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The Evolution of Spinosad Resistance in Colorado Potato Beetles (*Leptinotarsa decemlineata*)

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THE EVOLUTION OF SPINOSAD RESISTANCE IN COLORADO POTATO BEETLES (*LEPTINOTARSA DECEMLINEATA*)

by

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This manuscript has been read and accepted by the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

The Evolution of Spinosad Resistance in Colorado Potato Beetles (*Leptinotarsa decemlineata*)

by

Coby Michael Klein

Advisor: Dr. Mitchell Baker

Colorado potato beetles (*Leptinotarsa decemlineata*) are a major pest of cultivated potato plants worldwide. They are well-known for their ability to rapidly evolve resistance to all major classes of pesticides. Defoliation of potato plants by *L. decemlineata* can reduce potato yields by a considerable margin. The damage done by resistant beetles is steep and much research is focused on developing new chemical controls, especially those derived from naturally occurring compounds. Spinosad is a relatively new natural product insecticide, introduced approximately a decade ago, suitable for use in organic farming. Potato beetles on Long Island, NY developed very strong resistance to spinosad earlier in this decade. In order to assess the level of resistance and to what degree it has reverted towards susceptibility in the past half decade, A survey of spinosad resistance in *L. decemlineata* was conducted on Long Island. Strong resistance is tied to overuse of spinosad on several fields. Resistance has partially reverted since beetle control using spinosad failed and its use was discontinued. This finding implies that there may be evolutionary trade-offs associated with spinosad resistance. A set of traits were examined to attempt to determine if those potential trade-offs were related to reproductive fitness. There was a negative relationship between resistance level and egg development time, providing evidence for

pleiotropic effects of resistance. Evidence was also found against pleiotropy from fitness indices that showed no relationship between resistance and general reproductive fitness. Dominance of resistance is an important factor in determining pest control strategies. So to clarify the level of dominance of spinosad resistance in separate populations, we investigated colonies of *L. decemlineata* in Michigan, Maine, and Long Island. There was considerable variation in dominance between each site in 2010 but none of the sites were significantly different from additive resistance. In 2012, resistance on Long Island was significantly different from additive but not significantly different from fully recessive. This finding could indicate the spread of resistance alleles from the most resistant fields across the eastern part of the Island. Widespread, recessive resistance to spinosad in *L. decemlineata* on Long Island is theoretically easier to manage as long as resistance is costly to the beetles.

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INTRODUCTION

The use of chemical pesticides to control arthropod pests is a large and still growing industry. In 2012, worldwide spending on all types of pesticides—including fungicides and herbicides—totaled nearly $56 billion (Atwood & Paisley-Jones, 2012). Sixteen percent of that total—approximately $9 billion of that—was spent in the United States. According to that report, spending on pesticide in the USA has remained fairly steady since 2008 but has risen steadily in the rest of the world. Part of the reason for that increase is likely an increase in resistance to pesticides, requiring higher, more frequent doses to control pests. Resistance is the development of a strain capable of a dose of pesticide lethal to a majority of the population (ffrench-Constant & Roush, 1990). In more practical terms, resistance is a genetic change, resulting from selection with pesticide, that impairs control in the field (Sawicki, 1987).

Pesticide resistance is typically observed first in the field when there is no significant reduction in pest numbers after treatment. In laboratory conditions, resistance is measured as an increase in the dose or concentration of pesticide required to kill 50% of the sample, referred to as the LD_{50} when the exact dose is known and the LC_{50} when it is not (e.g. when the toxin is directly applied to the subject vs. the subject feeding on a leaf treated with an insecticide solution) (Alyokhin, et al. 2008). Resistance ratios are obtained by calculating the ratio of LD_{50} or LC_{50} of the resistance population to the LD_{50} or LC_{50} of a control population known to be susceptible to the toxin of interest.

Resistance may be granted by one of several mechanisms. Some organisms have an increased metabolic capability to detoxify xenobiotic compounds. This is usually accomplished via the overexpression of P450 genes, glutathione *S*-trasferases, or the upregulation or amplification of esterases (Li, Schuler, & Berenbaum, 2007). Mutations to target sites that

prevent pesticides from binding to their targets are common as well. Thickening of the cuticle prevents toxins from penetrating and increased excretion also plays a role in insecticide tolerance. ATP binding cassette transporters have recently been identified as a likely instrument for this type of process (Gott et al., 2017; Alyokhin, et al. 2008). Some insects that show physiological tolerance also display avoidance behavior towards the same toxins (Hoy & Head, 1995; Alyokhin & Ferro, 1999).

There is a variety of ways to analyze the genetics of resistance. According to Alyokhin et al. (2008), a positive response to selection with a pesticide can show the presence of additive genetic variation for resistance within the population. They also suggest crosses between resistant and susceptible subjects and backcrosses to the parent strains can be used to determine dominance, sex-linkage, and to distinguish monogenic resistance from polygenic resistance. Quantitative genetic estimates of heritability can show potential for subsequent resistance evolution. And linkage mapping and cDNA sequencing are also used for identifying specific resistance genes.

The dominance of a resistant phenotype is a key factor that determines how resistance can spread. Dominance is the measure of the relative occurrence of the phenotype of the heterozygote relative to the phenotype of the two corresponding homozygotes (Bourguet, Genissel and Raymond 2000). The level of dominance of resistance to a single dose of insecticide is often inversely related to the concentration of the dose to which the insects are exposed and can also be altered by the environment (Liu and Tabashnik 1997, Sayyed, et al. 2000, Tabashnik, Gould and Carrière 2004, Szendrei, et al. 2011). Dominance can also vary based on the number of genetic loci responsible for conferring resistance. Lab selection for resistance starting from a susceptible baseline often results in polygenic resistance since the

starting population is relatively small compared to field populations. Resistance, therefore, must be built on common, existing variation, which is likely to utilize multiple genes of small effect to obtain the desired result (Roush & McKenzie, 1987). When resistance depends on having a set of alleles rather than a single allele, the spread of resistance will be more gradual and there will be a large additive component. Monogenic resistance is more likely to arise in the field where population sizes are much bigger. The probability of a single resistance mutation appearing in the field is much greater than in a small lab population. A single gene with a large effect will spread quickly and the trait will be less additive.

Colorado potato beetles (*Leptinotarsa decemlineata*) are the most widespread insect defoliator of potato plants. Their original home range is the western United States and Central Mexico. They feed on ten species of native and exotic solanaceous plants. Some populations are host specific. In Arizona, for example, *L. decemlineata* specialize on white horsenettle (*Solanum elaeagnifolium*) in their native range but can adapt to other hosts when necessary (Hsiao, 1978).

The origin of the pest populations of *L. decemlineata* has been in dispute. Because of the high diversity of *Leptinotarsa* in Mexico, it was presumed at one time that *L. decemlineata* originated there and spread northward as first the Spanish explorers and then later cattle ranching pushed the beetles' chief host plant, *S. rostratum,* northward. It was these immigrant populations that jumped host plants to cultivated potato (*Solanum tuberosum*) (Casagrande, 1985). More recent research has revealed that L. decemlineata is, in fact, indigenous to the eastern slope of the Rocky Mountains in the United States, where it fed on *S. rostratum* (or possibly another *Solanum* species) before the arrival of European explorers and pest populations apparently developed from these western plains beetles (Izzo, Chen, & Hawthorne, 2018).

Once the host plant switch was made, *L. decemlineata* rapidly spread across the United States. It was first reported on cultivated potato in 1859 in Nebraska (Jacques, 1988). It reached the east coast of the US in 15 years (Casagrande, 1987). It completed the colonization of North America by 1919 (Ivanschik & Izhenvsky, 1981). *L. decemlineata* began colonizing Europe less than 20 years after becoming potato pests in the US. It quickly became firmly established there around the time of World War II and spread from France, to the Soviet Union, and across Asia (Ivanschik & Izhenvsky, 1981).

Adult *L. decemlineata* overwinter in the soil at forest/field edges at depths between approximately 7.5 and 12.5 cm. Those that dig deeper burrows tend to survive at a slightly better rate (Lashomb, et al., 1984). Newly emerged adults in the spring disperse by foot or by flight to find food and mate (Voss & Ferro, 1990). Upon finding food, mating may commence right away. Females who mated the previous fall, prior to diapause, may be able to lay viable eggs without mating again in the spring (Tauber, et al., 1988). Females lay egg masses of generally between 20-60 eggs on the undersurface of potato foliage. Once hatched, larvae pass through four instars before pupation (Hare, 1990). They pupate in the soil for approximately ten days. Upon emerging as adults, they may breed right away, migrate to a new location and breed, or to enter diapause immediately, depending upon temperature, seasonality, and the plant quality (Tauber, et al.1988; Voss & Ferro, 1990). The number of generations produced per year generally varies by latitude. Warmer locales could produce as many as three generations annually, while colder climates may produce just one (Hiisaar, et al., 2016). Defoliation of potato by *L. decemlineata* during the middle of the growing season can reduce potato yields by as much as 64% (Hare J. D., 1980).

Chemical controls have been used on *L. decemlineata* since the late 19th century. Early pesticides were arsenicals, which were difficult to use and sometimes toxic to the plants themselves (Kuhar, et al., 2013). DDT was introduced in the 1940's and remained effective for nearly a decade, although *L. decemlineata* became one of the first pests to develop resistance to it, as well as to the other chlorinated hydrocarbons (Gauthier, Hofmaster, & Semel, 1981). Over a 40 year span, *L. decemlineata* developed resistance to more than 25 different insecticides, in every class of compounds (Kuhar, et al. 2013).

It is difficult to quantify the extent of the damage done by *L. decemlineata*. Estimates twenty years ago placed the figure at as much as $1.4 million a year just in the state of Michigan (Grafius, 1997). More recently, the annual losses caused by *L. decemlineata* in China were estimated at $3.2 million and could climb as high as $235 million if they colonize a larger area (Liu, Li, & Zhang, 2012).

Much of the recent research into chemical pest control has been directed towards compounds derived from natural products. That focus is necessary in part due to stricter regulations by the Environmental Protection Agency (EPA) regarding the registration of new pesticides and in part because of evolved resistance to existing ingredients (Dayan, Cantrell, & Duke, 2009). Natural products are an important source of new pesticides on the market. Between 1997 and 2010, nearly 30% of all new pesticide registrations were natural products or derived from natural products (Cantrell, Dayan, & Duke, 2012). According to Mann & Kaufman (2012) these chemicals are generally considered safer because they tend to break down faster than synthetic pesticides and tend to have a lower toxicity to non-target species. They also provide a potential economic advantage since they are easier to register than synthetics. They add that advantage may be cancelled out though by the difficulty of identifying the chemicals in the first

place and the perception within the industry that the development of natural products pesticides would not provide a big enough return on investment. Nevertheless, a variety of natural pesticides have been successfully tested and marketed in the past several decades.

Spinosad is a relatively new naturally derived pesticide, obtained from the fermentation of a soil actinomycete, *Saccharopolyspora spinosa*. It was first identified in 1985 in a soil sample from the Caribbean (Sparks, Crouse, & Durst, 2001). The active ingredients in spinosad are the spinosyns A & D, the two most active naturally occurring metabolites of S. spinosa (Thompson, Dutton, & Sparks, 2000). Spinosad acts by depolarizing insect neurons by activating nicotinic acetylcholine receptors and GABA receptors. That leads to hyperactivity of the neurons which causes involuntary muscle contractions and tremors (Wang, et al., 2006; Salgado, 1998). Prolonged spinosyn-induced hyperexcitation results in paralysis associated with neuromuscular fatigue (Salgado, 1998). Although spinosad acts on similar receptors to the neonicotinoids, spinosyn A does not appear to directly interact with GABA or nicotinic receptor sites, rather it operates by binding to a target site distinct from neonicotinoid pesticides or ivermectins (Orr, et al., 2009).

Spinosad offers a number of benefits over conventional pesticides. According to Thompson, et al. (2000), spinosad is readily degraded by the process of photolysis, especially when suspended in water so it poses little risk to groundwater systems when used properly. They also add that it has low toxicity to mammals and birds and only a moderate toxicity to fish. It is also not highly active against beneficial insects.

Environmental change may, from time to time, eliminate or weaken a source of selection pressure on the maintenance of certain traits. Those traits are then said to be under relaxed selection. When one source of selection is relaxed, other sources may act on the trait, potentially

leading to alterations of the phenotype (Lahti, et al., 2009). Traits may be eliminated once mutations that result in the loss of those traits are no longer under selective disadvantage (Coss, 1999). There may be multiple mechanisms through which evolutionary reductions of traits may take place. Traits may be lost over time through the accumulation of neutral mutations or more rapidly through indirect selection for energy economy or because of evolutionary trade-offs (Fong, Kane, & Culver, 1995). In some cases, though, traits may be retained for many generations under relaxed selection conditions. California ground squirrels (*Otospermophilus beecheyi*) innately recognize snakes as dangerous and exhibit anti-snake behaviors even in locations where serpentine predators have not lived for tens of thousands of years (Coss, 1991).

Reversion from a condition of pesticide resistance to susceptibility is fairly common once the use of chemical control measures is stopped. Laboratory studies demonstrate that reversal of resistance can often be nearly as rapid as its initial appearance. For example, it took just five generations without positive selection for cotton bollworms (*Helicoverpa armigera*) to lose 99% of their resistance to cypermethrin (Achaleke & Brévault, 2010).

Historically, relaxed selection has not received the same attention from biologists as positive selection and adaptation. Identifying the causes and mechanisms by which traits evolve and the need to demonstrate those in the absence of selective forces has until recently been a fairly challenging proposition (Lahti, et al., 2009).

Long Island, NY has been a literal breeding ground for pesticide resistance over the past century. Since the 1940s, every new class of pesticide used against *L. decemlineata* has failed, often after just a few years or less. In 2010, extremely high levels of spinosad resistance were found on two potato fields on Long Island's South Fork. Those fields subsequently discontinued spinosad use because it had become totally ineffective as a control agent. This presented an

opportunity to examine the resistance to spinosad on Long Island's East End under the lens of relaxed selection.

I studied the spread of resistance and its persistence under relaxed selection conditions. It was apparent from preliminary work that resistance had reverted on the South Fork in the absence of spinosad use. I attempted to measure the stability of spinosad resistance on the South Fork over time. It was expected that resistance would be highly unstable. Resistance to spinosad on the North Fork was not previously documented so I surveyed fields that had not been sampled before. Conversations with the growers revealed that spinosad use had not been as intensive there and some had not used it at all. I expected that beetles on the North Fork fields would not be as resistant as those on the South Fork.

The results of the survey described above indicated that spinosad resistance was highly unstable and declined on both the North and South Forks over the course of the survey period. Therefore I also looked at whether pleiotropic effects of resistance might be driving a reduction in the degree of resistance. I hypothesized that there would be a relationship between the measures of reproductive fitness traits and resistance.

Finally, since aspects of resistance evolution can differ in disparate populations, we examined the dominance of the resistance trait in three different geographic areas, and on Long Island in different seasons, to try to track the spread of resistance alleles and predict the effects of relaxed selection on these populations. We expected resistance to be most recessive on the most resistant fields, and most dominant on least resistant fields.

CHAPTER 1: A survey of resistance to spinosad in Colorado potato beetles (*Leptinotarsa decemlineata*) on Long Island

Long Island, NY is known as a hotbed of pesticide resistance in Colorado potato beetles (*Leptinotarsa decemlineata*). Beetles there have rapidly developed resistance to all major classes of pesticides. Most recently, Long Island beetles have developed resistance to spinosad, a relatively new compound that is derived from a soil actinomycete and is used in organic farming. Resistance was particularly strong on two fields on the Island's South Fork, leading to a complete failure of spinosad to control *L. decemlineata* within a few years. Use of spinosad was subsequently discontinued there, and the relaxation of selection pressure would likely bring about changes in patterns of resistance. In order to assess how resistance to spinosad on Long Island has changed over time, particularly since its failure on the South Fork, I conducted a survey of four organic fields on the North Fork of Long Island and two on the South Fork. Bioassay revealed that resistance was strongest on the South Fork fields every year the survey was conducted. Resistance ratios were between 12% and 95% lower in fields on the North Fork than fields on the South Fork. Resistance declined significantly over the course of the study. Resistance ratios declined by as much as 80% on both the North and South Fork. This reversion of resistance suggests that there could be pleiotropic effects associated with spinosad resistance.

