RNA Interference as a Tool for the Functional Analysis of Genes in the Colorado Potato Beetle, *Leptinotarsa decemlineata* (Say)

Ashley Danielle Yates  
*University of Nebraska-Lincoln*

Follow this and additional works at: [http://digitalcommons.unl.edu/entomologydiss](http://digitalcommons.unl.edu/entomologydiss)  
Part of the [Entomology Commons](http://digitalcommons.unl.edu/entomologydiss), and the [Genetics and Genomics Commons](http://digitalcommons.unl.edu/entomologydiss)

[http://digitalcommons.unl.edu/entomologydiss/33](http://digitalcommons.unl.edu/entomologydiss/33)

This Article is brought to you for free and open access by the Entomology, Department of at [DigitalCommons@University of Nebraska - Lincoln](http://digitalcommons.unl.edu). It has been accepted for inclusion in Dissertations and Student Research in Entomology by an authorized administrator of [DigitalCommons@University of Nebraska - Lincoln](http://digitalcommons.unl.edu).
RNA Interference as a Tool for the Functional Analysis of Genes in the Colorado Potato Beetle, *Leptinotarsa decemlineata* (Say)

By

Ashley Danielle Yates

A THESIS

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Master of Science

Major: Entomology

Under the Supervision of Professor Nicholas J. Miller

Lincoln, Nebraska

August 2014
RNA interference (RNAi) is a naturally occurring phenomenon in eukaryotes in which a double-stranded RNA (dsRNA) suppresses the expression of a target gene. RNAi has markedly changed the way in which functional genetics studies are performed, especially in non-model organisms. In insects, the efficacy of RNAi is influenced by several factors, including the species and the methods of dsRNAs delivery.

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say), is a pest of the plant family Solanaceae. RNAi in the CPB is of interest for potential use in insect management and as a tool to study the interaction with host plants. The efficacy of different methods of dsRNA delivery for the functional analysis of genes in the CPB was evaluated. As a proof of concept, the Laccase2 gene, which is responsible for pigmentation and sclerotization in beetles, was targeted for knockdown. The potential for systemic RNAi was evaluated for several dsRNA delivery methods. Results indicated that the potential for systemic and persistent RNAi exists for each of the methods evaluated here. A slight phenotypic effect of Laccase2 knockdown was observed in larvae fed dsRNAs on artificial diet, and a stronger phenotypic effect was exhibited by larval microinjection of dsRNAs. Additionally, qRT-PCR indicated RNAi knockdown by microinjection of embryos.
To gain an understanding of how RNAi genes respond to dsRNA, gene expression in response to dsRNA challenge was assessed using Illumina high throughput sequencing. Core RNAi genes were not differentially expressed following dsRNA challenge, although a handful of genes involved in the RNAi pathway were found to be upregulated.

Establishing dsRNA delivery methods for RNAi is a useful tool for studying insect-plant interactions in this system. Additionally, this research provides a glimpse into the RNAi pathway of the CPB, which can contribute to our understanding of RNAi variability among different insect species, and identify genes that are likely to respond to selection pressure if RNAi is employed for insect management.
Acknowledgements

First and foremost, I would like to express my sincerest gratitude to my advisor, Dr. Nick Miller, for granting me the opportunity to pursue a master’s degree in his lab. Dr. Miller has devoted countless hours of his advice and support, which has helped to ensure my success as a graduate student. I would also like to express my appreciation to the other members of my advisory committee, Drs. Tiffany Heng-Moss and Blair Siegfried. My committee has challenged me with thought-provoking questions and provided valuable input and resources for this research.

I would like to express my appreciation to past and present members of Dr. Miller’s lab: Dr. Laramy Enders, Leslie Rault, Jelfina Alouw and Zixiao Zhao. Together the members of Dr. Miller’s lab discuss research strategies, interpret results and teach one another laboratory techniques. I am very grateful to be a part of such a close-knit team. Additionally, I would like to extend my thanks to Dr. Haichuan ‘John’ Wang, for helping me understand the details of many of the molecular protocols.

I would like to thank all students in Entomology for welcoming me into such a friendly department. I would like to specifically acknowledge those who I have leaned on most- including Ashley Foster, Crystal Ramm, Laramy Enders and Nick Eurek. Their friendship and understanding support is so important to me. I am also grateful for those who have played sand volleyball and taken yoga class with me, as these times were not only fun but also served as stress relievers.

Finally, I would like to thank my family- dad and mom, John and Kelly Yates, and brother and sister, Nick and Jackie Yates, for their unwavering support from two states away. My family has known that attending graduate school has always been one of
my highest priorities, and without their support I would not have succeeded. Influence from my dad, who provided me with a background in agriculture and instilled in me a hard-working attitude, led me to pursue a master’s in Entomology. I would also like to acknowledge my Grandma Nancy, whose phone calls and cards always served as pick-me-ups. I’m grateful for my best friends- Casey DeGroot, Julia Fromme, Kara Burger and my sister, Jackie, for making trips to Nebraska to visit me and for keeping me involved in a group message that has lasted for years. Finally, I would like to thank my boyfriend, David Stewart, for his support, patience and understanding, as seeing me has always meant driving 14 hours in a weekend. He has cheered me on when I was feeling down and made me laugh when I was feeling stressed. Our phone calls were always the best part of my day, and I’m lucky to have a person like him in my life.
TABLE OF CONTENTS

Acknowledgements ........................................................................................................... i
List of Tables ...................................................................................................................... v
List of Figures .................................................................................................................... vi

CHAPTER 1: Literature Review ......................................................................................... 1
  Introduction ...................................................................................................................... 2
  RNAi Discovery .............................................................................................................. 2
  RNAi Pathways and Mechanism ................................................................................... 5
  Methods of dsRNA Delivery to Insects .......................................................................... 6
  RNA Interference for Functional Genetics ..................................................................... 7
  RNAi for Species-Specific Insecticide ........................................................................... 8
  Colorado Potato Beetle: Insect Pest ............................................................................. 11
  Plant-Insect Interactions .............................................................................................. 11
  Insecticide Resistance in CPB ....................................................................................... 13
  CPB Genetic Characterization ..................................................................................... 15
  Objectives ...................................................................................................................... 15

CHAPTER 2: Evaluation of Methods for dsRNA Delivery for RNA Interference as a Tool for the Functional Analysis of Genes in the Colorado Potato Beetle, *Leptinotarsa decemlineata* (Say) ......................................................................................... 17
  Introduction ...................................................................................................................... 18
  Materials and Methods ................................................................................................. 21
  Results ........................................................................................................................... 33
  Discussion ....................................................................................................................... 35
CHAPTER 3: Expression of RNA Interference Genes in Response to Double-Stranded RNA in the Colorado Potato Beetle, *Leptinotarsa decemlineata* (Say) ...... 63

Introduction.................................................................................................................. 64

Materials and Methods................................................................................................ 66

Results.......................................................................................................................... 73

Discussion..................................................................................................................... 76

REFERENCES: ................................................................................................................. 89
LIST OF TABLES

CHAPTER 1:

Table 1. A list of primers used for Laccase2 isolation, PCR and qRT-PCR in the first set of RNAi experiments. ................................................................. 42

Table 2. Primers and probes used for qRT-PCR in the second set of RNAi experiments. 43

Table 3. geNorm evaluation of potential reference genes for qRT-PCR......................... 44

Table 4. Parameters for Laccase2 and B-actin primers for qRT-PCR............................ 45

Table 5. Egg hatch rates and survival for the first and second RNAi egg microinjection experiments. ........................................................................................................ 46

Table 6. Second set of RNAi experiments: larval microinjection. Larvae exhibiting phenotypic loss of pigmentation on day 3 post-injection of dsRNAs. ......................... 47

CHAPTER 2:

Table 1. A list of primers used in PCR and for dsRNA synthesis.................................. 81

Table 2A. Genes upregulated at 6 hr and 24 hr. .............................................................. 82

Table 2B. Genes downregulated at 6 hr and 24 hr...................................................... 82
LIST OF FIGURES

CHAPTER 1:

Figure 1. Experimental materials and designs for dsRNA delivery methods .......... 48

Figure 2. First set of RNAi experiments: phenotypic effect of feeding dsLac2 on artificial diet .............................................................................................................. 49

Figure 3. Second set of RNAi experiments: phenotypic effect of larval dsLac2 injection. ........................................................................................................ 50

Figure 4. First set of RNAi experiments: expression of Laccase2 following microinjection of dsRNAs into embryos ........................................................................ 51

Figure 5. First set of RNAi experiments: expression of Laccase2 following injection of dsRNAs into larvae ........................................................................ 52

Figure 6. First set of RNAi experiments: expression of Laccase2 following feeding dsRNAs on artificial diet ................................................................. 53

Figure 7. Second set of RNAi experiments: expression of Laccase2 following injection of dsRNAs into larvae ........................................................................ 54

Figure 8. Second set of RNAi experiments: expression of Laccase2 following injection of dsRNAs into embryos ........................................................................ 55

Figure 9. Second set of RNAi experiments: expression of Laccase 2 following continuous feeding of dsRNAs on leaf surface .................................................. 56

Figure 10. Second set of RNAi experiments: expression of Laccase 2 following pulsed feeding of dsRNAs on leaf surface ...................................................... 57
Figure 11. A summary of Laccase2 knockdown, phenotypic effect, and observations and suggestions for each dsRNA delivery method evaluated. ........................................... 58

CHAPTER 2:

Figure 1. Differentially expressed genes at 6 hr and 24 hr. ................................................. 84

Figure 2. The distribution of log2 fold change in RNAi genes at 6 hr and 24 hr. ........... 85
CHAPTER 1:

Literature Review
**Introduction**

The discovery of RNA interference in 1997 by Fire and Mello markedly changed the way in which functional genomics studies in eukaryotes are performed. RNA interference (RNAi) has enabled the functional analysis of genes in non-model organisms without the requirement of a mutant. RNAi, also known as dsRNA-mediated gene silencing, is a naturally occurring phenomenon in eukaryotes in which a double-stranded RNA knocks down or suppresses the expression of a target gene. RNAi as an entomological research tool has been used to elucidate genes involved in physiological processes, embryogenesis, reproduction and behavior in model and non-model insects (Bellés, 2010). An emerging area for RNAi research is its use as a species-specific insecticide in insect pest management (Baum *et al*., 2007; Whyard *et al*., 2009). RNAi efficacy is highly variable depending upon the insect species (Bellés, 2010; Terenius *et al*., 2011), the method of RNAi delivery (Scott *et al*., 2013; Terenius *et al*., 2011) and the gene targeted by RNAi (Bellés, 2010). By gaining a better understanding of the relationship between RNAi delivery and efficacy, as well as identifying the genes that are responsive to RNAi we will better understand mechanisms associated with RNAi in insect systems.

**RNAi Discovery**

Prior to the elucidation and characterization of RNA interference (Fire *et al*., 1998), several studies were published that described the phenomenon of gene silencing by homologous sequences across several eukaryotic kingdoms. These phenomena became known as quelling in fungi (Romano and Macino, 1992), co-suppression (Napoli...
et al., 1990) or post-transcriptional gene silencing (PTSG) in plants, and RNA interference in animals.

The first studies to exploit nucleic acids for analysis of gene function relied on the introduction of homologous DNA or antisense RNA into the organism for gene inhibition (Fire et al., 1991; Rosenberg et al., 1985). The Krüppel gene, which controls segmentation during Drosophila egg development, was inactivated following embryonic injection with antisense RNA (Rosenberg et al., 1985). Rosenberg and colleagues (1985) were unable to determine the mechanism in which antisense RNA inhibited the expression of the Krüppel gene, but speculated that antisense RNAs were binding to the homologous RNAs within the nucleus and either causing degradation or preventing exportation to the cytoplasm. Additionally, they considered that the antisense RNAs were acting within the cytoplasm to prevent translation of the gene. Fire and colleagues (1991) hypothesized that the introduction of homologous DNA into C. elegans resulted in the production of antisense RNAs, and by examining protein levels inferred that the antisense RNAs interfered with gene expression during a late step in gene expression.

As gene silencing via anti-sense RNA was being explored in animals, a similar phenomenon was discovered in the plant kingdom. Jorgensen found that the introduction of an anti-sense transgene into a petunia flower led to a reduction or a total loss in pigmentation of the flower (Napoli et al., 1990). This result was unexpected, as the transgene was introduced to deepen the pigment of the petunia. Jorgensen and colleagues termed this phenomenon “co-suppression,” as the addition of the transgene seemed to interact and suppress both the transgene and the homologous gene(s) (Napoli et al., 1990).
Fire and Mello discovered that injecting double-stranded RNAs into *C. elegans* would result in more “potent and specific interference” than injecting single-stranded sense or antisense RNAs alone (Fire *et al.*, 1998). In addition to the dramatic reduction or elimination of the mRNA transcript following injection of dsRNA, they also found that the dsRNA could be injected into the head or tail of the nematode and the dsRNA silencing effect was exhibited in the animal’s progeny. Andrew Fire and Craig Mello won the Nobel Prize in Physiology or Medicine in 2006 for these findings (nobelprize.org). Gene silencing by introduction of exogenous dsRNA became known as RNA interference. Kennerdell and Carthew (1998) found that similar gene-suppression effects of RNAi seen in *C. elegans* could be translated to insects. Kennerdell and Carthew demonstrated RNAi knockdown in *Drosophila* embryos. This was one of the first studies to demonstrate RNAi in an insect, and found commonalities with RNAi in the *C. elegans* nematode, including that dsRNA was more effective in causing gene silencing than either sense or anti-sense ssRNA (Kennerdell and Carthew, 1998). However, as Kennerdell and Carthew (1998) were conducting RNAi in early blastoderm stage *Drosophila* embryos lacking cell membranes, they were unable to determine the potential for systemic RNAi in insects.

Following the initial discovery of RNAi, the mechanism for this phenomenon remained to be elucidated. One of the first mediators of RNAi-induced silencing to be characterized was an enzyme/protein complex called RISC, or RNA-induced silencing complex (Hammond *et al.*, 2000). In *Drosophila*, components of RISC include Argonaute-2 (Hammond *et al.*, 2001), fragile X mental retardation protein (FMRP), Vasa intronic gene (VIG) (Caudy *et al.*, 2002), and Tudor-SN (Caudy *et al.*, 2003; Hammond,
5

The nuclease activity of RISC destroys target mRNA, resulting in knockdown of the target gene. The efficacy of the RISC is variable depending on the length of dsRNA (S M Hammond et al., 2000).

**RNAi Pathways and Mechanism**

Three major RNAi pathways found have been characterized for small non-coding RNAs. These pathways are microRNA (miRNA), piwiRNA (piRNA) and small interfering RNA (siRNA). The three RNAi pathways employ several distinct but related core RNAi proteins. The miRNA and siRNA pathways function as negative regulators of gene expression, while the piRNA pathway functions to defend against transposable elements (Aravin, et al., 2007). All RNAi pathways are evolutionarily conserved, although core genes of the siRNA pathway are more variable among insects. Two studies (Shreve et al., 2013; Swevers et al., 2013) have examined the conservation of core RNAi proteins between different insect species and *C. elegans* in the miRNA and siRNA pathways. Core RNAi genes implicated in the miRNA pathway (Shreve et al., 2013; Swevers et al., 2013) and piRNA pathway (Swevers et al., 2013) are more conserved between species than core RNAi genes of the siRNA pathway.

The siRNA pathway is activated by exogenous dsRNA, and this pathway serves to defend the genome against invading nucleic acids. RNAi experiments exploit the siRNA pathway by delivering dsRNA to induce knockdown of the target gene. When a dsRNA is introduced into a cell, it is processed into ~21bp small interfering RNAs (siRNAs) by an enzyme called Dicer-2. The siRNAs are incorporated into an RNA-Induced Silencing Complex (RISC), which is coupled with the argonaute-2 (Ago-2) protein. The double-stranded siRNA is unwound, the passenger strand is degraded and
the RISC complex uses the guide strand to guide the complex to the homologous mRNA. The RISC complex has endonuclease activity and cleaves the mRNA. The mRNA is destroyed, and the protein for which the mRNA is coding is not expressed.

