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Characterization of Cry3Bb1 resistance in field-derived strains of western corn rootworm (Coleoptera: Chrysomelidae)

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Characterization of Cry3Bb1 resistance in field-derived strains of western corn rootworm
(Coleoptera: Chrysomelidae)

by

David Adam Ingber

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Entomology
Program of Study Committee
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Iowa State University
Ames, Iowa
2014

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CHAPTER 1: INTRODUCTION

Resistance is defined as being a genetically base decrease in susceptibility of an insect population to an insecticide (Tabashnik 1994). The evolution of resistance in insect populations has long plagued the agricultural industry across the globe. The tools which have been developed to combat insect pests have inadvertently aided in the evolution of resistance. The harsh environment set forth by pesticide application creates a strong selective pressure favoring insects able to cope with the chemicals. Coping mechanisms can take many forms. Adaptations such as decreased sensitivity, decreased cuticular or membrane permeability, conversion to an inert, excretable compound, increased excretion, behavioral avoidance of the chemical in question, or any combination thereof can all give insects a distinct advantage for surviving pesticides (Mallet 1989, McKenzie and Batterham 1994). Fundamentally, the evolution of resistance provides for an almost ideal selection model in which to study insect adaptation to an environmental stress. Practically and economically, however, the study of resistance is of great significance due to the increasing number and severity of resistance events in agricultural settings across the world (Denholm and Rowland 1992).

The western corn rootworm *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) is one of the foremost agricultural pests in the United States today (Gray et al. 2009). Larval feeding on corn root tissue results in decreased yields with one node of root injury (the root injury threshold for classifying a field planted with Bt corn (*Zea mays* L) expressing a single toxin as having greater than expected feeding injury (EPA 2011)) measured on the 0-3 nodal scale (a node or roots is denoted as a circle of roots around the stalk of the plant) causing approximately a 17% reduction in yield (Dun et al. 2010). Larval feeding also weakens the structural integrity of the plant resulting in their developing a severe lean (lodging), which makes
harvesting difficult. To exacerbate the problem, western corn rootworm has evolved resistance to several management practices. Populations in Illinois have developed a resistance to traditional crop rotation schemes. Adult insects have exhibited decreased fidelity to oviposition in corn fields and will instead move into neighboring fields that may not contain corn. The eggs will then diapause until the next planting season, becoming established in fields that rotate to corn (Levine et al. 2002, Gray et al. 2009). In Nebraska the adults of western corn rootworm populations have exhibited significant resistance to the foliar insecticides Penncap M (whose active ingredient is the organophosphate Methyl parathion) and Sevin 4-Oil (active ingredient is the carbamate Carbaryl) (Meinke et al. 1998). Additionally, the hemolymph of larval western corn rootworm contains allomones including coagulants and deterents, which makes them undesirable targets for predation (Lundgren et al. 2009). Allomone defenses are, however, not exclusive to the western corn rootworm. They are commonly found across most insect taxa and cover a wide range of chemicals (Laurent et al. 2005). For example, larvae of sawflies in the family Argidae have similar hemolymphic defenses to predation as the western corn rootworm (Petre et al. 2007), and larvae of moths in the family Arctiidae sequester pyrrolizidine alkaloids from their host plant in their hemolymph as a predation deterrent (Naumann et al. 2002).

Genetically modified crops producing insecticidal toxins derived from the bacterium Bacillus thuringiensis (hereby to be referred to as Bt) have played a key role in the control of many agricultural pests. The mode of action for Bt involves crystalline (Cry) proteins that are ingested and subsequently bind to receptors in the target insect’s midgut, causing pore formation and the eventual lysing of the gut, killing the insect (Schnepf et al. 1998). In 2003 transgenic Bt corn was approved for commercial sale by the Environmental Protection Agency (EPA) (EPA 2003). In 2012, 67% of the corn planted in the United States was of Bt hybrids for the
management of pest insects (ERS 2012). Unfortunately, the efficacy of current commercially available hybrids of Bt corn is in danger due to the evolution of resistance to the Cry3Bb1 Bt toxin by western corn rootworm (Gassmann et al. 2011, Gassmann 2012, Gassmann et al. 2012b, 2014).

Resistance to Bt toxins may occur from a variety of different mechanisms. Bt receptors include aminopeptidases, cadherin like proteins, alkaline phosphatase, and glycolipid receptors (Ma et al. 2005). Mutations resulting in the inactivation or reduced activity of Bt receptors is one of the best characterized resistance mechanisms (Ma et al. 2005), and results in “Mode 1” resistance, which is characterized by high levels of resistance (over 500 fold compared to susceptible insects), recessive inheritance of resistance, and no cross resistance with other Bt toxins (Tabashnik et al. 1998). Mode 1 resistance is prevalent in lepidopteran pests such as tobacco budworm (*Heliothis virescens* Fabricius, Lepidoptera: Noctuidae), pink bollworm (*Pectinophora gossypiella* Saunders, Lepidoptera: Gelechiidae), cotton bollworm (*Helicoverpa armigera* Hübner, Lepidoptera: Noctuidae), and diamondback moth (*Plutella xylostella* Linnaeus, Lepidoptera: Plutellidae) (Heckel et al. 2007). The Bt resistance of each pest, with the exception of *P. xylostella*, is caused by decreased binding of Bt proteins to cadherin domains (Heckel et al. 2007).

