Molecular characterization of insecticide resistance mechanisms in populations of *Leptinotarsa decemlineata*: potential for pest control using RNA interference and understanding natural phenology of insecticide resistance

By

Justin Clements

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The dissertation is approved by the following members of the Final Oral Committee:

Russell L. Groves, Professor, Department of Entomology
Christopher A. Bradfield, Professor, Department of Oncology
Warren P. Porter, Professor, Department of Zoology
Lyric C. Bartholomay, Associate Professor, Department of Pathobiological Sciences
Sean D. Schoville, Assistant Professor, Department of Entomology

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FORWARD

This dissertation is comprised of four chapters that summarize the research conducted during my doctoral work in the Molecular and Environmental Toxicology Center in the Department of Entomology. The research focuses on the invasive agricultural pest, the Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say) and understanding the underlying factors that give rise to imidacloprid insecticide resistance. The first three chapters represent published and submitted scientific manuscripts in refereed journals. The fourth and final chapter represents graduate research conducted under Dr. Sean Schoville with efforts to help annotate the *L. decemlineata* genome.

Chapter 1, entitled "Characterizing molecular mechanisms of imidacloprid resistance in select populations of *Leptinotarsa decemlineata* in the Central Sands region of Wisconsin", focuses on phenotypic classification of imidacloprid resistance and susceptible populations of *L. decemlineata* in the Central Sands region of Wisconsin. It then examines possible mechanisms of imidacloprid resistance by comparing mRNA transcript abundance in an imidacloprid susceptible population versus an imidacloprid resistant populations through the use of RNA-sequencing.

Chapter 2, entitled "RNA interference of three up-regulated transcripts associated with insecticide resistance in an imidacloprid resistant population of *Leptinotarsa decemlineata"*, focuses on using double-stranded RNA (dsRNA) to suppress transcript abundance that encode proteins with a putative rule in imidacloprid resistance as identified in Chapter 1. By disrupting the production of proteins involved in imidacloprid resistance, we expected to observe an induced imidacloprid susceptible phenotype.

Chapter 3, entitled “Phenotypic response of *Leptinotarsa decemlineata* to imidacloprid and changes in transcript abundance spanning a growing season in the Central Sands region of Wisconsin”, focuses on the phenotypic classification of imidacloprid resistance throughout a growing season in both an imidacloprid susceptible and resistant population. It then classifies transcript abundance through the use of RNA sequencing during more resistant time points during the growing season compared to time points where imidacloprid susceptibility is observed.

Chapter 4, entitled “Understanding the demographic history of *Leptinotarsa decemlineata* through the use of Dadi and Treemix”, focuses on using SNP allele frequency from RNA sequencing aligned to genomic scaffolds to determine a mutation rate in *L. decemlineata*. It then shifts its focus to using SNP allele frequency from RNA sequencing aligned to genomic scaffolds to determine patterns of population splits in *L. decemlineata.*

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ABSTRACT

The Colorado potato beetle, *Leptinotarsa decemlineata* (Say), is a major agricultural pest of potatoes. Previous studies have shown that populations of *L. decemlineata* have become resistant to many classes of insecticides, including imidacloprid, and demonstrated that the median lethal concentration to kill 50% of the test organisms (LC_{50}) in different field populations varies greatly, which may suggest that resistance of *L. decemlineata* is heritable and involves genetic changes. Furthermore, *L. decemlineata* possesses multiple mechanisms of resistance, including enhanced metabolic detoxification by cytochrome p450s and glutathione S-transferases to combat an insecticide insult.

We examined imidacloprid resistance among different field populations in the Central Sands region of Wisconsin. The LC_{50} values collected in 2008-2011, and 2013-2014, indicate some field locations remain susceptible, while nearby fields (<100km) have developed resistance. We compiled a transcriptome for 'susceptible' and 'resistant' populations by isolating mRNA from adult beetles and analyzed differences in transcript abundance to uncover potential mechanisms of resistance. Differences were observed in constituently up- and down-regulated transcripts among different populations, including the up-regulation of 3 cytochrome p450s and a glutathione synthetase related protein in multiple resistant populations.

We further hypothesize that a portion of the up-regulated transcripts encode for pesticide resistance and may be suppressed to re-establish a susceptible phenotype. Following the successful suppression of transcripts encoding for a cytochrome p450, a cuticular protein, and a glutathione synthetase protein using RNA interference, we observed reductions in measured resistance to imidacloprid.

Finally, we examined temporal changes in phenotypic response to imidacloprid between a susceptible and an imidacloprid-resistant population. Estimated LC_{50} values varied throughout the growing season in the resistant population, with increased susceptibility among over-wintered, recently emerged adult beetles compared with heightened resistance in the second generation. The abundance of mRNA transcript was compared between multiple time points, and showed that transcripts encoding cuticular proteins and cytochrome p450s were highly up-regulated during peaks of resistance.

INTRODUCTION: Neonicotinoid resistance in Wisconsin populations of the Colorado potato beetle; mechanisms of insecticide resistance

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say), is a major agricultural pest. This phrase has been used in countless scientific manuscripts and is usually, in some form or another, the first introduction to this unique invertebrate species. To growers, this species is an invasive pest whose colonization of commercial crop fields in the family Solanaceae including potatoes (*Solanum tuberosum*), tomatoes (*Solanum lypcopersicum*), eggplants (*Solanum melongena*) and peppers (*Solanum annuum),* can have long lasting consequences. Often, *L. decemlineata* infestation results in a significant expenditure of time and money and, without the proper insecticidal treatment, can lead to complete field failure. Pest managers face many unique challenges combating populations of *L. decemlineata.* Unlike many other pests of commercial agricultural crops which possess specific windows of insecticidal treatment, *L. decemlineata* is controlled throughout the entire growing season with a combination of foliar and systemic insecticides (1). The necessity to adequately achieve season-long control can lead to many challenges for pest managers, including continuous pest monitoring to determine the timing of insecticidal applications and selection of the appropriate chemistry for the specific life stages of *L. decemlineata*. Another challenge facing pest managers is the ability of *L. decemlineata* to develop insecticidal resistance at an alarming rate across significant spatial scales and to several chemical modes of action. It has been documented that populations of *L. decemlineata* have developed some form of resistance to 56 different active ingredients, including many classes of insecticides which are still commonly used to control field populations

(2,3). This rapid development of resistance to multiple insecticides influences pest managers to use multiple, insecticidal chemistries to handle problematic populations of *L. decemlineata*.

To academics, *L. decemlineata* is a fascinating species which possesses significant phenotypic variation, together with the ability to develop resistance to multiple insecticide modes of action, only a portion of which has been explained. Multiple possible mechanisms of resistance have been suggested, but it appears that these represent only a fraction of the entire story. Briefly, insecticidal resistance is defined as a heritable change in a pest population that is reflected by repeated field failure when the insecticidal product is used within its regulatory guidelines (4). Yu et al. describes insecticide resistance in further detail and suggests that true resistance does not occur unless there are structural genetic changes resulting from insecticidal pressures that are passed from one generation to the next and further that insecticide resistance is an evolutionary phenomenon (5). This review will examine the life history of the *L. decemlineata* with a focus on the history of colonization which has established this species as a dominant pest, together with current pest management strategies. Next, chemical resistance will be explored, with a focus on resistance to neonicotinoid insecticides, a group which is commonly used to control *L. decemlineata* in agricultural settings, and examine new studies that attempt to classify how this pest develops resistance at such an alarming rate. Finally, a discussion of new and emerging techniques that could lead to potentially effective control of *L. decemlineata* will be discussed.

1. The life history of *Leptinotarsa decemlineata* and historical pest management strategies

Currently, in the United States (U.S.), there are more than 200 known agricultural pests of commercial potatoes, but none has a larger impact than *L. decemlineata* (1). Adult forms of the insect are described as small (3/8 inch), and brightly colored with yellow bodies and black

stripes on their dorsal side. This species belongs to the Coleopteran (beetles and weevils) family. Under optimal conditions, adults can consume up to 10 cm^2 of potato foliage daily (6,7). Native to northern Mexico and with a natural diet composed of Buffalo Burr (*Solanum rostratum*), a member of the Solanaceae family (nightshades) which produces glycoalkaloid toxins, *L. decemlineata* has adapted to survive by consuming a portions of this plant family which is toxic to most mammals; e.g. the plant's foliage (2,8–11) . It has been hypothesized that colonization and invasion of *L. decemlineata* in the U.S. began when Mexican vaqueros unintentionally brought the beetles across the Mexican border with livestock, crossing into Texas in the 1680's (11). From there, *L. decemlineata* spread throughout the U.S., with the first record of the beetles becoming an agricultural pest occurring in 1859, 100 miles west of Omaha, Nebraska on a potato crop (2). Beetles spread quickly throughout the U.S., reaching the East Coast by 1880 and traveling to Europe by 1922 (12). To date, *L. decemlineata* has reached pest status in North America, Europe, and Asia, encompassing more than 16 million km^2 (2). It has further been suggested that as climates warm, beetle expansion will only increase in the coming years, resulting from new habitat formation for the beetles in northern climates (13).

The life history of *L. decemlineata* has been studied in depth in both field settings and laboratory cultures, where individuals can have a life expectancy of approximately 1 year (14). *L. decemlineata* has a high fecundity rate, and females can lay an excess of 500 eggs in their lifetime (7). With very few natural enemies, *L. decemlineata* populations are not regulated by predation. The few natural enemies found in agricultural fields range from, but are not limited to, other insects, such as the two spotted stink bug (*Perillus bioculatus*) and the spotted pink lady beetle (*Coleomegilla maculata*), and to fungal pathogens such as *Beauveria bassiana* (10,15).

Given the lack of natural regulatory influences, large and rapidly expanding populations can result.

In an agricultural setting, the beetle's holometabolous life cycle begins in the egg stage, followed by 4 larval instars, a pupal stage, and an adult stage that generally spans two growing seasons, with 1 to 2 full generations per year in northern potato growing regions (1). Beetles overwinter in diapause a few inches (2-8 in.) underground, within grassy edges and wooded areas next to potato fields (7). *L. decemlineata* emerge from diapause in late May through early June to start mating, with peak egg-laying in mid-June (1). While beetles tend to migrate to potato fields after they emerge from diapause, they can also be found on other members of the Solanaceae family, including tomatoes, eggplants, and other nightshades. Most of the damage seen in agricultural fields is not a result of the over-wintered, colonizing beetles (post-diapause) but, instead, is due to their offspring (1). Further, potato defoliation usually goes unnoticed until the third instar stage of this first generation (7). *L. decemlineata* is incredibly adept at colonizing potato fields, and if agricultural potato fields are not effectively treated with insecticides, complete field failure may occur. Many theories have been suggested as to why *L. decemlineata* was easily able to adapt to, and become a pest of potatoes, including the fact that the beetles' natural host plant, Buffalo Burr, is in the same family as potatoes (2). Further, it has been shown that larvae have a unique symbiotic relationship with bacteria found in their oral secretions to avoid anti-herbivore defenses of potato plants, leading to improved population fitness (16).

The history of the pest management of *L. decemlineata* is a story of insecticidal resistance. Before the introduction of insecticides, growers used a combination of crop rotation, early planting dates, reduced nitrogen, and other tactics to manage beetle populations (17). With the introductions of chemical xenobiotics, it appeared that beetle populations could be effectively

controlled, although this observation was short-lived. Paris green and other arsenical compounds were some of the first insecticides used to successfully control beetle populations until the 1940's, although there were recorded cases of arsenical insecticide resistance as early as 1912 (17). Since then, beetles have developed resistance to all major classes of insecticides, encompassing 56 different chemistries and over 18 unique modes of action (2,3).

Transgenic potatoes engineered to express proteins produced by *Bacillus thuringiensis* (Bt), an insecticidal bacterium that is naturally found in the environment, were developed in the 1990s (18). The use of genetically modified potatoes has shown past success to combat *L. decemlineata*, but they were only commercially available for a short period of time between 1996-1999 (1,19,20). Commercialization was discontinued in the U.S. due to concerns over the use of genetically modified organisms (1). To date, the most common method to control field populations of CPB is a combination of foliar and systemic insecticides used throughout the growing season (21). Besides insecticides, there has been some evidence that other non-insecticide chemistries, including fungicides, have negative developmental effects on *L. decemlineata* (22), suggesting that pest managers might soon be able to consider new insecticides and insecticidal technologies when controlling field populations of *L. decemlineata*.

2. Resistance to insecticides with a focus on imidacloprid

Approximately three decades ago, a new insecticide was registered for the control of *L. decemlineata.* Belonging to the insecticide resistance action committee's (IRAC), mode of action (MoA) class 4A, the neonicotinoids were initially registered in 1994, and now encompass 7 active ingredients, including clothianadin, imidacloprid and thiamethoxam, which have become the core of neonicotinoid use since their initial registration (21,23). Neonicotinoids were initially registered and viewed as a safer class, and organo-phosphate-alternative, insecticide due to high target site specificity between the insect and the vertebrate nicotinic acetylcholine receptor (nAChR) , giving it extremely low mammalian toxicity when compared to legacy insecticides (24). Neonicotinoids are agonists of the nAChR, causing the accumulation of acetylcholine, which results in paralysis and ultimately death of susceptible insects (24).

Unfortunately for growers, within 3 years of initial registration and introduction of the neonicotinoid insecticides, resistance was first observed in a Long Island, NY population (25). This observation was supported through the use of median lethal concentration assays (LC_{50}), where imidacloprid was topically applied to *L. decemlineata* followed by dose-response analyses. Since the initial study, multiple studies have demonstrated that field populations of *L. decemlineata* have developed resistance throughout the U.S., including populations located in New York, Maine, Michigan, and Wisconsin (26–31).

Four possible modes of insecticide resistance have been previously suggested, including enzymatic detoxification, target site insensitivity, increased excretion and reduced penetration, and behavioral resistance. The general enzymatic mechanisms that insects possess to break down and metabolize the IRAC MoA, Group 4A chemicals has been examined. Using a combination of phase 1 and phase 2 enzymes, beetles can detoxify and eliminate neonicotinoids. Enzymatic

detoxification pathways include molecules such as aldehyde oxidase, cytochrome p450, and glutathione S-transferase (24,32). Other imidacloprid resistant invertebrates, such as *Drosophila melanogaster,* have been shown to have developed resistance through over-expression of cytochrome P450 6g1 (33,34). In specific studies involving *L. decemlineata,* piperonyl butoxide (PBO), a cytochrome p450 inhibitor, has been used to demonstrate a phenotypic reversal back to susceptibility in resistant populations, suggesting that cytochrome p450 plays a role in insecticide resistance (30). Multiple studies have demonstrated that target site insensitivity is another possible mechanism of increased resistance and these studies have demonstrated that beetles have developed S291G mutations in acetylcholinesterase, giving rise to organophosphate resistance in *L. decemlineata* (35–37). Reduced penetration to the target site can be the result of many processes, including cuticular hardening, lipid reservoir trapping of insecticides in the cuticle, and degradation enzymes in the cuticle itself (38). In a pyrethroid resistant population of *Anopheles funestus,* cuticular thickness was measured with scanning electron microscopy (SEM), uncovering the association of increased mean cuticular thickness and pyrethroid resistance (39). In *Cimex lectularius,* the over-expression of multiple cuticular proteins has been associated with pyrethroid resistance(40). Increased expression of mRNA transcripts encoding for cuticular proteins in *L. decemlineata* have been observed in adult beetles and associated with environmental stressors such as dry environments and insecticidal exposure (29,41). Through the use of RNA sequencing, Clements et al. observed a statistically significant increase in transcript abundance encoding for cuticular proteins in an imidacloprid-resistant population of *L. decemlineata* when compared to an imidacloprid-susceptible population, giving support to the theory that cuticular proteins play a role in imidacloprid resistance (29). The final, and least understood, proposed mechanism of neonicotinoid resistance is behavioral resistance. Behavioral

resistance involves the ability of beetles to avoid areas of insecticidal treatment (5). Behavioral resistance in *L. decemlineata* has been examined in association with Bt, where a Bt-resistant laboratory strain of *L. decemlineata* had increased flight activity in the presence of Bt-expressing, transgenic potatoes. These observations lead Alyokhin et al. to conclude that beetles were moving away from areas of high insecticidal activity (42). While an imidacloprid-resistant laboratory strain did not have the same reaction, it is still possible that beetles could exhibit behavioral resistance through other mechanisms (43). In the case of imidacloprid resistance, it is important to determine the mechanisms by which *L. decemlineata* have developed resistance. Multiple modes of action may give rise to resistance in select populations and modes of resistance can vary between populations (29). The need to classify both the environmental and enzymatic mechanisms of resistance has led to great strides in understanding the fundamental concepts of insecticide resistance and potentially the principal factors that drive these phenotypes.

3. Transcript abundance studies

To preserve and utilize effective modes of action for pest managers, researchers will need to uncover the fundamental processes which lead to insecticide resistance. Scientists have turned to the use of RNA sequencing to examine transcript abundance within and among insecticide-resistant populations. Transcript abundance can be used as a tool to examine the genetic response to certain stressors and to provide insight into how organisms interact with such stressors. It should not be interpreted as examining gene regulation directly, as these studies are examining mRNA transcripts and not protein activity, but is instead a proxy for glimpsing into the genetic mechanisms of response to insecticides.

The products of RNA sequencing are nucleotide sequences representative of mRNA transcripts, which can be aligned to known genomic scaffolds or other classified biological macromolecules to determine their identity. To date, *L. decemlineata* does not have an annotated genome for comparison of mRNA sequences, so many studies use reference sequences from other invertebrate species to identify transcripts. Transcriptomic studies have attempted to identify mechanisms involved in insecticide resistance by classifying all transcripts in *L. decemlineata* that could be responsible for insecticide detoxification (44). With the use of larval and adult transcriptomic data, Kumar et al. used 454-FLX pyrosequencing to assemble genomic contigs which were analyzed to classify possible mechanisms of insecticidal resistance, including the classification of 621 contigs which are possibly involved in insecticide resistance. These mechanisms ranged from cytochrome p450s and glutathione S-transferases to catalases. Other studies have examined transcript regulation between susceptible and imidacloprid-resistant populations to classify possible mechanisms of detoxification. Zhu et al. compared transcript abundance between an imidacloprid-susceptible and resistant population. They demonstrated the resistant population had elevated transcript levels of multiple cytochrome p450s that could play a role in imidacloprid resistance (45). Clements et al. demonstrated the increased transcript abundance in resistant field populations of *L. decemlineata* which possessed multiple, potential modes of insecticide resistance, including cytochrome p450s, glutathione synthetase, carboxylesterases, and cuticular proteins (29). While these studies give an important glimpse into the genetic mechanisms of resistance, it is difficult to definitively classify these genetic mechanisms as the cause of resistance without a corresponding phenotypic change back to a susceptible population. This may be accomplished in future studies through the use of RNA interference.

4. New Technologies targeting *Leptinotarsa decemlineata*

With insecticidal resistance to neonicotinoids on the rise, new advances in pest management strategies need to be considered. One such strategy is to find new insecticides from the natural environment, including examining natural products produced by higher fungi (46). Smid et al. demonstrated that when *L. decemlineata* larvae were fed macrocypins, a protease inhibitor, significant effects on larval development, weight gain, and growth rates were observed, and further suggested that macrocypines should be further studied as a method for *L. decemlineata* management (46). Besides naturally occurring products, new technologies developed in the laboratory have also shown great promise. One such technique is suppressing transcript expression through the use of small, non-coding RNA molecules, also referred to as RNA interference (RNAi) (47–49). Ongoing research has demonstrated that RNAi has the potential to become a viable approach for insect control when fundamental genes of insecticide detoxification and essential genes for organism function are targeted. RNAi has been studied in depth in *L. decemlineata,* and the process by which RNA is transported into the cell to silence complementary mRNA is being unraveled (50). RNAi can also be used as a tool to determine the function of specific genes by observing phenotypic expression after RNAi suppression in *L. decemlineata* (51–54). Zhu et al. demonstrated the suppression of fundamental biological elements using long, double-stranded (ds) RNA fed to larval stages of *L. decemlineata*, and this suppression triggered mortality, validating the uptake and suppression of targeted dsRNA by ingestion (55). Revuelta demonstrated the importance of acetylcholinesterase activity with dsRNA suppression of *Ldace* 1, and this suppression, together with organophosphate insecticidal exposure, resulted in high observed mortality (75.6%) compared to control mortality (40.3%) in

adults insects (56). Further studies have targeted and suppressed specific receptors through the use of targeted dsRNA to determine their function in the phenotypic resistance response, including the role that the ryanodine receptor plays in chlorantraniliprole resistance. Wan et al. successfully suppressed the ryanodine receptor through the use of dsRNA, but observed little fitness cost due the suppression (52). The question of how pest managers could effectively administer this new technology in the field is of great importance. One clever solution includes the use of transgenic plants, which may be engineered to express dsRNA in the chloroplast of potatoes in order to effectively target *L. decemlineata* (57). Zhang et al. developed a transgenic potato plant that produced dsRNA encoding for β-actin in the chloroplast resulting in lethality and protection of potato foliage (57).

5. Conclusion

As an agricultural pest of potatoes, *L. decemlineata* has had a long history of insecticide resistance. Pest managers face current and future challenges, as the insecticides which are currently being used are becoming less and less effective. For growers who rely on pest managers to minimize pest damage for an optimal agricultural yield and minimize pest damage, the realization of new technologies cannot come soon enough. For academic scientists, *L. decemlineata* has revealed itself as a fascinating system to be explored, including the mechanisms by which beetles develop insecticide resistance and the fact that there might be multiple biological process and environmental factors that need to be examined to better understand the entire life history of resistance in *L. decemlineata.*

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CHAPTER 1: Characterizing molecular mechanisms of imidacloprid resistance in select populations of *Leptinotarsa decemlineata* in the Central Sands region of Wisconsin

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Authors: Justin Clements, Sean Schoville, Nathan Peterson, Que Lan, and Russell L. Groves

Abstract

The Colorado potato beetle, *Leptinotarsa decemlineata* (Say), is a major agricultural pest in the Central Sands region of Wisconsin. Imidacloprid, a neonicotinoid insecticide, has commonly been used for control of *L. decemlineata* since its registration in 1995. In the last 10 years, many field populations of *L. decemlineata* have begun to show increasing imidacloprid resistance. We studied resistance phenotype as a phenomenon that reduces neonicotinoid efficacy and has practical consequences for potato pest management. Although we have not observed complete field failure following the use of these products, multiple studies have demonstrated that the lethal concentration to kill 50% of the test organisms (LC_{50}) in different field populations of *L. decemlineata* varies greatly which may suggest that resistance of *L. decemlineata* is heritable and involves genetic changes. An important challenge in understanding resistance is assessing the genetic mechanisms associated with resistance and classifying up-regulated genes that may be involved in combating an insecticide insult. In this study we uncovered trends in imidacloprid phenotypic response that have developed in the region by

estimating the LC_{50} values among different field populations against a range of imidacloprid doses. The LC_{50} values collected in 2008-2011, and more recently in 2013 and 2014, show that some field locations remain susceptible to imidacloprid, while nearby fields (<100km) have developed high levels of resistance. We also sought to uncover potential mechanisms of resistance at each field location. We compiled a transcriptome for populations, characterized as phenotypically 'susceptible' and 'resistant', by isolating mRNA from adult beetles and analyzing gene expression level differences. Strong differences were observed in constituently up and down-regulated genes among different field populations. Most significantly, the up-regulation of 3 cytochrome p450s and a glutathione synthetase related protein in multiple resistant populations provide a mechanistic explanation of resistance evolution in *L. decemlineata.*

Keywords: Colorado potato beetle, neonicotinoid, resistance mechanisms, transcriptomics

1. Introduction

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say), is a key agricultural pest infesting commercial potato (*Solanum tuberosum*), as well as tomatoes (*S. lypcopersicum*), eggplants (*S. melongena*) and peppers (*S. annuum)* [1]. *L. decemlineata* is thought to have expanded from the southwestern United States and Mexico to a global range encompassing the United States, Mexico, Europe, and Asia, inclusive of over 16 million km^2, and switching from its native host plant buffalobur (*Solanum rostratum)* to agricultural crops [1,2]. With very few natural enemies, the beetles can be devastating on crops if an insecticide treatment is not used. Adult beetles consume as much as 10 cm^2 of foliage daily [2], leading to 50-100% crop loss in unmanaged situations. Potato fields throughout the United States are regularly treated with a combination of at-plant, systemic and foliar applications of neonicotinoid insecticides throughout the growing season to control *L. decemlineata*. Since its initial registration in 1995, Imidacloprid has been the most widely used neonicotinoid insecticide for crop protection. Imidacloprid affects the nicotinic acetylcholine receptor (nAChR) in insects and causes the accumulation of acetylcholine at nerve synapses, which ultimately leads to paralysis and death of the target organism [3]. Due to its unique structure, imidacloprid has high specificity to insect nAChR and low specificity to vertebrate nAChR, leading to low mammalian toxicity [3] and permitting a variety of uses on many crop and non-crop plant species.

A number of studies have shown that select populations of *L. decemlineata* are developing resistance to neonicotinoid insecticides, including field populations in Wisconsin, Maine, Michigan and New York [2,4–7]. Resistant populations of beetles are typically classified using LC_{50} assays, where the estimated ratio (RR) is calculated as the ratio of the estimated LC_{50} of the test population over the LC_{50} of a reference susceptible population [8]. If the RR value is

greater than 10, a population is considered to have (or to be trending towards) a resistant phenotype. Measured levels of imidacloprid resistance have increased in select populations of *L. decemlineata* from the Central Sands region of Wisconsin since 2008 [4]. This is similar to observations of imidacloprid resistance found in field populations of *L. decemlineata* from other states [9]. While RR values are only a snapshot of a population's resistance phenotype, a similar study from 2011 revealed large increases in resistance in field populations of *L. decemlineata* in Michigan over consecutive years [9]. Increasing levels of observed resistance have also coincided with an increasing frequency of neonicotinoid applications to obtain effective control of *L. decemlineata* populations, evidenced by higher LC_{50} values in commercial potato fields using imidacloprid [4], which may further exacerbate the potential for increased resistance. Understanding the population dynamics and underlining genetic resistance mechanisms that give rise to a resistant phenotype in *L. decemlineata* is of critical importance in managing beetle populations. With an improved understanding of the genetic mechanisms that play a role in *L. decemlineata* resistance development, we will be able to better monitor field populations for resistance and may also have the capacity to predict the spread and prevalence of resistance in the future.

Previous studies have suggested that *L. decemlineata*, as well as other insects, possess four candidate mechanisms of resistance to toxic chemicals [2,10]. The first is described as target site insensitivity. In the case of neonicotinoid resistance, this would involve a mutation in the nAChR, similar to that which has been found in the brown planthopper [11]. A second mechanism is enhanced metabolic breakdown, whereby increased metabolism by enzymes purge cells of toxic compounds. Proteins such as monooxygenases, which include the cytochrome p450s, are primary agents (Phase I metabolism) enhancing metabolic breakdown. cytochrome

p450s have been shown to play a vital role in insect metabolism of imidacloprid [12,13] as well as other neonicotinoid insecticides. Other secondary agents (Phase 2 metabolism) include enzymes such as glutathione S-transferases [12] which conjugate neonicotinoid molecules to increase water solubility and facilitate bodily excretion. The third potential mechanism is increased excretion, which can also be performed by some metabolic mechanisms or reduced penetration which would inhibit the pesticide from reaching its target site. This can be accomplished by a suite of cellular mechanisms. The fourth mechanism is simply classified as behavioral resistance, whereby beetles avoid exposure to pesticide by modifying their phenology, foraging behavior, or habitat choices, avoiding leaves with higher concentrations of pesticide and even avoidance of areas that have been treated [2]. These four mechanisms can be classified into two categories of protection: toxicokinetic and toxicodynamic mechanisms [14]. Toxicokinetic mechanisms play a role in absorption of the insecticide into the organism and to the fate of the insecticide after it enters the organism (biotransformation and excretion), while toxicodynamic mechanisms involve the interactions of the pesticides with their target sites [14]. These interactions can be complex in nature and multiple interactions can take place simultaneously.

In the current study, our primary aim was to uncover any genes that were involved in enhanced molecular breakdown or reduced penetration of imidacloprid. Specifically, we attempted to determine if genes in resistant populations were constitutively active or were differently expressed compared to a susceptible reference population. We composed a transcriptome of all the genes expressed in both resistant and susceptible populations using RNA extracted from field populations of beetles collected in 2013. This approach allowed us to observe gene regulation across both the susceptible and resistant populations collected from a

similar geographic region and similar time points during the growing season. Site selection for these experimental populations was based upon prior knowledge gained from earlier LC_{50} bioassays performed by Huseth and Groves [4] together with newly collected LC_{50} estimates. This approach provided a strong foundation to choose from among several resistant populations in the Central Sands Region of Wisconsin previously identified as resistant over successive growing seasons. Our results are principally focused on transcript expression in these *L. decemlineata* populations of the Central Sands Region of Wisconsin using RNA sequencing technologies. Expression studies were further validated with quantitative polymerase chain reaction (PCR). Based upon previous assessments of neonicotinoid resistance among populations in Wisconsin [4], we selected two candidate resistant populations and one nearby susceptible population to analyze in this study. This study uncovered many up-regulated genes in the two resistant populations that have the ability to combat insecticides in *L. decemlineata.* The two resistant populations showed some similarities in the up-regulated genes, but many of the up-regulated genes were differentially expressed between the two populations, suggesting that different populations of *L. decemlineata* may cope with insecticides in different manners.

2. Materials and Methods

Ethics Statement:

No specific permits were required for field collection of *L. decemlineata* for the study described here. Access to all field sites was granted by landholders.

Leptinotarsa decemlineata Collection

Two generations of *L. decemlineata* were collected from fields in the Central Sands region of Wisconsin in the spring and summer of 2013 and 2014. Approximately 700

overwintered adult beetles were randomly chosen from newly emerged potato plants at each of the pre-selected field locations. Adult beetles were hand-collected from plants at 4 different field locations including the Hancock Agricultural Research Station (HARS, 44.120430, -89.539149), plus three nearby agricultural fields in the Central Sands region, henceforth referred to as Systemic-1, Systemic-2, and Systemic-3. An additional set of 700 adult *L. decemlineata* were obtained from the Arlington Agricultural Research Station (AARS, 43.315527, -89.334545), and this population was regarded as a susceptible population. All adult beetles collected from the canopy of plants at all locations did not show any symptoms of acute poisoning at the time of collection. After collection, adult beetles were additionally maintained for 72 hours on untreated potato foliage, which was grown in an insecticide-free greenhouse on the campus of the University of Wisconsin-Madison. Following the 72 hour feeding interval, a randomly selected subset of 350-400 adults were used for LC_{50} assays, and another smaller, randomly selected subset (N=60) was used for RNA extraction and transcriptome assembly. A second collection of approximately 200 adult *L. decemlineata* (termed 2^{nd} generation adults) were collected from the same field locations after they had completed their first full generation and had emerged as adults from the soil in mid-July. Here again, the adult *L. decemlineata* were caged for 72 hours on untreated potato foliage after collection and were later used in RNA extraction and transcriptome construction.

