRNA in an insect. Consequently, we tested the role of reduced uptake of dsRNA into the midgut cells of a dsRNA-resistant *L. decemlineata* population (CEAS), and its role in resistance. Results from monitoring the uptake of fluorescently labeled dsRNA using

confocal microscopy supports reduced uptake of dsRNA in midgut cells of CEAS compared to susceptible larvae GC. This is in part supported by results from sRNA sequencing, which also suggest the existence of a potential second mechanism involving up-regulation of the target gene. Further experiments are required to make a compelling statement regarding the resistance mechanism in CEAS.

Chapter Five
Conclusions

Suppression of vital genes via RNA-interference (RNAi) provides a novel mode of action for insecticides. Delivery of dsRNA targeting essential genes can induce RNAi in insects and result in inhibition of growth, abnormalities during development process or mortality. Though the efficacy of RNAi response varies within different insect orders, researchers globally are actively working on developing dsRNA products to control insects belonging to different orders, in the form of transgenics that produce dsRNA (transformative RNAi) or dsRNA sprays (non-transformative RNAi) for foliar application, root drench or trunk injections. The first commercial product based on RNAi technology, a maize event expressing three insecticidal crystal (Cry) genes from *Bacillus thuringiensis* and a novel RNAi-based trait, DvSnf7, has already been approved for commercialization by the US Environmental Protection Agency (Head, Carroll et al. 2017). With the presence of the DvSnf7 trait, the plant expresses dsRNA that is complimentary to a 240 bp segment of *D. v. virgifera* sucrose-non-fermenting (*DvSnf7*) gene. When an insect feeds on such a dsRNA producing plant, it's RNAi machinery recognizes that dsRNA and induces a RNAi response resulting in down regulation of *DvSnf7* gene, and eventually insect mortality. Similar dsRNA based insecticidal products are expected to reach market in the coming years. With the commercialization of these products, it becomes important to address the issue of resistance development, since insects are expected to develop resistance against insecticidal RNAi technology, just like every other novel insect control strategy. However, there is a dearth of knowledge regarding resistance mechanisms that insects may develop against the RNAi technology.

Several potential mechanisms of resistance based on the RNAi pathway have been proposed. These include increased nuclease activity in insect gut leading to reduced stability of dsRNA, reduced or impaired uptake of dsRNA into target cells, mutation(s) in RNAi machinery genes or mutation(s) in the target gene (Baum and Roberts 2014). A recent study demonstrated impaired uptake of dsRNA into the midgut cells of a dsRNA-resistant *D. v. virgifera* population (Khajuria, Ivashuta et al. 2018) and provided the first evidence for development and mechanism of resistance to dsRNA in an insect.

Through this thesis, we provide insights into the mechanism of dsRNA resistance in another coleopteran insect, *L. decemlineata* (Colorado potato beetle), when selected through non-transformative RNAi delivery using V-ATPase subunit A dsRNA. The selection resulted in the development of a resistant population, named CEAS, that demonstrates >5,000-fold resistance

against V-ATPase subunit A dsRNA when compared to the susceptible population GC (Dee et. al., in preparation). Comparison of dsRNA stability in digestive fluids from susceptible (GC) and resistant (CEAS) populations support that degradation of dsRNA by nucleases is not involved in resistance. Lack of target gene silencing in the resistant population after treatment with an alternative dsRNA confirm that the resistance is not sequence-specific and target site mutations are not involved in the resistance mechanism, as confirmed by cross-resistance to diverse dsRNA targets in CEAS. This observation suggests limitations in the development and use of multiple dsRNA targets to delay resistance, since resistance against one dsRNA may confer resistance against multiple dsRNAs, irrespective of the target gene.

Surprisingly, we observed up-regulation of V-ATPase subunit A transcript in CEAS when treated with dsRNA targeting the V-ATPase subunit A gene. This up-regulation appears specific to this treatment and may represent a feedback mechanism contributing to resistance. Thus, we hypothesize the presence of multiple, at least two, mechanisms of resistance in CEAS. One general mechanism conferring resistance and cross-resistance to multiple dsRNAs and other, more specific mechanism, related to the up regulation of V-ATPase subunit A. We plan to test this hypothesis by establishing resistance levels in CEAS against an alternative dsRNA. If our hypothesis is true, we expect to observe lower levels of resistance against the alternative dsRNA compared to >5,000-fold resistance observed against V-ATPase A dsRNA.

Monitoring uptake of fluorescently labeled dsRNA supports reduced uptake of dsRNA by the midgut cells of larvae from CEAS when compared to susceptible population. Reduced dsRNA uptake inside the midgut cells in CEAS is also partly supported by results from sRNA sequencing, which also suggested the presence of a second mechanism of resistance specific to up-regulation of the target gene in response to treatment with V-ATPase subunit A dsRNA. Comparing these results with the essentially completely impaired uptake of dsRNA observed in the *D. v. virgifera* resistant population (Khajuria, Ivashuta et al. 2018), suggests that alterations in dsRNA uptake may present a common mechanism for developing resistance against insecticidal dsRNA in insects. Performing sRNA sequencing after treatment with an alternative dsRNA, whose target transcript is not up regulated in response to dsRNA treatment, will allow to get a clearer picture regarding reduced uptake as the resistance mechanism. Recently, Christiaens et. al. demonstrated that formulating dsRNA with guanylated polymers increased RNAi efficiency in *Spodoptera exigua*, due to increased stability and cellular uptake of dsRNA (Christiaens, Tardajos et al. 2018).

Thus, our results will aid in the optimization of RNAi technology and instigate the development of modified dsRNA products with improved efficiency.