Bt resistance may also occur as a result of inducible tolerance mechanisms, which consist of immune responses (Ma et al. 2005). Immune responses that may impart Bt resistance include increased activity of proteases in the gut, resulting in “over-digestion” of ingested toxins, increased ability for gut cells to recover from Bt activity, or increased coagulation responses which prevent toxins from reaching the gut lining (Gould 1998, Ma et al. 2005). For example, Loeb et al. (2001) found greatly increased cell recovery rates in gut cell cultures of a strain of *H.*
*virescens* when exposed to the spores of two strains of *Bacillus thuringiensis*, and Loseva et al. (2002) found increased aminopeptidase activity in a Cry3Aa resistant strain of Colorado potato beetle (*Leptinotarsa decemlineata* Say, Coleoptera: Chrysomelidae). Mode 1 resistance is characterized by a loss of activity. However, resistance mechanisms that involve inducible tolerance are the result of increased activity or gain of function (Gould 1998). Subsequently, inducible tolerance resistance mechanisms are more likely to result in non-recessive inheritance of resistance and cross resistance, as well as generally lower levels of Bt resistance (Heckel 1994, Gould 1998, Tabashnik et al. 1998). Inducible tolerance mechanisms are also less commonly observed in field settings (Ma et al. 2005). This may be due to increased prevalence of fitness costs (decreased fitness of resistant insects compared to susceptible insects in the absence of Bt (Gassmann et al. 2009)) associated with inducible tolerance mechanisms of Bt resistance (Ma et al. 2005).

Because Mode 1 and inducible response resistance mechanisms are each associated with certain resistance traits (recessive or non-recessive inheritance, likelihood of cross-resistance, and potential for associated fitness costs), the early determination of the mode of resistance may be useful for the development of IRM strategies for populations of Bt resistant insects (Gould 1998, Tabashnik et al. 1998). However, care should be taken, as it is possible for different populations of a single pest species to exhibit differing resistance mechanisms (Heckel 1994, Tabashnik et al. 1998). Because of this phenomenon the characterization of many populations of a Bt resistant insect species is important in order to fully understand the species’ Bt resistance and develop appropriate IRM strategies (Tabashnik et al. 1998).

The EPA requires resistance monitoring programs as part of its insect resistance management (IRM) program for Bt corn products. Resistance monitoring programs must
include bioassay evaluations of resistance conducted by the registrants of the Bt hybrid, and encourage growers to report cases of unexpected damage of Bt crops (EPA 2006). The purpose of these monitoring programs is the early identification of resistance in order for the timely implementation of remedial actions to delay further resistance evolution (Devos et al. 2013). Effective programs should also include data on baseline susceptibility to allow for monitoring of changes in the pest’s susceptibility to Bt (EPA 2003, Devos et al. 2013), and include bioassays that are reliable and accurate (Siegfried et al. 2007). Data from resistance monitoring provides evidence for the effectiveness of IRM programs and the monitoring techniques used as part of them, as well as evidence for or against the model parameters used in designing IRM plans (EPA 2003, Glaser and Matten 2003).

Bioassays are experiments that measure the biological activity of a chemical on live organisms or cell cultures. Bioassays used in western corn rootworm resistance monitoring had been mostly diet-based (EPA 2011), but has shifted to include more plant-based methods in recent years (EPA 2013a, b). Diet-based bioassays are conducted by overlaying chemicals or pathogens onto artificial insect diet (Siegfried et al. 2005, Siegfried et al. 2007). Western corn rootworm larvae are subsequently placed onto the diet and monitored for mortality or sub-lethal effects. Plant-based bioassays involve either the growth of single corn plants in containers (Gassmann et al. 2011, Gassmann et al. 2012b, Meihls et al. 2012), or the growth of many corn seeds in containers into seedling-mats (Notwatzki et al. 2008, Petzold-Maxwell et al. 2012). Both single-plant and seedling-mat plant-based bioassays then involve the placement of western corn rootworm eggs or larvae onto or next to the roots of the corn plants, and subsequent monitoring.
Wild type western corn rootworm exhibit a univoltine life cycle (Gray et al. 2009, Spencer et al. 2009), which makes measuring their evolutionary response to selection pressures a slow process. The development of non-diapausng laboratory strains of the insect (Branson 1976) alleviated this issue, allowing for the assessment of many generations of the pest per year. Introgression of field derived alleles into non-diapauasing strains has also been used to select for Bt resistance in laboratory colonies of western corn rootworm (Meihls et al. 2008, Owsald et al. 2011, Meihls et al. 2012). Non-diapauasing strains of western corn rootworm are often reared in the laboratory for many generations prior to use in experiments, which creates the potential for loss of genetic diversity over time (Kim et al. 2007, Lefko et al. 2008). However, it may take exceedingly large numbers of generations for this to occur (Kim et al. 2007). This effect may be eliminated by the occasional introgression of new alleles into populations reared in the laboratory for extended periods (Kim et al. 2007, Li et al. 2014). The overall fitness of laboratory colonies of insects may also change over time due to adaptation to laboratory conditions (Rossler, 1975). Such changes may negatively affect the direct comparability of laboratory and field colonies (Li et al. 2014). Therefore, caution should be taken when applying the results of bioassays with laboratory reared colonies to field settings, in using laboratory colonies for behavioral studies (Li et al. 2014), and in the overall selection of bioassay methods.

All fields planted with commercially registered Bt corn hybrids for the management of western corn rootworm are required to be planted with a non-Bt refuge (EPA 2010). The proportion of a field that must be devoted to refuge may vary depending on the number of Bt toxins targeting western corn rootworm a hybrid produces, and whether it is planted as a seed blend or block. A 5% refuge is required for a pyramid of two or more toxins whose refuge is planted as either a block or seed blend, 20% for a single trait hybrid whose refuge is planted in a
blocked manner, or 10% for a single trait hybrid whose refuge is planted as a seed blend (EPA 2010). The non-Bt plants serve as a safe haven for the development of susceptible individuals. The susceptible insects then mate with their resistant counterparts producing heterozygous progeny. The degree to which heterozygotes have lower fitness compared to homozygous resistant insects can result in delaying the evolution of resistance (Gould 1998, Tabashnik et al. 2003, Gassmann et al. 2011, Gassmann 2012, Tabashnik et al. 2013). Additionally, it is possible to achieve longer delays by increasing the field percentage devoted to refuge (Tabashnik et al. 2008).

The refuge strategy’s effectiveness is highly dependent on the effective dominance of resistance, which is the fitness of heterozygotes relative to resistant homozygotes given the ecological conditions present in the environment (Gould 1998, Tabashnik et al. 2004). As effective dominance increases, the fitness of heterozygote insects on Bt plants will more closely resemble that of the homozygous resistant parent, resulting in increased survival and a subsequent increase in the transmission of resistance alleles. Importantly, the effective dominance will decrease as the concentration of the toxin increases (Tabashnik et al. 2004, Taylor and Georghiou 1979, Roush and McKenzie 1987, Gould 1998).