Keywords: spinosad, resistance, Colorado potato beetle, *Leptinotarsa decemlineata*, resistance ratio, dose response, reversal of resistance

Colorado potato beetles (*Leptinotarsa decemlineata*) are the most widespread insect defoliator of cultivated potatoes worldwide. Potato beetles are specialists on solanaceous plants, particularly from the genus *Solanum*. The species may have originated in the highlands of south-central Mexico and historically ranged as far north as the Great Plains of the United States (Hsiao, 1978). In the mid-19th Century beetles from the plains near the eastern slope of the Rocky Mountains in the United States switched host plant from buffalo bur, *S. rostratum* to cultivated potato, *S. tuberosum* (Izzo, Chen, & Hawthorne, 2018). Since then the potato beetle has spread worldwide on potatoes and continues to expand its range as climate change opens more northern latitudes to potato cultivation.

Potato beetles have gained a reputation over the past century for rapidly becoming resistant to all types of chemical insecticides. That is especially true on Long Island, NY where very high levels of resistance to insecticides have been found in potato beetles and on average, insecticides fail within about 2 years (Georghiou, 1986). Evolution of resistance to the neonicotinoid pesticide imidacloprid, for example, was rapid but comparatively slower. This may be due to costs of imidacloprid resistance or changes in patterns of use by potato growers (Alyokhin, et al., 2015).

Several mechanisms may be responsible for granting pesticide resistance. Behavioral resistance refers to avoidance of treated plants by resistant individuals. After exposure to *Bt* potatoes, one group of *L. decemlineata* increased flight activity while a susceptible strain did not (Alyokhin & Ferro, 1999a). Physiological mechanisms prevent pesticide residues from entering the organism in the first place or remove residues from the body before taking effect. ATP binding cassette transporters have recently been identified as a likely instrument for this type of process (Gott, et al., 2017). Rapid excretion of several types of pesticide has been implicated in

resistance of *L. decemlineata* (Rose & Brindley, 1985; Olson, Dively, & Nelson, 2000; Mota-Sanchez, 2002; Krishnan, et al., 2007). Metabolic resistance is the result of enhanced enzymatic activity that targets pesticide compounds in the body. Most frequently this is due to activity of esterases, monooxygenases, and carboxylesterases (Alyokhin, Baker, Mota-Sanchez, Dively, & Grafius, 2008). Kaplanoglu et al. (2017) found that multiple genes coding for the production of cytochrome P450 (CYP) proteins and other detoxifying enzymes were significantly over expressed in imidacloprid-resistant *L. decemlineata* compared to a susceptible strain. Clements et al. (2016) also found CYP genes upregulated in imidacloprid-resistant *L. decemlineata* but not the susceptible strain. Genetic changes that prevent a pesticide from binding to its intended receptor is called target-site insensitivity. Changes to the α-subunit of the sodium channel were responsible for conferring permethrin resistance to *L. decemlineata* and may also play a part in imidacloprid resistance (Kim, et al., 2005; Tan, et al., 2005).

A number of factors have the greatest contribution to rapid evolution of resistance. The presence of resistance genes in the population before selection, the dominance of resistance alleles, strong selection pressure for resistance, and potential costs of resistance all play a role.

Unless the population already possesses what Georghiou (1972) describes as the potentiality for the development of resistance, then it is very unlikely that resistance could arise spontaneously. Georghiou failed to induce resistance in small populations of mosquitoes in lab studies even over 20-30 generations (Georghiou, 1963; Georghiou, 1969a; Georghiou, 1969b). Kikkawa was able to induce resistance to parathion in the lab by using x-rays (Kikkawa, 1964) and McKenzie et al. (1992) used chemical mutagens to create monogenic diazinon-resistant Australian sheep blowflies (*Lucilia cuprila*). In field conditions, the physical stress brought on

by sub-lethal doses of pesticide may increase the rate of mutation and facilitate the evolution of resistance (Gressel, 2011).

If resistance genes are present in a population then the rate of resistance evolution depends on the initial frequency of resistance genes in the population, the dominance of the resistance alleles, strength of selection and the presence or absence of pleiotropic effects of resistance (Roush & McKenzie, 1987). Population modelling has demonstrated that greater initial resistance allele frequency (IRAF) leads to faster resistance evolution when the population is sufficiently large to allow resistant individuals to reproduce with one another (Roush, 1994; Georghiou & Taylor, 1977). The dominance of resistance depends on the dose of pesticide that is used. A high enough dose can theoretically delay resistance evolution by killing heterozygotes, which effectively makes resistance recessive (Roush & McKenzie, 1987).

Pleiotropic effects of resistance, also referred to as costs or trade-offs, are seen in many resistant insect populations. These effects can affect reproductive fitness, survival, behavior, and other aspects of biology or natural history (Roush & McKenzie, 1987). Other factors that can contribute to rapid resistance are previous exposure to pesticides, and gene flow (Georghiou, 1972; Roush & McKenzie, 1987).

Resistance can be reversed once pesticide use is discontinued. Laboratory studies demonstrate that reversal of resistance can often be nearly as rapid as its initial appearance. The effectiveness of the organophosphate pesticide chlorpyrifos increased by over 50% for green lacewings (*Chrysoperla carnea*) from Pakistan in just three generations (Sayyed, et al., 2010). Tobacco cutworms (*Spodoptera litura*), also from Pakistan, became 55%-86% more susceptible to abamectin, indoxacarb, and acetamiprid after 4 generations without exposure to toxins (Shad, Sayyed, & Saleem, 2010). Resistance of cotton bollworms (*Helicoverpa armigera*) from

Cameroon to the pyrethroid cypermethrin declined by 99% after 5 generations (Achaleke & Brévault, 2010). The diamondback moth (*Plutella xylostella*), a major worldwide crop pest, has developed resistance to every major class of insecticides but resistance may be reversed once insecticide use is terminated, and in some cases may restore almost complete susceptibility (Hama, Suzuki, & Tanaka, 1992; Tabashnik, et al., 1994; Sayyed, Ferre, & Wright, 2000).

Environmental change may, from time to time, eliminate or weaken a source of selection pressure on the maintenance of certain traits. Those traits are then said to be under relaxed selection. When one source of selection is relaxed, other sources may act on the trait, potentially leading to alterations of the phenotype (Lahti, et al., 2009). Traits may be eliminated once mutations that result in the loss of those traits are no longer under selective disadvantage (Coss, 1999). For example, dark forms of the peppered moth (*Biston betularia*) were under selective disadvantage prior to the industrial revolution because the melanic forms are more easily seen by predators than the light forms. But when soot from coal-burning industries began to cover the surfaces of trees and buildings, melanic forms were no longer selected against, so the melanic alleles proliferated in the population (Berry, 1990).

Pleiotropic effects may play a role in reversing resistance when selection is relaxed. Roush & McKenzie (1987) assert that in the absence of treatment, resistance can be reversed if there is migration into the resistant population from a susceptible population or if resistance is somehow costly to fitness or survival. Tabashnik et al. (1994), for example, found that the reproductive fitness of resistant diamondback moths and the population's rate of increase was lower than in a strain that had reverted to susceptibility.

Spinosad is a relatively new insecticide, derived from a naturally occurring, soil-dwelling, Caribbean actinomycete, *Saccharopolyspora spinosa*. Because of its natural origin, it

is formulated for use in organic as well as conventional management. Spinosad was first brought to the market in 1997 (Salgado & Sparks, 2005). The active ingredients in spinosad are spinosyns A & D, which act on the insect's nervous system, primarily interacting with the nicotinic acetylcholine receptors (nACHr) at a site distinct from that of the neonicotinoids (Sparks, et al., 2012). Spinosad was first used by organic farms on Long Island between 2004 and 2006. A small number of conventional growers used it in 2008-09. Extremely high-level resistance appeared on organic fields on Long Island's South Fork in 2009 and 2010. Spinosad use was discontinued on those fields thereafter.

While spinosad has been shown to be effective against many lepidopteran and other insect pests, resistance can evolve quickly, especially when used more often than recommended by the manufacturer (Zhao, et al., 2006). In a 2012 review, resistance to spinosad was documented in at least a dozen insect species, but only half of them have shown resistance in field populations, most notably *P. xylostella* and the western flower thrip (*Frankliniella occidentalis*).Target-site resistance is the most common mechanism associated with spinosad resistance and resistance is typically monogenic and recessive (Sparks, et al., 2012). The only observation of spinosad resistance in potato beetles to date shows that resistance evolved rapidly on Long Island, with less than 5 years until total failure on several South Fork fields (Schnaars-Uvino, 2013).

Resistance to spinosad can be reversed in some cases. Several strains of leafminers (*Lyriomyza trifolii*) from the USA became much more susceptible to spinosad in a laboratory setting without exposure. Two strains were 84% and >99% less resistant when unexposed for 5 generations. A third strain was 93% less resistant after 10 generations (Ferguson, 2004). Very high resistance was 99% reversed in 8 generations in a population of tomato borers (*Tuta*

absoluta) in Brazil (Campos, et al., 2014). However, spinosad resistance did not decline significantly after 8 generations in cotton mealybugs (*Phencoccus solenopsis*) (Afzal & Shad, 2017).

There is little documentation of reversal of resistance in Colorado potato beetles. *Bt*-resistant beetles from Michigan became 76% less resistant after 11 generations (Whalon & Wierenga, 1994). There have been no previous observations of rapid evolution and reversal of resistance in potato beetles on Long Island, a focal point for beetle resistance. Following an outbreak of highly resistant beetles in 2010, the growers with the two most resistant populations on the South Fork of Long Island ceased using spinosad altogether. I conducted surveys of these South Fork fields as well as several organic fields on the North Fork to assess the level of resistance to spinosad on Long Island and to what degree resistance has been reversed.

Methods

Field Surveys

From 2012-2014 beetles were sampled annually from a single field on the South Fork of LI, NY. That field was treated with Entrust™, whose active ingredient is spinosad, (Dow AgroSciences, Indianapolis, IN) starting in 2010. Entrust™ was applied weekly, rather than the monthly recommended application, and the pesticide soon lost its effectiveness as the beetles evolved a strong resistance to Entrust™. Subsequently, Entrust™ use was discontinued for several years, from 2011-2013. Since 2013, that field has used a combination of a single annual Entrust™ application and mechanical (suction) removal of beetles from potato plants. Beetle

sampling was conducted in June, before pesticide application and, if additional beetles were needed to replenish the population in the lab, in July after application.

In 2016 beetles were also collected from four additional organic farms on Long Island; one on the South Fork and three on the North Fork. Three of those fields were treated annually with Entrust™ (Dow AgroSciences, Indianapolis, IN), supplemented with manual removal. One field on the North Fork was not treated with any chemical insecticide at any time in its history. Beetles were collected from one additional untreated field on the North Fork in 2017.

There are no truly susceptible field populations of Colorado potato beetles on Long Island (Alyokhin, et al., 2015) so from 2012-16, a susceptible strain (NJ)—originally from the USDA research station in Rutgers NJ—was used as the baseline for larval assays. This line has been maintained in captivity for decades and has often been used as a reference for resistance studies (e.g. Olson et al. 2000, Mota-Sanchez et al. 2006). In the fall of 2016, we identified a field in Ithaca, NY that did not use any chemical insecticides and was geographically isolated from other conventional and organic potato farms, so beetles were collected there in order to determine if this population could serve as a suitable field susceptible strain to use as the baseline for larval assays in 2017 instead of the lab-raised, NJ strain.

Adult beetles were collected by hand from plants and were housed in 46 x 61 x 51 cm cages on potted, untreated potato foliage. Field-collected clutches and clutches from caged adults were collected and reared to second instar for bioassay.

Larval Assays

Second-instar larvae weighing 6-8.5 mg were assayed by direct topical application on the abdomen of a 1 µl drop of spinosad dissolved in HPLC-grade (0.995) acetone. Spinosad was

extracted from Entrust™ (Dow AgroSciences LLC) by first diluting 1:9 using HPLC-grade (0.995) acetone, then vacuum filtered twice to remove remaining particulates. Up to 9 concentrations from 4.2×10^{-3} to 1.35×10^{-1} µg/larva, plus an acetone control were used. Following application, larvae were placed on a potato leaf cutting and held at 25°C for 24 hours until scoring. Mortality was defined as failure to move a leg for 10 seconds after the larva was placed on its back.

Stability

The stability of resistance of resistance was calculated for Fields 1 and 2, which were sampled in 2010 (Schnaars-Uvino, 2013), after which spinosad use was discontinued on those fields, and again during the present study. The formula is given in Tabashnik et al. (1994) as:

$$R = \frac{\log[\text{final } LD_{50}] - \log[\text{initial } LD_{50}]}{n}$$

where R is the stability of resistance in the absence of selection, n is the number of generations not exposed to pesticide, final LD_{50} is the LD_{50} after n generations without selection, and initial LD_{50} is the LD_{50} before n generations without selection. Negative values reflect a decrease in the LD_{50} and therefore an increase in susceptibility. The inverse of R is the number of generations required for a 10-fold change in LD_{50}.

Analyses

Dose-mortality curves were analyzed using Log-Logistic analysis to estimate the slope and the confidence intervals around the estimate of the dose necessary to achieve 50% mortality (LD50). I attempted to assay at least 20 individuals at each dose. Resistance ratios were calculated as the LD50 relative to that of the susceptible colonies.

Statistical analyses were performed in R, using the *drc* and *stats* packages, and in Microsoft Excel.

Results

The resistance of field populations is presented in Tables 1-3 and Figures 1-4. The least resistant field population in both 2016 and 2017 was Field 5, on the North Fork, with an LD_{50} of 0.016 μg/mL in 2016 and 0.009 in 2017. Tables 1-3 present resistance ratios of the field populations to both the lab-raised and field susceptible populations. Resistance was highest on the South Fork. The resistance ratio of South Fork fields to the lab strain were 213.33 (Field 1) and 156.46 (Field 2) in 2016. Resistance ratios declined by approximately 80% and 70% in 2017 to 35.94 (Field 1) and 49.37 (Field 2) respectively.

Resistance was generally much lower on the North Fork. There, resistance ratios ranged from 138.37 to 4.07 in 2016 and from 23.76 to 2.24 in 2017. The resistance ratios of two North Fork fields declined from 2016 to 2017. Field 3's RR fell from 138.37 to 23.76, a decline of over 80%. The resistance ratio of Field 5 fell by more than half from 5.99 to 2.24. Field 6, from which beetles were only collected in 2017, had a resistance ratio of 8.44. Field 4 was the only field that was more resistant in 2017 than 2016, its RR increasing from 5.99 to 8.44, an increase of approximately 40%.

The resistance ratios of field populations to the least resistant field population (Field 5) also declined sharply from 2016 to 2017 but less so because the resistance of the Field 5 beetles declined as well. The South Fork fields declined 70% (Field 1) and 43% (Field 2) respectively. On the North Fork, Field 3 declined by 31%. Field 4's resistance ratio increased 2.5-fold, from 1.47 to 3.77.

Resistance ratios of spinosad to the lab susceptible beetle strain for the most resistant field, Field 1, ranged from 242.39 in 2013 to 35.94 in 2017, a nearly sevenfold decrease in four years. The resistance ratio of the most resistant field to the most susceptible field population decreased from 2016 to 2017 by two thirds.

Stability of resistance was calculated by estimating the number of generations from the cessation of spinosad use in 2010. Field 1, which was extremely resistant in 2010, was resampled at the beginning of the present study in 2013, an estimated 9 generations (3 generations per year) between the samples. Field 2 was sampled again only in 2016, an estimated 18 generations. The stability of resistance for Field 1 was calculated to be -0.42 and for Field 2 it was -0.17. This indicates that resistance on Field 1 was much more unstable than on Field 2. On average, approximately just two and a half generations were required for a 10-fold decrease in resistance on Field 1, while it took about 6 generations to achieve the same magnitude decrease on Field 2.

Discussion

Resistance declined sharply from between 2013 and 2017. Beetles from the highly resistant Field 1 were nearly seven times more resistant in 2013 than in 2017 and 24 times more resistant in 2010 than in 2013 (Schnaars-Uvino 2013). Resistance on Field 2 was 11 times stronger in 2010 than in 2016. Beetles in the other fields sampled in both 2016 & 2017 were on

average 3 times more resistant in 2016 than 2017. This trend may be the result of pleiotropic effects of resistance on the reproductive fitness or survival of potato beetles. Negative impacts on fitness and survival may increase with the degree of resistance across populations exposed to different pesticide regimes (Carrière, et al., 1994). Once pesticide use is discontinued, the level of resistance may revert towards susceptibility. If the duration and intensity of treatment is sufficiently short, then the reversion to susceptibility will be relatively quick. If the duration and intensity of treatment is higher, then selection pressure or simply time can encourage the evolution of modifier genes that counteract the costs to fitness or survival (McKenzie, Whitten, & Adena, 1982).