**Methods of dsRNA Delivery to Insects**

Various methods of dsRNA delivery to insects may be employed, depending on the application of RNAi, insect species or life stage of the insect, and the expression of the target gene. Many entomological RNAi experiments rely on extracellular RNAi, whereby the dsRNAs are delivered into the hemolymph or midgut, and the cells must take up the dsRNAs (Yu et al., 2012). Extracellular RNAi is classified as environmental and/or systemic (Huvenne and Smagghe, 2010; Whangbo and Hunter, 2008). Environmental RNAi is the phenomenon in which dsRNAs are taken up from the cells’ environment, and the gene knockdown effect is exhibited in these cells (Huvenne and Smagghe, 2010; Whangbo and Hunter, 2008). Systemic RNAi occurs when the silencing effect is passed from cell to cell (Huvenne and Smagghe, 2010; Whangbo and Hunter, 2008). While the mechanism for extracellular RNAi is poorly understood, there are two mechanisms proposed for the uptake of dsRNAs: an endocytic pathway (Saleh et al., 2006) and a transmembrane protein, SID-1 (Feinberg and Hunter, 2003).

The three most common methods of delivering dsRNAs to insects include microinjection, feeding, and soaking (Scott et al., 2013; Yu et al., 2012). One of the first methods used for delivering dsRNAs was microinjection. This was done in both a nematode (*Caenorhabditis elegans*) (Fire et al., 1998) and an insect (*Drosophila melanogaster*) (Kennerdell and Carthew, 1998). Microinjection is used to deliver dsRNAs directly into insect hemolymph or into an insect embryo. Delivery of dsRNAs
by microinjection has advantages over other delivery methods, as the site of delivery and the dose of dsRNAs are controlled.

Delivering dsRNAs to the midgut cells via feeding requires the uptake of dsRNAs by the cells. Feeding dsRNAs is effective in insects that exhibit a robust RNAi response. RNAi by feeding can be utilized to deliver dsRNAs in a high-throughput manner, while avoiding the mechanical damage caused by microinjection (Scott et al., 2013). Additionally, RNAi delivery by feeding is of particular interest for insect control (Baum et al., 2007).

In insects, RNAi by soaking has been mainly conducted in cell lines. However, this method has also been shown to be valuable as a high-throughput tool for large-scale gene expression studies in *C. elegans* (Maeda et al., 2001).

**RNA Interference for Functional Genetics**

In classical or forward functional genetics, a gene associated with the phenotype is determined (Bellés, 2010), usually via a mutant organism. While forward functional genetic practices have proven invaluable for the functional analysis of genes, there are several drawbacks to this method. Forward genetics typically rely on a mutant organism, but a naturally variant or an induced mutant can be difficult to obtain in the laboratory (Brown et al., 1999). Additionally, forward functional genetic practices are largely limited to model organisms, such as *Drosophila melanogaster* or *Tribolium castaneum* (Brown et al., 1999). RNAi enables the application of reverse functional genetics, whereby a gene of interest is chosen and the phenotype associated with the gene is discovered (Bellés, 2010). This has opened the door to our understanding of gene function in non-model organisms. RNAi has advantages over classical functional
genomics studies, as the only genetic information required is the DNA sequence of the target gene. Additionally, RNAi allows for a phenotypic evaluation of gene function without the requirement of a mutant organism.

RNAi has been used in functional genetics to elucidate genes involved in many insect processes, including embryonic development, physiology and behavior (Bellés, 2010). Embryonic RNAi was utilized to determine the role of two genes, frizzled and frizzled 2, in the *Drosophila* wingless pathway (Kennerdell and Carthew, 1998). Two previously known insect chitin synthase genes were determined to have separate roles by RNAi: CHS1 was found to function solely in the formation of chitin found in the insect cuticle, while CHS2 was found to function mainly in the formation of chitin in the peritrophic membrane (Arakane et al., 2005a). Similarly, RNAi was used in *T. castaneum* to distinguish the phenoloxidase gene(s) thought to be involved in cuticle tanning (Arakane et al., 2005b). Arakane and colleagues (2005) found that RNAi of the Laccase2 gene caused a loss of pigmentation and sclerotization in the beetle, indicating that Laccase2 was the gene that is required for these biochemical processes. A review of functional RNAi conducted in *Tribolium castaneum* (Noh et al., 2012) reveals that RNAi has been used in more than 15 studies to further our understanding of many insect processes.

**RNAi for Species-Specific Insecticide**

As part of the integrated pest management (IPM) strategy, we seek to effectively utilize novel means of insect control to delay the onset of resistance evolution. In order for insecticides to remain effective, novel modes of action must be discovered and various target sites exploited. *Bacillus thuringiensis* (Bt) is a gram-positive bacterium
that undergoes a sporulation phase which results in the production of proteins that have insecticidal activity against certain insect groups. The insecticidal proteins have been engineered in crops to protect from insect feeding. The introduction of Bt as a novel method for insect control was instrumental in managing insects that were rapidly becoming resistant to pesticides and other methods of control. However, there are limitations to the current widespread use of Bt in IPM. Bt is not effective against some insect groups, and resistance to Bt has been reported in the field in *Diabrotica virgifera virgifera* (western corn rootworm) (Gassmann et al., 2011), *Helicoverpa armigera* (cotton bollworm) (Zhang et al., 2011) and *Pectinophora gossypiella* (pink bollworm) (Tabashnik et al., 2002) demonstrating the need for novel means of insect control.

After a decade of utilizing RNAi as a method for studying gene function, the potential for RNAi emerged in entomology as a novel tool for the control of insect pests (Baum et al., 2007) (Mao et al., 2007). RNAi can be used to cause mortality in insects by silencing genes that are essential to the insect’s survival. RNAi possesses desirable qualities for insect control, such as a novel mode of action and species specificity.

One of the first RNAi studies in an insect utilized microinjection of dsRNAs into *Drosophila* to induce RNAi-mediated gene silencing (Kennerdell and Carthew, 1998). Microinjection of dsRNAs is a useful method for delivering dsRNAs to a particular cell or region of the body in laboratory experiments. However, microinjection is not an appropriate method of delivery for the purpose of insect pest control. Therefore, the potential of other methods of dsRNA delivery, such as feeding, were evaluated. Wang and Granados (2001) suggested the insect midgut as a target site for insect control
because it is the only region of the gut that is not protected by a cuticle covering and is an active interface with the environment.

Baum and colleagues (2007) demonstrated the potential of western corn rootworm control by oral administration of RNAi. Transgenic corn expressing dsRNAs targeting an essential gene of the western corn rootworm (vATPase) caused a reduction in insect feeding (Baum et al., 2007). Baum and colleagues (2007) indicate that the unique mode of action of RNAi could be used to complement the current strategy of expressing Bt toxin(s) in crops for control of insect pests.

In addition to its novel mode of action, RNAi is also of interest for insect control as it can act as a species-specific insecticide. RNAi specificity was demonstrated within the Drosophila genus when feeding dsRNAs selectively caused mortality in one species, without affecting three other species (Whyard, et al., 2009). Species-specificity is a desired trait for insect control, as current practices include the use of broad-spectrum insecticides. RNAi should reduce the effects on non-target organisms relative to broad-spectrum approaches.

Zhu and colleagues (2011) evaluated the potential of insecticidal RNAi by feeding bacteria expressing dsRNAs that targeted essential genes of the Colorado potato beetle. By feeding dsRNAs targeting 5 essential genes in the CPB, they demonstrated a significant knockdown in target gene mRNA, as well as a significant increase in mortality. This was the first study to demonstrate effective RNAi via feeding dsRNAs synthesized and delivered in a bacterial vector. Delivering dsRNAs in bacteria will likely increase the environmental stability of the dsRNAs, while lowering the overall production cost. In vitro dsRNA synthesis is expensive, and dsRNA synthesis and
delivery via bacteria is likely more practical for field applications (Zhu et al., 2011). The first studies to demonstrate the potential for RNAi in insect control included feeding dsRNAs expressed in planta that targeted essential insect genes (Baum et al., 2007; Mao et al., 2007) or by spraying dsRNAs synthesized in bacteria (Zhu, et al., 2011)

**Colorado Potato Beetle: Insect Pest**

The Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) is native to Mexico and the southern United States, and earned its common name after establishing as a pest species in North America. The Colorado potato beetle’s (CPB) primary host was wild *Solanum* species in the Solanaceae family, including *Solanum rostratum*, or buffalobur. After the potato was introduced to North America for cultivation, the CPB quickly adapted and remains one of the world’s most destructive defoliators of the plant family Solanaceae (Hare, 1990). CPB larvae and adults consume leaf material at an alarming rate, quickly defoliating entire plants. The adults consume 10cm$^2$ of leaf tissue per day, while the larvae consume up to 40 cm$^2$ of leaf tissue per day. The CPB also attacks other members of the Solanaceae, including tomato and eggplant.

**Plant-Insect Interactions**

Plants have evolved a series of direct and indirect plant defenses to protect against herbivory. Plant tolerance enables the plant to sustain some level of insect attack, with little to no fitness cost to the plant (Kessler and Baldwin, 2002). Direct and indirect plant defenses can be constitutive or inducible. (Kessler and Baldwin, 2002). Indirect plant defenses are plant traits that do not directly exhibit adverse effects on herbivores, but rather exhibit effects via a third party. An example of an indirect plant defense is the
production of a volatile to attract a natural enemy or predator of an insect (Chen, 2008; Mello and Silva-filho, 2002). Constitutive defenses are barriers that are always present, including physical barriers, such as trichomes or thorns (Mithöfer and Boland, 2012), and chemical barriers. Constitutively expressed chemical plant defenses are usually stored in an inactive form, in order to prevent toxicity to the plant itself (Mithöfer and Boland, 2012). Inducible defenses are not continuously expressed, but are induced in response to a stimulus, such as insect feeding. By maintaining inducible defenses that respond to insect attack, the plant is able to retain energy, and elicit a defense that can be pest-specific (Chen, 2008).

The Solanaceae plant family has more than 12,000 specialized chemical defense compounds (Mithöfer and Boland, 2012). One very large group of specialized Solanaceae defense compounds is glycoalkaloids. Glycoalkaloids are induced in response to wounding, and are very toxic to animals, including insects. Glycoalkaloids do not serve a primary metabolic function in plants, and toxicity of glycoalkaloids to insects is caused by interference with insect enzymes or the insect nervous system (Mithöfer and Boland, 2012). In the Solanaceae, herbivory-induced wounding triggers jasmonate, a plant phytohormone, which regulates the biosynthesis of several defense compounds (Creelman and Mullet, 1995; Mithöfer and Boland, 2012; Turner et al., 2002). Defense compounds regulated via the jasmonic acid pathway to help protect plants from insect predators include glycoalkaloids and proteinase inhibitors (Creelman and Mullet, 1995; Mithöfer and Boland, 2012).

The role of proteinase inhibitors (PIs) in plant defense is to inhibit insect digestive enzymes, which prevents the insect from digesting proteins and obtaining the
required amino acids from the plant. In addition to serving as a defense mechanism, proteinase inhibitors are essential to plant physiological processes (Jongsma and Beekwilder, 2011) and can be constitutively expressed, while others are induced in response to wounding (Green and Ryan, 1972). Although there are several classes of PIs, the mechanism by which they inhibit insect digestive enzymes is mainly the same (Bode and Huber, 2000). PIs inhibit digestive enzymes by interacting with the active site and preventing the insect enzyme from degrading plant proteins. PIs and other toxic chemical defenses induced in response to wounding help protect Solanaceous plants from damage caused by insect feeding.

The CPB has co-evolved with Solanaceous plants, mediating toxic plant defenses such as glycoalkaloids and proteinase inhibitors and establishing as a destructive pest. The CPB can adapt to proteinase inhibitors by altering the constitution of the protease digestive enzymes in the midgut. While feeding on Solanaceous plants, the CPB can replace PI-sensitive proteases with PI-insensitive proteases (Bolter and Jongsma, 1995; Bolter and Jongsma, 1997; Gruden et al., 2004; Petek et al., 2012; Rivard, et al., 2004) in order to continue feeding on PI-enriched tissue. Differential regulation of CPB enzymatic midgut digestive proteases is an evolutionary adaptation to allow the insect to eat plant tissues high in PIs. Five groups of CPB digestive cysteine proteases have been characterized that have evolved to adapt to proteinase inhibitors (Gruden et al., 2004; Petek et al., 2012).

**Insecticide Resistance in CPB**

Insecticides were very effective against the CPB until the 1960s, when resistance to DDT became widespread among insects. Since the 1960s, additional insecticides have
been developed to control the CPB, but it has become resistant to all of them. During the 1970s and 1980s, the CPB became resistant to several insecticides in all major classes, including organophosphates, pyrethroids, and carbamates (Forgash, 1985). As new insecticides are developed to control this pest, its resistance to each one occurs progressively faster, with some insecticides failing to control the CPB in as little as one year after their introduction (Forgash, 1985). Since 1960, CPB resistance to insecticides has been reported for more than 50 chemicals (Alyokhin et al., 2008). Resistance to these insecticides is linked to major genes in the beetle, including a sex-linked gene associated with cross-resistance to several pyrethroid insecticides (Argentine et al., 1989). Therefore, it is expected that the CPB possesses the ability to develop resistance against most synthetic insecticides. Two strains of Bt produce toxins that have been demonstrated to be effective against the CPB in the laboratory and in the field. However, CPB resistance to Bt toxins in laboratory populations has been documented (Whalon et al., 1993) (Rahardja and Whalon, 1995), indicating that management by Bt toxin is likely not a sustainable method for control of CPB.

The CPB’s rapid development of insecticide resistance is likely attributed to several factors involving CPB biology, ecology, and coevolution with Solanaceous plants (Alyokhin et al., 2008). One of the likely reasons for CPB resistance is likely that the CPB has evolved to detoxify defense glycoalkaloids produced by the Solanum host species (Ferro, 1993; Alyokhin et al., 2008). A predisposition to detoxifying these compounds may enable the CPB to readily detoxify or tolerate insecticidal compounds (Ferro, 1993). Additionally, as cultural control of CPB has primarily relied on the use of insecticides, this has placed an intense selection pressure on CPB resistance (Alyokhin et
al., 2008). The ability of this insect to adapt to toxic plant defenses and insecticides, and the current lack of control of this pest demonstrate a need for an enhanced understanding of the CPB at the molecular level.

**CPB Genetic Characterization**

Although the CPB has remained a devastating pest of the plant family Solanaceae for more than 50 years, relatively little genetic information has been available on this insect until recently. Prior to 2013, genetic information was only available for several specific sets of genes in the CPB. Some of the first CPB genes to be characterized included intestain genes in the CPB midgut that are differentially regulated in response to plant defense (Gruden *et al.*, 2004) (Petek *et al.*, 2012) as well as an acetylcholinesterase (Revuelta *et al.*, 2011). In 2014, sequencing and *de novo* assembly of the CPB transcriptome was performed and the annotation was made publicly available (Kumar *et al.*, 2014). In 2013, the gut transcriptome of the CPB was analyzed and RNAi-related genes were characterized (Swevers *et al.*, 2013). A genome sequence is now available for the CPB (www.hgsc.bcm.edu), enabling further genetic analyses in this insect.

**Objectives**

The discovery of RNA interference has enabled functional gene analysis in non-model organisms. Another emerging area for RNAi is its use as a species-specific insecticide in insect pest management. There are several common methods for delivering dsRNAs to insects in any life stage, including feeding, injecting and soaking. The method by which dsRNAs are delivered to insects is dependent upon several factors, including the application of RNAi, insect species or life stage of the insect, and the expression of the target gene. While the general mechanism of the siRNA pathway is known, the
manner in which insect species respond to RNAi is highly variable. This research seeks to explore the potential of different methods of dsRNA delivery for the functional analysis of genes, and to understand the role of core RNAi genes in response to dsRNA in the CPB. As a proof of concept, the Laccase2 gene was targeted for RNAi knockdown. The role of the Laccase2 gene is for pigmentation and sclerotization of the cuticle in beetles (Arakane et al., 2005). RNAi-targeted knockdown of this gene was evaluated phenotypically and by gene expression analysis. Establishing the potential of RNAi as a tool to analyze gene function in the CPB will provide means to enhance our understanding of this devastating pest at the molecular level. The specific objectives of this research are:

1. To evaluate different methods of dsRNA delivery for the functional analysis of genes in the Colorado potato beetle. These methods include feeding dsRNAs on artificial diet, feeding on a leaf surface, injecting eggs and injecting larvae. Gene expression analysis of the Laccase2 gene was evaluated using reverse-transcription quantitative real time PCR (qRT-PCR).

