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## RNA Interference of MDR Genes in *Leptinotarsa decemlineata* and *Trichoplusia ni* by Ingestion of Double-Stranded RNA

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biology

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## Abstract

Insecticide resistance is often linked to the expression of detoxification genes such as *MDRs* which code for ATP-binding cassette efflux transporters with broad substrate specificity. To investigate the role of *MDR* genes in *Leptinotarsa decemlineata* and *Trichoplusia ni* tolerance for the insecticide ivermectin, ingested dsRNA was used to attempt silencing of various *MDR* genes in these insects through RNA interference. Silencing was effective in *L. decemlineata*, but not *T. ni*. No change in ivermectin tolerance was found in *L. decemlineata* after *MDR* gene silencing. Because RNAi efficiency was different between *L. decemlineata* and *T. ni*, the stability of dsRNA in midgut lumen and hemolymph was compared between the two species and another insect, *Manduca sexta*. The dsRNA was least stable in *T. ni* body fluids, providing a likely cause for the ineffectiveness of ingested dsRNA.

## Keywords

Multidrug-resistance, MDR, insecticide resistance, xenobiotic metabolism, ATP-binding cassette transporter, ABC, RNA interference, RNAi, pest management, *Leptinotarsa decemlineata*, Colorado potato beetle, CPB, *Trichoplusia ni*, cabbage looper

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## Chapter 1

### 1 Introduction

Insects from many different orders have been agricultural pests, either directly through feeding or as vectors of diseases, ever since humans cultivated plants (Oerke, 2006) and the Animal Plant and Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) estimated losses amount to \$120 billion each year in the USA alone (Montalvo, 2015). Such losses take on a greater significance when one considers that food production must increase by 70% by 2050 to accommodate global food demands (FAO, 2009). Meeting such goals will require better agriculture practices, including more effective pest management strategies that do not place as much reliance on chemical insecticides, since they can negatively impact ecological systems and human health (Fairbrother et al., 2014; Pimentel, 2005; Weston et al. 2013).

Furthermore, as the widespread use of insecticides has also resulted in a rise in resistant strains of insects, there has been an increase in research studying insecticide resistance and alternative methods of pest management (Jensen, 2015; Kogan, 1998). One approach that has gained popularity is the use of double-stranded RNA (dsRNA)-mediated RNA-interference (RNAi) where dsRNA with sequence complementarity to that of a targeted mRNA is introduced into a cell such that the dsRNA then reduces the expression of the respective gene. This approach has been used to investigate the function of individual gene products but also has potential as a novel pest management technique (Mamta & Rajam, 2017). If a gene is determined to be involved in insecticide metabolism, downregulating it through RNAi can lower the dose of insecticide that is required to be effective. This can allow for less intensive use of insecticides and reversal of insecticide resistance by targeting resistance-related genes. The success of RNAi as a pest control method has varied between different insect species (Katoch et al, 2013), but when successful it has often targeted genes related to the metabolism of xenobiotics, including insecticides. Considering that metabolic genes often provide a multidrug resistance (MDR) phenotype, this could allow for decreased use of multiple insecticides. Consequently, identifying the genes involved in insecticide resistance and the MDR

phenotype will not only provide valuable information regarding the development of insecticide resistance, but also provide targets for manipulation to increase insecticide lethality. Transgenic crop plants that express dsRNA for targeted RNAi of these genes are an ideal end product that would increase insecticide effectiveness without having significant ecological drawbacks.

The *MDR* genes are strong candidates for targeting with RNAi as they code for MDR proteins that are transmembrane efflux transporters with broad substrate specificity. They have been intensively studied in humans because upregulation of these genes confers the MDR phenotype to cancer cells (Gottesman et al., 2002), but since they are highly conserved across many organisms their function is being more actively explored with respect to insecticide resistance in insects (Dean et al., 2001; Dermauw & Van Leeuwen, 2014; Liu et al., 2011b; Roth et al., 2003).

It will prove invaluable to investigate and refine RNAi as a pest management tool and identify appropriate gene targets for manipulation. There is still limited knowledge regarding which insects are responsive to RNAi and the reasons there are differences between species. Identifying which species are responsive or refractory will elucidate which insects can be easily managed with RNAi. Additionally, while *MDR* genes are excellent candidates for RNAi-mediated knockdown, their role in insecticide resistance is not yet thoroughly investigated in many insects. As such, targeting *MDR* genes for RNAi-mediated knockdown in species where their function is not yet known will both serve to investigate *MDR* gene function and test *MDR* knockdown as a pest management technique.

The two species examined in this study are the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) the most important insect defoliator of potato plants (Alyokhin et al., 2008), and the cabbage looper, *Trichoplusia ni* (Hübner), a persistent polyphagous pest of Brassicaceae plants, like cabbage, as well as others including tomato, lettuce, and cucumber (Soo Hoo et al., 1984). Both species are resistant to different chemical insecticides and transgenic plants producing toxins derived from the bacterium *Bacillus thuringiensis* (Bt) (Alyokhin et al., 2008; Janmaat & Myers, 2003), but currently the

extent to which *MDR* genes are implicated in their resistance is unknown. Furthermore, RNAi has been shown to be successful in *L. decemlineata*, but has had limited success in lepidopterans like *T. ni*. Investigating these two species allowed for a comparison of RNAi between responsive and refractory insects, as well as an assessment of *MDR* genes as insecticide resistance genes and potential targets for RNAi-mediated pest management.

## 1.1 Xenobiotic Metabolism and the Multidrug Resistance Phenotype

### 1.1.1 Xenobiotic Metabolism and Detoxification

Almost all known organisms use common intracellular structures, processes, and enzymes for xenobiotic metabolism and toxin defense. Semi-permeable membranes prevent most hydrophilic molecules from diffusing into cells, but many hydrophobic molecules freely pass through. Therefore, metabolic proteins and mechanisms are used to detoxify and actively transport harmful hydrophobic molecules out of the cells. The metabolism of foreign toxins is divided into three phases: Phase I (Modification), Phase II (Conjugation), and Phase 3 (Excretion), although depending on the chemical properties of the molecule in question, it may or may not go through all three (Le, 2017). In Phase I, enzymes such as cytochrome P450 monooxygenases (CYPs) add reactive groups to the xenobiotic molecule through reactions such as oxidation, hydrolysis, hydroxylation, typically adding an oxygen atom or removing a hydrogen atom. These reactions either create a site for Phase II enzymes to catalyze, or make the target molecule polar enough to be excreted directly. Phase II enzymes, such as glutathione S-transferases (GSTs), use the newly added reactive groups to catalyze a reaction that conjugates the xenobiotic molecule with a charged species like glutathione or sulfate. This reduces the toxicity of the molecule and further polarizes it which facilitates the excretion process. Phase II enzymes tend to have broader substrate specificity and substrate overlap than those of Phase I. Phase III enzymes, also noted for having particularly broad substrate specificities, are typically ATP-binding cassette (ABC) transporters, like MDRs, that use ATP to actively transport their targets across the cell membrane. If the targets are large molecules that have been polarized through Phase I or Phase II reactions, they will

remain outside of the cell. However, if not polarized, the molecule will enter and be re-excreted by other cells until it is excreted from the body.

### 1.1.2 Insecticide Resistance and the Multidrug Resistance Phenotype

Mutations that increase the activity of detoxification enzymes, usually through increased gene expression, can increase an organism's resistance to xenobiotics. In insects, these mutations can arise if they are continually challenged by insecticides for many generations. The selective pressure from continued insecticide exposure may increase the prevalence of mutations that protect against those insecticides. There are many examples of enzymes from all three phases contributing to xenobiotic metabolism and insecticide resistance. *CYP* genes that directly increase metabolism of insecticides are overexpressed in some resistant species, like the housefly, *Musca domestica* (Zhu et al. 2008), a malaria vector, *Culex quinquefasciatus* (Liu et al., 2011a) and the cotton bollworm, *Helicoverpa armigera* (Brun-Barale et al., 2010). Overexpression of *GST* genes has been linked to DDT resistance in *M. domestica* (Clark & Shamaan, 1984) and pyrethroid resistance in the maize weevil, *Sitophilus zeamais* (Fragoso et al, 2003). ABC transporters, like MDRs, have been identified as resistance factors in the tobacco budworm, *Heliothis virescens*, (Lanning et al., 1996) and a Bt-resistant leaf beetle, *Chrysomela tremula* (Pauchet et al, 2016). In some cases multiple resistances can result, depending on the substrate specificity of the encoded enzyme, by a single upregulated gene or by co-regulation (Alyokhin et al., 2008; Edi et al., 2014). Genes for enzymes with broad-substrate specificity, such as *MDR* genes, are commonly upregulated in organisms displaying the MDR phenotype. A single mutation in such a gene is often sufficient to support multiple resistances, as their enzyme products can interact with multiple insecticides (Sun et al., 2017). This can be particularly problematic for pest control as insect populations can become resistant to multiple chemically unrelated insecticides, sometimes after being exposed to only one chemical. Therefore, it is important to understand the nature of the MDR phenotype in insects, so that it can be prevented or circumvented by management techniques.

## 1.2 Multidrug Resistance Proteins (MDRs)

### 1.2.1 ATP-binding Cassette Superfamily

MDRs are members of the ATP-binding cassette transporter superfamily, a very large and ancient protein family consisting of transmembrane transporters that are present in archaea (Albers et al., 2004), bacteria (Davidson & Chen, 2004) and eukaryotes (Dassa & Bouige, 2001). They are defined by their ATP-binding cassette domain used to acquire energy from ATP for active transport. ABC transporters, responsible for transporting a large variety of substrates across membranes, including steroids, phospholipids, ions, peptides, bile acids, and xenobiotics (Klein et al., 1999), act exclusively as exporters in eukaryotes, but can be either importers or exporters in archaea and prokaryotes. They are divided into 7 subfamilies (ABCA-G) in humans and 8 in arthropods (ABCA-H) based on structural similarities, and many ABC proteins in other non-human organisms are named for their homologous human counterparts. MDRs are part of the ABCB subfamily, also known as the MDR/TAP subfamily as it also includes transporters associated with antigen processing (TAP) proteins.

### 1.2.2 Structure and Mechanism

MDRs, like all ABC transporters, are composed of four domains: two nucleotide binding domains (NBDs) and two transmembrane domains (TMDs) that can be organized in various combinations: one NBD with one TMD (half transporter), or all four domains together (full transporter). However, all four domains are required for the protein to be functional so the polypeptides for half transporters are combined after translation as homodimers or heterodimers to form full transporters. The NBDs are also considered ATP-binding cassette domains as they contain Walker-A and Walker-B motifs that are shared with other non-ABC proteins, and a signature amino acid sequence of LSGGQ unique to ABC proteins (Dean et al., 2001). The NBDs are situated towards the cytosol and bind with two ATP molecules in a “sandwich dimer” structure having both ATP molecules enclosed between the two NBD domains. Hydrolysis of these ATP molecules provides energy that drives conformational changes in all domains of the protein, moving the substrate across the membrane. The TMDs contain multiple alpha

helices that span the membrane into which the protein is embedded, in addition to sequences that recognize substrates for transport. As the TMDs are responsible for substrate recognition, their sequences can vary significantly between different ABC proteins that transport different substrates. The alpha helices are oriented to provide a pore across the membrane through which the substrate is passed during transport. In resting conformation, the TMD pore is oriented to be open towards the side of the membrane from which the substrate is to be transported. Several mechanisms have been proposed for ABC transport: the “alternating site” (Senior et al., 1995), “switch” (Higgins & Linton, 2004), and “constant contact” (Sauna et al., 2007) models. All of these models include the steps of ATP binding, ATP hydrolysis, ATP release, NBD dimerization, TMD conformational changes from inward facing to outward facing (or vice versa), and a return to resting state. However, they differ in the specific order of actions, and in which action provides the “power stroke” to push the substrate across the membrane. It is also possible that multiple mechanisms are valid and simply vary between different transporters (Wilkins, 2015).

### 1.2.3 Function

MDRs are well studied because of their involvement in drug-resistant human cancer cells and xenobiotic resistance in other organisms, particularly insects. The first MDR protein was identified and characterized in drug-resistant hamster ovary cells and termed a permeability glycoprotein (P-glycoprotein) for its role in affecting drug permeation (Juliano & Ling, 1976). Similar proteins and their corresponding genes were later found in human multidrug-resistant cancer cells, and their gene expression and protein activity were correlated with drug resistance (Roninson et al., 1986). Since then, MDR proteins and *MDR* genes have been identified and linked to xenobiotic tolerance in many different organisms including dogs (Roulet et al., 2003), mice (Chin et al., 1990), moths (Aurade et al., 2012), mosquitos (Buss et al., 2002; Figueira-Mansur et al., 2013), flies (Mayer et al., 2009; Tapadia & Lakhota, 2005) and nematodes (James & Davey, 2009). They protect against xenobiotics by transporting the compounds out of the cell to reduce their accumulation and toxic effects. *MDR* genes are typically expressed in barrier and entry tissues like digestive and blood-brain barrier tissues, as well as in tissues performing

detoxification or regulatory functions (Croop et al., 1989; Fojo et al., 1987; Fromm, 2004; Huai-Yun et al., 1998; Schinkel, 1999; Simmons et al., 2013; Tapadia & Lakhota, 2005; Tsuji, 1998). In insects these tissues include midgut as a barrier to ingested compounds, central nervous system tissue as a blood-brain barrier equivalent, and Malpighian tubule tissue for detoxification. *MDR* genes within each organism are not always expressed ubiquitously amongst these tissues, indicating that MDR transporters serve different purposes and that each transporter may act on a different collection of xenobiotics. Regardless of their localization, MDR proteins expressed by these genes have broad substrate specificity and are capable of transporting a wide variety of toxins. While most research interest is in their capacity for drug resistance, MDR transporters can also perform basic metabolic functions. Some MDRs transport non-toxic molecules such as cholesterol (Aurade et al., 2012; Garrigues et al., 2002), lipids, bile salts (van Helvoort et al., 1996), and peptides (Momburg et al., 1994). Thus, they can be essential for normal cellular and physiological processes instead of, or in addition to functioning as detoxification transporters. Mutations in MDRs that are responsible for such processes often result in diseases such as, progressive familial intrahepatic cholestasis (PFIC) and immune suppression in humans.