Alternatively, the sharp decline in resistance may be the result of migration by susceptible beetles (Georghiou, 1972). More susceptible North Fork beetles may be dispersing to the South Fork fields. More likely is the possibility that beetles from conventional farms—which would use a neonicotinoid like imidacloprid instead of spinosad—on the South Fork are dispersing onto the organic South Fork fields.

Resistance was highly unstable on both highly resistant South Fork fields, Field 1 and Field 2 since spinosad use was discontinued after 2010, although it declined approximately 3 times faster on Field 1. That is likely because after spinosad use failed the more highly resistant Field 1 switched to a primarily mechanical control method in 2011, while Field 2 changed to a different chemical pesticide, perhaps maintaining some selection pressure for resistance in that population.

This decline in resistance may have implications for future pest management. Georghiou & Taylor (1986) suggest that previous selection for resistance may facilitate the evolution of resistance to new insecticides due to cross-resistance, as long as the resistance mechanism is

similar. Potato beetles that are resistant to the neonicotinoid pesticide imidacloprid are also resistant to other neonicotinoids as well as spinosad. Both types of pesticide act on the nicotinic acetylcholine receptors, albeit at different loci (Mota-Sanchez, et al., 2006). Beetles may also rapidly regain resistance to spinosad if selection pressure is once again intensified (Georghiou, 1972) because resistance genes will likely persist in the population in heterozygotes for many generations after spinosad use is ceased. Tabashnik, et al. (1994) found just that in a population of *P. xylostella*, formerly resistant to *bt*, but not exposed to it for approximately 15 generations.

The data suggest that resistance to spinosad is costly for potato beetles. Exactly what that cost or costs might be remains unknown. I examined a series of potential fitness costs (detailed in Chapter 2) that have been found in previous studies of potato beetles and studies of spinosad and other, similar insecticides. The potential costs I investigated were fecundity, hatch rate, egg development time, larval development time, adult emergence rate, and adult size. Baker et al. (2007) found that imidacloprid-resistant potato beetles in Massachusetts exhibited lower fecundity and a decreased rate of hatching success as well as increased egg development time. Potato beetles resistant to *Bt* toxins showed reduced fecundity and slower larval development (Trisyono & Whalon, 1997; Alyokhin & Ferro, 1999b). Spinosad-resistant *P. xylostella* failed to produce viable offspring in unfavorable temperature conditions while susceptible moths were able to reproduce successfully (Li et al., 2007). Spinosad-resistant *H. armigera* showed reduced fecundity and hatching success and increased development time compared to susceptible strains (Wang, et al., 2010). Rehan & Freed (2015) found costs to fecundity, larval development, and pupation in spinosad-resistant *S. litura*. Other costs not measured in the present study may be responsible for the reversal of resistance observed on LI.

The results of that study are presented in Chapter 2. But if resistance is costly—and it certainly appears to be both in the present study and in previous work—then the cost may not be to reproductive fitness. Another possibility might be a cost to survival rate, be it larval, adult or adult overwintering survival. Overwintering potato beetles resistant to *bt* toxin survive diapause at half the rate of susceptible beetles (Alyokhin & Ferro, 1999b). Another possibility is that spinosad resistance affects the ability of beetles to avoid predation or parasitism, an effect found in both resistant aphids and mosquitoes (Foster, et al., 1999; Beticat, et al., 2004).

Resistance of Colorado potato beetles to spinosad can be related to the degree and duration of usage. The fields that used spinosad the most showed very high levels of resistance. Rotation away from spinosad can help recover some susceptibility. Resistance declined rapidly after spinosad use was discontinued, implying that spinosad resistance carries some pleiotropic effects or that migration of susceptible beetles into the resistant population has been taking place. Spinosad use in the field may be sustainable as long as it is used infrequently and in combination with other pest management strategies.

Table 1. Larval resistance of Long Island field populations and a susceptible lab population to spinosad in 2016

Field	Location	N	LD_{50}[a]	Lower[b]	Upper[c]	Std. Error	t-value	p-value	RR lab[d]	RR field[e]
1	South Fork	1347	0.878	0.536	1.22	0.175	5.03	< 0.001	213.33	52.4
2	South Fork	1384	0.614	0.406	0.822	0.106	5.78	< 0.001	156.46	38.43
3	North Fork	221	0.543	0.281	0.805	0.134	4.06	< 0.001	138.37	33.99
4	North Fork	233	0.024	0.011	0.036	0.007	3.61	0.003	5.99	1.47
5	North Fork	129	0.016	0.006	0.026	0.005	3.01	0.0026	4.07	1

[a] The lowest dose that kills half the sample

[b] Lower bound of the 95% confidence interval around the LD_{50}

[c] Upper bound of the 95% confidence interval around the LD_{50}

[d] Ratio of LD_{50} of the field collected strain to the lab strain

[e] Ratio of LD_{50} of the field collected strain to the least resistant field collected strain

Table 2. Larval resistance of Long Island field populations and a susceptible lab population to spinosad in 2017

Field	Location	N	LD_{50}[a]	Lower[b]	Upper[c]	Std. Error	t-value	p-value	RR lab[d]	RR field[e]
1	South Fork	555	0.141	0.106	0.177	0.019	7.78	< 0.001	35.94	16.03
2	South Fork	192	0.194	0.09	0.296	0.052	3.71	< 0.001	49.37	22.02
3	North Fork	269	0.093	0.064	0.122	0.015	6.30	< 0.001	23.76	10.60
4	North Fork	145	0.033	0.014	0.052	0.010	3.41	< 0.001	8.44	3.77
5	North Fork	199	0.009	0.006	0.011	0.001	6.93	< 0.001	2.24	1
6	North Fork	230	0.018	0.008	0.028	0.005	3.66	< 0.001	4.62	2.06
7	Ithaca	239	0.012	0.009	0.015	0.002	7.47	< 0.001	3.03	1.35

[a] The lowest dose that kills half the sample

[b] Upper bound of the 95% confidence interval around the LD_{50}

[c] Lower bound of the 95% confidence interval around the LD_{50}

[d] Ratio of LD_{50} of the field collected strain to the lab strain

[e] Ratio of LD_{50} of the field collected strain to the least resistant field collected strain

Table 3. Larval resistance of beetles collected from Field 1 on the South Fork of Long Island from 2013-17

Year	N	LD_{50}[a]	Lower[b]	Upper[c]	Std. Error	t-value	p-value	RR lab[d]	RR field[e]
2013	465	0.951	0.559	1.34	0.2	4.75	< 0.001	242.39	NA
2014	354	0.761	0.437	1.08	0.165	4.6	< 0.001	193.96	NA
2016	1336	0.837	0.509	1.17	0.168	4.99	< 0.001	213.33	52.4
2017	555	0.141	0.106	0.177	0.018	7.86	< 0.001	35.94	16.03

[a] The lowest dose that kills half the sample

[b] Upper bound of the 95% confidence interval around the LD_{50}

[c] Lower bound of the 95% confidence interval around the LD_{50}

[d] Ratio of LD_{50} of the field collected strain to the lab strain

[e] Ratio of LD_{50} of the field collected strain to the least resistant field collected strain

Figure 1: Dose-response curves from larval bioassays (µg/larva) for beetles taken from Field 1 showing a steady decline in resistance from 2013-2017.

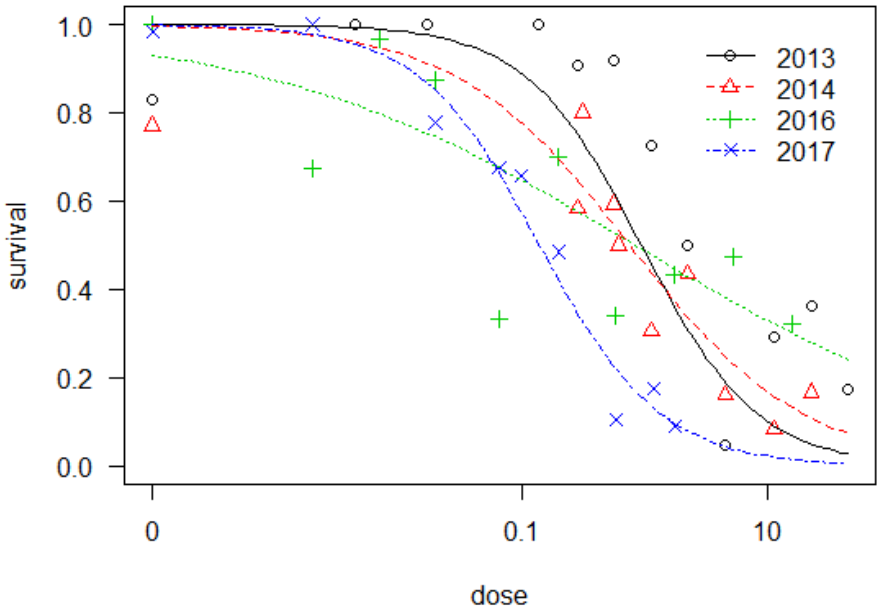


Figure 7: Dose-response curves from larval bioassays (µg/larva) from five organically managed fields in 2016, showing the highest resistance on the South Fork and generally lower resistance on the North Fork.

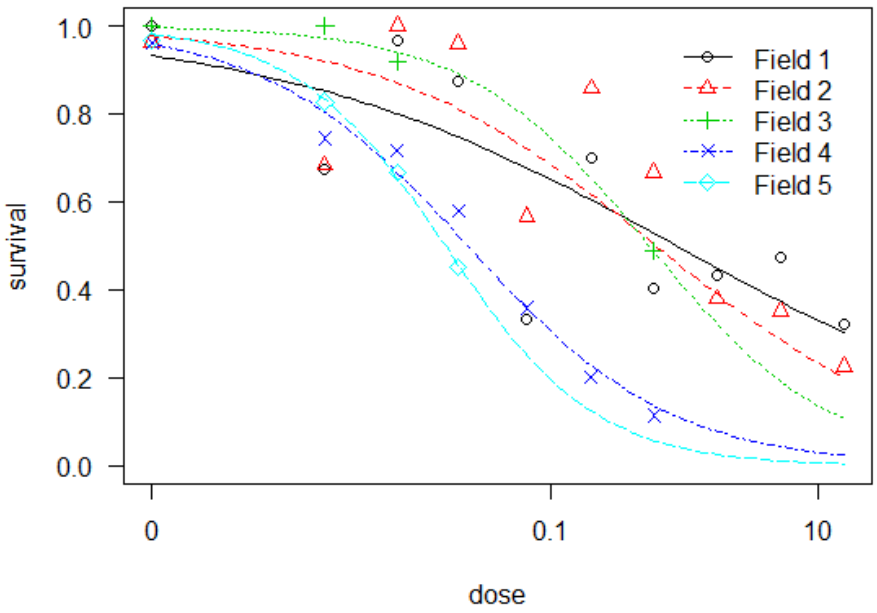


Figure 8: Dose-response curves from larval bioassays (µg/larva) for seven organically managed fields in 2017, showing generally higher resistance on the South Fork but not as high as in previous years.

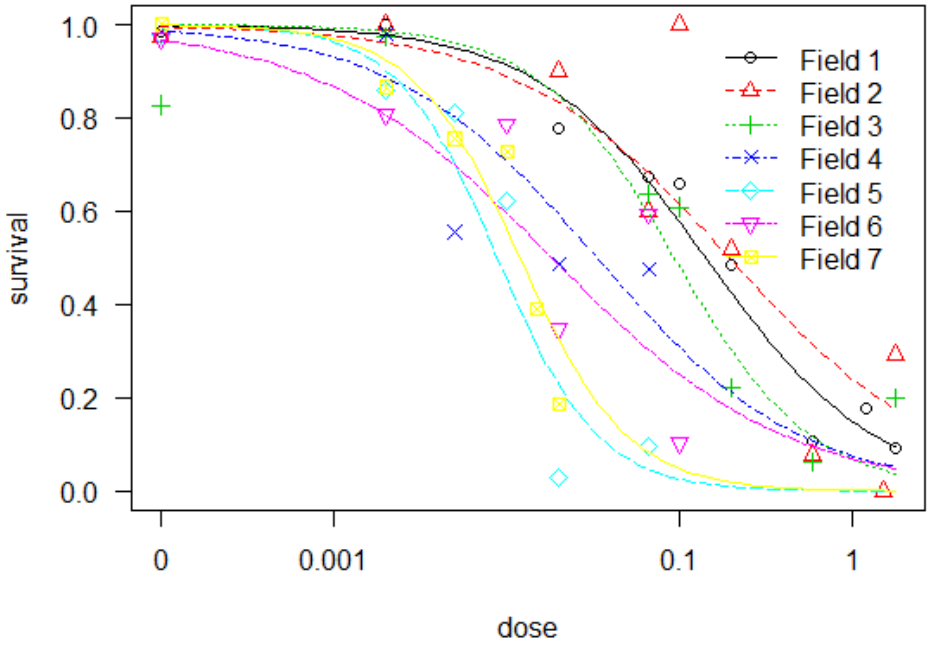
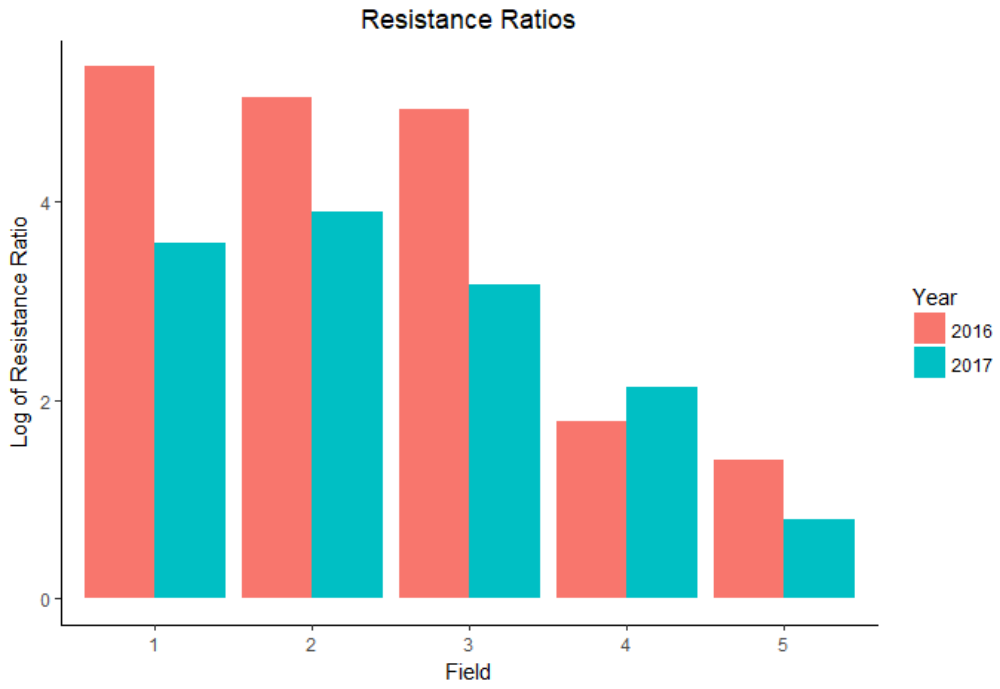


Figure 4: Bar graph of resistance ratios (RRs) of the five fields that were sampled in both 2016 and 2017. The RRs are displayed as logarithms to better illustrate the year to year decline in resistance. Only Field 4's RR increased from 2016-2017.