Additionally, the dsRNA-resistant population appears to be >3-fold less susceptible to *Bacillus thuringiensis* Cry3Aa protoxin. These results signify the need for more thorough assessment of risks associated with the use of the two technologies together, before bringing products to the market. Taken together, the results in this work provide a partial and preliminary understanding of the mechanism of resistance against insecticidal dsRNA in *L. decemlineata*. These results help resolve the scarcity of information on the topic of dsRNA resistance in insects and will help in careful assessment of resistance risks to ensure durability of the RNAi technology. It will also aid to the development of effective resistance management strategies and optimization of RNAi technology.

References

Ahammadsahib, K. I., R. M. Hollingworth, M. E. Whalon, P. M. Ioannidis and E. J. Grafius (1994). "Polysubstrate Monooxygenases and Other Xenobiotic-Metabolizing Enzymes in Susceptible and Resistant Colorado Potato Beetle." Pesticide Biochemistry and Physiology **49**(1): 1-12.

Ajiro, M., R. Jia, R.-H. Wang, C.-X. Deng and Z.-M. Zheng (2015). "Adapted Resistance to the Knockdown Effect of shRNA-Derived Srsf3 siRNAs in Mouse Littermates." International journal of biological sciences **11**(11): 1248-1256.

Allen, M. L. and W. B. Walker (2012). "Saliva of *Lygus lineolaris* digests double stranded ribonucleic acids." Journal of Insect Physiology **58**(3): 391-396.

Almeida Garcia, R., L. Lima Pepino Macedo, D. Cabral do Nascimento, F.-X. Gillet, C. E. Moreira-Pinto, M. Faheem, A. M. Moreschi Basso, M. C. Mattar Silva and M. F. Grossi-de-Sa (2017). "Nucleases as a barrier to gene silencing in the cotton boll weevil, *Anthonomus grandis*." PLOS ONE **12**(12): e0189600.

Alyokhin, A., M. Baker, D. Mota-Sanchez, G. Dively and E. Grafius (2008). Colorado Potato Beetle Resistance to Insecticides.

Alyokhin, A. and Y. H. Chen (2017). "Adaptation to toxic hosts as a factor in the evolution of insecticide resistance." Current Opinion in Insect Science **21**: 33-38.

Alyokhin, A. V. and D. N. Ferro (1999). "Modifications in dispersal and oviposition of Bt-resistant and Bt-susceptible Colorado potato beetles as a result of exposure to *Bacillus thuringiensis subsp. tenebrionis* Cry3A toxin." Entomologia Experimentalis et Applicata **90**(1): 93-101.

Andrade, E. and W. Hunter (2016). RNA Interference – Natural Gene-Based Technology for Highly Specific Pest Control (HiSPeC).

Andrews, S. (2014). FastQC A Quality Control tool for High Throughput Sequence Data.

Argentine, J. A., S. H. Lee, M. A. Sos, S. R. Barry and J. M. Clark (1995). "Permethrin Resistance in a Near Isogenic Strain of Colorado Potato Beetle." Pesticide Biochemistry and Physiology **53**(2): 97-115.

Argentine, J. A., K. Y. Zhu, S. H. Lee and J. M. Clark (1994). "Biochemical Mechanisms of Azinphosmethyl Resistance in Isogenic Strains of Colorado Potato Beetle." Pesticide Biochemistry and Physiology **48**(1): 63-78.

Arimatsu, Y., T. Furuno, Y. Sugimura, M. Togoh, R. Ishihara, M. Tokizane, E. Kotani, Y. Hayashi and T. Furusawa (2007). "Purification and Properties of Double-stranded RNA-degrading Nuclease,dsRNase, from the Digestive Juice of the Silkworm, *Bombyx mori*." Journal of Insect Biotechnology and Sericology **76**(1): 1_57-51_62.

Arimatsu, Y., E. Kotani, Y. Sugimura and T. Furusawa (2007). "Molecular characterization of a cDNA encoding extracellular dsRNase and its expression in the silkworm, *Bombyx mori*." Insect Biochemistry and Molecular Biology **37**(2): 176-183.

Armer, C. A. (2004). "Colorado Potato Beetle Toxins Revisited: Evidence the Beetle Does Not Sequester Host Plant Glycoalkaloids." Journal of Chemical Ecology **30**(4): 883-888.

Aronstein, K. and E. Saldivar (2005). "Characterization of a honey bee Toll related receptor gene Am18w and its potential involvement in antimicrobial immune defense." Apidologie **36**(1): 3-14.

Åsman, A. K. M., C. Dixelius, J. Fogelqvist, R. R. Vetukuri, S. N. Jahan and P. Corcoran (2015). "Plant-mediated gene silencing restricts growth of the potato late blight pathogen *Phytophthora infestans*." Journal of Experimental Botany **66**(9): 2785-2794.

Asokan, R., S. Chandra, M. Maligeppagol and N. Kumar (2013). Effect of diet delivered various concentrations of double-stranded RNA in silencing a midgut and a non-midgut gene of *Helicoverpa armigera*.

Auer, C. and R. Frederick (2009). "Crop improvement using small RNAs: applications and predictive ecological risk assessments." Trends in Biotechnology **27**(11): 644-651.

Bachman, P. M., R. Bolognesi, W. J. Moar, G. M. Mueller, M. S. Paradise, P. Ramaseshadri, J. Tan, J. P. Uffman, J. Warren, B. E. Wiggins and S. L. Levine (2013). "Characterization of the spectrum of insecticidal activity of a double-stranded RNA with targeted activity against Western Corn Rootworm *(Diabrotica virgifera virgifera LeConte*)." Transgenic Research **22**(6): 1207-1222.

Baum, J. A., T. Bogaert, W. Clinton, G. R. Heck, P. Feldmann, O. Ilagan, S. Johnson, G. Plaetinck, T. Munyikwa, M. Pleau, T. Vaughn and J. Roberts (2007). "Control of coleopteran insect pests through RNA interference." Nature Biotechnology **25**: 1322.