#### 1.2.4 MDRs and Insecticide Resistance

The capacity of *MDR* genes and MDR proteins to protect insects from xenobiotics like insecticides is of particular interest to agricultural research. Changes to *MDR* gene expression, rather than changes to the MDR amino acid sequence are much more common as resistance mechanisms and have been linked to insecticide resistance in multiple insect species. Dermauw and Van Leeuwen (2014) reported that ABCB transporters contributed to resistance of a diverse array of arthropod species to carbamates, macrocyclic lactones, organochlorines, organophosphates, pyrethroids, and Cry1A toxin. A variety of assays including *in vivo* knockdown of gene expression and the use of protein inhibitors were used to link these genes and proteins to various resistances. Verapamil, a competitive inhibitor, is commonly used to inhibit ABC transporter activity before challenging the insect with an insecticide. Macrocyclic lactones, such as

ivermectin, are often used to test the relationship between *MDR* genes and insecticide resistance because they are substrates for many MDR transporters.

## 1.3 RNA Interference (RNAi)

### 1.3.1 Mechanism

RNAi is the process by which dsRNA molecules with sequence complementarity to a mRNA transcript of a gene reduce expression of that gene by targeted degradation of the transcript. RNAi was first reported by Fire *et al.* (1998) by microinjecting dsRNA into *Caenorhabditis elegans* and has since been investigated in bacteria, nematodes, insects and plants (Hannon, 2002). The RNAi process begins when precursor dsRNA molecules are either produced or acquired by a cell. These molecules are then cleaved into 21-23 nucleotide long small interfering RNA (siRNA) fragments by the RNase enzyme Dicer (Bernstein *et al.*, 2001). One strand from each siRNA molecule, referred to as the guide strand, is incorporated into the multi-protein RNA-induced silencing complex (RISC) (Hannon *et al.*, 2000), while the other, the passenger strand, is discarded (Filipowicz, 2005). The RISC uses the complementary sequence of the guide strand to identify and bind to the target mRNA molecules and then the catalytic protein of the RISC, Argonaute, cleaves the mRNA, preventing it from being translated (Hammond *et al.*, 2001). In insects, RNAi can occur through either the micro-RNA (miRNA) or the small interfering-RNA (siRNA) pathways, which has separate purposes and proteins. The miRNA pathway uses endogenously transcribed dsRNA from the genome to regulate cellular gene expression, while the siRNA pathway uses exogenous dsRNA and is believed to be a defense mechanism against foreign dsRNA molecules (Tomari *et al.*, 2007). This makes the siRNA pathway of particular interest in pest management if exogenous dsRNA can be used as a pesticide by downregulating vital genes, particularly because the specificity of the dsRNA sequences can minimize or even completely eliminate effects on non-target organisms.

RNA interference can be divided into four major categories: cell-autonomous, non-cell-autonomous, systemic, and environmental (Whangbo & Hunter, 2008). Cell-autonomous refers to RNAi that occurs within the cell that also produced the precursor dsRNA. Non-

cell-autonomous refers to cases where RNAi occurs in a cell that acquired the dsRNA either from the environment or from other cells. Systemic RNAi refers to interference that is spread from cell to cell through intercellular transport and signaling.

Environmental RNAi refers to interference that occurs when cells uptake the precursor RNA from the environment. These different categories are not mutually exclusive, and very often occur together. Environmental and systemic RNAi are the most important with respect to pest management as any dsRNA used will come from external sources. In addition it is usually crucial for the target gene to be adequately downregulated throughout the whole body of the insect to have an appropriate effect (Huvenne & Smagghe, 2010).

### 1.3.2 dsRNA Uptake and Propagation in Insects

Acquiring dsRNA molecules from an external source requires an uptake mechanism that ensures the dsRNA reaches the target cells. Two different cellular pathways have been identified for this: the SID-1/SID-2 channel protein-mediated pathway and the receptor-mediated endocytosis pathway. The SID-1/SID-2 pathway uses the transmembrane channel SID-1, which allows for passive transport of dsRNA through the membrane (Feinberg & Hunter, 2003; Winston et al., 2002). The role apical intestinal membrane protein SID-2 has yet to be elucidated (McEwan et al., 2012; Winston et al., 2007). These proteins were first identified in *C. elegans* using knockout mutants and gene orthologs have been identified in many insect species (Huvenne & Smagghe, 2010). However, *sid-1* and *sid-2* genes may not be required for successful RNAi. *Drosophila melanogaster* S2 cells responded to environmental dsRNA even though this species has no known *sid-1* or *sid-2* gene orthologs (Saleh et al., 2006) and downregulation of a *sid-1* ortholog in *Locusta migratoria*, did not inhibit RNAi (Yuan Luo et al., 2012). Insects that demonstrate an RNAi response to environmental RNA without *sid* genes are presumed to take up the dsRNA through receptor-mediated endocytosis. For example, the clathrin heavy chain gene, vacuolar H<sup>+</sup> ATPase, and other genes related to endocytosis are necessary for uptake of dsRNA in *D. melanogaster* S2 cells when both downregulation of these genes and pharmacological inhibition of endocytosis inhibited dsRNA uptake (Saleh et al., 2006; Ulvila et al., 2006). These two forms of uptake are also not mutually

exclusive, as inhibition of either *sid* genes or endocytosis genes in *C. elegans* and the beetle *L. decemlineata* reduced RNAi efficiency (Cappelle et al., 2016; Saleh et al., 2006). Many insects do not respond efficiently to environmental dsRNA (Whangbo & Hunter, 2008). Whether this is due to a lack of a proper uptake pathway or other physiological factors, such as gut pH or RNase activity, is not yet clear.

For systemic RNAi, both SID-1/SID-2 mediated and endocytosis-mediated uptake are used by cells to receive dsRNA from neighbouring cells, but an extra element is also required to copy the dsRNA fragments for use in other cells. In nematodes and plants, this is accomplished by RNA-dependent RNA polymerases (RdRPs) which amplify the RNAi effect by creating new dsRNA molecules (Dalmay et al., 2000; Sijen et al., 2001). Neither RdRPs nor equivalent mechanisms have been identified in insects, so while their cells may be able to spread existing dsRNA to other cells, they cannot reproduce the silencing signal. Despite the lack of RdRPs, some insects still have strong systemic silencing when exposed to dsRNA (Tomoyasu et al., 2008). Absence of RdRPs does, however, imply that while systemic RNAi is possible, it is also transient, so insects must continuously receive environmental dsRNA to have a persistent downregulatory effect (Price & Gatehouse, 2008).

### 1.3.3 RNAi in Pest Management

While RNAi has not yet been used in the field, it is seen as a promising new form of pest management (Huvenne & Smaghe, 2010; Katoch et al., 2013; Zhu et al., 2011) as the specificity of the mRNA targeting allows it to selectively affect the target species with minimal or non-existent ecological side effects on other animals. Furthermore, as the dsRNA sequence used for its insecticidal activity is assumed to have robust plasticity, it could be easily altered if the target insect develops resistance. The interspecific variability in receptiveness to RNAi has hindered development of management techniques for certain insects. However, the use of dsRNA-mediated RNAi in transgenic plants that produce insecticidal dsRNA has proved successful in reducing survival and reproduction of the potato peach aphid (Mao & Zeng, 2014), cotton bollworm (Mao et al., 2007), western corn rootworm (Baum et al., 2007), and Colorado potato beetle (Zhang et al., 2015). Thus the use of such transgenic plants offers an ideal end product

for field crops as there would be no additional costs, such as spraying, for growers. However, consumer acceptance remains a challenge.

In the absence of a complete understanding of the mechanisms of dsRNA uptake and spread in insects, success of RNAi can be difficult to predict when considering its use in pest management. It is difficult to know whether or not a particular species will respond to environmental dsRNA, even when genome-wide analysis is performed to check for relevant genes such as *sid-1* or *sid-2* orthologs. Even with putative RNAi uptake genes present, there are other physiological obstacles that can hinder RNAi such as pH, and RNase enzyme activity in the gut lumen, which are potential barriers for ingested dsRNA (Arimatsu et al., 2007; Price & Gatehouse, 2008). Similarly, hemolymph degrades dsRNA at different rates in different species (Shukla et al., 2016). Even if the dsRNA is taken up by cells, there is still no guarantee that the cell will be able to use the dsRNA (Shukla et al., 2016).

## 1.4 *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae): The Colorado Potato Beetle

### 1.4.1 Description and Life Cycle

The small, yellow or orange, oblong eggs are often laid in clusters (20-100) on the underside of leaves. After 4-15 days they hatch into reddish-brown larvae with black spots along their sides and a black head capsule. They feed on the foliage and pass through four larval instars in 6-10 days. The fully mature, yellowish orange, larvae burrows several centimeters into the soil where they pupate then 5-10 days later emerge as adults. Variation in life stages is caused by environmental factors including temperature and humidity. The oval-shaped beetles have orange-yellow elytra with 10 black lines oriented lengthwise, the source of the species name; *decemlineata* for “ten lines”. Adult beetles feed for several days before mating and females can lay 200-500 eggs over their life (Capinera, 2001). In the fall, in response to environmental cues (short days, cool temperatures and declining food quality) adults enter reproductive diapause and will remain in the soil until the following spring.

### 1.4.2 Distribution and Damage

*Leptinotarsa decemlineata* are native to Mexico, but spread across America and into Canada after an outbreak in 1859 (Casagrande, 1987). The beetle established in France in 1922 and spread throughout most of Europe, the Middle-East, central Asia and parts of China (Weber, 2003). Its total geographic range was estimated to be 16 million km<sup>2</sup> and is still increasing (Weber, 2003). Although the insect primarily attack potatoes they may also be pests on other Solanaceae, such as eggplant and tomato (Weber, 2003). Larvae consume approximately 40 cm<sup>2</sup> of leaf tissue during their development while adults consume approximately 10 cm<sup>2</sup> per day (Ferro et al., 1985). They will also consume stems and tubers, when foliage is no longer available. Losses due to defoliation will depend on many factors, such as the species of plant and the timing of the infestation, but total crop loss is common when the beetles are left uncontrolled. One adult beetle, or 1-4 larvae per plant is considered an acceptable economic threshold, above which treatment for control is required (Weber, 2003).

### 1.4.3 Control and Resistance

Different management techniques are used against *L. decemlineata*. Cultural techniques such as crop rotation and trap crops are useful when potato growers are able to do so. Physical controls, such as flaming and vacuuming the insects, have also proven effective in limited cases (Weber, 2003). The transgenic potato plant “Newleaf”, developed by Monsanto Company to express a *L. decemlineata*-specific *Bt* toxin, was briefly used in the 1990s, but discontinued to avoid public backlash due to poor public opinion regarding genetically modified organisms (Gianessi et al., 2002). However, insecticides are the most common control technique, with over 30 chemical insecticides currently registered for use against *L. decemlineata* (Whalon et al., 2008). Due to the extensive use of insecticides as a management technique, *L. decemlineata* populations have developed resistance to 55 different insecticides in 13 different chemical groups (Whalon et al., 2008). In many cases there are also MDR phenotypes in the resistant strains (Alyokhin et al., 2008), making the use of chemical controls increasingly difficult.

#### 1.4.4 *MDR* Genes in *L. decemlineata*

No *MDR* genes had been specifically identified or characterized in *L. decemlineata* prior to the research conducted for this thesis. However a published *L. decemlineata* transcriptome was available online (Kumar et al., 2014) and an unpublished transcriptome was available at the London Research and Development Centre (London, Ontario, Canada) for use in identifying *MDR* transcripts.

#### 1.4.5 RNA Interference in *L. decemlineata*

Coleopterans in general respond well to RNAi, making them easy organisms to study using the technique (Katoch et al., 2013). Recent research has specifically identified several physiological factors in *L. decemlineata*, which are likely common to other coleopterans, that make them more responsive to RNAi than most lepidopterans, mainly that their gut contents and hemolymph do not degrade dsRNA as quickly and their cells more easily process long dsRNA into siRNA fragments (Shukla et al., 2016). Ingested dsRNA has successfully been used in *L. decemlineata* to downregulate multiple vital genes, causing mortality and demonstrating its potential use as an insecticide (Zhu et al., 2011). Transgenic plants producing dsRNA lethal to *L. decemlineata* have also been tested in a lab setting and were effectively protected from herbivory by the insects (Zhang et al., 2015).

### 1.5 *Trichoplusia ni* (Hübner) (Lepidoptera:Noctuidae): The cabbage looper

#### 1.5.1 Description and Life Cycle

The small, yellowish-white, hemispherical eggs, usually laid individually or in small clusters on the undersurface of leaves, hatch within 2–5 days depending on the temperature (Capinera, 1999). The caterpillar, generally green with a white lateral stripe running the length of the body, passes through five larval instars over 9-14 days before pupating within a thin, white cocoon on the plant or in other secluded locations. The pupal stage lasts for 4-13 days, giving rise to a mottled brown and gray moth which may live for up to 14 days. The female moths can produce 300-600 eggs during their life. On each forewing next to a white dot there is a white U-shaped mark resembling the lower case

Greek letter ni, which is the source of their species name *ni*. The common name “cabbage looper” is derived from the looping mode of walking deployed by the larvae. *T. ni* prefer subtropical climates as they do not survive in low temperatures and do not enter diapause to survive inhospitable seasons (Capinera, 1999). If winter temperatures do not reach below 10°C, they may overwinter by pupating on plant debris and extending their pupal stage until environmental conditions are more favourable (Chalfant et al., 1974). In colder climates, they can overwinter by taking refuge in greenhouses (Cervantes et al., 2011; Franklin et al., 2010, 2011).