CHAPTER 2: Potential costs of spinosad resistance in Colorado potato beetles (*Leptinotarsa decemlineata*)

Pleiotropic effects of resistance are frequently observed in insect populations resistant to pesticide. These costs often manifest as decreases in reproductive fitness, survival or other aspects of the species' natural history. These effects are generally understood to be the result of the production and maintenance of resistance mechanisms possibly diverting resources away from reproductive systems. Colorado potato beetles (*Leptinotarsa decemlineata*), are known for rapidly developing resistance to all the major classes of insecticides. These agricultural pests have shown pleiotropic effects of resistance to pyrethroid and neonicotinoid pesticides. I measured reproductive fitness of *L. decemlineata* from five fields on Long Island, NY and one in Tompkins County, NY to attempt to identify potential costs of resistance to the organic pesticide spinosad. Previous research has shown that when the selection pressure for resistance to spinosad was relaxed, resistance began to revert from a very high level, indicating that trade-offs may be present. There was a negative relationship between resistance level and egg development time, providing evidence for pleiotropic effects of resistance. However, adult emergence rate was highest in the most resistant populations and may provide a fitness benefit. General fitness indices showed no cost of resistance. These findings could indicate that any potential costs present in these populations do not affect reproductive fitness, that the beetles have evolved modifier genes that mitigate the pleiotropic effects of resistance, or that costs may be related to environmental conditions.

Keywords: spinosad, resistance, Colorado potato beetle, *Leptinotarsa decemlineata*, evolutionary trade-offs, pleiotropy, pleiotropic effects, resistance costs, relaxed selection

Pleiotropic effects of resistance, also referred to as evolutionary tradeoffs, are seen in many resistant insect populations. Resistant insects may suffer reduced reproductive fitness, survival, behavior, detrimental impacts on other aspects of biology or natural history (Roush and McKenzie 1987). Evolutionary trade-offs are usually assumed to be present in most natural, co-evolved systems—such as insect resistance to natural plant toxins but insecticide resistance may be more costly than commonly thought. Costs may be present even when evidence for them is lacking because it is assumed that the production and maintenance of the resistance mechanisms divert resources from fitness-enhancing characters or because the system is at genetic equilibrium. If the system was at equilibrium, resistance alleles would have opposite effects on fitness depending on whether or not selection pressures were applied (Carrière, et al. 1994). Furthermore, researchers may be looking at different populations whose levels of fitness may vary from region to region in ways unrelated to resistance. They may not be examining all of the components of insect fitness, and that they may be looking at systems with long histories of pesticide treatment, where the costs may have been somewhat mitigated over time (Oliveira, et al. 2007, Carrière, et al. 1994). For example, fitness costs in diazinon-resistant Australian sheep blowflies (*Lucilia cuprina*) were reduced over time by the evolution of a modifier gene (McKenzie and Clarke 1988).

Similar resistance mechanisms may produce different costs in different species. The mosquito *Culex pipiens* shows a strong overwintering survival cost, increased risk of predation, and increased risk of infection from the alleles that grant resistance to organophosphate insecticides (Chevillon, et al. 1997, Bourguet, et al. 2004, Arnaud and Haubruge 2002, Berticat, et al. 2004). A similar allele however does not seem to carry any of the costs that the other alleles do (Duron, et al. 2006). Peach-potato aphids, (*Myzus persicae* Sulzer), that carry the same

organophosphate resistance alleles show reduced movement from older leaves to newer, fresher ones and this species also has a reduced alarm response in carriers of a separate resistance gene (Foster, et al. 1997, Foster, et al. 1999).

Fitness costs have been found in a variety of insect pests. Both Reissig, Stanley and Hebding (1986) and Carriere, et al. (1994) found a clear correlation between increased resistance to several compounds and reduced fitness components in the oblique-banded leafroller (*Choristoneura rosaceana*). Resistant individuals had smaller mass on average and longer development times. *Bt*-resistant cabbage loopers (*Trichoplusia ni*) are also smaller than susceptibles, are less fecund, and grow more slowly (Janmaat and Myers 2004). Pyrethroid-resistant maize weevils (*Sitophilus zeamais*) also have a slower population growth rate as well as delayed emergence and higher mortaily (Fragoso, Guedes and Peternelli 2009). Survival costs are found in a number of other species, including pink bollworm (*Perctinophora gossypiella*) and diamondback moth (*Plutella xylostella*) (Carrière, et al. 2001, Groeters, et al., 1994).

Sometimes different populations exhibit different costs of resistance to the same treatment. *Bt*-resistant Indianmeal moth (*Plodia intetrpunctella*), colonies exhibit slower development times or lower survival rates but rarely both (Oppert, et al. 2000). Some fitness costs can be enhanced or mitigated by parasitic infection. Agnew et al. (2004) found that a particular parasite carried by *C. pipiens* mosquitoes can either increase or decrease the fitness of resistant strains relative to susceptible ones, depending on the population and the fitness trait being measured. This finding is in line with the idea that there is a trade-off between different intrinsic defense mechanisms in insects (Cotter, Kruuk and Wilson 2004). Resistance to insecticides can take resources away from defense against parasites and predators.

Potential reproductive costs of resistance can take the form of reduced fecundity or decreased mating opportunities. Colorado potato beetles, brown planthoppers (*Nilaparvata lugens* Stål), and cabbage loopers all show reduced fecundity (Baker, et al. 2007, Liu and Han 2006, Janmaat and Myers 2004). Male *Culex* mosquitoes, pink bollworms, cotton bollworms (*Helicoverpa armigera*), diamondback moths, and *L. decemlineata* mate with decreased frequency, duration, and success than susceptible males (Berticat, et al. 2002, Higginson, et al. 2005, Anilkumar, Pusztai-Carey and Moar 2008, Groeters, et al. 1993, Alyokhin & Ferro 1999b). Some species, such as the *S. zeamais*, have energetic costs as well. Guedes, et al. (2006) found trade-offs between energy allocation and resistance and other resistant species have lower respiration rates (Pimentel, et al. 2007).

Resistance is not always costly. Resistance to DDT in *Drosophila melanogaster* continued to spread globally despite DDT being off the market for many years. The absence of pleiotropic effects helps explanation this phenomenon (McCart, Buckling and ffrench-Constant 2005). Of the three resistance genes analyzed by Chevillon, et al. (1997) only one had a constant severe fitness cost. Some maize weevil populations show a fitness cost but others do not (Oliveira, et al. 2007). Male red flour beetles (*Tribolium castaneum*) actually show a fitness benefit of resistance, apart from the resistance itself; they mate more often than susceptible males (Arnaud and Haubruge 2002). A similar situation is seen in female western flower thrips (*Frankiniella occidentalis*). Resistant females are more fecund than susceptible females and those resistant to spinsosad were more fertile (Bielza, et al. 2008). German cockroaches (*Blatella germanica*) show no energetic cost of resistance (Hostetler, Anderson and Lanciani 1994). Boivin, et al. (2003) found that resistant codling moths (*Cydia pomonella*) had an altered phenology, i.e. higher propensity to diapause, which some consider to be a cost but which these

authors described it as a potential advantage since diapausing individuals might better survive the winter. A lack of fitness costs may be the result of modifier genes that mitigate the costs of resistance, non-deleterious resistance genes, long-term exposure to pesticides, or fixation of resistance alleles in the population. Non-genetic reasons for a lack of costs might be female mate choice of susceptible males or sperm competition (Arnaud and Haubruge 2002, Oliveira, et al. 2007, Fragoso, Guedes and Peternelli 2009). Castañeda et al. (2011) found no metabolic or resistance costs in *M. persicae,* which they attributed to interactions between resistance mechanisms. A summary of these studies may be found in Table 1.

Pleiotropic effects may play a role in reversing resistance when selection is relaxed. Roush & McKenzie (1987) assert that in the absence of treatment, resistance can be reversed if there is migration into the resistant population from a susceptible population or if resistance is somehow costly to fitness or survival. Tabashnik et al. (1994), for example, found that the reproductive fitness of resistant diamondback moths and the population's rate of increase was lower than in a strain that had reverted to susceptibility.

Colorado potato beetles (*Leptinotarsa decemlineata*) are the most widespread insect defoliator of cultivated potatoes worldwide. The species likely originated in the highlands of south-central Mexico with populations also occurring in the western United States (Hsiao 1978). *L. decemlineata* are specialists on solanaceous plants, particularly form the genus *Solanum*. In the mid-19th century beetles from the plains near the eastern slope of the Rocky Mountains in the United States switched host plant from buffalo bur, *S. rostratum* to cultivated potato, *S. tuberosum* (Izzo, Chen and Hawthorne 2018). Since then the potato beetle has spread worldwide on potatoes and continues to expand its range as climate change opens more northern latitudes to potato cultivation.

L. decemlineata has gained a reputation over the past century for rapidly becoming resistant to all types of chemical insecticides. That is especially true on Long Island, NY where very high levels of resistance to insecticides have been found in *L. decemlineata* and on average, insecticides fail within about 2 years (Georghiou 1986). Evolution of resistance to the neonicotinoid pesticide imidacloprid, for example, was rapid but a change in the pattern of use, from comprehensive pest control to a more targeted measure against aphids and potato beetle larvae, has slowed the rate of evolution somewhat (Alyokhin, et al. 2015).

L. decemlineata are not immune from pleiotropic effects of resistance. A colony of permethrin and azinphosmethyl-resistant beetles from Massachusetts showed reduced fecundity and increased development time compared to a susceptible strain (Argentine, Clark and Ferro 1989). Imidacloprid-resistant beetles on Long Island showed a cost to overwintering survival (Baker and Porter 2008). Huseth & Groves (2013) found evidence of a potential cost of imidacloprid resistance to body size, which they related to reproductive fitness. Imidacloprid-resistant beetles in two New England populations laid significantly fewer eggs and took significantly longer to hatch than the susceptible strain (Baker, et al. 2007). However, a hatching success cost that was present in 1999 was not in evidence when that population was tested again five years later, suggesting that the gene or genes responsible for the cost were modified or replaced. Huseth & Groves (2013) also propose a similar explanation for the lack of reduced fecundity in their resistant population. Chen et al. (2014) failed to tie differences in reproductive fitness to resistance and Baker et al. (2008) found that mating competition is not reduced by imidacloprid-resistance.

Spinosad is a relatively new insecticide, derived from a naturally occurring, soil-dwelling, Caribbean actinomycete, *Saccharopolyspora spinosa*. Because of its natural origin, it

is used as an insecticide in organic farming. Spinosad was first brought to the market in 1997 (Salgado and Sparks 2005). The active ingredients in spinosad are spinosyns A & D, which act on the insect's nervous system, primarily interacting with the nicotinic acetylcholine receptors (nACHr) at a site distinct from that of the neonicotinoids (Sparks, et al. 2012).

Fitness costs associated with spinosad resistance have been documented in a number of species. Resistant male tobacco budworms (*Heliothis virescens*) from North Carolina develop more slowly as larvae and emerge from pupae later than susceptible individuals and weigh slightly less (Wyss, et al. 2003). A resistant budworm population in Mississippi had lower fecundity and a lower hatch rate than susceptible strains (Sayyed, Ahmad and Crickmore 2008). Resistant cotton bollworms of both sexes develop slower, weigh less, and lay fewer eggs than susceptible ones (Wang, et al., 2010). Unstable levels of resistance to Spinosad in diamondback moths in Pakistan indicate the presence of significant costs. Resistance in this population was accompanied by a reduction in fecundity, hatch rate, development time, and larval size (Sayyed, Saeed, et al. 2008). In a Chinese population of *P. xylostella* the fitness costs are temperature dependent. At medium temperatures there was no fitness cost but at high and low temperature conditions the moths produced significantly fewer viable offspring (Li et al., 2007).

Spinosad has been used against *L. decemlineata* with some success (Azimi, et al. 2009, Kowalska 2010). In many places however, they have developed high levels of resistance to other compounds rather quickly (Mota-Sanchez, et al., 2006) and evidence from Long Island indicates that is also happening with spinosad. Schnaars-Uvino (2013) found high levels of resistance to spinosad on Long Island, NY in 2010 and its use was subsequently stopped. Beetles collected from the same farm three years later, when the selection pressure had been relaxed, showed much lower levels of resistance (Chapter 1), which may be evidence of significant

resistance costs. This study attempts to determine if fitness costs of Spinosad are present on Long Island by comparing fecundity, egg development time, hatch rate, larval development time, adult emergence rate, and adult body size between populations with differing levels of resistance. If reproductive fitness costs are present, the highly resistant populations should perform worse in some or all fitness measures than the moderately resistant and field susceptible populations.

Methods

Field Collection

Adult Colorado potato beetles were collected from five organic potato fields on the eastern part of Long Island, NY in June and July 2017. Two of the fields were located on the South Fork of Long Island and three on the North Fork. Since there are no truly susceptible field populations of *L. decemlineata* on Long Island, a relatively new field in Ithaca, NY that did not use any chemical insecticides and was geographically isolated from other conventional and organic potato farms was identified in 2016 as a potential susceptible field strain to use as the baseline for larval assays and reproductive fitness measures. Beetles were collected from the Ithaca field in the fall of 2016. All adult beetles were collected by hand from plants and were housed in 46 x 61 x 51 cm cages on potted, untreated potato foliage.

Larval Assays

Bioassays were conducted to determine the LD_{50} of each field-collected population. Second-instar larvae weighing 6-8.5 mg were assayed by direct topical application on the abdomen of a 1 µl drop of spinosad dissolved in HPLC-grade (0.995) acetone. Spinosad was extracted from Entrust™ (Dow AgroSciences LLC) by first diluting 1:9 using HPLC-grade (0.995) acetone, then vacuum filtered twice to remove remaining particulates. Up to 9

concentrations from 4.2×10^{-3} to 1.35×10^{-1} μg/larva, plus an acetone control were used. Following application, larvae were placed on a potato leaf cutting and held at 25°C for 24 hours until scoring. Mortality was defined as failure to move a leg for 10 seconds after the larva was placed on its back.

Screening for Resistance

Preliminary bioassays revealed that resistance declined rapidly in the most resistant field populations (see Chapter 1). To draw a sharper contrast between the possible differences in reproductive fitness among the field populations, one generation of selection was performed on the highly resistant South Fork populations. Individuals were screened for resistance with a 0.1 μg/mL dose, approximately two-thirds of the 2017 LD_{50} of Field 1 and one half the 2017 LD_{50} of Field 2. It is possible that this action may have selected for higher overall body condition and not resistance. If that were the case it would be expected that the LD_{50} of the screened population would be higher than the field populations.

Fitness Costs

Twenty adult beetles (ten males and ten females) from each field population were collected shortly after emergence from pupation and placed in 5 x 7 x 12 cm containers and fed with fresh, untreated potato foliage. Eggs were collected from the cages daily and placed in clean petri dishes. Upon hatching, the larvae were transferred to larger containers and supplied with fresh, untreated potato foliage.

Fecundity

The number of eggs in each new clutch was counted before being transferred to petri dishes. Fecundity was calculated as the average number of eggs laid by females per day.

Egg development

Egg development was measured by hatch rate and time to hatching. Hatchlings were removed to prevent egg cannibalism. Hatch rate was calculated as the of the proportion of eggs that hatched out of each daily clutch. Time to hatching was simply the number of days from laying to hatching. One outlier, which took almost twice as long to emerge as the median adult, was removed from the sample.

Larval development

Larval development was measured by adult emergence rate, time to emergence, and body size. Adult emergence rate was calculated as the proportion of larvae that entered pupation and successfully emerged as adults. Time to emergence was the number of days from hatching to emergence from pupation. The length of each newly emerged adult was measured to determine body size.

Net Replacement Rate and Intrinsic Rate of Population Increase

The variables above were integrated into a single population growth rate after Chen (2014). Net replacement rate (R_0) was defined as in Birch (1948) as the number of daughters that replace the mother over a generation. Mathematically, it was calculated using the formula given in Chen et al. (2014):

$$R_o = \frac{m \text{ X } n \text{ X } l_e \text{ X } l_a \text{ X } l_r}{s}$$

where *m* is the fraction of reproductive females, *n* is the average clutch size, l_e is the hatch rate, l_a is the adult emergence rate, and l_r is the fraction of adults surviving to reproductive maturity, and *s* is the sex ratio coefficient, here assumed to be 50:50 (Alyokhin & Ferro, 1999). The intrinsic rates of population increase (Birch, 1948) were calculated based on the value of net replacement rate for all strains as follows:

$$r_m = \frac{ln\, R_0}{DT}$$

where *DT* represented the total development time of one generation in days, from egg laying to adult development, including the preoviposition period of adulthood, here estimated for all populations as 5 days. Based on the calculated intrinsic growth rates, I estimated increase over a 50-day period for hypothetical initial populations of 100 beetles of each strain as follows:

$$N_t = N_0 e^{trm}$$

where N_0 was initial population size, N_t was the number of individuals in the population after t units of times, and r_m was the intrinsic rate of population increase as described above (Chen, Alyokhin, Mota-Sanchez, Baker, & Whalon, 2014).