Overwintered adult *L. decemlineata* were again collected from the same populations in 2014 using similar methods and used for LC_{50} assays. Due to crop rotation in the spring of 2014, overwintered adult beetles were collected in fields that were adjacent to the previously-sampled field (less than <0.8 km apart) in 2013 where adult insects had previously been collected. Because adult *L. decemlineata* are not known to disperse over great distances [15] from their

previous field locations, adult insects collected at these adjoining field locations were considered to be from the same field populations.

LC_{50} Assessments within Populations

In 2013, 350 to 400 overwintered adult beetles from each population were used to determine the lethal concentration required to kill 50% of the test insects (LC_{50}) using serial dilutions of technical imidacloprid (Bayer, Kansas City, Mo) in acetone. Specifically, 350 to 400 adult beetles were divided into 7 or 8 groups (n=50) and treated with a 1µl solution of imidacloprid in acetone on the first abdominal sternite. Imidacloprid concentrations ranged between 0-2 ppm and dilutions were prepared taking into consideration the specific density of acetone. A 0 ppm acetone concentration was used as a no insecticide control. Adult beetles were placed in petri dishes with fresh foliage that was changed every day for 7 days in an incubator held at 26°C, 70% humidity, and light and dark cycle of 16 hours light and 8 hours dark. After 7 days, the number of live beetles, incapacitated beetles, and dead beetles was recorded, as measured by the pencil test [6]. Briefly, adult beetles were presented with the opportunity to climb a pencil: if they could move a full body length they were considered alive, if they appeared alive, but could not move a body length then they were considered incapacitated, and if they had no movement, even after pinching their back legs with tweezers, they were considered dead. Incapacitated and dead beetles were pooled and LC_{50} values for each population were calculated. Abbott's correction was used to normalize the data and a $\log_{10}$ probit regression analysis (PROC PROBIT, SAS Institutes) [4,16] was used to calculate the LC_{50} for each first generation population. In 2014, a similar protocol was followed, but 450-500 beetles were used to determine the LC_{50} with 10 groups (n=50) ranging from 0-100ppm.

RNA Extraction and Illumina Sequencing

Total RNA was extracted for RNA sequencing from beetles collected at the AARS and the systemic-1 and systemic-3 populations in 2013. The 2 systemic populations were chosen because they were found to be the most resistant in the LC_{50} assays while the AARS population served as the susceptible comparison. At least 60 healthy, adult female beetles were randomly selected from each population and used for RNA extraction. Briefly, total RNA was extracted from each beetle with Trizol (Life Technology, Grand Island, NY) and stored at -80C for later analysis. Total RNA was pooled into three biological replicates composed of 60 individuals for each population (1 replicated representing overwintered adult beetles and 2 replicates representing 2^{nd} generation beetles) and treated with TurboDNase (Life Technology, Grand Island, NY). The quality of the RNA was examined using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA concentrations were measured using a Nanodrop (Thermo Fisher Scientific, Waltham, MA) and 500 ng of total RNA per replicate was submitted for RNA-sequencing (Beckman Coulter Genomics, Danvers, MA). Beckman Coulter Genomics was contracted to isolate mRNA using an Illumina automated RNA-seq platform to generate 338 million high quality reads of 2X100bp. Beckman Coulter Genomics was also contracted to perform quality checks and *de novo* assembly of RNA-seq data into a whole transcriptome encompassing all populations. Assembly was performed using Trinity Bioinformatics [17,18]. Beckman Coulter used the default settings in Trinity Bioinformatics software release 2013-11-10 with an initial input of reads from Arlington, Systemic-1, and Systemic-3 with 12 CPUs to generate the transcriptome. Raw reads were uploaded to NCBI Sequence Read Archive (SRA) SRP064192. The Transcriptome Shotgun Assembly project has been deposited at

DDBJ/EMBL/GenBank under the accession GEEF00000000. The version herein described is the first version, GEEF01000000. The Transcriptome was submitted following NCBI submission criteria, and the assembled transcriptome was uploaded after removal of contamination identified by blast searched against the Univec database and contigs smaller than 200 basepairs. Gene expression was calculated in units of fragments per kilobase of exon per million fragments mapped (FPKM) using Trinity Bioinformatics for each separate biological replicate.

Annotation of Trinity Contigs

BLAST standalone [19] was used to classify Trinity assembled contigs into possible genes. Reference protein sequences (Refseq) from *Tribolium castaneum*, *Acyrthosiphon pisum*, *Anopheles gambiae*, *Drosophila melanogaster*, and *Pediculus humanus* were downloaded from NCBI for a total of 80,498 sequences. A reference database was created with the protein sequences to blast against the transcriptome. Using Blast standalone with BLASTx, the total unique trinity contigs in the transcriptome were compared to the reference proteins (*E* value $<10^{-3}$). Transcripts were classified based on the NCBI nomenclature returned by BLASTx. BLASTx results were uploaded into Blast2Go [20] for further data analysis. The entire transcriptome was first analyzed and the components were mapped to the corresponding GO terms, then the annotation step was run with a cutoff of E_{value} < 1E-3, annotation cut off > 45, and GO weight >5.

Differential Gene Expression

Transcript abundance between the putative resistant and susceptible populations in 2013 was calculated using Trinity bioinformatics software (RSEM). FPKM were calculated for each

set of unique contigs in each population (1 was added to each FPKM value). Gene abundance was compared between the 2nd generation populations and confirmed with qPCR. A linear regression between the two biological replicates of 2nd generation for each of the three different populations was conducted along with determining the FDR (false discovery rate) ($P \leq 0.059$) to eliminate outlying data in R commander [21]. The FPKM of the biological replicates in each population were averaged and the FPKM's between the two putative resistant and the single susceptible populations were compared. Genes with a fold change greater than 2 were considered up- or down-regulated. An enrichment analysis between the annotated transcriptome and the upregulated genes found using Trinity was run using a two-tailed FDR test with 0.05 cut-off in order to determine if any group of GO terms were differentially expressed in the up-regulated components. The up-regulated GO terms were then categorized using CateGOrizer [22] with GO_slim.

Quantitative PCR

Three biological replicates of pooled cDNA from each population from 2013 were used in cDNA synthesis for quantitative, real-time PCR (qPCR). Total RNA from each population was quantified using a Nanodrop (Thermo Fisher Scientific, Waltham, MA). DNA contamination was removed using TurboDNase (Life Technology, Grand Island, NY). DNA-free RNA was purified to remove any possibility of PCR inhibition with a MasterPure Complete DNA and RNA Purification Kit (Epicentre, Madison, WI). Following purification, RNA was suspended in 20µl of water, and the integrity was checked on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). All RNA concentrations were equalized before input into the cDNA synthesis kit and the subsequent cDNA was generated with a high capacity cDNA reverse

transcription kit (Applied Biosystems, Foster City, CA). The cDNA was diluted to a final concentration of 5ng/µl of RNA equivalent input for qPCR. β-actin was used as a reference gene. The β-actin primers were shortened versions previously used by Zhu [23]. The qPCR reaction was run on a CFX-96 platform (Bio-Rad Laboratories, Hercules, CA) with a master mix of Bullseye EverGreen (MIDSCI, Valley Park, MO). Genes of interest (GOI) were selected based on their relevance to this study and primers were designed to contigs found in the transcriptome. Primer and primer efficiency are found in Table 1**.** Primer specificity was checked against the transcriptome using BLAST. Primer efficiencies were calculated and optimized. Triplicate reactions were run at 95°C for 10min followed by 95°C for 15 s, 62°C for 60 s for 40 cycles. Data were collected for each biological replicate and relative expression of resistant strains to susceptible strains was calculated using the Pfaffl methodology [24], as seen in Equation 1. The Pfaffl methodology takes into consideration the efficiency of the primer sets and gives the ratio of the target gene to the reference population.

Table 1: qPCR primers and primer efficiency

	Forward Primer (5'-3')	**Reverse Primer (5'-3')**	**Primer Efficiency**
B-actin (Reference)	CATCCAAGCTGTACTCTCCTTG	GGAAGAGCGTAACCTTCGTAG	1.92
Comp115309 (Cytochrome P450)	CGAGAAATGCGACCTATTCTCAG	ACACAGTCTTGGTCTTTCTTGAG	1.98
Comp105889 (Cuticular protein)	CTCCAGTGGTTCCGTTATTACAC	AGCGTAGTCGTGGAAATGTTG	1.94
Comp114026 (Glutathione synthetase)	CAGAGCAGGGTATGAACCTAATC	CCAGCCAAGTGATACTGAATCG	1.97

Equation 1: $\text{ratio} = \frac{(E_{\text{target}})^{\Delta \text{CP}_{\text{target}}(\text{Control}-\text{samples})}}{(E_{\text{reference}})^{\Delta \text{CP}_{\text{ref}}(\text{control}-\text{samples})}}$ [24]

3. Results

Topical Bioassay

Resistance (LC_{50}) estimates generated in the 2013 and 2014 growing seasons closely approximate trends from previously reported data over the interval 2008- 2011 in the same geographic region of the state [4]. Table 2 presents regression estimates for populations including three putative resistant populations (systemic-1, systemic-2, and systemic-3), a moderately resistant population from the HARS, and a susceptible population from the AARS over two years in the current study. The current study uses the AARS population as the reference control strain with an estimated LC_{50} value of 0.09ppm in 2013 and 4.7ppm in 2014. The field population of *L. decemlineata* at the AARS has never been exposed to a season-long, at-plant use of imidacloprid. As expected, the data demonstrate that the field populations at the central Wisconsin locations, which have annually received successive, at-plant neonicotinoid applications, possess higher estimated LC_{50} values, found in Table 2, and higher resistance ratios, ranging from 10.11 to 18.18 in 2013 and 1.84 to 11.16 in 2014 (a resistance ratio exceeding 10 was considered a resistant population [5] in previous investigations). The *L. decemlineata* populations collected from the 3 commercial potato fields in the Central Sands region had the highest overall levels of measured resistance in 2013. In 2014, only one of the field locations (systemic-3) was classified as resistant according to this designation, while the remaining two field locations had estimated resistance ratios < 10. The HARS population was classified as resistant in 2013, but also dropped below this ratio in 2014. It should be noted, however, that while resistance ratios decreased from 2013 to 2014, the LC_{50} values for all field populations substantially increased (Table 2).

Table 2: Regression estimates resulting from topical bioassays of adult *Leptinotarsa decemlineata* at the Arlington (AARS) and Hancock Agricultural Research Stations (HARS), plus agricultural fields classified as systemic-1, 2 and 3 in 2011, 2013 and 2014.

Population	Year[1]	N[2]	Slope(SEM)[3]	LC_{50} (PPM)	95% CI	Resistance Ratio[4]
Arlington	2011	600	3.13(0.33)	0.027	(0.028-0.34)	NA
Hancock	2011	525	1.47(0.11)	0.48	(0.4-0.6)	17.77
systemic-1	2011	425	1.63(0.14)	0.72	(0.59-0.87)	26.66
systemic-2	2011	524	1.9(0.27)	0.62	(0.41-0.94)	22.96
systemic-3	2011	500	2.03(0.73)	0.73	(0.51-1.04)	27.03
Arlington[5]	2013	400	2.23(.25)	0.09	(.08-.12)	NA
Hancock	2013	300	0.73(.23)	0.91	(.49-4.30)	10.11
systemic-1[5]	2013	350	0.69(.26)	1.83	(.21-12.36)	18.18
systemic-2	2013	350	1.49(.37)	1.10	(.58-1.61)	11.11
systemic-3[5]	2013	350	2.08(.30)	1.20	(.94-1.57)	12.12
Arlington	2014	500	0.55(.12)	4.72	(1.57-10.74)	NA
Hancock	2014	500	0.80(.09)	8.69	(5.00-15.19)	1.84
systemic-1	2014	500	1.05(1.0)	12.81	(8.73-19.96)	2.71
systemic-2	2014	500	0.71(.09)	43.67	(23.60-109.27)	9.25
systemic-3	2014	500	0.66(.09)	52.68	(25.31-154.56)	11.16

[1] 2011 data is reported regression estimates obtained from Huseth and Groves (4).
[2] Total number of adult beetles used in biological replicates at each location and year combination.
[3] Mean slope estimates of the probit linearized dose response function plus standard error
[4] Resistance ratio estimates comparing test populations to the reference control population (AARS) in each year.
[5] Populations of *Leptinotarsa decemlineata* used in transcriptome assembly

Transcriptome Assembly

Illumina short-read sequencing was used to sequence mRNA collected from adult female *L. decemlineata* and subsequently assemble a transcriptome using Trinity bioinformatics software [17,18]. A summary of data from the assembled transcriptome from all three Wisconsin *L. decemlineata* populations is presented in Table 3. The transcriptome was composed of RNA isolated in 2013. RNA sequencing was performed on nine samples of pooled RNA, n=3 from each population (AARS, systemic-1 and systemic-3). The transcriptome revealed 98,002 possible unique transcripts for investigation and presumably some portion of these genes encode the aforementioned mechanisms of pesticide resistance. Subcomponents of Trinity were then

grouped together and classified as individual components. From the 98,002 unique transcripts, we used 80,498 reference sequences from NCBI [25] to identify 23,860 known transcripts in our transcriptome.

Table 3: Summary of *Leptinotarsa decemlineata* transcriptome assembled from the 2013 beetle populations collected from the Arlington Agricultural Research Station and two field populations, termed systemic-1 and systemic-3.

Summary of *L. decemlineata* transcriptome	
Total assembled bases	197,128,499
Total contigs	208,754
Unique transcripts	98,002
median contig length	479 bp
Average contig	944.31 bp
NC50	1742 bp
Percent GC	36.49
Blastx hits(*e* value $\geq$ 0.001)	23,860

Differential Expression Analysis

An overall goal of this study was to investigate gene expression differences between the resistant and susceptible populations. From the 23,860 transcripts identified in the analysis, we chose to focus on transcripts that encoded for genes associated with the known mechanisms of resistance. Specifically, we investigated changes in the expression levels of 8 classes of enzymes and proteins (cytochrome p450, glutathione related proteins, cuticular proteins, carboxylesterases, nicotinic acetylcholine receptors, ABC transporters, superoxide dismutase enzymes and catalase enzymes) to determine if patterns emerged in the up- or down-regulation of specific transcripts in the two resistant populations compared to the susceptible population. The transcriptome of *L. decemlineata* revealed 107 transcripts that encoded cytochrome p450s, 96 that encoded cuticular proteins, 21 that encoded glutathione related proteins, 16 that encoded carboxylesterases, 59 that encoded ABC transporter proteins (including multi drug resistant proteins), 7 that encoded superoxide dismutase enzymes, and 6 that encoded catalase enzymes. A

portion of these may constitute possible resistance mechanisms (Table 4). The transcriptome also revealed 20 possible nAChRs transcripts, some of which are known targets of imidacloprid.

Table 4: Differentially expressed up- and down-regulated transcripts observed between two resistant populations compared to the Arlington Agricultural Research Station population.

	Whole Transcriptome	Systemic-1 Up-regulated	Systemic -1 Down-regulated	Systemic-3 Up-regulated	Systemic-3 Down-regulated
Total Transcripts	98,002	394	195	562	632
Total Transcripts with Blastx results evaluate(0.001)	23,860	290	78	399	405
Cytochrome p450	107	6	1	13	6
Cuticular	96	0	1	23	2
ABC transporters	59	4	0	7	0
Glutathione related proteins	21	1	0	1	0
Nicotinic acetylcholine receptor	20	0	0	0	0
Catalase	6	1	1	0	0
Superoxide dismutase	7	1	0	0	0
Carboxylesterase	16	1	0	2	0

Transcript expression was compared between the AARS susceptible population and two of the three previously described resistant populations from agricultural fields (systemic -1 and -3). For the comparison, we used two pooled samples (biological replicates) of adult *L. decemlineata* collected from the second generation (mid-July collections) from each field. After the transcriptome was assembled, we calculated the fragments per kilobase of exon per million fragments mapped (FPKM) of each transcript to estimate expression levels between the populations. We then identified significantly up- and down –regulated genes by adjusting levels according to a false discovery rate (FDR) of 0.059 between the populations. Gene regulation between the susceptible AARS population and the two resistant field populations (systemic-1, systemic-3) can be seen in Table 4. In the systemic-1 population there were a total of 394 transcripts, of which 290 share homology to known proteins. In the systemic-3 population, there

were 562 transcripts, of which 399 share homology to known proteins. The up-regulated components of the systemic-1 and -3 populations can be seen in S1 Table and S2 Table.

Using Blast2go we mapped Trinity components to Gene Ontology (GO) terms. We then classified the up- regulated genes into GO terms to examine if there was any difference in GO terms between up- regulated populations and the whole transcriptome. The analysis revealed 290 up-regulated and annotated transcripts that correlated to known genes that were classified into 349 GO terms and then grouped into 65 GO_slim classes in the systemic-1 population. The systemic-3 population had 399 up-regulated and annotated transcripts encoding for genes which were classified into 400 GO terms and were grouped in to 45 GO_slim classes. The up-regulated GO terms in both systemic-1 and -3 populations had similar GO_slim classes with 13.68% of GO terms of systemic 1 population falling into molecular functions and 16.72% of GO terms falling into biological processes. The systemic-3 population had 21.78% of GO terms falling into molecular functions and 12.34% categorized into biological processes. An enrichment analysis of the GO terms between the compiled transcriptome and the up-regulated GO terms in the systemic-1 and -3 populations showed different trends between the two resistant populations. The enrichment analysis revealed three sets of GO terms were significantly up-regulated (catalytic activity, single-organism metabolism processes, oxidoreductase activity) in the systemic-1 population, while in the systemic-3 population the enrichment analysis revealed several up-regulated GO terms including structural constituents of the cuticle, monooxygenase activity, oxidoreductase activity and oxidation-reduction processes. Significantly different GO terms (FDR > 0.05) between the up-regulated genes in the resistant populations and the entire transcriptome can be found in S3 Table and S4 Table.

The up-regulated transcripts were then examined in both populations. In the up-regulated transcripts of the two resistant populations, we observed 8 shared transcripts that may play a role in insecticide resistance, including 3 cytochrome p450s, 1 carboxylesterase, 1 glutathione synthetase, and 3 proteins that are related to ABC transporters, as seen in Table 5. When we examined the fold change calculated in RSEM, we found that the 8 shared transcripts had a fold change of around two, which was the cut off for up-regulation. One of the cytochrome p450s in the systemic-3 population was up-regulated three fold. This observation could indicate that these genes are constitutively active, but not significantly over-expressed due to the fitness cost of activating multiple mechanisms of resistance simultaneously. Trends in the down-regulated transcripts were observed between the susceptible population and the two resistant populations. The two populations shared a total of 129 down-regulated transcripts. Two of these transcripts, a cytochrome p450 and a cuticular protein, encode a known mechanism of resistance.

Table 5: Fold changes in the up-regulated genes observed between the two resistant populations, Systemic-1 and Systemic-3.

Transcript	Systemic-1 (Fold Change)	Systemic-3 (Fold Change)	Predicted Genes from Blastx against 80,498 reference sequence[1] in NCBI database	NCBI accession number
comp118021_c0	2.11	2.06	atp-binding cassette transporter	XP_969849
comp117821_c0	2.06	2.42	atp-binding sub-family c (cftr mrp) member 4	XP_971632
comp114343_c0	2.07	2.25	PREDICTED: similar to carboxylesterase	XP_969104
comp117371_c0	2.43	2.18	multi drug resistance 50 cg8523-pa	XP_001810982
comp114026_c0	2.08	2.66	glutathione synthetase	XP_968070
comp103658_c0	2.37	2.34	cytochrome p450 9z4	NP_001164248
comp106072_c0	2.5	2.38	cytochrome p450 9z4	NP_001164248
comp111691_c1	2.77	3.25	cytochrome p450 monooxygenase	XP_972348

[1] Reference sequences complied from NCBI in January 2014

We then confirmed the gene regulation between the samples by using quantitative PCR (qPCR) (Table 6). From among the total up-regulated transcripts studied, we focused on

transcripts that could play a role in insecticide resistance. Additionally, β-actin expression was used as a reference and confirmed the expression of 3 transcripts, an up-regulated cytochrome p450 in the systemic-3 population (not classified as up-regulated in systemic-1 due to an FDR value greater than 0.059), a cuticular protein that was up-regulated in the systemic-3 population, and an up-regulated glutathione synthetase protein that was also up-regulated in both resistant populations. The qPCR results in the systemic-1 and -3 populations closely resembled the fold changes calculated with FPKM.

Table 6: Gene expression determined by quantitative PCR.

	Arlington	Systemic-3			Systemic-1		
	Mean CT ±SD	Mean CT ±SD	FPKM Fold Change	qPCR Expression ratio	Mean CT ±SD	FPKM Fold Change	qPCR Expression ratio
ß-actin (Reference)	20.3±.30	19.39±.37	N/A	N/A	19.72±.49	N/A	N/A
comp115309 (cytochrome p450)	22.4±.38	20.04±.03	3.5	2.77	20.42±.31	2.85	2.65
comp105889 (cuticular protein)	23.9±.62	20.92±.39	6.48	3.96	22.31±1.70	1.72	1.96
comp114026 (glutathione synthetase)	27.1±1.02	24.68±.23	2.66	2.9	25.52±.39	2.08	2.04

4. Discussion

The ability of *L. decemlineata* to become resistant to insecticides has become a major agricultural problem with wide-reaching consequences. Although previous studies have indicated that populations of *L. decemlineata* have developed some form of resistance to imidacloprid [2, 4–7], the molecular determinants of this resistance within or between populations of *L. decemlineata* remain unknown. Understanding the genetic mechanisms and whether genetic changes are shared among populations is important in predicting the evolution of resistance in other potato growing regions. Topical bioassays have been used to demonstrate resistance to

neonicotinoids as early as 1997 [6]. Field populations of *L. decemlineata* in the Central Sands region of Wisconsin demonstrate variable levels of resistance that are maintained across years. When comparing the data between field populations collected in the same year, trends in resistance remain consistent, with treated fields having higher LC_{50} values than non-treated fields.

Although topical bioassays have considerable annual variation, with data collected in 2013 closely resembling estimates generated in a previous study [4] and data collected in 2014 resulting in much higher LC_{50} values, the trend towards increasing LC_{50} values is consistent across the field populations investigated. There is the possibility that resistance is increasing throughout Wisconsin, including at the AARS site previously considered susceptible due to its low resistance ratios and lack of pesticide exposure. However, variation in the estimated LC_{50} values is not unexpected, as subtle differences in sampling times after beetles emerged from diapause, as well as fitness costs associated with overwintering conditions, could have influenced our experimental results. Modest variations in collection times of overwintered beetles could play a large role in the fitness of the beetles and how they respond to topical bioassays. Variation between LC_{50} estimates is expected to increase as field populations of beetles become more resistant to insecticides [9]. The inter-annual variation between populations can clearly be seen as reported by Huseth and Groves [4] with some mixed populations of beetles varying in LC_{50} values as much as 20 fold between years.

A transcriptome was assembled from RNA extracted from *L. decemlineata* in an effort to identify the potential mechanisms of resistance. The transcriptome itself encompassed 208,754 transcripts, of which 98,002 were unique. Recently, an annotated transcriptome was published in 2014 [26] that distinguished the activity of genes in different developmental stages of *L.*

decemlineata. This study revealed a transcriptome that contained 121,912 transcripts from both adult and larval data using 454 sequencing technology. Interestingly, they found more than twice the number of cytochrome p450 and glutathione related proteins when compared to our study (221 cytochrome p450 and 45 glutathione S-transferase proteins compared to 107 cytochrome p450 and 21 glutathione related proteins). The use of different sequencing and assembly methods, reference gene set, and the use of only adult *L. decemlineata* in our study may explain this discrepancy. Additionally, although Trinity uses a complex set of equations to try to generate full length transcripts from contings, there may be fragmentation of genes with low coverage, including the cytochrome p450 family if genes are weakly expressed. The closest genome that has been annotated to *L. decemlineata* is *Tribolium castaneum*, and its genome revealed 132 Cyp genes [13] which is more similar in number to our annotation.

We also observed differences in gene expression between two second generation *L. decemlineata* populations through analysis of gene expression from whole *L. decemlineata* samples. We focused our study on previously described mechanisms of resistance, including phase one metabolism (cytochrome p450s) and phase two metabolism (glutathione S–transferases), as well as the metabolic signature of reduced penetrance and increased excretion. Previous studies have demonstrated the importance of cytochrome p450 in imidacloprid resistant populations of insects, including the overexpression of Cyp6g1 in *Drosophila melanogaster* [27] and Cyp6CM1 in *Bemisia tabaci* [28]. Another study demonstrated that cytochrome p450 inhibition in *L. decemlineata* using piperonyl butoxide re-establishes a more susceptible population [8]. When we examined up-regulated genes in two resistant populations, we found many possible mechanisms of resistance that could play a role in detoxification of imidacloprid including multiple up-regulated cytochrome p450s.

Without an annotated genome, we classified genes based on their similarities to known reference sequences. Our study revealed that the systemic-1 population had 14 transcripts which could be classified among the 8 molecular mechanism classes investigated, and 6 of these were similar in homology to one of the reference protein sequences encompassing the cytochrome p450 family. In comparison, the systemic-3 population had 46 transcripts that could be classified in one of the 8 classes. We also found that the systemic-3 population expressed large numbers of proteins which are functionally relevant with cuticular proteins (23 out of the 46 up-regulated molecular mechanisms), while the systemic-1 population had no up-regulated cuticular proteins. The enrichment analysis between the whole transcriptome and the systemic populations also revealed that both populations overexpressed oxidoreductase activity and the systemic-3 population overexpressed monooxygenase activity. Finally, we examined whether there were genes that were up-regulated in both resistant populations. When we compared genes across resistant populations we found 8 shared transcripts that could code for some type of metabolic resistance, including 3 cytochrome p450s and a single glutathione synthetase related protein. We also examined if there were any trends in the shared, up-regulated transcripts that encoded for a known mechanism of resistance. The three cytochrome p450 genes were all from the Cyp 9 family and matched best to a cytochrome p450 from *T. castaneum*. The 3 ABC transporter proteins, were also compared to each other. Two belonged to Subfamily C, and all three transcripts that encoded for ABC transporter had a closest match to *T. castaneum*. Possible mutations in the nAChR between the susceptible and resistant populations were also examined, but were omitted from this study due to the low depth of coverage of RNA sequencing at the sites. The RNA sequencing data is also from pooled individuals, so conclusions about individual genotypes from this data regarding mutations in the nAChR could be misleading.

Our study suggests that different populations of *L. decemlineata* are most likely using a mixture of similar and dissimilar genes to combat pesticide insults. Despite the fact that the resistant populations of *L. decemlineata* were collected from similar geographic and agricultural regions of the state of Wisconsin, we speculate that field populations of *L. decemlineata* have adapted to the unique trends in pesticide application at each field location, leading to differences in up-regulated transcripts in each resistant population. Naturally, some of the genes could be similar because *L. decemlineata* host plants contain high levels of glycoalkaloids and *L. decemlineata* has adapted to become an effective pest. This speculation is further supported because it has been previously shown that most *L. decemlineata* do not migrate more than 400m from their overwintering range in a given year [29]. Although some population mixing can certainly occur over agricultural landscapes, these observations further suggest that *L. decemlineata* genetics might be discrete in their distribution.

5. Conclusions

The purpose of this study was to uncover potential genes responsible for imidacloprid resistance in spatially explicit *L. decemlineata* populations in the Central Sands region of Wisconsin. We used knowledge acquired from Huseth and Groves [4], together with dose-response generated regression estimates that we calculated in 2013 and 2014, to identify 2 candidate imidacloprid resistant field populations and a single susceptible population. With this data, we attempted to establish the mode of imidacloprid resistance within these selected populations of *L. decemlineata* by uncovering genes that were differentially expressed in association with imidacloprid exposure. We found that both of the resistant populations investigated had many up-regulated genes that were constitutively activated, including many

cytochrome p450s. Interestingly cuticular proteins were found up-regulated in only the systemic-3 population. One limitation of this study is that it did not integrate the effects that other pesticides and environmental stressors have had on beetles. It is possible that some of the genes that are being up-regulated are the product of other stressors. We chose to use field populations of *L. decemlineata* to examine relevant field conditions that give rise to insecticide resistance that farmers face.

This study also aimed to better understand the beetle's genome by creating a transcriptome from extracted RNA. We hypothesized that resistant populations would have differentially regulated gene expression when compared to susceptible populations. RNA sequencing and assembly technology provided us a comprehensive picture of genes that are expressed differently between selected populations of *L. decemlineata* in this region of Wisconsin. Even though populations of beetles are collected from fields within the same geographical region, the modes of action for insecticide resistance seem to vary in space, as multiple up-regulated genes vary among populations.

In particular, we demonstrated that multiple genes, or modes of action that could encode for pesticide resistance, are up-regulated in each of the two resistant populations. Studies have suggested that cytochrome p450 may play a role in the detoxification of imidacloprid and indeed we found evidence that these genes are up-regulated in resistant Wisconsin populations. The information provided from this study clarifies possible resistance mechanisms and can assist us in future efforts to determine good candidate genes for gene targeting pesticides (i.e. RNAi technology), such as the cytochrome p450 and cuticular proteins.

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8. Supplemental Information

S1Table: Up-regulated components in the Systemic-1 population determined by a fold change of greater than 2 and a FDR of less than 0.059.