### 1.5.2 Distribution and Damage

*T. ni* are native to southwestern North America, but are now present in South America, Africa and Asia (CAB International, 2013; Capinera, 1999; Infonet-Biovision, 2017). As this species is not cold tolerant, annual populations observed in northern areas of North America are the result of immigrants from further south or from populations that successfully overwintered in local greenhouses. They feed on crucifers such as cabbage, broccoli and cauliflower, but are sporadic pests on a wide variety of other crops (Andeloro & Shelton, 1981). Feeding can directly reduce yield while contamination with frass can render the crop unacceptable to buyers. The threshold established for control measures to be applied intervention is approximately 0.3 larvae per plant (Kirby & Slosser, 1984).

### 1.5.3 Control and Resistance

The looper is generally controlled using *Bacillus thuringiensis* (Bt) toxins, chemical insecticides (Caron & Myers, 2008; Cervantes et al., 2011; Franklin & Myers, 2008; Janmaat & Myers, 2003; Kain et al., 2004) or the naturally occurring nucleopolyhedroviruses (NPVs) (Milks et al., 1998). Resistance to *Bt* is becoming increasingly problematic, especially when it develops in the overwintering refuge populations in greenhouses and the moths emigrate into other susceptible populations in the summer (Caron & Myers, 2008; Franklin & Myers, 2008; Janmaat & Myers, 2003; Kain et al., 2004).

#### 1.5.4 MDR Genes in *Trichoplusia ni*

Three different *MDR* genes, *trnMDR1*, *trnMDR2*, and *trnMDR3*, have been identified and characterized in *T. ni* (Simmons et al., 2013). *TrnMDR1* is expressed primarily in midgut tissue, with lesser expression in the Malpighian tubules and nervous tissue. *TrnMDR2* and *trnMDR3* are both expressed in nerve tissue, with the latter present in much higher abundance. Small changes in mRNA transcript abundance of *trnMDR1* and *trnMDR2* occur when the insect was exposed to the insecticide deltamethrin, being downregulated in some tissues and upregulated in others. These somewhat contradictory results demonstrate the need for additional research to clarify their potential involvement in insecticide susceptibility.

#### 1.5.5 RNA Interference in *Trichoplusia ni*

RNA interference in *T. ni* has previously been examined using cell cultures, as well as through injection or ingestion. In cell cultures, transfection of dsRNA with complementarity to *Tn-caspase-1* mRNA decreased transcript abundance and prevented apoptosis (Heber et al., 2009). Injection of dsRNA used to downregulate endogenous developmental genes in *T. ni* larvae (Kim et al., 2007; Kramer, 2003) found that that cells are capable of taking up dsRNA and causing a silencing response indicative of a full RNAi pathway. In their review Terenius et al. (2013) reported that feeding dsRNA to *T. ni* larvae can cause downregulation: however the expression reduction was low and the data have not been published in the primary literature. In general, Lepidoptera are less amenable to dsRNA-mediated RNAi than other orders, so it is improbable that ingested RNAi will be as successful in *T. ni* as in insects such as *L. decemlineata* (Shukla et al., 2016; Terenius et al., 2011).

### 1.6 Objectives

Crop losses by pest insects significantly impacts global food production and the emergence of insecticide resistance clearly exacerbates the problem. Understanding the biological mechanisms behind resistance will allow us to develop methods to prevent, eliminate or circumnavigate this problem, which would be valuable for the agriculture industry and global food safety. *MDR* genes are associated with xenobiotic resistance in a

multitude of organisms, including insects, making them likely contributors to insecticide resistance and important targets for research. RNAi is often used to investigate gene function and is now being adapted as a pest control technique, but has varied success in different insect species. Therefore two hypotheses were used to guide my experiments: *L. decemlineata* and *T. ni* tolerance for ivermectin correlate with expression levels of their *MDR* genes; and RNAi is more effective in *L. decemlineata* than in *T. ni* because of higher RNase enzyme activity in *T. ni* body fluids. The objectives of my research were to (i) identify and characterize *MDR* genes by their tissue expression in *L. decemlineata*, (ii) test and compare ingested dsRNA as a delivery method for RNAi of *MDR* genes in *L. decemlineata* and *T. ni*, and (iii) provided that RNAi is successful, use it to silence *MDR* genes in *L. decemlineata* and *T. ni* to investigate their involvement in insecticide tolerance.

## Chapter 2

### 2 Materials and Methods

#### 2.1 Insect Rearing

All insects used came from laboratory colonies reared in the London Research and Development Centre in London, Ontario. Insect strains used in experiments showed no previous resistance to insecticides. The *L. decemlineata* were reared on potato plants (*Solanum tuberosum* var. Kennebec), *T. ni* were reared on cabbage plants (*Brassica oleracea* var. Golden Acre) and *Manduca sexta*, used for dsRNA degradation assays, were reared on tobacco plants (*Nicotiana tabacum*). All were reared at 25°C, 50% relative humidity under a 16L:8D photoperiod, and all bioassays were conducted under these same conditions.

#### 2.2 Identification of *MDR* Genes in *L. decemlineata*

*MDR* genes in *L. decemlineata* were identified using the BLAST program and the QIAGEN® CLC Genomics Workbench by comparing known *MDR* transcripts from *T. ni* to two *L. decemlineata* transcriptomes to identify potential *MDR* sequences. The transcriptomes were acquired from Kumar et al. (2014) and an unpublished transcriptome from the London Research and Development Center (London, Ontario, Canada). Three different sequences were acquired and compared to translated *MDR* protein sequences from the Asian long-horned beetle, *Anoplophora glabripennis*, the leaf beetle, *Chrysomela tremula*, and the red flour beetle, *Tribolium castaneum*, using BLAST.

#### 2.3 Tissue Expression of *MDR* Genes in *L. decemlineata*

Adult beetles that were less than 7 days old were anesthetized on ice then pinned in wax-bottomed 100 mm Petri dish filled with Calpode's insect saline solution (pH 7.2, 10.7 mM NaCl, 25.8 mM KCL, 90 mM glucose, 29 mM CaCl<sub>2</sub>, 20 mM MgCl<sub>2</sub>, and 5 mM HEPES). The head was removed to obtain brain tissue while midgut and Malpighian tubule tissues were obtained via an anterior-posterior incision along the ventral side of the body. Each replicate of brain or midgut tissues used material from three insects, while

a replicate of Malpighian tubule tissue used material from 12 insects. All tissue for a replicate was combined in a 1.5 mL microcentrifuge tube containing 300  $\mu$ L of RNAlater®, stored at 4°C overnight then held at -20°C for long term storage until total RNA was extracted using the QIAGEN® RNeasy® Mini Kit. This RNA was used for cDNA synthesis and qRT-PCR to measure the relative expression of each gene in each tissue (Fig. 1). Three replicates of each tissue type were used.

## 2.4 L4440 Plasmid and HT115(DE3) *E. coli*

### 2.4.1 L4440 Plasmid

The L4440 plasmid confers ampicillin resistance for bacterial selection and its multi-cloning site (MCS) is flanked by two convergent T7 RNA polymerase promoters for RNA synthesis of both strands of an insert simultaneously. The L4440 vector was a gift from Andrew Fire (Addgene plasmid # 1654). For *in vivo* dsRNA production, fragment + L4440 plasmid constructs were transformed into HT115(DE3) strain *E. coli*.

### 2.4.2 HT115(DE3) *E. coli*

The HT115(DE3) *E. coli* strain has the genotype *F*-, *mcrA*, *mcrB*, IN(*rrnD-rrnE*)1 *rnc14::Tn10*(DE3) lysogen: *lacUV5* promoter-T7 polymerase (IPTG-inducible T7 polymerase) (RNase III minus). The strain is tetracycline resistant, RNase deficient and has isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG)-inducible T7 polymerase for dsRNA synthesis from cloned DNA fragments that have been inserted into the L4440 plasmid MCS. Reduced RNase levels in this bacterial host allow greater yields of dsRNA to be produced.

## 2.5 *In vivo* Transcription of dsRNA for *L. decemlineata*

### 2.5.1 Fragment Design and Synthesis

*L. decemlineata* dsRNA was produced *in vivo* through bacterial expression because the method was logistically preferable. It was cheaper and easier to use than *in vitro* synthesis for creating high volumes of dsRNA.

Pairs of primers (Table 2) for PCR amplification of a fragment from each of *LedMDR1*, *LedMDR2*, and *LedMDR3* mRNA transcripts in *L. decemlineata* were designed using sequences acquired from the unpublished London Research and Development Center *L. decemlineata* transcriptome. The fragments were chosen from areas that had low homology between each transcript to minimize possible cross-target effects on different *MDR* transcripts. The primers included recognition sites for Not1 and Sal1 restriction enzymes on the forward and reverse primers, respectively, to facilitate ligation into the L4440 plasmid. Each fragment was amplified by PCR using cDNA synthesized from total RNA taken from head, midgut and Malpighian tissue as a template in four 50  $\mu$ L reactions using 25 ng of cDNA per reaction. The following PCR cycle was used: an initial step at 94°C for 3 min, then 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, then a final step at 72°C for 7 min. The PCR reactions were purified and concentrated using Amicon® Ultra 30k filters. The purified PCR products and the L4440 plasmid were digested with Not1 and Sal1 enzymes for 16 h at 37°C, which were then deactivated at 65°C for 5 minutes. Successful digestion was verified by visualizing and comparing the digested products to undigested products through gel electrophoresis using a 1.5% agarose gel. The successfully digested products were then isolated through electrophoresis on a 1.2% agarose gel and extracted using the QIAGEN® Gel Extraction Kit. A fragment of *GFP*, acquired from a pre-constructed *GFP* + L4440 plasmid that was used in subsequent transformation and induction protocols, was used as a control for non-specific dsRNA

### 2.5.2 Ligation and Transformation

Each digested fragment was then ligated into the digested L4440 plasmid over 72 h at 15°C. The fragment + L4440 constructs were transformed into competent HT115(DE3) *E. coli* cells which were incubated on LB + agar + 100  $\mu$ g/mL ampicillin + 12.5  $\mu$ g/mL tetracycline plates at 37°C for 16 h. Transformants were screened for the insert by using them as templates in PCR amplifications with the fragment-specific primers to amplify the fragment if it was present. The transformants were inoculated into 100  $\mu$ L of low salt LB and 2  $\mu$ L of that was used as template in PCR using the same fragment-specific

primers and protocol as before. The PCR products were then visualized on a 1.2% agarose gel using electrophoresis to verify that the fragments were present.

### 2.5.3 Induction and Transcription

Transformants were induced to produce T7 RNA polymerase using IPTG to promote synthesis of dsRNA fragments. For each transformant, 20 mL of an overnight culture of the transformed bacteria were inoculated into 1 L of LB + 100 µg/mL ampicillin + 12.5 µg/mL tetracycline and 400 µL of an overnight culture were inoculated into 20 mL of LB + 1 µM ampicillin + 12.5 µg/mL tetracycline. Both cultures were incubated at 37°C while shaking at 180 rpm. The 1 L culture was induced with 1 mL of 1 M IPTG at an OD<sub>600</sub> of 0.5, while the 20 mL culture was not, and both were incubated again for 4 h. A 500 µL sample was taken from each culture and centrifuged at 10 000g for 10 min to pellet the cells. The supernatants of each sample were removed and replaced with 50 µL of RNAlater®. The samples were stored at 4°C overnight and then stored at -20°C until having total RNA extracted from them using the MasterPure™ Complete DNA and RNA Purification Kit. The RNA was visualized using electrophoresis on a 1.2% agarose gel comparing induced to non-induced samples to verify that the dsRNA fragment was successfully synthesized (Fig. 2). The remainder of the 1 L induced culture was separated into four 250 mL sterile centrifuge tubes and centrifuged at 10 400 g, 10°C to pellet the cells. The supernatants were removed and the pellets were washed with 5 mL of PBS, resuspended in 25 mL of PBS, combined, divided into 10 mL aliquots, and then stored at -80°C until being used for feeding assays.

## 2.6 *In vitro* Transcription of dsRNA for *T. ni*

*T. ni* dsRNA was produced *in vitro* instead of *in vivo* as it allowed for easily quantifiable dsRNA. Quantifiable amounts of dsRNA allowed for controlled dosing of dsRNA to ensure that high quantities were being ingested by the *T. ni* larvae. *TrnV-ATPaseA* was targeted to serve as a positive control to further evaluate how effective ingested dsRNA is in *T. ni*. A *V-ATPase* gene was chosen because similar genes have been successfully silenced in the lepidopterans *M. sexta* (Whyard et al., 2009) and *H. armigera* (Mao et al., 2015), with observable phenotypic changes. Multiple dsRNA fragments for each of

*TrnV-ATPaseA* and *TrnMDR1* were also used to target each transcript to increase the chance that at least one would be effective in case sequence choice affected silencing efficiency. A *GFP* fragment was also used, similar to *L. decemlineata*.

Pairs of primers (Table 2) for PCR amplification of 3 different fragments of the *TrnV-ATPase A* and *TrnMDR1* mRNA transcripts were designed using sequences acquired from Simmons et al. (2013) and a *T. ni* transcriptome assembly (Chen et al., 2014). The fragment sequences were chosen to target different areas of each gene. The primers for *TrnV-ATPaseA* included T7 RNA polymerase transcription promoter sequences on their ends, so that each fragment would contain convergent promoters for dsRNA transcription. They were amplified by PCR using cDNA synthesized from total RNA of whole second instar larvae as template in four 50  $\mu$ L reactions using 25 ng of cDNA per reaction. The *GFP* and *TrnMDR1* fragments were amplified using primers consisting of the sequence of the T7 RNA polymerase promoter region to amplify them from fragment + L4440 constructs created previously. The same protocol was followed as for fragments from *L. decemlineata*. The same protocols for PCR amplification and purification as the *L. decemlineata* fragments were also used. These purified products were used as templates in the Promega™ T7 Ribomax™ Express RNAi System to synthesize dsRNA. Successful synthesis was verified by visualizing the dsRNA products on a 1.2% agarose gel using electrophoresis (Fig. 3).