Baum, J. A. and J. K. Roberts (2014). Chapter Five - Progress Towards RNAi-Mediated Insect Pest Management. Advances in Insect Physiology. T. S. Dhadialla and S. S. Gill, Academic Press. **47:** 249-295.

Bellés, X. and M.-D. Piulachs (2007). "Structural and RNAi characterization of the German cockroach lipophorin receptor, and the evolutionary relationships of lipoprotein receptors." BMC molecular biology **8**(1): 53.

Bolognesi, R., P. Ramaseshadri, J. Anderson, P. Bachman, W. Clinton, R. Flannagan, O. Ilagan, C. Lawrence, S. Levine, W. Moar, G. Mueller, J. Tan, J. Uffman, E. Wiggins, G. Heck and G. Segers (2012). "Characterizing the Mechanism of Action of Double-Stranded RNA Activity against Western Corn Rootworm (*Diabrotica virgifera virgifera LeConte*)." PLoS ONE **7**(10).

Bucher, G., J. Scholten and M. Klingler (2002). "Parental RNAi in *Tribolium* (Coleoptera)." Current Biology **12**(3): R85-R86.

Cappelle, K., C. F. R. De Oliveira, B. Van Eynde, O. Christiaens and G. Smagghe (2016). "The involvement of clathrin-mediated endocytosis and two Sid-1-like transmembrane proteins in double-stranded RNA uptake in the Colorado potato beetle midgut." Insect Molecular Biology **25**(3): 315-323.

Cárdenas, P. D., P. D. Sonawane, U. Heinig, S. E. Bocobza, S. Burdman and A. Aharoni (2015). "The bitter side of the nightshades: Genomics drives discovery in Solanaceae steroidal alkaloid metabolism." Phytochemistry **113**: 24-32.

Carthew, R. W. and E. J. Sontheimer (2009). "Origins and Mechanisms of miRNAs and siRNAs." Cell **136**(4): 642-655.

Chi, Y., X. Wang, X. Le, Y. Ju, T. Guan and H. Li (2016). "Exposure to double-stranded RNA mediated by tobacco rattle virus leads to transcription up-regulation of effector gene Mi-

vap-2 from *Meloidogyne incognita* and promotion of pathogenicity in progeny." International Journal for Parasitology **46**(2): 105-113.

Christiaens, O., L. Swevers and G. Smagghe (2014). DsRNA degradation in the pea aphid (*Acyrthosiphon pisum*) associated with lack of response in RNAi feeding and injection assay.

Christiaens, O., M. G. Tardajos, Z. L. Martinez Reyna, M. Dash, P. Dubruel and G. Smagghe (2018). "Increased RNAi Efficacy in *Spodoptera exigua* via the Formulation of dsRNA With Guanylated Polymers." Frontiers in Physiology **9**(316).

Dalzell, J. J., N. D. Warnock, M. A. Stevenson, A. Mousley, C. C. Fleming and A. G. Maule (2010). "Short interfering RNA-mediated knockdown of drosha and pasha in undifferentiated *Meloidogyne incognita* eggs leads to irregular growth and embryonic lethality." International Journal for Parasitology **40**(11): 1303-1310.

Dowling, D., T. Pauli, A. Donath, K. Meusemann, L. Podsiadlowski, M. Petersen, R. Peters, C. Mayer, S. Liu, X. Zhou, B. Misof and O. Niehuis (2016). Phylogenetic Origin and Diversification of RNAi Pathway Genes in Insects.

Dykxhoorn, D. M. and J. Lieberman (2005). "The Silent Revolution: RNA Interference as Basic Biology, Research Tool, and Therapeutic." Annual Review of Medicine **56**(1): 401-423.

Egli, M. and M. Manoharan (2019). "Re-Engineering RNA Molecules into Therapeutic Agents." Accounts of Chemical Research **52**(4): 1036-1047.

Ferro, D. N. (1993). "Potential for Resistance to *Bacillus thuringiensis*: Colorado Potato Beetle (Coleoptera: Chrysomelidae)—A Model System." American Entomologist **39**(1): 38-44.

Ferro, D. N., and W. D. Gelernter (1989). " Toxicity of a new strain of *Bacillus thuringiensis* to Colorado potato beetle (Coleoptera:Chrysomelidae)." J.Econ.Entomol: 750-755.

Ferro, D. N., J. A. Argentine and M. J. Clark (1989). "Genetics and Synergism of Resistance to Azinphosmethyl and Permethrin in the Colorado Potato Beetle (Coleoptera: Chrysomelidae)." Journal of Economic Entomology **82**(3): 698-705.

Fire, A., D. Albertson, S. W. Harrison and D. G. Moerman (1991). "Production of antisense RNA leads to effective and specific inhibition of gene expression in *C. elegans* muscle." Development **113**(2): 503-514.

Fire, A., S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver and C. C. Mello (1998). "Potent and specific genetic interference by double-stranded RNA in *caenorhabditis elegans*." Nature **391**(6669): 806-811.

Forgash, A. J., C. A. Silcox and G. M. Ghidiu (1985). "Laboratory and Field Evaluation of Piperonyl Butoxide as a Pyrethroid Synergist Against the Colorado Potato Beetle (Coleoptera: Chrysomelidae)." Journal of Economic Entomology **78**(6): 1399-1405.

Garbutt, J. S., X. Bellés, E. H. Richards and S. E. Reynolds (2013). "Persistence of double-stranded RNA in insect hemolymph as a potential determiner of RNA interference success: Evidence from *Manduca sexta* and *Blattella germanica.*" Journal of Insect Physiology **59**(2): 171-178.