Components	FDR	Fold Change	Component Description
comp100892_c0	0.001	9.730	---NA---
comp100923_c0	0.001	2.674	PREDICTED: similar to alpha-esterase
comp101093_c0	0.006	11.940	---NA---
comp101549_c0	0.005	7.215	caseinolytic peptidase b protein homolog (suppressor of potassium transport defect 3)
comp101816_c0	0.001	2.364	juvenile hormone-inducible
comp101841_c0	0.040	2.525	---NA---
comp101855_c0	0.054	3.017	---NA---
comp101939_c0	0.001	2.443	---NA---
comp102061_c0	0.001	3.174	cathepsin b-like like proteinase
comp102186_c0	0.001	2.629	AGAP009328-PA
comp102187_c1	0.026	2.241	Actin, muscle
comp102265_c1	0.017	2.474	---NA---
comp102305_c0	0.041	2.263	cg9090 cg9090-pa
comp102424_c0	0.001	2.422	translocator protein-like
comp102425_c0	0.001	3.388	---NA---
comp102476_c0	0.001	21.655	---NA---
comp102528_c0	0.041	2.013	---NA---
comp102657_c0	0.011	2.389	PREDICTED: similar to AGAP006424-PA
comp102690_c0	0.001	4.905	PREDICTED: hypothetical protein
comp102761_c0	0.020	2.457	28 kda desiccation stress protein
comp102762_c0	0.003	2.846	---NA---
comp102853_c0	0.001	2.304	PREDICTED: similar to 3-oxoacyl-
comp102935_c0	0.001	7.199	---NA---
comp103119_c0	0.001	3.230	sodium-dependent phosphate transporter
comp103228_c0	0.053	2.091	PREDICTED: hypothetical protein LOC100570164
comp103236_c0	0.008	2.487	AGAP007368-PA

comp103555_c0	0.001	4.097	---NA---
comp103556_c0	0.057	4.272	cg9427 cg9427-pa
comp103650_c0	0.001	13.195	defensin 1
comp103658_c0	0.001	2.373	cytochrome p450 9z4
comp103760_c0	0.001	3.113	beta- -glucan recognition protein 2
comp103910_c0	0.001	3.282	---NA---
comp103967_c0	0.001	2.168	PREDICTED: similar to AGAP001449-PA
comp104160_c0	0.015	2.940	---NA---
comp104308_c0	0.002	2.207	PREDICTED: similar to GA15997-PA
comp104384_c0	0.001	2.369	---NA---
comp104411_c0	0.001	2.528	29-kda galactose-binding lectin
comp104533_c0	0.001	7.084	acid phosphatase 1
comp104646_c0	0.001	2.164	defensin 1
comp104702_c0	0.020	2.398	---NA---
comp104711_c0	0.022	2.206	cg6870 cg6870-pa
comp104713_c0	0.034	2.197	niemann-pick type c-1b
comp104806_c0	0.001	2.003	PREDICTED: similar to conserved hypothetical protein
comp104861_c0	0.003	2.326	---NA---
comp104933_c0	0.001	3.334	glucose dehydrogenase
comp104970_c0	0.001	2.164	---NA---
comp104984_c0	0.001	8.539	allergen bla g
comp105039_c1	0.032	2.567	cathepsin l-like protein cysteine proteinase
comp105223_c0	0.001	2.291	serine protease 2
comp105323_c0	0.019	2.118	---NA---
comp105434_c0	0.001	2.130	juvenile hormone-inducible
comp105457_c0	0.058	2.066	---NA---
comp105517_c0	0.051	2.057	---NA---
comp105537_c2	0.001	3.163	---NA---
comp105581_c0	0.037	2.038	purine biosynthesis protein pur6 isoform 1
comp105731_c1	0.001	2.325	cg7675 cg7675-pb
comp105917_c0	0.005	3.144	---NA---
comp105956_c0	0.013	2.012	---NA---
comp105973_c0	0.001	3.302	PREDICTED: similar to AGAP005972-PA
comp105989_c0	0.001	2.420	AGAP011167-PA
comp106035_c1	0.001	6.524	tryptophan -dioxygenase
comp106072_c0	0.001	2.504	cytochrome p450 9z4
comp106118_c0	0.003	2.143	PREDICTED: similar to conserved hypothetical protein
comp106220_c0	0.001	2.867	cg6870 cg6870-pa
comp106244_c0	0.001	4.736	12 kda hemolymph protein b
comp106294_c2	0.001	2.061	atp-binding cassette transporter

comp106296_c0	0.015	2.594	---NA---
comp106421_c0	0.026	2.346	AGAP011167-PA
comp106543_c1	0.001	2.747	poils au dos
comp106548_c0	0.001	2.517	xanthine dehydrogenase
comp106668_c0	0.001	2.061	cg6084 cg6084-pa
comp106677_c0	0.009	2.567	AGAP011630-PA
comp106711_c0	0.001	2.163	acid phosphatase-1
comp106832_c0	0.006	2.289	PREDICTED: similar to AGAP001553-PA
comp106834_c0	0.001	2.182	AGAP003206-PB
comp107125_c0	0.001	2.385	conserved hypothetical protein
comp107157_c0	0.021	2.248	pacifastin-related serine protease inhibitor isoform 2
comp107172_c0	0.001	2.076	---NA---
comp107182_c0	0.001	2.169	zinc-containing alcohol dehydrogenase
comp107277_c0	0.019	2.027	variable lymphocyte receptor a
comp107290_c0	0.001	3.318	PREDICTED: hypothetical protein LOC100165870
comp107322_c0	0.001	2.375	hemoglobin c1 polymer
comp107451_c0	0.020	2.460	---NA---
comp107507_c0	0.001	2.123	cg1673 cg1673-pa
comp107523_c0	0.030	2.675	hypothetical protein Phum_PHXM454910
comp107656_c0	0.001	3.503	---NA---
comp107703_c0	0.001	5.077	cg34115 cg34115-pa
comp107706_c0	0.001	4.201	PREDICTED: similar to AGAP005839-PA
comp107777_c0	0.025	2.291	---NA---
comp107842_c0	0.001	2.050	seven in absentia 1b
comp107865_c0	0.006	2.013	aldo-keto reductase
comp107875_c0	0.018	3.357	PREDICTED: similar to AGAP010241-PA
comp107926_c0	0.001	3.099	---NA---
comp108155_c0	0.001	5.841	PREDICTED: hypothetical protein LOC100574147
comp108241_c0	0.001	2.913	PREDICTED: similar to AGAP003785-PA
comp108250_c0	0.001	2.027	cg15105 cg15105-pa
comp108251_c0	0.001	5.033	PREDICTED: hypothetical protein LOC100571634
comp108262_c0	0.001	2.930	3-hydroxy-3-methylglutaryl- reductase
comp108539_c0	0.050	2.378	cathepsin b-like cysteine proteinase-like
comp108541_c1	0.001	2.178	---NA---
comp108561_c0	0.006	2.484	cg3108 cg3108-pa
comp108640_c0	0.001	2.469	---NA---
comp108695_c0	0.001	3.965	---NA---
comp108705_c0	0.020	2.044	Xracil-DNA glycosylase, putative
comp108777_c0	0.001	2.634	acid phosphatase-1
comp108826_c0	0.006	2.470	peritrophic matrix protein 3 precursor
comp108902_c0	0.001	2.389	PREDICTED: similar to AGAP001894-PA

comp108962_c1	0.003	2.358	---NA---
comp109050_c0	0.001	3.063	---NA---
comp109194_c0	0.020	2.015	isoform a
comp109331_c0	0.010	2.519	---NA---
comp109356_c0	0.013	2.340	eukaryotic translation initiation factor 4e binding protein
comp109435_c0	0.001	2.349	29-kda galactose-binding lectin
comp109504_c1	0.001	2.412	chitinase 3 precursor
comp109529_c0	0.001	2.328	sodium-dependent phosphate transporter
comp109623_c0	0.001	14.366	PREDICTED: hypothetical protein
comp109630_c0	0.001	2.468	PREDICTED: hypothetical protein
comp109633_c0	0.005	2.203	PREDICTED: similar to putative esterase
comp109639_c0	0.001	2.486	sodium-dependent phosphate transporter
comp109647_c0	0.001	2.064	PREDICTED: similar to conserved hypothetical protein
comp109680_c0	0.020	2.918	juvenile hormone-inducible
comp109791_c1	0.001	2.495	uncharacterized protein LOC662961
comp109815_c1	0.033	2.313	NA
comp109849_c0	0.001	2.018	cg14275 cg14275-pa
comp109878_c0	0.001	3.644	phosphodiesterase 9 cg32648-pa
comp109936_c0	0.001	2.954	NA
comp109941_c0	0.010	2.597	---NA---
comp110160_c0	0.002	2.068	cg17664 cg17664-pb
comp110161_c0	0.001	3.175	antennal-enriched xdp-glycosyltransferase
comp110172_c0	0.012	2.056	---NA---
comp110251_c0	0.001	2.129	cg9119 cg9119-pa
comp110258_c0	0.039	2.370	cg6084 cg6084-pa
comp110286_c1	0.011	2.380	equilibrative nucleoside
comp110308_c0	0.001	2.375	PREDICTED: hypothetical protein LOC100570299
comp110336_c0	0.002	2.103	PREDICTED: similar to AGAP002198-PA
comp110381_c0	0.001	7.377	---NA---
comp110446_c0	0.003	3.330	peptidoglycan recognition protein short class (agap006343-pa)
comp110599_c0	0.002	2.048	propionyl- carboxylase alpha mitochondrial precursor (pccase subunit alpha) (propanoyl- :carbon dioxide ligase subunit alpha)
comp110691_c0	0.024	2.247	PREDICTED: similar to GA11424-PA
comp110698_c2	0.001	2.174	---NA---
comp110718_c0	0.001	5.725	alcohol dehydrogenase
comp110734_c0	0.001	2.713	alcohol dehydrogenase
comp110813_c0	0.005	2.193	glucosyl glucuronosyl transferases
comp110885_c0	0.001	2.620	amino acid transporter
comp110906_c0	0.001	23.437	PREDICTED: hypothetical protein

comp110935_c0	0.001	3.993	pancreatic lipase
comp110977_c0	0.018	2.132	lysosomal thiol reductase ip30 precursor
comp110997_c1	0.001	2.505	cg3106 cg3106-pa
comp111069_c0	0.001	2.372	peroxidasin homolog
comp111132_c0	0.001	2.439	PREDICTED: similar to GA12046-PA
comp111231_c1	0.001	2.148	---NA---
comp111255_c0	0.007	2.465	---NA---
comp111326_c0	0.002	2.239	transposable element p transposase (p-element transposase)
comp111372_c0	0.001	2.447	PREDICTED: similar to AGAP002559-PA
comp111513_c0	0.038	2.254	clip domain serine protease
comp111616_c0	0.001	2.759	PREDICTED: similar to predicted protein
comp111631_c0	0.001	2.380	---NA---
comp111641_c0	0.006	3.581	AGAP002387-PA
comp111653_c0	0.001	3.354	PREDICTED: similar to GA13362-PA
comp111660_c0	0.004	2.378	---NA---
comp111665_c0	0.006	2.088	AGAP002799-PA
comp111667_c1	0.001	2.802	cg1213 cg1213-pa
comp111672_c0	0.001	2.971	PREDICTED: similar to AGAP012156-PA
comp111691_c1	0.001	2.775	cytochrome p450 monooxygenase
comp111701_c1	0.003	2.055	cg5044 cg5044-pa
comp111719_c0	0.001	2.280	cationic amino acid transporter
comp111768_c1	0.001	2.298	juvenile hormone-inducible
comp111850_c0	0.001	2.931	isoform a
comp111874_c0	0.001	2.042	beta 1-like 2
comp111910_c0	0.025	2.027	PREDICTED: similar to AGAP006427-PA
comp111916_c1	0.051	2.048	uncharacterized protein LOC662961
comp111937_c0	0.001	3.705	---NA---
comp111971_c0	0.001	2.037	PREDICTED: similar to AGAP001553-PA
comp111980_c0	0.011	2.095	PREDICTED: hypothetical protein LOC100570299
comp111987_c1	0.001	2.145	dimeric dihydrodiol dehydrogenase isoform 1
comp112067_c1	0.001	2.100	cytochrome p450 monooxygenase
comp112077_c0	0.001	2.031	isoform a
comp112104_c0	0.001	2.261	---NA---
comp112120_c0	0.002	2.009	---NA---
comp112148_c0	0.001	3.030	PREDICTED: similar to cystathionine-beta-synthase
comp112200_c0	0.018	2.113	---NA---
comp112222_c0	0.010	2.145	juvenile hormone-inducible
comp112294_c0	0.001	4.511	---NA---
comp112295_c0	0.001	2.069	tpr repeat-containing protein c9orf52
comp112376_c0	0.002	2.017	PREDICTED: hypothetical protein LOC100573156
comp112427_c1	0.001	2.031	PREDICTED: similar to F28G4.5

comp112459_c0	0.001	2.906	ventral nervous system defective
comp112482_c0	0.005	2.831	---NA---
comp112570_c0	0.001	2.138	PREDICTED: similar to chrysoptin
comp112604_c0	0.001	3.249	PREDICTED: similar to GA19585-PA
comp112608_c0	0.001	2.056	antennal-enriched xdp-glycosyltransferase
comp112638_c0	0.001	3.741	---NA---
comp112664_c0	0.005	2.489	---NA---
comp112685_c0	0.001	6.499	AGAP005332-PC
comp112752_c0	0.001	2.116	PREDICTED: similar to AGAP006569-PA
comp112759_c0	0.001	2.492	PREDICTED: similar to AGAP000973-PA
comp112816_c0	0.001	5.786	antennae-rich cytochrome p450
comp112968_c0	0.001	2.625	glucosyl glucuronosyl transferases
comp113028_c1	0.001	2.176	aldo-keto reductase
comp113119_c1	0.002	2.181	PREDICTED: similar to chrysoptin
comp113204_c0	0.009	2.061	steroid dehydrogenase isoform 1
comp113238_c1	0.001	2.352	cathepsin b
comp113270_c0	0.001	2.655	AGAP007074-PA
comp113283_c0	0.005	2.058	PREDICTED: hypothetical protein LOC100573212, partial
comp113360_c0	0.001	3.071	equilibrative nucleoside transporter 1 cg11907-pa
comp113424_c0	0.001	2.794	PREDICTED: hypothetical protein LOC100575395
comp113436_c1	0.001	2.040	PREDICTED: hypothetical protein LOC100570299
comp113475_c0	0.001	2.435	PREDICTED: hypothetical protein
comp113507_c0	0.003	2.046	lipase 3
comp113542_c1	0.001	2.676	scavenger receptor acting in neural tissue and majority of rhodopsin is absent cg12789-pb
comp113584_c0	0.002	2.057	PREDICTED: similar to GA18316-PA
comp113592_c0	0.001	2.974	lysosomal acid lipase
comp113607_c1	0.001	2.034	inebriated protein
comp113625_c1	0.001	3.771	bifunctional protein fold
comp113636_c0	0.001	3.098	glucose dehydrogenase
comp113703_c0	0.001	2.560	---NA---
comp113704_c0	0.001	2.140	beta 1-like 2
comp113764_c0	0.001	2.434	sodium-dependent phosphate transporter
comp113913_c0	0.001	2.302	ankyrin repeat
comp113948_c0	0.001	6.193	PREDICTED: similar to Luciferase
comp113982_c0	0.005	2.046	PREDICTED: hypothetical protein LOC100569635
comp114026_c0	0.001	2.089	glutathione synthetase
comp114049_c1	0.001	2.526	---NA---
comp114076_c0	0.001	2.530	ornithine decarboxylase
comp114081_c0	0.001	3.623	PREDICTED: similar to alpha-esterase
comp114139_c0	0.012	2.192	---NA---

comp114166_c0	0.005	2.234	PREDICTED: similar to AGAP008487-PA
comp114174_c0	0.001	2.211	PREDICTED: similar to 4-nitrophenylphosphatase
comp114295_c0	0.001	2.132	cgmp-dependent protein kinase
comp114343_c0	0.056	2.079	PREDICTED: similar to carboxylesterase
comp114363_c0	0.004	2.772	---NA---
comp114539_c1	0.055	2.171	---NA---
comp114570_c0	0.001	2.381	cg12340 cg12340-pa
comp114596_c0	0.020	3.412	PREDICTED: similar to AGAP002559-PA
comp114629_c0	0.012	2.416	histone-lysine n-methyltransferase setmar-like
comp114643_c0	0.001	2.891	PREDICTED: similar to AGAP009114-PA
comp114658_c0	0.001	7.200	PREDICTED: hypothetical protein
comp114838_c0	0.001	2.406	cad88c cg3389-pa
comp114870_c0	0.001	3.529	PREDICTED: similar to beta-glucosidase
comp114950_c0	0.052	2.003	---NA---
comp114996_c1	0.001	2.064	cg4382 cg4382-pa
comp115030_c0	0.001	3.015	---NA---
comp115076_c0	0.001	2.401	PREDICTED: hypothetical protein
comp115119_c0	0.001	2.290	---NA---
comp115141_c0	0.015	2.300	PREDICTED: similar to AGAP012043-PA
comp115178_c0	0.001	5.170	sodium solute symporter
comp115194_c0	0.001	4.482	sodium solute symporter
comp115223_c0	0.015	2.238	---NA---
comp115261_c0	0.001	2.075	e3 ubiquitin-protein ligase siah2 (seven in absentia homolog 2-like) (siah-2)
comp115479_c0	0.001	2.682	---NA---
comp115507_c0	0.001	3.920	PREDICTED: similar to conserved hypothetical protein
comp115654_c0	0.001	2.512	matrix metalloproteinase
comp115666_c0	0.003	2.328	farnesyl pyrophosphate synthase
comp115689_c0	0.001	2.472	sodium solute symporter
comp115735_c0	0.001	3.236	kaz1-orfb cg1220-pe
comp115775_c0	0.001	2.250	sodium nucleoside cotransporter
comp115842_c1	0.001	2.481	chitinase 2 precursor
comp115848_c0	0.001	2.882	sodium-dependent phosphate transporter
comp115889_c0	0.053	2.334	rna-directed dna polymerase from mobile element jockey-like
comp115891_c0	0.005	2.002	---NA---
comp115908_c0	0.001	2.165	PREDICTED: similar to C25F9.2, partial
comp115954_c0	0.001	2.277	antennal-enriched xdp-glycosyltransferase
comp115994_c0	0.053	2.609	PREDICTED: similar to AGAP004918-PA
comp116046_c1	0.001	2.926	equilibrative nucleoside transporter 1 cg11907-pa
comp116113_c0	0.001	2.397	PREDICTED: similar to AGAP003785-PA

comp116205_c0	0.001	2.036	ankyrin unc44
comp116246_c0	0.001	2.020	PREDICTED: similar to GA10859-PA
comp116262_c0	0.001	3.195	cg1213 cg1213-pa
comp116489_c0	0.001	2.052	chitin synthase 2
comp116495_c1	0.001	2.086	sid-1-related a precursor
comp116505_c0	0.001	2.069	---NA---
comp116539_c1	0.001	2.310	probable galactose-1-phosphate uridylyltransferase-like
comp116612_c0	0.018	2.058	---NA---
comp116616_c0	0.007	2.034	PREDICTED: similar to AGAP012154-PA
comp116671_c0	0.001	2.251	---NA---
comp116743_c1	0.001	2.284	lysosomal alpha-mannosidase (mannosidase alpha class 2b member 1)
comp116803_c0	0.001	3.867	PREDICTED: similar to AGAP008487-PA
comp116806_c2	0.015	2.054	membrane alanyl aminopeptidase
comp116830_c1	0.030	2.361	---NA---
comp116900_c0	0.001	2.120	cgmp-dependent protein kinase
comp116927_c1	0.038	3.190	---NA---
comp116958_c0	0.001	3.200	labial
comp116970_c0	0.016	2.002	forked cg5424-pb
comp117027_c0	0.007	2.044	cg8709 cg8709-pb
comp117070_c0	0.001	2.877	transposable element p transposase (p-element transposase)
comp117074_c0	0.001	2.526	diphosphomevalonate decarboxylase
comp117076_c0	0.001	9.178	glucose dehydrogenase
comp117132_c0	0.001	2.729	PREDICTED: similar to phosphoribosylformylglycinamidine synthase, putative
comp117214_c0	0.001	2.673	reverse transcriptase homolog
comp117241_c0	0.006	2.305	ras-like gtp-binding protein rho1
comp117263_c0	0.050	2.208	---NA---
comp117365_c0	0.001	2.008	npc1 protein
comp117371_c0	0.001	2.433	multi drug resistance 50 cg8523-pa
comp117423_c1	0.001	2.479	amp dependent ligase
comp117438_c0	0.023	2.088	isoform a
comp117469_c0	0.001	2.356	sugar transporter
comp117494_c0	0.046	2.177	laccase 1
comp117534_c0	0.001	2.082	---NA---
comp117547_c0	0.001	2.057	PREDICTED: similar to conserved hypothetical protein
comp117554_c0	0.027	2.037	fatty acid synthase-like
comp117597_c0	0.001	3.503	elongation of very long chain fatty acids protein aael008004-like

comp117622_c0	0.001	2.257	cg7044 cg7044-pa
comp117771_c0	0.001	7.446	---NA---
comp117805_c0	0.001	3.615	---NA---
comp117812_c0	0.034	2.272	kynurenine alpha-aminoadipate aminotransferase mitochondrial precursor (kat) (kynurenine--oxoglutarate transaminase ii) (kynurenine aminotransferase ii) (kynurenine--oxoglutarate aminotransferase ii) (2-aminoadipate transaminase) (
comp117821_c0	0.001	2.068	atp-binding sub-family c (cftr mrp) member 4
comp117866_c0	0.001	2.046	PREDICTED: similar to AGAP006427-PA
comp117882_c0	0.001	2.695	AGAP012173-PA
comp117934_c0	0.001	4.610	xanthine dehydrogenase oxidase
comp118021_c0	0.001	2.119	atp-binding cassette transporter
comp118072_c0	0.001	2.649	xanthine dehydrogenase
comp118086_c0	0.001	2.762	zinc finger bed domain-containing protein 5-like
comp118104_c1	0.001	3.141	3-hydroxy-3-methylglutaryl coenzyme a synthase
comp118166_c0	0.034	2.020	cg41538 cg41538-pa
comp118239_c0	0.001	2.222	tripsin, putative
comp118287_c1	0.012	2.221	AGAP012074-PA
comp118327_c0	0.016	2.268	agap008849-pa isoform 1
comp118337_c0	0.035	2.481	hemoglobin c1 polymer
comp118465_c0	0.001	2.435	PREDICTED: similar to beta-glucosidase
comp118468_c0	0.001	2.200	PREDICTED: similar to AGAP005839-PA
comp118479_c0	0.001	2.256	xanthine dehydrogenase
comp118549_c0	0.002	2.165	---NA---
comp118656_c0	0.001	2.076	cg31116 cg31116-pe
comp118737_c0	0.021	2.392	fatty acid
comp118743_c0	0.001	2.331	fatty acid synthase
comp118800_c0	0.002	4.100	agap002830-pa isoform 5
comp118846_c0	0.010	2.360	glycosyltransferase 25 family member
comp118914_c0	0.002	2.329	PREDICTED: similar to polyprotein
comp118999_c0	0.001	2.762	PREDICTED: similar to AGAP006427-PA
comp119092_c0	0.057	2.084	oxidase peroxidase
comp119311_c1	0.001	2.099	PREDICTED: similar to AGAP012156-PA
comp119434_c0	0.030	2.371	PREDICTED: hypothetical protein LOC100573963
comp119437_c0	0.002	2.052	AGAP004533-PA
comp119500_c0	0.017	2.770	---NA---
comp119526_c0	0.009	2.040	muscle-specific protein 300 cg33715-pd
comp119545_c0	0.001	2.360	glucosyl glucuronosyl transferases
comp119560_c1	0.030	2.051	sugar transporter
comp119617_c1	0.032	2.053	juvenile hormone-inducible
comp119690_c0	0.001	2.915	---NA---

comp119777_c0	0.003	2.180	cathepsin d isoform 1
comp119857_c0	0.051	3.065	xbiquitin-conjugating enzyme e2-17 kda (xbiquitin-protein ligase) (xbiquitin carrier protein) (protein effete)
comp119907_c0	0.016	3.255	---NA---
comp119921_c0	0.007	5.425	---NA---
comp119922_c0	0.031	3.110	---NA---
comp119955_c0	0.013	4.895	---NA---
comp120081_c0	0.032	4.448	---NA---
comp120251_c0	0.031	2.400	---NA---
comp46473_c0	0.046	2.375	adenine nucleotide translocase isoform a
comp46590_c0	0.001	2.235	---NA---
comp57556_c0	0.047	2.180	---NA---
comp61890_c0	0.042	3.395	AGAP000462-PA
comp62119_c0	0.059	2.690	---NA---
comp62480_c0	0.006	5.220	---NA---
comp63542_c0	0.032	3.640	---NA---
comp63545_c0	0.001	3.190	elongation factor isoform a
comp65111_c0	0.006	2.695	PREDICTED: similar to putative esterase
comp65689_c0	0.043	2.240	---NA---
comp66456_c0	0.006	2.845	AGAP010885-PA
comp67283_c0	0.031	4.580	AGAP009623-PA
comp68119_c0	0.058	2.100	---NA---
comp68160_c0	0.034	2.930	cg6084 cg6084-pa
comp79274_c0	0.010	3.955	PREDICTED: similar to AGAP003584-PA
comp80300_c0	0.051	3.210	---NA---
comp80468_c0	0.006	7.450	tripartite motif-containing protein 2-like
comp81149_c0	0.028	2.860	cytochrome p450 isoform 1
comp82505_c0	0.041	2.020	isoform a
comp82707_c0	0.008	4.660	---NA---
comp85114_c0	0.048	3.030	PREDICTED: hypothetical protein LOC100166108 isoform 3
comp85230_c0	0.001	14.250	---NA---
comp87864_c0	0.017	2.178	calcium-transporting atpase type 2c
comp88149_c0	0.006	5.040	---NA---
comp88335_c0	0.006	8.220	small heat shock protein 21 isoform 1
comp88374_c0	0.048	3.567	---NA---
comp89182_c0	0.001	3.049	---NA---
comp91024_c0	0.025	3.530	---NA---
comp92340_c0	0.006	2.805	2-hydroxyphytanoyl-CoA lyase, putative
comp92480_c0	0.031	3.950	superoxide dismutase 2
comp93486_c0	0.009	3.695	isoform e

comp93921_c0	0.006	6.235	lethal essential for life l2efl
comp94687_c0	0.009	5.442	---NA---
comp94693_c0	0.006	5.155	Catalase, putative
comp95046_c0	0.006	3.810	AGAP006958-PA
comp95711_c0	0.001	4.987	---NA---
comp95983_c0	0.001	7.315	PREDICTED: hypothetical protein
comp96315_c0	0.039	3.531	AGAP002599-PA
comp96596_c0	0.050	2.425	---NA---
comp97310_c0	0.002	6.450	heat shock 70 kda protein
comp97809_c0	0.051	4.040	---NA---
comp97937_c0	0.011	2.535	AGAP004936-PA
comp98499_c0	0.059	2.045	---NA---
comp99067_c0	0.001	3.568	---NA---
comp99795_c0	0.023	2.030	pro-phenol oxidase subunit 2
comp99864_c0	0.058	2.162	13 kda hemolymph protein a
comp99927_c0	0.001	7.737	---NA---

S2Table: Up-regulated components in the Systemic-3 population determined by a fold change of greater than 2 and a FDR of less than 0.059.