2. To understand how genes for core RNAi machinery in the Colorado potato beetle respond to dsRNA. Double-stranded RNAs were delivered to the CPB by injection, and differentially expressed genes between control and RNAi-treated larvae were evaluated using Illumina next-generation sequencing.
CHAPTER 2:

Evaluation of Methods for dsRNA Delivery for RNA Interference as a Tool for the
Functional Analysis of Genes in the Colorado Potato Beetle,

*Leptinotarsa decemlineata* (Say)
Introduction

The discovery of RNA interference (Fire et al., 1998) has markedly changed the way in which functional genetics studies in eukaryotes are performed, enabling the functional analysis of genes in non-model organisms without the requirement of a mutant. RNAi, also known as double-stranded RNA (dsRNA)-mediated gene silencing, is a naturally occurring phenomenon in which a dsRNA suppresses or silences the expression of a homologous target gene.

RNA interference is initiated when a dsRNA is introduced into a cell. The dsRNA is processed into several 21-23bp small-interfering RNAs (siRNAs) by an enzyme called Dicer-2 (Bernstein et al., 2001). The siRNAs are incorporated into an RNA-Induced Silencing Complex (RISC) (S M Hammond et al., 2000), which is coupled with the argonaute-2 (Ago-2) protein (Hammond et al., 2001). The double-stranded siRNA is unwound, the passenger strand is degraded and the RISC complex uses the guide strand to guide the complex to the homologous mRNA (Nykänen et al., 2001). The RISC complex has endonuclease activity and cleaves the mRNA (Hammond et al., 2000; Nykänen et al., 2001; Zamore, et al., 2000). The mRNA is destroyed, and the protein for which the mRNA is coding for is not expressed. However, RNAi is not a knockout method for a gene (Huvenne and Smagghe, 2010), but rather a knockdown method, as suppression of the target gene is transient, and not 100%. RNAi generates a loss-of-function-phenotype, and this result can be exploited in order to study gene function (Bellés, 2010; S M Hammond et al., 2000).

Gene suppression by RNAi is broadly classified as cell autonomous or non-cell autonomous (Whangbo and Hunter, 2008; Huvenne and Smagghe, 2010). Cell
autonomous RNAi results in suppression of the target gene solely in the cell in which the dsRNAs are delivered. However, many gene function studies in entomology rely on non-cell autonomous RNAi, where suppression of the target gene is observed in cells and tissues other than the location where the dsRNAs are delivered (Whangbo and Hunter, 2008; Huvenne and Smagghe, 2010). Non-cell autonomous delivery methods can be performed with relative ease in most laboratories, whereas delivering dsRNAs to a single cell can prove far more challenging. Therefore, non-cell autonomous RNAi delivery methods are desirable in order to utilize RNAi as a functional tool in insects. Non-cell autonomous RNAi is classified as environmental and/or systemic. An environmental RNAi response occurs when dsRNAs are taken up from the cells’ environment and suppression of the target gene occurs in these cells (Huvenne and Smagghe, 2010; Whangbo and Hunter, 2008). A systemic RNAi response occurs when knockdown of the target gene is spread to other cells and/or tissues (Whangbo and Hunter, 2008; Huvenne and Smagghe, 2010).

Systemic RNAi is influenced by many factors, including the life stage of the insect (Araujo et al., 2006) and method of dsRNA delivery (Rajagopal et al., 2002; Araujo et al., 2006; Yao et al., 2013; Yang and Han, 2014). Systemic non-cell autonomous RNAi in insects can be achieved by delivering dsRNAs to the midgut or hemocoel using microinjection, feeding, and soaking (Scott et al., 2013; Yu et al., 2012). These methods can be performed in any of the insect life stages from egg to adult.

The Colorado potato beetle (CPB) is a devastating insect pest in Solanum plant species, which includes tomato and potato. Current options for insect control are largely restricted to cultural control methods, such as crop rotation and early planting of short-
season varieties (Hare, 1990) as this insect has become resistant to more than 50 insecticides (Forgash, 1985; Alyokhin et al., 2008). Additionally, the CPB is a non-model organism for which relatively few genes were characterized until the recent assemblies of a gut and whole body transcriptome (Swevers et al., 2013; Kumar et al., 2014). RNAi is a potential tool that can be used to study insect-plant interaction in this system. Establishing dsRNA delivery methods for RNAi as a functional tool in the CPB will provide means to enhance our understanding of this devastating pest at the molecular level and provide insights into novel management options.

Evaluation of dsRNA delivery methods in other insects has indicated that the method in which dsRNAs are delivered directly influences the efficacy of systemic RNAi. Double-stranded RNA delivery by microinjection results in a faster and more dramatic reduction of vATPase mRNA transcript than by feeding dsRNAs in the corn planthopper, *Peregrinus maidis* (Yao et al., 2013). In the cotton bollworm, *Helicoverpa armigera*, RNAi knockdown of the ultraspiracle gene was found to be more effective with feeding dsRNAs synthesized in bacteria than dsRNAs synthesized *in vitro* (Yang and Han, 2014). RNAi knockdown of a *Bacillus thuringiensis* (Bt) receptor, Animopeptidase N (APN), in *Spodoptera litura* was achieved by injection of dsRNAs into 5th instar larvae (Rajagopal et al., 2002). Preliminary experiments by Rajagopal et al. (2002) indicated that knockdown of APN, a gene expressed in the *S. litura* midgut, was not successful by feeding or soaking neonate larvae with dsRNAs.

This research seeks to explore the efficacy of different methods of dsRNA delivery for the functional analysis of genes in the CPB. Desired RNAi methods for gene function analyses should result in RNAi knockdown with systemic and persistent effects.
As a proof of concept for systemic RNAi in the CPB, the Laccase 2 gene was targeted for knockdown. The Laccase 2 (Lac2) gene is a phenoloxidase gene that is responsible for pigmentation and sclerotization of the cuticle in *Tribolium castaneum* (Arakane *et al.*, 2005) and other Coleoptera, including *Diabrotica virgifera virgifera* (Alves *et al.*, 2010) and *Monochamus alternatus* (Cerambycidae) (Niu *et al.*, 2008). Knockdown of the Lac2 gene results in a reduction of pigmentation and sclerotization, which can be evaluated phenotypically (Arakane *et al.*, 2005; Alves *et al.*, 2010; Niu *et al.*, 2008; Futahashi *et al.*, 2011) and by gene expression analysis (qRT-PCR). In this study, the efficacies of several dsRNA delivery methods were evaluated in CPB embryos and larvae for systemic and persistent RNAi knockdown of the Laccase2 gene. RNAi-mediated knockdown of the Laccase 2 gene was evaluated phenotypically and by quantitative reverse-transcription real time PCR (qRT-PCR).

**Materials and Methods**

**cDNA synthesis and degenerate PCR**

Total RNA was isolated from a pool of 18 neonate larvae using Trizol reagent (Invitrogen, Grand Island, NY). cDNA was synthesized using the Mint-2 cDNA synthesis kit (Evrogen, Moscow, Russia) and purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA). Degenerate primers (Alves *et al.*, 2010) designed to amplify Laccase 2A in the western corn rootworm, *Diabrotica virgifera virgifera* LeConte, were used to amplify a putative 1082bp Laccase2 in *Leptinotarsa decemlineata*. The forward primer (Table 1) was prepended with the T7 promoter sequence at the 5’ end, and the reverse primer was prepended with SP6 on the 3’ end,
allowing direct sequencing of PCR products. All PCR primers were synthesized by Sigma-Aldrich, (St. Louis, MO).

Degenerate PCR was performed with an initial denaturation cycle of 3 minutes at 94°C, followed by 20 cycles of denaturation for 30 seconds at 94°C, 30 seconds annealing at 53°C, 30 seconds of extension at 72°C, with a final extension of 12 minutes at 72°C. PCR reactions were conducted in 20µl of 1X Taq Buffer with MgCl₂ (Amresco, Solon, OH) containing 1µl of 10µM forward and reverse primers, each dNTP at 2.5mM, 0.5U Taq polymerase (Amresco) and 1ng cDNA. Degenerate PCR resulted in the production of a 600bp amplicon in addition to the putative 1082bp Lac2. The 1082bp putative Laccase2 was excised from a Sybr green-stained agarose gel and purified using the MinElute Gel Extraction Kit (Qiagen). The extraction and purification of the 1082bp fragment from a gel was repeated in order to isolate only the 1082bp fragment, and not the 600bp amplicon. The 1082bp PCR product (Appendix A) was subjected to DNA sequencing in both directions using the T7 forward primer and SP6 reverse primer (Eurofins MWG Operon, Huntsville, AL). Sequence alignment was performed in Bioedit (v 7.1.3.0). The consensus sequence of the alignment was used in NCBI blastx and blastn searches to confirm hits to Laccase2 homologues in other insects.

**Laccase 2A Amplification and Cloning**

*Leptinotarsa decemlineata* Laccase 2A gene-specific primers (Table 1) were designed using Primer3 (v0.4.0) to amplify an 800bp fragment. PCR was performed with an initial denaturation of 3 minutes at 94°C, followed by 25 cycles of denaturation for 30 seconds at 94°C, 30 seconds annealing at 65°C, 30 seconds of extension at 72°C,
followed by a final extension of 12 minutes at 72°C. The concentrations of PCR components were the same as specified for the degenerate PCR.

The PCR product was purified and ligated into plasmid vector pCR 2.1 using the TA cloning kit (Invitrogen). The construct was transformed into competent *E. coli* cells (INVαF’) and positive clones were selected using Ampicillin. Twelve bacterial colonies were isolated and grown in LB broth. Plasmid DNA was recovered from the bacteria using the High Speed Plasmid Mini Kit (IBI Scientific, Peosta, IA). A glycerol stock of bacteria was stored at -80°C.

PCR was performed using the CPB gene-specific primers to amplify 800bp plasmid DNA template in 10 of the 12 clones. An initial denaturation cycle of 3 minutes at 94°C was followed by 25 cycles of denaturation for 30 seconds at 94°C, 30 seconds annealing at 65°C, 30 seconds of extension at 72°C, followed by a final extension of 12 minutes at 72°C. The concentrations of PCR components were the same as specified for the gene-specific PCR with cDNA.

The ten bacterial colonies were inoculated into fresh LB broth, allowed to grow for 16 hours, and plasmid DNA was extracted from the bacteria for DNA sequencing with M13 forward and reverse primers (Eurofins). Sequence alignment and blast searches were performed as described before. Of the 10 clones sequenced, all clones contained some nucleotides that varied from the consensus sequence. Clone 4 (Appendix B) had only one nucleotide difference (nucleotide 172) from the consensus sequence of all the clones, and was selected for use for dsRNA synthesis.

*Primer design for RNAi and in vitro dsRNA synthesis*
Primers were designed (Primer3) to generate a 442bp amplicon to serve as a template for Lac2 dsRNA synthesis, which is within the recommended range of dsRNA length (Huvenne and Smagghe, 2010). The T7 promoter sequence was prepended to the 5’ end of the forward and reverse primers to create a 442bp amplicon (Table 1). The 442bp amplicon was designed to avoid the nucleotide (172) that differed from the consensus sequence of the ten clones. PCR was performed with an initial denaturation of 95°C for 3 minutes, followed by 25 cycles of 95°C denaturation for 30 seconds, 65°C annealing for 30 seconds, and 72°C extension for 30 seconds, followed by a final extension of 72°C for 12 minutes. The concentrations of PCR components were the same as specified for the gene-specific PCR with cDNA. The PCR product was purified using the MinElute PCR Purification kit (Qiagen). Double-stranded RNA for green fluorescent protein (dsGFP) was synthesized to serve as a dsRNA control that is not homologous to the insect mRNA. GFP plasmid DNA was obtained from Dr. John Wang in Dr. Blair Siegfried’s lab, and PCR was performed to generate a template for dsGFP. PCR for GFP was performed in 50µl reactions, with 5µl 10X buffer with MgCl₂, 4µl 2.5mM each dNTPS, 1µl each of forward and reverse primers, and 0.25µl taq polymerase with 100ng plasmid DNA.

Double-stranded RNA synthesis for Lac2 (dsLac2) and GFP (dsGFP) was performed using the MEGAscript Transcription Reaction kit (Ambion). The dsRNA was treated with TURBO DNase (Ambion), and purified using the RNeasy cleanup kit (Qiagen). Double-stranded RNA quality was confirmed by gel electrophoresis on a 1.2% agarose gel, and the concentration was obtained using a Nanodrop spectrophotometer (model ND-1000).
**First Set of RNAi Experiments**

A first set of RNAi experiments was performed to establish methods of dsRNA delivery, dsRNA doses and desired time points for gene expression analysis of Lac2. The first RNAi experiments consisted of microinjection of dsRNAs for embryonic RNAi, and feeding dsLac2 on artificial diet and microinjection of dsRNAs for larval RNAi. The second set of RNAi experiments consisted of microinjection of dsRNAs into embryos and larvae, and two assays with feeding on a leaf surface. In the second set of RNAi experiments, the expression of Lac2 was evaluated at multiple time points after dsRNA delivery.

**Insects and Plants**

*Leptinotarsa decemlineata* eggs were obtained from French Agriculture in Lamberton, MN. Upon delivery, the eggs were 2 days old. After 4 days the eggs hatched, and larvae were reared on artificial diet (Bio Serv, Frenchtown, NJ) or tomato plants as specified for each experiment. Tomato plants, *Lycopersicon esculentum* "Martian Giant" were grown in a growth chamber at 34°C with a 16:8 hour (L:D) photoperiod. Artificial diet for *L. decemlineata* (Bio Serv) consisted of a casein base, agar and KOH solution.

**Feeding dsRNAs on artificial diet**

In order to evaluate the efficacy of systemic RNAi by feeding in the Colorado potato beetle, dsRNAs were applied as a coating on artificial diet. Fifty mL of diet was poured into a large Petri dish, and diet plugs were made using a #2 cork borer. Five newly emerged (<24 hours old) larvae were confined in 2 oz. plastic cups for a total of 50 larvae per treatment (Figure 1B). Each cup contained three diet plugs that were treated
with 1000ng dsGFP, 500ng dsLac2 or 1000ng dsLac2 diluted in 5µl water. Double-stranded RNA-treated diet was replaced each day for 10 days. Following 10 days of dsRNA treatment, larvae were fed with untreated tomato leaves for an additional 3 days (Figure 1H). On the fourteenth day, larvae were flash-frozen in liquid nitrogen and stored at -80°C until further processing. Twelve larvae from each treatment were selected for Lac2 gene expression by qRT-PCR analysis. One biological replicate was removed from the 1000ng dsLac2 analysis, as it was an obvious outlier.

**Egg Microinjection of dsRNAs**

The potential for embryonic RNAi in CPB was evaluated using microinjection of dsRNAs into CPB eggs. *Leptinotarsa decemlineata* eggs were injected at four time points: at the ages of 24 hours after oviposition, 48, 72 and 96 hours. Prior to injection, eggs were soaked in 2% bleach for 5 minutes for surface sterilization. The eggs were positioned on a microscope slide (Figure 1C) and placed inside a desiccator for 2-4 hours so that when injected, the amount of yolk that exuded would be reduced. Microinjection needles were pulled from 3” thin wall glass capillaries (1.0mm OD/ 0.75mm ID) using a Shutter Instrument Co. needle puller (Model P-30), and the needle was shaped using the Micropipette Beveler (Shutter Instrument Co. Model BV-10). Injections were performed with an Eppendorf Transjector 5246 (Figure 1A).