Biotic Potential

Biotic potential, as described in Roush & Plapp (1982), is a measure of the relative reproductive potential of resistant and susceptible insects. The formula is as follows:

$$B_p = \frac{lnF}{DT_r}$$

where F is the average daily fecundity of each female and DT_r is the development time ratio, calculated as the ratio of time from egg laying to adult emergence of each field population to the most susceptible field population, Field 5.

Statistical Analyses

Simple linear regression analyses were conducted in order to determine if any individual measures of reproductive fitness could be predicted by the LD_{50}s of the beetle populations. Analyses were done in *R* using the *stats* package.

Results

Larval Assays

The results of the larval assays are presented in Table 2 & Figure 1. The least resistant population was Field 5, located on the North Fork, which was used as the baseline for testing fitness costs. Field 2 had the largest resistance ratio before screening. After screening for resistance, the RR for Field 1 increased by nearly 600%.

Reproductive fitness

The average values for each of the measures of reproductive fitness are presented in Tables 3 & 4. The selected Field 1 population, which was most resistant, had the highest average fecundity, the highest emergence rate, the highest replacement rate, and the greatest biotic potential. That population also had the highest adult emergence time. The least resistant population, Field 5 had the highest hatch rate, the lowest fecundity, the largest body size, and the smallest biotic potential.

The highest population growth rate belongs to the screened Field 1 population (0.089) with Field 6 the next highest (0.083). The growth rates for the unscreened Field 1 population (0.056), Field 2 (0.053), & Field 7 (0.70) were moderately high. The growth rates for Fields 4 (0.042) and 5 (0.043) were the lowest, less than half that of Field 1 screened and Field 6.

Regression analyses

The results of the regression analyses are presented in Table 5 and Figures 3-10. Statistically significant regression equations were found in two of the analyses. A significant regression equation predicting egg development time from LD_{50} was found ($F(1,5) = 10.06$, $p < 0.05$) with an R^2 of 0.6. Egg development time could be predicted from LD_{50} with the following formula: egg development time = $0.65(LD_{50}) + 4.51$. Egg development time increased by approximately two-thirds of a day for each μg/mL of LD_{50}.

A significant regression equation predicting emergence rate from LD_{50} was also found ($F(1,5) = 8.11$, $p < 0.05$) with an R^2 of 0.54. Emergence rate could be predicted from LD_{50} with the following formula: emergence rate = $0.33(LD_{50}) + 0.22$. Emergence rate increased by approximately one-third for each μg/mL of LD_{50}.

Discussion

If spinosad resistance was costly to the reproductive fitness of *L. decemlineata* then one might expect that the most resistant populations would exhibit lower fecundity, take longer to develop, and have smaller body size than the more susceptible populations, or some combination of those variables. The statistical analyses revealed a potential fitness cost with regard to egg development time. Eggs belonging to the Field 1 population selected for resistance took, on average, longer to hatch than the less resistant populations. Egg development costs have been

observed in *bt*-resistant *C. rosaceana* (Carrière, et al., 1994) as well as imidacloprid-resistant *L. decemlineata* (Baker, et al., 2007). An egg development cost was not present in *bt*-resistant *P. gossypiella* (Carrière, et al., 2001).

Interestingly, the analysis of emergence rate uncovered a potential fitness benefit of resistance. The emergence rate of new adults tended to increase with increasing resistance. Resistance almost by definition carries a benefit, as the resistant individual experiences increased survival compared to susceptible individuals. The absence of trade-offs could be interpreted as a benefit since the resistant individuals would be fitter than if costs were present. While rare, positive benefits to reproductive fitness have been observed in malathion-resistant red flour beetles and spinosad-resistant western flower thrips (Arnaud & Haubruge, 2002; Bielza, et al., 2008).

In the absence of pesticide use any potential benefit of increased survival is often outweighed by the cost to other aspects of life history. And small differences in fitness can combine to have a greater effect than each measure. Therefore, it is important to weigh the benefits of resistance against the costs. Measures like the net placement rate, intrinsic rate of population growth and biotic potential, which combine several reproductive fitness variables into a single measurement, can detect those combined influences. What these indices show is that there does not seem to be any relationship between resistance and reproductive potential. Beetles from the most resistant fields tended to have higher fitness levels than those from the least resistant fields.

It is possible that other reproductive fitness costs may become evident in more extreme circumstances. Environmental conditions interact with genes to determine costs of resistance in different populations. At normal temperatures *P. xylostella* show a marginal fitness cost of

Spinosad resistance but at higher and lower than average temperatures there were significant resistance costs (Li, et al. 2007). Gassman, Carriére, & Tabashnik (2009) found that costs of resistance to *Bt* toxin were typically recessive and that non-recessive costs acted more strongly against resistance. Different field populations of *P. xylostella* respond differently to different feeding conditions, indicating that resistance costs can appear under stressful conditions and that resistance can depend on the particular interaction between genes and the environment (Raymond, Sayyed and Wright 2005). The beetles in this study were kept at a constant 24° C and were fed ad libidum with fresh foliage. Had they been kept at more extreme temperatures or given less food, or lower quality food, fitness costs may have been observed. Further study of overwintering costs and environmental conditions is necessary to confirm this possibility.

Strong selective forces, which favor resistance evolution, cancel out even high costs, at most delaying the evolution of resistance only slightly (Groeters and Tabashnik 2000). The South Fork fields were exposed to high levels of spinosad for an extended period of time. The strong selection pressure may have cancelled out whatever costs may have existed. That does not explain the apparent absence of costs however in the North Fork populations.

The effective absence of fitness costs may also be the result of the evolution of modifier genes that mitigate against costs. Resistance tends to revert quickly after selection is relaxed but become more stable with every cycle of selection and relaxation. That is typically explained as the coadaptation of resistance genes at other loci that ameliorate the deleterious effects of resistance (Roush and McKenzie 1987). Long Island beetles may have developed these modifier genes, which would eliminate any evidence of costs. This hypothesis assumes though that the genes that grant resistance were not already present in the population before the treatment began and that after the start of treatment resistance arose *de novo* from a new mutation. The

maintenance of resistance alleles in the population before treatment—even at low densities—would indicate that any costs that may have existed at one point have been mitigated. ffrench-Constant & Bass (2017) argue that since, in most cases, it is not known if resistance genes are present in a population before treatment is started, the possibility cannot be ruled out. If pre-existing resistance takes the form of a balanced polymorphism—which favors the heterozygous condition—then we would not expect to see costs in the absence of pesticide use because the resistance genes arose in the absence of any selection pressure for resistance. That appears to be the case for malathion-resistant *L. cuprina*. Preserved specimens, collected before the introduction of malathion use, possessed mutations that conferred malathion resistance and no fitness costs have been measured for resistance at those alleles (Hartley, et al. 2006). It is possible that a similar situation may be present on Long Island.

Spinosad resistance genes may have been present in the population before it was introduced in 2010. Since *L. decemlineata* was not screened for pre-resistance before spinosad use began, there is no way to rule out that possibility at this time. If there were any resistance alleles present on Long Island prior to the introduction of spinosad, it would likely be related to the use of neonicotinoid insecticides on large, conventional farms, namely imidacloprid. Like spinosad, imidacloprid binds to nicotinic acetylcholine receptors and interferes with chemical signal transmission, although at a different target site than spinosad (Abbink, 1991; Sparks, Crouse, & Durst, 2001). Imidacloprid has been used extensively on Long Island since 1996. Resistance was documented right away, and it has increased steadily since then, with resistance ratios climbing as high as 80.7 in 2010 (Alyokhin, et al., 2015). Cross-resistance has been documented between spinosad and imidacloprid but to a lesser degree than cross-resistance to other neonicotinoid insecticides (Mota-Sanchez, et al., 2006). Alyokhin, et al. (2015) describe

asymmetric cross-resistance between imidacloprid and spinosad, where previous resistance to imidacloprid grants some resistance to spinosad but spinosad resistance does not grant resistance to imidacloprid.

Strong resistance may be reversed under relaxed selection if costs of resistance are present. Although a potential cost to egg development time was found in this study it was likely counterbalanced by a potential benefit to adult emergence. The overall patterns observed in the fitness indices does not indicate the presence of fitness costs of spinosad resistance. Future research on this subject should determine whether fitness costs may be found in resistant populations of *L. decemlineata* under adverse temperature or feeding conditions. Lack of fitness costs on Long Island may be the result of strong selection pressure or the presence of modifier genes. Further genetic examinations can confirm or refute those possibilities. Looking backward, if preserved specimens from Long Island exist, it may be possible to determine whether *L. decemlineata* possessed spinosad resistance alleles before it was ever used. A lack of resistance costs can have a major impact on resistance management decisions. Strategies that rely on alternations will not work in the long term if there are no costs to resistance. In that case pesticide resistance can only be overcome by introducing new classes of chemicals for which there is no pre-existing mechanism of cross-resistance (ffrench-Constant and Bass 2017).

Table 1 Examples of studies that did not find evolutionary trade-offs between resistance and fitness.

Species	Ingredient	Costs Present	Costs Absent	Citation
Drosophila melanogaster	DDT	None	Egg, larval, & pupal viability, fecundity	(McCart, Buckling, & ffrench-Constant, 2005)
Culex pipiens	Organophosphates	Survival	Overwintering	Chevillon et al. 1997
Sitophilus zeamais	Deltamethrin	Competition	Competition	Oliveira et al. 2007
Tribolium castaneum	Malathion	None	Male mating	Arnaud & Haubruge 2002
Frankiniella occidentalis	Spinosad, acrinathrin	None	Fecundity, egg development	Bielza et a. 2008
Blatella germanica	Organophosphates, pyrethroids	None	Metabolic rate	Hostetler, Anderson, & Lanciani 1994
Cydia pomonella	Diflubenzuron	None	Diapause	Boivin et al. 2003
Myzus persicae	Various	None	Metabolism, population growth rate	Castañeda et al. 2011

Table 2: Larval resistance of Long Island field populations and a susceptible lab population to spinosad in 2017

Field	Location	N	LD_{50}[a]	Lower[b]	Upper[c]	Std. Error	t-value	p-value	RR lab[d]	RR field[e]
1 Unsel	South Fork	555	0.141	0.106	0.177	0.019	7.78	< 0.001	35.94	16.03
1 Sel	South Fork	109	0.813	0.409	1.212	0.206	3.94	<0.001	207.23	90.33
2	South Fork	192	0.194	0.09	0.296	0.052	3.71	< 0.001	49.37	22.02
4	North Fork	269	0.033	0.014	0.052	0.010	3.41	< 0.001	8.44	3.77
5	North Fork	145	0.009	0.006	0.011	0.001	6.93	< 0.001	2.24	1
6	North Fork	230	0.018	0.008	0.028	0.005	3.66	< 0.001	4.62	2.06
7	Ithaca	239	0.012	0.009	0.015	0.002	7.47	< 0.001	3.03	1.35

[a] The lowest dose that kills half the sample

[b] Upper bound of the 95% confidence interval around the LD_{50}

[c] Lower bound of the 95% confidence interval around the LD_{50}

[d] Ratio of LD_{50} of the field collected strain to the lab strain

[e] Ratio of LD_{50} of the field collected strain to the least resistant field collected strain

Table 3: Average values for individual measures of reproductive fitness

Field	LD50[a]	Fecundity[b]	Egg Dev. Time[c]	Hatch Rate[d]	Larval Dev. Time[e]	Adult Emerg. Rate[f]	Body Size[g]
1 Unsel	0.141	25	4.67	0.19	22.42	0.25	8.29
1 Sel	0.813	35.24	5.02	0.14	23.13	0.49	8.23
2	0.194	31.95	4.67	0.15	22.13	0.31	8.62
4	0.033	19.76	4.26	0.22	23.29	0.23	8.44
5	0.009	9.78	4.67	0.48	24.27	0.31	8.7
6	0.018	29.09	4.5	0.15	22.5	0.28	8.35
7	0.012	17.79	4.55	0.26	24.5	0.07	8.46

[a] The lowest dose that kills half the sample

[b] Average number of eggs laid per female per day

[c] Average number of days from laying to hatching

[d] Proportion of eggs that hatched

[e] Average number of days from hatching to adult emergence

[f] Proportion of 4^{th} instar larvae that successfully emerge from pupation

[g] Average body length (mm)

Table 4: Values of reproductive fitness indices for each field

Field	R_0[a]	R_m[b]	B_P[c]
1 Unsel	6.10	0.06	3.48
1 Sel	19.11	0.09	3.74
2	5.68	0.05	3.80
4	4.06	0.04	3.11
5	4.45	0.04	2.28
6	13.44	0.08	3.64
7	10.69	0.07	2.85

[a] Net replacement rate

[b] Intrinsic rate of population increase

[c] Biotic potential

Table 5: Linear regression results for individual measures and indices of fitness

		Estimate	Lower CI	Upper CI	Std. Error	t value	p value
Fecundity	(Intercept)	20.493	12.16	28.83	3.24	6.32	0.00146**
	LD50	20.62	-5.37	46.61	10.11	2.04	0.097
Egg Development	(Intercept)	4.51	4.34	4.68	0.065	69.076	1.2e-08***
	LD50	0.65	0.122	1.17	0.203	3.17	0.025*
Hatch Rate	(Intercept)	0.256	0.119	0.392	0.53	4.83	0.005**
	LD50	-0.17	-0.597	0.252	0.165	-1.043	0.345
Larval Development	(Intercept)	23.291	22.148	24.43	0.44	52.407	4.78e-08***
	LD50	-0.652	-4.21	2.91	1.39	-0.471	0.658
Emergence Rate	(Intercept)	0.22	0.12	0.31	0.04	5.67	0.002**
	LD50	0.34	0.03	0.65	0.12	2.85	0.04*
Body Size	(Intercept)	8.594	8.311	8.679	0.07	118.84	8e-10***
	LD50	-0.31	-0.88	0.27	0.22	-1.395	0.222
Replacement Rate	(Intercept)	6.69	1.68	11.69	1.95	3.43	0.19*
	LD50	13.73	-1.88	29.35	6.07	2.26	0.07
Biotic Potential	(Intercept)	3.10	2.49	3.71	0.236	13.16	4.52e-05***
	LD50	0.98	-0.90	2.87	0.73	1.34	0.24

Figure 1: Dose-response curves from larval bioassays (µg/larva) from five organically managed fields in Eastern Long Island and one from Central New York in 2017.

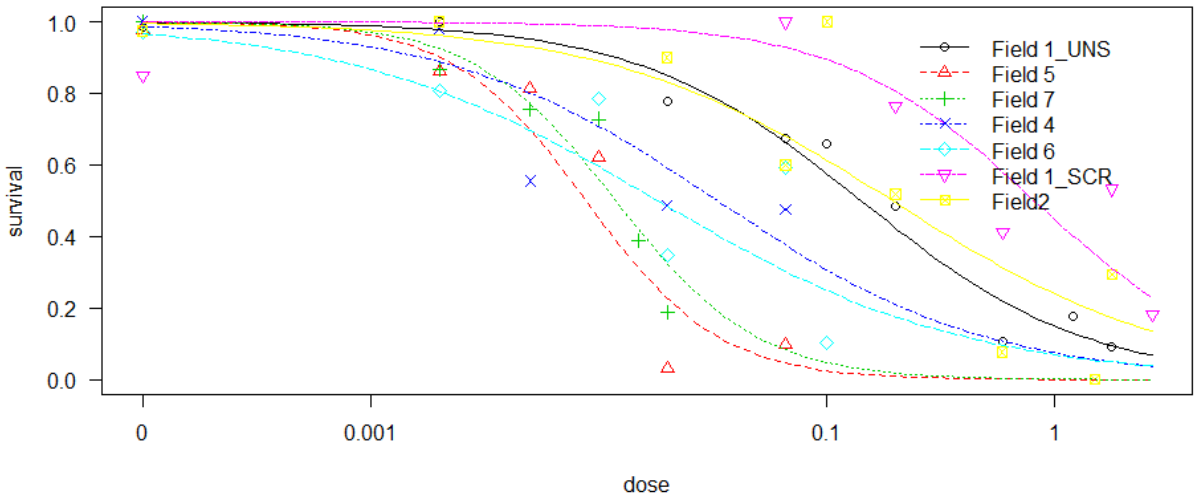


Figure 2: Estimated population growth curves for Colorado potato beetle strains from Long Island over a 50-day period. Intrinsic rates of population growth were calculated based on demographic parameters determined in this study.