Components	FDR	Fold Change	Seq. Description
comp100068_c0	0.001	3.304	---NA---
comp100083_c0	0.001	4.260	CG32603
comp100494_c0	0.001	4.684	immune-related hdd11
comp100635_c0	0.008	2.370	PREDICTED: hypothetical protein
comp100776_c0	0.001	6.073	---NA---
comp100892_c0	0.019	3.983	---NA---
comp100923_c0	0.001	2.946	PREDICTED: similar to alpha-esterase
comp101002_c0	0.057	2.070	---NA---
comp101214_c0	0.001	3.252	---NA---
comp101314_c0	0.001	2.580	PREDICTED: similar to AGAP007667-PA
comp101651_c0	0.003	2.677	---NA---
comp101692_c0	0.001	2.981	pupal cuticle
comp101721_c0	0.001	4.930	allergen aca s 13
comp101742_c0	0.056	2.484	---NA---
comp101816_c0	0.001	2.535	juvenile hormone-inducible
comp101926_c0	0.005	2.542	---NA---
comp101927_c0	0.001	2.466	---NA---
comp101938_c0	0.002	2.447	lysosomal thiol reductase ip30 precursor
comp101939_c0	0.001	2.309	---NA---
comp101978_c0	0.001	4.332	25d cg6514-pa
comp101995_c0	0.001	4.813	---NA---
comp102065_c0	0.034	2.235	cg31760 cg31760-pa
comp102070_c0	0.039	2.543	---NA---
comp102166_c0	0.001	3.314	---NA---
comp102181_c0	0.001	4.701	---NA---
comp102186_c0	0.025	2.151	AGAP009328-PA
comp102265_c1	0.001	13.051	---NA---
comp102272_c0	0.024	2.280	AGAP011412-PA
comp102291_c0	0.001	8.210	---NA---
comp102305_c0	0.007	2.606	cg9090 cg9090-pa
comp102387_c0	0.035	2.125	---NA---
comp102424_c0	0.001	3.294	translocator protein-like
comp102425_c0	0.001	2.950	---NA---
comp102476_c0	0.001	9.864	---NA---
comp102545_c0	0.001	2.252	PREDICTED: similar to conserved hypothetical protein
comp102636_c0	0.006	2.629	pheromone binding protein
comp102657_c0	0.010	2.345	PREDICTED: similar to AGAP006424-PA
comp102733_c0	0.010	2.092	AGAP001409-PA
comp102743_c0	0.058	2.233	---NA---

comp102762_c0	0.034	2.110	---NA---
comp102853_c0	0.001	3.005	PREDICTED: similar to 3-oxoacyl-
comp102935_c0	0.028	3.623	---NA---
comp102949_c0	0.020	2.175	cg34184 cg34184-pa
comp103172_c0	0.001	2.549	---NA---
comp103236_c0	0.001	4.159	AGAP007368-PA
comp103269_c0	0.001	2.437	---NA---
comp103293_c0	0.033	2.363	---NA---
comp103300_c0	0.043	2.522	---NA---
comp103326_c0	0.001	5.995	chemosensory protein 19 precursor
comp103377_c0	0.001	3.275	chemosensory protein 1 precursor
comp103527_c0	0.010	2.271	cral trio domain-containing protein
comp103555_c0	0.012	2.660	---NA---
comp103556_c0	0.002	6.068	cg9427 cg9427-pa
comp103564_c0	0.001	3.489	cuticle protein cp5
comp103650_c0	0.001	6.988	defensin 1
comp103658_c0	0.001	2.340	cytochrome p450 9z4
comp103662_c0	0.001	2.498	AGAP010929-PA
comp103663_c0	0.001	2.588	PREDICTED: similar to AGAP012342-PA
comp103671_c0	0.001	2.530	PREDICTED: similar to alpha-esterase
comp103717_c0	0.001	6.594	---NA---
comp103824_c0	0.040	2.810	---NA---
comp103893_c0	0.003	2.250	---NA---
comp103910_c0	0.001	14.484	---NA---
comp103926_c0	0.001	3.171	CG12947
comp103954_c0	0.031	2.105	AGAP011167-PA
comp103967_c0	0.001	2.086	PREDICTED: similar to AGAP001449-PA
comp104036_c0	0.001	4.070	---NA---
comp104056_c0	0.012	2.077	methyltransferase 1
comp104142_c0	0.004	2.033	---NA---
comp104213_c0	0.001	2.641	cg2837 cg2837-pd
comp104218_c0	0.001	2.445	---NA---
comp104411_c0	0.002	2.400	29-kda galactose-binding lectin
comp104425_c0	0.001	2.790	cytochrome p450 cyp9z1
comp104506_c0	0.001	7.893	---NA---
comp104517_c0	0.052	2.370	antennal-enriched xdp-glycosyltransferase
comp104533_c0	0.001	4.664	acid phosphatase 1
comp104559_c0	0.007	3.595	atp-dependent dna helicase pif1-like
comp104596_c1	0.024	3.143	---NA---
comp104679_c0	0.003	2.523	PREDICTED: hypothetical protein LOC100163076
comp104714_c1	0.005	2.699	---NA---

comp104719_c2	0.002	2.229	---NA---
comp104806_c0	0.001	2.571	PREDICTED: similar to conserved hypothetical protein
comp104861_c0	0.001	2.916	---NA---
comp104933_c0	0.001	3.560	glucose dehydrogenase
comp104984_c0	0.001	23.172	allergen bla g
comp105013_c0	0.001	4.171	---NA---
comp105039_c1	0.001	3.717	cathepsin l-like protein cysteine proteinase
comp105058_c0	0.001	3.335	---NA---
comp105087_c0	0.014	2.121	protein takeout-like
comp105133_c0	0.010	2.041	---NA---
comp105180_c0	0.017	2.230	---NA---
comp105213_c0	0.006	2.551	---NA---
comp105223_c0	0.001	2.292	serine protease 2
comp105250_c0	0.001	3.505	---NA---
comp105273_c0	0.001	2.981	cg9427 cg9427-pa
comp105274_c0	0.001	10.018	cg30101 cg30101-pa
comp105406_c0	0.007	2.245	PREDICTED: similar to AGAP011167-PA
comp105412_c0	0.001	3.775	AGAP000697-PB
comp105434_c0	0.001	2.240	juvenile hormone-inducible
comp105537_c2	0.001	2.333	---NA---
comp105555_c0	0.051	2.301	PREDICTED: hypothetical protein LOC100575767
comp105589_c0	0.001	4.511	resilin precursor
comp105656_c0	0.001	3.109	protein takeout
comp105686_c0	0.003	2.085	sodium-dependent phosphate transporter
comp105691_c0	0.035	2.262	oxidase peroxidase
comp105731_c1	0.001	2.675	cg7675 cg7675-pb
comp105797_c1	0.045	2.367	---NA---
comp105859_c0	0.002	2.812	AGAP007368-PA
comp105889_c0	0.001	6.484	cuticular protein 92f cg5494-pa
comp105908_c0	0.025	2.014	---NA---
comp105972_c0	0.026	2.450	---NA---
comp105989_c0	0.001	3.066	AGAP011167-PA
comp106035_c1	0.001	8.811	tryptophan -dioxygenase
comp106072_c0	0.001	2.237	cytochrome p450 9z4
comp106188_c0	0.001	4.339	cytochrome p450 cyp18a1
comp106193_c0	0.001	2.979	PREDICTED: similar to AGAP002559-PA
comp106220_c0	0.001	10.482	cg6870 cg6870-pa
comp106242_c0	0.001	2.431	PREDICTED: similar to AGAP001552-PA
comp106244_c0	0.001	5.616	12 kda hemolymph protein b
comp106245_c0	0.059	2.682	valyl-trna synthetase
comp106259_c0	0.021	3.186	---NA---

comp106283_c0	0.001	2.066	CG17571
comp106293_c0	0.002	2.055	PREDICTED: similar to conserved hypothetical protein
comp106395_c0	0.003	2.431	uncharacterized protein LOC662961
comp106483_c0	0.001	4.137	---NA---
comp106539_c0	0.001	2.639	anterior fat body protein
comp106543_c1	0.001	2.413	poils au dos
comp106548_c0	0.001	2.771	xanthine dehydrogenase
comp106611_c0	0.001	3.663	PREDICTED: similar to AGAP002557-PA
comp106668_c0	0.001	2.347	cg6084 cg6084-pa
comp106677_c0	0.008	2.478	AGAP011630-PA
comp106688_c2	0.040	2.146	---NA---
comp106704_c0	0.001	2.342	PREDICTED: similar to predicted protein
comp106740_c1	0.002	2.386	---NA---
comp106758_c0	0.001	2.747	cuticular protein rr-1 family (agap009876-pa)
comp106772_c1	0.001	4.530	---NA---
comp106810_c0	0.001	4.594	cg13024 cg13024-pa
comp106832_c0	0.001	2.782	PREDICTED: similar to AGAP001553-PA
comp106834_c0	0.001	2.270	AGAP003206-PB
comp106883_c0	0.001	2.360	equilibrative nucleoside transporter 1 cg11907-pa
comp106947_c0	0.001	2.057	---NA---
comp106953_c1	0.001	4.347	---NA---
comp107026_c0	0.001	6.918	---NA---
comp107049_c0	0.001	2.189	chitin deacetylase 4 precursor
comp107059_c0	0.005	7.422	---NA---
comp107072_c0	0.001	3.914	---NA---
comp107080_c0	0.001	3.412	---NA---
comp107106_c0	0.001	2.115	cathepsin b precursor
comp107125_c0	0.001	2.312	conserved hypothetical protein
comp107160_c0	0.023	2.543	---NA---
comp107182_c0	0.001	3.087	zinc-containing alcohol dehydrogenase
comp107230_c0	0.001	2.173	---NA---
comp107286_c0	0.001	2.272	---NA---
comp107290_c0	0.001	4.174	PREDICTED: hypothetical protein LOC100165870
comp107294_c0	0.001	2.089	PREDICTED: hypothetical protein
comp107322_c0	0.001	2.387	hemoglobin c1 polymer
comp107323_c0	0.008	2.566	allergen aca s 13
comp107326_c0	0.001	2.541	---NA---
comp107348_c0	0.001	3.400	glucose dehydrogenase
comp107354_c1	0.011	2.015	ecdysteroid regulated 16 kda
comp107401_c0	0.026	2.456	---NA---
comp107418_c0	0.001	5.426	cuticle protein precursor

comp107435_c0	0.001	9.305	PREDICTED: hypothetical protein
comp107462_c1	0.004	2.314	---NA---
comp107494_c1	0.001	2.390	retinol dehydrogenase 11
comp107507_c0	0.001	2.397	cg1673 cg1673-pa
comp107534_c2	0.001	2.193	alcohol dehydrogenase
comp107589_c0	0.001	2.267	transmembrane protein 195
comp107656_c0	0.001	14.979	---NA---
comp107703_c0	0.001	4.532	cg34115 cg34115-pa
comp107706_c0	0.001	3.185	PREDICTED: similar to AGAP005839-PA
comp107777_c0	0.017	2.317	---NA---
comp107779_c0	0.001	8.553	---NA---
comp107865_c0	0.001	2.268	aldo-keto reductase
comp107899_c0	0.001	2.063	ribosomal protein l36e
comp107917_c0	0.002	2.102	PREDICTED: similar to AGAP008125-PA
comp107926_c0	0.001	3.397	---NA---
comp107960_c0	0.001	3.519	cg8927 cg8927-pa
comp107984_c0	0.017	2.383	---NA---
comp108102_c0	0.001	5.555	cytochrome p450
comp108174_c0	0.022	2.057	---NA---
comp108181_c0	0.019	2.037	---NA---
comp108220_c1	0.001	2.423	tyrosine-protein kinase
comp108225_c0	0.014	2.305	conserved hypothetical protein
comp108241_c0	0.001	4.197	PREDICTED: similar to AGAP003785-PA
comp108251_c0	0.001	15.79	PREDICTED: hypothetical protein LOC100571634
comp108257_c1	0.001	2.647	luciferin-regenerating enzyme
comp108273_c1	0.001	2.035	---NA---
comp108285_c0	0.001	2.280	yellow-c precursor
comp108290_c1	0.001	2.127	low density lipoprotein receptor adapter protein 1 (autosomal recessive hypercholesterolemia protein) isoform 1
comp108325_c0	0.058	2.000	---NA---
comp108333_c0	0.001	5.926	cuticular protein 49aa cg30045-pb
comp108385_c0	0.001	2.342	---NA---
comp108398_c0	0.013	2.426	cuticular protein 47ef cg13214-pa
comp108416_c0	0.008	2.138	---NA---
comp108460_c0	0.010	2.019	PREDICTED: similar to AGAP001652-PA
comp108539_c0	0.058	2.243	cathepsin b-like cysteine proteinase-like
comp108640_c0	0.001	2.300	---NA---
comp108656_c0	0.001	3.588	AGAP002583-PA
comp108663_c0	0.001	2.492	cg6178 cg6178-pa isoform 1
comp108669_c0	0.001	2.389	cuticular protein analogous to peritrophins 3-b precursor

comp108734_c0	0.010	2.105	PREDICTED: hypothetical protein LOC100575723, partial
comp108777_c0	0.001	2.281	acid phosphatase-1
comp108825_c1	0.037	2.565	---NA---
comp108826_c0	0.032	2.142	peritrophic matrix protein 3 precursor
comp108857_c0	0.023	2.337	kal-1 protein
comp108882_c1	0.001	2.708	---NA---
comp108902_c0	0.001	2.644	PREDICTED: similar to AGAP001894-PA
comp108962_c1	0.001	2.615	---NA---
comp108997_c1	0.001	2.385	atp-binding cassette transporter
comp109009_c0	0.038	2.887	---NA---
comp109050_c0	0.001	3.003	---NA---
comp109087_c0	0.001	2.661	tan cg12120-pa
comp109117_c0	0.001	2.316	alcohol dehydrogenase
comp109180_c0	0.017	2.275	reverse transcriptase, putative
comp109194_c0	0.008	2.061	isoform a
comp109227_c0	0.001	3.974	PREDICTED: similar to GA14281-PA
comp109230_c0	0.001	3.124	AGAP012988-PA
comp109270_c0	0.001	2.305	prophenoloxidase activating factor
comp109291_c0	0.001	2.413	PREDICTED: similar to four-jointed protein, putative
comp109356_c0	0.001	6.731	eukaryotic translation initiation factor 4e binding protein
comp109406_c0	0.002	3.356	---NA---
comp109418_c1	0.001	2.559	cytochrome p450 monooxygenase
comp109449_c0	0.001	2.015	---NA---
comp109498_c0	0.001	2.713	---NA---
comp109522_c0	0.001	3.706	PREDICTED: similar to GA14040-PA
comp109529_c0	0.001	2.577	sodium-dependent phosphate transporter
comp109623_c0	0.004	3.966	PREDICTED: hypothetical protein
comp109630_c0	0.004	2.109	PREDICTED: hypothetical protein
comp109633_c0	0.002	2.233	PREDICTED: similar to putative esterase
comp109639_c0	0.001	2.517	sodium-dependent phosphate transporter
comp109645_c0	0.001	2.695	cytochrome p450
comp109709_c1	0.001	5.467	cuticular protein
comp109774_c0	0.001	4.200	PREDICTED: hypothetical protein LOC100574204
comp109791_c1	0.001	4.851	uncharacterized protein LOC662961
comp109841_c0	0.001	2.149	---NA---
comp109849_c0	0.001	2.404	cg14275 cg14275-pa
comp109878_c0	0.001	2.382	phosphodiesterase 9 cg32648-pa
comp109936_c0	0.001	8.132	---NA---
comp109975_c1	0.001	2.739	phosphatidylinositol transfer protein
comp109993_c0	0.001	2.173	PREDICTED: similar to GA18075-PA

comp110056_c0	0.001	4.191	tyrosine hydroxylase
comp110071_c0	0.021	2.090	cg7675 cg7675-pb
comp110160_c0	0.001	2.685	cg17664 cg17664-pb
comp110161_c0	0.001	3.260	antennal-enriched xdp-glycosyltransferase
comp110229_c0	0.009	2.575	---NA---
comp110251_c0	0.001	2.544	cg9119 cg9119-pa
comp110258_c0	0.001	3.682	cg6084 cg6084-pa
comp110286_c1	0.001	3.559	equilibrative nucleoside
comp110295_c0	0.001	2.906	cuticular protein ld-cp3
comp110333_c2	0.001	2.423	PREDICTED: similar to AGAP011736-PA
comp110336_c0	0.001	2.171	PREDICTED: similar to AGAP002198-PA
comp110381_c0	0.002	3.000	---NA---
comp110386_c0	0.001	2.663	---NA---
comp110444_c0	0.007	2.385	probable c-5 sterol desaturase-like
comp110568_c0	0.001	2.221	cuticle protein cp5
comp110572_c0	0.001	5.599	cuticular protein precursor
comp110592_c0	0.001	4.551	PREDICTED: similar to copia-type polyprotein, putative
comp110599_c0	0.001	2.241	propionyl- carboxylase alpha mitochondrial precursor (pccase subunit alpha) (propanoyl- :carbon dioxide ligase subunit alpha)
comp110625_c0	0.001	2.241	---NA---
comp110686_c0	0.023	2.152	carrier protein
comp110718_c0	0.001	8.646	alcohol dehydrogenase
comp110766_c0	0.013	2.083	---NA---
comp110802_c1	0.024	2.394	endonuclease and reverse transcriptase-like protein
comp110813_c0	0.001	2.745	glucosyl glucuronosyl transferases
comp110852_c0	0.001	2.159	PREDICTED: similar to AGAP008839-PA
comp110885_c0	0.001	3.478	amino acid transporter
comp110906_c0	0.001	8.107	PREDICTED: hypothetical protein
comp110912_c0	0.004	2.212	cytochrome p450 monooxigenase cyp4q3
comp110933_c0	0.001	2.889	AGAP013476-PA
comp110935_c0	0.001	14.210	pancreatic lipase
comp110977_c0	0.001	2.629	lysosomal thiol reductase ip30 precursor
comp110990_c0	0.014	2.068	PREDICTED: similar to AGAP010241-PA
comp110991_c0	0.001	2.680	AGAP001009-PA
comp110997_c1	0.001	2.715	cg3106 cg3106-pa
comp111132_c0	0.001	2.320	PREDICTED: similar to GA12046-PA
comp111171_c0	0.042	2.160	chitinase 3
comp111182_c1	0.001	3.673	copper-zinc superoxide dismutase
comp111205_c3	0.030	2.142	---NA---
comp111326_c0	0.001	3.533	transposable element p transposase (p-element

			transposase)
comp111372_c0	0.001	3.052	PREDICTED: similar to AGAP002559-PA
comp111380_c0	0.002	2.033	PREDICTED: similar to Hibadhb
comp111467_c0	0.001	2.118	cuticular protein analogous to peritrophins 3-a1 precursor
comp111553_c1	0.047	2.410	---NA---
comp111560_c0	0.001	2.058	cuticular precursor
comp111573_c0	0.001	2.403	---NA---
comp111590_c0	0.001	3.088	cg3625 cg3625-pc
comp111616_c0	0.001	2.952	PREDICTED: similar to predicted protein
comp111617_c0	0.001	2.825	---NA---
comp111631_c0	0.001	2.772	---NA---
comp111641_c0	0.041	2.760	AGAP002387-PA
comp111653_c0	0.001	4.176	PREDICTED: similar to GA13362-PA
comp111660_c0	0.007	2.316	---NA---
comp111667_c1	0.001	2.825	cg1213 cg1213-pa
comp111672_c0	0.001	2.660	PREDICTED: similar to AGAP012156-PA
comp111691_c1	0.001	3.253	cytochrome p450 monooxygenase
comp111701_c1	0.001	2.214	cg5044 cg5044-pa
comp111719_c0	0.001	3.256	cationic amino acid transporter
comp111753_c0	0.001	2.522	PREDICTED: similar to conserved hypothetical protein
comp111768_c1	0.001	3.036	juvenile hormone-inducible
comp111824_c0	0.001	2.608	dimethylaniline monooxygenase
comp111867_c0	0.001	4.083	---NA---
comp111874_c0	0.001	2.113	beta 1-like 2
comp111900_c0	0.001	2.123	monoacylglycerol lipase abhd12-like isoform 1
comp111910_c0	0.004	2.210	PREDICTED: similar to AGAP006427-PA
comp111912_c0	0.001	3.577	cytochrome p450 9z4
comp111916_c1	0.001	2.822	uncharacterized protein LOC662961
comp111951_c0	0.001	2.209	---NA---
comp111971_c0	0.001	2.195	PREDICTED: similar to AGAP001553-PA
comp111980_c0	0.001	3.758	PREDICTED: hypothetical protein LOC100570299
comp111989_c0	0.001	2.159	ribosomal protein l4e
comp112010_c0	0.001	2.021	cg15786 cg15786-pa
comp112148_c0	0.001	3.299	PREDICTED: similar to cystathionine-beta-synthase
comp112222_c0	0.001	2.980	juvenile hormone-inducible
comp112295_c0	0.001	3.378	tpr repeat-containing protein c9orf52
comp112419_c0	0.001	5.354	PREDICTED: hypothetical protein
comp112427_c1	0.001	2.731	PREDICTED: similar to F28G4.5
comp112459_c0	0.001	3.604	ventral nervous system defective
comp112465_c0	0.001	2.687	phenylalanine hydroxylase
comp112482_c0	0.001	12.77	---NA---

comp112509_c0	0.003	2.158	---NA---
comp112538_c1	0.049	2.586	thymus-specific serine protease
comp112604_c0	0.001	4.063	PREDICTED: similar to GA19585-PA
comp112622_c0	0.001	4.079	PREDICTED: similar to AGAP005515-PA
comp112640_c0	0.001	2.189	xdp-n-acetylglucosamine pyrophosphorylase 1
comp112652_c0	0.001	4.071	agap000696-pa isoform 1
comp112658_c0	0.003	2.142	---NA---
comp112685_c0	0.001	5.663	AGAP005332-PC
comp112720_c0	0.001	2.458	seven in absentia 1b
comp112723_c0	0.001	2.472	argininosuccinate synthetase
comp112725_c0	0.001	2.063	cg6847 cg6847-pa
comp112737_c0	0.001	2.067	PREDICTED: similar to AGAP010734-PA
comp112759_c0	0.001	3.163	PREDICTED: similar to AGAP000973-PA
comp112915_c0	0.001	2.040	antennal-enriched xdp-glycosyltransferase
comp112968_c0	0.001	2.540	glucosyl glucuronosyl transferases
comp113028_c1	0.001	2.497	aldo-keto reductase
comp113083_c0	0.001	3.186	---NA---
comp113105_c0	0.001	2.939	---NA---
comp113157_c0	0.013	2.331	---NA---
comp113238_c1	0.001	2.452	cathepsin b
comp113270_c0	0.001	2.065	AGAP007074-PA
comp113327_c2	0.001	2.672	tyrosine aminotransferase
comp113360_c0	0.001	2.448	equilibrative nucleoside transporter 1 cg11907-pa
comp113362_c0	0.001	2.409	cg3999 cg3999-pa
comp113364_c0	0.001	4.744	PREDICTED: similar to AGAP003782-PA
comp113369_c0	0.001	2.104	sugar transporter
comp113433_c1	0.001	2.177	conserved hypothetical protein
comp113458_c0	0.001	2.889	PREDICTED: similar to conserved hypothetical protein
comp113504_c0	0.001	2.674	---NA---
comp113507_c0	0.001	2.182	lipase 3
comp113528_c0	0.019	2.228	---NA---
comp113542_c1	0.001	2.539	scavenger receptor acting in neural tissue and majority of rhodopsin is absent cg12789-pb
comp113545_c0	0.001	2.015	PREDICTED: similar to AGAP004793-PA
comp113584_c0	0.001	2.259	PREDICTED: similar to GA18316-PA
comp113592_c0	0.001	3.077	lysosomal acid lipase
comp113595_c0	0.001	2.010	abc transporter
comp113607_c1	0.001	2.102	inebriated protein
comp113610_c0	0.001	2.503	PREDICTED: hypothetical protein LOC100575357
comp113625_c1	0.001	7.566	bifunctional protein fold
comp113682_c0	0.001	2.385	choline ethanolamine kinase
comp113703_c0	0.001	2.318	---NA---

comp113705_c0	0.001	2.319	---NA---
comp113764_c0	0.001	2.443	sodium-dependent phosphate transporter
comp113845_c2	0.001	2.778	kruppel-homolog 1
comp113862_c2	0.057	2.050	---NA---
comp113948_c0	0.001	4.702	PREDICTED: similar to Luciferase
comp113982_c0	0.005	2.017	PREDICTED: hypothetical protein LOC100569635
comp114026_c0	0.001	2.669	glutathione synthetase
comp114040_c0	0.007	2.345	---NA---
comp114049_c0	0.010	2.477	PREDICTED: hypothetical protein LOC100570299
comp114062_c0	0.036	2.000	timeless isoform b
comp114076_c0	0.001	2.224	ornithine decarboxylase
comp114081_c0	0.001	2.722	PREDICTED: similar to alpha-esterase
comp114159_c0	0.001	2.611	---NA---
comp114163_c1	0.001	2.574	pxphosphoserine phosphatase
comp114166_c0	0.001	2.304	PREDICTED: similar to AGAP008487-PA
comp114174_c0	0.001	2.251	PREDICTED: similar to 4-nitrophenylphosphatase
comp114247_c0	0.001	2.029	PREDICTED: similar to alpha-esterase
comp114328_c0	0.001	3.721	cg32645 cg32645-pb
comp114330_c0	0.001	2.738	arylsulfatase b
comp114338_c0	0.001	4.968	---NA---
comp114343_c0	0.014	2.257	PREDICTED: similar to carboxylesterase
comp114346_c0	0.001	2.445	cytochrome p450
comp114347_c0	0.001	2.186	beta-n-acetylglucosaminidase nag3 precursor
comp114363_c0	0.001	2.951	---NA---
comp114384_c0	0.001	2.014	PREDICTED: similar to AGAP006427-PA
comp114489_c0	0.036	2.369	---NA---
comp114525_c2	0.001	2.976	PREDICTED: hypothetical protein LOC100573898
comp114565_c1	0.001	3.448	PREDICTED: similar to F28G4.5
comp114570_c0	0.001	2.084	cg12340 cg12340-pa
comp114639_c0	0.001	2.287	serine proteinase
comp114643_c0	0.001	2.888	PREDICTED: similar to AGAP009114-PA
comp114684_c0	0.001	2.254	zinc finger protein 77
comp114697_c0	0.001	2.467	uncharacterized protein LOC663557
comp114714_c0	0.001	3.032	PREDICTED: similar to carboxylesterase
comp114774_c3	0.001	2.568	PREDICTED: similar to trehalase
comp114782_c0	0.001	2.332	uncharacterized protein LOC662961
comp114842_c0	0.001	2.903	knickkopf cg6217- partial
comp114861_c0	0.001	2.397	---NA---
comp114870_c0	0.001	3.201	PREDICTED: similar to beta-glucosidase
comp114950_c0	0.014	2.106	saposin- isoform a
comp115026_c0	0.032	3.014	---NA---

comp115030_c0	0.001	3.958	---NA---
comp115076_c0	0.001	2.135	PREDICTED: hypothetical protein
comp115119_c0	0.001	2.623	---NA---
comp115127_c0	0.001	2.231	xanthine dehydrogenase
comp115141_c0	0.014	2.230	PREDICTED: similar to AGAP012043-PA
comp115178_c0	0.001	3.141	sodium solute symporter
comp115194_c0	0.001	2.833	sodium solute symporter
comp115223_c0	0.001	2.967	---NA---
comp115233_c0	0.001	2.019	PREDICTED: similar to AGAP010534-PA
comp115284_c0	0.057	2.244	---NA---
comp115309_c0	0.020	3.509	cytochrome p450 9z4
comp115600_c0	0.001	2.066	conserved hypothetical protein
comp115654_c0	0.001	5.789	matrix metalloproteinase
comp115775_c0	0.001	2.210	sodium nucleoside cotransporter
comp115807_c0	0.001	3.375	PREDICTED: similar to AGAP004961-PA
comp115838_c0	0.005	2.159	---NA---
comp115842_c1	0.001	2.341	chitinase 2 precursor
comp115889_c0	0.001	3.223	rna-directed dna polymerase from mobile element jockey-like
comp115924_c0	0.001	2.429	chitin synthase 1
comp115994_c0	0.001	3.688	PREDICTED: similar to AGAP004918-PA
comp116046_c1	0.001	4.351	equilibrative nucleoside transporter 1 cg11907-pa
comp116104_c1	0.001	2.417	acid phosphatase 1
comp116113_c0	0.001	2.462	PREDICTED: similar to AGAP003785-PA
comp116153_c0	0.001	2.344	cuticular protein analogous to peritrophins 1-h precursor
comp116178_c0	0.001	3.317	hypothetical protein Phum_PHXM433170
comp116218_c1	0.001	2.576	cuticular protein analogous to peritrophins 3-c5 isoform 2 precursor
comp116262_c0	0.005	2.111	cg1213 cg1213-pa
comp116267_c0	0.011	2.867	PREDICTED: similar to alpha-esterase
comp116310_c0	0.001	3.069	cuticular protein analogous to peritrophins 3-a2 precursor
comp116431_c0	0.001	2.248	AGAP009200-PA
comp116539_c1	0.001	2.677	probable galactose-1-phosphate uridylyltransferase-like
comp116551_c0	0.001	2.516	---NA---
comp116560_c0	0.001	2.450	cytochrome p450
comp116625_c0	0.001	2.067	PREDICTED: similar to conserved hypothetical protein
comp116671_c0	0.001	2.503	---NA---
comp116743_c1	0.001	3.198	lysosomal alpha-mannosidase (mannosidase alpha class 2b member 1)
comp116755_c1	0.001	2.252	collagen alpha-2 chain
comp116782_c0	0.001	2.928	glucose dehydrogenase

comp116790_c0	0.001	2.009	AGAP005223-PA
comp116803_c0	0.001	4.242	PREDICTED: similar to AGAP008487-PA
comp116810_c0	0.002	2.118	PREDICTED: similar to GA20668-PA
comp116830_c1	0.047	2.181	---NA---
comp116842_c0	0.001	2.167	---NA---
comp116927_c1	0.007	3.553	---NA---
comp116958_c0	0.001	3.178	labial
comp116970_c0	0.001	5.788	forked cg5424-pb
comp117027_c0	0.001	3.114	cg8709 cg8709-pb
comp117070_c0	0.001	3.860	transposable element p transposase (p-element transposase)
comp117081_c0	0.053	6.079	---NA---
comp117090_c0	0.009	2.360	---NA---
comp117095_c0	0.037	2.105	cg10440 cg10440-pa
comp117132_c0	0.001	2.959	PREDICTED: similar to phosphoribosylformylglycinamidine synthase, putative
comp117214_c0	0.002	2.044	reverse transcriptase homolog
comp117237_c0	0.001	3.043	---NA---
comp117241_c0	0.031	2.056	ras-like gtp-binding protein rho1
comp117260_c0	0.001	5.618	AGAP013007-PA
comp117266_c0	0.001	2.512	cuticular protein analogous to peritrophins 3-d1 precursor
comp117328_c0	0.001	2.946	---NA---
comp117364_c0	0.001	2.297	PREDICTED: hypothetical protein
comp117371_c0	0.001	2.189	multi drug resistance 50 cg8523-pa
comp117423_c0	0.001	2.157	amp dependent ligase
comp117463_c1	0.001	3.954	---NA---
comp117469_c0	0.001	2.836	sugar transporter
comp117538_c0	0.001	2.211	atp-binding cassette transporter
comp117567_c0	0.001	3.008	myc proto-oncogene
comp117573_c0	0.001	4.112	CG11409
comp117595_c0	0.001	3.674	cad88c cg3389-pa
comp117597_c0	0.001	2.129	elongation of very long chain fatty acids protein aael008004-like
comp117605_c0	0.001	2.028	sphingomyelin phosphodiesterase
comp117622_c0	0.001	2.547	cg7044 cg7044-pa
comp117632_c0	0.001	2.306	fibrillin 2
comp117706_c0	0.001	2.378	collagen alpha-1 chain-like
comp117719_c1	0.001	3.693	cuticular protein 100a cg12045-pa
comp117821_c0	0.001	2.423	atp-binding sub-family c (cftr mrp) member 4
comp117866_c0	0.001	2.038	PREDICTED: similar to AGAP006427-PA
comp117934_c0	0.001	4.857	xanthine dehydrogenase oxidase
comp117944_c0	0.041	2.321	4-hydroxyphenylpyruvate dioxygenase

comp118021_c0	0.001	2.064	atp-binding cassette transporter
comp118050_c0	0.001	3.257	---NA---
comp118072_c0	0.001	2.725	xanthine dehydrogenase
comp118086_c0	0.001	2.792	zinc finger bed domain-containing protein 5-like
comp118098_c0	0.027	2.592	lysosomal thiol reductase ip30 precursor
comp118149_c0	0.001	2.376	isoform a
comp118190_c0	0.001	2.628	---NA---
comp118327_c0	0.001	3.293	agap008849-pa isoform 1
comp118337_c0	0.001	3.606	hemoglobin c1 polymer
comp118393_c0	0.001	5.051	PREDICTED: hypothetical protein LOC100162732
comp118465_c0	0.001	2.380	PREDICTED: similar to beta-glucosidase
comp118479_c0	0.001	2.376	xanthine dehydrogenase
comp118489_c0	0.001	2.086	cg32645 cg32645-pb
comp118505_c0	0.001	2.449	PREDICTED: similar to AGAP006427-PA
comp118539_c1	0.001	2.070	dimmed cg8667-pa
comp118576_c0	0.016	2.528	glycogen synthase
comp118656_c0	0.001	2.457	cg31116 cg31116-pe
comp118672_c0	0.001	2.212	PREDICTED: similar to AGAP000521-PA
comp118740_c0	0.001	2.396	PREDICTED: similar to conserved hypothetical protein
comp118791_c0	0.001	3.126	cuticular protein precursor
comp118800_c0	0.001	4.298	agap002830-pa isoform 5
comp118907_c0	0.001	2.325	chitinase 7 precursor
comp118911_c0	0.001	2.897	reverse transcriptase, putative
comp118914_c0	0.003	2.273	PREDICTED: similar to polyprotein
comp118980_c0	0.001	2.532	sp1070 cg9138-pa
comp118999_c0	0.001	3.198	PREDICTED: similar to AGAP006427-PA
comp119029_c0	0.001	2.096	PREDICTED: similar to AGAP003205-PA
comp119069_c1	0.015	2.248	conserved hypothetical protein
comp119130_c0	0.001	2.053	PREDICTED: similar to conserved hypothetical protein
comp119203_c1	0.001	2.067	---NA---
comp119236_c0	0.001	2.293	glt8d3 protein
comp119311_c1	0.001	2.145	PREDICTED: similar to AGAP012156-PA
comp119317_c0	0.001	2.620	PREDICTED: similar to conserved hypothetical protein
comp119437_c0	0.001	2.308	AGAP004533-PA
comp119437_c2	0.028	2.326	cathepsin b-like like proteinase
comp119545_c0	0.001	2.268	glucosyl glucuronosyl transferases
comp119576_c0	0.001	2.115	elongation of very long chain fatty acids protein 4
comp119576_c1	0.053	2.727	---NA---
comp119617_c1	0.001	2.746	juvenile hormone-inducible
comp119631_c0	0.002	2.559	PREDICTED: hypothetical protein
comp119659_c0	0.001	2.953	PREDICTED: similar to AGAP003493-PC

comp119777_c0	0.001	2.513	cathepsin d isoform 1
comp119952_c0	0.001	7.370	---NA---
comp122972_c0	0.001	4.152	---NA---
comp136756_c0	0.001	2.746	AGAP003277-PA
comp46898_c0	0.001	6.162	---NA---
comp61647_c0	0.045	3.645	---NA---
comp67468_c0	0.001	6.552	---NA---
comp78210_c0	0.014	3.300	---NA---
comp80499_c0	0.001	3.074	ccp84ad cg2341-pa
comp80899_c0	0.016	2.205	---NA---
comp81107_c0	0.001	2.862	---NA---
comp82445_c0	0.006	4.783	multidrug resistance
comp85230_c0	0.001	118.341	---NA---
comp88333_c0	0.035	2.904	---NA---
comp88590_c0	0.008	2.369	---NA---
comp89053_c0	0.016	2.452	---NA---
comp89314_c0	0.024	2.672	cg30380 cg30380-pa
comp91122_c0	0.034	2.462	---NA---
comp92892_c0	0.042	2.897	---NA---
comp92895_c0	0.001	2.925	AGAP006261-PA
comp93153_c0	0.053	3.022	---NA---
comp93642_c0	0.018	4.973	peritrophic matrix protein 3 precursor
comp93996_c0	0.001	3.465	---NA---
comp95355_c0	0.059	3.013	PREDICTED: similar to AGAP006427-PB
comp95983_c0	0.009	3.319	PREDICTED: hypothetical protein
comp96052_c0	0.001	2.788	cuticular protein ld-cp1v1
comp96355_c0	0.003	2.073	PREDICTED: hypothetical protein
comp96687_c0	0.001	2.632	PREDICTED: ovochymase-1-like
comp96761_c0	0.044	2.125	---NA---
comp97937_c0	0.008	2.498	AGAP004936-PA
comp98478_c0	0.028	2.573	---NA---
comp98501_c0	0.003	3.981	---NA---
comp99067_c0	0.001	3.831	---NA---
comp99107_c0	0.004	2.822	cuticular protein 62bc cg1919-pa
comp99207_c0	0.001	2.103	---NA---
comp99330_c0	0.001	3.102	---NA---
comp99498_c0	0.009	2.340	drosulfakinins precursor, putative
comp99743_c0	0.049	2.336	---NA---
comp99795_c0	0.001	7.050	pro-phenol oxidase subunit 2
comp99854_c0	0.001	2.942	---NA---
comp99927_c0	0.003	3.862	---NA---

comp99940_c0	0.027	2.559	cuticular protein 62bc cg1919-pa

S3Table: Enrichment analysis between GO terms from the up-regulated transcripts of the systemic-1 population compared to the whole transcriptome.