Gauthier, N. L., Hofmaster, R. N. & Semel, M (1981). "History of Colorado potato beetle control." Advances in potato pest management 13-33.

Gordon, K. H. J. and P. M. Waterhouse (2007). "RNAi for insect-proof plants." Nature Biotechnology **25**: 1231.

Grafius, E. (1997). "Economic Impact of Insecticide Resistance in the Colorado Potato Beetle (Coleoptera: Chrysomelidae) on the Michigan Potato Industry." Journal of Economic Entomology **90**(5): 1144-1151.

Guo, Y., J. Zhang, R. Yu, K. Y. Zhu, Y. Guo and E. Ma (2012). "Identification of two new cytochrome P450 genes and RNA interference to evaluate their roles in detoxification of commonly used insecticides in *Locusta migratoria*." Chemosphere **87**(7): 709-717.

Hare, J. D. (1990). "Ecology and management of the Colorado potato beetle." Annual review of entomology **35**(1): 81-100.

Harris, C. R. and S. A. Turnbull (2012). "Contact toxicity of some pyrethroid insecticides, alone and in combination with piperonyl butoxide, to insecticide-susceptible and pyrethroid-

resistant strains of the Colorado potato beetle (Coleoptera: Chrysomelidae)." The Canadian Entomologist **118**(11): 1173-1176.

He, Z.-B., Y.-Q. Cao, Y.-P. Yin, Z.-K. Wang, B. Chen, G.-X. Peng and Y.-X. Xia (2006). "Role of hunchback in segment patterning of *Locusta migratoria manilensis* revealed by parental RNAi." Development, Growth & Differentiation **48**(7): 439-445.

Head, G. P., M. W. Carroll, S. P. Evans, D. M. Rule, A. R. Willse, T. L. Clark, N. P. Storer, R. D. Flannagan, L. W. Samuel and L. J. Meinke (2017). "Evaluation of SmartStax and SmartStax PRO maize against western corn rootworm and northern corn rootworm: efficacy and resistance management." Pest Management Science **73**(9): 1883-1899.

Hirai, M., O. Terenius, W. Li and I. Faye (2004). "Baculovirus and dsRNA induce Hemolin, but no antibacterial activity, in *Antheraea pernyi*." Insect Molecular Biology **13**(4): 399-405.

Hong, J., Z. Qian, S. Shen, T. Min, C. Tan, J. Xu, Y. Zhao and W. Huang (2005). "High doses of siRNAs induce eri-1 and adar-1 gene expression and reduce the efficiency of RNA interference in the mouse." Biochemical Journal **390**(3): 675-679.

Hoy, C. W. and G. Head (1995). "Correlation Between Behavioral and Physiological Responses to Transgenic Potatoes Containing *Bacillus thuringiensis* δ-Endotoxin in *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae)." Journal of Economic Entomology **88**(3): 480-486.

Hsiao, T. H. and G. Fraenkel (1969). "Properties of leptinotarsin: A toxic hemolymph protein from the Colorado potato beetle." Toxicon **7**(2): 119-130.

Hu, X., N. M. Richtman, J.-Z. Zhao, K. E. Duncan, X. Niu, L. A. Procyk, M. A. Oneal, B. M. Kernodle, J. P. Steimel, V. C. Crane, G. Sandahl, J. L. Ritland, R. J. Howard, J. K. Presnail, A. L. Lu and G. Wu (2016). "Discovery of midgut genes for the RNA interference control of corn rootworm." Scientific Reports **6**: 30542.

Hunter, W., J. Ellis, D. vanEngelsdorp, J. Hayes, D. Westervelt, E. Glick, M. Williams, I. Sela, E. Maori, J. Pettis, D. Cox-Foster and N. Paldi (2010). "Large-Scale Field Application of RNAi Technology Reducing Israeli Acute Paralysis Virus Disease in Honey Bees (*Apis mellifera*, Hymenoptera: Apidae)." PLOS Pathogens **6**(12): e1001160.

Hunter, W., E. Glick, N. Paldi and B. R. Bextine (2012). Advances in RNA interference: dsRNA Treatment in Trees and Grapevines for Insect Pest Suppression.

Huvenne, H. and G. Smagghe (2010). "Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: A review." Journal of Insect Physiology **56**(3): 227-235.

Inwood, S., M. J. Betenbaugh, M. Lal and J. Shiloach (2018). Genome-Wide High-Throughput RNAi Screening for Identification of Genes Involved in Protein Production. Recombinant Protein Expression in Mammalian Cells: Methods and Protocols. D. L. Hacker. New York, NY, Springer New York**:** 209-219.

Ioannidis, P. M., E. J. Grafius, J. M. Wierenga, M. E. Whalon and R. M. Hollingworth (1992). "Selection, inheritance and characterization of carbofuran resistance in the Colorado potato beetle (Coleoptera: Chrysomelidae)." Pesticide Science **35**(3): 215-222.

Jacques, R. L. (1988). "The potato beetles ".

Khajuria, C., S. Ivashuta, E. Wiggins, L. Flagel, W. Moar, M. Pleau, K. Miller, Y. Zhang, P. Ramaseshadri, C. Jiang, T. Hodge, P. Jensen, M. Chen, A. Gowda, B. McNulty, C. Vazquez, R. Bolognesi, J. Haas, G. Head and T. Clark (2018). "Development and characterization of the first dsRNA-resistant insect population from western corn rootworm, *Diabrotica virgifera virgifera* LeConte." PLoS ONE **13**(5).

Khajuria, C., A. M. Vélez, M. Rangasamy, H. Wang, E. Fishilevich, M. L. F. Frey, N. P. Carneiro, P. Gandra, K. E. Narva and B. D. Siegfried (2015). "Parental RNA interference of genes involved in embryonic development of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte." Insect Biochemistry and Molecular Biology **63**: 54-62.