## 2.7 *L. decemlineata* RNAi Silencing and Mortality Assays

### 2.7.1 RNAi Silencing Assays

Prior to feeding treatments, beetles were kept in groups of at least 20 and fed untreated potato leaves *ad libitum*. For each replicate of each treatment (HT115; *GFP* dsRNA; *MDR1* dsRNA; *MDR2* dsRNA; and *MDR3* dsRNA), leaf clippings from potato plants were submerged in PBS containing the appropriate induced cells and allowed to air dry. Untreated leaf clippings were used for a control treatment. The leaf clippings were placed in a 150 mm Petri dish with air holes in the lid and moist filter paper on the floor of the dish. Four or five beetles less than 7 days old were placed in the dish with the enough leaf clippings to feed *ad libitum*. Each dish was considered a replicate and six replicates were

performed for each treatment. The filter paper was re-moistened and the food replaced every 24 h for 72 h, at which point 3 beetles were removed and dissected for head, midgut, and Malpighian tubule tissues. All tissues from the 3 insects were pooled together and stored in RNAlater® at 4°C overnight, then moved to -20°C until RNA was extracted from them. Total RNA was extracted from these tissues using the QIAGEN® RNeasy® Mini Kit. This RNA was used for cDNA synthesis and qRT-PCR to measure the expression levels of each gene (Fig. 4).

### 2.7.2 dsRNA + Ivermectin Survival Assays

To test for ivermectin toxicity after gene silencing the same protocol was used except 13 or 14 beetles were placed in each Petri dish rather than 4 or 5. Ten beetles were placed in individual 100 mm Petri dishes with moistened filter paper and an 8 mm diameter leaf that had been treated with 2 microlitres of 5 ppm ivermectin. The dose was chosen as an approximate LD50 based on toxicity tests performed on *L. decemlineata* (Appendix A1). The insects were allowed to feed on the disc overnight after which they received no food. They were assessed daily for 7 days for paralysis (unable to exhibit normal walking behaviour) or death. No paralyzed beetles recovered so they were deemed moribund and counted as deaths when analyzing the data. Each beetle was considered a replicate. Kaplan-Meier estimators were used to model the survival of the insects in each treatment and a log-rank test was performed to compare the survival rates (Fig. 5).

### 2.7.3 Verapamil + Ivermectin Survival Assays

Verapamil was used to inhibit all ABC transporter activity at the protein level to verify if phenotypic changes caused by direct transporter inhibition would be similar to those caused by transcript downregulation.

Sixty beetles that were less than 7 days old were chilled on ice, and then 30 had 1 µL of 1 mM verapamil dissolved in acetone topically applied to the underside of their abdomen. The remaining 30 had acetone topically applied and served as controls. The verapamil dosage was based off of a similar assay performed by Hou et al. (2016). Each beetle was then fed a dose of ivermectin and then observed for 7 days using the same protocol as in the dsRNA + ivermectin survival assays (Fig. 6).

## 2.8 *T. ni* RNAi Silencing Assays

For each replicate of each treatment (*GFP* dsRNA; *V-ATPaseA* Frag 1, 2, 3; *MDR1* Frag 1, 2, 3), 2 µg of dsRNA were applied to three or four 6 mm diameter cabbage leaf discs and allowed to air dry. Untreated cabbage leaf discs were used as a control treatment. One second instar *T. ni* larva was placed with each leaf disc in a 50 mm petri dish lined with moist filter paper. The disc was replaced and the filter paper re-moistened every 24 h for 72 h. Six replicates were performed for each treatment. Two or three larvae from each replicate were placed in a 1.5 mL centrifuge tube, chilled on ice for 5 minute then submerged in 150 µL of *RNAlater*®, held overnight at 4°C then stored at -20°C until total RNA was extracted using the *QIAGEN*® *RNeasy*® Kit. This RNA was used for cDNA synthesis and qRT-PCR to measure the relative expression levels of each gene (Fig. 7 and 8).

## 2.9 cDNA Synthesis and Real-time Quantitative PCR (qRT-PCR)

### 2.9.1 cDNA Synthesis

Total RNA samples were DNase-digested using the *Ambion*™ *Turbo DNA-free*™ Kit prior to synthesis in order to remove any contaminating DNA. cDNA was then synthesized using the *Invitrogen*™ *SuperScript*® III First-Strand Synthesis System for RT-PCR. Successful synthesis of cDNA was verified by using the cDNA products as templates in PCR. The same PCR protocol for amplification of *L. decemlineata* RNAi fragments was used, with 25 ng of cDNA used as template and qPCR primers for *L8e* and *EIF4α* for *L. decemlineata* and *T. ni*, respectively, were used for amplification. The products were visualized by electrophoresis on a 1.2% agarose gel to verify successful amplification, indicating the presence of cDNA.

### 2.9.2 qRT-PCR

The *Bio-Rad* *SsoFast*™ *EvaGreen*® *Supermix* kit, *Bio-Rad* *C1000*™ *Thermal Cycler*, and *Bio-Rad* *CFX96*™ *Real-Time Detection System* were used for all qPCR reactions. Primer pairs for each gene of interest were designed such that each amplicon was located

outside of the sequences used for the dsRNA fragments to avoid false positives. Primer pairs for the *L8e* and *EIF4a* transcripts were designed for use as reference genes in *L. decemlineata* and *T. ni*, respectively. Amplification efficiency of these primers was determined by using 2x dilution series starting with a concentration of 2.5 ng/ $\mu$ L and diluting down to 0.078 ng/ $\mu$ L of cDNA template in 10  $\mu$ L reactions. The following PCR cycle was used: an initial step of 95°C for 3 min, then 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s. Fluorescence was measured at the end of every cycle and a melt curve from 95 to 65°C performed to ensure no off-target products were amplified. Primer pairs were only used if their amplification efficiency was within 90-110%. No- template controls were included for every primer pair and all reactions were performed in duplicate. When measuring the genes of interest in insect cDNA samples, the same PCR cycle was used, 2.5 ng/ $\mu$ L of cDNA template was used, no-template controls were included, and each reaction was performed in triplicate. Expression of *L8e* was measured in all *L. decemlineata* samples and *EIF4a* was measured in all *T. ni* samples. The  $\Delta\Delta$ Ct method was used to analyze the expression data with all Ct values in *L. decemlineata* being normalized to *L8e* Ct values in the same sample, and *T. ni* Ct values being normalized to *EIF4a*. Ct values to generate the  $\Delta$ Ct values. When measuring localized expression of *MDR* transcripts in *L. decemlineata*,  $\Delta\Delta$ Ct values were calculated relative to the tissue with the highest expression for each gene. When measuring expression of genes after attempted silencing in both *L. decemlineata* and *T. ni*,  $\Delta\Delta$ Ct values were calculated relative to the expression in insects fed untreated potato or cabbage. To determine statistical significance, one-way ANOVA and Tukey's HSD tests ( $p < 0.05$ ) were used to determine differences between  $\Delta$ Ct values.

**Table 1:** Primers used for dsRNA fragment synthesis and qPCR expression measurement of genes in dsRNA-fed Colorado potato beetles and cabbage loopers. Sequences added for restriction enzyme digestion or for T7 transcription are indicated by square brackets.

Primer	Sequence
<b>Primers for Fragment + L4440 Construction</b>	
<i>LedMDR1</i> For	[GAGCGGCCGC]TGTTTCATGATTTATTCTAGT
<i>LedMDR1</i> Rev	[GCAGGTCGAC]ATCTGGTCTGACGGATAG
<i>LedMDR2</i> For	[GAGCGGCCGC]AAGTTAGCCGTGGAAGCCAT
<i>LedMDR2</i> Rev	[GCAGGTCGAC]TCCCACGCACAATTCACTGG

<i>LedMDR3</i> For	[TAGCGGCCGC]AGTGGGAAGACGCCATCAGT
<i>LedMDR3</i> Rev	[GCAGGTCGAC]TGCCATACCACCAACATAACGA
<i>TrnMDR1</i> Frag 1 For	[GAGCGGCCGC]CTCCAGACGTTCCGTTTACA
<i>TrnMDR1</i> Frag 1 Rev	[GCAGGTCGAC]TTACGAAGGTCAGGGCGACTA
<i>TrnMDR1</i> Frag 2 For	[GAGCGGCCGC]ATACCCAACCTCGTCCGATGG
<i>TrnMDR1</i> Frag 2 Rev	[GCAGGTCGAC]TACATCTTGGGGCTCGTATG
<i>TrnMDR1</i> Frag 3 For	[GAGCGGCCGC]ATACCCAACCTCGTCCGATGG
<i>TrnMDR1</i> Frag 3 Rev	[GCAGGTCGAC]TACATCTTGGGGCGTCTGATG
<b>Primers for <i>in vitro</i> dsRNA Synthesis</b>	
T7	TAATACGACTCACTATAGGG
<i>TrnV-ATPaseA</i> Frag 1 For	[TAATACGACTCACTATAGGG]CGGGCCACAACATCGCATA
<i>TrnV-ATPaseA</i> Frag 1 Rev	[TAATACGACTCACTATAGGG]AACGTGGAATGCAAGAGGGT
<i>TrnV-ATPaseA</i> Frag 2 For	[TAATACGACTCACTATAGGG]GTTCTACAAGACCGTGGGCA
<i>TrnV-ATPaseA</i> Frag 2 Rev	[TAATACGACTCACTATAGGG]AATATGCGATGTTGTGGCCC
<i>TrnV-ATPaseA</i> Frag 3 For	[TAATACGACTCACTATAGGG]CGAGACCGACAAGATCACCC
<i>TrnV-ATPaseA</i> Frag 3 Rev	[TAATACGACTCACTATAGGG]ATATGCGATGTTGTGGCCC
<b>qPCR Primers</b>	
q <i>LedL8e</i> For	GGTAACCATCAACACATTGG
q <i>LedL8e</i> Rev	TCTTGGCATCCACTTTACC
q <i>LedMDR1</i> For	TAGACCTCACATGGTTCAGG
q <i>LedMDR1</i> Rev	TTAGACTTCCGTTGACTTCTTC
q <i>LedMDR2</i> For	TAGTTTCCCAGGAGCCGAAC
q <i>LedMDR2</i> Rev	TTCGCACTCTTGCAGCTTTC
q <i>LedMDR3</i> For	TCGTTGGTATCTGCTCTCTTCG
q <i>LedMDR3</i> Rev	TGAGGTGCCATTATTCGATCTG
q <i>TrnEIF4<math>\alpha</math></i> For	ACCTTGCGGCGAGTGTT
q <i>TrnEIF4<math>\alpha</math></i> Rev	CTGGATACGCTGTGTGAC
q <i>TrnV-ATPaseA</i> For	TTCCATCTTTGTCGTCCTCCGT
q <i>TrnV-ATPaseA</i> Rev	CTTCAAACCGCCTTTGGTTCG
q <i>TrnMDR1</i> For	GCTGCCTGTTCTACGCCTAT
q <i>TrnMDR1</i> Rev	CACCTTCCCACACTGACCTC

## 2.10 Comparison of dsRNA Degradation in Midgut Lumen and Hemolymph

Degradation of dsRNA in hemolymph and midgut lumen of *L. decemlineata*, *M. sexta*, and *T. ni* was compared to determine if gut dsRNase enzymes could be a mitigating factor for RNAi in *T. ni*. *Manduca sexta* was included as it is a lepidopteran where RNAi

has had some success. Thus, *T. ni* could be compared to multiple relevant species to establish its relative refractoriness to ingested dsRNA.

### 2.10.1 Midgut Lumen and Hemolymph Extraction

The hemolymph and lumen content were obtained from <7 day old *L. decemlineata* adults, 4<sup>th</sup> instar *T. ni* and 3<sup>rd</sup> instar *M. sexta* larvae that were first held at -20°C for 5 minutes. Hemolymph was pipetted from a pinhole made in the ventral surface of the *L. decemlineata* abdomen, while for the caterpillars it was obtained from an incision made in one of the prolegs. In all three, the midgut content was obtained by removing the midgut via a lengthwise incision along the ventral surface, placing it in a 1.5 mL microcentrifuge tube and then gently pressing with a pestle. The samples of hemolymph and midgut contents were kept on ice and hemolymph was preserved with 2 mg of N-Phenylthiourea/10 µL. The hemolymph from 3-4 insects was combined and centrifuged at 16 000 g for 7 min, while the gut contents from 2-3 insects were combined and centrifuged at 6 000 g for 7 min. Only the supernatants were used in degradation assays that were conducted the same day as the extractions were made.

### 2.10.2 Comparison of dsRNA Degradation

Serial dilutions were created starting at 100% hemolymph and 10% midgut lumen then diluting 2x using PBS until reaching concentrations of 1.56% hemolymph and 0.156% midgut lumen. One microgram of *GFP* dsRNA synthesized *in vitro* was added to 10 µL of each dilution and incubated at room temperature for 90 minutes. One microgram of *GFP* dsRNA was added to 10 µL of PBS as a control. The remaining dsRNA from each reaction was then visualized on a 1.2% agarose gel using gel electrophoresis (Fig. 9 and 10).