GO Term	Name	FDR	Over/Under expressed GO term in systemic-1 population
GO:0044464	cell part	0.001	under
GO:0005623	cell	0.001	under
GO:0044260	cellular macromolecule metabolic process	0.001	under
GO:0005622	intracellular	0.001	under
GO:0044424	intracellular part	0.002	under
GO:0044710	single-organism metabolic process	0.023	over
GO:0003824	catalytic activity	0.023	over
GO:0043170	macromolecule metabolic process	0.023	under
GO:0016491	oxidoreductase activity	0.023	over
GO:0043226	organelle	0.023	under
GO:0043229	intracellular organelle	0.023	under

S4Table: Enrichment analysis between GO terms from the up-regulated transcripts of the systemic-3 population compared to the whole transcriptome.

GO Term	Name	FDR	Over/Under expressed GO term in systemic-3 population
GO:0044464	cell part	0.001	under
GO:0005623	cell	0.001	under
GO:0005622	intracellular	0.001	under
GO:0044424	intracellular part	0.001	under
GO:0044260	cellular macromolecule metabolic process	0.001	under
GO:0043229	intracellular organelle	0.001	under
GO:0043226	organelle	0.001	under
GO:0016491	oxidoreductase activity	0.001	over
GO:1901564	organonitrogen compound metabolic process	0.001	over
GO:0044710	single-organism metabolic process	0.001	over
GO:0006030	chitin metabolic process	0.001	over
GO:0055114	oxidation-reduction process	0.001	over
GO:0004497	monooxygenase activity	0.001	over
GO:0043231	intracellular membrane-bounded organelle	0.001	under
GO:0043227	membrane-bounded organelle	0.001	under
GO:1901071	glucosamine-containing compound metabolic process	0.001	over
GO:0006040	amino sugar metabolic process	0.001	over

GO:0008061	chitin binding	0.001	over
GO:0006022	aminoglycan metabolic process	0.001	over
GO:0032991	macromolecular complex	0.001	under
GO:0005737	cytoplasm	0.001	under
GO:0003824	catalytic activity	0.001	over
GO:0005506	iron ion binding	0.001	over
GO:0003676	nucleic acid binding	0.001	under
GO:0044267	cellular protein metabolic process	0.001	under
GO:0043234	protein complex	0.001	under
GO:0020037	heme binding	0.001	over
GO:0046906	tetrapyrrole binding	0.001	over
GO:0006685	sphingomyelin catabolic process	0.001	over
GO:0006684	sphingomyelin metabolic process	0.001	over
GO:0009308	amine metabolic process	0.001	over
GO:0044106	cellular amine metabolic process	0.001	over
GO:0044422	organelle part	0.001	under
GO:1901565	organonitrogen compound catabolic process	0.002	over
GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	0.002	over
GO:0071840	cellular component organization or biogenesis	0.002	under

GO:0044446	intracellular organelle part	0.002	under
GO:0050896	response to stimulus	0.003	under
GO:0009987	cellular process	0.003	under
GO:0009395	phospholipid catabolic process	0.003	over
GO:0090304	nucleic acid metabolic process	0.003	under
GO:0016043	cellular component organization	0.005	under
GO:0044444	cytoplasmic part	0.008	under
GO:0044281	small molecule metabolic process	0.008	over
GO:0005634	nucleus	0.014	under
GO:0006520	cellular amino acid metabolic process	0.014	over
GO:0042439	ethanolamine-containing compound metabolic process	0.014	over
GO:1901616	organic hydroxy compound catabolic process	0.014	over
GO:0046164	alcohol catabolic process	0.014	over
GO:0009072	aromatic amino acid family metabolic process	0.014	over
GO:0050794	regulation of cellular process	0.014	under
GO:0042302	structural constituent of cuticle	0.014	over
GO:0044707	single-multicellular organism process	0.014	under
GO:0009169	purine ribonucleoside monophosphate catabolic	0.014	over

	process		
GO:0009158	ribonucleoside monophosphate catabolic process	0.014	over
GO:0009128	purine nucleoside monophosphate catabolic process	0.014	over
GO:0009125	nucleoside monophosphate catabolic process	0.014	over
GO:0006200	ATP catabolic process	0.014	over
GO:0006576	cellular biogenic amine metabolic process	0.017	over
GO:0030149	sphingolipid catabolic process	0.020	over
GO:0046466	membrane lipid catabolic process	0.020	over
GO:0044712	single-organism catabolic process	0.020	over
GO:0044282	small molecule catabolic process	0.020	over
GO:0043170	macromolecule metabolic process	0.024	under
GO:0032502	developmental process	0.027	under
GO:0019538	protein metabolic process	0.028	under
GO:1901615	organic hydroxy compound metabolic process	0.033	over
GO:0019752	carboxylic acid metabolic process	0.033	over
GO:0005576	extracellular region	0.035	over
GO:0051716	cellular response to stimulus	0.035	under
GO:0032501	multicellular organismal	0.035	under

	process		
GO:0006066	alcohol metabolic process	0.035	over
GO:0065007	biological regulation	0.035	under
GO:0043436	oxoacid metabolic process	0.035	over
GO:0044699	single-organism process	0.035	under
GO:0006082	organic acid metabolic process	0.036	over
GO:0006665	sphingolipid metabolic process	0.037	over
GO:0004767	sphingomyelin phosphodiesterase activity	0.037	over
GO:0055085	transmembrane transport	0.038	over
GO:1901605	alpha-amino acid metabolic process	0.042	over
GO:0007154	cell communication	0.044	under
GO:0009055	electron carrier activity	0.044	over
GO:0007275	multicellular organismal development	0.047	under
GO:0043603	cellular amide metabolic process	0.050	over

CHAPTER 2: RNA interference of three up-regulated transcripts associated with insecticide resistance in an imidacloprid resistant population of *Leptinotarsa decemlineata*

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Authors: Justin Clements, Sean Schoville, Nathan Peterson, Anders S. Huseth, Que Lan and Russell L. Groves

Abstract

The Colorado potato beetle, *Leptinotarsa decemlineata* (Say), is a major agricultural pest of potatoes in the Central Sands production region of Wisconsin. Previous studies have shown that populations of *L. decemlineata* have become resistant to many classes of insecticides, including the neonicotinoid insecticide, imidacloprid. Furthermore, *L. decemlineata* has multiple mechanisms of resistance to deal with a pesticide insult, including enhanced metabolic detoxification by cytochrome p450s and glutathione S-transferases. With recent advances in the transcriptomic analysis of imidacloprid susceptible and resistant *L. decemlineata* populations, it is possible to investigate the role of candidate genes involved in imidacloprid resistance. A recently annotated transcriptome analysis of *L. decemlineata* was obtained from select populations of *L. decemlineata* collected in the Central Sands potato production region, which revealed a subset of mRNA transcripts constitutively up-regulated in resistant populations. We

hypothesize that a portion of the up-regulated transcripts encoding for genes within the resistant populations also encode for pesticide resistance and can be suppressed to re-establish a susceptible phenotype. In this study, a discrete set of three up-regulated targets were selected for RNA interference experiments using a resistant *L. decemlineata* population. Following the successful suppression of transcripts encoding for a cytochrome p450, a cuticular protein, and a glutathione synthetase protein in a select *L. decemlineata* population, we observed reductions in measured resistance to imidacloprid that strongly suggest these genes control essential steps in imidacloprid metabolism in these field populations.

Keywords: Colorado potato beetle; RNA interference; imidacloprid; cytochrome p450; pest management

1. Introduction

The Colorado potato beetle, *Leptinotarsa decemlineata* (Say), is a major agricultural pest of potatoes (*Solanum tubersum*) encompassing the United States, Mexico, Europe, and Asia, inclusive of over 16 million km^2 [1]. Studies have shown that many populations of *L. decemlineata* obtained from agricultural fields with a prior history of imidacloprid use have measurable levels of resistance [2, 3, 4, 5, 6]. Imidacloprid, a neonicotinoid insecticide (IRAC MoA 4A), became commercially available in 1995 when it was registered in potatoes [6, 7, 8], and quickly gained popularity with pest managers because of its flexibility in application patterns (at-plant, foliar spray, side-dress, etc.). As an at-plant application, neonicotinoids have a long duration residual activity against many pest species and are relatively safe for humans and other mammals [9]. Relatively soon after its initial registration, studies indicated that the effectiveness of imidacloprid against target populations of *L. decemlineata* started to decline, and field populations of beetles developed resistance in select locations of the United States [2,4,5,6]. Even as populations of *L. decemlineata* continue to increase in their relative levels of insensitivity to imidacloprid, it remains as one of the most widely used insecticides for control of problematic populations of *L. decemlineata*. In the Central Sands region of Wisconsin specifically, most potato producers rely on an at-plant (in-furrow or seed treatment) application of a neonicotinoid (e.g. imidacloprid, thiamethoxam) for control of *L. decemlineata*, as well as other key pests of the potato crop. As levels of product sensitivity continue to decline in *L. decemlineata*, producers and pest managers are facing new and emerging challenges to control this pest, resulting in repeated foliar applications of alternate mode of action insecticides, reduced grower profit, and a larger environmental footprint of potato pest management [10,11]

The pathways in which neonicotinoids are detoxified in insects have been studied in-depth [12, 13], highlighting the role of cytochrome p450 and glutathione S-transferase enzymes as principle agents in neonicotinoid metabolism. Recently, three studies described transcriptomic resources that enhance our ability to classify modes of resistance in *L. decemlinea*ta [3, 14, 15]. Using transcriptomic data as a template to measure genetic responses to imidacloprid, experimental assays of mRNA transcript level regulation in *L. decemlineata* [3] found multiple, up-regulated mRNA transcripts (statistically significant increases in mRNA transcript abundance levels) that were constitutively active in beetles from two resistant field populations in Wisconsin when compared to a reference susceptible strain. In the present study, we attempt to assess the role of these up-regulated mRNA transcripts that encode for genes responsible for insecticide resistance by knock-down (suppression of transcripts that encode for targeted genes) experiment using RNA interference (RNAi). We hypothesize that after reduction of transcript abundance (gene knock-down) utilizing RNAi of key genes involved in imidacloprid resistance, we can re-establish a susceptible phenotype in a resistant population of beetles. Successful re-establishment of a susceptible phenotype from a resistant *L. decemlineata* population would strongly suggest which genes encode for essential steps in imidacloprid metabolism in these field populations.

In recent years, RNAi has come into widespread use for *in vivo* studies of insects, to specifically target a gene of interest and assay phenotypic impacts [16]. This technique is tractable in *L. decemlineata.* Zhu et al. [17] used double-stranded RNA (dsRNA) produced in bacteria to knock-down housekeeping genes that encode for essential biological functions in *L. decemlineata*. Following ingestion of bacteria producing an RNAi trigger, knock-down of the target genes was confirmed; therefore, *per os* RNAi is effective in *L. decemlineata*. Additionally,

RNAi triggers (e.g., β-actin) can be produced in *planta* to effectively deliver dsRNA and induce lethality in early instars of *L. decemlineata* [18]. Successful RNA interference can be influenced by several factors including delivery of the RNA, size of the target transcript and associated dsRNA, and the ability to observe or accurately measure the phenotypic effects of the specific knock-down on the target. In the current study, we used a micro-injection approach to deliver dsRNA directly to the hemocoel of adult *L. decemlineata*, to develop proof of concept in the role of three candidate genes in imidacloprid resistance. More specifically, the current investigations focused on individuals from a specific resistant field population (Systemic-3) of *L. decemlineata* which has an estimated resistance ratio of 11.16 (imidacloprid resistance when compared to a reference, susceptible field population) measured in the 2014 crop year [3], and is a population which has been monitored for imidacloprid resistance since 2007 [4]. Moreover, this population possessed elevated transcript levels for genes, including 13 cytochrome p450s and 23 cuticular proteins, which were involved in several potential modes of insecticide resistance when compared to a susceptible population [3].

2. Experimental Methods

Confirmation of mRNA Transcripts Encoding Genes of Interest

Among a set of 562 up-regulated, mRNA transcripts found to be significantly more abundant in an imidacloprid resistant population (Systemic-3) [3] compared to an imidacloprid susceptible population, three transcripts encoding for genes were chosen as candidate targets for dsRNA-based, RNA interference; a cytochrome p450 (NCBI accession number GEEF01131148), a cuticular protein (NCBI accession number GEEF01064138), and a glutathione synthetase (NCBI accession number GEEF01119768). A 2.77, 3.96, and 2.90 fold

change in constitutively up-regulated transcript abundance was measured in this resistant *L. decemlineata* population and was determined to be statistically significant in a whole-transcriptome assay. Their potential role in insecticide resistance, and their abundant transcript levels, highlights them as candidates for functional validation. These transcripts were subjected to RACE PCR to generate full-length sequences, using the Smarter Rapid Amplification of cDNA Ends (RACE) cDNA amplification kit (Clontech, Mountain View, CA) and primers designed from the transcriptome sequences. The resulting products from RACE were inserted into pCR^{tm}-Blunt II-TOPO$^{®}$ vector and cloned into One Shot$^{®}$ *E.coli* Chemically Competent cells (Life Technology, Grand Island, NY) and delivered for sequencing (University of Wisconsin-Madison Biotech Center, Madison, WI). Once obtained, sequence data was combined with transcriptomic data to obtain full length sequences (Supplementary Table S1). RACE primers are illustrated in **Table 1**.

Table 1: Rapid Amplification of cDNA End primers used for cloning and sequencing of up-regulated transcripts of interest in the resistant (Systemic-3) *Leptinotarsa decemlineata* population.

	NCBI Accession number	**3' RACE primer**	**5' RACE primer**
Comp115309 (Cytochrome P450)	GEEF01131148	TGCGACCTATTCTCAGCC CATCGTTCAC	GTGAATCGACTTTCACACC AAAGGCAGAGGTG
Comp105889 (Cuticular protein)	GEEF01064138	CCAGGTGGTCCACCAGCC CAAATCG	CCGATTTGGGCTGGTGGAC CACCTG
Comp114026 (Glutathione Synthetase)	GEEF01119768	ATGGGGCCTCGATGAGAT CAAGACGCAAC	CTTGGAAAGTAGCCTGTAC GTCCACCACCTTC

dsRNA Generation

A set of T7 tagged primers (**Table 2**) were designed to amplify ~250-500bp lengths of the genes of interest to generate PCR products for *in vitro* transcription and production of dsRNA (Supplementary Table S2). Primer specificity was checked against the transcriptome using standalone BLAST [19]. Specific gene segments were amplified using RT-PCR from RNA

converted to cDNA extracted from adult, female *L. decemlineata* collected from the Systemic-3 population [3]. A heterologous control, used to induce RNAi machinery, was also generated for the gene that encodes enhanced green fluorescent protein (EGFP). The amplified segments were run on a 1.2% agarose gel, extracted and purified with a Gel/PCR DNA fragment Extraction Kit (IBI scientific, Peosta, IA). The resulting fragments were inserted into pCR™-Blunt II-TOPO® vector and cloned into One Shot® *E.coli* Chemically Competent cells (Life Technology, Grand Island, NY). Confirmation of the correct insert was evaluated by sequencing plasmids for the gene segments (Biotech center, Madison, WI). To generate large quantities of template, plasmids with the correct inserts were used for Phusion high fidelity amplification RT-PCR (New England Biolabs, Ipswich, MA) with T7 tagged primers. The amplification products were cleaned with a Gel/PCR DNA Fragment Extraction Kit (IBI scientific, Peosta, IA) and used in T7 RiboMax Express Large Scale RNA Production System (Promega, Madison, WI). The single stranded RNA products were cleaned using an Illustra™ MicroSpin™ G-25 Column (GE Healthcare, Piscataway, NJ, USA). Single stranded RNA were annealed to the complimentary strand by incubation at 94°C/1 min followed by 26°C/10 min. The concentration of dsRNA was measured using a Nanodrop (Thermo Fisher Scientific, Waltham, MA) and all samples were brought to a concentration of 500 ng/µl prior to injection into the hemocoel.

Table 2: T7 tagged primers used to generate dsRNA for RNAi in adult *Leptinotarsa decemlineata*. T7 sequence is provided in uppercase letters.

	Forward	**Reverse**
Enhanced Green Fluorescent Protein (EGFP)	TAATACGACTCACTATAGGGcca caagttcagcgtgtc	TAATACGACTCACTATAGGGct cgttggggtctttgctc
Comp115309 (Cytochrome P450)	TAATACGACTCACTATAGGGctaa cgatgttatagccacctctg	TAATACGACTCACTATAGGGat caatagatgaatcatatcaggtcg
Comp105889 (Cuticular protein)	TAATACGACTCACTATAGGGgag tctaccctctccattatccag	TAATACGACTCACTATAGGGgt ggttcctgttgagacacatatag
Comp114026 (Glutathione Synthetase)	TAATACGACTCACTATAGGGtacg ctgatattcaaagtggagagc	TAATACGACTCACTATAGGGc agtgaattcgtctacttggatg

RNAi though dsRNA knock-down

In late July and early August of 2014, approximately 1,500 2nd generation, adult *L. decemlineata* (Systemic-3) with a documented history of imidacloprid insensitivity [3] were collected from an agricultural field in the Central Sands region of Wisconsin. All adult beetles were caged for at least 72 hours in insect-proof cages in a greenhouse and fed fresh, untreated potato foliage before any studies were conducted. A preliminary diagnostic assay was initiated to first determine an appropriate concentration of dsRNA that would result in suppression of target gene transcript abundance in *L. decemlineata* using dsRNA for the Comp115309 cytochrome p450 gene. Specifically, dsRNA was diluted to produce a variety of concentrations from (100 ng/µl-500 ng/µl) and injected into adult female *L. decemlineata* between the 1st and 2nd abdominal sternite using a 10 µl Hamilton syringe (Hamilton Company, Reno, NV). At 24 and 48 hours after injection, RNA was extracted from whole insect preparations using Trizol (Life Technology, Grand Island, NY) and cDNA was generated with a high capacity, cDNA reverse transcription kit (Applied Biosciences, Foster City, CA). A preliminary RT-PCR was conducted to observe knock-down of transcript abundance at 24 and 48 hours after exposure to different concentrations of dsRNA. The reaction was performed at 98°C for 30 sec followed by 98°C for 10 sec, 63°C for 30sec, and 72°C for 30 sec, totaling 18 cycles, followed by a final extension step of 72°C for 10 min. PCR product was visualized on a 1.2% agarose gel. The preliminary results demonstrated reduced transcript abundance levels at a concentration as low as 190 ng/µl and 24 hours after injection. However, using quantitative PCR diagnostic assays, the greatest knock-down was not observed until the concentration of dsRNA had reached, or surpassed, 500 ng/µl and 48 hours had passed after injection (Supplementary Fig. S3). Based on this data, approximately 100 adult beetles were similarly injected per group for each gene target. As

previously described, individual beetles were injected with 1µl of the 500 ng/µl dsRNA concentrate using a 10 µl Hamilton syringe (Hamilton Company, Reno, NV). All adult beetles were injected on the ventral surface of the abdomen between the 1st and 2nd sternites (**Fig. 1**). Beetles were placed back into their respective cages (based upon gene target) for a period of 48 hours to accommodate the RNAi process.

Figure 1: Picture of *Leptinotarsa decemlineata* injections targeting the tergum between the 1st and 2nd abdominal sternite in adult beetles using a 10µl Hamilton syringe.

Phenotypic Assays of dsRNA Knock-Down

Assays were preformed to observed median lethal concentration (LC_{50}) on knock-downs for each gene to determine if the transcript knock-down resulted in a phenotypic change in the imidacloprid resistant population. Forty eight hours post-injection, beetles were bioassayed with a 1µl solution of imidacloprid (Technical grade 98.80%) carried in acetone ranging in concentrations between 0-1000 ppm applied to the dorsal surface between the first and second sternites. Specifically, beetles that survived dsRNA injections were divided into 5 equal groups ranging from approximately 9 to 15 individuals and treated with a range of imidacloprid concentrations. All adult *L. decemlineata* were held in an incubator at 26° C, 70% humidity, a light and dark cycle of 16:8 hours (light:dark), and fed untreated potato foliage. The median

lethal concentration (LC_{50}) of the adult beetles was estimated for each specific gene knock-down at both 48 and 168 hour time points after topical application of imidacloprid, Abbott's correction was used to normalize the data and a probit regression analysis was conducted [PROC PROBIT,[20]] and was used to assess the phenotypic response to an insecticide exposure. Two additional control groups were also included and represented by a no injection group and a dsRNA EGFP group. At the time of imidacloprid topical application, total RNA was extracted with Trizol (Life Technology, Grand Island, NY) from a set of 4 randomly selected adult female *L. decemlineata* from each gene knock-down group and controls to confirm knock-down with quantitative PCR (qPCR).

Quantitative PCR

Quantitative PCR was used to quantify transcript levels in whole insects after RNAi. Three biological replicates of cDNA from each gene knock-down group were used in cDNA synthesis for qPCR. Total RNA from each group was initially quantified using a Nanodrop (Thermo Fisher Scientific, Waltham, MA) and any DNA contamination was removed using TurboDNase (Life Technology, Grand Island, NY). The resulting DNA-free RNA was further purified to remove any possibility of PCR inhibition using a MasterPure Complete DNA and RNA Purification Kit (Epicentre, Madison, WI). All RNA concentrations were equalized before input into the cDNA synthesis kit. A high capacity cDNA reverse transcription kit (Applied Biosciences, Foster City, CA) was used to generate cDNA which was subsequently diluted to a final concentration of 5 ng/µl of RNA equivalent input for qPCR. Replicate sets of a reference control gene (β-actin) were also evaluated; the β-actin primers used were shortened versions of those previously described by Zhu 2010 [17]. The qPCR reaction was run on a CFX-96 platform (Bio-Rad Laboratories, Hercules, CA) with a master mix of Bullseye EverGreen (MIDSCI,

Valley Park, MO). Similar primers to those used in a previous study [3] were selected for the current experiments and the corresponding primer efficiency can be found in **Table 3**. Triplicate reactions were run at 95°C for 10 min followed by 95°C for 15 sec, 62°C for 60 sec for a total of 40 cycles. Data were collected for each biological replicate and relative expression of resistant strains in comparison to susceptible strains was calculated using the Pfaffl method [21], as seen in **Equation 1**. The Pfaffl methodology takes into consideration the efficiency of the primer sets and provides an estimate of the ratio of the target gene to the reference population.

Table 3: Quantitative PCR primers and primer efficiency used for quantification of transcript abundance after dsRNA injection.

	Forward Primer (5'-3')	Reverse Primer (5'-3')	Primer Efficiency
β-actin (Reference)	CATCCAAGCTGTACTCTCCTTG	GGAAGAGCGTAACCTTCGTAG	1.92
Comp115309 (Cytochrome P450)	CGAGAAATGCGACCTATTCTCAG	ACACAGTCTTGGTCTTTCTTGAG	1.98
Comp105889 (Cuticular protein)	CTCCAGTGGTTCCGTTATTACAC	AGCGTAGTCGTGGAAATGTTG	1.94
Comp114026 (Glutathione Synthetase)	CAGAGCAGGGTATGAACCTAATC	CCAGCCAAGTGATACTGAATCG	1.97

Equation 1: $\text{ratio} = \frac{(E_{\text{target}})^{\Delta \text{CP}_{\text{target}}(\text{Control}-\text{samples})}}{(E_{\text{reference}})^{\Delta \text{CP}_{\text{ref}}(\text{control}-\text{samples})}}$ [21]

3. Results

The RACE amplification produced full transcript sequences as illustrated in Supplementary Table S1. Following a BLAST search using elongated sequence, Comp 115309 (cytochrome p450) had the highest query coverage of 97% to *L. decemlineata* cytochrome p450 (cyp9Z26, accession KJ476503.1) and with an estimated identity of 97%. Comp 105889 (cuticular protein) had the highest query coverage of 41% to *Tribolium castaneum* cuticle protein 18.7 (accession XM 964708.2) and an estimated identity of 66%. Finally, Comp 114026

(glutathione synthetase) had a query coverage of 79% to *T. castaneum* glutathione synthetase-like transcript variant X1 (accession XM 008196868.1) with an estimated identity of 64% as of March 2016.

An appropriate concentration of dsRNA required for successful gene knock-down was determined to be 500 ng/µl (Supplementary Fig. S3). Mortality from injection ranged from 18% in the cytochrome p450 knock-down to 49% in the cuticular protein knock-down. Forty-eight hours after the beetles were injected with dsRNA, expression levels of the transcripts were measured with qPCR; the outcome of which resulted in successful gene knock-down. β-actin was used as a reference gene in the knock-downs to ensure that the injections themselves did not affect the levels of transcript expression. Knock-down efficiency varied among the target genes, with glutathione synthetase resulting in 27.56X less expression than the control, the cytochrome p450 resulting in 14.97X less expression, and the cuticular protein resulting in 5.83X less expression **Table 4**.

Table 4: Quantitative PCR confirmation of transcripts levels that encode for genes, plus mean percent survival estimates of adult *Leptinotarsa decemlineata* 48 hours post injection.

	Cytochrome p450	Cuticular protein	Glutathione synthetase	EGFP
β-actin expression in control	21.30±0.36	21.30±0.36	21.30±0.36	NA
β-actin expression in knock-down	21.18±0.07	21.30±0.18	20.24±1.8	NA
Targeted expression value in control	20.45±.1.07	25.30±1.17	27.14±1.70	NA
Targeted expression value in knock-down	24.33±1.27	27.90±0.21	30.91±2.17	NA
Gene knock-down detected by quantitative PCR	14.97	5.83	27.56	NA
Survival rate after 48 hours	82%	51%	69%	78%

The phenotypic effects of a reduction in transcript abundance (gene knock-down) was assessed in *L. decemlineata* using an LC_{50} topical bioassay. Gene knock-downs were shown to

re-establish a level of susceptibility, as shown in the resulting LC_{50} experiments, and effective knock-down was confirmed by qPCR. Results of the LC_{50} topical bioassays are presented in **Table 5** and measured 48 and 168 hours after topical application. The no injection control had an estimated LC_{50} of 732.24 ppm after 48 hours and 495.33 ppm after 168 hours, representing the mean LC_{50} of the resistant Systemic-3 field population. The EGFP injection control had a marginally significant effect on *L. decemlineata* mortality, with an estimated mean LC_{50} of 274.23 after 48 hours and 335.53 after 168 hours. The results from the three target gene knock-downs showed lower mean LC_{50} estimates than either the no injection or the EGFP control after 48 hours, while the cytochrome p450 and glutathione synthetase protein knock-downs were both lower than controls after 168 hours.

Table 5: Median lethal concentration (LC_{50}) estimates resulting from imidacloprid bioassays at 48 and 168 hours after topical application.