The use of plant hybrids that produce extremely high concentrations of Bt toxins can bolster the efficacy of refuges, this is referred to as the high-dose refuge strategy (Gould 1998). According to the EPA, in order for a Bt event to be considered high-dose it must produce toxins whose concentrations are 25 times greater than that required to kill a susceptible individual, or result in at least 99.99% mortality (EPA 1998). The excessively high concentrations of Bt toxin serve a dual purpose. The first is to cause increased heterozygote mortality, forcing the inheritance of resistance into effective susceptibility due to the increased fitness difference
between heterozygotes and resistant homozygotes (Taylor and Georgiou 1979, Tabashnik and Croft 1982, Roush and McKenzie 1987, Denholm and Rowland 1992). The second is to ensure nearly 100% mortality for both susceptible homozygotes and heterozygotes (Tabashnik et al. 2013). This ensures that the only insects that will survive are those possessing the homozygous resistant genotype (Tabashnik et al. 2004, Carrière et al. 2010). Surviving resistant insects that mate with susceptible refuge insects will then produce heterozygous progeny that will subsequently be killed by Bt toxins (Gould 1998).

Both of the approaches to the refuge strategy are greatly affected by the initial frequency of resistance alleles in the insect population. As the frequency of alleles in the breeding pool increases so does the frequency of the homozygous resistant genotype (assuming random mating the frequency of the genotype will be the square of the frequency of the resistance allele). Thus if the initial frequency of the allele is 0.001, the initial frequency of homozygous resistant individuals will be $10^{-6}$. Due to the square relationship between the allele and genotype frequencies, even a small increase in the initial frequency of the resistance allele will result in a relatively large increase in the presence of the genotype, increasing the rate of resistance evolution (Gould 1998). These effects can be mitigated via increased refuge sizes (Tabashnik et al. 2013, Gassmann et al. 2014). However, increased refuge percentages are widely considered to be impractical by most farmers (Gould 1998).

The high-dose approach coupled with the refuge strategy is a powerful tool in delaying the evolution of resistance. However, in the case of western corn rootworm and Cry3Bb1 corn, the concentration of Bt toxin being produced by corn plants is insufficient to constitute high-dose, which limits the effectiveness of current refuge strategies (Meihls et al. 2008, Gassmann 2012, Tabashnik and Gould 2012). This lack of high-dose promotes non-recessive inheritance of
resistance that heightens the risk of resistance evolution. It is therefore pertinent to determine the effective dominance for resistance of western corn rootworm to Cry3Bb1 corn in order to better understand the effectiveness of the refuge strategy currently being employed as part of western corn rootworm IRM.

The refuge strategy assumes that there is some degree of plant-to-plant movement by adult insects (Gould 1998). In western corn rootworm, this movement can be described as being either trivial or sustained (Barcic et al. 2007). Trivial movement occurs over short distances (Coats et al. 1986), and serves to disperse susceptible individuals throughout a field, which in turn will may promote mating between resistant insects that developed on Bt plants and susceptible insects that developed in the refuge (Gould 1998). Contrasting, sustained movement is migratory flight over long distances, which is conducted primarily by gravid females in search of oviposition sites (Coats et al. 1986, Naranjo 1990, Spencer et al. 2009). However, the proportion of insects that partake in sustained flight is low (Naranjo 1990).

Refuges planted in a block may promote assortative mating between resistant and susceptible western corn rootworm due to the special separation of Bt and non-Bt plants in corn fields. Blended refuge strategies may alleviate this by facilitating random mating due to the close proximity of Bt and non-Bt plants (Pan et al. 2011). Random mating promotes the generation of larger proportions of heterozygote progeny. If the inheritance of resistance in a Bt resistant insect population is recessive, or the Bt toxins produced by plants in a field are high-dose the heterozygotes will be subsequently killed (Gould 1998). The established lack of high-dose in Bt corn hybrids for the management of western corn rootworm may, however, limit the effectiveness of blended refuge strategies.
It is possible for western corn rootworm larvae to move up to 100 cm from their site of hatching to that of pupation (Short and Luedtke 1970, Hibbard et al. 2004), or approximately three plants down a row (Hibbard et al. 2003). This movement is largely inconsequential in a block refuge, but in a blended refuge it creates a potential for heterozygote larvae to move from a corn plant that produces Bt to one which does not (Hibbard et al. 2005, Spencer et al. 2009, Zukoff et al. 2012). This could potentially result in increased heterozygote survival that would increase the effective dominance of resistance and subsequently increase the rate of resistance evolution (Mallet and Porter 1992, Spencer et al. 2009). Overall, results comparing block and blended refuge strategies are mixed (Mallet and Porter 1992, Pan et al. 2011), making it difficult to determine which method is more efficacious.

It is important to note when considering the refuge strategy that resistant insects also will disperse into and reproduce in the refuge, which diminishes its effectiveness in delaying Bt resistance (Comins, 1977, Caprio 2001). However, the selective pressure imposed by fitness costs of Bt resistance can act to remove resistance alleles from insect populations inhabiting the refuge (Crowder and Carrière 2009, Gassmann et al. 2009, Carrière et al. 2010). As a result, their presence will aid in delaying the development of resistance to Bt crops. Quantifying the magnitude of fitness costs that accompany resistance traits can therefore be used to determine the risk of resistance developing (Gassmann et al. 2008, Gassmann et al. 2012b).