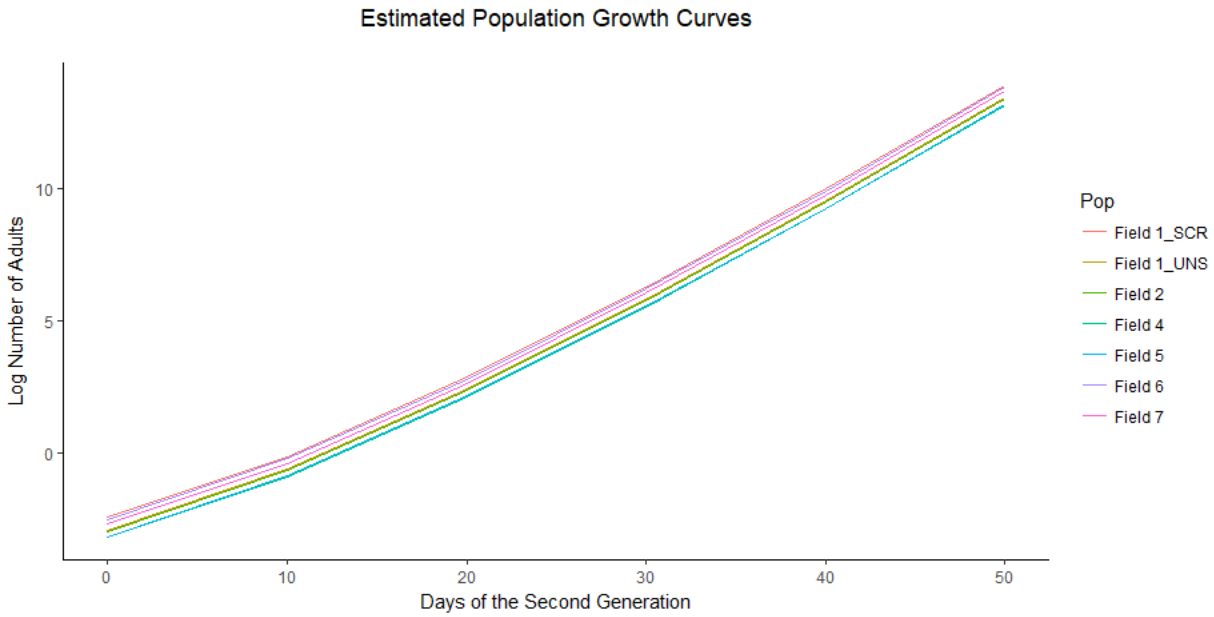


Figure 3: Scatter plot illustrating the relationship between LD50 and fecundity.

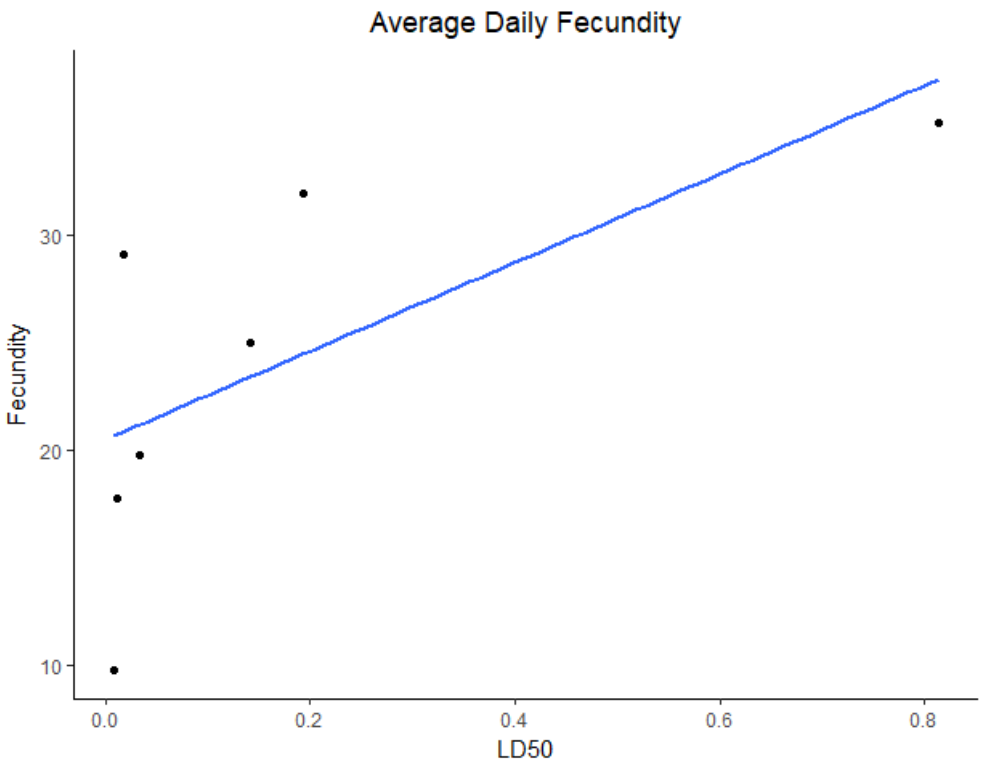


Figure 4: Scatter plot illustrating the relationship between LD50 and egg development time.

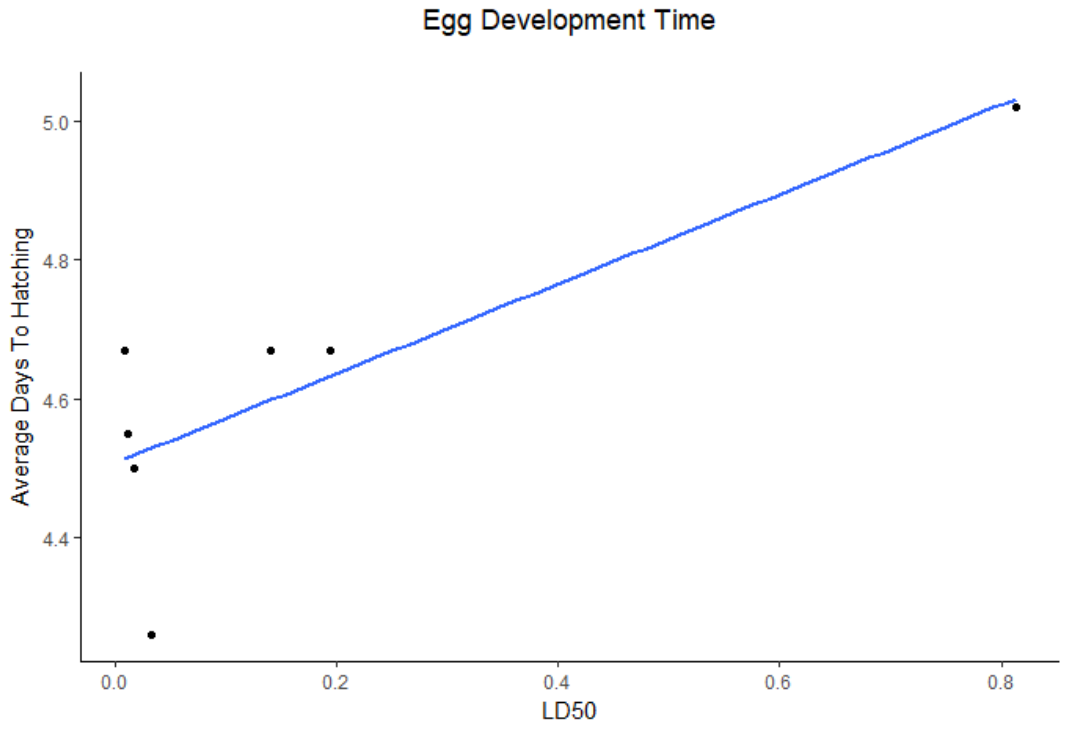


Figure 9: Scatter plot illustrating the relationship between LD50 and hatch rate.

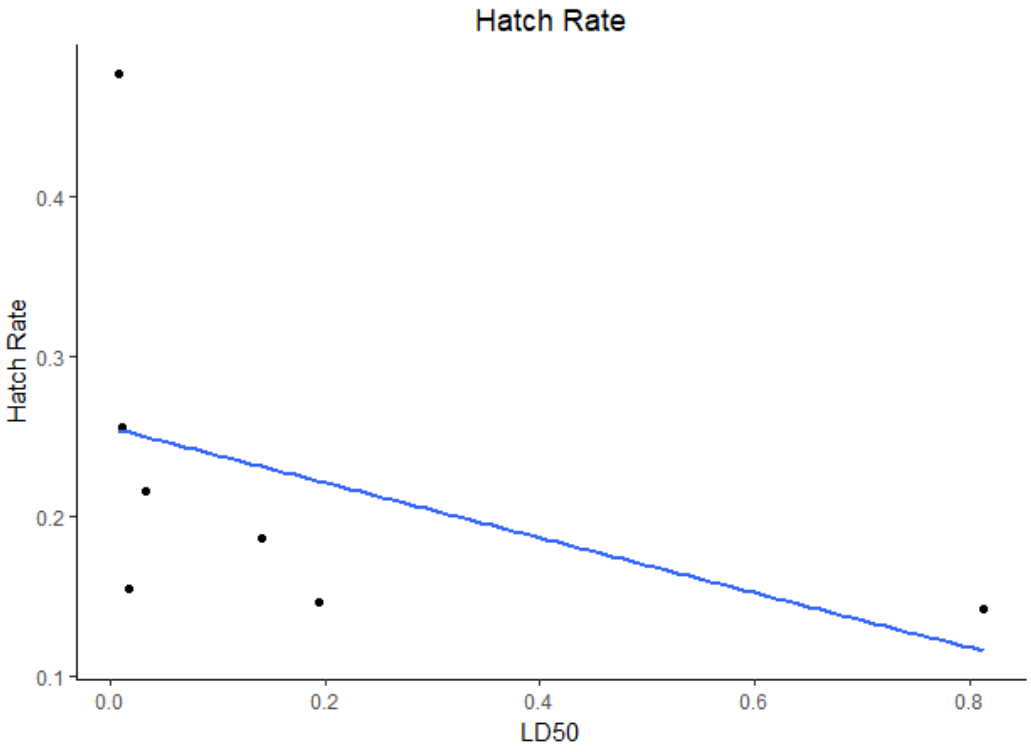


Figure 10: Scatter plot illustrating the relationship between LD50 and larval development.

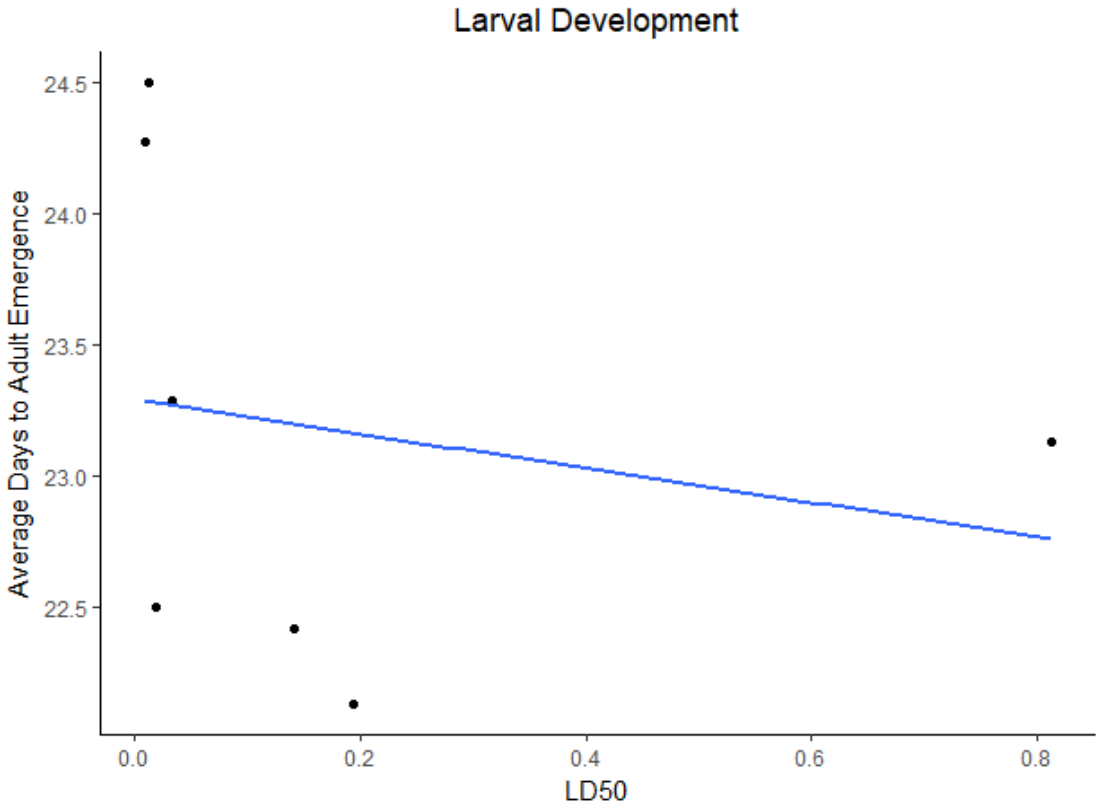


Figure 11: Scatter plot illustrating the relationship between LD50 and adult emergence rate.

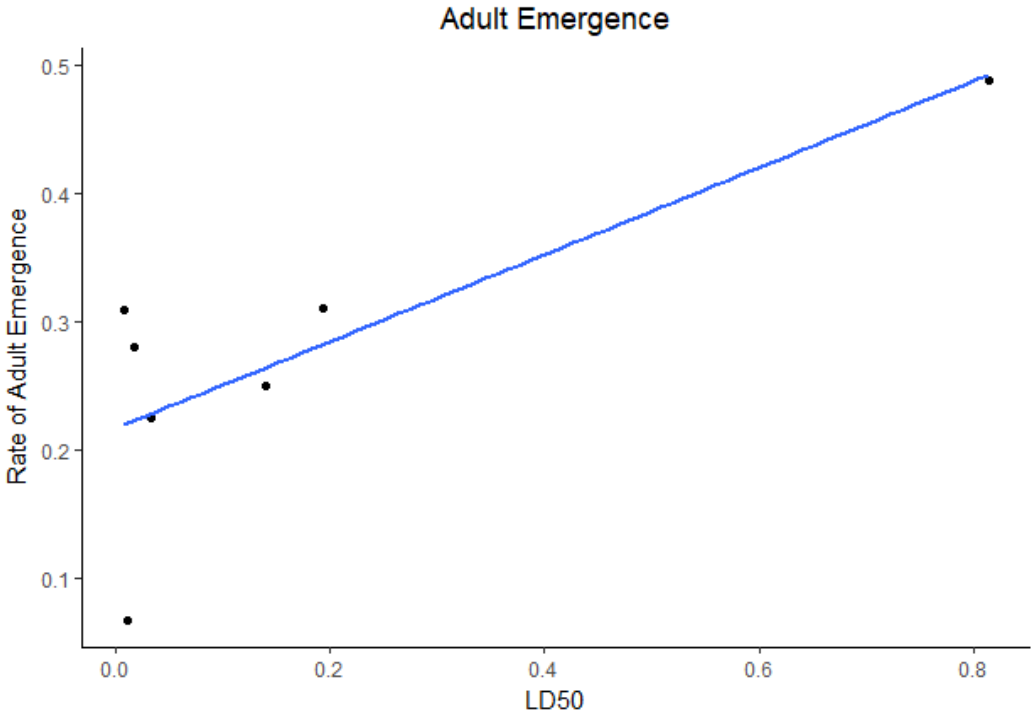


Figure 12: Scatter plot illustrating the relationship between LD50 and body size.