Target	Time Point (Hours)	N	[1]Slope(SEM)	LC_{50} (PPM)	[2]95% CI	Chi-square	PR>Chi-square
Control no injection	48	75	2.06 (0.60)	732.24	(486.09-1799)	11.61	0.0007
EGFP	48	65	2.31 (0.62)	274.23	(167.41-404.18)	13.79	0.0002
Comp115309 (Cytochrome P450)	48	71	1.75 (0.55)	203.23	(75.45-320.23)	9.88	0.0017
Comp105889 (Cuticular protein)	48	45	1.57 (0.69)	240.75	(25.13-530.86)	5.14	0.0234
Comp114026 (Glutathione Synthetase)	48	65	2.07 (0.60)	231.78	(121.98-351.07)	11.97	0.0005
Control no injection	168	75	2.45 (0.59)	495.33	(350.38-788.49)	11.17	<.0001
EGFP	168	65	3.42 (0.75)	335.53	(244.78-449.31)	20.78	<.0001
Comp115309 (Cytochrome P450)	168	71	1.38 (0.53)	145.60	(9.73-262.52)	6.59	0.0103
Comp105889 (Cuticular protein)	168	45	1.82 (0.69)	340.65	(156.93-822.69)	6.96	0.0084
Comp114026 (Glutathione Synthetase)	168	65	1.92 (0.57)	258.45	(134.92-407.55)	11.16	0.0008

[1] Slope ± standard error of the mean (SEM) estimates of the estimated probit regression function.
[2] 95% confidence interval (CI) estimates around mean LC_{50} estimates.
[3]Chi-square analysis of effects of the proc probit regression

4. Discussion

Select populations of *L. decemlineata* throughout the United States have recently developed resistance to many of the major classes of insecticides, including the Group 4A neonicotinoid class, in several potato production regions [2, 4, 5, 6, 11]. One hypothesis to explain *L. decemlineata's* capability to rapidly develop resistance to imidacloprid relies on the pre-formed molecular mechanisms used to metabolize plant secondary metabolites (alkaloids), including cytochrome p450s and glutathione-s-transferases [1, 15]. The purpose of the current study was to determine whether three sets of transcripts encoding for genes which are up-regulated in a resistant population are at least partially responsible for increased imidacloprid resistance and whether RNAi could be used to knock-down putative resistance genes with the goal of re-establishing a susceptible phenotype. Clements [3] identified candidate, resistance genes that had mRNA transcripts highly up-regulated when comparing imidacloprid susceptible and resistant populations in Wisconsin. In the current study, we knocked-down three genes including an up-regulated glutathione synthetase (partially responsible for the production of glutathione used by glutathione S-transferase to carry out phase 2 metabolisms of neonicotinoids), the most up-regulated cuticular protein, and one of the most up-regulated cytochrome p450s in the resistant population, compared to a susceptible population. Furthermore, after the RACE generation of full-length sequences, the cytochrome P450 had the highest query coverage of 97% to a *L. decemlineata* cytochrome p450 (cyp9Z26, accession KJ476503.1). Zhu 2016 demonstrated that cyp9Z26 was also significantly up-regulated in a Long Island, NY imidacloprid resistant population [15], which suggests that in both a Wisconsin and New York population, the up-regulation of this important detoxification mechanism could be responsible for imidacloprid resistance.

A principle goal of the current study was to re-establish susceptibility to the insecticide imidacloprid within a resistant population of *L. decemlineata* as a possible component of pest management. Although RNAi possesses considerable potential for studying gene function, RNAi may also possess some drawbacks, including potential off-target effects on genes that have similar homology, and it is possible to knock-down non target genes and genes of other non-target species [22]. We took care when designing primers and creating unique dsRNA for each gene. Confirmation of RNAi knock-down was measured 48 hours after injection with qPCR. To establish that no general knock-down took place, we used an internal control of β-actin to compare transcript expression. A successful knock-down of genes of interest was observed to varying degrees; the expression of glutathione synthetase was knocked down 27.56X compared to the control, the cytochrome p450 was knocked down 14.97X, and the cuticular protein had the smallest knockdown of only 5.83X when compared to the control. A wide range of variation among gene targets was observed in the current study using LC_{50} bioassays to evaluate the effects of gene knock-down and a possible return to a susceptible phenotype. Baseline LC_{50} estimates for the resistant field population (Systemic-3) was estimated to be 495.33 ppm after 168 hours, whereas the cytochrome p450 knock-downs had an estimated LC_{50} value of 145.60 ppm, the cuticular protein had an estimated LC_{50} value of 340.65ppm and the glutathione synthase LC_{50} had an estimated LC_{50} value of 258.45 ppm. The estimated LC_{50} value of the EGFP injected control was found to be 335.53ppm. This value suggests that the dsRNA EGFP itself may have had a potential effect on insect survivorship. Although EGFP is not present within the genome of *L. decemlineata,* it may be possible that the EGFP dsRNA had off target effects resulting in lower LC_{50} values. Although EGFP is commonly used as a control in RNAi studies, previous research has demonstrated that dsEGFP can have off target effects and may

have substantial indirect effects on transcript levels associated with multiple biological processes [23]. For example, a study conducted in *Bactericera cockerelli* demonstrated relativity high mortality in individuals injected with dsRNA encoding for GFP. When injected with 200nl of 100ng/µl dsRNA, mortality of 58% was observed after 6 days [24]. Nevertheless, all of our target gene knock-downs showed a decrease in resistance in the LC_{50} assays. This suggests that the down-regulation of these genes can result in a more susceptible phenotype from an imidacloprid resistant population and that up-regulation may be partially responsible for increased imidacloprid resistance. The knock-down that showed the largest decrease in resistance was the cytochrome p450, further suggesting that this gene may play a significant role in imidacloprid resistance in this select population of *L. decemlineata.*

Although the results of the LC_{50} studies had large confidence intervals, we expect that this reflects natural variation among members of a beetle population from an agricultural field. This field population is most likely composed of a heterogeneous group of highly (homozygotic) and moderately (heterozygotic) resistant individuals. As a result, the genes that encode for detoxification are likely differentially expressed between individuals in this highly resistant population, leading to large confidence intervals. This observation has been previously described in similar field populations for 1^{st} generation beetles in 2014, where the LC_{50} value was estimated to be 52.68 ppm (95% CI: 25.31-154.56) [3]. This data further suggests the possibility that not all beetles in each treatment are homozygous for the genes that encode resistance and certain beetles can be more susceptible to imidacloprid without ever knocking-down resistant genes, leading to variation in LC_{50} within groups. Moreover, imidacloprid resistance could be polygenic and knocking-down just a single gene may have only limited effects on *L. decemlineata* susceptibility.

RNA interference is becoming an increasingly common method to knock-down genes of interest in insects and may have important application in developing gene-targeted pesticides. Previous studies have demonstrated the ability to knock-down specific genes in larval and adult *L. decemlineata* by microinjection and feeding assays using target dsRNA [17, 18, 25]. Zhang [18] demonstrated a transgenic plant that could express dsRNA of *L. decemlineata* using a dsRNA homolog of β-actin which resulted in mortality of immature stages of *L. decemlineata*. Previous work by Katoch (2013) illustrates the importance of correctly choosing a delivery method for RNAi, and suggests that *per os* delivery of dsRNA is less effective than injecting dsRNA [22], presumably because many insect taxa do not possess the appropriate systemic interfering defective (SID) proteins necessary for dsRNA transport across midgut barriers [26]. The transcript abundance assays conducted by Clements (2016) [3] extracted RNA from whole insect preparations. To limit the effects of localized dsRNA, it was concluded that injections into the hemocoel was appropriate method to deliver dsRNA to suppress transcripts of targeted genes. Although injections were used in this study and would be impractical in an agricultural field setting, it was determined that injecting beetles with dsRNA was appropriate to demonstrate proof of concept. Successful knock-down was demonstrated by qPCR for all three genes, and was a requirement to determine whether the up-regulation of mRNA transcript levels found in the resistant population was responsible for an increase in imidacloprid resistance. Determining genes that are responsible for imidacloprid resistance through injections is one step to develop new methods for controlling beetle populations and is the building block to generate new technologies.

Injections of dsRNAs may impact insect survivorship; which is often observed within 48 hours after injection as the knock-down takes place. In our investigation, mortality from the

injection of dsRNA before insecticidal exposure ranged from 18 to 49%, depending on the dsRNA target, with an observed mortality of 22% in the EGFP control. The highest mortality of 49% after injection resulted from the cuticular protein. One plausible explanation may result from the fact that this cuticular protein may be multi-functional and could play important roles in other metabolic functions, inducing far greater mortality than other dsRNA injections. Work performed by Jasrapuria (2012) on *Tribolium castaneum* demonstrated the diverse functions of cuticular proteins, including the suppression of TcCPAP3-A1 by dsRNA injections leading to a lethal phenotype in mature adults [27].

Resistance of *L. decemlineata* to imidacloprid is now common throughout many portions of the United States [11, 28]. However, neonicotinoids are still commonly used for control of this problematic pest [11]. Out of necessity, novel approaches are warranted to increase the longevity of this important class of insecticide chemistry, as well as developing an understanding of how this insect copes with insecticides and accurately determining the genes involved in resistance development. Many researchers are developing RNAi to find viable gene targets for agricultural settings and urban pests, as available insecticides are becoming less effective as pests develop resistance to insecticides [16, 29, 30, 31]. The potential for RNAi to be used on up-regulated, molecular mechanisms of resistance could be a feasible option for pest management and needs to be studied in-depth. Knocking-down specific genes in insect taxa that have the ability to metabolize insecticides may open new doors in pest management practices, for example by allowing limited, short-duration pesticide applications when coupled with RNAi treatment. Many studies in the past decade have investigated possible RNAi targets, including genes such as β-actin [17, 18]. Still other studies have used a variety of gene targets, including genes responsible for essential metabolic processes; one such case focuses on the disruption of metabolic processes

corresponding to insect development and wing formation, resulting in the inability of flight, and an ultimate outcome of insect death [32]. Some of these RNAi targets appear to require long duration exposure or repeated exposure in the field to effectively knock-out essential metabolic processes until mortality results. Further, previous studies have targeted specific receptors to determine their function in resistance [33]. Our study is unique in that we targeted metabolic detoxifying enzymes that were up-regulated in an imidacloprid resistant agricultural field population. By targeting genes that are up-regulated due to imidacloprid resistance in specific populations (classified through mRNA transcript abundance), we suggest that efficacy of conventional insecticides could be improved when used in combination with targeted RNAi technology to control resistant *L. decemlineata* populations . However, further research is needed to determine whether knock-down of one or a few key resistance genes is sufficient to increase susceptibility in an agricultural setting and improve the sustainability of *L. decemlineata* management.

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7. Supplementary Materials

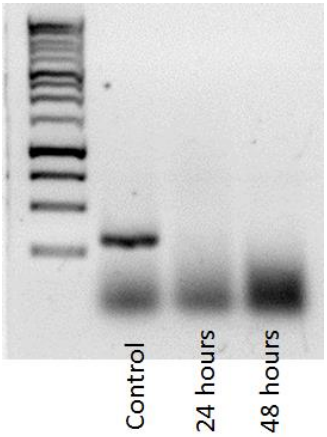
Supplementary Table S1: 3' and 5' RACE sequences overlade illustrating extended gene transcripts of interest.

Cytochrome p450	acatgggaaccaatttcaaggcacaagatattatgggatctatgaatccggtcaacctactttattgataaaggatccagaaatactgaga aaaatcactgttaaagattttgatcacttcactgaccacagaagctttttttcagaagagactgatcctctatgggacaaaaatttatttacatt gagaggtagaaaatggcgagaaatgcgacctattctcagcccatcgttcactagtagcaaaatgcgaacaatgtttgttctgatttcgaa atgttctaaggacttcgtcaatcattttctcaagaaagaccaagactgtgtagaaatggaaatgaaagacacattcacccgttttactaacg atgttatagccacctctgcctttggtgtgaaagtcgattcacttgaagaaccaaataatgaattttattcgatgggaaagagggcaacagat ttttcaggattcgcaatgatgttgaagttcataggatttttcgtggctcctgatcttttaaagtatctgcggatatcattcatggattcagcagtt aacagatttttctgcgatctggttgataataccatacaagttcgggaaaaaaaacaatataatacgacctgatatgattcatctattgatgga agcgaaaaaaggagttcatcacaaagatgaaaattcaggcgtggatacaggatttgccacagttgaggaagctgaccttggagaagg gcacgtcaccgaaataactaacttagatatcacagctcaggccttgatatttttctttgccgggtttgattcggtgtcttcgctcatgtgcttc atgtcccatgagttagctgtcaatccaaacattcaaacaaaactaagaaatgaaattgaagagacactagcggggatgtggaggagaa ataacatatgaagcattactgaaaatgaaatatatggatatggnttatatcagaatcattgaggaaatggccaagtgctgtagcaacagg acagaagtttgtacgaaacccttatacaatanaggccaatgaaattcngnatgaaaaaaccncntaccttatcgaaaaaggncncaac attgattganaccccntaggtnaggaaatacatccctggatccccaaaatata
Cuticular protein	acatggggtgtttaggaggttcataactaatatcaacaccatgatgattttggttttaatatcctgtgccctcgccatctgtgaagctggcta cgcatcaccttactactactcacaacataactcagttattctacccaacggagctcctttagacactcccgaggtccaactagctaaagca gcccatttcgctgctcacgctaaagcaaggtatggcgctccagtggtaccgttattacactatggtcaaccccttgcccataacgcccttc ctttggacactcccgaagtagcagcagccaaagctcaacatttccacgactacgctgtagccgctcaacgcaacggagtctccgttgg acaccttcaactcgccccaggtcttccagtcgatactcccgaagtccagcatgcgaaagctgctcacttcgctgcccatgctgaagctg cagctcgtgcccatggatctcactacaggaggcgtcgcggagtctaccctctccattatccagtcattgaccacaatggcgtaccagta gagaccccagaagtgcaagcagcaaaagctcatcactttgctgaatatgctaaagttgccagtcgtccaggtcagaatattcctgaagc tgtgaactactatggtcatcctcagacaattcagtatggcgcaccagaatattaccgcggcccaggtggtccaccagcccaaatcggac cagacggacagcccattgaaacccatgaagttcaagctgctaaggccgcgcattttgcagctcatgcagctgtcagaggaaataactat ggtcatttcgggcactattaaaaccaattctctgtgaaagctacaaattggagtttgtagttactgatgagaaactatatgtgtctcaacagg aaccacatagcctctttaaagttttataaaataggcgtgataccttagtttactgtaaatattgtttggaaataaacggcaaaacatatgatgt taatgaaactgtgaaaaaaaaaaaa
Glutathione Synthetase	acatggggacaatacgctgatattcaaagtggagagcattaaacatattttcctgaacagagtagcttgaagaaaaaaatcaacatgtgc tcagtttctgtaactcccgcttgtgtacctttgcctttgccagaccaacaactgaaagacttggtgaggaaaactagagactgggcgatctt gcatggggcctcgatgagatcaagacgcaacttcagcgaggattcgatacaattcgctcctttcaatctgtttccgtctactttcctcaaga aagattttcagaaggtggtggacgtacaggctactttccaagagttgatacacaaagttgcccatgatggggagtttttgaggaaatgtct caaagataccatccaagtagacgaattcactggaaatttgttcaaaatatatgagacggtgcaaaaagaaggaatagtgcagcctttgaa cctgggacttttcagatgcgattacatgttagagtctggcaacgctaagaatcaatcttcccatgagaattatccatgttgttgttggaaaca agtagaatataataccatcgcatcgggatttggatggcttggacctatttctgctgcaattcagaggtatgctctgcaagaactaggagatt tgaaaaggctggaaaacttacctgaaaataatgctcttgatgggttgtgtgagggaatgttagaagcatggaaagcatacggaaatcca gaagccataattttatttattatcgaaaatgtctcctataatattgccgaccagagattccatgaattcagacttagagaattaaggccagac attaaagttgttagaaaaactctgaccgaaatcagtgatagaggcaggatcagctcgacnaaaacatttacgatagatgaagatgaagtt gcagtagtttacttcagagcagggtatgaacctaatcactatccatctaaagcggaatgggacgccngactgttaatcnaaaggtcgaa ggcaatcnaatgccccaacgatttcagtatccncttgggctgggtacccaaaaagttccancnaagaattntccnaaacccngggacc tgtttggaatatttttttttaaaggganncccaatnnaagntcn

Supplementary Table S2: T7 tagged dsRNA sequences used in the knock-down assay.

Enhanced green fluorescent protein	TAATACGACTCACTATAGGGccacaagttcagcgtgtccggcgagggcgagggcgatgccacctacggcaagct gaccctgaagttcatctgcaccaccggcaagctgcccgtgccctggcccaccctcgtgaccaccctgacctacggcgtgcagtgcttc agccgctaccccgaccacatgaagcagcacgacttcttcaagtccgccatgcccgaaggctacgtccaggagcgcaccatcttcttca aggacgacggcaactacaagacccgcgccgaggtgaagttcgagggcgacaccctggtgaaccgcatcgagctgaagggcatcg acttcaaggaggacggcaacatcctggggcacaagctggagtacaactacaacagccacaacgtctatatcatggccgacaagcag aagaacggcatcaaggtgaacttcaagatccgccacaacatcgaggacggcagcgtgcagctcgccgaccactaccagcagaaca cccccatcggcgacggccccgtgctgctgcccgacaaccactacctgagcacccagtccgccctgagcaaagaccccaacgagC CCTATAGTGAGTCGTATTA
Cytochrome p450	TAATACGACTCACTATAGGGctaacgatgttatagccacctctgcctttggtgtgaaagtcgattcacttga Agaaccaaataatgaattttattcgatgggaaagagggcaacagatttttcaggattcgcaatgatgttgaagttc Ataggatttttcgtggctcctgatcttttaaagtatctgcggatatcattcatggattcagcagttaacagatttttctg Cgatctggttgataataccatacaagttcgggaaaaaaaacaatataatacgacctgatatgattcatctattgat CCCTATAGTGAGTCGTATTA
Cuticular protein	TAATACGACTCACTATAGGGgagtctaccctctccattatccagtcattgaccacaatggcgtaccagtag Agaccccagaagtgcaagcagcaaaagctcatcactttgctgaatatgctaaagttgccagtcgtccaggtcaga Atattcctgaagctgtgaactactatggtcatcctcagacaattcagtatggcgcaccagaatattaccgcggccc Aggtggtccaccagcccaaatcggaccagacggacagcccattgaaacccatgaagttcaagctgctaaggccg Cgcattttgcagctcatgcagctgtcagaggaaataactatggtcatttcgggcactattaaaaccaattctctgtg aaagctacaaattggagtttgtagttactgatgagaaactatatgtgtctcaacaggaaccacCCCTATAGTGAGTCGTA TTA
Glutathione Synthetase	TAATACGACTCACTATAGGGtacgctgatattcaaagtggagagcattaaacatattttcctgaacagagt Agcttgaagaaaaaaatcaacatgtgctcagtttctgtaactcccgcttgtgtacctttgcctttgccagaccaacaa Ctgaaagacttggtgaggaaaactagagactgggcgatcttgcatggggcctcgatgagatcaagacgcaacttca Gcgaggattcgatacaattcgctcctttcaatctgtttccgtctactttcctcaagaaagattttcagaaggtggtggac Gtacaggctactttccaagagttgatacacaaagttgcccatgatggggagtttttgaggaaatgtctcaaagatac catccaagtagacgaattcactgCCCTATAGTGAGTCGTATTA

Supplementary Figure S3: PCR reaction of RNA converted into cDNA from cytochrome P450 gene knock-down using 500ng/µl dsRNA at 24 and 48 hours compared to control.



CHAPTER 3: Phenotypic response of *Leptinotarsa decemlineata* to imidacloprid and changes in transcript abundance spanning a growing season in the Central Sands region of Wisconsin

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Authors: Justin Clements, Sean Schoville, Nathaniel Clements, Scott Chapman, and Russell L. Groves

Abstract

The Colorado potato beetle, *Leptinotarsa decemlineata* (Say), is a major agricultural pest of commercial potatoes. Pest managers use a combination of control tactics to limit populations, including multiple insecticides. Finding a window of insecticide susceptibility and understanding genetic responses to insecticide exposure during a growing season may provide novel management recommendations for *L. decemlineata*. We examined temporal changes (during one growing season) in phenotypic response between a susceptible population and an imidacloprid-resistant population. Beetles remained more susceptible to imidacloprid in the susceptible population throughout the growing season. Estimated mean LC_{50} values varied throughout the growing season in the resistant population, with increased susceptibility among over-wintered, and recently emerged adult beetles compared with a heightened level of resistance in the second generation. RNA transcript abundance was compared among multiple time points through the growing season, showing that cuticular proteins and cytochrome p450s were highly up-regulated during peaks of resistance. Temporal variation in imidacloprid susceptibility of *L. decemlineata*

was observed, which included early time points of susceptibility and later peaks in resistance. Heightened resistance occurred during the second generation and correlated to increased transcript abundance of multiple mechanisms of resistance, including multiple cuticular protein and cytochrome p450 transcripts.

1. Introduction

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say), is a key agricultural pest of potatoes (*Solanum tuberosum*), tomatoes (*Solanum lypcopersicum*), eggplants (*Solanum melongena*) and peppers (*Solanum annuum)*[1], causing significant crop loss and direct damage that can lead to loss of revenue for commercial growers. *L. decemlineata* has become a global pest, occupying over 16 million square kilometers[1,2] and impacting potato production in North America and Eurasia. According to the United Nations Food and Agricultural Organization, the United States (US) produced 19.8 million tons of potatoes in 2013, and it is one of the leading vegetable crops in the country[3]. The impact of *L. decemlineata* on individual state agricultural markets is also significant, especially in Wisconsin where potato production accounts for more than $310 million annually[4].

The history of insecticidal inputs for control of *L. decemlineata* is a story retold in many potato producing areas of the country, where many classes of insecticides have been effective for short periods of time, before the beetles become resistant[2]. Recent estimates suggest that populations of *L. decemlineata* have now become resistant to more than 56 insecticides[2,5]. More recently (1995), the registration and introduction of the neonicotinyl insecticide class (IRAC Classification, Group 4A, nicotinic acetylcholine receptor (nAChR) agonists) has resulted in the use of active ingredients which includes, but are not limited to, imidacloprid, thiamethoxam, clothianadin, and dinotefuran [2,6]. Since the initial introduction of this insecticide class in the mid-1990s, populations of *L. decemlineata* have steadily developed resistances, but it remains the principal insecticidal tool used for crop protection in potato [2,7–11].

Temporal patterns of phenotypic variation in resistance to insecticides within populations of *L. decemlineata* has been previously suggested between generations in this pest species[2,7].

Specifically, these studies suggest that the second generation population is significantly more resistant when compared to its first generation counterpart[12]. In the current investigation, we hypothesize that temporal patterns in phenotypic variation in imidacloprid resistance may not be limited to only differences between first and second generation, but may also vary at additional time points throughout the growing season. Uncovering new information about the specific time points of increased susceptibility, or conversely increased resistance, would provide insight into pest management strategies and enhance the effectiveness of insecticide deployment.

Recent investigations have examined transcriptomic data to classify possible mechanisms of pesticides resistance in *L. decemlineata* [11,13,14]. These studies examined transcript abundance in relation to insecticide resistant populations independent of collection time, with the goal of classifying over expressed transcripts and contigs in resistant populations. These up-regulated transcripts provide an initial glimpse into enzymatic detoxification mechanisms, such as those mediated by cytochrome p450s and glutathione S-transferases, taking place within a select set of adult *L. decemlineata*, but are limited to a discrete time point over the growing season. We hypothesize that an increase in the abundance of transcripts which encode for known mechanisms of resistance, such as cytochrome p450s and glutathione S-transferase, will correlate with time points of increased resistance. Furthermore, these peaks in resistance and the associated up-regulated transcripts are known to differ between resistant and susceptible populations[11]. Uncovering up-regulated transcripts provides important new information on how this species combats insecticide inputs and could lead to improvements in our ability to manage resistant populations of this damaging pest.

2. Experimental Methods

Beetle collection

Two populations of *L. decemlineata* were collected from two field locations in the Central Sands region of Wisconsin in the spring and summer of 2015. The first population represents a documented imidacloprid-susceptible population collected from the University of Wisconsin's Arlington Agricultural Research Station (AARS), Arlington, WI. The second population was collected from a commercial agricultural field with a previously documented history of imidacloprid resistance and termed 'Systemic-3'[7]. From the two populations, adult beetles were collected at 4 time points representing; (1) early emergence from diapause (28 May – 1 June), (2) late emergence from diapause (16-20 June), (3) conclusion of first generation (26-30 June), and (4) emergence of second generation (10 July – 10 September). Efforts were made to ensure that each collection represented the aforementioned distinct subgroups of adult beetles at each of the two experimental locations by staggering collections due to the natural differences in phenology of emergence. This staggered collection resulted from the fact that the two locations differed only very slightly in longitude (0.153^{o}), but did depart more significantly in latitude (0.796^{o}). On the first collection dates, approximately 2000 adult beetles were collected from each field within the first 48 hours of initial adult emergence and field colonization. On the second collection dates, an estimated 2000 adult beetles were again collected from the Systemic-3 population and approximately 1900 adult beetles were collected from the AARS site. On the third range of dates of adult collection, approximately 500 beetles were obtained from both sites. Taken together, these first three collection dates effectively encompass the first-generation (post-diapause) of *L. decemlineata* present at each experimental field location. The fourth and final set of collections represented adult emergence and colonization by the second generation of *L.*

decemlineata, where approximately 300 beetles were obtained from each location. Due to the low number of beetles in the second generation populations, we were unable to collect more than 300 beetles at this time. Furthermore, scouting of the beetles was performed regularly through each of the field locations in order to ensure that adult beetles were collected at appropriate times to represent the categories described previously. Due to the low number of beetles present in the second generation population at the AARS, we were unable to collect beyond the forth collection date (10 July). However, due to the more abundant populations at the Systemic-3 field site, we continued collections at 3 additional time points (20 July, 27 August, and 10 September) during the late summer and collectively refer to these additional collections as the fourth collection interval for this specific location. Following collection from the field, all adults representing unique phenological time points were held on pesticide-free, field grown potato plants located in an agricultural field and secured within separate 1 m^3 mesh cages (BioQuip Products, Inc., Rancho Dominguez, CA).

LC_{50} assays

After beetles were placed in their respective cages, median lethal concentration (LC_{50}) assays were performed as previously described by Zhoa et al.[9] and results were used to characterize variation in resistant phenotypes throughout the growing season. Every 4 days, 90-270 adult beetles were randomly selected from each field cage representing a different field collection date and location, to conduct LC_{50} assays. Initially we aimed to assay approximately 225-270 adult beetles for each LC_{50} assay, but as the season progressed and mortality increased, only assays with smaller sample sizes were possible on later assay dates. To establish an initial dose range for the study, a pre-screening assay was conducted on 1 June from among a randomly

selected set of adults from each collection site and were dosed with a concentration gradient of imidacloprid (Technical grade 98.80%) carried in acetone (imidacloprid contents of 0.0034 – 1.74 µg/µl). Specifically, one microliter of solution was placed on the 1st abdominal sternite of a subset of adult beetles and the material was absorbed within 3-5 seconds following topical application. From the pre-screening assays, we determined that the adult beetles from the AARS would be serially dosed with concentrations of 0, 0.00034, 0.0034, 0.034, and 0.17 µg/µl of imidacloprid in acetone in order to accurately estimate LC_{50} values, whereas adult beetles from the imidacloprid-resistant, Systemic-3 population were dosed with concentrations of 0, 0.034, 0.17, 0.69, and 1.74 µg/µl. The pre-screening assay process was intermittently performed throughout the growing season as beetles became more or less responsive to the initially pre-determined dose ranges. Prior to the full screening assay with the full range of serial imidacloprid doses, adult beetles were first placed into petri dishes (5 beetles per dish) and equally divided for the assay containing 5 serial concentrations. One microliter of the imidacloprid solution was topically applied to replicate sets of 5 adult beetles/dose, and adults were held dorsal side down until the solution had been completely absorbed (e.g. 3-5 sec) and were then placed back into their respective petri dishes. Following topical application, all petri dishes containing adults, plus fresh, untreated potato foliage, were held in an incubator at 26^{o}C, 72% relative humidity and a photoperiod of 16:8 hours (light:dark). Adult beetles were maintained in these conditions for 7 days before any response (e.g. mortality) was assessed (Proc Probit, SAS)[15]. In total, 15 time points were assessed throughout the growing season for the imidacloprid-susceptible, AARS population, whereas a total of 17 time points were assessed for the imidacloprid-resistant Systemic-3 population.

RNA extraction and RNA sequencing

At similar time points for which LC_{50} assays were performed, randomly selected subgroups of (N=3 each site) untreated beetles were similarly collected and later used for RNA extraction. Total RNA was extracted using Trizol (Life Technology, Grand Island, NY) and stored at -80^{0}C for later analysis. The University of Wisconsin-Madison, Biotechnology Center was contracted to isolate and generate mRNA libraries and run Illumina HiSeq 2500 1X100bp sequencing. We conducted RNA sequencing (RNA-seq) to examine transcript abundance throughout the growing season in the imidacloprid-resistant population, although available funding only allowed us to examine 11 of the 17 time points that corresponded to times when LC_{50} measurements were performed. Prior to submitting samples to the Biotechnology Center, RNA was initially pre-treated with TurboDNase (Life Technology, Grand Island, NY), and the DNA-free RNA was cleaned from protein with a phenol-chloroform extraction, and an EtOH precipitation was conducted to remove any other contaminates. Approximately 1500 ng of RNA was submitted to the Biotechnology Center, and the RNA was analyzed with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) before RNA sequencing was conducted.

Differential transcript comparison and enrichment analysis

After high quality reads were generated, the University of Wisconsin-Madison, Biotechnology Center further cleaned and aligned the raw reads to unannotated *L. decemlineata* genomic scaffolds available from Baylor College[16]. As a research participant, the Biotechnology Center also examined transcript abundance and the difference in transcript abundance between different collections through the use of RSEM[17], EdgeR,[18] and EBSeq[19]. This was quantified using FPKM (fragments per kilobase of exon per million fragments mapped), TPM (transcripts

per million), and read counts to generate a "gene count" to examine differentially expressed transcripts. Finally, the Biotechnology Center compared transcript abundance between first and second generations of collected adult *L. decemlineata* in three contrasts: early emergence from diapause vs. second generation, late emergence vs. second generation, conclusion of first generation vs. second generation. These comparisons were conducted by examining all the "gene counts" of the first generation collection versus all the "gene counts" of the second generation. Fold change and a FDR (false discovery rate) was also calculated for each transcript. Transcripts that had a fold change greater than 2 and a FDR less than ($P \leq 0.049$) were considered differentially expressed and up-regulated. Using standalone BLAST with BLASTx, all transcripts including the up-regulated transcripts were compared to reference proteins (*E* value $<10^{-3}$). Transcripts were classified based on the NCBI nomenclature returned by BLASTx. The database of Reference Protein Sequences (Refseq) from *Tribolium castaneum*, *Acyrthosiphon pisum*, *Anopheles gambiae*, *Drosophila melanogaster*, and *Pediculus humanus* was downloaded from NCBI for a total of 80,498 sequences and used to classify transcripts. BLASTx results were examined for up-regulated transcripts known to play a role in insecticide resistance including cuticular proteins, cytochrome p450's, glutathione S-transferase, ABC transporters, and carboxylesterases. BLASTx results were uploaded into Blast2Go[20] for further data analysis. Up-regulated transcripts were first analyzed and the components were mapped to the corresponding GO terms. The annotation step was run with a cutoff of $E_{value} < 1E\text{-}3$, annotation cut off > 45, and GO weight >5. An enrichment analysis was performed between all the up-regulated transcripts in the second generation collection and all aligned transcripts were run using a two-tailed FDR test with a 0.005 cut-off in order to determine if any group of GO terms were differentially expressed in the up-regulated components.