Fitness costs of Bt resistance have been found to affect a variety of life history traits such as developmental rate, survival, fecundity, adult lifespan, and male mating success (Carrière et al. 2001, Gassmann et al. 2009, Miehls et al. 2012, Jakka et al. 2014). Additionally, fitness costs may impose greater delays to the evolution of resistance depending on their dominance (Carrière and Tabashnik 2001, Gassmann et al. 2009). If fitness costs are recessive, then resistant
homozygote insects will have reduced fitness compared to susceptible homozygotes when developing on non-Bt plants, but the fitness of heterozygotes will not significantly differ from that of susceptible homozygotes. Non-recessive fitness costs also result in decreased heterozygote fitness compared to susceptible homozygotes as well, which subsequently results in greater delays of resistance (Carrière and Tabashnik 2001, Pittendrigh et al. 2004). While non-recessive fitness costs are more desirable than recessive costs from an IRM standpoint as they impose greater selective pressures, recessive fitness costs may still delay or even reverse the development of resistance if refuge proportions are sufficiently large (Gassmann et al. 2009).

There are positive relationships between the presence and magnitude of fitness costs and the magnitude of Bt resistance. Gassmann et al. (2009) determined that resistance ratios (the fitness of a resistant strain of an insect divided by that of a corresponding susceptible strain) were significantly larger in Bt resistant insect strains for which fitness costs were identified than in those where none were detected. Additionally, Liang et al. (2008) studied a laboratory strain of Cry1Ac resistant *H. armigera* over many generations and found that as the magnitude of the strain’s resistance ratio increased over time from selection on artificial diet treated with Cry1Ac, so did the magnitude of corresponding fitness costs in fecundity and larval weight. However, the number of alleles associated with the Cry1Ac resistance in the *H. armigera* strain also increased over the generations which makes it unclear as to whether the increased fitness cost magnitudes were due to larger resistance ratios or the presence of additional resistance alleles (Liang et al. 2008, Carrière et al. 2010).

Many ecological factors have the potential to amplify fitness costs. The suitability of an insect’s host plant may affect the magnitude of fitness costs, with feeding on less suitable hosts tending to increase the effects of fitness costs (Raymond et al. 2007). It has also been
demonstrated that secondary metabolites produced by host plants may increase the dominance and magnitude of costs (Carrière et al. 2004). Finally, the presence of entomopathogens such as nematodes and fungi may also magnify the effects of fitness costs (Gassmann et al. 2009). The inclusion of ecological factors that affect fitness costs in non-Bt refuges may bolster resistance management (Carrière et al. 2004, Raymond et al. 2007, Gassmann et al. 2009).

To date, all data on fitness costs of Bt resistance in western corn rootworm are from strains that were selected for resistance in the laboratory (Miehls et al. 2012, Oswald et al. 2012, Petzold-Maxwell et al. 2012, Hoffmann et al. 2014). In addition, few fitness costs of Bt resistance have been identified in western corn rootworm overall (Meihls et al. 2012, Oswald et al. 2012, Petzold-Maxwell et al. 2012, Hoffmann et al. 2014). Subsequently, fitness costs data on strains collected from the field or laboratory strains with field-evolved resistance are of value for the assessment of fitness costs in western corn rootworm IRM.

For this Master’s thesis I performed studies involving Cry3Bb1 resistant, non-diapausng strains of western corn rootworm with field-introgressed alleles. Adult male western corn rootworm were collected from fields reported as having greater than expected western corn rootworm damage to Cry3Bb1 corn. The field-collected male insects were crossed with females from a non-diapausng, Bt susceptible strain of western corn rootworm. The progeny of the crosses were selected on Cry3Bb1 corn several times to eliminate susceptible insects in the breeding population and backcrossed to the susceptible strain several times to increase the genetic similarity between the resistant strains and the susceptible strain.

We conducted laboratory and greenhouse plant-based bioassays with a resistant strain and corresponding susceptible strain to identify an optimal bioassay approach for measuring Cry3Bb1 resistance in a non-diapausng laboratory strain of western corn rootworm compared to
a corresponding susceptible strain. We then used the identified plant-based bioassay and additional diet-based bioassays to measure the magnitude and inheritance of Cry3Bb1 resistance in two resistant strains of western corn rootworm. Lastly, we measured life history characteristics of the two resistant strains compared to the susceptible strain reared on non-Bt corn with growth chamber experiments to identify any fitness costs of Cry3Bb1 resistance. Data from this thesis will aid in the development and assessment of IRM policies for the management of Bt resistance in western corn rootworm.

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CHAPTER 2: A COMPARISON OF METHODOLOGIES FOR MEASURING RESISTANCE TO BT CORN BY WESTERN CORN ROOTWORM

(COLEOPTERA: CHrysomelidae)

A paper for submission to the Journal of Economic Entomology

Authors to be determined

Abstract

The western corn rootworm, Diabrotica virgifera virgifera LeConte, is a serious pest of corn. Populations of western corn rootworm have developed resistance to several management strategies, including transgenic corn producing the toxin Cry3Bb1 derived from the bacterium Bacillus thuringiensis (Bt). The Environmental Protection Agency requires resistance monitoring programs that must include bioassay assessments of Bt resistance conducted by registrants as part of its insect resistant management program for Bt corn. Here we examine the discriminatory power of five bioassay methods for detecting statistically significant differences in the magnitude of resistance to Cry3Bb1 in two non-diaposing strains of western corn rootworm. The resistant strain (Hopkinton) was generated by introgressing alleles from a field population with Cry3Bb1 resistance into a non-diapousing susceptible strain (Standard). The Standard strain was never exposed to Bt corn and served as the susceptible control in the bioassays. The bioassay methods studied were: a single-plant assay for larval survival, seedling-mat assay for larval survival, small single-plant assay for survival to adulthood, large single-plant assay for survival to adulthood, and seedling-mat assay for survival to adulthood. In general, seedling-mat assays tended to distinguish between resistant and susceptible strains on Bt corn better than single-plant assays, and whole-plant assays conducted in the greenhouse showed the least discriminatory power. Future research will be needed to determine how these bioassay
methods perform with diapausing strains of western corn rootworm and other Bt toxins. Data from this study will aid in the development of more effective resistance monitoring techniques for western corn rootworm.