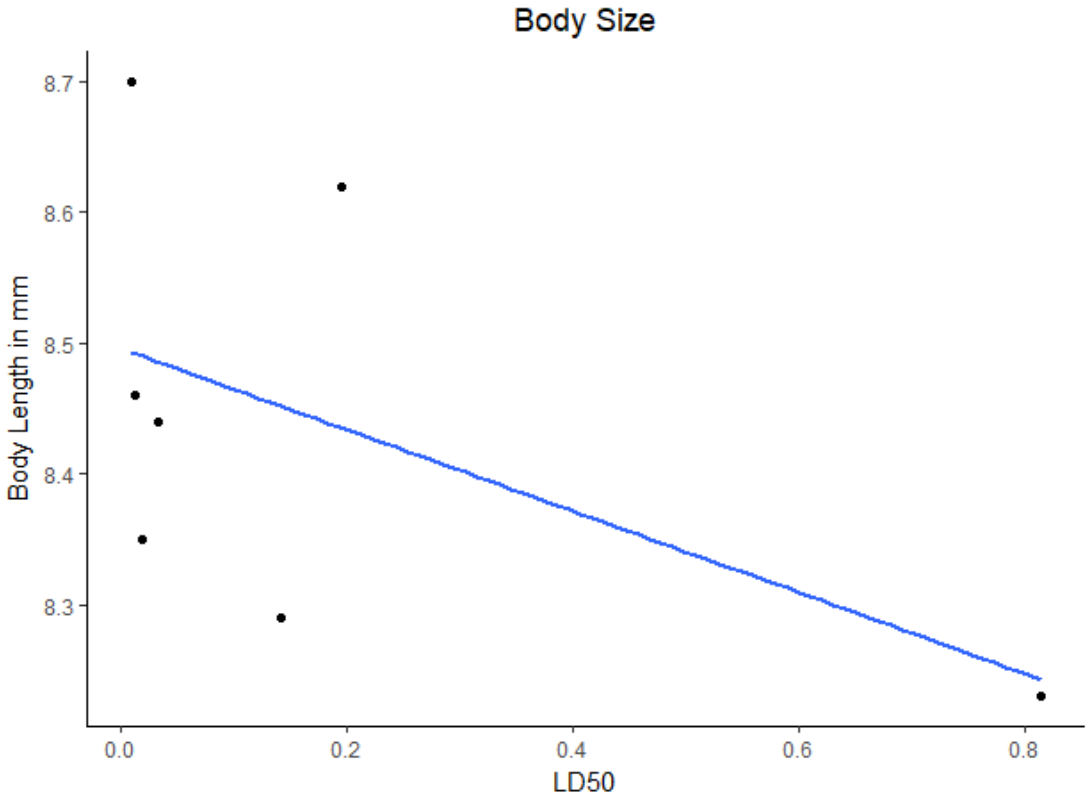


Figure 13: Scatter plot illustrating the relationship between LD50 and net replacement rate.

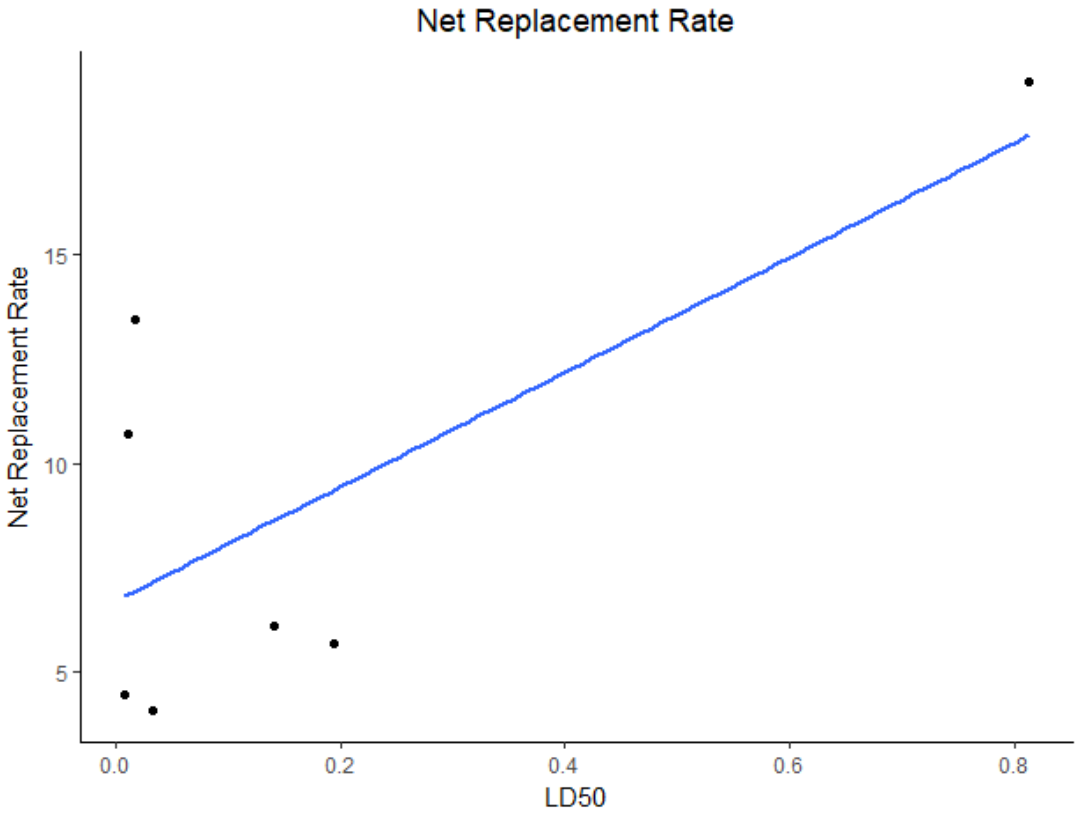
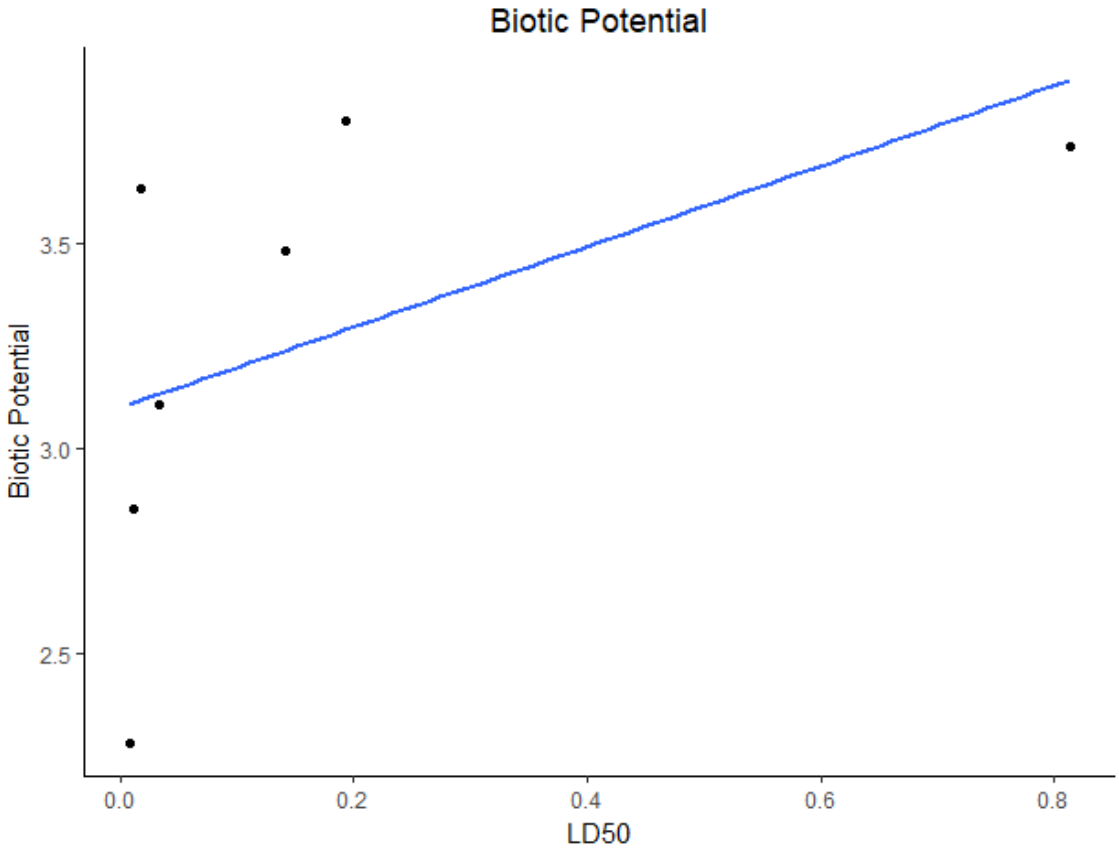


Figure 14: Scatter plot illustrating the relationship between LD50 and biotic potential.



CHAPTER 3: Geographic variation in dominance of spinosad resistance in Colorado potato beetles (*Leptinotarsa decemlineata*)

Potato farms on Long Island have been coping with insecticide resistance since the 1940's. Since then, every new insecticide that has been introduced to combat the pest species Colorado potato beetle (*Leptinotarsa decemlineata*) has failed, often in a very short time frame. In some instances, the beetles were pre-adapted for resistance to certain products before they were even available commercially. The dominance of a resistant phenotype is a key factor that determines how resistance can spread. Ascertaining the level of dominance at field concentrations is also crucial in developing plans to manage resistance. On organic farms on Long Island, *L. decemlineata* has evolved a high level of resistance to spinosad in a short period of time and that resistance has spread across the eastern part of the Island. Resistance has also emerged in other parts of the country as well. In order to clarify the level of dominance or recessiveness of spinosad resistance in different parts of the United States and how resistance differs in separate beetle populations, we sampled beetle populations from Maine, Michigan, and Long Island, in addition to the highly resistant population from Long Island identified in 2012 and hybridized them with a lab susceptible strain to determine dominance. None of the populations sampled in 2010 were significantly different from additive resistance but the population sampled in 2012 was not significantly different from fully recessive. This finding could be evidence of the spread of resistance alleles from the most resistant fields across Long Island. Widespread, recessive resistance to spinosad in *L. decemlineata* on Long Island could be easier to manage under the right circumstances.

Keywords: spinosad, resistance, Colorado potato beetle, *Leptinotarsa decemlineata*, dominance, recessive, geographic variation

Insecticide resistance is very costly in terms of crop losses and potential health effects. Oerke (2006), estimates that, left unprotected, 75% of potential potato crops could be lost to pests, both insect and otherwise. In dollar figures, Grafius (1997) projects that insect damage to potato crops in Michigan cost upward of $1.4 million each year. Worldwide assessments put that figure at $9.6 billion during a 3-year span from 1988-1990 (Rosenzweig, et al., 2001). Resistance affects health and safety because increasing pesticide use in response to resistance damages agricultural land and livestock and can cause illness and death in people who are exposed to these chemicals (Wilson & Tisdell, 2001). The key to managing resistance is understanding how it develops.

Under strong selection pressure from insecticides, resistance can evolve rapidly. For example, Australian sheep blowflies (*Lucilia cuprina*), developed dieldrin resistance within two years of initial use (J. A. McKenzie 1987) and diazinon resistance in the same species took less than a decade to emerge (Hughes and McKenzie 1987). Colorado potato beetles (*Leptinotarsa decemlineata*) in Michigan developed resistance to multiple insecticides in widespread areas in a span of about five years (Grafius 1997) and became resistant to several insecticide types on Long Island in a similar time frame (Georghiou 1986). Intensification of potato farming and the accompanying intensification of pesticide use are major factors in the rapid development of resistance. Increasing the area and frequency of potato fields increases resistance to imidacloprid, for example (Huseth, et al., 2015).

The dominance of a resistant phenotype is another key factor that determines how resistance can spread. Dominance is the measure of the relative occurrence of the phenotype of the heterozygote relative to the phenotype of the two corresponding homozygotes (Bourguet, Genissel and Raymond 2000). The level of dominance of resistance to a single dose of

insecticide is often inversely related to the concentration of the dose to which the insects are exposed and can also be altered by the environment (Liu and Tabashnik 1997, Sayyed, et al. 2000, Tabashnik, Gould and Carrière 2004, Szendrei, et al. 2011). Dominance can also vary based on the number of genetic loci responsible for conferring resistance. Lab selection for resistance starting from a susceptible baseline often results in polygenic resistance since the starting population is relatively small compared to field populations. Resistance, therefore, must be built on common, existing variation, which is likely to utilize multiple genes of small effect to obtain the desired result (Roush & McKenzie, 1987). When resistance depends on having a set of alleles rather than a single allele, the spread of resistance will be more gradual and resistance alleles in that situation are additive rather than a single allele being dominant or recessive. Monogenic resistance is more likely to arise in the field where population sizes are much bigger. The probability of a single resistance mutation appearing in the field is much greater than in a small lab population. A single gene with a large effect will spread quickly and the trait will be less additive.

The high dose-refuge strategy of resistance management is built on the idea that recessive, monogenic resistance can be delayed if treated properly. Development of resistance can theoretically be delayed if fields are initially treated with a high enough dose of pesticides to kill heterozygotes and untreated refuge areas can provide a source of susceptible genes to the treated fields (Roush and McKenzie 1987). Over time however, dominance can change with the evolution of dominance modifiers. These modifiers can act on one or more traits and effectively increase the dominance level of resistance (Bourguet, Genissel and Raymond 2000). For example, a dominance modifier has been documented in DDT-resistant house flies (*Musca*

domestica). When the modifier gene is present, the levels of both resistance and dominance are increased (Grigolo & Oppenoorth, 1966).

Potato farms on Long Island have been dealing with insecticide resistance for decades. The first documented failure of pesticides on the Island was in the 1940s (Gauthier, Hofmaster and Semel 1981). Since then, every new product that has been used has failed, often in just a few years. In certain cases, *L. decemlineata* showed signs of resistance to pesticides before they were even available for use by the public (Grafius 1997, Olson, Dively and Nelson 2000). High levels of resistance to neonicotinoid insecticides have been present on Long Island farms for more than 20 years. In fact, imidacloprid resistance first appeared on Long Island before spreading across the Midwest (Szendrei, Grafius, Byrne, & Ziegler, 2011). Resistance has been shown in different studies to be autosomal and either partially recessive or partially dominant (Zhao, Bishop and Grafius 2000, Baker, et al. 2007, Alyokhin, et al. 2008). Resistance to bt toxin by L. decemlineata was found to be incompletely dominant by Rahadja & Whalon (1995) and Miyo, et al. (1999) described esfenvalerate resistance as semi-recessive.

Spinosad is a relatively new natural insecticide compound, derived from the soil actinomycete *Saccharopolyspora spinosa*. It acts on the nicotinic acetylcholinesterase receptors of insects but at a different target site than the neonicotinoids (Sparks, et al. 2012). Spinosad resistance has been detected in a diverse group of insect species. According to a review by Sparks et al. (2012), resistance to spinosad across species is typically due to monogenic, recessive mutations, although there are examples of polygenic resistance as well as incomplete dominance. More recent research has shown that the LD_{50} of spinosad-resistant tomato borers (*Tuta absoluta*) and fall armyworms (*Spodoptera frugiperda*) were incompletely recessive (Campos, et al., 2014; Okuma, et al., 2018). Cross-resistance has been documented between

spinosad and imidacloprid. Mota-Sanchez, et al. (2006) found cross-resistance to spinosad but to a lesser degree than cross-resistance to other neonicotinoid insecticides. Alyokhin, et al. (2015) describe asymmetric cross-resistance between imidacloprid and spinosad, where previous resistance to imidacloprid grants some resistance to spinosad but spinosad resistance does not grant resistance to imidacloprid.

Long Island, NY has been a hotbed of L. decemlineata resistance for decades. Every pesticide that has been used to control beetles since the middle of the 20th century has failed, on average within 2 years (Georghiou 1986). It is theorized that high selection pressure, low gene flow due to geographic isolation, and a favorable climate—both in terms of high reproductive capabilities and low overwintering mortality—contribute to this phenomenon (Alyokhin, et al. 2015). Extremely high level (>5000-fold increase in LD_{50} relative to a laboratory susceptible colony) resistance to spinosad has been documented on Long Island starting in 2010 (Schnaars-Uvino 2013).

In this study we analyze the genetics of spinosad resistance and geographic variation in dominance. We sampled beetle populations from Maine, Michigan, and Long Island, in addition to the highly resistant population from Long Island identified in 2012. We hybridized each to a laboratory susceptible colony to measure the dominance of resistance from each population. This study will clarify the level of dominance or recessiveness of such mutations as well as showing how resistance differs in separate populations of potato beetles.