Statistical Analysis

Statistical analyses were conducted to determine if the three biological comparisons were dissimilar using a Bray Curtis dissimilarity index to examine transcripts up-regulated by a fold change of 2 and a fold change of 100. In order to incorporate a "gene count" dissimilarity comparison between the 3 biological conditions, we initially subtracted the second generation "gene count" from that of the first generation to obtain an absolute value. The absolute value of the resulting difference in gene count was then divided by the average of both gene counts, resulting in a value that ranged between 0 and 2; with 0 representing identical transcript expression values, and 2 representing completely different values. All of the up-regulated transcripts were then assigned as similar or dissimilar, using a cut off of < 0.5 to represent similar values. Finally, estimates were input into a Bray-Curtis analysis to examine dissimilarities between time points, which provides an output between 0 and 1, with 0 being completely similar and 1 being completely dissimilar (UW Statistical Consulting, Bray Curtis analysis[21]).

Quantitative PCR

To confirm transcript abundance from RNA-seq data, quantitative PCR (qPCR) was conducted. Three technical replicates of pooled cDNA from each unique collection time point in 2015 were used in cDNA synthesis for qPCR. Total RNA from each population was quantified using a Nanodrop (Thermo Fisher Scientific, Waltham, MA), and DNA contamination was removed using TurboDNase (Life Technology, Grand Island, NY). An EtOH precipitation was conducted to purify RNA, after which time RNA was suspended in 100µl of water. All RNA

concentrations were equalized before input into the cDNA synthesis kit and the subsequent cDNA was generated with a Super Script III kit (ThermoFisher Scientific, Waltham, MA). The cDNA was diluted to a final concentration of 5 ng/µl of RNA equivalent input for qPCR. *β*-actin was used as a reference gene in these investigations, and the *β*-actin primers were shortened versions of those previously described by Zhu 2011[22]. The qPCR reaction was run on a CFX-96 platform (Bio-Rad Laboratories, Hercules, CA) with a master mix of Bullseye EverGreen (MIDSCI, Valley Park, MO). Genes of interest (GOI) were selected based on their relevance to this study and primers were designed to contigs found in the generated transcripts. Primer and primer efficiency are found in (**Table 1).** Primer specificity was checked against the transcriptome using BLAST. Triplicate reactions were run at 95°C for 10min followed by 95°C for 15 s, 62°C for 60 s for a total of 40 cycles. Data were collected for each biological replicate and relative expression of resistant strains to susceptible strains was calculated using the Pfaffl methodology[23], which takes into consideration the efficiency of the primer sets and provides the ratio of the target gene to the reference population.

Table 1: Quantitative PCR primers and primer efficiency

	Forward Primer (5'-3')	**Reverse Primer (5'-3')**	**Primer Efficiency**
β-actin (Reference)	CATCCAAGCTGTACTCTCCTTG	GGAAGAGCGTAACCTTCGTAG	1.92
LDEC003961 (Cuticular protein)	ACCTGCTGCCGGTATTATTG	TACAGTTCCAGAGGGTCCAG	1.96
LDEC014400 (Cuticular protein)	GCCCATTGAAACCCATGAAG	TAGTGCCCGAAATGACCATAG	2.01
LDEC003423 (Cuticular protein)	CCAACAAGAAACCGGACAAC	CGTTCCATGTGAATTGATAAGATCC	1.96
LDEC016769 (Cytochrome p450)	CAGGTCTGACAAGGATATGGTTAG	TCCAGAGCTTTCGGATGATTC	1.98

3. Results

LC_{50} comparison

Median lethal concentration (LC_{50}) assays were conducted on both an imidacloprid susceptible and a resistant population of *L. decemlineata* to determine temporal patterns of phenotypic variation in imidacloprid susceptibility throughout a growing season. During the growing season, 4 collections were conducted on both field populations (early emergence from diapause, late emergence, conclusion of first generation, and second generation). From these collections, LC_{50} assays were conducted at 15 time points for the imidacloprid susceptible (AARS) population and 17 time points for the imidacloprid-resistant (Systemic-3) population (**Tables 2 and 3, respectively**). The mean LC_{50} estimates showed considerable phenotypic variation in imidacloprid-susceptibility over the four collection intervals, specifically representing the 3 time points of first generation emergence (e.g. early, late, and conclusion emergence periods) compared to the second generation emergence (**Fig. 1**). With few exceptions, the LC_{50} estimates were statistically higher during the growing season in the imidacloprid-resistant population when compared to the susceptible population over the entire sampling season (**Tables 2 and 3)**. Further examination of the mean LC_{50} values observed in the imidacloprid-resistant population demonstrate that adult beetles appear to be more susceptible immediately after emergence and colonization, and again later in the development of their adult lifecycle before they reach the end of their adult lives.

Table 2. Regression estimates for median lethal concentration assays (LC_{50}) resulting from topical bioassays of adult *L. decemlineata* for the Arlington Agricultural Research Station (AARS), imidacloprid-susceptible population during summer 2015.

Population	Assay Date	N[1]	LC_{50} (µg/µl)	95% Confidence Interval
Arlington (Early Emergence)	June 9th	225	0.0052	(0.00051-0.021)
Arlington (Early Emergence)	June 13th	225	0.058	(0.0015-0.61)
Arlington (Early Emergence)	June 21st	270	0.15	(0.012-0.72)
Arlington (Early Emergence)	June 25th	270	0.099	(NA*)
Arlington (Early Emergence)	June 29th	180	0.029	(NA*-0.22)
Arlington (Late Emergence)	June 25th	270	0.18	(0.13-0.24)
Arlington (late Emergence)	June 29th	270	0.23	(0.068-0.68)
Arlington (Late Emergence)	July 3rd	270	0.073	(NA*-0.24)
Arlington (Late Emergence)	July 6th	270	0.099	(0.0059-0.40)
Arlington (Late Emergence)	July10th	270	0.11	(0.015-0.55)
Arlington (Late Emergence)	July14th	90	0.19	(0.021-13)
Arlington (Conclusion of First Generation)	July10th	180	0.18	(0.12-0.27)
Arlington (Conclusion of First Generation)	July14th	180	0.14	(0.063-0.36)
Arlington (Conclusion of First Generation)	July18th	90	0.030	(NA*)
Arlington (Second Generation)	July21st	270	0.28	(0.016-1.3)

[1] Total number of adult beetles used in biological replicates at each location and year combination.
*NA represents a probit mortality regression estimate without a 95% confidence interval (CI).

Table 3: Regression estimates for median lethal concentration assays (LC_{50}) resulting from topical bioassays of adult *L. decemlineata* collected from the Systemic-3, imidacloprid-resistant field population during summer 2015.

Population	Assay Date	N[1]	LC_{50} (µg/µl)	95% Confidence Interval
Systemic-3 (Early Emergence)	June 9th	225	1.5	(.18-19)
Systemic-3 (Early Emergence)	June 13th	225	3.5	(1.1-53)
Systemic-3 (Early Emergence)	June 21st	270	7.5	(3.0-49)
Systemic-3 (Early Emergence)	June 25th	270	7.5	(4.5-19)
Systemic-3 (Early Emergence)	June 29th	180	2.4	(1.6-3.8)
Systemic-3 (Late Emergence)	June 25th	270	6.8	(5.5-10)
Systemic-3 (Late Emergence)	June 29th	270	8.7	(6.3-30)
Systemic-3 (Late Emergence)	July 3rd	270	8.7	(6.0-13.9)
Systemic-3 (Late Emergence)	July 6th	270	11	(4.9-38)
Systemic-3 (Late Emergence)	July10th	270	9.0	(1.5-15)
Systemic-3 (Late Emergence)	July14th	180	4.1	(3.1-5.5)
Sytemic-3 (Conclusion of First Generation)	July10th	180	5.7	(4.2-8.9)
Sytemic-3 (Conclusion of First Generation)	July14th	180	8.7	(.50-23)
Sytemic-3 (Conclusion of First Generation)	July18th	90	5.3	(3.7-8.3)
Systemic-3 (Second Generation)	July21st	270	8.4	(4.1-20)
Systemic-3 (Second Generation)	August 27th	270	15	(7.0-28)
Systemic-3 (Second Generation)	September 10th	270	14	(4.9-28)

[1] Total number of adult beetles used in biological replicates at each location and year combination.

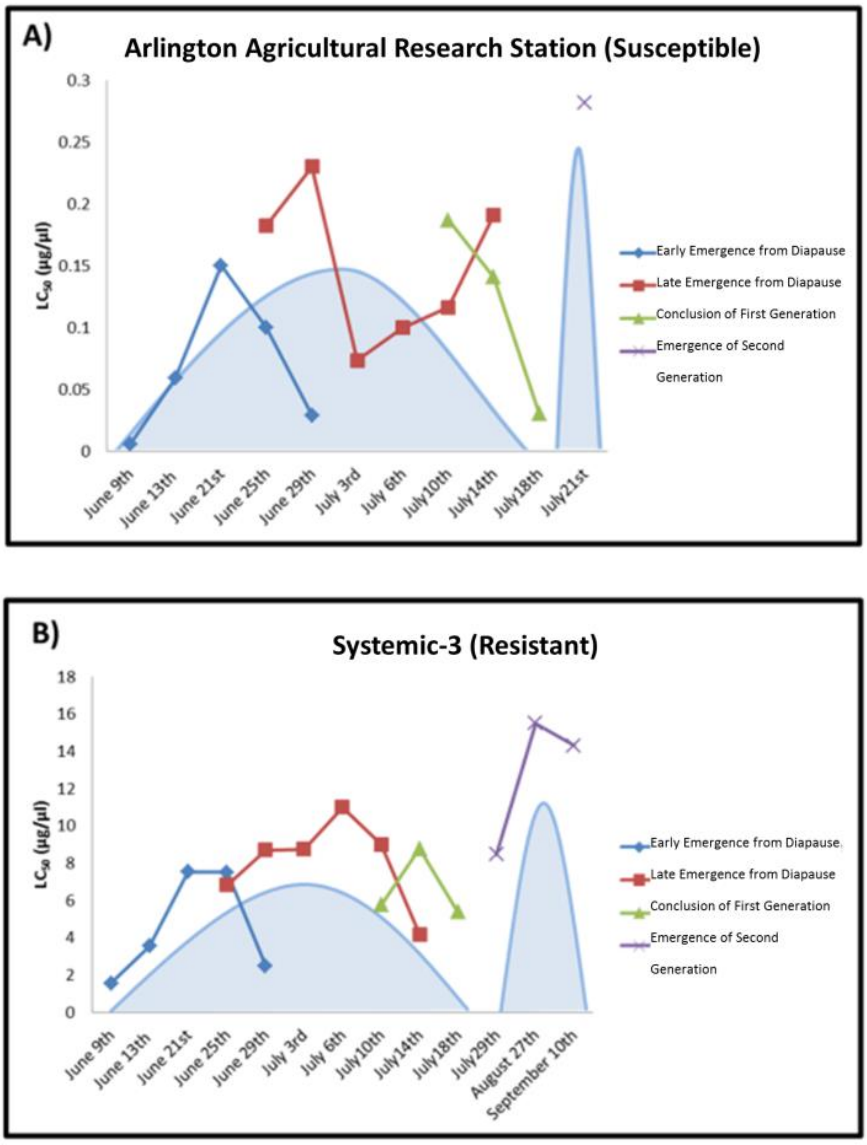

Figure 1. Median lethal concentration (LC_{50}) estimates representing the four different collection intervals plotted over the season for the imidacloprid-susceptible (A) and resistant (B) populations of *L. decemlineata*. Blue shaded areas represent the time period over which the separate first and second generation emergence was observed to occur. Note significant differences in the scale of median LC_{50} (µg/µl) estimates for each population.

Differential transcript comparison

From the imidacloprid-resistant, Systemic-3 population, 11 unique time points (9 from first generation and 2 from the second generation), were sequenced to examine transcript abundance (Fig. 2). Time points were selected to cover all 4 collection intervals including time

points with high and low imidacloprid susceptibility. We conducted three transcript abundance comparisons between the specific emergence periods in the first generation versus the second generation (Fig. 3) to determine up-regulated transcripts in the second generation population that could partially explain the phenotypic increases in levels of measured resistance. These comparisons examined all "gene counts" across generational comparisons. From the three comparisons, candidate molecular mechanisms of resistance were classified (Table 4). These mechanisms of resistance were classified based on significant levels of fold change and FDR. Here again, a transcript was considered up-regulated if there was a fold change greater than 2 and a FDR ≥ 0.049. A candidate list of possible mechanisms of resistance can be seen in (Supplementary Table 1). Similarly, highly up-regulated transcripts were classified in the second generation population to uncover trends in transcript abundance. This was done by examining transcripts encoding possible mechanisms of resistance which were up-regulated greater than 100 fold and FDR ≥ 0.049 (Table 5). The results from the three comparisons demonstrate a set of 13 cuticular proteins and a cytochrome p450 which were highly up-regulated in the second generation when compared with the discrete emergence intervals (early, middle and late) of the first generation. We further conducted an enrichment analysis between the transcripts that were up-regulated in the second generation population and all the transcripts assembled from RNA sequencing to determine if there were any apparent trends in the up-regulated transcripts (Supplementary Table 2). Both over and under expression of 51 GO terms was observed in this analysis, including over expression in the levels of oxidoreductase activity, monoooxygenase activity, and structural constituents of the cuticle.

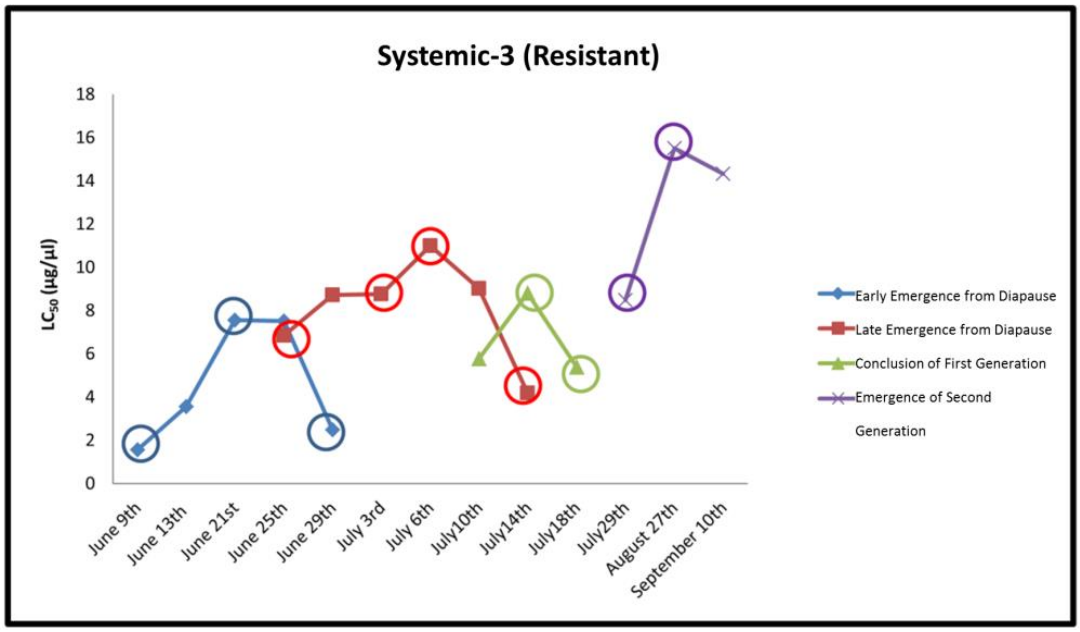

Figure 2. Time points of adult *L. decemlineata* collection chosen from the Systemic-3 field location for RNA sequencing. Colored circles correspond to the specific time points chosen for RNA sequencing.

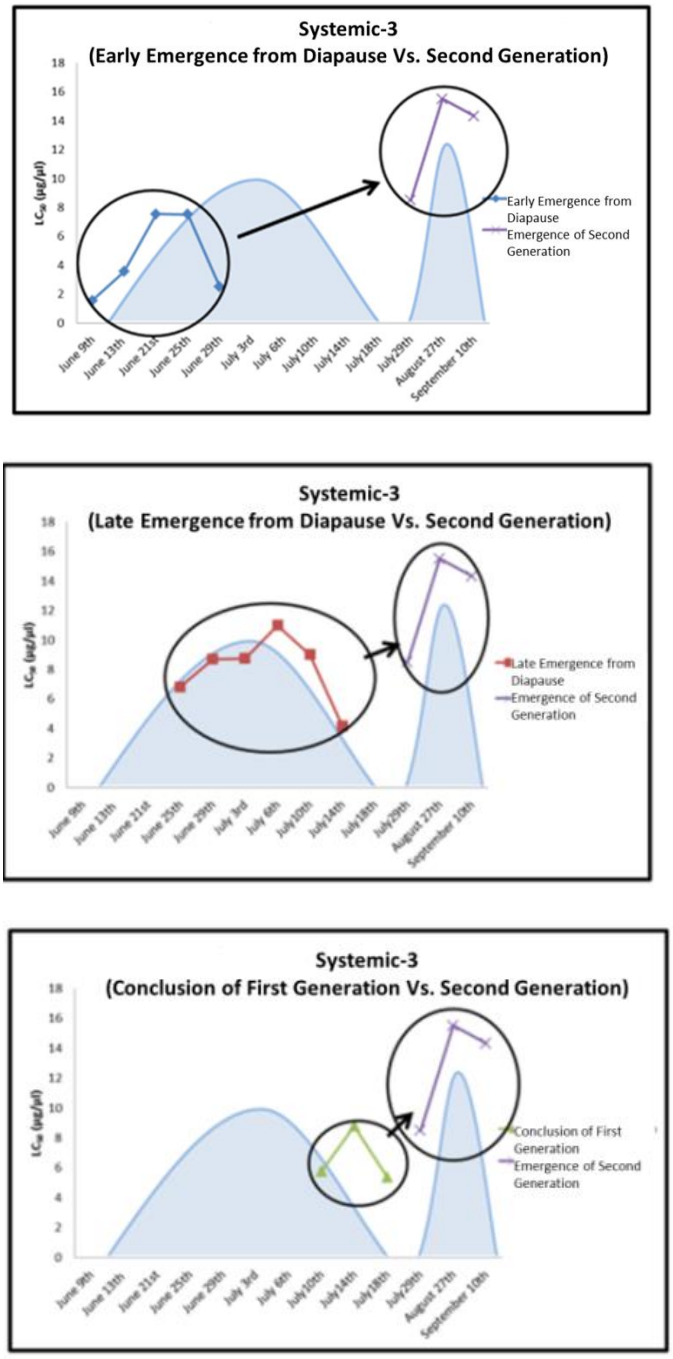

Figure 3. Early, late and conclusion-emergence time periods of the first generation of *L. decemlineata* used for discrete comparisons against second generation beetles using RNA sequencing to examine transcript abundance.

Table 4. Number of differentially expressed transcripts between the three discrete emergence time points of the first generation compared to the second generation collection time points in the imidacloprid-resistant field population. Transcripts were considered up-regulated if there was a fold change of 2 and FDR value ≥ 0.049.

	Total Up-regulated Transcripts in the Second generation population compared to the first generation	**Transcripts encoding for Cuticular proteins**	**Transcripts encoding for Cytochrome P450**	**Transcripts encoding for Glutathione S-transferase**	**Transcripts encoding for ABC transporters**	**Transcripts encoding for Carboxylesterase**
Generation 1 Collection 1 (early) vs. Generation 2	469	38	10	2	0	0
Generation 1 Collection 2 (late) vs. Generation 2	624	44	11	2	2	1
Generation 1 Collection 3 (conclusion) vs. Generation 2	423	40	13	2	2	0
Total Unique Genes	728					

Table 5. Up-regulated transcripts with a log fold change > 10 and FDR ≥ 0.049 that could encode for increased imidacloprid resistance in the second generation of *L. decemlineata* at the Systemic-3 location. NCBI accession numbers represent the BLAST hit.

Transcript Blast x Result	Fold Change	NCBI accession number	Transcript I.D.
Early Emergence from Diapause Vs. Second Generation			
cuticular protein	993.839	XP_966639.1	LDEC003961
cuticular protein ld-cp1v1	473.248	XP_970573.1	LDEC005679
cuticular protein isoform a	437.191	NP_647668.1	LDEC024510
cuticular protein 62bc cg1919-pa	226.513	XP_968445.1	LDEC003211
cuticular protein 92f cg5494-pa	202.820	XP_969801.1	LDEC014400
cytochrome p450	167.761	XP_973153.1	LDEC016769
cuticle protein cp5	166.128	XP_973942.1	LDEC006896
cuticle protein 1	110.520	XP_970381.1	LDEC003423
cuticular protein rr-1 family (agap000344-pa)	108.965	XP_971011.1	LDEC013734
Late Emergence from Diapause Vs. Second Generation			
cuticular protein ld-cp1v1	1804.609	XP_970573.1	LDEC005679
cuticular protein	1411.250	XP_966639.1	LDEC003961
cuticular protein ld-cp3	1212.622	XP_973909.1	LDEC006898
cuticular protein precursor	835.095	NP_001161316.1	LDEC013733
cuticular protein 92f cg5494-pa	655.360	XP_969801.1	LDEC014400
cuticle protein 1	631.133	XP_970381.1	LDEC003423
cuticular protein rr-1 family (agap000344-pa)	549.102	XP_971011.1	LDEC013734
cytochrome p450	199.748	XP_973153.1	LDEC016769
cuticular protein precursor	133.291	NP_001161313.1	LDEC014399
Conclusion of First Generation Emergence from Diapause Vs. Second Generation			
cuticular protein 92f cg5494-pa	1399.634	XP_969801.1	LDEC014400
cuticular protein	1166.062	XP_966639.1	LDEC003961
cuticular protein 62bc cg1919-pa	624.162	XP_968445.1	LDEC003211
cuticular protein ld-cp3	536.339	XP_973909.1	LDEC006898
cuticular protein ld-cp1v1	399.977	XP_970573.1	LDEC005679
cuticular protein rr-1 family (agap000344-pa)	371.136	XP_971011.1	LDEC013734
cuticular protein isoform a	355.026	NP_647668.1	LDEC024510
cytochrome p450	271.828	XP_973153.1	LDEC016769
cuticle protein 1	260.013	XP_970381.1	LDEC003423
cuticular protein precursor	202.230	NP_001161316.1	LDEC013733
cuticle protein cp5	141.040	XP_973942.1	LDEC006896
cuticle protein 20	105.933	XP_968593.1	LDEC017994

Statistical Analysis

To determine if there were dissimilarities in transcript abundance from among the three time point comparisons to the second generation collection, a Bray Curtis dissimilarity analysis was performed. The analysis was conducted at a fold change greater than 2 and a fold greater than 100 (**Table 6**). At a fold change of greater than 2, we concluded that the comparison results were similar with values between 0.25 and 0.32 for the three comparisons, suggesting that the up-regulated transcripts were rather similar at a fold change greater than 2. The Bray Curtis analysis of transcripts with a fold change greater than 100 demonstrated substantially more dissimilarities with values between 0.30 to 0.73, suggesting that the up-regulated transcripts with a fold change greater than 100 were much more dissimilar, suggesting that as the fold change increases, the transcripts become more dissimilar.

Table 6. Bray Curtis analysis conducted to observe the dissimilarities between the three comparisons for investigating fold changes greater than 2 or fold changes greater than 100. Bray Curtis analysis output is expressed as a dissimilarity constant between 0 and 1, with 0 being completely similar and 1 completely different.

	Comparison 1 Vs. Comparison 2	**Comparison 1 Vs. Comparison 3**	**Comparison 2 Vs. Comparison 3**
Dissimilarity constant FC (2)	0.321428571	0.256868132	0.281593
Dissimilarity constant FC (100)	0.735849057	0.301886792	0.641509

Comparison 1 (Early Emergence from Diapause Vs. Second Generation)
Comparison 2 (Late Emergence Vs. Second Generation)
Comparison 3 (Conclusion of First Generation Vs. Second Generation)

Confirmation with Quantitative PCR

To confirm transcript abundance generated through the use of RNA sequencing, qPCR assays were performed between the late emergence time point of the first generation and the second generation collections in the Systemic-3, imidacloprid-resistant, population to confirm up-regulated transcripts. We specifically focused on 4 transcripts that have previously been implicated as important components in imidacloprid resistance, and *β*-actin expression was used as a reference. We confirmed the transcripts were up-regulated by calculating the transcript expression ratio with the Pfaffl methodology and the fold change of the "gene count" (**Table 7)**.

Table 7. Transcript expression determined by quantitative PCR

	Late Emergence	**Second Generation**		
	Mean CT ±SD	Mean CT ±SD	Fold Change "Gene Count"	qPCR expression ratio
β-actin (Reference)	21.32±.14	19.96±0.01	N/A	N/A
LDEC003961 (Cuticular protein)	32.28±.25	21.12±0.01	1411.250	752.12
LDEC014400 (Cuticular protein)	30.79±.32	20.86±0.06	655.360	422.13
LDEC003423 (Cuticular protein)	31.42±1.5	21.24±0.03	631.133	388.94
LDEC016769 (Cytochrome p450)	37.48±1.0	27.22±0.17	199.748	325.65

4. Discussion

The life cycle of *L. decemlineata* has been previously described in detail, including investigations into which specific developmental stages (e.g. eggs, larvae, adults) are the best targets for insecticide treatments[24,25]. However, the phenotypic variation in imidacloprid resistance over the growing season in adult *L. decemlineata* has yet to be examined. In this study, we examined phenotypic changes in imidacloprid resistance throughout a growing season in both an imidacloprid susceptible and resistant population through the use of imidacloprid LC_{50} assays. Huseth et al. observed staggered, post-diapause emergence of *L. decemlineata* in agricultural settings, which he hypothesized could partially explain the beetles' capacity to cope with systemic insecticides[7]. Here, we have examined the effects of temporal patterns of phenotypic variation in insecticide resistance throughout a growing season using distinct collections of adult *L. decemlineata* with staggered emergence dates. Furthermore, we utilized RNA-sequencing to classify candidate transcripts that could explain these temporal patterns in phenotypic variation in an imidacloprid-resistant population.

Our study demonstrates that there is phenotypic variation in imidacloprid resistance during the growing season in adult *L. decemlineata*. In describing the temporal patterns in mean LC_{50} estimates over the growing season in the first generation set of collection time points (early, late and conclusion), an imidacloprid-resistant population generally followed a bell shaped distribution in susceptibility as the growing season progressed, with newly emerged and aging adults being the most susceptible. Dramatic levels of overall resistance were obvious in LC_{50} estimates that were two orders of magnitude (100x) higher in the resistant population compared to the susceptible population. With the use of LC_{50} assays we were able to describe trends in resistances that give valuable insight to pest managers, including optimal windows of

susceptibility to insecticides. Huseth et al. further hypothesized that trends in insecticide resistance are tied to the dynamic life history of *L. decemlineata*. In this study we observed that individuals in the late emergence time period of the first generation were considerably more resistant than the early emergence period, suggesting that staggered and later emerging beetles are potentially more resistance. The differential effects of pesticides applied as either systemic or foliar protectants may have an impact in these trends and need to be examined further.

Mean LC_{50} values of the Systemic-3, imidacloprid-resistant population show that first generation adults are consistently more susceptible than second generation adults, indicating that overwintering diapause might influence the relative fitness of first generation adults. However, there is significant variation in the estimated confidence intervals (CIs), potentially due to the differing sample sizes of adult beetles included in these assays. We also noted that field populations inherently contain a heterogeneous mixture of both resistant and susceptible individuals and variance is frequently high[7,11]. Despite significant variation in estimated CI's, several time points in the first generation remain statistically more susceptible than their second generation counterparts. Multiple factors could explain these findings, including the increased expression of enzymatic detoxification mechanisms in the second, and more resistant generation.

Recent investigations have classified potential mechanisms by which insecticide resistance develops in *L. decemlineata*[11,13,14] . The general processes by which imidacloprid can be metabolized and broken down relies on multiple detoxification enzymes, including cytochrome p450 and glutathione S- transferases[26]. We therefore chose to conduct comparisons between multiple groups of the first generation collection to the second generation collection of the imidacloprid resistant population (early emergence from diapause vs. second generation, late emergence vs. second generation, conclusion of first generation vs. second generation) to

determine if difference in resistance could be partially explained by transcript abundance of enzymatic detoxification mechanisms.

Examining up-regulated transcripts that corresponded to imidacloprid resistance in the second, more resistant, generation uncovered multiple mechanisms of resistance that were up-regulated in the second generation population compared to the first generation counterpart. Moreover, we classified highly up-regulated transcripts (fold change greater than 100) in the second generation population. This revealed interesting trends in highly up-regulated cuticular proteins and a single cytochrome p450. The cytochrome p450 had the highest BLAST match to *L. decemlineata* cytochrome P450 412a2 (NCBI accession KF044265.1). Both cuticular and cytochrome p450 have been previously suggested to play a large role in insecticide resistance in multiple insect taxa. In *Anopheles funestus*, increases in cuticular thickness was associated with pyrethroid resistance[27], and in *Cimex lectularius* the over expression of multiple cuticular proteins was observed in association with pyrethroid resistance[28]. In *L. decemlineata,* elevated expression of mRNA transcripts encoding for cuticular proteins have been observed in adult beetles and has previously be associated with environmental stressors such as a dry environments and insecticidal exposure [11,29]. Mota-Sanchez et al. demonstrated a phenotypic change back to susceptibility in a neonicotinoid resistant population with the use of piperonyl butoxide, an inhibitor of cytochrome p450 suggesting the importance in cytochrome p450 in neonicotinoid resistance[8]. The elevated transcript abundance data indicates that both cuticular and cytochrome p450 have a role in the increased resistance of the second generation collections. Moreover, it is possible that some of the trends in the up-regulated cuticular proteins and cytochrome p450 could be due to other, non-insecticidal exposures. Previous studies classified similar mechanisms of resistance in *L. decemlineata* using RNA sequencing in imidacloprid resistant populations.

Zhu et al. classified up-regulated cytochrome p450 in an imidacloprid resistant population; many of the cytochrome p450 classified in our study belong to the same clans that Zhu found to be import in imidacloprid resistance[14]. Clements et al. also used RNA sequencing to classify up-regulated transcripts in an imidacloprid resistant population, in both studies the up-regulation of similar mechanisms was observed including the up-regulation of transcripts encoding for cytochrome p450 9z4[11].