During the embryo injection procedure, 20 eggs were placed on double-sided tape adhered to a microscope slide. Twenty eggs were injected with one of the three treatments: 1000ng dsGFP, 500ng dsLac2 or 1000ng dsLac2. After injection, the eggs on the microscope slide were placed into a large Petri dish containing 2% agar. The Petri dish was wrapped with parafilm to prevent dehydration, and placed in a growth chamber.
until the eggs hatched. After the larvae hatched, they were collected from the petri dishes and placed into 2 oz. cups and fed fresh tomato leaves every day. Larvae were flash frozen in liquid nitrogen at the age of 10 days, and stored at -80°C until further processing. Lac2 expression was evaluated using qRT-PCR in eggs that were injected with dsRNAs 24 hours before hatch, as eggs injected prior to this did not survive in sufficient numbers, or altogether failed to hatch.

**Larval Microinjection of dsRNAs**

To evaluate the potential of systemic RNAi by microinjection in CPB larvae, dsRNAs were delivered by microinjection into the hemocoel of first and second instar larvae. Needles for microinjection were fashioned as described previously. Larvae were positioned on double-sided tape adhered to a microscope slide, and anesthetized on ice for several minutes. Larvae were injected on the dorsal side of the abdomen for each of the three treatments: 250ng dsGFP, 100ng dsLac2, and 250ng dsLac2 for a total of 10 first and 10 second instar larvae injected for each treatment. Following injection, larvae were maintained in 2 oz. cups and fed fresh tomato leaves. The larvae were flash frozen in liquid nitrogen 7 days post-injection of second instars. Lac2 expression of second instar larvae was evaluated using qRT-PCR, as larvae injected as first instars did not survive in sufficient number. One biological replicate was removed from the 250ng dsGFP and 250ng dsLac2 analyses, as they were obvious outliers.

**RNA Extraction, Reference Gene Selection and Reverse-Transcription Quantitative Real-Time PCR (qRT-PCR)**

Prior to RNA extraction, photos were taken of each larva to document cuticle pigmentation. Total RNA was isolated from individual larvae using the RNeasy Mini Kit
RNA was treated with DNaseI (Qiagen) and stored at -80°C. RNA quality was confirmed using a 1% denaturing agarose gel (Formaldehyde-free RNA gel kit, Amresco) and the concentration was obtained using the Qubit fluorometer. RNA was treated with DNaseI (Qiagen) and 1μg of RNA was used for cDNA synthesis using the iScript kit (Bio-Rad, Hercules, CA).

Reverse-transcription quantitative PCR was performed in a CFX Connect Real-Time System. The stability of three potential reference genes, B-actin, RP4 and RP18 (Zhu, et al., 2011) was evaluated in order to select a stably-expressed reference gene for Lac2 normalization. Gene expression for each of the three potential reference genes was evaluated among 3 larvae from each of the feeding dsRNA treatments. The stability of the potential reference genes from the three biological replications was evaluated using the geNorm application within Qbase plus (Biogazelle, Zwijnaarde, Belgium) and B-actin (Appendix D) was selected for use as a reference gene. Prior to qRT-PCR analysis, serial dilutions of cDNA were used to generate a standard curve to establish primer efficiencies for the Lac2 and B-actin. Each 20μl qRT-PCR reaction consisted of 10μl iTaq Universal SYBR Green Supermix (Bio-Rad), 2μl undiluted cDNA template and 0.6μl each of forward and reverse primers (10μM concentration). The same Lac2 primers were used for qRT-PCR as for dsRNA synthesis (Table 1, Appendix C). Each biological replicate was performed in triplicate for both the Laccase-2 and B-actin primer sets. A no-template negative control and a no-reverse transcriptase negative control were performed on each plate. Reverse-transcription quantitative PCR was performed with an initial incubation of 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds and 65°C for 30 seconds, with a final extension of 70°C for 5 seconds, followed by 5 seconds of
95°C.

Statistical Analysis

Gene expression values for Lac2 were reported as CNRQs for each biological sample by Qbase (Biorad, Hellemans, et al., 2007). CNRQ gene expression values are defined as the relative quantity of the target gene normalized to the reference gene, and calibrated to account for samples on multiple plates and gene-specific efficiencies. Gene expression analysis of Lac2 for each treatment was performed using a general linear model with an exponential distribution by proc glimmix (general linearized mixed model) in SAS v.9.2. Significant differences between treatments was determined at p< 0.05.

Second Set of RNAi Experiments

Upon establishing methods for dsRNA delivery and doses for gene expression evaluation of Lac2, a second set of RNAi experiments were performed in order to further explore Lac2 gene expression at multiple time points after dsRNA delivery. The second set of RNAi experiments included embryonic and larval RNAi experiments by microinjection of dsRNAs as performed before, but expression of Lac2 was evaluated at multiple time points after dsRNAs were delivered. The concentrations of dsRNAs injected into second instar larvae were increased in attempt to achieve the desired phenotype involving loss of pigmentation and sclerotization. Additionally, the second set of RNAi experiments included two experiments with feeding dsRNAs on a leaf surface to larvae.

Insects and Plants

Newly emerged larvae were reared on tomato plants L. esculentum "Martian Giant" that were grown in a greenhouse. The petioles of newly excised leaves were
placed in 0.6mL tubes containing 0.4% agar to maintain freshness. Leaves and agar were replaced as needed, every 1-3 days. Individual larvae were kept in 2 oz. cups with the tomato leaf (Figure 1G), and were reared in the growth chamber with conditions as previously described. For qRT-PCR analysis, four biological replications from each treatment were harvested from each experiment at days 1, 3, 6 and 10 after administration of dsRNAs, unless otherwise noted. The larvae were flash-frozen and stored at -80°C until further processing.

**Egg Microinjection of dsRNAs**

Eggs were injected 24 hours before hatch as previously described. Thirty eggs were injected for each of the three treatments: 1000ng dsGFP, 500ng dsLac2, 1000ng dsLac2 in the volume of 1.5µl. After egg hatch, larvae were maintained individually on tomato leaves in 2oz cups and were flash-frozen for qRT-PCR analysis as previously described.

**Larval Microinjection of dsRNAs**

Second instar larvae were injected as previously described. Twenty-eight larvae were injected with each of the three treatments in the amount of 1µl: 1000ng dsGFP, 500ng dsLac2, and 1000ng dsLac2. After injection, larvae were individually maintained on tomato leaves in 2 oz. cups. Laccase 2 qRT-PCR analysis was performed only on larvae harvested on days 1 and 6 post-injection. However, only 2 biological replications were evaluated for qRT-PCR analysis day 6 post-injection in larvae treated with 1000ng dsLac2 due to errors during RNA extraction. Larvae were not harvested on day 3 due to high mortality exhibited in all treatments on Day 2. Results are not shown for day 10 as mortality resulted in lack of biological replication in larvae treated with 1000ng dsLac2.
Continuous Feeding of dsRNAs on Leaf

The potential for systemic RNAi in the CPB by feeding dsRNAs on a leaf surface was evaluated. Double-stranded RNA was delivered as a leaf treatment to larvae for 10 days. Individual cages were constructed for each leaf to confine the larva only to the region where dsRNA was applied (Figure 1 D, E, F). The overall structure of the cage consisted of a top, an arena fastened to both the anterior and posterior of the leaf, and a bottom structure. All the pieces of the cage were held to the leaf using one small rubber band (Goody’s). The top structure of each cage was made of four 2.54 cm X 2.54 cm sticky foam squares (Office Max) stuck together, with a hole cut out of the center of each using a #12 cork corer. The bottom structure was made of two 2.54 cm X 2.54 cm sticky foam squares with a hole cut out of the center. Organdy mesh (Joann Fabric) was adhered to the top and bottom structures of the cage to create an enclosure. The arena is the area on the top and bottom of the leaf where the dsRNA treatment was applied and larva was confined to. Arenas were constructed from one 2.54cm X 2.54 cm sticky foam square (Office Max, Hobby Lobby) with a hole cut out of the center using a #12 corer. Each leaf with individual cage was placed in a 2 oz. cup with the petiole placed in 0.4% agar. One larva was placed on the leaf arena for a total of 30 larvae per treatment. The 3 RNAi treatments were 1000ng dsGFP, 500ng dsLac2 and 1000ng dsLac2. Leaves were treated with dsRNA by applying half of the dose to the top of the leaf in the amount of 25µl, and half of the dose onto the bottom of the leaf in the amount of 30µl. An inoculating loop was used to spread the dsRNA over the leaf surface. Larvae were flash-frozen for qRT-PCR analysis as previously described. To ensure that lack of Lac2 knockdown was not due to reduced biological replications for qRT-PCR, at least 8 larvae
were evaluated for each treatment at day 10. One biological replicate was removed from the 1000ng dsGFP analysis at day 3, as it was an obvious outlier.

**Pulsed Feeding of dsRNAs on Leaf**

Double-stranded RNAs were delivered on the leaf surface to larvae as a “pulsed” treatment to evaluate the efficacy of systemic and persistent RNAi following an initial exposure to dsRNAs. Double-stranded RNA was delivered as a leaf treatment to larvae for 2 days and the same experimental procedure as Continuous Feeding on Leaf was used. Larvae were flash-frozen for qRT-PCR analysis as previously described.

**RNA Extraction and Reverse-Transcription Quantitative Real-Time PCR (qRT-PCR)**

Prior to RNA extraction, photos were taken of each larva to document cuticle pigmentation. Total RNA was isolated from individual larvae harvested at Days 10 and 6 from the larval injection experiment using Trizol. However, the RNA exhibited a brown color, and was subjected to re-precipitation by 5M ammonium acetate. RNA from these individuals was then purified (RNeasy, “RNA Cleanup” protocol) and treated with DNaseI (Invitrogen). Due to this issue, total RNA was isolated from all remaining samples using the RNeasy Mini Kit (Qiagen), treated with DNaseI (Qiagen) and stored at -80°C. RNA quality was confirmed using a denaturing agarose gel, and the concentration was obtained using the Qubit fluorometer. cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) and 1µg of DNaseI-treated RNA was used as template.

Primers and probes were designed for qRT-PCR using the PrimerQuest Tool (IDT) to amplify a 97bp amplicon for B-actin and an 114bp amplicon for Lac2 (Table 2). Tm for B-actin and Lac2 probes were ~6°C above the Tm of the forward and reverse
primers. Reference and target gene primer concentrations were optimized to run as a
duplex assay. The standard deviations of single and duplex reactions were compared,
and amplification curves were evaluated. Serial dilutions of cDNA were used to generate
a standard curve for each primer/probe pair for the target and reference gene as a duplex
reaction in order to establish primer efficiencies. For qRT-PCR, technical replication and
negative controls were performed as described before. Each 20µl qRT-PCR duplex
reaction consisted of 10µl iTaq Universal Probes Supermix (Bio-Rad), 2µl cDNA
template, 150nM B-actin forward and reverse primers, 500nM Lac2 forward and reverse
primers, and 250nM each B-actin and Lac2 probe. Reverse-transcription quantitative
PCR was performed with an initial incubation of 95°C for 30 seconds, followed by 40
cycles of 95°C for 5 seconds and 60°C for 30 seconds, followed by 5 seconds at 70°C and
a final incubation of 95°C for 5 seconds.

**Statistical Analysis**

Gene expression analysis of Lac2 CNRQs was conducted for each treatment
within each day using a general linear model with an exponential distribution by proc
glimmix in SAS v. 9.2. Statistical significance was determined at p< 0.05.

**Results**

**DNA Sequencing- Degenerate PCR**

An NCBI blastx search (http://blast.ncbi.nlm.nih.gov) with a high quality *L.
decemlineata* Laccase2 consensus sequence of 983bp (Appendix A) indicated 90% amino
cacid sequence identity with a *Laccase 2* precursor of *T. castaneum* (GenBank accession
NP_001034487.2). An NCBI blastn search indicated 76% nucleotide identity to *T.
castaneum* Laccase 2A mRNA (GenBank accession NM_001039398.2).
**DNA Sequencing of Laccase2 Clone**

An NCBI blastn search of the consensus of all clones (Appendix B) indicated a 76% nucleotide identity to *T. castaneum* Laccase2A mRNA (GenBank accession NM_001039398.2). A blastx search indicated 91% amino acid identity to *T. castaneum* Laccase 2A mRNA (GenBank accession NP_001034487.2).

**Reference Gene Selection for qRT-PCR**

Potential reference genes for Laccase 2 normalization in qRT-PCR gene expression analysis were evaluated using the geNorm function within Qbase (Table 3). The stability values (M) for each gene across dsGFP, 500ng and 1000ng of dsLac2 treatments were as follows: 0.958, 1.106 and 1.059 for B-actin, RP18, and RP4 respectively. The coefficient of variation (CV) for each gene was 0.298, 0.511 and 0.402 for B-actin, RP18, and RP4 respectively. Guidelines for this software recommend selection of a reference gene with M < 1 and CV < 50% (biogazelle.com). B-actin was selected for further use as a reference gene for Laccase 2 normalization in qRT-PCR.

**Phenotypic Effect of Laccase 2 Knockdown by RNAi**

A slight phenotypic effect was exhibited in larvae exposed to 500ng and 1000ng dsLac2 on artificial diet in the first set of RNAi experiments (Figure 2). This effect was slight, and change in pigmentation alone was not enough of a phenotypic change to indicate knockdown of Laccase 2. However, when 500ng and 1000ng of dsLac2 were injected into 2nd instar larvae in the second set of RNAi experiments, the phenotypic effect was apparent beginning at 3 days post-injection (Figure 3). At day 3 post-injection, the phenotype was exhibited in 61% of larvae injected with 500ng dsLac2 and
33% of larvae injected with 1000ng dsLac2 (Table 6). No phenotypic change in pigmentation was observed in larvae injected with dsGFP that molted to 3rd instar. At 7 days post-injection, mortality in these two treatments increased to over 50% each, and mortality from days 7-10 only occurred in larvae that were exhibiting the phenotypic change.

qRT-PCR Detection of Laccase 2 Knockdown by RNAi

Delivery of dsLac2 resulted in a significant reduction in Laccase 2 expression using all dsRNA delivery methods except for feeding dsLac2 on a leaf surface. In the first set of RNAi experiments, expression of Laccase 2 was reduced in a concentration-dependent manner by feeding dsRNAs on artificial diet (Figure 6). Additionally, reduction of Laccase 2 was achieved by injecting 500ng dsLac2 into embryos (Figure 4) and by injecting 250ng into second instar larvae (Figure 5). In the second set of RNAi experiments, Laccase 2 expression was evaluated at multiple time points after dsRNA delivery. Significant knockdown of Laccase 2 occurred in second instar larvae injected with 1000ng dsLac2 1 day post-injection, but Lac2 knockdown was not observed at 6 days post-injection (Figure 7). Knockdown of Laccase 2 was not observed in the second set of RNAi experiments that fed dsRNA on a leaf surface (Figures 9,10) or injected dsLac2 into embryos (Figure 8).

Discussion

RNA interference is a valuable tool to evaluate gene function in non-model organisms and is rapidly being adapted for use to study gene function in many insect species. In order to evaluate gene function, it is desirable that the effects of RNAi-mediated knockdown of the target gene are systemic and persistent. However,
establishing methods of systemic and persistent RNAi is variable among insect species. In this study, a variety of dsRNA delivery methods were evaluated in two life stages of the CPB in order to determine the potential of systemic and persistent RNAi. The Laccase 2 gene, which is responsible for insect cuticular pigmentation and sclerotization, was targeted for RNAi knockdown as a proof of concept. Systemic and persistent RNAi was tested by microinjection of dsRNAs into embryos and microinjection and feeding dsRNAs on diet in the larval stages. No knockdown of Lac2 was observed by feeding dsLac2 on the leaf surface. It is worth noting that different qRT-PCR methods were used to evaluate Lac2 expression for the first and second set of RNAi experiments. Sybr green was used in the first set of experiments, and hydrolosis probes were used in the second set of RNAi experiments. Hydrolosis probes were used in the second set of experiments in order to duplex the target and reference gene reaction. This can reduce the variability between the reference and target gene, as both genes are amplified from the same cDNA aliquot, while simultaneously conserving reagents. We believe that the lack of statistical significance obtained in the second set of RNAi experiments is due to the reduction of biological replications evaluated in qRT-PCR, as opposed to the different qRT-PCR method.