**Keywords:** bioassay, corn, *Diabrotica virgifera virgifera*, resistance management

**Introduction**

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte, (Coleoptera: Chrysomelidae) is one of the foremost agricultural pests in the United States (Gray et al. 2009). Larval feeding on corn roots (*Zea mays* L) results in direct yield loss (Dun et al. 2010), and can increase the susceptibility of plants to lodging, which makes harvesting difficult. The western corn rootworm has developed resistance to several management strategies including conventional insecticides (Meinke et al. 1998), crop rotation (Levine et al. 2002, Gray et al. 2009), and most recently, Bt corn (Gassmann et al. 2011, Gassmann 2012b, a, Gassmann et al 2014).

Genetically modified corn producing Bt toxins has played a key role in the management of many agricultural pests. The mode of action for Bt involves crystalline (Cry) proteins that are ingested and bind to receptors in the target insect’s midgut, causing pore formation and the eventual lysing of the gut that in turn kills the insect (Schnepf et al. 1998). In 2003, transgenic Bt corn producing Bt toxin Cry3Bb1 was approved for management of western corn rootworm by the Environmental Protection Agency (EPA) (EPA 2003). In 2012, 67% of the corn planted in the United States was of Bt hybrids for the management of pest insects (ERB 2012). However, the efficacy of some commercially available hybrids of Bt corn is in danger due to the evolution of resistance to Cry3Bb1 and mCry3A by western corn rootworm (Gassmann et al. 2011, Gassmann 2012, Gassmann et al. 2012, Gassmann et al. 2014).
Bioassays are experiments that expose live organisms or cell cultures to a chemical to measure its biological activity. Multiple bioassay methods are used in resistance monitoring for western corn rootworm. Plant-based bioassay methods may involve the growth of single corn plants in containers (Gassmann et al. 2011, Meihls et al. 2012, Gassmann et al. 2012, Meihls et al. 2012), or seedling-mats that are grown from many corn seeds (Notwatzki et al. 2008, Petzold-Maxwell et al. 2012). Western corn rootworm eggs or neonate larvae are then placed on the single-plants or seedling-mats and monitored for survival or sub-lethal effects. Diet-based bioassays are conducted using artificial insect diet onto which chemicals or pathogens are overlaid (Siegfried et al. 2007). Larvae are then placed onto the diet and monitored. Bioassays used in western corn rootworm resistance monitoring had been mostly diet-based (EPA 2011), but has shifted to include more plant-based methods in recent years (EPA 2013a, b).

Single-plant bioassays are plant-based bioassays that involve the growth of single corn plants in containers. Plants are typically grown in a greenhouse for a set period of time (Meihls et al. 2011, Meihls et al. 2012) or to a pre-determined growth stage (V-stage (Abendroth et al. 2011)) (Gassmann et al. 2011, Gassmann et al. 2014). Plant rearing may also occur in growth chambers (Vaughn et al. 2005). After plants are grown to the desired point, western corn rootworm eggs (Vaughn et al. 2005, Meihls et al. 2011, Meihls et al. 2012) or larvae (Gassmann et al. 2011, Gassmann et al. 2014) are placed onto or next to the roots of the corn plants. Plants with western corn rootworm are then transferred to growth chambers (Gassmann et al. 2011, Gassmann et al. 2014) or left in the greenhouse (Meihls et al. 2011, Meihls et al. 2012) for a set time period after which Berlese funnels (Gassmann et al. 2011, Meihls et al. 2011, Meihls et al. 2012, Gassmann et al. 2014) or other analogous methods are used to remove surviving larvae from the soil and capture them in a collection medium. An alternate model is to rear insects to
adulthood on single-plants, collecting emerging adult insects in mesh covers that are placed over the corn plants (Meihls et al. 2008, Meihls et al. 2011, Meihls et al. 2012).

Seedling-mat bioassays are another method of plant-based bioassay and are conducted in growth chambers. Many corn seeds are planted in a single container to form a layer of roots on which western corn rootworm larvae may feed. Western corn rootworm eggs (Nowatzki et al. 2008, Meihls et al. 2011, Oswald et al. 2011, Oswald et al. 2012) or larvae (Petzold-Maxwell et al. 2012, Hoffmann et al. 2014) are placed on the seedling-mats. Seedling-mats with western corn rootworm are then reared in growth chambers either for a pre-determined time period after which larvae are extracted (Nowatzki et al. 2008, Petzold-Maxwell et al. 2012, Hoffmann et al. 2014) or until adult emergence (Oswald et al. 2011, Oswald et al. 2012). Larvae and adults are collected using techniques similar to those in single-plant bioassays.

Diet-based bioassays are performed by overlaying Bt toxins onto artificial diet (Siegfried et al. 2005). Western corn rootworm larvae are then placed on the artificial diet for a set period of time after which their survival on each Bt concentration is scored using pre-determined criteria (Siegfried et al. 2005, Nowatzki et al. 2008). Data from such experiments can be analyzed with probit models to determine lethal concentration that causes 50% mortality ($LC_{50}$) mortality, the effective concentration that causes a 50% reduction in development or growth ($EC_{50}$) values for larval mass, or other similar values (Meihls et al. 2008, Nowatzki et al. 2008, Meihls et al. 2011).

Western corn rootworm has a univoltine life cycle (Gray et al. 2009, Spencer et al. 2009), which makes can make research on this pest a slow process. The development of non-diapausing laboratory strains of the insect has alleviated this issue (Branson 1976). Past work has introgressed field-derived alleles into non-diapausing strains and then selected for Bt resistance,
but uncertainty exists about the extent that such laboratory-selected resistance resembles resistance that may arise in the field (Meihls et al. 2008, Owsald et al. 2011, Meihls et al. 2012). Here we evaluated a strain in which alleles for field-evolved resistance to Cry3Bb1 corn were introgressed into a non-diapausing strain. We then compared the resistant strain to a companion susceptible strain across several plant-based bioassay methods to assess the sensitivity of each bioassay method for measuring resistance to Cry3Bb1 corn.