Examining the data further, we ran an enrichment analysis on the gene ontology terms for which the transcripts encoded. The enrichment analysis suggested that members of the second generation population over expressed multiple metabolic processes, including terms that correspond to increased oxidoreductase activity, monooxygenase activity, and structural constituents of the cuticle. This suggests that individuals representing the second generation up-regulate molecular mechanisms of resistance that, in turn, give rise to higher imidacloprid resistance. Although the stresses that beetles face in an agricultural setting are vast, including different xenobiotics and environmental stressors, the transcript abundance data clearly demonstrated that there are many differences in the gene regulation between the first and second generation population; many of which can be tied to insecticide resistance. Our results provide pest managers with valuable insight describing mechanisms by which beetles cope with insecticide insults, including the suggestion that to effectively control problematic populations of *L. decemlineata,* the genetic mechanisms of resistance must be consider including possible uses if enzymatic detoxification inhibitors.

5. Conclusions

Leptinotarsa decemlineata is a major agricultural pest of potatoes. It is of upmost importance to understand differences in levels of observed insecticide resistance that correspond to the phenology of *L. decemlineata* over a growing season. This study demonstrates that there is phenotypic variation in imidacloprid susceptibility within a resistant population over a growing season. The results of this study further demonstrate that researchers, producers, and pest management practitioners may benefit from an improved understanding of when, during the growing season, this insect may be better prepared to cope with insecticide inputs. This study also demonstrates the specific up-regulation of a unique set of transcripts, a portion of which may encode the dominant mechanisms of insecticide resistance. The differential expression, and overall abundance, of these transcripts provide us a glimpse into how these economically important pests deal with insecticide insults and aid in our ability to determine the specific mechanisms of resistance, which may lead to more precision delivery of pest management options that slow the pace of resistance development.

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8. Supplemental Material

Supplementary Table 1. List of up-regulated transcripts in the second generation population that could encode for increased imidacloprid resistance and transcript ID's. NCBI accession numbers represent the BLAST hit.

Transcript Blast x Result	Fold Change	NCBI accession number	Transcript I.D.
Emerging from Diapause Vs. Second Generation			
cuticle protein 1	110.520	XP_970381.1	LDEC003423
cuticle protein 1	7.894	XP_970381.1	LDEC011657
cuticle protein 20	20.500	XP_968593.1	LDEC017994
cuticle protein cp5	33.271	XP_974938.1	LDEC006894
cuticle protein cp5	37.573	XP_974938.1	LDEC006895
cuticle protein cp5	166.128	XP_973942.1	LDEC006896
cuticle protein cp5	28.851	XP_974938.1	LDEC021944
cuticle protein cp6	8.050	XP_973788.1	LDEC006905
cuticle protein precursor	21.469	NP_001156673.1	LDEC015503
cuticular protein	993.839	XP_966639.1	LDEC003961
cuticular protein 100a cg12045-pa	50.793	XP_001811600.1	LDEC005680
cuticular protein 49aa cg30045-pb	12.668	XP_968434.1	LDEC023002
cuticular protein 62bc cg1919-pa	226.513	XP_968445.1	LDEC003211
cuticular protein 65au	6.304	NP_652661.1	LDEC006899
cuticular protein 92f cg5494-pa	202.820	XP_969801.1	LDEC014400
cuticular protein analogous to peritrophins 1-g precursor	7.694	NP_001161924.1	LDEC013009
cuticular protein analogous to peritrophins 1-h precursor	11.608	NP_001161918.1	LDEC016579
cuticular protein analogous to peritrophins 1-i precursor	11.514	NP_001161900.1	LDEC007424
cuticular protein analogous to peritrophins 1-j precursor	14.474	NP_001161901.1	LDEC015701
cuticular protein analogous to peritrophins 3-a1 precursor	13.173	NP_001161910.1	LDEC015793
cuticular protein analogous to peritrophins 3-a2 precursor	12.845	NP_001161909.1	LDEC004878
cuticular protein analogous to peritrophins 3-b precursor	13.453	NP_001073566.1	LDEC010572
cuticular protein analogous to peritrophins 3-c5 isoform 1 precursor	37.278	NP_001073569.1	LDEC014321
cuticular protein analogous to peritrophins 3-d1 precursor	21.204	NP_001161908.1	LDEC004876
cuticular protein analogous to peritrophins 3-d2 precursor	4.279	NP_001073568.1	LDEC009655
cuticular protein analogous to peritrophins 3-e	5.416	NP_001161915.1	LDEC016473
cuticular protein isoform a	437.191	NP_647668.1	LDEC024510
cuticular protein ld-cp1v1	473.248	XP_970573.1	LDEC005679
cuticular protein ld-cp3	24.439	XP_973909.1	LDEC006897
cuticular protein ld-cp3	1821.342	XP_973909.1	LDEC006898
cuticular protein precursor	204.093	NP_001161316.1	LDEC013733
cuticular protein precursor	75.476	NP_001161313.1	LDEC014399

cuticular protein precursor	63.599	NP_001161313.1	LDEC021671
cuticular protein rr-1 family (agap000344-pa)	108.965	XP_971011.1	LDEC013734
cuticular protein rr-2 family (agap001664-pa)	9.113	XP_001816516.1	LDEC011784
cuticular protein rr-2 family (agap001669-pa)	7.221	XP_970955.1	LDEC022019
cuticular protein rr-2 family (agap012466-pa) partial	4.268	XP_971993.2	LDEC020133
cytochrome p450	7.558	XP_968884.1	LDEC008798
cytochrome p450	167.761	XP_973153.1	LDEC016769
cytochrome p450	14.069	XP_968884.1	LDEC016770
cytochrome p450	7.897	XP_973153.1	LDEC023589
cytochrome p450 cyp18a1	8.101	NP_001123908.1	LDEC011152
cytochrome p450 cyp6bk17	7.881	XP_969746.2	LDEC005602
cytochrome p450 cyp6bk17	9.667	XP_969813.1	LDEC005603
cytochrome p450 monooxigenase cyp4q3	5.266	NP_001107847.1	LDEC008923
cytochrome p450 monooxygenase	9.347	NP_001137200.1	LDEC005601
glutathione-s-transferase gst	3.334	XP_975048.1	LDEC015622
glutathione-s-transferase gst	3.224	XP_975048.1	LDEC023492
probable cytochrome p450 4aa1	3.405	XP_973810.2	LDEC007837
pupal cuticle protein	25.805	XP_969263.1	LDEC013799
Late Emergence Vs. Second Generation			
PREDICTED: similar to AGAP002051-PA (Highly similar to Atp-binding cassette sub family G with 98% similarity and 73% identity)	3.632	XP_968555.1	LDEC018634
abc transporter	4.480	XP_973444.1	LDEC003853
cuticle protein 1	631.133	XP_970381.1	LDEC003423
cuticle protein 1	18.038	XP_970381.1	LDEC003431
cuticle protein 1	10.693	XP_970381.1	LDEC011656
cuticle protein 1	9.724	XP_970381.1	LDEC011657
cuticle protein 1	6.890	XP_970381.1	LDEC011658
cuticle protein 20	83.819	XP_968593.1	LDEC017994
cuticle protein cp5	12.644	XP_974938.1	LDEC003447
cuticle protein cp5	3.736	XP_974938.1	LDEC003452
cuticle protein cp5	21.417	XP_974938.1	LDEC006894
cuticle protein cp5	57.061	XP_974938.1	LDEC006895
cuticle protein cp5	98.979	XP_973942.1	LDEC006896
cuticle protein cp5	51.360	XP_974938.1	LDEC021944
cuticle protein cp6	10.990	XP_973788.1	LDEC006905
cuticle protein precursor	16.997	NP_001156673.1	LDEC015503
cuticular protein	1411.250	XP_966639.1	LDEC003961
cuticular protein 100a cg12045-pa	52.652	XP_001811600.1	LDEC005680
cuticular protein 49aa cg30045-pb	63.296	XP_968434.1	LDEC023002
cuticular protein 62bc cg1919-pa	603.435	XP_968445.1	LDEC003211
cuticular protein 65au	7.753	NP_652661.1	LDEC006899

cuticular protein 92f cg5494-pa	655.360	XP_969801.1	LDEC014400
cuticular protein analogous to peritrophins 1-g precursor	7.774	NP_001161924.1	LDEC013009
cuticular protein analogous to peritrophins 1-h precursor	14.829	NP_001161918.1	LDEC016579
cuticular protein analogous to peritrophins 1-i precursor	17.544	NP_001161900.1	LDEC007424
cuticular protein analogous to peritrophins 1-j precursor	18.878	NP_001161901.1	LDEC015701
cuticular protein analogous to peritrophins 3-a1 precursor	15.046	NP_001161910.1	LDEC015793
cuticular protein analogous to peritrophins 3-a2 precursor	18.765	NP_001161909.1	LDEC004878
cuticular protein analogous to peritrophins 3-b precursor	28.417	NP_001073566.1	LDEC010572
cuticular protein analogous to peritrophins 3-c5 isoform 1 precursor	74.786	NP_001073569.1	LDEC014321
cuticular protein analogous to peritrophins 3-d1 precursor	17.483	NP_001161908.1	LDEC004876
cuticular protein analogous to peritrophins 3-d2 precursor	8.677	NP_001073568.1	LDEC009655
cuticular protein analogous to peritrophins 3-e	8.849	NP_001161915.1	LDEC016473
cuticular protein isoform a	246.784	NP_647668.1	LDEC024510
cuticular protein ld-cp1v1	1804.609	XP_970573.1	LDEC005679
cuticular protein ld-cp3	34.794	XP_973909.1	LDEC006897
cuticular protein ld-cp3	1212.622	XP_973909.1	LDEC006898
cuticular protein precursor	835.095	NP_001161316.1	LDEC013733
cuticular protein precursor	133.291	NP_001161313.1	LDEC014399
cuticular protein precursor	88.890	NP_001161313.1	LDEC021671
cuticular protein rr-1 family (agap000344-pa)	549.102	XP_971011.1	LDEC013734
cuticular protein rr-1 family (agap009876-pa)	5.127	XP_973663.1	LDEC003453
cuticular protein rr-2 family (agap001664-pa)	10.423	XP_001816516.1	LDEC011784
cuticular protein rr-2 family (agap001669-pa)	14.606	XP_970955.1	LDEC022019
cuticular protein rr-2 family (agap012466-pa) partial	8.283	XP_971993.2	LDEC020133
cytochrome p450	11.032	XP_968884.1	LDEC008798
cytochrome p450	199.748	XP_973153.1	LDEC016769
cytochrome p450	9.533	XP_968884.1	LDEC016770
cytochrome p450	11.912	XP_973153.1	LDEC023589
cytochrome p450 9z4	2.627	NP_001164248.1	LDEC015774
cytochrome p450 cyp18a1	10.824	NP_001123908.1	LDEC011152
cytochrome p450 cyp6bk17	23.897	XP_969746.2	LDEC005602
cytochrome p450 cyp6bk17	74.380	XP_969813.1	LDEC005603
cytochrome p450 monooxigenase cyp4q3	5.979	XP_311224.2	LDEC008874
cytochrome p450 monooxygenase	33.636	NP_001137200.1	LDEC005601
glutathione-s-transferase gst	3.265	XP_975048.1	LDEC015622
glutathione-s-transferase gst	3.302	XP_975048.1	LDEC023492
PREDICTED: similar to carboxylesterase	3.389	XP_968987.2	LDEC014844
PREDICTED: similar to Cyp49a1	6.804	XP_970738.1	LDEC001293
pupal cuticle protein	25.557	XP_969263.1	LDEC013799
End of First Generation Vs. Second Generation			

PREDICTED: similar to AGAP002051-PA (Highly similar to Atp-binding cassette sub family G with 98% similarity and 73% identity)	5.366	XP_968555.1	LDEC018634
abc transporter	4.404	XP_973444.1	LDEC003853
antennae-rich cytochrome p450	4.600	XP_970633.1	LDEC007220
cuticle protein 1	260.013	XP_970381.1	LDEC003423
cuticle protein 1	8.003	XP_970381.1	LDEC003431
cuticle protein 1	10.984	XP_970381.1	LDEC011656
cuticle protein 1	22.724	XP_970381.1	LDEC011657
cuticle protein 20	105.933	XP_968593.1	LDEC017994
cuticle protein cp5	7.037	XP_974938.1	LDEC003447
cuticle protein cp5	20.196	XP_974938.1	LDEC006894
cuticle protein cp5	46.171	XP_974938.1	LDEC006895
cuticle protein cp5	141.040	XP_973942.1	LDEC006896
cuticle protein cp5	60.310	XP_974938.1	LDEC021944
cuticle protein cp6	9.999	XP_973788.1	LDEC006905
cuticle protein precursor	7.936	NP_001156673.1	LDEC015503
cuticular protein	1166.062	XP_966639.1	LDEC003961
cuticular protein 100a cg12045-pa	67.658	XP_001811600.1	LDEC005680
cuticular protein 49aa cg30045-pb	54.686	XP_968434.1	LDEC023002
cuticular protein 62bc cg1919-pa	624.162	XP_968445.1	LDEC003211
cuticular protein 65au	6.886	NP_652661.1	LDEC006899
cuticular protein 92f cg5494-pa	1399.634	XP_969801.1	LDEC014400
cuticular protein analogous to peritrophins 1-h precursor	16.921	NP_001161918.1	LDEC016579
cuticular protein analogous to peritrophins 1-i precursor	20.837	NP_001161900.1	LDEC007424
cuticular protein analogous to peritrophins 1-j precursor	12.087	NP_001161901.1	LDEC015701
cuticular protein analogous to peritrophins 3-a1 precursor	15.383	NP_001161910.1	LDEC015793
cuticular protein analogous to peritrophins 3-a2 precursor	9.397	NP_001161909.1	LDEC004878
cuticular protein analogous to peritrophins 3-b precursor	24.784	NP_001073566.1	LDEC010572
cuticular protein analogous to peritrophins 3-c5 isoform 1 precursor	65.874	NP_001073569.1	LDEC014321
cuticular protein analogous to peritrophins 3-d1 precursor	24.258	NP_001161908.1	LDEC004876
cuticular protein analogous to peritrophins 3-d2 precursor	9.956	NP_001073568.1	LDEC009655
cuticular protein analogous to peritrophins 3-e	6.415	NP_001161915.1	LDEC016473
cuticular protein isoform a	355.026	NP_647668.1	LDEC024510
cuticular protein ld-cp1v1	399.977	XP_970573.1	LDEC005679
cuticular protein ld-cp3	10.131	XP_973909.1	LDEC006897
cuticular protein ld-cp3	536.339	XP_973909.1	LDEC006898
cuticular protein precursor	202.230	NP_001161316.1	LDEC013733
cuticular protein precursor	49.473	NP_001161313.1	LDEC014399
cuticular protein precursor	40.983	NP_001161313.1	LDEC021671

cuticular protein rr-1 family (agap000344-pa)	371.136	XP_971011.1	LDEC013734
cuticular protein rr-2 family (agap001664-pa)	16.363	XP_001816516.1	LDEC011784
cuticular protein rr-2 family (agap001669-pa)	13.749	XP_970955.1	LDEC022019
cuticular protein rr-2 family (agap012466-pa) partial	6.438	XP_971993.2	LDEC020133
cytochrome p450	5.647	XP_968884.1	LDEC008798
cytochrome p450	271.828	XP_973153.1	LDEC016769
cytochrome p450	12.544	XP_968884.1	LDEC016770
cytochrome p450	3.417	XP_973153.1	LDEC022076
cytochrome p450	6.872	XP_973153.1	LDEC023589
cytochrome p450 cyp18a1	8.365	NP_001123908.1	LDEC011152
cytochrome p450 cyp6bk17	10.185	XP_969746.2	LDEC005602
cytochrome p450 cyp6bk17	22.486	XP_969813.1	LDEC005603
cytochrome p450 mitochondrial precursor (protein shadow)	4.026	XP_970122.2	LDEC011422
cytochrome p450 monooxigenase cyp4q3	4.333	NP_001107847.1	LDEC008923
cytochrome p450 monooxygenase	13.312	NP_001137200.1	LDEC005601
glutathione-s-transferase gst	4.174	XP_975048.1	LDEC015622
glutathione-s-transferase gst	4.343	XP_975048.1	LDEC023492
probable cytochrome p450 4aa1	5.861	XP_973810.2	LDEC007837
pupal cuticle protein	17.758	XP_969263.1	LDEC013799

Supplementary Table 2. Enrichment analysis between GO terms from the up-regulated transcripts of the second generation population compared to all transcripts.

GO Term	Name	FDR	Over/Under expressed GO term in systemic-3 population
GO:0005576	extracellular region	0.0001	Over
GO:0006030	chitin metabolic process	0.0001	Over
GO:1901071	glucosamine-containing compound metabolic process	0.0001	Over
GO:0006040	amino sugar metabolic process	0.0001	Over
GO:0006022	aminoglycan metabolic process	0.0001	Over
GO:0042302	structural constituent of cuticle	0.0001	Over
GO:0044260	cellular macromolecule metabolic process	0.0001	Under
GO:0044424	intracellular part	0.0001	Under
GO:0043229	intracellular organelle	0.0001	Under
GO:0043226	Organelle	0.0001	Under
GO:0005622	Intracellular	0.0001	Under
GO:0044464	cell part	0.0001	Under
GO:0005623	Cell	0.0001	Under
GO:1901564	organonitrogen compound metabolic process	0.0001	Over
GO:0016491	oxidoreductase activity	0.0001	Over
GO:0044267	cellular protein metabolic process	0.0001	Under
GO:0009072	aromatic amino acid family metabolic process	0.0001	Over
GO:0009987	cellular process	0.0001	Under
GO:0043231	intracellular membrane-bounded organelle	0.0001	Under
GO:0043227	membrane-bounded organelle	0.0001	Under
GO:1901265	nucleoside phosphate binding	0.0001	Under
GO:0000166	nucleotide binding	0.0001	Under
GO:0036094	small molecule binding	0.0002	Under
GO:0044444	cytoplasmic part	0.0002	Under
GO:0055114	oxidation-reduction process	0.0003	Over
GO:0019538	protein metabolic process	0.0004	Under
GO:0097159	organic cyclic compound binding	0.0004	Under
GO:1901363	heterocyclic compound binding	0.0006	Under
GO:0032991	macromolecular complex	0.0006	Under
GO:0010467	gene expression	0.0007	Under
GO:0019752	carboxylic acid metabolic process	0.0008	Over
GO:0006082	organic acid metabolic process	0.0011	Over
GO:0043436	oxoacid metabolic process	0.0011	Over

GO:0044710	single-organism metabolic process	0.0013	Over
GO:0034645	cellular macromolecule biosynthetic process	0.0014	Under
GO:0009059	macromolecule biosynthetic process	0.0014	Under
GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	0.0022	Over
GO:0006139	nucleobase-containing compound metabolic process	0.0027	Under
GO:0032553	ribonucleotide binding	0.0027	Under
GO:0017076	purine nucleotide binding	0.0027	Under
GO:0001882	nucleoside binding	0.0027	Under
GO:0004058	aromatic-L-amino-acid decarboxylase activity	0.0031	Over
GO:0090304	nucleic acid metabolic process	0.0035	Under
GO:0035639	purine ribonucleoside triphosphate binding	0.0035	Under
GO:0001883	purine nucleoside binding	0.0035	Under
GO:0032555	purine ribonucleotide binding	0.0035	Under
GO:0032550	purine ribonucleoside binding	0.0035	Under
GO:0032549	ribonucleoside binding	0.0035	Under
GO:0005737	Cytoplasm	0.0036	Under
GO:0004497	monooxygenase activity	0.0040	Over
GO:0003676	nucleic acid binding	0.0049	Under

CHAPTER 4: Understanding the demographic history of *Leptinotarsa decemlineata* through the use of Dadi and Treemix

Authors: Justin Clements and Sean Schoville

1. Introduction:

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say) is a major agricultural pest of potatoes. Currently *L. decemlineata* has geographic ranges encompassing over 16 million km^2, including the United States, Europe, and Asia [1,2]. *L. decemlineata* is known to have originated in northern Mexico and spread into the United States with the introduction of agricultural crops in the family *Solanum* including *Solanum tuberosum* (potatoes) [1]. With the domestication and propagation of agricultural crops in the *Solanum* family, the beetles have spread and become a major agricultural pest globally. Using historical records, the route that the beetle migrated can be traced throughout the world [3]. Records suggest that the first major outbreak and agricultural damage due to *L. decemlineata* occurred in the United States in 1859, 100 miles west of Omaha, Nebraska [2]. The beetles then spread throughout regions of agricultural production of *Solanum* cultivars in the United States, reaching the Atlantic coast by 1880 [4]. After many small populations were unable to establish in Europe, a population was established in 1922 in France, which then migrated throughout the continent [4]. Though beetle populations have expanded throughout the world, little is known about the similarities and differences between these populations, including the genetic variation between populations in different geographic regions. A major problem facing growers is the ability of these beetles to

become resistant to pesticides, which is complicated by the differences in resistance between populations. Differences in resistance have been noted in different regions of the Unites States, including Michigan and Wisconsin [5]. One hypothesis is that this could be due to genetic differences between different populations of beetles. We sought to examine if invasive populations contain a considerable amount of genetic variation and exhibit genetic differences across their distribution. In addition, using the known invasion history, we attempt to estimate the underlying genome-wide mutation rate. A mutation rate estimate allows us to examine if the beetles have a high rate of evolution, possibly explaining the rapid evolution of resistance to pesticides.

2. Experimental Methods:

Genomic scaffolds for *L. decemlineata* were obtained and downloaded from NCBI (the genome assembly based on the i5K CPB genome project). Scaffolds were indexed using bowtie2 version 2.1.0 [6]. RNA sequencing (RNAseq) reads for 8 different populations were obtained: 2 Wisconsin populations, a Michigan population, 2 lab strains originating from a New Jersey field population, and 3 samples from European populations. All the RNA seq data came from pooled populations or was combined into a population sample after it was obtained from individual reads. For the Wisconsin, Michigan and lab strains from New Jersey, RNAseq data was aligned to the genome scaffolds, using Bowtie2 version 2.1.0 [6], to generate aligned SAM files. We used Burrows-Wheeler Aligner (BWA) version 0.7.5a [7] to align the RNAseq from the 3 populations from Europe. Using SAMtools version 0.1.19 [8] we converted the SAM files into BAM files. All BAM files were sorted and indexed with SAMtools. To identify single nucleotide polymorphisms (SNPs), SAMtools mpileup was used to generate a BCF file and was

converted into a VCF file with BCFtools within SAMtools. All SNP calls were filtered with VCFtools version 0.1.11[9]. We used a minimum quality score of 30 and minimum depth of 10 reads to filter SNPs. All indels were removed from this study. The resulting SNPs were then used for population genetic inference.

Treemix

All SNPs were sorted using vcf-sort in VCFtools version 0.1.11[9], zipped using Bgzip, and indexed using Tabix within SAMtools. We merged the VCF files together using vcf-merge within VCFtools. Using a custom perl script (UW Biotech center), the SNP allele counts were extracted at each SNP site and standardized according to the number of individuals sequenced in pooled samples. The data was formatted and analyzed in Treemix version 1.12 [10]. We ran Treemix with SNPs in groups of 1000 to estimate a population graph representing the genealogical relationship among populations, choosing to root the tree with a Wisconsin population.

Dadi

Dadi version 1.6.3 [11] was used to estimate the demographic history of the Midwestern populations (Wisconsin and Michigan) and the European populations. Dadi uses the genome-wide allele frequency spectrum to infer demographic parameters under a variety of population histories. We employed a one-dimensional expansion model found within Dadi to reconstruct the history and estimate population genetic parameters in *L. decemlineata*. This one-dimensional model was used to model exponential growth at a time point T in the past for both the Midwestern and European populations. Using the known population boom of *L. decemlineata* in

Europe, we employed Dadi to model an instantaneous population size change followed by exponential growth. This was used to estimate the genome-wide mutation rate, assuming a known expansion time of 89 years in the European populations [2]. We used the same model in the Midwest populations with an expansion time of 155 years since the first population boom was recorded in Nebraska [2], and the mutation rates of the two geographic regions were compared. Then we employed a two-dimensional split model in Dadi to estimate instantaneous size change followed by exponential growth and then a separation of the Midwestern and European populations to estimate the time of divergence of these populations. A visualization of these models can be seen in Figure 1.

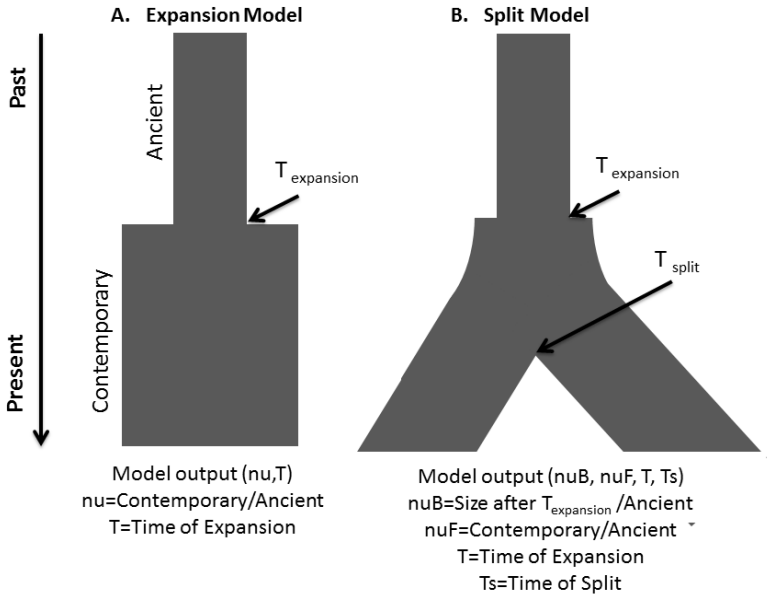

Figure 1: A) Dadi expansion model, B) Dadi split model

3. Results:

Treemix

Figure 2 shows the estimated drift parameter from a root in the Wisconsin populations, which was used to observe patterns in population splits. The output of Treemix shows a distinct grouping of beetles from European populations that differ from the United States populations, while the New Jersey strain and the Midwest populations fall into separate groups within the United States. The amount of drift that separates populations in the Midwestern region suggests a substantial amount of local genetic structure.

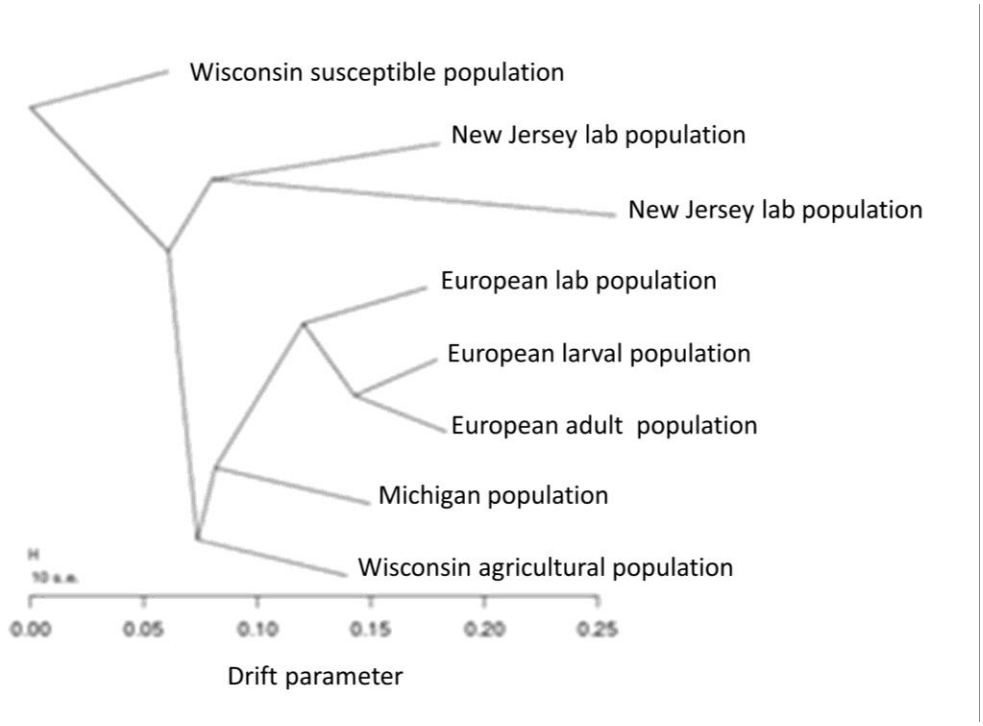

Figure 2: Treemix output of *Leptinotarsa decemlineata* pattern of population splits.

Dadi

Using Dadi's one-dimensional expansion model, along with known expansion times in the Midwest and Europe, we calculated a mutation rate in *L. decemlineata* for both the European

and Midwest populations (Equation 1). We compared the mutation rate between the populations and found a mutation rate of 1.73 x 10^{-8} mutations per base per year in Europe and 1.48 x10^{-8} mutations per base per year in the Midwest populations.

Equation 1:

$$Known\ time = 2 * (Generation\ time) * (Dadi\ Time) * \frac{(Theta\ generated\ by\ Dadi)}{4 * mutation\ rate * (RNAseq\ Coverage)}$$

$$Mutation\ rate = \frac{(Generation\ time)(Dadi\ Time)(Theta\ generated\ by\ Dadi)}{2 * Known\ time * (RNAseq\ Coverage)}$$

Next we used a split model in Dadi to model the time of divergence between the European populations and the Midwest populations. The split model gave us a time of divergence of 181.9 years.

4. Discussion:

The analysis of SNP allele frequencies from Treemix indicates a clear subset comprised of the European populations that grouped away from the Midwest and New Jersey populations. The European populations have the least amount of genetic drift from one another suggesting that they are very similar in their genetic makeup. The Midwest populations, on the other hand, exhibit substantial genetic structure over a small geographic region. The larger degree of structure is in agreement with historical accounts claiming that beetles in the United States have

migrated from the Great Plains, subsequently diverging into regional populations, as well as there being a single introduction to Europe.

Using Dadi in conjunction with known expansion times of *L. decemlineata* in the United States and Europe, we were able to calculate a mutation rate, as well as the time at which the European populations and the Midwest populations split from one another. From historical records, it is clear that there was a large population expansion in the Midwest in 1859[2], and another large expansion in Europe starting in 1922[4]. Using these time constraints, we were able to generate two separate mutation rates; one for each of the European and Midwestern populations. The European mutation rate was $1.73x10^{-8}$ while the Midwest mutation rate was $1.48x10^{-8}$, both of these rates being very similar to one another. Comparing this to the known mutation rate of *Drosophila melanogaster* of $8.4x10^{-9}$ [12], suggests *L. decemlineata* may be an order of magnitude faster in its evolutionary rate. Since there is less certainty in the age of expansion of Midwestern populations, we used the mutation rate calculated for the European population to infer the expansion time for the Midwest population with the starting date of 144.7 years ago. Next, we estimated the time that the European population and Midwest populations split from one another. Dadi's two-dimensional model indicated that the population split occurred 181.9 years ago, suggesting that the European population may have been derived from an as yet undetermined source population.

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