**Feeding dsRNAs on Artificial Diet**

Larvae fed dsRNAs on artificial diet exhibited a significant reduction of Laccase2 expression that was concentration dependent, as a higher concentration of dsLac2 resulted in more knockdown of the Laccase2 gene. However, the phenotypic effect of Laccase 2 knockdown in this assay was minimal, suggesting that sufficient RNAi knockdown of the Lac2 gene can be achieved without obtaining obvious loss of
pigmentation. A similar result occurred in western corn rootworm larvae where some larvae maintained their pigmentation yet still demonstrated Lac2 knockdown by qRT-PCR (Alves et al., 2010). The phenotypic effect of Lac2 knockdown in other insects was exhibited using microinjection to deliver dsRNAs (Arakane et al., 2005; Niu et al., 2008; Alves et al., 2010; Futahashi et al., 2011; Wu et al., 2013). The slight reduction in pigmentation caused by feeding may have been attributed to gradual ingestion of dsRNAs caused by gradual feeding on diet. Feeding dsRNAs on artificial diet is a useful delivery technique for the CPB. This method is less traumatic and invasive than microinjection, and knockdown of Laccase 2 using this method resulted in systemic and persistent RNAi effects.

**Larval Microinjection of dsRNAs**

Systemic and persistent RNAi was demonstrated in second instar larvae injected with dsLac2. The knockdown effect was exhibited 24 hours after injection, as seen in the second RNAi injection experiment, and was also seen one week after injection, as demonstrated in the first RNAi injection experiment. The first RNAi injection experiment indicates that 100ng of dsLac2 was not sufficient to cause RNAi knockdown. Injection of second instar larvae with 250ng dsLac2 was sufficient to cause systemic and persistent knockdown of Laccase 2, but not the visible phenotype. Injecting second instar larvae with 500ng and 1000ng of dsLac2 resulted in knockdown of Laccase 2 at 1 day post-injection, but not 6 days post-injection (second injection experiment). It is not entirely surprising that Lac2 knockdown was not observed at day 6, as larvae were randomly selected for sampling and none of the larvae harvested at that time point displayed a phenotypic loss of pigmentation. Additionally, only 2 biological replications
were evaluated in qRT-PCR in larvae injected with 1000ng dsLac2 at day 6. There were several larvae that displayed a loss of pigmentation 3 days after injection with 500ng and 1000ng dsLac2 in the second RNAi injection experiment. However, higher doses of dsLac2 may have contributed to mortality, as many of the larvae that were exhibiting the phenotype did not survive past day 7 and were therefore unable to be evaluated by qRT-PCR. RNAi by microinjection is more invasive and traumatic than other dsRNA delivery methods such as feeding, but ensures the delivery and timing of the dsRNA dose to each insect.

Egg Microinjection of dsRNAs

Embryonic RNAi by microinjection in the Colorado potato beetle yielded an interesting and unexpected result. Systemic and persistent RNAi knockdown of Lac2 was exhibited in the first RNAi injection experiment by delivering 500ng dsLac2 to eggs. CPB hatch rate was also lower in eggs injected with 500ng of dsLac2 in both the first and second set of RNAi experiments (Table 5). Surprisingly, no RNAi knockdown was achieved by injection of 1000ng dsLac2. Huvenne and Smagghe (2010) suggest dsRNA concentration directly affects gene knockdown, as a higher concentration of dsRNAs does not always result in more knockdown of the target gene (Meyering-Vos and Müller, 2007). Embryonic RNAi studies conducted in insects have been largely limited to genes that are expressed during the embryonic stage (Kennerdell and Carthew, 1998; Brown et al., 1999; Bolognesi et al., 2008). Although the role of Laccase 2 has been demonstrated for the very dark eggshell pigmentation in the mosquito Aedes albopictus (Wu et al., 2013), it is unknown if Laccase 2 is expressed in coleopteran embryos. Tomoyasu and Dennell (2004) suggest that it is possible that the effects of embryonic RNAi do not
persist through the developmental stages of the insect (Tomoyasu and Denell, 2004). More investigation is needed to evaluate the potential for embryonic RNAi targeting genes that are expressed in later life stages of the insect. Systemic and persistent embryonic RNAi was demonstrated in the CPB, although the effects were not dose-dependent. Embryonic RNAi is a useful method for targeting genes that are expressed very early, and optimization of dsRNA concentration is needed for genes expressed in later life stages.

No RNAi-mediated knockdown of the Laccase 2 gene was detected in the second RNAi embryonic injection experiment, where expression of Lac2 was evaluated at multiple time points. We believe that a reduction of the number of biological replicates evaluated for qRT-PCR may have contributed to this result. For the first RNAi embryonic injection experiment, knockdown of the Lac2 gene was detected by evaluating a minimum of 10 biological replicates/treatment, whereas 4 biological replicates/treatment were evaluated at each time point in the second RNAi embryonic injection experiment, and knockdown was not observed. In other studies, 6 or fewer biological replications have been evaluated using qRT-PCR to detect RNAi knockdown of an essential gene, such as vATPase (Li, et al., 2011; Zhu et al., 2011). Therefore, more biological replications may be necessary to detect knockdown in genes that are more variable in expression than essential housekeeping genes, such as vATPase.

**Feeding dsRNAs on Leaf**

The second set of RNAi experiments included two assays with feeding dsRNAs on a leaf surface. One experiment was conducted with continuous delivery of dsRNAs. The other experiment was conducted with a pulsed delivery of dsRNAs in order to see if
dsRNAs supplied on a leaf surface for 2 days would have a persistent knockdown effect. No RNAi knockdown effect was observed in either of these experiments. Feeding dsRNAs on a leaf surface has been successful by targeting a vATPase gene in the CPB (Zhu et al., 2011). It is possible that feeding dsLac2 on the leaf surface did not achieve knockdown because a high enough dose was not achieved. The dsRNA dose was applied to a leaf area that the larvae did not completely consume (which contained the dsRNA dose) until day 7-8 (Yates personal observation). The area of leaf required for consumption in order to acquire the dose was much larger than the area of the artificial diet (Figure 1E, 1B). It is possible that the acquisition of the dsRNA dose was much slower with this method than with any of the other methods evaluated. Perhaps this issue could have been circumvented by significantly increasing the dsRNA dose on the leaf surface. Zhu et al. (2011) achieved RNAi knockdown by feeding 50µg dsvATPase, which is 50x the highest dsLac2 dose evaluated in these experiments. Perhaps significant RNAi knockdown can be achieved by feeding dsRNAs on a leaf surface by applying equally high doses of dsLac2. To ensure that lack of Lac2 knockdown was not due to reduced biological replications for qRT-PCR, at least 8 larvae were evaluated for each treatment at day 10 of continuous feeding on leaf. Knockdown of Lac2 was still not exhibited after adding more biological replications, suggesting that a higher dsRNA dose is necessary for this method of dsRNA delivery. Establishing methodologies for feeding dsRNAs on a leaf surface would be a useful method for evaluating gene function in CPB insect-plant interactions.

Taken together, these results demonstrate the potential for RNA interference as a tool for the functional analysis of genes in the CPB. The results indicate that the
potential for systemic and persistent RNAi exists for injection and artificial diet assays, and potentially for feeding on a leaf surface, following further modifications. A slight phenotypic effect of Lac2 knockdown was observed in larvae fed dsRNAs on artificial diet, and a stronger phenotypic effect was exhibited by larval microinjection of dsRNAs, which may have contributed to mortality. Establishing dsRNA delivery methods for RNAi is a useful tool for studying insect-plant interaction in this system.
Table 1. A list of primers used for Laccase2 isolation, PCR and qRT-PCR in the first set of RNAi experiments.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Amplicon</th>
<th>Sequence 5’-3’</th>
<th>Ta °C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Degenerate Primers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lac2A_F+T7</td>
<td>1082bp</td>
<td>TAATACGACTCACTATAGGGCAYTTYTGGCAYGCNCAAYACNGG</td>
<td>53</td>
</tr>
<tr>
<td>Lac2A_R+SP6</td>
<td>1082bp</td>
<td>ATTTAGGTGACACTATAGCCRTGNARRRTGRAANNGGRTG</td>
<td>53</td>
</tr>
<tr>
<td><strong>Gene Specific Primers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPBLac2_F</td>
<td>800bp</td>
<td>GCACATGCAGTTTTGTCCAC</td>
<td>65</td>
</tr>
<tr>
<td>CPBLac2_R</td>
<td>800bp</td>
<td>CGGTGAATACCCGGTCAAGAT</td>
<td>65</td>
</tr>
<tr>
<td><strong>In vitro dsRNA Synthesis Primers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNAi_F + T7</td>
<td>442bp</td>
<td>TAATACGACTCACTATAGGGGAGCGTGCTGGTCTGATTTTGGT</td>
<td>65</td>
</tr>
<tr>
<td>RNAi_R + T7</td>
<td>442bp</td>
<td>TAATACGACTCACTATAGGGGAGCGTGCTGGTCTGATTTTGGT</td>
<td>65</td>
</tr>
<tr>
<td>GFP_F + T7</td>
<td>375bp</td>
<td>TAATACGACTCACTATAGGGGAGGTTGATGCTACATACGGAAG</td>
<td>63</td>
</tr>
<tr>
<td>GFP_R + T7</td>
<td>375bp</td>
<td>TAATACGACTCACTATAGGGGAGGTTGATGCTACATACGGAAG</td>
<td>63</td>
</tr>
</tbody>
</table>

*Primers used for in vitro dsRNA synthesis of Laccase2 and qRT-PCR of Lac2 in the first set of RNAi experiments. Ta denotes annealing temperature used in PCR cycling.
Table 2. Primers and probes used for qRT-PCR in the second set of RNAi experiments.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Amplicon</th>
<th>Sequence 5’-3’</th>
<th>Tm °C</th>
<th>Ta °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-actin_F</td>
<td>97bp</td>
<td>AAGCCAACAGGGAGAAGATG</td>
<td>54.8</td>
<td>60</td>
</tr>
<tr>
<td>B-actin_R</td>
<td>97bp</td>
<td>GAAGCGTACAAGGAGAGGTACAG</td>
<td>54.7</td>
<td>60</td>
</tr>
<tr>
<td>B-actin_probe</td>
<td>n/a</td>
<td>/5HEX/ACCTTCAAC/ZEN/ACACCGCCATGTAT/3IABkFQ/</td>
<td>60.9</td>
<td>60</td>
</tr>
<tr>
<td>Lac2_F</td>
<td>114bp</td>
<td>CCTCACACCACATTCTCATTCAA</td>
<td>54.4</td>
<td>60</td>
</tr>
<tr>
<td>Lac2_R</td>
<td>114bp</td>
<td>GTCAACACCGTCTATTTCTTCTCA</td>
<td>54.2</td>
<td>60</td>
</tr>
<tr>
<td>Lac2_probe</td>
<td>n/a</td>
<td>/56-FAM/TGAATCCAG/ZEN/TAAGCTCCAACCGGGT/3IABkFQ/</td>
<td>60.2</td>
<td>60</td>
</tr>
</tbody>
</table>

HEX and FAM are fluorophores and ZEN and IaBkFQ (Iowa Black Dark Quencher) are quenchers for probes. Tm denotes melting temperature of primers and probes. Ta denotes annealing temperature used in PCR cycling.
Table 3. geNorm evaluation of potential reference genes for qRT-PCR.

<table>
<thead>
<tr>
<th>Reference Target</th>
<th>M</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \beta)-actin</td>
<td>0.958</td>
<td>0.298</td>
</tr>
<tr>
<td>RP18</td>
<td>1.106</td>
<td>0.511</td>
</tr>
<tr>
<td>RP4</td>
<td>1.059</td>
<td>0.402</td>
</tr>
</tbody>
</table>

Evaluation of potential reference genes was conducted using the geNorm function within Qbase. M is the stability of the gene between treatments, where a lower M indicates a greater stability of the gene. CV is the coefficient of variation. The expression of three biological replicates in triplicate was evaluated for each of the three treatments by feeding on artificial diet: dsGFP, 500ng dsLac2, 1000ng dsLac2.
Table 4. Parameters for Laccase2 and B-actin primers for qRT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Efficiency</th>
<th>$R^2$</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>First Set of RNAi Experiments</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-actin</td>
<td>102.7%</td>
<td>0.96</td>
<td>-3.259</td>
</tr>
<tr>
<td>Lac2</td>
<td>99.9%</td>
<td>0.966</td>
<td>-3.325</td>
</tr>
<tr>
<td><em>Second Set of RNAi Experiments</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-actin</td>
<td>93.60%</td>
<td>1</td>
<td>-3.487</td>
</tr>
<tr>
<td>Lac2</td>
<td>93.70%</td>
<td>1</td>
<td>-3.483</td>
</tr>
</tbody>
</table>

Lac2 gene expression in the first set of RNAi experiments was evaluated using Sybr green. Lac2 gene expression in the second set of RNAi experiments was evaluated using hydrolysis probes. Parameters for the second set of RNAi experiments are expressed for the duplex reaction of B-actin + Lac2.
Table 5. Egg hatch rates and survival for the first and second RNAi egg microinjection experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>First RNAi Experiment</th>
<th>Second RNAi Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Hatch</td>
<td>% Survive</td>
</tr>
<tr>
<td>dsGFP</td>
<td>90%</td>
<td>66.6%</td>
</tr>
<tr>
<td>500ng dsLac2</td>
<td>65%</td>
<td>77%</td>
</tr>
<tr>
<td>1000ng dsLac2</td>
<td>90%</td>
<td>61%</td>
</tr>
</tbody>
</table>

For egg microinjections: n=20 for first RNAi experiment, n=30 for second RNAi experiment. % Survival is given as the number of larvae at the end of the experiment. In the second RNAi egg microinjection experiment, there were 3 prior harvest days performed before experiment concluded and % survival is given.
Table 6. Second set of RNAi experiments: larval microinjection. Larvae exhibiting phenotypic loss of pigmentation on day 3 post-injection of dsRNAs.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N Surviving</th>
<th># Slightly Pale</th>
<th># Pale</th>
<th>Total % Pale</th>
</tr>
</thead>
<tbody>
<tr>
<td>500ng dsLac2</td>
<td>13</td>
<td>1</td>
<td>7</td>
<td>61%</td>
</tr>
<tr>
<td>1000ng dsLac2</td>
<td>9</td>
<td>1</td>
<td>2</td>
<td>33%</td>
</tr>
</tbody>
</table>

Larvae injected with 500ng and 1000ng dsLac2 during the second set of RNAi experiments exhibited a loss of pigmentation beginning at day 3 post-injection. Phenotypic knockdown of Lac2 was classified as “pale” or “slightly pale.”
Figure 1. Experimental materials and designs for dsRNA delivery methods.

A. Microinjection apparatus used in egg and larval injection experiments.  B. Artificial diet with dsRNA treatment used in first feeding experiment.  C. Eggs aligned on double-sided tape and adhered to microscope slide for injection.  D. Arena used for leaf feeding experiments.  E. Area inside arena where dsRNA treatment was applied and larva was confined.  F. Individual leaf cages used in leaf feeding experiments.  G.,H. Leaf petioles placed in 0.4% agar to maintain freshness. Larvae reared in 2 oz. cups.
**Figure 2.** First set of RNAi experiments: phenotypic effect of feeding dsLac2 on artificial diet.

Larvae fed dsRNA on artificial diet for 10 days in first set of RNAi experiments.  

**A.** Control-treated (dsGFP) larva exhibiting a fully pigmented head, pronotum and spiracles.  

**B,C.** Reduction in head and/or pronotum and spiracle pigmentation exhibited in individuals fed 1000ng dsLac2.
Figure 3. Second set of RNAi experiments: phenotypic effect of larval dsLac2 injection.