Kim, H. J., J. B. Dunn, K. S. Yoon and J. M. Clark (2006). "Target site insensitivity and mutational analysis of acetylcholinesterase from a carbofuran-resistant population of Colorado potato beetle, *Leptinotarsa decemlineata* (Say)." Pesticide Biochemistry and Physiology **84**(3): 165-179.

Kim, H. J., D. J. Hawthorne, T. Peters, G. P. Dively and J. M. Clark (2005). "Application of DNA-based genotyping techniques for the detection of kdr-like pyrethroid resistance in field

populations of Colorado potato beetle." Pesticide Biochemistry and Physiology **81**(2): 85-96.

Kim, H. J., K. S. Yoon and J. M. Clark (2007). "Functional analysis of mutations in expressed acetylcholinesterase that result in azinphosmethyl and carbofuran resistance in Colorado potato beetle." Pesticide Biochemistry and Physiology **88**(2): 181-190.

Kobayashi, I., H. Tsukioka, N. Kômoto, K. Uchino, H. Sezutsu, T. Tamura, T. Kusakabe and S. Tomita (2012). "SID-1 protein of *Caenorhabditis elegans* mediates uptake of dsRNA into *Bombyx* cells." Insect Biochemistry and Molecular Biology **42**(2): 148-154.

Koch, A., D. Biedenkopf, A. Furch, L. Weber, O. Rossbach, E. Abdellatef, L. Linicus, J. Johannsmeier, L. Jelonek, A. Goesmann, V. Cardoza, J. McMillan, T. Mentzel and K.-H. Kogel (2016). "An RNAi-Based Control of *Fusarium graminearum* Infections Through Spraying of Long dsRNAs Involves a Plant Passage and Is Controlled by the Fungal Silencing Machinery." PLOS Pathogens **12**(10): e1005901.

Koch, A., N. Kumar, L. Weber, H. Keller, J. Imani and K.-H. Kogel (2013). "Host-induced gene silencing of cytochrome P450 lanosterol C14α-demethylase–encoding genes confers strong resistance to *Fusarium* species." Proceedings of the National Academy of Sciences **110**(48): 19324-19329.

Krishnan, N., D. Kodrík, F. Turanli and F. Sehnal (2007). "Stage-specific distribution of oxidative radicals and antioxidant enzymes in the midgut of *Leptinotarsa decemlineata*." Journal of Insect Physiology **53**(1): 67-74.

Lawrence, S. D., N. G. Novak, C. J. T. Ju and J. E. K. Cooke (2008). "Examining the molecular interaction between potato (*Solanum tuberosum*) and Colorado potato beetle *Leptinotarsa decemlineata*." Botany **86**(9): 1080-1091.

Lee, S. and J. M. Clark (1996). "Tissue Distribution and Biochemical Characterization of Carboxylesterases Associated with Permethrin Resistance in a Near Isogenic Strain of Colorado Potato Beetle." Pesticide Biochemistry and Physiology **56**(3): 208-219.

Lee, S. H., J. B. Dunn, J. Marshall Clark and D. M. Soderlund (1999). "Molecular Analysis ofkdr-like Resistance in a Permethrin-Resistant Strain of Colorado Potato Beetle." Pesticide Biochemistry and Physiology **63**(2): 63-75.

Lehmann, P., A. Lyytinen, S. Piiroinen and L. Lindström (2014). "Northward range expansion requires synchronization of both overwintering behaviour and physiology with photoperiod in the invasive Colorado potato beetle (*Leptinotarsa decemlineata*)." Oecologia **176**(1): 57-68.

Li, H., A. J. Bowling, P. Gandra, M. Rangasamy, H. E. Pence, R. E. McEwan, C. Khajuria, B. D. Siegfried and K. E. Narva (2018). "Systemic RNAi in western corn rootworm, *Diabrotica virgifera virgifera*, does not involve transitive pathways." Insect Science **25**(1): 45-56.

Li, H., R. Guan, H. Guo and X. Miao (2015). "New insights into an RNAi approach for plant defence against piercing-sucking and stem-borer insect pests." Plant, Cell & Environment **38**(11): 2277-2285.

Li, X., X. Dong, C. Zou and H. Zhang (2015). "Endocytic pathway mediates refractoriness of insect *Bactrocera dorsalis* to RNA interference." Scientific Reports **5**.

Li, X., M. Zhang and H. Zhang (2011). "RNA interference of four genes in adult *Bactrocera dorsalis* by feeding their dsRNAs." PloS one **6**(3): e17788-e17788.

Liao, Y. and L. Tang (2016). "Inducible RNAi system and its application in novel therapeutics." Crit Rev Biotechnol **36**(4): 630-638.

Liu, Y., Q. Ge, B. Chan, H. Liu, S. R. Singh, J. Manley, J. Lee, A. M. Weideman, G. Hou and S. X. Hou (2016). "Whole-animal genome-wide RNAi screen identifies networks regulating male germline stem cells in *Drosophila*." Nature Communications **7**: 12149.

Livak, K. J. and T. D. Schmittgen (2001). "Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method." Methods **25**(4): 402-408.

Lu, P. Y., F. Xie and M. C. Woodle (2005). "In vivo application of RNA interference: from functional genomics to therapeutics." Adv Genet **54**: 117-142.

M. Soderlund, D., C. W. Hessney and M. Jiang (1987). Metabolism of fenvalerate by resistant Colorado potato beetles.

Manisit, D., M. Sara and H. Leaf (2019). "RNA Interference-Based Cancer Drugs: The Roadblocks, and the “Delivery” of the Promise." Nucleic Acid Therapeutics **29**(2): 61-66.