**Materials and methods**

**Standard strain.** The standard strain of western corn rootworm is the non-diapausing strain of western corn rootworm (Branson 1976) that was originally obtained from the USDA-ARS North Central Agricultural Research Laboratory in Brookings, South Dakota. The F1 generation was brought into the Gassmann lab in Iowa State University in October, 2009. Throughout its rearing Standard was never exposed to Bt, and was reared solely on non-Bt corn with no seed treatment. The strain was reared on corn seedling-mats according to the methods of Jackson (1986) and Oswald et al. (2011), and maintained at a population size of 1,200 adults.

**Hopkinton strain.** On 20 August, 2010, adult western corn rootworm were sampled from two cornfields that had been planted to Cry3Bb1 corn for seven consecutive years. These fields constituted site P1 in Gassmann et al. (2011) and a nearby field on the same farm that was studied in Gassmann (2012). Fifty-three, male western corn rootworm were collected from these cornfields and crossed with 356 virgin females from Standard. Progeny of this cross (hereafter referred to as the Hopkinton strain) were selected on Cry3Bb1 corn (DKC6169 Monsanto Company, St. Louis, Missouri) three times (F1, F2, F5) and backcrossed to Standard three times (Parental, F1 and F4) to increase genetic similarity to Standard.
**Strain rearing.** Adult insects were maintained at a population size of 1,200 adults in cages (30x30x30cm, MegaView Science Co. Ltd., Taichung, Taiwan) housed in an incubator (I41-LL Percival Scientific, Perry, IA) (25°C; 16/8 L/D). Cages were provided with artificial western corn rootworm diet (Bio-Serv, Frenchtown, NJ), a 1.5% agar solid for water, and corn leaf tissue, all of which were replaced three times per week. Eggs were collected in an oviposition substrate of sieved soil (< 180 µm) placed in a Petri dish (diameter = 150 mm). Petri dishes for oviposition were replaced two times per week.

Larvae were reared in seedling mats grown plastic containers (volume = 1 L, C32DE; Dart Container Corporation, Mason, MI) following Jackson (1986) and Oswald (2011), with 600 western corn rootworm eggs dispensed onto each seedling mat. The seedling mats were prepared with 25 mL of non-Bt corn (Hybrid 34m94, DuPont Pioneer, Johnston, Iowa), 80 mL of tap water, and 0.5 L of a soil mixture consisting of 50% sieved field soil (particle size < 1 cm) and 50% potting soil (Sunshine LC1 Professional Growing Mix, SunGro Horticulture, Vancouver, British Columbia, Canada). Mesh fabric (Chiffon; Hobby Lobby Stores, Inc. Oklahoma City, OK) covered containers to prevent larvae from escaping. After 9 d seedling mats were transferred in a two to one ratio onto fresh seedling mats prepared in plastic bins (volume = 5.7 L, product #1642, Sterlite Corporation, Townsend, MA) and composed of 120 mL corn seed, 0.5 L of tap water, and 3 L of soil. Fabric covers and lids prevented larvae and adults from escaping. Hopkinton and Standard were reared identically with the exception of Hopkinton generations when larvae were selected on Cry3Bb1 corn, in which 900 eggs were used per ca. 1 L container instead of 600.

**Single-plant assay for larval survival.** A single-plant assay was conducted between 27 April and 26 June, 2012 and used generations F9 of Hopkinton and F16 of Standard. Corn plants
were grown singly in 1 L containers following Gassmann et al. (2011, 2012, 2014), except that plants were grown to the V7-8 stage (Abendroth et al. 2011) to produce a larger root mass, intended to more closely mimic the seedling-mat, and larvae were allowed to feed for 14 d instead of 17 d to compensate for faster larval development on the larger root masses. Two hybrids were tested, Cry3Bb1 corn (DKC6169, Monsanto Company, St. Louis, Missouri) and its non-Bt near isoline (DKC6172), neither of which contained any type of pesticidal seed treatment. Plants were grown in a greenhouse (mean temperature = 30°C; range 20°C to 46°C). A replication consisted of one plant for each of the four strain by hybrid combinations, 16 replications were planted.

After plants reached the V7-8 stage they were trimmed to 20 cm high. Twelve neonate western corn rootworm larvae less than 1 d old were placed onto or directly next to the roots of each plant. After receiving larvae, plants were placed in a growth chamber (I41-LL Percival Scientific, Perry, IA (Settings = 24°C, 60% RH, 16/8 L/D)). After the 14 d, plants containing larvae were removed from their containers, the soil broken up by hand, and placed on Berlese funnels for 4 d to extract surviving larvae, which were stored in 85% ethanol.

The number of surviving larvae were enumerated and used to calculate the proportion surviving on each plant (surviving larvae / total larvae placed). The instar of each surviving larvae was determined from larval head capsule widths following Hammack et al. (2003). Head capsule width was measured using a dissecting microscope (Leica Mz6; Leica Microsystems, Wetzlar, Germany), with a digital camera (Moticam 2500 5.0Mp; Meyer Instruments, Houston, Texas) and accompanying image analysis software (Motic Images Plus 2.0; Motic Images, Inc., Richmond, British Columbia, Canada).
Seedling-mat assay for larval survival. This experiment occurred from 19 April to 30 May 2012 and used F9 of Hopkinton and F16 and F17 of Standard. The assay followed methods described in Petzold-Maxwell et al. (2012) and Nowatzki et al. (2008). The experiment used a blocked design with a block consisting of one replication of each of the four strain by hybrid combinations, and a total of 16 blocks were run. Seedling-mats were prepared in 1 L, flat, plastic containers (Dart C32DE, Dart Container Corp., Mason, Michigan), with six 0.5 cm holes made in each lid with a soldering iron to provide air flow. Each seedling-mat was prepared with 25 mL of dry corn seed of either Cry3Bb1 corn (DKC6169) or its non-Bt near isolate (DKC6172), 80 mL water, and 0.5 L of a 50/50 mixture of sieved field soil (particle size < 1 cm) and potting soil (Sunshine LC1 Professional Growing Mix (SunGro Horticulture, Vancouver, British Columbia, Canada)). Field soil was collected from a field planted with soybeans in the previous season.