Mortality occurred in larvae injected with dsLac2 and exhibiting a phenotypic effect of Lac2 knockdown 7 days post-injection. **A.** Control-treated (dsGFP) larva exhibiting a fully pigmented head, pronotum and spiracles. Loss of pigmentation demonstrated in head, pronotum and spiracles of **B.** 500ng dsLac2 and **C.** 1000ng dsLac2- treated larvae.
**Figure 4.** First set of RNAi experiments: expression of Laccase2 following microinjection of dsRNAs into embryos.

Laccase2 expression in control and dsLac2-treated larvae after injection of dsRNAs into CPB embryos. Lac2 expression was evaluated using qRT-PCR and normalized to B-actin. Statistical analysis was performed using a general linearized model with an exponential distribution. Means with different letters represent statistical significance ($p<0.05$; treatment $n=12$ dsGFP and 1000ng dsLac2, $n=10$ 500ng dsLac2).
**Figure 5.** First set of RNAi experiments: expression of Laccase2 following injection of dsRNAs into larvae.

Laccase2 expression in control and dsLac2-treated larvae after injection of dsRNAs into 2\textsuperscript{nd} instar larvae. Lac2 expression was evaluated using qRT-PCR and normalized to B-actin. Statistical analysis was performed using a general linearized model with an exponential distribution. Means with different letters represent statistical significance (p <0.05, treatment n=7 dsGFP, n=9 100ng dsLac2, n=5 250ng dsLac2).
Figure 6. First set of RNAi experiments: expression of Laccase2 following feeding dsRNAs on artificial diet.

Laccase2 expression in control and dsLac2-treated larvae after feeding dsRNAs on artificial diet. Lac2 expression was evaluated using qRT-PCR and normalized to B-actin. Statistical analysis was performed using a general linearized model with an exponential distribution. Means with different letters represent statistical significance (p <0.05, treatment n=12, 1000ng dsLac2 n=11).
Figure 7. Second set of RNAi experiments: expression of Laccase2 following injection of dsRNAs into larvae.

Laccase2 expression in control and dsLac2-treated larvae 1 and 6 days post-injection. Gene expression was evaluated using qRT-PCR and normalized to B-actin. Statistical analysis was performed for each day using a general linearized model with an exponential distribution. Means with different letters represent statistical significance within the same number of days post-injection (p <0.05, treatment n=4, day 6 1000ng dsLac2 n=2).
**Figure 8.** Second set of RNAi experiments: expression of Laccase2 following injection of dsRNAs into embryos.

Laccase2 expression in control and dsLac2-treated embryos 1, 3, 6 and 10 days post-hatch. Gene expression was evaluated using qRT-PCR and normalized to B-actin. Statistical analysis was performed for each day using a general linearized model with an exponential distribution. No statistical significance between control and dsLac2 treated is observed (p <0.05, n=4/treatment).
Figure 9. Second set of RNAi experiments: expression of Laccase 2 following continuous feeding of dsRNAs on leaf surface.

Laccase2 expression in control and dsLac2-treated larvae at days 1, 3, 6 and 10 during continuous feeding of dsRNAs on leaf experiment. Gene expression was evaluated using qRT-PCR and normalized to B-actin. Statistical analysis was performed for each day using a general linearized model with an exponential distribution. There are no significant differences observed among treatments within each day at p <0.05. (N=4/treatment, Day 3: dsGFP n=3, additional biological replicates added for Day 10 dsGFP n=8, 500ng dsLac2 and 1000ng dsLac2 n=10.)
**Figure 10.** Second set of RNAi experiments: expression of Laccase 2 following pulsed feeding of dsRNAs on leaf surface.

Laccase2 expression in control and dsLac2-treated larvae at days 1, 3, 6 and 10 during pulsed feeding of dsRNA on leaf. Gene expression was evaluated using qRT-PCR and normalized to B-actin. Statistical analysis was performed for each day using a general linearized model with an exponential distribution. There are no significant differences observed among treatments within each day at p <0.05. (N=4/treatment, Day 1 dsGFP n=3.)
**Figure 11.** A summary of Laccase2 knockdown, phenotypic effect, and observations and suggestions for each dsRNA delivery method evaluated.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Knockdown?</th>
<th>Phenotype?</th>
<th>Observations/ Suggestions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed on diet</td>
<td>Yes</td>
<td>-</td>
<td>Diet may affect CPB</td>
</tr>
<tr>
<td></td>
<td>Yes- concentration dependent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inject larvae</td>
<td>Yes</td>
<td></td>
<td>Mortality associated with injection</td>
</tr>
<tr>
<td>Inject embryos</td>
<td>Yes- low dose</td>
<td>No</td>
<td>Increase biological replication for qRT-PCR</td>
</tr>
<tr>
<td>Feed on leaf</td>
<td>No</td>
<td>No</td>
<td>Increase dose Increase biological replication for qRT-PCR</td>
</tr>
</tbody>
</table>
Appendix A. 983bp sequence of Laccase 2A.

CAATCGCATTTAGGTATATCAACTTTTGAGTGACTGACATGGAGTTTGTCCA
CAATCGCAGTGGTCTGTTTATCTCCGTTGCAAGATTTTGGTTTTCAGATATC
GTACTGAGAAATCGTTGGTGGAGCCGACATGTATGAAATTCATCTATCC
AGACTTTATTACATGTGCTCCATTTCCGCGCAACTAAATGTCTGTTTATAAGTGTG
GGGGCGAAATAGACCTCTCTGGAGTATATAACATGGAATCTGAACGG
TAGGAA
AATTTTAACATCTGGCCTCTCAGCTATAAAATTCTCTGTCTATTCTCTTGTGCTATT
TTCAACTGACTTACGCAAATAGCGTCTGCTGTTATTTCTGTTACATCTTTGCA
CCAACTGGGTAAAGAACCCACCTCCTTTGTGTTATACCATACTGCTATATGTGGTGG
GGCGGTCGAAGGTGATATGGAACCTCTGGCATACGCGAGATGCGCCAACTGC
TGAACTCTCCCTCAACACCACATTCTCCTAAACCTCAGTGGATTTCCAGTAAAGC
TCCAACGGTGGATTGCACTGATAATAATGAAATCGTATCTTTTCTCCGAGAATG
AAATGACGCTGTGACTTTTACAGGATGACTCCCTCCTGTTGCGATA
AGAGTCAATCATGACCTTGGAGATGTAAATTGAGCCGACATACCGAAAGCGA
ATGAATTAATCATCTCTGAATCTATATCTTTTACGCCGGTAAATGGTGAAAAT
TCCAACGGTGGTTGCTGACAGGACTGGAGTGGGCTCCTGTAACACTGCTTT
ACCGTTTATAAGTAAACTCTCTGGATTACCCGAGTCATACCGCACTGCC
CAGGGTAGACCTTTCCAAATAGCGGTTCGCTGCAATCTCAGAAGTACGAGAC
ATGGGTGGTGAGATCAGTAGAGATGCTGTTTGAGATCTTTTGAAGGAA
GGTTGGCGAAGCGAGAATGCTACCATAAGATACCCATCC
Appendix B. 807bp consensus sequence of *Laccase 2A Clone 4.*

GCACATGCAGTTTTGTCCACAATCAGCTGTCTTTATCTCCGTGTTGCAGAAATTGTGCTGTTATTAGGTGTGGGGCAGGAAATAGATCCTCTGTGGAGATATAAACATGGGATCTGAGGATAGAAAAATTTAATCTCTGGCCCTCTCAGCTAAAAATTCTCTCTGTCTATTTCTTGTGCATTTTTTCAAACTGACTTACGCAAAATAGCGTCTGGTCTGATTTCGTTACATCTTGCATCCAATGGGTTAAGAACCACTCCTTGCTGGATACCCGAGATGGCCAACTGCTGAACTCTCCTCACACCACATTCTCTCTAAACCTCTCAGTTGAATCCAGTAAGCTCCAACGGGTTGATTTGCTAGTTAATAATGGAAATCTGATAGGTTTCTGCGATAAGAGTCAAAATCATGACCTTGGAATGTTAATTGAGCCGGACATACCGAAGCGAATGAATTAATCATCCTGAATCTATATCTTTTACCCGGGGTAATTGTGAAAACTTCCAACGGGTTGGCTCATGAAACCAGTGTTGGGTCTCTGAACTGACCTTTACCGTTTATAAGTAAACTCTCTGGATCTTGACCGGTATTCACCG
Appendix C. Lac2 442bp amplicon

AGCGTCTGGTCTGATTTACATCTTTGATCCGCATCAATGGGTAAGAACCACCTC
CTTGTGGTATAACCATAGTCATATGTGGGTGCGGCGGTCGAAGGTTGATATGG
ACCTCTGGCATAACGCAGGATGGCGAACTGCTGAACACTCTCCTCACCACCAT
TCTCCTAAACCTCTCCAGTTGAATCCAGTAAGCTTCAACCGGTTGATTTCATT
AATAATGAAATCGTATCTCTTTCTCCGGGAGAATGAAATGACGCTTGGACTTTGA
CAGGATGTACAGGGTCTCCATCTGTGCTGATAAGAGTCAATCGAACTCTTTGG
AATGTTAATTCGACCCGAGCATACCAGAAGCGAATGAATTAATCATCTCACAATC
TATATCCTTTACCCGGGTGAATTGGAAACTTCCACCGGTGTGGGCATGGAAACCAGTTGGGCTCTG
Appendix D. B-actin 573bp amplicon (GenBank acc EB761683)

GCACGAGGTTTTTCTGTCTAGTGAGCAGTGCTCAACCTCAAAAAAGACAACATG
TGTGACGACGATGTAGCGGTCTCTTGTGACAATGGATCCGGTATGTGCA
AAGCCCGTCTTCGAGGAGATGACGCACCCTCGTCCCTCCCTCAGACTGTC
GGTGCTCCAAGGCTATGAAGTGCTGCTGGGTAGGGACAAAGGACTCAT
ACGTAAGGAGATGACGCCCAAGGAAAGAGGGATCGACTCACCCTAGAAATACC
CCATCGAACACGGGTATCACACCAACTGGGATGACAGTGGAAAAGATCTGGCA
CCACACCTTTCTACAACGAACTCCGTTGCTCCAGAGACGACCCAGTCTCTCC
TCACTGAGCTCCAACCCCAAAGGCAACAGGGAGAAGATGACCCAAAT
CATGTGGAGACCTTCAACACACCCGCGATGTAGTGCCTCAGGAAGCTGTAC
TCTCCTTGTCGCTTCTGCCGTCACCCGCTATGCTTGGAGAT
GGTGTCACCACACCCTACCAATCTACGAAGGTTACGCTCTTTCCCACGC
CHAPTER 3:

Expression of RNA Interference Genes in Response to Double-Stranded RNA

in the Colorado Potato Beetle, Leptinotarsa decemlineata (Say)
Introduction

RNA interference, or double-stranded RNA (dsRNA)-mediated gene silencing, is a naturally occurring phenomenon in which a dsRNA suppresses or silences the expression of a homologous gene (Fire et al., 1998). RNA interference (RNAi) is employed as an entomological research tool in order to elucidate gene function in model and non-model insects. In addition to serving as a tool to study gene function, there is growing interest for RNAi as a means for insect control, whereby a dsRNA is designed to target and suppress an essential gene, resulting in mortality of the insect.

The small interfering RNA pathway (siRNA) is initiated when a dsRNA is introduced into a cell. The dsRNA is processed into ~21bp small interfering RNAs (siRNAs) by an enzyme called Dicer-2 (Bernstein et al., 2001). The siRNAs are incorporated into an RNA-Induced Silencing Complex (RISC) (S M Hammond et al., 2000), which is coupled with the Argonaute-2 (Ago-2) protein (S M Hammond et al., 2001). The double-stranded siRNA is unwound, the passenger strand is degraded and the RISC complex uses the guide strand to direct the complex to the homologous mRNA (Nykänen et al., 2001). The RISC complex has endonuclease activity and cleaves the mRNA (Hammond et al., 2000; Zamore et al., 2000; Nykänen et al., 2001). The mRNA is destroyed, and the protein for which the mRNA is coding is not expressed.

Although the general mechanism of the siRNA pathway has been characterized, the response to RNAi is variable among different insect orders. Coleoptera exhibit a robust RNAi response (Tomoyasu et al., 2008; Bellés, 2010), while Lepidoptera tend to be less susceptible to RNAi (Terenius et al., 2011). There is a growing interest to understand differences in RNAi response, especially in insect pests, in order to evaluate
the potential for RNAi-based insect control. Several studies have identified and characterized genes involved in the RNAi pathway to determine if variation in RNAi response is due to differences in core RNAi machinery. RNAi genes have been characterized for several species, including *Tribolium castaneum* (Tomoyasu et al., 2008), *Bombyx mori* (Swevers et al., 2011), soybean aphid *Aphis glycines* (Bansal and Michel, 2013), whitefly *Bemisia tabaci* (Upadhyay et al., 2013), hessian fly *Mayetiola destructor* (Shreve et al., 2013) and the Colorado potato beetle (gut) (Swevers et al., 2013). The two core components of the RNAi pathway, Dicer-2 and Ago-2, are shared among insect species and are essential for an RNAi response in *Tribolium* (Tomoyasu et al., 2008). Many species differ in the presence or number of genes involved in the RNAi pathway that are not considered core components. However, none of these differences have been exclusively linked to differences in RNAi sensitivity among species.

Bellés (2010) and Terenius (2011) suggest that varying RNAi sensitivity among insects could be attributed to differences in expression of RNAi genes in response to dsRNAs. In the tobacco hornworm, *Manduca sexta*, Dicer-2 and Ago-2 expression were upregulated in response to dsRNA injection (Garbutt and Reynolds, 2012). Upregulation of the core RNAi machinery in *M. sexta* was described as “rapid and transient,” as Dicer-2 was upregulated 6 hours post-injection and Ago-2 upregulated up to 18 hours post-injection. A similar result was found in *B. mori* where upregulation of Dicer-2 and Ago-2 was observed up to 6 hours following dsRNA injection (Liu et al., 2013). In the cockroach *Blattella germanica*, injection of dsRNAs resulted in upregulation of Dicer-2 six hours post-injection (Lozano et al., 2012).
In this study, we aimed to characterize the response of CPB RNAi genes following exposure to dsRNAs. The CPB is a devastating insect pest to *Solanum* plant species, which includes tomato and potato. Current options for insect control are largely restricted to cultural control methods, such as crop rotation and early planting of short-season varieties (Hare, 1990), as this insect has become resistant to more than 50 insecticides (Forgash, 1985; Alyokhin *et al*., 2008). The CPB exhibits a sensitive RNAi response, and the potential for RNAi-based control of this pest has been demonstrated by feeding dsRNAs (Zhu *et al*., 2011).

The goal of this study was to investigate overall changes in gene expression using Illumina high throughput sequencing technology in response to dsRNA challenge in the CPB. We hypothesized that core RNAi components will be upregulated in response to dsRNA, as exhibited in other insect species. In order to generate an RNAi response, dsRNA for Laccase 2 was delivered to the CPB. Laccase 2 is responsible for cuticular pigmentation and sclerotization in *Tribolium* (Arakane *et al*., 2005), and RNAi knockdown of this gene has been demonstrated using qPCR in Chapter 1. Differences in gene expression between control and dsRNA-treated were evaluated 6 and 24 hours after dsRNA delivery.

**Materials and Methods**

**cDNA synthesis and degenerate PCR**

Total RNA was isolated from a pool of 18 neonate larvae using Trizol reagent (Invitrogen, Grand Island, NY). cDNA was synthesized using the Mint-2 cDNA synthesis kit (Evrogen, Moscow, Russia) and purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA). Degenerate primers (Alves *et al*., 2010)
designed to amplify Laccase 2A in the western corn rootworm, *Diabrotica virgifera virgifera* LeConte, were used to amplify a putative 1082bp Laccase2 in *L. decemlineata*. The forward primer (Table 1) was prepended with the T7 promoter sequence at the 5’ end, and the reverse primer was prepended with SP6 on the 3’ end, allowing direct sequencing of PCR products. All PCR primers were synthesized by Sigma-Aldrich, (St. Louis, MO).