Mao, Y.-B., W.-J. Cai, J.-W. Wang, G.-J. Hong, X.-Y. Tao, L.-J. Wang, Y.-P. Huang and X.-Y. Chen (2007). "Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol." Nature Biotechnology **25**: 1307.

Maori, E., N. Paldi, S. Shafir, H. Kalev, E. Tsur, E. Glick and I. Sela (2009). "IAPV, a bee-affecting virus associated with Colony Collapse Disorder can be silenced by dsRNA ingestion." Insect Molecular Biology **18**(1): 55-60.

Martin, M. (2011). CUTADAPT removes adapter sequences from high-throughput sequencing reads.

Mello, C. C. and D. Conte (2004). "Revealing the world of RNA interference." Nature **431**(7006): 338-342.

Milner, S. E., N. P. Brunton, P. W. Jones, N. M. O’ Brien, S. G. Collins and A. R. Maguire (2011). "Bioactivities of Glycoalkaloids and Their Aglycones from Solanum Species." Journal of Agricultural and Food Chemistry **59**(8): 3454-3484.

Miyata, K., P. Ramaseshadri, Y. Zhang, G. Segers, R. Bolognesi and Y. Tomoyasu (2014). "Establishing an In Vivo Assay System to Identify Components Involved in Environmental RNA Interference in the Western Corn Rootworm." PLOS ONE **9**(7): e101661.

Moar, W., C. Khajuria, M. Pleau, O. Ilagan, M. Chen, C. Jiang, P. Price, B. McNulty, T. Clark and G. Head (2017). "Cry3Bb1-Resistant Western Corn Rootworm, *Diabrotica virgifera virgifera (LeConte)* Does Not Exhibit Cross-Resistance to DvSnf7 dsRNA." PLOS ONE **12**(1): e0169175.

Mota-Sanchez, D., R. M. Hollingworth, E. J. Grafius and D. D. Moyer (2006). "Resistance and cross-resistance to neonicotinoid insecticides and spinosad in the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae)." Pest Management Science **62**(1): 30-37.

Napoli, C., C. Lemieux and R. Jorgensen (1990). "Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans." The Plant Cell **2**(4): 279-289.

Novillo, C., P. Castañera and F. Ortego (1997). "Characterization and distribution of chymotrypsin-like and other digestive proteases in Colorado potato beetle larvae." Archives of Insect Biochemistry and Physiology **36**(3): 181-201.

Petek, M., N. Turnšek, M. B. Gašparič, M. P. Novak, K. Gruden, N. Slapar, T. Popovič, B. Štrukelj, K. Gruden, B. Štrukelj and M. A. Jongsma (2012). "A complex of genes involved in adaptation of *Leptinotarsa decemlineata* larvae to induced potato defense." Archives of Insect Biochemistry and Physiology **79**(3): 153-181.

Piiroinen, S., T. Ketola, A. Lyytinen and L. Lindström (2011). "Energy use, diapause behaviour and northern range expansion potential in the invasive Colorado potato beetle." Functional Ecology **25**(3): 527-536.

Preall, J. B. and E. J. Sontheimer (2005). "RNAi: RISC Gets Loaded." Cell **123**(4): 543-545.

Price, D. R. G. and J. A. Gatehouse (2008). "RNAi-mediated crop protection against insects." Trends in Biotechnology **26**(7): 393-400.

Rocheleau, Christian E. (2012). "RNA Interference: Systemic RNAi SIDes with Endosomes." Current Biology **22**(20): R873-R875.

Rose, R. L. and W. A. Brindley (1985). "An evaluation of the role of oxidative enzymes in Colorado potato beetle resistance to carbamate insecticides." Pesticide Biochemistry and Physiology **23**(1): 74-84.

Roush, R. T. (1998). "Two-toxin strategies for management of insecticidal transgenic crops: can pyramiding succeed where pesticide mixtures have not?" Philosophical Transactions of the Royal Society B: Biological Sciences **353**(1376): 1777-1786.

Saleh, M.-C., R. P. van Rij, A. Hekele, A. Gillis, E. Foley, P. H. O'Farrell and R. Andino (2006). "The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing." Nature Cell Biology **8**: 793.

San Miguel, K. and J. G. Scott (2016). "The next generation of insecticides: dsRNA is stable as a foliar-applied insecticide." Pest Management Science **72**(4): 801-809.

Schoville, S. D., Y. H. Chen, M. N. Andersson, J. B. Benoit, A. Bhandari, J. H. Bowsher, K. Brevik, K. Cappelle, M.-J. M. Chen, A. K. Childers, C. Childers, O. Christiaens, J. Clements, E. M. Didion, E. N. Elpidina, P. Engsontia, M. Friedrich, I. García-Robles, R. A. Gibbs, C. Goswami, A. Grapputo, K. Gruden, M. Grynberg, B. Henrissat, E. C. Jennings, J. W. Jones, M. Kalsi, S. A. Khan, A. Kumar, F. Li, V. Lombard, X. Ma, A. Martynov, N. J. Miller, R. F. Mitchell, M. Munoz-Torres, A. Muszewska, B. Oppert, S. R. Palli, K. A. Panfilio, Y. Pauchet, L. C. Perkin, M. Petek, M. F. Poelchau, É. Record, J. P. Rinehart, H. M. Robertson, A. J. Rosendale, V. M. Ruiz-Arroyo, G. Smagghe, Z. Szendrei, G. W. C. Thomas, A. S. Torson, I. M. Vargas Jentzsch, M. T. Weirauch, A. D. Yates, G. D. Yocum, J.-S. Yoon and S. Richards (2018). "A model species for agricultural pest genomics: the genome of the Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae)." Scientific Reports **8**(1): 1931.