Seedling-mats were grown in a growth chamber (25°C, 60% RH, 16/8 L/D) for 7 d after which any above ground vegetation was trimmed to the soil level and 20 neonate western corn rootworm larvae were placed directly onto or next to the roots of each seedling-mat. Fabric covers (194811 poly chiffon; Hobby Lobby Stores Inc., Oklahoma City, Oklahoma) were placed between the lids and seedling-mat in each container to prevent larvae from escaping. Seedling-mats were returned to the growth chamber, watered as needed, and larvae were allowed to feed for 12 d. After 12 d, seedling-mats, larvae and soil were transferred to Berlese funnels for 4 d and live larvae collected in vials with 85%. Larval survival and instar were scored in the same manner as in the single-plant assay for larval survival.

Small, single-plant assay for survival to adulthood. This experiment occurred from 23 March to 26 June 2012 following the methods of Meihls et al. (2011, 2012), and used F9 of
Hopkinton and F16 of Standard. Corn plants were grown in 2.4 L containers (Encore “Mix-n’-Measure” Encore Plastics Corp, Sandusky, Ohio) with one 1.6 cm hole drilled in the bottom for drainage. Holes were covered with fine mesh (716571 white organza fabric; Hobby Lobby Stores, Inc., Oklahoma City, Oklahoma) to prevent larvae from escaping. Each container received 2 L of a medium that was 80% potting soil (50% Sunshine LC1 Professional Growing Mix, 50% Sunshine MetroMix-900 Professional Growing Mix, SunGro Horticulture, Vancouver, British Columbia, Canada), and 20% sieved field soil. Each container received two to three seeds of either Cry3Bb1 corn (DKC 6169) or its non-Bt near isoline (DKC 6172). When plants reached the V1 stage they were thinned to one plant per container.

Plants were grown in the greenhouse (mean temperature = 30°C; range 17°C to 46°C) and sprayed with fungicide at the V1 stage (Infuse systemic disease control; Bonide Products Inc., Oriskany, New York). Plants were fertilized weekly with 200 ml of a 0.08 mg/mL fertilizer solution (Peters Excel 15-5-15 Cal-Mag Special; Evrirs International B.V. Geldermalsen, Netherlands). When plants reached the V8-9 stage they received 30 neonate larvae from either Hopkinton or Standard. Larvae were placed onto plants over a 3 d period, and a total of 13 plants were established for each combination of strain by hybrid for a total of 52 plants for the entire experiment. Fabric nets (194811 poly chiffon) were affixed to the bottom of each container with a rubber band, and the stem of the corn plant with a twist tie. Newly emerged adult western corn rootworm were collected with an aspirator (1135A Aspirator, BioQuip Products, Rancho Domingues, California). Plants were checked for adults three times per week beginning 28 days after larvae were placed on plants and ending 22 days later. Data collection was terminated after three consecutive collections with no adults collected from the experiment as a whole. Adults were stored in 85% ethanol. The number of surviving adults was enumerated.
and used to calculate the proportion of survival for each plant (surviving adults / total larvae placed). The sex of each adult was determined following Hammack and French (2007) and head capsule widths were measured using the same equipment employed in the bioassays measuring larval survival.

**Large, single-plant assay for survival to adulthood.** This experiment occurred from 5 June to 27 August, 2013 following Meihls et al. (2011), and used F15 of Hopkinton and F22 of Standard. Plants were grown in a greenhouse (mean temperature = 33°C; range 21.8°C to 42.4°C) in 14 L containers (Classic 1600; Nursery Supplies Inc. Chambersburg, Pennsylvania) that contained 13 L of the same soil medium used in the small, single-plant assay for survival to adulthood. Five 1.6 cm holes were drilled in the bottom each container for drainage, and each hole was covered with fabric (716571 white organza) to prevent larval escape. Each container initial received two seeds of either Cry3Bb1 corn (DKC 6169) or non-Bt near isoline (DKC 6172). Containers were thinned to one plant per container when plants reached the V1. Corn plants were sprayed with fungicide (Infuse systemic disease control) at the V1 stage and fertilized weekly with 400 mL of a 0.08 mg/mL fertilizer solution (Peters Excel 15-5-15 Cal-Mag Special). When plants reached the V5 to V6 stage 25 they received 25 neonate larvae from either Hopkinton or Standard. Plants received larvae following a blocked design, with one block receiving larvae the same day. A block consisted of one container for each of the four combinations of corn hybrid by western corn rootworm strain, and a total of 12 blocks received larvae. Fabric nets (194811 poly chiffon) were affixed to each plant approximately 10 days after larvae were placed onto the plants. Nets were attached to the bottom of each container with a rubber band, and the stem of the corn plant with a twist tie, and emerging adult insects were collected in the same manner as in the small, single-plant assay for survival to adulthood. Plants
were checked for adult emergence three times per week beginning 34 d after larvae were placed on plants and ending 29 days later. All plants were disposed of after six consecutive collection attempts with no adult emergence detected. The sex and head capsule width of each adult were measured using the same techniques and equipment as in the small, single-plant assay for survival to adulthood.

**Seedling-mat assay for survival to adulthood.** This experiment occurred from 30 July to 3 October, 2012 and used F10 of Hopkinton and F18 of Standard. Seedling mats were prepared following standard WCR rearing procedures (Jackson (1986), Oswald et al. (2011)), and were based on previously developed bioassay methods Hoffmann (2013). Seedling-mats of either Cry3Bb1 corn (DKC 6169) or non-Bt near isoline (DKC 6172) were planted in ca. 0.5 L containers (RD-16; Placon Corporation, Madison, Wisconsin). Fifteen mL of corn, 50 mL of tap water, and 200 mL potting medium (equal parts Sunshine LC1 Professional Growing Mix and sieved field soil) were placed in each container. Containers were then covered with a lid that was left ajar to allow for airflow. Ten replications were prepared on the same day for each of the four strain by hybrid treatment groups. Seedling-mats were grown for 7 d in a growth chamber (25°C, 60% RH, 16/8 L/D), after which any aboveground vegetative tissue was trimmed to the soil level, removing all leaf tissue, and 15 neonate larvae <1 d old from either the Hopkinton strain or Standard strain were placed onto each seedling mat. Seedling-mats with neonates were then held in the growth chamber for 12 d.