Degenerate PCR was performed with an initial denaturation cycle of 3 minutes at 94°C, followed by 20 cycles of denaturation for 30 seconds at 94°C, 30 seconds annealing at 53°C, 30 seconds of extension at 72°C, with a final extension of 12 minutes at 72°C. PCR reactions were done in 20µl of 1X Taq Buffer with MgCl₂ (Amresco, Solon, OH) containing 1µl of 10µM forward and reverse primers, each dNTP at 2.5mM, 0.5U Taq polymerase (Amresco) and 1ng cDNA. Degenerate PCR resulted in the production of a 600bp amplicon in addition to the putative 1082bp Lac2. The 1082bp putative Laccase2 was excised from a Sybr green-stained agarose gel and purified using the MinElute Gel Extraction Kit (Qiagen). The extraction and purification of the 1082bp fragment from a gel was repeated in order to isolate only the 1082bp fragment, and not the 600bp amplicon. The 1082bp PCR product (Appendix A) was subjected to DNA sequencing in both directions using the T7 forward primer and SP6 reverse primer (Eurofins MWG Operon, Huntsville, AL). Sequence alignment was performed in Bioedit (v 7.1.3.0). The the consensus sequence of the alignment was used in NCBI blastx and blastn searches to confirm hits to Laccase2 homologues in other insects.

**Laccase 2A Amplification and Cloning**
L. decemlineata Laccase 2A gene-specific primers (Table 1) were designed using Primer3 (v0.4.0) to amplify an 800bp fragment. PCR was performed with an initial denaturation of 3 minutes at 94°C, followed by 25 cycles of denaturation for 30 seconds at 94°C, 30 seconds annealing at 65°C, 30 seconds of extension at 72°C, followed by a final extension of 12 minutes at 72°C. The concentrations of PCR components were the same as specified for the degenerate PCR.

The PCR product was purified and ligated into plasmid vector pCR 2.1 using the TA cloning kit (Invitrogen). The construct was transformed into competent E. coli cells (INVαF’) and positive clones were selected using Ampicillin. Twelve bacterial colonies were isolated and grown in LB broth. Plasmid DNA was recovered from the bacteria using the High Speed Plasmid Mini Kit (IBI Scientific, Peosta, IA). A glycerol stock of bacteria was stored at -80°C.

PCR was performed using the CPB gene-specific primers to amplify 800bp plasmid DNA template in 10 of the 12 clones. An initial denaturation cycle of 3 minutes at 94°C was followed by 25 cycles of denaturation for 30 seconds at 94°C, 30 seconds annealing at 65°C, 30 seconds of extension at 72°C, followed by a final extension of 12 minutes at 72°C. The concentrations of PCR components were the same as specified for the gene-specific PCR with cDNA.

The 10 bacterial colonies were inoculated into fresh LB broth, allowed to grow for 16 hours, and plasmid DNA was extracted from the bacteria for DNA sequencing with M13 forward and reverse primers (Eurofins). Sequence alignment and blast searches were performed as described before. Of the 10 clones sequenced, all clones contained some nucleotides that varied from the consensus sequence. Clone 4 (Appendix
B) had only one nucleotide difference (nucleotide 172) from the consensus sequence of all the clones, and was selected for use as template for dsRNA synthesis.

**Primer design for RNAi and in vitro dsRNA synthesis**

Primers were designed (Primer3) to generate a 442bp amplicon to serve a template for Lac2 dsRNA synthesis, which is within the recommended range of dsRNA length (Huvenne and Smagghe, 2010). The T7 promoter sequence was prepended to the 5’ end of the forward and reverse primers to create a 442bp amplicon (Table 1). The 442bp amplicon (Appendix C) was designed to avoid the nucleotide (172) that differed from the consensus sequence of the ten clones. PCR was performed with an initial denaturation of 95°C for 3 minutes, followed by 25 cycles of 95°C denaturation for 30 seconds, 65°C annealing for 30 seconds, and 72°C extension for 30 seconds, followed by a final extension of 72°C for 12 minutes. The concentrations of PCR components were the same as specified for the gene-specific PCR with cDNA. The PCR product was purified using the MinElute PCR Purification kit (Qiagen).

Double-stranded RNA synthesis for Lac2 (dsLac2) was performed using the MEGAscript Transcription Reaction kit (Ambion). Double-stranded RNA was treated with TURBO DNase (Ambion), and purified using the RNeasy cleanup kit (Qiagen). Double-stranded RNA quality was confirmed by gel electrophoresis on a 1.2% agarose gel, and the concentration was obtained using a Nanodrop spectrophotometer (model ND-1000).

**Insects and Plants**

*Leptinotarsa decemlineata* eggs were obtained from Lee French Agriculture in Lamberton, MN. Eggs were maintained until hatch in a growth chamber at 34°C with a
16:8 hour (L:D) photoperiod. Potatoes (Solanum tuberosum var. Atlantic) were obtained from Dr. Alexander Pavlista at the UNL Panhandle Research and Extension Center in Scottsbluff, NE. Potatoes were grown for 6 weeks in a greenhouse, and excised potato leaves were used as a food source for the larvae. To maintain freshness, the leaf petiole was placed into a 0.6mL tube containing 0.4% agar. Larvae were reared on excised potato leaves in the growth chamber until second instar molt occurred (three days).

**Larval Microinjection and RNA Extraction**

Microinjection needles were pulled from 3” thin wall glass capillaries (1.0mm OD/ 0.75mm ID) using a Shutter Instrument Co. needle puller (Model P-30), and the needle was shaped using the Micropipette Beveler (Shutter Instrument Co. Model BV-10). Second instar larvae were positioned on double-sided tape adhered to a microscope slide, and placed on ice for several minutes for anesthesia. Twelve larvae were injected on the dorsal side of the abdomen with 1µl for each treatment: RNase-free water (control) or 1000ng dsLac2. Following injection, larvae were maintained in the growth chamber in 2 oz. cups and fed fresh potato leaves. Three larvae from each treatment were flash frozen in liquid nitrogen 6 and 24 hours post-injection (for a total of 12 samples) and stored at -80C until further processing.

Total RNA was isolated from individual larvae using the RNeasy Mini Kit (Qiagen) and treated with DNaseI (Qiagen). RNA quality was confirmed using a 1% denaturing agarose gel, and the concentration was obtained using the Qubit fluorometer (Life Technologies, Carlsbad, CA). A quantity of 3µg of RNA from each sample was sent to the University of Nebraska Medical Center (UNMC) Next Generation Sequencing Core Facility for RNA sequencing.
RNA Sequencing and Data Processing

RNA sequencing was performed on an Illumina Hi-Seq 2000 (UNMC, Omaha, NE). RNA was assessed for potential degradation and genomic contamination using an Agilent Bioanalyzer (UNMC). cDNA from the 12 samples was ligated to paired-end (PE) adapters and run on one lane.

Paired-end adapters were trimmed from reads using Trimmomatic v 0.32. Reads were mapped to the *L. decemlineata* genome (www.hgsc.bcm.edu) (Richards *et al.*) and analyzed for differential gene expression using the Tuxedo package, which includes Bowtie 2 (Langmead and Salzberg, 2012), TopHat (Trapnell *et al.*, 2009), Cufflinks (Trapnell *et al.*, 2010) and Cuffdiff (Trapnell *et al.*, 2013). An index file of the *L. decemlineata* genome was generated using Bowtie2-read v. 2.2.1, which allows the genome file to be searched rapidly. Tophat v. 2.0.11 was used to align forward and reverse paired reads to the genome. The acceptedhits.bam files generated by Tophat were used by Cufflinks v. 2.2.0 to calculate the expression level of each transcript by normalizing the reads that map to each transcript to the number of fragments per kilobase of transcript per million mapped fragments, or FPKM. After Cufflinks assembled transcripts for each sample individually, Cuffmerge v. 1.0.0 was used to generate a merged.gtf file, which contains a single merged transcriptome from all samples. Cuffdiff v. 2.2.0 was used to determine differential gene expression between control and dsLac2 treatments at 6hr and 24hr using an adjusted p value of <0.05. As the primary goal of the study was to characterize the response of RNAi genes to dsRNA, an additional alignment was performed using RNAi genes characterized by Swevers *et al.* (2013) to ensure identification of all RNAi genes from the transcriptome.
Gene Annotation

All *L. decemlineata* transcripts were functionally annotated using databases employed by the Trinotate annotation program (http://trinotate.sourceforge.net). Trinotate performs searches for each transcript in established databases such as SwissProt and PFAM, using tools such as NCBI Blast (blastx, blastp), HMMER, and SignalP/TmHMM, and assigns eggNOG and gene ontology (GO) annotation terms. All annotation information is incorporated into an SQLite database, which allows rapid searching of all acquired annotation information for each transcript.

To begin functional annotation of all genes using Trinotate, a fasta file (transcripts.fa) was generated containing the DNA sequence and associated gene and transcript ids. This was performed using the “gffreads” command (http://cufflinks.cbcb.umd.edu/gff.html) and the genome and merged.gtf files from cufflinks. Transdecoder v. 20131110 (http://transdecoder.sourceforge.net) was used to generate a .pep file, which contained likely coding regions within each transcript. The SwissProt database was used to search homologous transcripts by performing a blastx search using the transcripts.fa file, and to search predicted proteins with a blastp search using the .pep file. The .pep file was also employed in HMMER v. 3.161, SignalP v. 4.1 and tmHMM v. 2.0c searches to identify protein domains, predict signal peptides and transmembrane helices in proteins, respectively. To load all annotation results into a Trinotate SQLite database, a transcripts.fasta.gene_trans_map file was generated by obtaining gene and transcript identifiers using “grep” and “cut” programs, and were combined into the trans_map file using the “paste” program. A Trinotate annotation report was generated in tab-delimited text format. The SQLite Manager add-on in
Mozilla Firefox 29.0.1 was used to filter the Trinotate annotation report for differentially expressed genes at 6 hr and 24 hr.

**Results**

*Overall Changes in Gene Expression*

Analyses of differential gene expression were conducted for each of the two time points, and log2 fold changes for differentially expressed (DE) genes are expressed relative to controls. Log2 fold changes were converted to fold change. Gene expression analysis indicated a total of 245 DE genes at 6 hr and 345 DE genes at 24 hr. Of these, 69 genes were DE at both time points (Figure 1). Six genes were upregulated at both time points (Table 2A), and three of these genes were not assigned a fold change, as the FPKM of the control condition was 0. Two of these genes were not annotated. The FPKM values for the third gene, Acetyl-CoA acetyltransferase, were 166 and 151 for 6 hr and 24 hr, respectively. A hypothetical protein with a retro transposon-like region was upregulated 2.8 fold at both 6 hr and 24 hr. Two diapause-associated transcripts were upregulated by a fold change of 2.5- 26 and 3.5 – 5.7 fold at 6 and 24 hr, respectively.

Ten genes were downregulated at both time points (Table 2B). Four of the ten genes were not assigned a fold change, as the FPKM of the dsLac2 treated was 0. One of these genes was not annotated, and high FPKM values (189 and 494) were observed for the two time points. The other three genes not assigned a log2 fold change were two retrovirus related pol polyprotein from transposons, and a trypsin inhibitor. Three Laccase2 and Laccase2 precursor genes were downregulated in dsLac2-treated larvae at 6 hr and 24 hr by approximately the same fold change (~5.6), indicating that Laccase2 was downregulated at 6 hr and remained downregulated at 24 hr. Another unannotated gene
was downregulated by a fold change of 9.8 and 21 at 6 hr and 24 hr, respectively. Finally, cytochrome b5 was downregulated by fold change of 2.5 and 4.9 at each of the time points.

Four genes that were upregulated at 6 hr were also found to be downregulated at 24 hr. These genes consisted of 2 haemolymph juvenile hormone binding proteins, regulacin and a glucose dehydrogenase protein. Forty-nine genes that were downregulated at 6 hr were found to be upregulated at 24 hr. Of these 49 genes, eight are cuticle proteins.

**Expression of RNAi Genes**

Colorado potato beetle genes involved in the RNAi pathway were identified via the annotation procedure and also by aligning CPB RNAi genes characterized by Swevers and colleagues (2013) to the transcriptome produced in this study. Unexpectedly, the core RNAi machinery of the RNAi pathway, Dicer-2 and Ago-2, was not differentially expressed at either 6 hr or 24 hr. No differential expression of any of the RNAi genes was exhibited at 6 hr. At 24 hr, 5 genes that are implicated in the RNAi pathway (but are not core RNAi genes) were upregulated in response to dsRNA (although isoform of CG4572 was downregulated 1.9 fold.) These genes were characterized by Swevers et al. (2013) from homologues, and include Armitage, CG4572, Neuron-specific Staufen, and 2 non-specific DNA/RNA nucleases, which were upregulated by a fold change of 2.3-4, 1.9-2.1, 2-3.2, and 2.3, respectively.

The distribution of the log2 fold changes for the CPB RNAi genes at 6 hr (Figure 2A) and 24 hr (Figure 2B) reveal that the expression of most RNAi genes remain unchanged following injection of dsRNA, as they are clustered around log2 fold change
of 0. Although not significant, the expression of Dicer-2 was upregulated by a log2 fold change of 0.25-1.4 at 24 hr. Interestingly, the trend of Ago-2 expression was only slightly upregulated at 24 hr (log2 fold change 0.2-0.7, not significant). Log2 fold changes of Dicer-2 and Ago-2 (-0.05-0.23 and -0.34-0.66, not significant) at 6 hr indicated that the core RNAi machinery was expressed at levels similar to controls.

**Differential Expression of Non-RNAi Related Genes**

Many of the strongest fold changes were exhibited in genes that do not play a role in the RNAi pathway. Interestingly, strong changes in expression of several cuticle proteins were exhibited at 6 hr and 24 hr. At 6 hr 13 cuticle proteins were downregulated by a fold change of 2-6.5. Although cuticle proteins were significantly downregulated at 6 hr, they were upregulated at 24 hr. Several of the differentially regulated cuticle proteins showed overlap between the two time points, as eight of the proteins that were downregulated at 6 hr were in turn upregulated at 24 hr. The expression of 22 cuticle proteins was upregulated by a fold change of 1.7-52 at 24 hr.

In addition to differential expression of cuticle proteins, other genes also exhibited larger fold changes than genes involved in the RNAi pathway. At 6 hr, the gene with the largest upregulation (27 fold) was unannotated, followed by cathepsin b (11.3 fold). The strongest downregulated genes at 6 hr include an unannotated gene (-104 fold) and an E1-tetrahydrofolate synthase (-26 fold). At 24 hr, an unannotated gene was upregulated (16 fold) and genes with strong downregulation include polyprotein P3 (-32 fold) and a cytochrome P450 (-14.9 fold).
Discussion

RNA interference has been adopted as a tool to study gene function in insects. Additionally, there is significant interest in utilizing RNAi as a means for pest control. Unfortunately, not all insects are sensitive to gene specific knockdown by RNAi. In order to better understand the variability of RNAi sensitivity among insect species, several studies have characterized RNAi genes in different insect species, and a few studies have characterized the response of core RNAi genes in response to dsRNA challenge. In this study, we found that to our surprise CPB core RNAi genes were not differentially regulated in response to dsRNA.

It should be noted that a high number of transcripts were not assigned a blast hit or GO term in this study. Trinotate performed annotation of transcripts produced by cufflinks. Trinotate utilizes the SwissProt and PFAM databases, and annotates transcripts with eggnog and GO terms using several databases. Cufflinks assembled a total of 32,423 transcripts. The annotation yielded 12,639 that were not assigned a GO term, and 10,695 that did not return a blastx hit or a GO term. A high number of transcripts without annotation could be related to the annotation tool. Trinotate provides an annotation for each transcript by searching genes in well-characterized model organisms, and is therefore restricted to a smaller database than other annotation programs, such as Blast-2-GO.

Differential Expression of Non-RNAi Related Genes

The gene targeted for RNAi knockdown in this experiment was Laccase2, the phenoloxidase gene required for cuticular sclerotization and pigmentation in Coleoptera (Arakane et al., 2005). RNAi of Laccase2 resulted in significant downregulation of Lac2
to an expression level that was similar at both 6 hr and 24 hr. This suggests that RNAi in CPB larvae occurs very rapidly, prior to 6 hr after injection with dsRNAs.