Shi, X.-Q., W.-C. Guo, P.-J. Wan, L.-T. Zhou, X.-L. Ren, T. Ahmat, K.-Y. Fu and G.-Q. Li (2013). "Validation of reference genes for expression analysis by quantitative real-time PCR in *Leptinotarsa decemlineata* (Say)." BMC Research Notes **6**(1): 93.

Shukla, J. N., M. Kalsi, A. Sethi, K. E. Narva, E. Fishilevich, S. Singh, K. Mogilicherla and S. R. Palli (2016). "Reduced stability and intracellular transport of dsRNA contribute to poor RNAi response in lepidopteran insects." RNA biology **13**(7): 656-669.

Skryabin, K. (2010). "Do Russia and Eastern Europe need GM plants?" New Biotechnology **27**(5): 593-595.

Sledz, C. A., M. Holko, M. J. de Veer, R. H. Silverman and B. R. G. Williams (2003). "Activation of the interferon system by short-interfering RNAs." Nature Cell Biology **5**(9): 834-839.

Spit, J., A. Philips, N. Wynant, D. Santos, G. Plaetinck and J. Vanden Broeck (2017). "Knockdown of nuclease activity in the gut enhances RNAi efficiency in the Colorado potato beetle, *Leptinotarsa decemlineata*, but not in the desert locust, *Schistocerca gregaria*." Insect Biochemistry and Molecular Biology **81**: 103-116.

Stanković, S., A. Zabel, M. Kostic, B. Manojlovic and S. Rajkovic (2004). "Colorado potato beetle [*Leptinotarsa decemlineata* (Say)] resistance to organophosphates and carbamates in Serbia." Journal of Pest Science **77**(1): 11-15.

Tenllado, F., B. Martínez-García, M. Vargas and J. R. Díaz-Ruíz (2003). "Crude extracts of bacterially expressed dsRNA can be used to protect plants against virus infections." BMC Biotechnology **3**(1): 3.

Terenius, O., A. Papanicolaou, J. S. Garbutt, I. Eleftherianos, H. Huvenne, S. Kanginakudru, M. Albrechtsen, C. An, J.-L. Aymeric, A. Barthel, P. Bebas, K. Bitra, A. Bravo, F. Chevalier, D. P. Collinge, C. M. Crava, R. A. de Maagd, B. Duvic, M. Erlandson, I. Faye, G. Felföldi, H. Fujiwara, R. Futahashi, A. S. Gandhe, H. S. Gatehouse, L. N. Gatehouse, J. M. Giebultowicz, I. Gómez, C. J. P. Grimmelikhuijzen, A. T. Groot, F. Hauser, D. G. Heckel, D. D. Hegedus, S. Hrycaj, L. Huang, J. J. Hull, K. Iatrou, M. Iga, M. R. Kanost, J. Kotwica, C. Li, J. Li, J. Liu, M. Lundmark, S. Matsumoto, M. Meyering-Vos, P. J. Millichap, A. Monteiro, N. Mrinal, T. Niimi, D. Nowara, A. Ohnishi, V. Oostra, K. Ozaki, M. Papakonstantinou, A. Popadic, M. V. Rajam, S. Saenko, R. M. Simpson, M. Soberón, M. R. Strand, S. Tomita, U. Toprak, P. Wang, C. W. Wee, S. Whyard, W. Zhang, J. Nagaraju, R. H. ffrench-Constant, S. Herrero, K. Gordon, L. Swevers and G. Smagghe (2011). "RNA interference in Lepidoptera: An overview of successful and unsuccessful studies and implications for experimental design." Journal of Insect Physiology **57**(2): 231-245.

Tomoyasu, Y., S. C. Miller, S. Tomita, M. Schoppmeier, D. Grossmann and G. Bucher (2008). "Exploring systemic RNA interference in insects: a genome-wide survey for RNAi genes in *Tribolium*." Genome biology **9**(1): R10-R10.

Travella, S., T. E. Klimm and B. Keller (2006). "RNA Interference-Based Gene Silencing as an Efficient Tool for Functional Genomics in Hexaploid Bread Wheat." Plant Physiology **142**(1): 6-20.

Vachon, V., R. Laprade and J.-L. Schwartz (2012). "Current models of the mode of action of *Bacillus thuringiensis* insecticidal crystal proteins: A critical review." Journal of Invertebrate Pathology **111**(1): 1-12.

Vélez, A. M. and E. Fishilevich (2018). "The mysteries of insect RNAi: A focus on dsRNA uptake and transport." Pesticide Biochemistry and Physiology **151**: 25-31.

Vélez, A. M., C. Khajuria, H. Wang, K. E. Narva and B. D. Siegfried (2016). "Knockdown of RNA Interference Pathway Genes in Western Corn Rootworms (*Diabrotica virgifera virgifera* Le Conte) Demonstrates a Possible Mechanism of Resistance to Lethal dsRNA." PLOS ONE **11**(6): e0157520.

Walawage, S. L., M. T. Britton, C. A. Leslie, S. L. Uratsu, Y. Li and A. M. Dandekar (2013). "Stacking resistance to crown gall and nematodes in walnut rootstocks." BMC Genomics **14**(1): 668.

Walsh, B. D. (1865). "The new potato bug and its natural history." Practical Entomol **1**: 1-4.

Wang, K., Y. Peng, J. Pu, W. Fu, J. Wang and Z. Han (2016). "Variation in RNAi efficacy among insect species is attributable to dsRNA degradation in vivo." Insect Biochemistry and Molecular Biology **77**: 1-9.

Whalon, M. E., D. Mota-Sanchez, and R.M. Hollingworth (2019). "The MSU arthropod pesticide resistance database, http://www.pesticideresistance.org."

Whangbo, J. S. and C. P. Hunter (2008). "Environmental RNA interference." Trends in Genetics **24**(6): 297-305.