Methods

Populations and rearing

In 2010 three field populations and one susceptible lab-raised line were used. *L. decemlineata* clutches or adults were collected from commercial potato fields in Riverhead, NY, Mecosta Co., MI, and Fryeburg, ME, in June 2010. All populations were reared for one to two generations in 74 by 61 by 46 cm cages under 25° C and a 16:8 hr light: dark (L:D) cycle. Cages were provisioned with potted whole potato plants, and pots were replaced or watered as needed. Egg masses were collected daily; leaves with clutches were removed from the plants and incubated at 25° C and a 16:8 hr L:D cycle in a Percival® model I-36 VL incubator (Percival Scientific, Inc., Perry, IA). Populations were initiated with either 50 field-collected clutches or 200 field-collected adults, and individual populations were maintained in the lab by collecting 50 clutches or 150 adults from the previous generation to inoculate a new cage. The laboratory susceptible reference strain was used that has been reared in captivity for over 20 years since its establishment using beetles collected in New Jersey (French Agricultural Research Inc., Lamberton, MN).

Mating pairs were established using virgin adults from each colony. As adults first emerged from each colony, they were collected daily, visually sexed, segregated by gender to prevent mating, and held in single-sex groups of up to five per 325 ml vented Nalgene boxes until used to establish pairs. To measure the fecundity and offspring resistance of hybrids of each field population and the reference strain, reciprocal colonies were first established using one each of field males and virgin susceptible reference females, or susceptible reference males and virgin field females, for each field population. Larvae from those colonies were reared in separate cages and as virgin adults emerged from pupation, they were collected daily for use in this study.

In 2012 a highly resistant population on Long Island was used to assess the dominance of high-level resistance. This was a field that in 2010 showed high level resistance to spinosad (Schnaars 2013), with a resistance ratio for LD_{50} of 5,750, relative to the New Jersey laboratory susceptible strain. Adults were collected from that field on the South Fork of Long Island—located approximately 38 km from the 2010 field and separated by the Peconic Bay—in June of 2012 and reared in the lab for one generation. Hybrids were generated by stocking cages with 10-20 resistant Long Island males with a matched number of laboratory susceptible females or 10-20 Long island females with a matched number of laboratory susceptible males.

Experimental design

12-18 pairs of virgin adults from each source population, the susceptible reference strain, and hybrids between each source population and the susceptible population were housed by pair in 325 mL vented Nalgene boxes (12.5 by 7 by 5.5 cm) with mesh windows for ventilation in an incubator at 25 °C and a photoperiod of 16:8 hr L:D. Each pair's cage was maintained with fresh potato clippings mounted in floral pics changed daily. Clutches were collected and eggs counted 1-2 times daily for two weeks from the first laid clutch. Individual clutches were transferred to 3.5cm Petri dishes. Hatchlings were removed 1-2 times per day and pooled by population and reared to 2nd day-old 2nd instar for bioassay. The source population was coded so their it would be unknown when scored for bioassay.

Bioassay and analysis

Two day old second-instar larvae (weighing 5-8.5 mg) were assayed by direct topical application on the abdomen of a 1 µl drop of spinosad dissolved in HPLC-grade (0.995) acetone. Spinosad was extracted from SpinTor 2SC Naturalyte® (Dow AgroSciences LLC) by first

diluting 1:9 using HPLC-grade (0.995) acetone, then vacuum filtering twice to remove remaining particulates. The extraction used to create all solutions used in this study was confirmed by a specific immunosorbent assay performed by Environmental Micro Analysis, INC, Woodland, CA to have 0.59 efficiency from the stated concentration of active ingredient in the product label. Up to 7 concentrations of spinosad from 4.2×10-3 to 1.35×10-1 µg/larva in 2010, and up to 12 concentrations from 4.2×10-3 to 42 µg/larva in 2012, plus an acetone control, were used. Following application, larvae were placed on a potato leaf cutting and held at 25°C for 24 hours until scoring.

Dose-mortality curves were analyzed using Polo-Plus (LeOra Software 2007). Larvae were scored after 24 hours with mortality defined as failure to move a leg for 10 seconds after the larva is placed on its back. We attempted to assay at least 30 individuals from each population at each dose, but if fewer were available at the correct size, we analyzed the mortality data and included the LD50 results if the index of significance for potency estimation, g (Finney 1971), was less than 0.7 at the 0.95 confidence level. The degree of dominance (D) of resistance was calculated as in Stone (1968) on a -1 to 1 scale, where -1 is fully recessive, 0 additive, and 1 fully dominant:

$$D = \frac{(2RS - RR - SS)}{(RR - SS)}$$

where RS, RR, and SS are the logarithms of the LD50's for heterozygotes, homozygote resistant and homozygote susceptible strains. There were no differences between resistant female- or resistant male-hybrid colonies, so bioassay results were pooled for each colony type. Variance of D was calculated as in Preisler et al. (1990), to allow calculation of a standard error,

$\mathrm{SE} = \sqrt{\mathrm{var}(D)}$, allowing a confidence interval to test whether completely recessive (-1) or dominant (1) inheritance fell within the range of estimation.

Results

Resistance to spinosad varied among populations, and dominance appeared negatively associated with local resistance (Table 1). Resistance ratios varied from 12.5 to 58.6 in 2010. The Michigan assays were a poor fit to the logit model and were not significantly different from any population but the control. Riverhead, NY, and Freyburg, ME, were significantly different. The least resistant population, Freyburg, ME, showed the highest level of dominance, 0.66 on a -1 to +1 scale. Riverhead, NY, the most resistant population was also the least dominant, though poor fits of either the pure or hybrid strains from each geographical location prevented any of the dominance estimates from being significantly different from additive or dominant inheritance.

In contrast, higher level resistance from the South Fork of Long Island was much more, perhaps fully, recessive (Table 2). The resistance ratio was fifteen times greater than seen in the Riverhead population sampled in 2010. Dominance, D, of -0.73 had confidence limits of -1.02 to -0.43, so not significantly different from fully recessive and significantly less than additive, and also less than the -0.35 seen from Riverhead NY, two years earlier.

Discussion

Although there was considerable variation between strains in 2010, none of them were significantly different from additive resistance. The most resistant strain—Long Island—was also the most recessive. Two years later, the Long Island strain was much more recessive, being significantly different from additive but not from fully recessive. The fields from which resistant beetles were collected are approximately 38 km apart. This change in dominance on Long Island

suggests that perhaps the spinosad resistance trait was present in 2010 and had spread across the eastern part of the island by 2012. This would theoretically make resistance easier to control, as long as there are costs to high level resistance since the beetles are trading resistance for some diminution of overall fitness.

There is considerable disagreement about the best way to estimate dominance of resistance. Stone (1968) calculated a quantitative estimate of the degree of dominance of the LD_{50}. Roush & MacKenzie (1987) however, argued that it is difficult to apply estimates of dominance based on laboratory studies to field conditions. Differences may arise due to differing conditions between lab and field. An alternative is to assesses the relative mortality level at a given pesticide concentration, which is usually referred to as effective dominance (Bourguet, Genissel, & Raymond, 2000). That figures dominance at a single high dose, in order to more closely simulate actual field conditions in the lab and find a single high dose that would kill heterozygotes. This approach assumes that insects in the field are exposed to a single, high dose and that dose is the same as would be applied in the field but that is difficult to achieve. Pesticides can be washed away by rainfall and they break down over time. Spinosad, especially breaks down very quickly (Saunders & Bret, 1997). Even a plant that takes up pesticide systemically will vary in concentration over the life of that plant. Smaller plants have higher concentrations of pesticide while in older, larger plants the pesticide is more diffuse (Alford & Krupke, 2017). In this case, we wanted to show how quantitatively different homozygotes are relative to the heterozygotes, so we calculated the dominance of the LD_{50}. What's interesting about the results of our 2012 survey is that they show actual dominance of spinosad resistance, not just effective dominance in the field. This suggests a change to the underlying genetic architecture of the different *L. decemlineata* strains.

Dominance modifier genes may also play a role in the pattern of dominance uncovered in this study. Bourguet, et al. (1997) found evidence for such modifier genes in organophosphate and carbamate-resistant *Culex pipiens*. Those genes, they speculated, boosted acetylcholinesterase activity in resistant strains, enough that heterozygotes were able to survive bioassays in the lab, and making resistance more dominant. A similar phenomenon could be taking place with these populations of *L. decemlineata*. Although the exact mechanism of spinosad resistance in *L. decemlineata* is unknown, around half of the studies to date have identified target site mutations in other species—like organophosphate-resistant *C. pipiens*—that confer spinosad resistance (Sparks, et al., 2012). One reason to think this might not be the case here is that each resistant strain of *C. pipens* in the above-mentioned study exhibited identical mortality curves and differed only in dominance (Bourguet, et al., 1997). The different strains of *L. decemlineata* in the present investigation differ in both resistance level and dominance, perhaps indicating that the differences seen here are more likely due to environmental conditions than genetic modifications.

Varying levels of dominance of spinosad resistance present challenges to developing effective management strategies. Recessive resistance can be managed with the high-dose refuge strategy that uses high enough doses of pesticide to kill heterozygotes and spatial refuges that allow resistant individuals to develop free from selection pressure and then mix with the resistant population, so long as the resistant individuals are relatively rare (Tabashnik B. , 2008). Resistance in the Long Island strains has declined sharply since this investigation began (Chapter 1). Spinosad use was discontinued on the South Fork after this investigation (Calder-Piedmont, personal communication; Chasky, personal communication), creating a temporal refuge for susceptible individuals to migrate onto the farms and mate with the resident individuals. Since

resistance on Long Island was recessive, the result would be more heterozygotes that are less resistant to spinosad.

Managing dominant resistance is more difficult. One method is to increase the concentration of the insecticide to a level that would kill heterozygotes (Tabashnik, Gould, & Carrière, 2004). That raises some practical problems though. It is not feasible in all cases to estimate the appropriate concentration to kill heterozygotes and it also poses increased risk to non-target, potentially beneficial species (Roush & McKenzie, 1987). Denholm and Rowland (1992) suggest that denying refuges to insect pests—i.e. not rotating to other types of pesticide and not maintaining untreated fields—may be a more effective strategy to decrease dominance. That prevents resistant individuals from having the opportunity to migrate to an untreated area and mix with susceptible individuals (Preisler, Hoy, & Robertson, 1990).

The choice of management strategy is ideally based on local resistance and life history traits. A thorough understanding of those characteristics between geographically isolated populations provide the ability to customize control techniques based on local conditions (Chen, Alyokhin, Mota-Sanchez, Baker, & Whalon, 2014). There is often a great deal of variation in resistance, dominance, and fitness between populations of *L. decemlineata.* Imidacloprid resistance was first documented in the eastern United States in the late 1990s. Northeastern populations remained susceptible until around 2003 and only became established in the Midwest around 2009 (Szendrei, et al., 2011). A number of studies have documented differences in imidacloprid resistance between different regions of the US. Chen et al. (2014) found variation between populations in the Northeast, Midwest, & Mid-Atlantic regions. Crossley et al. (2018) found differences in resistance between populations in the Upper Midwest and Pacific Northwest. Huseth et al. (2015) & Crossley et al. (2017) both found considerable variation

between populations within the Upper Midwest region that might be related to the intensity of farming between different fields.

The level of dominance of insecticide resistance is an important factor in the determining the types of strategies that are used in controlling insect pests. Dominance can vary between geographically isolated populations and even between interconnected populations. Spinosad resistance was found to be incompletely dominant in Maine and Michigan but incompletely recessive on a large, conventional farm on Long Island. Resistance on a smaller, organic farm on Long Island was found to be almost completely recessive. Recessive resistance can be managed using spatial or temporal refuges to allow susceptible individuals to migrate into the population and fitness costs to take effect. Dominant resistance, like that found in the more northern fields, is more difficult to manage and may require the denial of refuges. Knowledge of the genetic structure of a population can provide growers with the tools to design customized management strategies.

Table 1: Resistance to spinosad of *L. decemlineata* from New York, Maine, and Michigan, a susceptible laboratory colony, and hybrids of each field population and the laboratory susceptible line

Population	N[a]	ld_{50}[b]	Lower[c]	Upper[c]	Slope	χ^2	df	P	RR[d]	D[e]
ME Freyburg	224	0.0863	0.0228	0.2807	0.623	9.99	8	0.27	12.5	0.66
ME Hybrid	506	0.0366	0.0035	0.1494	0.569	36.55	8	<0.001	5.3	
Riverhead NY	342	0.4042	0.2258	0.6826	1.068	12.01	9	0.21	58.6	-0.35
NY Hybrid	245	0.0266	0.0130	0.0571	1.238	6.42	4	0.17	3.9	
Michigan	250	0.1779	0.0100	1.9172	2.031	18.64	8	0.02	25.8	0.31
MI Hybrid	247	0.1034	0.0061	0.7411	0.393	4.84	6	0.56	15.0	
Susceptible	217	0.0069	0.0014	0.0137	1.57	13.14	4	0.01	1.0	

[a] Number of 2nd instar larvae tested

[b] LD_{50}s are in units of µg/larva

[c] 95% fiducial limits

[d] LD_{50} of a given field population / LD_{50} of the susceptible laboratory line

[e] Dominance as in Stone (1968) ranging from fully recessive (-1) to fully dominant (1)

Table 2: Resistance to spinosad of *L. decemlineata* from the most resistant population in eastern New York in 2012, a susceptible laboratory colony, and hybrids of between the two.

Population	N[a]	LD_{50}[b]	Lower[c]	Upper[c]	Slope	c^2	df	P	RR[d]	D[e]
LI Resistant	266	1.1406	0.5467	5.0625	0.629	7.03	7	0.43	877.3	-0.73
Hybrids	144	0.0032	0.0038	0.0074	2.4	2.58	8	0.96	2.5	
NJ	354	0.0013	0.0016	0.0029	2.05	1.62	5	0.90	1.0	

[a] Number of 2nd instar larvae tested

[b] LD_{50}s are in units of µg/larva

[c] 95% fiducial limits

[d] LD_{50} of a given field population / LD_{50} of the susceptible laboratory line

[e] Dominance as in Stone (1968) ranging from fully recessive (-1) to fully dominant (1)

SUMMARY

Overuse of spinosad to control Colorado potato beetles (*Leptinotarsa decemlineata*) on two organic fields on the South Fork of Long Island in 2010 led to the rapid evolution of extremely high levels of resistance and the failure of spinosad to control the pests. Resistance of *L. decemlineata* to spinosad on one of those fields was found to be highly recessive, while resistance on a nearby conventional field and two fields in Maine and Michigan was much less recessive (and not significantly different from additive). After spinosad use was discontinued on the South Fork fields and selection pressure for resistance was relaxed, I surveyed *L. decemlineata* on these, four additional organic potato fields on the North Fork, and one organic field in Tompkins County, NY for spinosad resistance. Resistance on the South Fork fields was higher than the North Fork or Tompkins County but lower than in previous seasons. Resistance dropped sharply from 2016 to 2017 on all fields, suggesting that evolutionary tradeoffs were affecting resistance in the absence of selection pressure.

I assessed whether these potential tradeoffs were associated with reproductive fitness. The results indicate that may be a cost to egg development time, as the most resistant population's eggs took the longest to hatch. This cost may be counterbalanced by a potential fitness benefit to emergence time. None of the fitness indices that I computed showed evidence of reduced general reproductive fitness in highly resistant populations. The decline in resistance without strong evidence for costs might be explained by the gradual migration of susceptible individuals into the study area over time. It may be that costs are present in these populations but do not affect reproductive fitness. Costs might also emerge only under field conditions but not in the lab.

The dominance of spinosad resistance varies within and between geographic regions. Resistance was incompletely dominant in Maine and Michigan and incompletely recessive on one Long Island field and almost completely recessive on another Long Island field. Knowing the dominance of resistance in a given area should allow growers to implement customized control strategies. Dominant and recessive resistance should not be treated the same. Recessive resistance, like that found on Long Island can be managed by providing refuges to susceptible pest insects and allowing costs to select against resistance. I have provided evidence of the effectiveness of temporal refuges, as spinosad resistance on Long Island has decreased since its intensive use was discontinued.

It is not yet clear whether the decline of resistance is due to evolutionary trade-offs or migration of susceptible individuals onto these potato fields. Further research should focus on answering that question by examining whether costs may be found under different conditions than in the present studies. The exact mechanism of resistance to spinosad has not yet been determined and future investigation can help solve that mystery as well.

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