Of the 6 common genes upregulated at 6 hr and 24 hr, two were annotated as diapause-associated transcript-2 (DAT-2). Insects enter diapause as a means to cope with stressful environmental conditions. DAT-2 was upregulated by 2.5-25 fold and 3.5-5.7 fold, at 6 hr and 24 hr, respectively. DAT-2 was isolated and characterized by Yocum (2003), and is expressed in adult CPB at the onset of and during diapause. Sequence analysis of DAT-2 has indicated that it may also play a role in protection from desiccation (Yocum, 2003). Serving as a desiccation protein could complement DAT-2’s role in diapause (Yocum, 2003), as diapausing insects retain less water than non-diapausing insects (Danks, 2000). Interestingly, in this study DAT-2 was upregulated at both time points following knockdown of Laccase2 by RNAi. The role of Laccase2 in cuticle sclerotization has been demonstrated (Arakane et al., 2005), and the insect cuticle serves as a barrier against environmental stresses (Neven, 2000). Perhaps the upregulation of DAT-2 is associated with the downregulation of Laccase2, in attempt to prevent desiccation that could occur as a result of reduction in cuticular sclerotization.

Cuticle proteins were differentially expressed at 6 hr and 24 hr. At 6 hr, cuticle proteins (13) were downregulated 2-6.5 fold. Enders et al. (in review) reports a similar finding in the soybean aphid Aphis glycines in response to heat and starvation stressors. Downregulation of cuticle proteins in the soybean aphid may serve as a stress response (Enders, personal communication). Cuticle proteins in the CPB are in turn upregulated at 24 hr (1.7-52 fold), with eight proteins in common between the two time points. It is possible that cuticle proteins at 24 hr are upregulated in response to RNAi knockdown of
the Laccase2 cuticle protein. As Laccase2 is required for pigmentation and sclerotization, perhaps other cuticle proteins are upregulated in order to compensate for reduced Laccase2 expression.

**Expression of RNAi Genes**

Core RNAi genes were not differentially regulated at either 6 hr or 24 hr. Upregulation of core RNAi genes in response to dsRNA challenge has been documented in other insect species, including *M. sexta* (Garbutt and Reynolds, 2012), *B. germanica* (cockroach) (Lozano et al., 2012) and *B. mori* (J. Liu et al., 2013). In these studies, upregulation of Dicer-2 was observed up to 6 hr after dsRNA administration. Additionally, upregulation of Ago-2 was also observed in *M. sexta* and *B. mori* 6 hr after dsRNA administration. Significant upregulation of Dicer-2 in the whole body was similar at 6 hr between *B. mori* and *B. germanica*, with 2 fold and 5 fold upregulation, respectively. These insect species display sensitive responses to RNAi. Additionally, a significant 2.3 fold upregulation of Dicer-2 was observed in *B. mori* 3 hr after dsRNA administration, and significant upregulation of Ago-2 was observed 1.6 and 1.5 fold at 3 and 6 hr, respectively. In *M. sexta*, an insect with variable response to RNAi, an 80 fold, 362 fold and 395 fold upregulation of Dicer-2 was observed in the fat body, hemocytes and midgut, respectively. Garbutt and Reynolds (2012) describe the upregulation of Dicer-2 in *M. sexta* in response to dsRNA as “rapid and transient.” If the response of core RNAi genes to dsRNA is very quick, it is possible that differential expression of CPB core RNAi genes had already occurred by 6 hr. It is also worth noting that the experiments in other insects delivered an alien dsRNA (for a gene not present in the insect), which could account for differences seen in the CPB.
Although core RNAi genes were not differentially regulated, a handful of genes involved in the RNAi pathway were found to be upregulated at 24 hours. These genes include the following homologues characterized by Swevers et al. (2013): Armitage, two non-specific RNA nucleases, CG4572, and Neuron-specific Staufen. In *Drosophila*, a role for Armitage has been demonstrated as a RISC auxiliary factor, with roles in the piRNA and siRNA pathways (Tomari *et al.*, 2004). Non-specific RNA nucleases play a role in digestion, but are also capable of dsRNA degradation (Arimatsu *et al.*, 2007; Liu *et al.*, 2013). It is thought that the presence of non-specific RNases could contribute to the overall reduced sensitivity of some insect species to RNAi (Christiaens *et al.*, 2014). It is curious that these RNases are upregulated in CPB in response to dsRNA, as the CPB does display sensitivity to RNAi (Zhu *et al.*, 2011) (this study). CG4572, also upregulated, is required for dsRNA uptake and systemic RNAi response to viruses in *Drosophila*. Finally, a Neuron-specific Staufen homolog was upregulated in response to dsRNA. *Drosophila* Neuron-specific Staufen has the same type of dsRNA binding motif as the R2D2 protein. The *Drosophila* R2D2 protein has two dsRNA-binding domains, and associates with Dicer-2 to generate siRNAs from dsRNA, which are then loaded into the RISC complex (Liu *et al.*, 2003). Interestingly, R2D2 was not identified or expression was too low to be identified in the CPB gut by Swevers *et al.* (2013), although R2D2 was annotated in a whole body CPB transcriptome (Kumar *et al.*, 2014). Two R2D2 genes have been identified in *Tribolium*, R2D2 and C3PO (Tomoyasu *et al.*, 2008). When R2D2 (Genbank EU273920.1) and C3PO (Genbank EU273921.1) were aligned to the CPB transcripts produced by cufflinks, there were no significant hits. Swevers (2013) suggests further analysis of the role of this gene in the RNAi pathway.
In conclusion, CPB core RNAi genes are not differentially regulated 6 hr and 24 hr following dsRNA challenge. Studies in other insects have demonstrated a rapid upregulation of core RNAi genes following dsRNA challenge. In the CPB and perhaps other insects that are sensitive to RNAi, it would be worthwhile to investigate the regulation of genes involved in the RNAi pathway prior to the 6 hr time point. In this study, a handful of genes involved in the RNAi pathway were upregulated at 24 hr. The upregulation of cuticle proteins and diapause-associated transcript-2 (DAT-2) occurred at 24 hr, which could be a consequence of Laccase 2 knockdown by RNAi. This research provides a glimpse into the RNAi pathway of the CPB, which can contribute to our understanding of RNAi variability among different insect species. Additionally, this can help to identify genes that are likely to respond to selection pressure if RNAi is employed for pest management of the CPB.
Table 1. A list of primers used in PCR and for dsRNA synthesis.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Amplicon</th>
<th>Sequence 5’-3’</th>
<th>Ta °C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Degenerate Primers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lac2A_F+T7</td>
<td>1082bp</td>
<td>TAATACGACTCACTATAGGGCAYTTYTGGCAYGCNCAYACNGG</td>
<td>53</td>
</tr>
<tr>
<td>Lac2A_R+SP6</td>
<td>1082bp</td>
<td>ATTTAGGTGACACTATAGCCRTGNARRTGRAANGGRTG</td>
<td>53</td>
</tr>
<tr>
<td><strong>Gene Specific Primers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPBLac2_F</td>
<td>800bp</td>
<td>GCACATGCAGTTTTGTCCAC</td>
<td>65</td>
</tr>
<tr>
<td>CPBLac2_R</td>
<td>800bp</td>
<td>CGGTGAATACCGGTCAAGAT</td>
<td>65</td>
</tr>
<tr>
<td><strong>In vitro dsRNA Synthesis Primers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNAi_F + T7</td>
<td>442bp</td>
<td>TAATACGACTCACTATAGGGAGCGCTCTGTCTGATTTGC</td>
<td>65</td>
</tr>
<tr>
<td>RNAi_R + T7</td>
<td>442bp</td>
<td>TAATACGACTCACTATAGGGCAGAGACCCCCAACACTGTT</td>
<td>65</td>
</tr>
</tbody>
</table>

Ta denotes annealing temperature used in PCR cycling.
Table 2A. Genes upregulated at 6 hr and 24 hr.

<table>
<thead>
<tr>
<th>Gene</th>
<th>NCBI Accession/UniProt Identifier</th>
<th>E value</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>(blastx) hypothetical protein TcasGA2_TC001995 (<em>Tribolium castaneum</em>), region = “RT- like”</td>
<td>EFA12688.1</td>
<td>5.00E-04</td>
<td>2.8 2.6</td>
</tr>
<tr>
<td>(blastx) diapause-associated transcript-2 (2) (<em>Leptinotarsa decemlineata</em>)</td>
<td>AAN05630.1</td>
<td>1.00E-53</td>
<td>2.5- 26 3.5- 5.6</td>
</tr>
<tr>
<td>Acetyl-CoA acetyltransferase</td>
<td>Q5BKN8</td>
<td>5.00E-09</td>
<td>n/a n/a</td>
</tr>
<tr>
<td>No annotation (2)</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a n/a</td>
</tr>
</tbody>
</table>

Table 2B. Genes downregulated at 6 hr and 24 hr.

<table>
<thead>
<tr>
<th>Gene</th>
<th>NCBI Accession/UniProt Identifier</th>
<th>E value</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>(blastx) Laccase 2</td>
<td>ABU68466.1</td>
<td>1.00E-99</td>
<td>7.2 8.7</td>
</tr>
<tr>
<td>(blastx) Laccase 2A [1]. Laccase 2B [1]</td>
<td>NP_001034487.2 AAX84203.2</td>
<td>0 0</td>
<td>4.5 5.4</td>
</tr>
<tr>
<td>(blastx) Laccase 2 precursor</td>
<td>NP_001034487.2</td>
<td>5.00E-171</td>
<td>6.4 7.3</td>
</tr>
<tr>
<td>(blastx) Cytochrome b5</td>
<td>XP_967809.1</td>
<td>6.00E-53</td>
<td>2.6 4.9</td>
</tr>
<tr>
<td>No annotation (3)</td>
<td>n/a</td>
<td>n/a</td>
<td>2.3- 9.8 2.5- 20.8</td>
</tr>
<tr>
<td>Trypsin inhibitor</td>
<td>P81902</td>
<td>4.00E-15</td>
<td>n/a n/a</td>
</tr>
<tr>
<td>Retro-virus related pol polyprotein from transposon (2)</td>
<td>P04323</td>
<td>2.00E-19 5.00E-06</td>
<td>n/a n/a</td>
</tr>
</tbody>
</table>

Gene duplications indicated in parentheses, and transcripts indicated in brackets. Fold change information is not available for genes where FPKM of the control or treated
condition is 0. Genes annotated with Trinotate are assigned a UniProt Identifier, genes annotated with NCBI blastx are assigned an NCBI Accession number.
Figure 1. Differentially expressed genes at 6 hr and 24 hr.
Figure 2. The distribution of log2 fold change in RNAi genes at 6 hr and 24 hr.

A.

Distribution of RNAi Genes at 6 Hours

Distribution of RNAi Genes at 24 Hours

Distribution of Log2 fold changes of RNAi genes at A) 6 hours and B) 24 hours. All RNAi genes at 6 hours and most RNAi genes at 24 hours are not differentially expressed, as the mode is clustered around log2 fold change 0.
Appendix A. 983bp sequence of *Laccase 2A*.

CAATCGCATTGAGTGTATATCAACTTTTGAGTGACTGTCAACCATGTTTTGTCCA
CAATCAGCTGGTCTGTATTATCTCCCGTTGAGAATTGGTTCGGGTGATATCATC
GTACTGAGAATACGTGGTGGAGCCCGACATGTATGAAATTTCATCTCTCATG
AGACTTATTACATGGTCTCCATTCCGCGCAACTAATGTCTGTTATAGATGT
GGGGGCAAATAGATCCTCCTGAGTATAAACATGGAATCTGAACGGTAGGAA
AATTAAACATCCTGGCCTCCTGACTTACAAATTCCTCTGCTGTATATTCTGTCAT
TTTCAACTGACCTACGCAAATAGCGTCTGGTCTGATTTCTGTTACATCTTTCAT
CCAATGGGTAAAGAACCACCTCCTTGTGATACCATAAGTCATATGTGGGTTGG
GGCGGTCAAGGTTGATATGGACCTCTGCGATAACGCAGGATGGCCAACTGC
TGAACTCTCCTCAACCACATTCTCTCTAAACCTCTGTTGAAATCCAGTAAGC
TCCAACCGGTTGATTGCAATATAATGAAATCGTATCTTTTCCTCCGAGAATG
AAATGACGGGTGTGACTTTGCAACAGGTATGCACAGTTTCCTCTCCATGTTGGCA
TAGTCAAAATCATGACCTTGGAATGTTAATTGAGCCCGACATACCGAAGCA
ATGACTACTCTCTCAATGCTATATCTCTCTTACCCGGGTTGAAATTTGTGAAAAC
TCTCAACGGGTGTGTTGTCATGAAACCAGTGTTGGGTCTCTGAACCTGACCTTT
ACCCGTTTATAGTAACATCTCCTGCTGACCCGTTATGACCCGCTACTCCGCCC
CAGGGGTATCTTCTCAATAGCAGTTTCTGTCATCCAAATCACTGAGAAGTACG
ATGGGTGTGGAGATCAAATGACAGATAGTGGTCTGTTGATCTTTTGAAGGA
GGTTGGCGAAGCAATGCTACCATAAGATACCCATCC
Appendix B. 807bp consensus sequence of Laccase 2A Clone 4.

GCACATGCAGTTTTGTCCACAATCAGCTGTCTTATCTCCGTGGCAGAATT
GTTCGGGGTTGATATCATCGTACTGAGAAATCAGTGGGGTTGGAGCCGACAT
GTATGAAATTTCTATCTATCAGACCTTTATTACATGCTCCATTCCATCGGCGCAACTA
AATGTTCTGTATAGGTGTGGGATGGGGGCAAATAGATCCTCTGGAGTATAACATG
GAATCTGAACGTTAGAAAAATTTTTAATCTCTGGCCTCTAGCTAAAAATTCCTC
TGTCATATTCTTGTGCAATTTTTCACACTGACTTTAGCAAATAGCTGCTGTTAGA
TTTCGTACATCTTTGCATCCAATAGGTAAAAGACACTTCTTGTTGGAAGAATT
TAGTCATATGTTGGGCGGTCAAGGTTGATATGGACCTCTGGCATAAC
GCAGGATGGGCAACTGCTGAATCTCTCCTACAGGCAATTCCTCTCTAAACCTCTC
AGTTGAATCCAGTAAGCTCCACGCGGTTTGAATTGCGATTAATAATGAAATCTCT
ATCTTTCTCCGGAATGAAATGACGGTGGTTGACTTTTCAGGATGACAGG
TTCTCCATCTGTGTCGATAAGAGCTCAAATCATGACCTTGGAATGTTAATTGAG
CCGGACATAACCGAAGCAGAATGAAATTAATATCATCCGATCTATATCTTTTACC
CGGGTAATTGTGAAAACCTCCTCAACCGGTGTGTTTGTCATGAAAACGATGTTGG
GGTCTCTGAACCTGACCTTTACCCTTTATAAGTAAACTCTCTGGATCTTGAGCCG
GTATTCACCG
Appendix C. Lac2 442bp amplicon.

AGCGTCTGGTCTGATTTCGTTACATCTTTGGCATCAAATGGGTTAAGAACCACTCC
TTGTTGGTATACCAGTAGCATATGTGAGGGGCTGGAAGGATATGGACCTCTGGCATAAAA
GCGATGACGGATGGCTTGATAAACCTGAACTCCTCAGTCCCACACATCTCCTAAA
CTCTAGTTGAAATCATCAATCCTTTCATTTCTCGGAGAATGAAATGACCGGTGTTGACTTTGA
CAGGATGTACAGGGTCTTCTTACTGTTTGCGATAAGAGTCAATGCTGACCTTGG
AATGTTAATTGGACCGGACATACCGAAGCGGATGAAATTAATCATCCTGAAATC
TATATCTTTTACCGGGTAATTGTGAAAACCTCCAACCGGTGTGTTGGTCATGAACCAGGTGTGGGCTCTG
REFERENCES:


https://www.hgsc.bcm.edu/arthropods/colorado-potato-beetle-genome-project