## Chapter 3

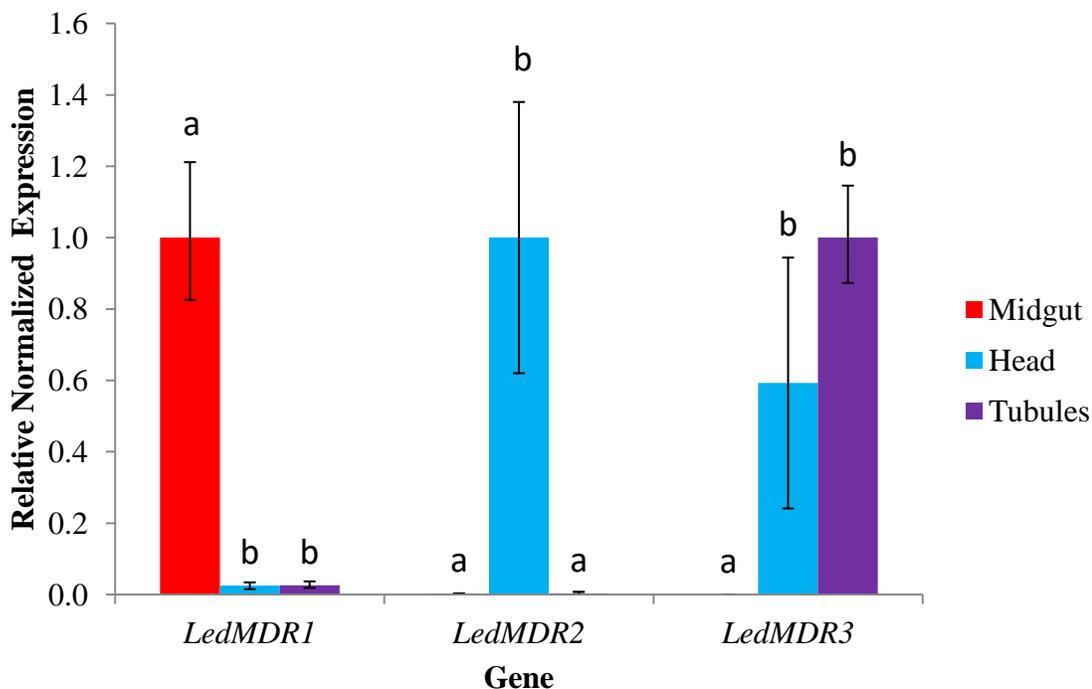
### 3 Results

#### 3.1 Identification of *MDR* Genes in *L. decemlineata*

Three potential *MDR* genes were identified in *L. decemlineata* after comparison of *L. decemlineata* transcriptomes to the *T. ni* transcriptome. Each putative *MDR* gene in the beetle had moderate to high identity at the amino acid level (52-74%) with the predicted *MDR* and *MDR*-like proteins from *A. glabripennis*, *C. tremula*, and *T. castaneum*. Each sequence also showed the conserved domains typical of *MDR* proteins: ABC transmembrane domains, ATP-binding cassettes, Walker A and B motifs; and ABC transporter signature motifs. The genes were named *LedMDR1*, *LedMDR2*, and *LedMDR3*.

#### 3.2 Tissue Expression of *MDR* Genes in *L. decemlineata*

To characterize the expression profiles of each of the three new *LedMDR* genes in *L. decemlineata*, qPCR was used to measure their relative expression levels in three tissues in which *MDR* genes are commonly expressed: midgut, nervous, and Malpighian tubule tissues. Dissected heads were used to serve as nervous tissue. *LedMDR1* had an approximately 40-fold higher expression in midgut tissue than head and Malpighian tubule tissues (Fig. 1,  $p < 0.05$ ), while *LedMDR2* had 300-500 fold higher expression in head tissue when compared to the other tissues (Fig. 1,  $p < 0.05$ ). *LedMDR3* had similar levels of expression in head and Malpighian tubule tissues that were significantly high than in the midgut tissue in which there was no detectable expression (Fig. 1,  $p < 0.05$ ).

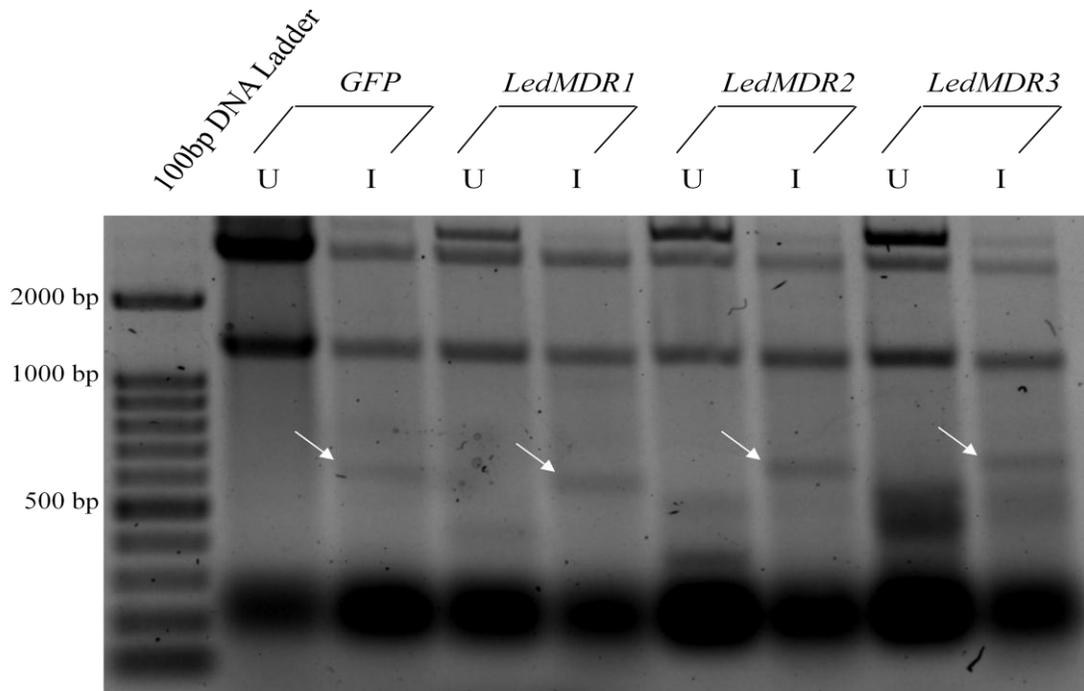


**Figure 1:** Expression levels of each *MDR* gene in midgut, head, and Malpighian tubule tissue of adult *L. decemlineata*. Bars represent the mean normalized fold expression of each gene ( $\pm$  SEM,  $n=3$ ) relative to the tissue in which each gene is most highly expressed. Different letters represent significantly different expression levels within each gene (Tukey's HSD,  $p<0.05$ ).

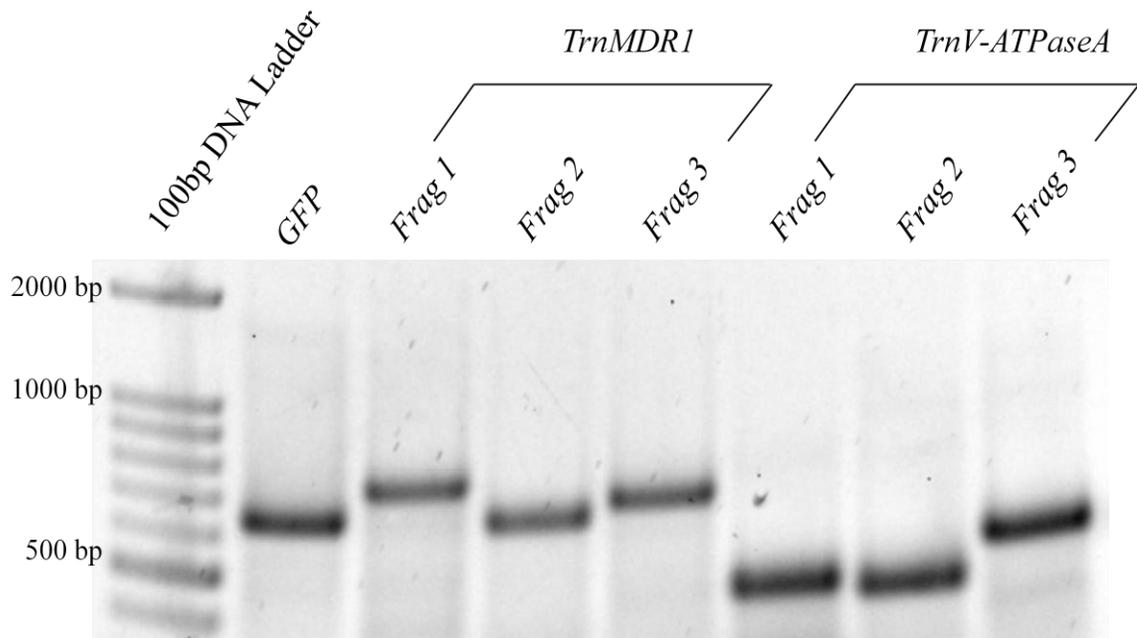
### 3.3 Verification of dsRNA Synthesis

The dsRNA fragments used for RNAi in *L. decemlineata* were produced in HT115 *E. coli* cells designed for inducible expression of dsRNA molecules. The dsRNA fragments used for RNAi in *T. ni* were produced *in vitro* from PCR templates or plasmids. Synthesis of the fragments was verified by visualizing the fragments using gel electrophoresis. Each of the HT115 *E. coli* strains transformed with a fragment + L4440 plasmid construct successfully synthesized the dsRNA fragments in high quantities upon induction with IPTG (Fig. 2). Each of the induced strains produced high quantities of nucleic acid fragments of the correct size. Each of the *T. ni* dsRNA fragments were also successfully synthesized *in vitro* (Fig. 3). Fragments of the correct size were able to be produced at

high concentrations (1.5  $\mu\text{g}/\mu\text{L}$  or greater), so that *T. ni* could be given high doses during feeding assays.



**Figure 2:** Gel visualization of nucleic acid extracts from bacteria transformed with *L. decemlineata* fragment + L4440 plasmids. Each lane contains total nucleic acids extracted from one of the transformed strains, as indicated by the labels. Each strain was either uninduced (U) or induced (I) to produce dsRNA before extraction. The synthesized dsRNA fragments are indicated by white arrows.

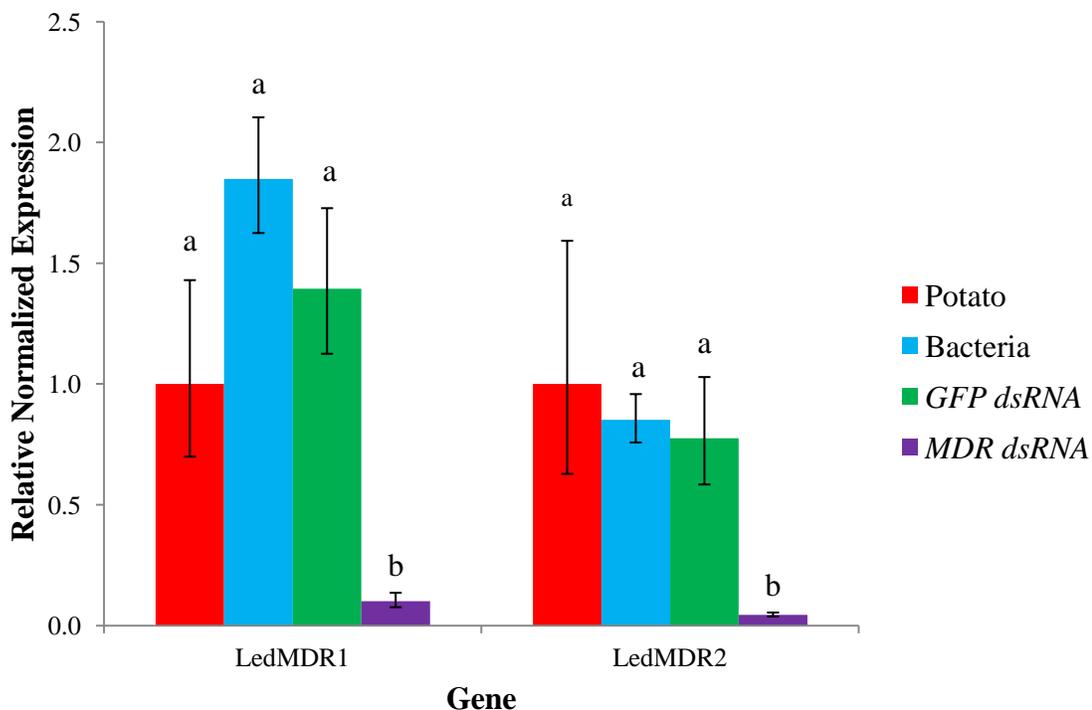


**Figure 3:** Gel visualization of *in vitro* synthesized *T. ni* dsRNA fragments. Labels indicate the target gene for each fragment as well as each fragment's name. Each lane contains 1  $\mu$ g of synthesized dsRNA.

### 3.4 *L. decemlineata* RNAi Silencing Assays

RNAi through feeding of bacterially-expressed dsRNA fragments was attempted for downregulation of *LedMDR1*, *LedMDR2*, and *LedMDR3* in *L. decemlineata* beetles. The insects were fed one of the following treatments: untreated potato foliage, potato foliage treated with non-dsRNA-expressing bacteria, potato foliage treated with *GFP* dsRNA-expressing bacteria, or potato foliage treated with bacteria expressing dsRNA specific to *LedMDR1*, *LedMDR2*, or *LedMDR3*. The relative expression levels of each gene were measured using qPCR after each feeding treatment. *LedMDR1* and *LedMDR2* were significantly downregulated in adults after ingestion of bacteria containing respective gene-specific dsRNA fragments compared to those fed with potato only, dsRNA-free bacteria, or *GFP* dsRNA-producing bacteria. (Fig. 4) Compared with controls *LedMDR1* and *LedMDR2* expression in the three tissues combined was 90% and 96% silenced, respectively, with no significant differences between the other treatments. *LedMDR3*

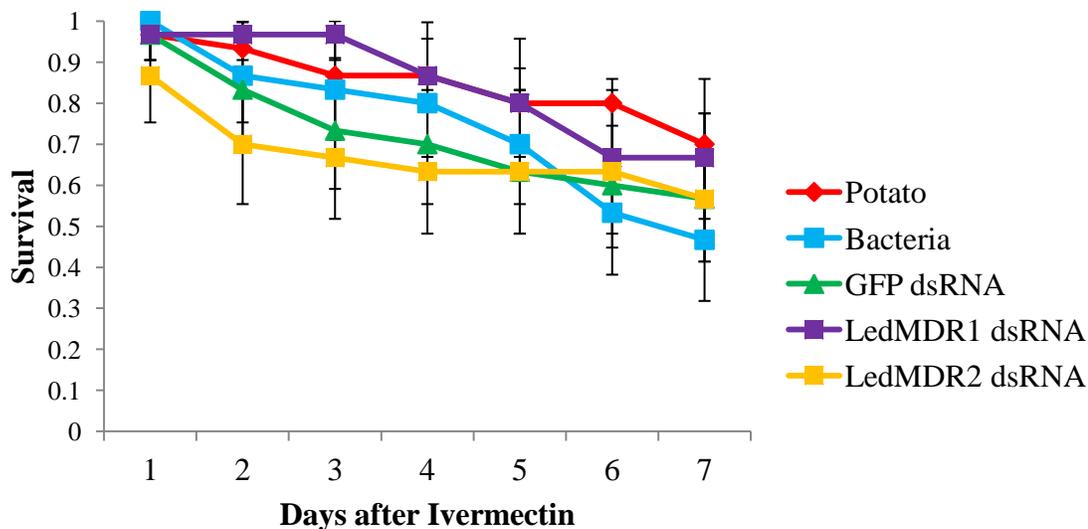
expression couldn't be measured in the silencing assays as the transcript abundance was too low for qPCR to provide consistently accurate measurements.