Wierenga, J. M. and R. M. Hollingworth (1993). "Inhibition of Altered Acetylcholinesterases from Insecticide-Resistant Colorado Potato Beetles (Coleoptera: Chrysomelidae)." Journal of Economic Entomology **86**(3): 673-679.

Wingard, S. A. (1928). "Hosts and symptoms of ring spot, a virus disease of plants." J Agric Res **37**(3): 127-153.

Wynant, N., D. Santos, P. Van Wielendaele and J. Vanden Broeck (2014). "Scavenger receptor-mediated endocytosis facilitates RNA interference in the desert locust, *Schistocerca gregaria*." Insect Molecular Biology **23**(3): 320-329.

Xiao, D., X. Gao, J. Xu, X. Liang, Q. Li, J. Yao and K. Y. Zhu (2015). "Clathrin-dependent endocytosis plays a predominant role in cellular uptake of double-stranded RNA in the red flour beetle." Insect Biochemistry and Molecular Biology **60**: 68-77.

Yoo, B., V. C. Jordan, P. Sheedy, A.-M. Billig, A. Ross, P. Pantazopoulos and Z. Medarova (2019). "RNAi-Mediated PD-L1 Inhibition for Pancreatic Cancer Immunotherapy." Scientific Reports **9**(1): 4712.

Yoon, J.-S., D. Gurusamy and S. R. Palli (2017). "Accumulation of dsRNA in endosomes contributes to inefficient RNA interference in the fall armyworm, *Spodoptera frugiperda*." Insect Biochemistry and Molecular Biology **90**: 53-60.

Yoon, J.-S., K. Mogilicherla, D. Gurusamy, X. Chen, S. C. R. R. Chereddy and S. R. Palli (2018). "Double-stranded RNA binding protein, Staufen, is required for the initiation of RNAi in coleopteran insects." Proceedings of the National Academy of Sciences of the United States of America **115**(33): 8334-8339.

Yoon, J.-S., J. N. Shukla, Z. J. Gong, K. Mogilicherla and S. R. Palli (2016). "RNA interference in the Colorado potato beetle, *Leptinotarsa decemlineata*: Identification of key contributors." Insect Biochemistry and Molecular Biology **78**: 78-88.

Yoon, J. S., J. N. Shukla, Z. J. Gong, K. Mogilicherla and S. R. Palli (2016). "RNA interference in the Colorado potato beetle, *Leptinotarsa decemlineata*: Identification of key contributors." Insect Biochemistry and Molecular Biology **78**: 78-88.

Yoon, K. S., J. O. Nelson and J. Marshall Clark (2002). "Selective induction of abamectin metabolism by dexamethasone, 3-methylcholanthrene, and phenobarbital in Colorado potato beetle, *Leptinotarsa decemlineata* (Say)." Pesticide Biochemistry and Physiology **73**(2): 74-86.

Youssef, R. M., K.-H. Kim, S. A. Haroon and B. F. Matthews (2013). "Post-transcriptional gene silencing of the gene encoding aldolase from soybean cyst nematode by transformed soybean roots." Experimental Parasitology **134**(2): 266-274.

Zhang, J., J. Zhang, M. Yang, Q. Jia, Y. Guo, E. Ma and K. Y. Zhu (2011). "Genomics-based approaches to screening carboxylesterase-like genes potentially involved in malathion

resistance in oriental migratory locust (*Locusta migratoria manilensis*)." Pest Management Science **67**(2): 183-190.

Zhang, X., J. Zhang and K. Y. Zhu (2010). "Chitosan/double-stranded RNA nanoparticle-mediated RNA interference to silence chitin synthase genes through larval feeding in the African malaria mosquito (*Anopheles gambiae*)." Insect Molecular Biology **19**(5): 683-693.

Zheng, Z.-M., S. Tang and M. Tao (2005). "Development of resistance to RNAi in mammalian cells." Annals of the New York Academy of Sciences **1058**: 105-118.

Zhou, X., F. M. Oi and M. E. Scharf (2006). "Social exploitation of hexamerin: RNAi reveals a major caste-regulatory factor in termites." Proceedings of the National Academy of Sciences of the United States of America **103**(12): 4499-4504.

Zhou, X., M. M. Wheeler, F. M. Oi and M. E. Scharf (2008). "RNA interference in the termite *Reticulitermes flavipes* through ingestion of double-stranded RNA." Insect Biochemistry and Molecular Biology **38**(8): 805-815.

Zhu, F., J. Xu, R. Palli, J. Ferguson and S. R. Palli (2011). "Ingested RNA interference for managing the populations of the Colorado potato beetle, *Leptinotarsa decemlineata*." Pest Management Science **67**(2): 175-182.

Zotti, M., E. A. dos Santos, D. Cagliari, O. Christiaens, C. N. T. Taning and G. Smagghe (2018). "RNA interference technology in crop protection against arthropod pests, pathogens and nematodes." Pest Management Science **74**(6): 1239-1250.

Zotti, M. and G. Smagghe (2015). RNAi Technology for Insect Management and Protection of Beneficial Insects from Diseases: Lessons, Challenges and Risk Assessments.

Vita

Swati Mishra is originally from India and graduated with a B.Sc. degree in Biotechnology form Punjab Agricultural University (PAU), Ludhiana, India. Upon completion of her undergraduate degree at PAU, she was offered the position of Graduate Research Assistant in the Jurat-Fuentes laboratory at the University of Tennessee, Knoxville. Her masters research addresses the mechanisms of resistance to insecticidal dsRNA in *Leptinotarsa decemlineata* (Colorado potato beetle). She is continuing her education with a Doctor of Philosophy (PhD) degree in Jurat-Fuentes laboratory.