**Figure 4:** Expression levels of each *LedMDR* gene in adult *L. decemlineata* fed with one of the indicated treatments. Bars represent the mean normalized fold expression ( $\pm$  SEM, n=6) relative to each gene's expression in insects fed with potato. Different letters represent significantly different expression levels within each gene (Tukey's HSD,  $p < 0.05$ ).

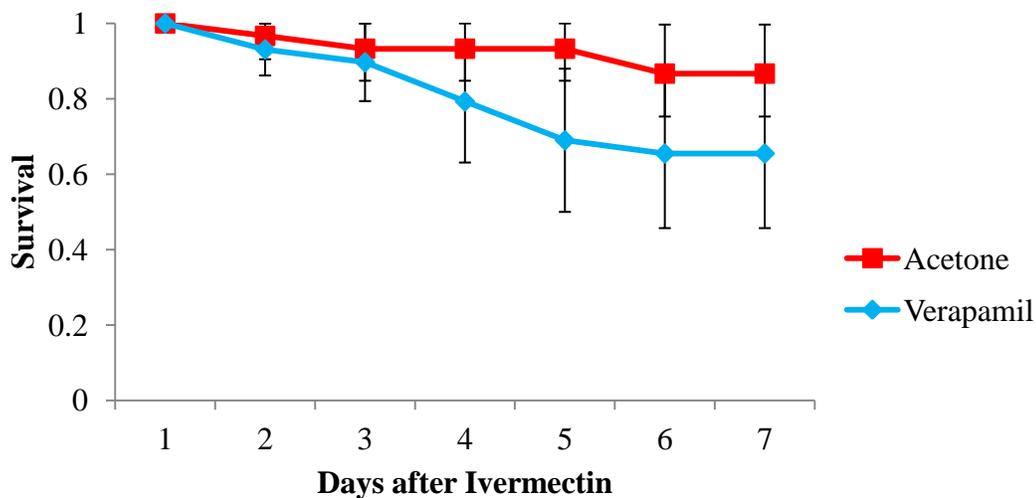
### 3.5 *L. decemlineata* Survival Assays

To test if downregulation of *LedMDR* genes had an effect on *L. decemlineata* tolerance for the insecticide ivermectin, *L. decemlineata* beetles were fed a dose of ivermectin after being fed one of the dsRNA feeding treatments. When challenged with ivermectin the survival of the beetles feeding on foliage treated with bacteria or bacteria expressing dsRNA did not differ significantly from those fed with untreated potato leaves (Fig. 5, log-rank test,  $p = 0.372$ ).



**Figure 5:** Proportion of *L. decemlineata* adults surviving in different dsRNA feeding treatments after receiving 2  $\mu$ L of 5 ppm ivermectin. Error bars represent 95% confidence intervals (n=30). No significant differences were observed between treatments (Log-rank test, p=0.372).

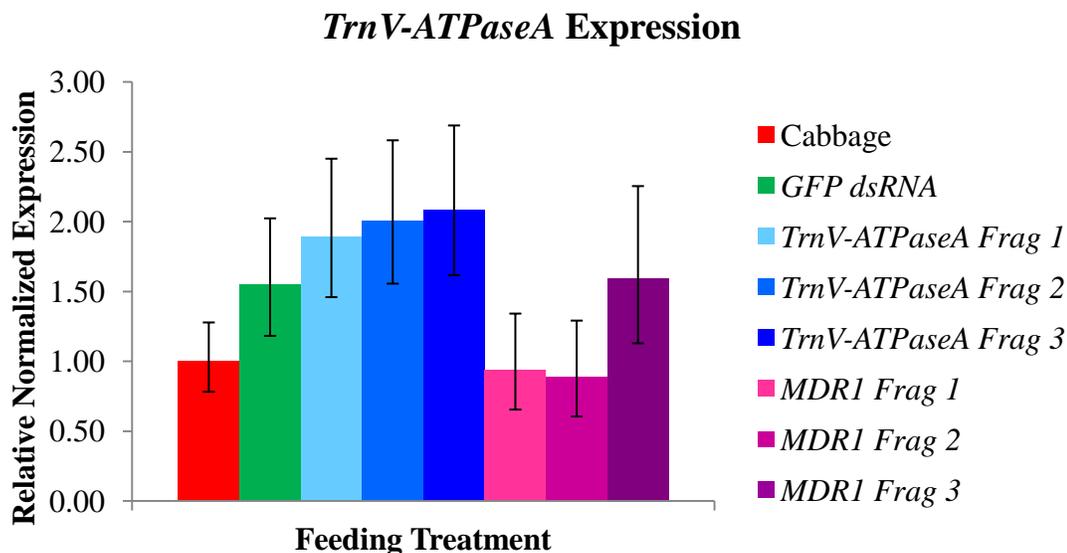
Since no significant changes in ivermectin tolerance were caused by downregulation of *LedMDR1* or *LedMDR2*, another survival assay was performed using verapamil to inhibit all MDR transporters in *L. decemlineata*. This would reveal if any MDR transporters other than *LedMDR1* or *LedMDR2* are involved in ivermectin tolerance. *L. decemlineata* beetles were given a topical treatment of either acetone to serve as a control, or verapamil before being fed a dose of ivermectin. There was no significant difference in survival between beetles treated with verapamil compared to those treated with acetone after both were challenged with ivermectin (Fig. 6, log-rank test, p=0.0547).



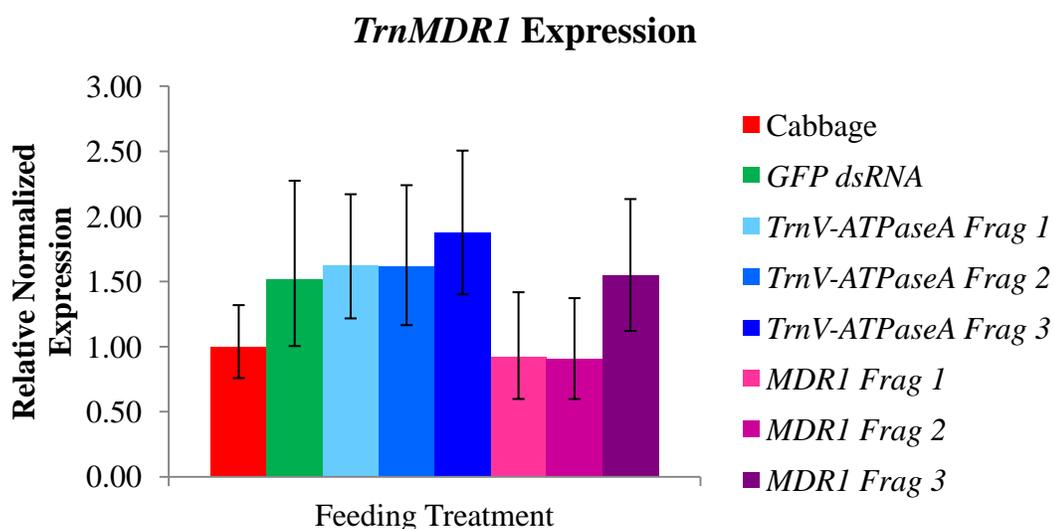
**Figure 6:** Proportion of *L. decemlineata* adults surviving in different feeding treatments each day after receiving 2  $\mu$ L of 5 ppm ivermectin. Error bars represent 95% confidence intervals (n=30). A significant difference was not observed between the two treatments (Log-rank test,  $p=0.0547$ ).

### 3.6 *T. ni* RNAi Silencing Assays

RNAi through feeding of *in vivo*-synthesized dsRNA fragments was attempted for downregulation of *TrnV-ATPaseA* and *TrnMDR1* in second instar *T. ni* larvae. The larvae were fed untreated cabbage foliage, foliage treated with *GFP* dsRNA, or foliage treated with dsRNA fragments specific to *TrnV-ATPaseA* or *TrnMDR1*. The relative expression levels of each gene were measured using qPCR after the feeding treatments. No significant differences in *TrnV-ATPaseA* (Fig. 7, Tukey's HSD,  $p>0.05$ ) or *TrnMDR1* (Fig. 8, Tukey's HSD,  $p>0.05$ ) expression were found between insects in any of the different feeding treatments.



**Figure 7:** Expression levels of *TrnV-ATPaseA* in *T. ni* larvae fed different foliage treatments. Bars represent mean normalized fold expression levels ( $\pm$  SEM,  $n=6$ ) relative to the expression of *TrnV-ATPaseA* in *T. ni* fed with cabbage. No significant differences were found between feeding treatments (Tukey's HSD,  $p>0.05$ ).

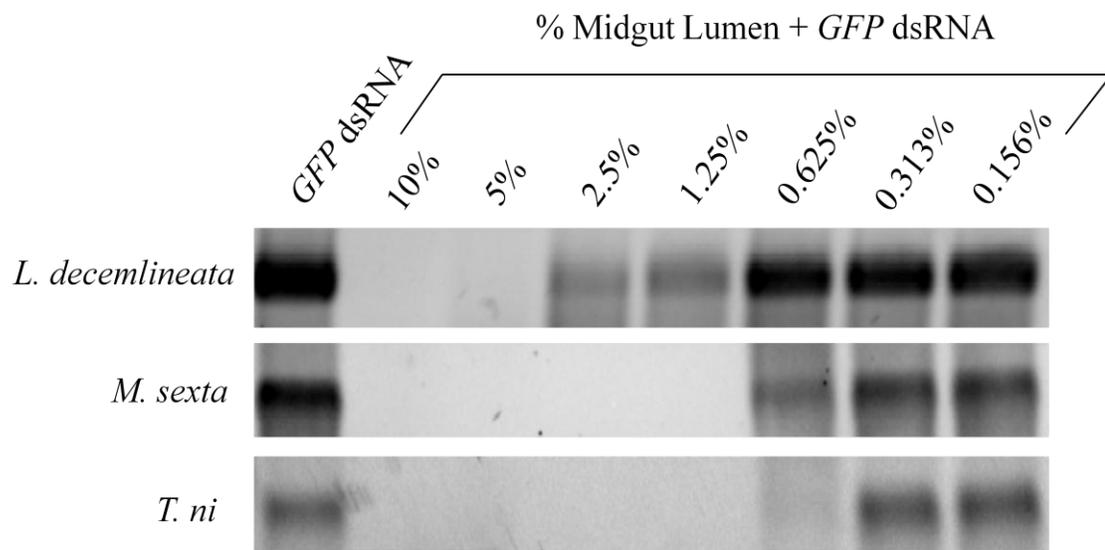


**Figure 8:** Expression levels of *TrnMDR1* in *T. ni* larvae fed different foliage treatments. Bars represent mean normalized fold expression levels ( $\pm$  SEM,  $n=6$ ) relative to the expression of *TrnMDR1* in *T. ni* fed with cabbage. No significant differences were found between feeding treatments (Tukey's HSD,  $p>0.05$ ).

### 3.7 Comparison of dsRNA Degradation in Midgut Lumen and Hemolymph

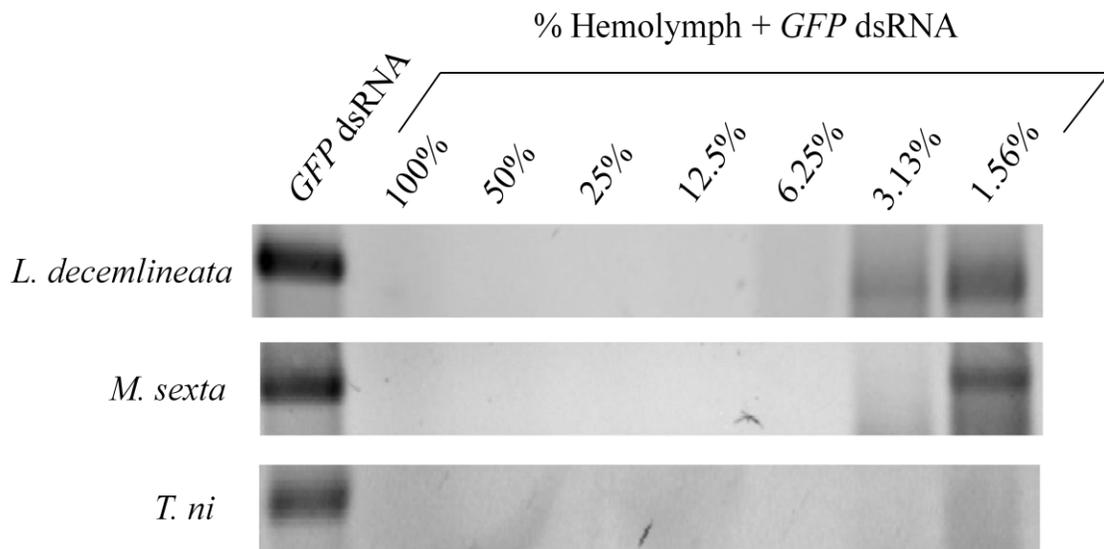
To determine the relative stability of dsRNA molecules in the body fluids of *L. decemlineata* and *T. ni*, *GFP* dsRNA was incubated in serial dilutions of midgut lumen contents or hemolymph from each insect before being visualized by gel electrophoresis. *GFP* dsRNA was also incubated in midgut lumen contents or hemolymph from *M. sexta* to provide a comparison to another lepidopteran insect.

dsRNA degradation capacity of midgut contents from the lepidopteran larvae was much greater (*M. sexta* fluids degraded the dsRNA slightly less than *T. ni*) than in *L. decemlineata* adults (Fig. 9). In both *M. sexta* and *T. ni* dsRNA was fully degraded at concentrations of 1.25% while it required 5% in *L. decemlineata*.



**Figure 9:** Gel visualization of *GFP* dsRNA incubated for 90 minutes in midgut lumen contents of *L. decemlineata*, *M. sexta*, and *T. ni*. The first lane of each image contains 1 µg of *GFP* dsRNA incubated in PBS. Each other lane contains 1 µg *GFP* dsRNA incubated in the indicated concentration of midgut lumen contents diluted in PBS.

The dsRNA degradation in hemolymph followed a similar pattern to the midgut lumen contents (Fig. 10). In *L. decemlineata*, dsRNA was fully degraded at hemolymph concentrations of 6.25% compared with 3.13% in *M. sexta* and 0.156% in *T. ni*.



**Figure 10:** Gel visualization of *GFP* dsRNA incubated for 90 minutes in hemolymph of *L. decemlineata*, *M. sexta*, and *T. ni*. The first lane of each image contains 1  $\mu$ g *GFP* dsRNA incubated in PBS. Each other lane contains 1  $\mu$ g *GFP* dsRNA incubated in the indicated concentration of hemolymph diluted in PBS.

## Chapter 4

### 4 Discussion

Investigating the nature of insecticide resistance and developing novel pest management techniques will be crucial for formulating future agricultural practices. The data presented in this thesis provide knowledge about the role of *MDR* genes in *L. decemlineata* tolerance for insecticides as well as elucidating differences between insects that are responsive to RNAi compared to those that are not.

Three *L. decemlineata* genes; *LedMDR1*, *LedMDR2*, and *LedMDR3* were identified and their tissue expression characterized across midgut, nervous, and Malpighian tubule tissues. Each gene had a different tissue expression profile, indicating that the functions of their protein products likely differ and may target different substrates for transportation across their respective membranes. Downregulation of *LedMDR1* and *LedMDR2* genes through RNAi did not significantly affect *L. decemlineata* tolerance for ivermectin, demonstrating that neither of them are strong components of ivermectin detoxification in this insect. Inhibition of MDR proteins using a chemical inhibitor, verapamil, had an effect on *L. decemlineata* ivermectin tolerance that was nearly statistically significant, but did not provide clear enough evidence to prove that ivermectin tolerance is correlated with MDR activity.

RNAi in *T. ni* was not successful when targeting either *TrnV-ATPaseA* or *TrnMDR1* with any of the dsRNA fragments utilized. This suggests that *T.ni* have a physiological barrier that prevents RNAi. Subsequent comparisons of dsRNA stability in the hemolymph and midgut lumen of *L. decemlineata*, *M. sexta*, and *T. ni* showed that dsRNA degrades faster in *T. ni* when compared to the other two insects. This result, along with similar published research, solidifies the conclusion that the physiological barrier is at least partly due to high RNase enzyme activity in lepidopteran insects.

## 4.1 Tissue Expression of *L. decemlineata* MDR Genes

The levels of expression of the *MDR* genes in *L. decemlineata* differed between the three tissues examined as reported in other organisms. In *Chrysomela populi*, a leaf beetle similar to *L. decemlineata*, the transcript abundance of 65 potential ABC transporter genes was examined between gut, Malpighian tubule, fat body, and glandular tissue (Strauss et al., 2014). Most of the genes in *C. populi* varied in expression by tissue type with some having markedly higher expression in a single tissue and others being more evenly expressed throughout all tissues. Likewise, three *MDR* genes previously investigated in *T. ni* had varied expression; one was primarily expressed in the midgut and two were almost exclusively expressed in the nervous system (Simmons et al., 2013). The differences in tissue expression, which are common across organisms, imply that the *MDR* proteins may differ in their substrates and function.

There is a high excretion of unmetabolised secondary metabolites in mammalian feces (Sorensen et al., 2004) and insect frass (Gómez et al., 1999), indicating that excretion itself is actually the primary mode of defense. Due to their general function of effluxing xenobiotics and their broad substrate specificity (Sorensen and Dearing, 2006), it has been postulated that ABC transporters are involved in this adaptive defense mechanism. While the research examining the role of ABC transporters in secondary metabolite excretion is limited, there are some examples that show direct correlations between *MDR* expression and xenobiotic tolerance. For example, *D. melanogaster* with *MDR* knockout mutations has increased susceptibility to cardenolides (Groen et al., 2017) and *MDR* expression in *Mayetiola destructor* larvae increases when they feed on resistant strains of wheat (Shukle et al., 2008). Similarly, when *H. armigera* larvae were fed different plant secondary metabolites several ABC transporter genes were upregulated in gut tissue (Bretschneider et al., 2016). Interestingly in *Chrysomela populi* ABC transporters play a role in sequestering secondary metabolites that the beetle subsequently uses in its defense against predators (Strauss et al., 2013) providing additional evidence that ABC transporters do indeed transport secondary metabolites. Given that *L. decemlineata* excretes glycoalkaloids from solanaceous plants without metabolizing them (Armer, 2004) and *LedMDR1* is almost exclusively expressed in midgut tissue would lend support

to the idea that it plays a role in the first line of defense against ingested compounds. If the substrate specificity of *LedMDR1* also includes insecticides, it could play an equally important role in insecticide resistance.

*LedMDR2* probably serves to protect against neurotoxins as it was primarily expressed in the head, which contains a concentration of nervous tissue. Multiple *MDRs* have been identified in other animals as a barrier against xenobiotics that accumulate in or attack the nervous system. Collie dogs with *MDR1* mutations compromising transporter function are specifically vulnerable to the neurotoxin ivermectin (Roulet et al., 2003), and *mdr1a* expressed in brain capillary and endothelial cells of mice prevents accumulation of a variety of drugs including ivermectin in the brain (Schinkel, 1999). Similar *MDR* genes have been identified in *D. melanogaster* (Mayer et al., 2009) and *M. sexta* (Murray et al., 1994) in which they are expressed in the CNS-humoral interface, the blood-brain barrier equivalent in insects. The expression of these genes and their corresponding proteins was linked to reduced penetration of nerve-targeting xenobiotics like nicotine. As *LedMDR2* shares a similar expression pattern to these examples, it is probably involved in the blood-brain barrier.

*LedMDR3* may serve a more general function to help excrete a wider variety of toxins as it was expressed in both the Malpighian tubules and head tissue. *MDR* genes expressed in Malpighian tubules have been linked to detoxification of plant secondary metabolites in *D. melanogaster* (Groen et al., 2017) and *M. destructor* (Say) (Shukle et al., 2008), including neurotoxins. The *MDR* in *M. sexta* that protected against nicotine was expressed in the CNS-humoral interface and the Malpighian tubules, similar to *LedMDR3*. As Malpighian tubules are the primary excretory tissue for filtering insect hemolymph, *LedMDR3* may serve as a second barrier, excreting xenobiotics against which the gut transporters don't protect.

## 4.2 RNAi Silencing Assays

### 4.2.1 *L. decemlineata* Silencing Assays

In feeding assays using *LedMDR1* and *LedMDR2* dsRNA, the targeted gene was significantly downregulated compared to controls. The insects fed with HT115 bacteria

showed no significant changes in gene expression, indicating that *MDR* expression is not affected by the ingestion of bacterial cells. Therefore using bacterial cells as a delivery method for dsRNA will not confound expression results. Similarly *GFP* dsRNA-fed insects showed no significant change in either *LedMDR1* or *LedMDR2* expression, indicating that *MDR* expression did not respond to a non-specific dsRNA molecule. As there are minimal off target effects from non-target dsRNA molecules any effects seen from *MDR*-complementary dsRNA molecules are likely due to specific targeting. It is also important to note that in *L. decemlineata* there was no upregulation of either *LedMDR1* or *LedMDR2* in response to ingested dsRNA yet in the Chinese tussar moth, *Antheraea pernyi*, injection of *hemolin*-specific dsRNA actually caused an increase rather than a decrease in the target mRNA abundance. This was interpreted as an immune response because similar results were found with baculovirus infection (Hirai et al., 2004). Large scale changes to gene expression are also possible, as feeding dsRNA to the honey bee, *Apis mellifera*, caused expression changes in genes related to RNA processing, immunity, stress response, and response to external stimulus (Nunes et al., 2013). While it is possible, even likely, that similar such changes occurred in *L. decemlineata*, neither *LedMDR1* nor *LedMDR2* were affected. The efficient downregulation of *LedMDR1* by ingested dsRNA demonstrates the ability of *L. decemlineata* to absorb and uptake dsRNA from the gut lumen into gut cells, which is consistent with other research (Cappelle et al., 2016). *LedMDR2* was also effectively downregulated, despite not being expressed in midgut tissue, showing that *L. decemlineata* are capable of spreading the RNAi effect to other tissues as seen in a study by (Zhu et al., 2011) in which five vital genes expressed in multiple tissues showed significant downregulation, and an increase in insect mortality. It should be noted that both *LedMDR1* and *LedMDR2* were successfully downregulated despite using a delivery method that did not allow for direct quantification of how much dsRNA the insects were consuming. As expression of dsRNA using HT115 *E. coli* cells is often cheaper and easier than synthesizing dsRNA *in vitro*, it is a useful method for dsRNA delivery whenever precise control of dosing is not necessary, as in this case with *L. decemlineata*.

Unfortunately *LedMDR3* expression could not be measured accurately by qPCR as I obtained inconsistent and highly variable Ct values within some samples, probably

caused by low transcript concentrations. The low concentrations were likely due to *LedMDR3* transcripts being primarily localized to Malpighian tubule tissue. During RNA extraction for the silencing assays, midgut, head, and Malpighian tubule tissues were combined from 3 insects instead of using solely Malpighian tubule tissue as for the tissue expression assay. Since the Malpighian tubule tissue has a smaller mass and volume than the other tissues, this lowered the concentration of Malpighian tubule-specific mRNA in the samples and consequently, the concentration of *LedMDR3* transcript. The expression of *LedMDR3* was already low compared to the other *LedMDR* transcripts, so this reduced the accuracy of qPCR measurements enough that transcript levels could not be consistently measured.

#### 4.2.2 *T. ni* Silencing Assays

In the case of *T. ni*, neither *TrnV-ATPaseA* nor *TrnMDR1* were downregulated by any of the dsRNA fragments used. This lack of downregulation was not likely due to inefficient dsRNA fragments, because varied amounts of silencing between the fragments would have likely been observed if that was the case. It is more likely that an inherent physiological inhibition exists in the *T. ni* larvae. These results are not unexpected, as RNAi is difficult with lepidopteran species and there is little evidence to suggest *T. ni* respond well to ingested dsRNA. However, *T. ni* cells are capable of taking up dsRNA from the hemocoel and show an interference response when the dsRNA is taken up or directly transfected into cells as shown in other research (Hebert et al., 2009; Kim et al., 2007). Unsuccessful interference from ingested dsRNA, even when targeting transcripts expressed in midgut tissue, indicate that there are barriers that prevent ingested dsRNA from being absorbed from the gut. One possibility is RNase activity or other factors within the gut that degrade the dsRNA fragments before they can be absorbed. Higher concentrations of dsRNA-targeting nucleases in *T. ni* gut lumen compared to other insects such as *L. decemlineata* could explain why RNAi through ingestion has varying effectiveness based on the species. In fact, dsRNase enzymes that are specifically excreted into the gut lumen have been identified in the silkworm, *Bombyx mori* (Arimatsu et al., 2007; Liu et al., 2012), another lepidopteran, as well as *Locusta migratoria* (Song et al., 2017). Notably, reduced effectiveness of ingested dsRNA compared to injected

dsRNA was found in an earlier study of *L. migratoria* (Luo et al., 2013). A review by Terenius et al. (2011) also noted reduced success with ingested dsRNA in the lepidopteran *Spodoptera frugiperda* compared to injected dsRNA. Information regarding insect gut nucleases is currently limited, but they have been identified in other publications as major obstacles for ingested dsRNA in pest management (Katoch & Thakur, 2012).

### 4.3 Comparison of dsRNA Degradation in Midgut Lumen and Hemolymph

To determine if dsRNA stability in body fluids is a determinant for the success of RNAi in the species studied here, relative degradation rates of dsRNA in midgut lumen and hemolymph were compared between *L. decemlineata*, *M. sexta*, and *T. ni*. The relative degradation of dsRNA in midgut lumen between *L. decemlineata*, *M. sexta*, and *T. ni* correlated with the relative success of ingested dsRNA between the three species. *L. decemlineata* had the least amount of degradation between all three species, which was expected as there are many published results successfully using ingested dsRNA in this species. *M. sexta* and *T. ni* lumen had much greater degradation than *L. decemlineata*, but similar degradation to each other, with dsRNA surviving slightly better in *M. sexta*. Use of ingested dsRNA in *M. sexta* has largely been successful, but the efficiency of silencing was not always high (Terenius et al., 2011), while in *T. ni* there are no published examples of successful RNAi through ingestion. These results agree with the current knowledge of RNAi in insects as environmental RNAi is generally more successful in coleopteran species than in lepidopteran species and imply that an insect's midgut lumen environment is a strong indicator of how successful ingested dsRNA will be. How quickly dsRNA fragments are degraded in the gut of the target insect seems to be an important factor for predicting environmental RNAi effects.

The hemolymph degradation assays followed a similar pattern to the lumen assays. There was a clear order of hospitability with the least degradation in *L. decemlineata* hemolymph and the most degradation in *T. ni* hemolymph. However, the difference between species was less pronounced in the hemolymph than in the midgut lumen. Whereas dsRNA survived in *L. decemlineata* lumen at 4 times the concentration of *M.*

*sexta* lumen, it only survived up to 2 times the concentration in hemolymph. This suggests that the hemolymph composition does not explain species differences in RNAi efficacy as strongly as midgut lumen composition. Notably there have also been successful RNAi studies using injected dsRNA in all three species, so the hemolymph is not as significant a barrier as the midgut lumen.

A similar experiment was performed by Shukla et al. (2016) comparing the lumen and hemolymph of *L. decemlineata* larvae to the larvae of another lepidopteran, *Heliothis virescens*. They found similar results in that dsRNA survived at higher concentrations for both fluids in *L. decemlineata* compared to *H. virescens*. The dsRNA also survived at higher concentrations of both fluids in the *L. decemlineata* larvae in their experiment compared to adults used for this thesis, which again agrees with current knowledge about RNAi in insects as earlier life stages of insects seem to be more susceptible to RNAi effects than later stages (Katoch et al., 2013). Survivability of dsRNA in the gut and hemolymph seem to be a factor in this difference between developmental stages.

Because evidence of the effects of the gut lumen on dsRNA are becoming more prevalent, some researchers have started looking for techniques to circumvent or protect against degradation in the gut. An experiment in *S. frugiperda* specifically timed dsRNA feeding assays for when insect guts have lower concentrations of dsRNAses, such as when they recently molted or were starved (Rodríguez-Cabrera et al., 2010). Timing dsRNA delivery around gut chemical properties can be useful in the lab for gene studies, but would be more difficult to execute in a field for pest management. Other researchers are using delivery techniques designed to protect the dsRNA fragments long enough for them to be absorbed by cells. Lin et al. (2017) delivered dsRNA to *Blattella germanica* by encasing it in lipoplexes which proved more effective than using naked dsRNA.

#### 4.4 *L. decemlineata* Survival Assays

Despite successful silencing of *LedMDR1* and *LedMDR2*, the subsequent survival assays using dsRNA with ivermectin showed no statistically significant changes due to the downregulation. Similarly, while the survival of insects dosed with verapamil prior to ivermectin had reduced survival compared to those dosed with the acetone control, the

difference was not statistically significant. Survival assays with verapamil returned a p-value of 0.0547 with a critical p-value of 0.05. It is possible that verapamil does in fact have a significant biological effect, but the assay wasn't powerful enough to capture it. If further replication of the assay were to provide a lower p-value, then it would be appropriate to conclude that there is a significant difference and that MDR proteins play a significant role in ivermectin detoxification. Repetition of the assay could also include multiple different doses of ivermectin or verapamil. Despite the use of an ivermectin concentration estimated to be approximately the LD50 for the insecticide, only 10% of the control insects were killed by the end of the assay, indicating that ivermectin tolerance can vary significantly between cohorts of insects. The use of multiple doses of ivermectin and verapamil would ensure that any assay captures a broader view of the effects of each variable. Higher concentrations of verapamil might also cause a larger biological effect that is more likely to be captured by statistical analysis. However, given the results obtained, it cannot be clearly concluded that *MDR* genes and proteins are significant components of ivermectin tolerance in *L. decemlineata*.

An initial concern is that downregulation was transient and did not persist after dsRNA feeding to have a measurable effect on the beetles' survival; however downregulation through feeding has been shown to have persistent phenotypic effects in *L. decemlineata* for up to 6 days after dsRNA feeding was halted (Zhu et al., 2011). Additionally, there is evidence that continuous or multiple applications of dsRNA in insects, as was performed for my research, have persistent silencing effects on the target transcript, unlike single applications (Asokan et al., 2013). Another concern is that even if transcript silencing was persistent during the assay, protein abundance wasn't reduced enough to create a phenotypic change. This could occur if post-transcriptional or post-translational regulatory processes were more important for controlling MDR protein abundance. Particularly, if MDR proteins have a slow turnover rate and remain active for several days after translation, reducing mRNA transcript abundance would have a delayed effect on the phenotype that might not have been captured by the survival assay. A third possibility is that downregulation of just *LedMDR1* or *LedMDR2* is not sufficient for a phenotypic change. Another *MDR* gene, such as *LedMDR3* for which downregulation could not be verified, may code for the primary transporter for ivermectin efflux. It is

also possible that multiple *MDRs* are responsible for ivermectin efflux, so singular downregulation of any of them would be insufficient. If the substrate specificity of multiple MDR proteins overlapped, then multiple genes may have to be silenced to significantly impair ivermectin efflux. If the dosage of verapamil was insufficient to have a strong enough effect on transporter activity, then these issues remain. This is a possibility, but it is not likely as the *L. decemlineata* beetles were given a dose originally designed for the German cockroach, *Blattella germanica* (Hou et al., 2016), which is a larger insect. However, provided that it was sufficient and effective, the assay should have accounted for these possibilities by directly inhibiting protein activity of all MDR transporters at once, in which case the similar results between the dsRNA and verapamil assays indicate that neither protein activity nor the number of inhibited transporters was an issue in affecting survival.

The lack of significant differences in survival from the treatments implies that the MDR transporters in *L. decemlineata* are not important detoxification enzymes for insecticides like ivermectin. It is possible that they aren't significantly involved in ivermectin metabolism in *L. decemlineata* at all, but current research in MDR proteins doesn't support that conclusion. *MDR* gene expression and MDR protein activity have been identified as important factors in detoxification of ivermectin and other macrocyclic lactones in a multitude of species: dogs (Roulet et al., 2003) and mice (Schinkel et al., 1995; Schinkel et al., 1996) use MDRs to prevent ivermectin penetration through the blood-brain barrier and into nerve tissue; *MDR* expression was linked to ivermectin resistance in the nematodes *Caenorhabditis elegans* (James & Davey, 2009) and *Haemonchus contortus*; and many arthropods like the tick *Rhipicephalus (Boophilus) microplus* (Pohl et al., 2011), the model insect *D. melanogaster* (Luo et al., 2013), and the agricultural pests *H. armigera* (Srinivas et al., 2004) and *Spodoptera exigua* (Zuo et al., 2017) all had *MDR* expression or MDR protein activity linked to ivermectin or abamectin resistance. The current knowledge of *MDR* genes indicates that they have highly conserved functions and similar substrate specificities across species, so it is unlikely that *L. decemlineata* *MDRs* play no role in ivermectin tolerance when *MDRs* in so many other species are clearly involved. A much more likely explanation is that other enzyme-coding genes involved in ivermectin metabolism in *L. decemlineata* have more

significant effects on the rate of metabolism, making inhibition of MDR activity insufficient to significantly increase susceptibility. While *MDRs* themselves are often involved in xenobiotic metabolism and resistance, the process includes more than just efflux transporters because avermectins, like many other compounds, are subjected to metabolic modifications before being excreted. Other detoxification genes that code for enzymes like P450 monooxygenases, glutathione S-transferases, or other ABC transporters have also been identified as part of the ivermectin detoxification pathway, and can also be upregulated in avermectin resistant strains of insects. Polymorphisms in the human cytochrome P450 genes CYP3A4 and CYP3A5 are associated with differential ivermectin metabolism and response to the drug (Kudzi et al., 2010). Likewise, a cytochrome P450 was identified as a major enzyme in avermectin metabolism in rats (Zeng et al., 1996). Cytochrome gene expression and enzyme activity have been implicated in avermectin resistance in the diamondback moth, *Plutella xylostella* (Qian et al., 2008), the mite, *Tetranychus urticae* (Riga et al., 2014), and in *L. decemlineata* as well (Yoon et al., 2002). While the *L. decemlineata* strain used in for my research was not resistant, it is clear that cytochromes are integral to metabolism of avermectins. Increased activity of glutathione S-transferase enzymes has also been connected to avermectin resistance in *T. urticae* (Stumpf & Nauen, 2002) and the scabies mite, *Sarcoptes scabiei* var. *hominis* (Mounsey et al., 2010). The ABCC transporter gene *mrp-1* was also upregulated alongside the ABCB transporter gene *pgp-1* in ivermectin resistant *C. elegans*, demonstrating that ABC transporters from multiple subfamilies are involved in efflux of avermectins. However, verapamil should inhibit activity of all ABC transporters, so it is not likely that they are preserving ivermectin tolerance in my assays..

## 4.5 Future Directions

While I was unable to prove a relationship between *MDR* gene expression and tolerance to ivermectin in *L. decemlineata*, this does not indicate that *LedMDR* genes aren't involved in xenobiotic metabolism. MDR substrate specificity is broad and varies between transporters. As such, the *LedMDR1* and *LedMDR2* transporters could be more important in detoxification of insecticides other than ivermectin. Challenging *L. decemlineata* with alternate insecticides after knockdown of *LedMDR1* or *LedMDR2*

would reveal their role. Additionally, multiple enzymes are usually involved in detoxification of a xenobiotic compound, so it is possible that expression of another detoxification gene, such as a cytochrome P450, is a greater determinant of ivermectin tolerance than *MDR* expression. To explore this, different potential detoxifying genes should be downregulated individually or in tandem with *MDR* genes before challenging the beetles with ivermectin to determine the involvement of each gene. The p-value of 0.0547 for the verapamil + ivermectin assay also suggests that further investigation is warranted. Further optimization of this assay could clarify whether or not there is a statistically significant effect that simply was not captured by the current experiments.

My results for *T. ni* RNAi clearly show that this insect is refractory to ingested dsRNA like many other lepidopterans. For continued use of RNAi in *T. ni* to silence select genes, injected dsRNA should be used when possible to have a greater chance of success. However, this isn't an option when studying RNAi for pest management as the dsRNA will need to be ingested. Techniques for protecting the dsRNA while it is in the *T. ni* midgut lumen will have to be developed to ensure it is absorbed and can cause silencing, such as encasing the dsRNA in lipoplexes similar to a method used in *B. germanica* (Lin et al., 2017). New transgenic plants in which RNAi-inducing dsRNA is expressed in chloroplasts instead of the nuclei may also serve as a solution (Zhang et al., 2017). Expression of dsRNA in plant chloroplasts has proven to be more effective at causing RNAi in insects than when it is expressed in nuclei, as the dsRNA is protected from plant Dicer enzymes. The protection of the chloroplast could also help shield the dsRNA from dsRNase enzymes in insect guts. If this proves true, then it would open up RNAi as a pest management tool for insects that are currently resistant to the practice.

## 4.6 Summary and Conclusions

My research examined three different *MDR* genes in *L. decemlineata*, establishing that *LedMDR1* is primarily expressed in midgut tissue, *LedMDR2* in nervous tissue, and *LedMDR3* in Malpighian tubule and nervous tissue. *LedMDR1* and *LedMDR2* were successfully downregulated in *L. decemlineata* beetles using ingested dsRNA, however this did not result in higher susceptibility to the insecticide ivermectin, contrary to expectation. Inhibition of ABC transporter activity by verapamil also failed to

significantly increase susceptibility to ivermectin, indicating that ABC transporters may not be a significant factor in *L. decemlineata* ivermectin metabolism compared to other pathways.

*T. ni* did not have an RNAi effect on either *TrnV-ATPaseA* or *TrnMDR1* after ingesting dsRNA. Investigating the relative degradation rates of dsRNA in *T. ni* midgut lumen and hemolymph compared to *L. decemlineata* and *M. sexta* revealed that dsRNA degraded faster in both body fluids compared to the other species. Therefore it is suspected that dsRNAase enzymes in their body fluids are responsible for *T. ni* resistance to ingested dsRNA.

These results provided interesting insights into how *MDR* genes are involved in *L. decemlineata* insecticide metabolism. Specifically, they may not be as important in detoxification as previously believed. A comparison of dsRNA stability in *T. ni* midgut lumen and hemolymph compared to *L. decemlineata* and *M. sexta* also demonstrated dsRNAses as a major factor for why *T. ni* are refractory to ingested dsRNA. These conclusions have improved the general understanding of insect insecticide detoxification and RNAi as a potential insect control mechanism.

## 5 References

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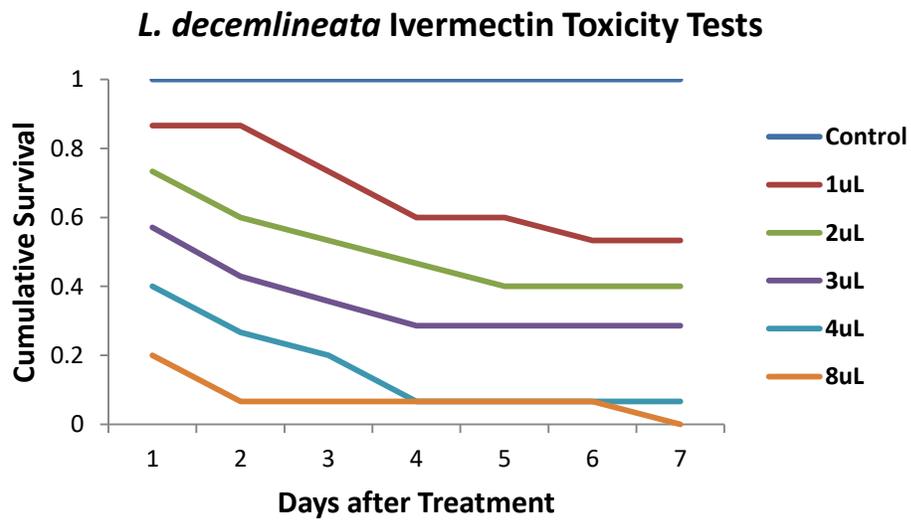
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## Appendices

**Appendix A:** Figure of ivermectin toxicity tests performed on *L. decemlineata*.

*L. decemlineata* beetles were treated with different doses of ivermectin to determine the lethality of ivermectin over 7 days. These tests were used to estimate LD50s for experiments. Fourteen to fifteen insects were used for each treatment following the same protocol as the dsRNA + ivermectin mortality assays.



**Figure A-1:** Cumulative survival over 7 days of *L. decemlineata* beetles fed different doses of 5ppm ivermectin to estimate lethality.

## Curriculum Vitae

**Name:** Grant Favell

**Post-secondary Education and Degrees:** University of Western Ontario  
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2010-2015 B.Sc.

**Honours and Awards:** Dean's Honor List  
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**Related Work Experience** Favell, G., and Donly, C. 2017. RNA interference of *MDR* genes in *Leptinotarsa decemlineata* and *Trichoplusia ni* by ingestion of double-stranded RNA. Poster Presentation, Biology Graduate Research Forum, London, Ontario, Canada

Favell, G., and Donly, C. 2017. RNA interference of *MDR* genes in *Leptinotarsa decemlineata* and *Trichoplusia ni* by ingestion of double-stranded RNA. Oral Presentation, Insect Biotechnology Conference, Niagara-on-the-Lake, Ontario, Canada