Six d after larvae were placed on the 0.5 L seedling-mats; larger, 1 L seedling-mats were prepared in the same manner as the seedling-mat assay for larval survival. Six d after the preparation of the 1 L seeding mats, the 0.5 L seedling-mats were transferred, upside down, in a 1:1 ratio onto the 1 L seedling-mats. 0.5 L seedling-mats of Cry3Bb1 corn were always
transferred to 1 L seedling-mats of Cry3Bb1 corn and 0.5 L seedling-mats of non-Bt corn were always transferred to 1 L seedlings mats of non-Bt corn. Seedling-mats were checked for adult emergence beginning 28 d after larvae were placed on the 0.5 L seedling-mats, and were checked for 30 d later, with collection ending after three consecutive collections in which no adults were collected from any seedling-mat. Data were collected in the same manner as the small, single-plant assays for survival to adulthood.

**Data analysis.** Data from each bioassay were analyzed in SAS 9.3 (SAS Institute Inc., Carry, North Carolina). SAS procedures used were PROC GLM for model I analysis of variance (ANOVA), and PROC MIXED for mixed-model ANOVA. If an interaction effect was significant, subsequent pairwise comparisons were made using an LSMEANS statement in PROC GLM and the PDIFF statement in PROC MIXED. Significance levels of pairwise comparisons were adjusted with Bonferroni corrections. In mixed-model ANOVA, the significance of random effects were tested with a log-likelihood statistic (-2 RES Log Likelihood in SAS) based on single-tailed $\chi^2$ tests with one degree of freedom (Littell et al. 2006). Random effects that were not significant at $P = 0.25$ were removed from the model to increase the overall statistical power (Littell et al. 2006). Non-significant lower order terms were retained in the models if their associated higher order terms were significant in the $\chi^2$ tests.

Data on proportion of survival in the single-plant assay for larval survival, the small, single-plant assay for survival to adulthood, and the seedling-mat assay for survival to adulthood, as well as data on the proportion of third instar larvae in the single-plant assay for larval survival were analyzed with two-way, model I ANOVA that included the factors of strain, hybrid, and their interaction. Proportion survival and proportion of third instar larvae data in the seedling-mat assay for larval survival and proportion survival data in the large single-plant assay for
survival to adulthood were analyzed with two-way, mixed model ANOVA. Fixed effects in the full model were strain, hybrid, and their interaction; random effects were block and all of its interactions with fixed effects. In all analyses with a significant strain by hybrid interaction, pairwise comparisons were conducted among means with a significance level of 0.0083 based on six pairwise comparisons. Data on survival to adulthood in the small, single-plant assay for survival to adulthood and on the proportion of third instar larvae in the seedling-mat assay for larval survival were transformed using the arcsine of the square root to normalize the residuals.

Data on adult head capsule widths from the small, single-plant assay for survival to adulthood and the seedling-mat assay for survival to adulthood were analyzed with model 1, three-way ANOVA that included the factors of strain, hybrid, sex, and all of their interactions. Data on adult head capsule from the large, single-plant assay for survival to adulthood were analyzed with a three-way, mixed model ANOVA that included the fixed effects of strain, hybrid, sex, and all of their interactions; random effects were block and all of its interactions with fixed effects.

Two power analyses were performed for each bioassay to compare the sensitivity of each method at detecting differences between Hopkinton and Standard. For both analyses, we set $\beta = 0.80$ and $\alpha = 0.05$. In the first analysis, we tested the number of replications that would be needed to detect a significant interaction between strain and hybrid using PROC GLMPOWER. In the second analysis, we tested the sample size that would be needed to detect a significant difference between a resistant and susceptible strain on Cry3Bb1 corn using PROC POWER. Because the power analysis was based on general linear model, block and its interactions were not included in the analysis.
Results

**Single-plant assay for larval survival.** Eight of the 16 replications prepared had no larval recovery due to insufficient breaking up of the soil during placement on Berlese funnels and were subsequently not included in the final data set. There was a significant strain by hybrid interaction for the proportion of larval survival (Table 1), however, none of the subsequent pairwise comparisons were significant (Fig. 1a). For the proportion of third instar larvae, there was a significant effect of hybrid with a greater proportion of third instar larvae on non-Bt corn than Cry3Bb1 corn (Table 2; Fig. 2a).

**Seedling-mat assay for larval survival.** Larval survival was affected by a significant strain by hybrid interactions (Table 1). Survival on Cry3Bb1 corn was significantly greater for Hopkinton than Standard, but no differences were detected among either strain on non-Bt corn and Hopkinton on Cry3Bb1 corn (Fig. 1b). There was also a significant interaction between hybrid and strain for the proportion of third instar larvae (Table 2). Similar to survival, the proportion of third instar larvae on Cry3Bb1 corn was significantly less for Standard compared with Hopkinton (Fig. 2b).

**Small, single-plant assay for survival to adulthood.** The strain by hybrid interaction was significant for survival (Table 1), however, none of the subsequent pairwise comparisons were significant (Fig. 1c). There was a significant strain by sex interaction for head capsule width (Table 3) with male insects of Hopkinton having significantly smaller head capsule widths than males from Standard (Table 4).

**Large, single-plant assay for survival to adulthood.** A significant effect of hybrid was present with fewer adults emerging from Cry3Bb1 corn than non-Bt corn (Table 1; Fig. 1d). There were no significant effects for size of surviving adults (Table 3; Table 4).
Seedling-mat assay for survival to adulthood. There was a significant strain by hybrid interaction for the proportion of survival to adulthood (Table 1). Survival of the Standard strain was significantly lower on Cry3Bb1 corn compared to the remaining three treatment groups, which did not differ significantly from each other (Fig. 1e). There were no significant effects for head capsule widths of surviving adult insects (Table 3; Table 4).

Power analyses. In the power analyses for detecting a significant strain by hybrid interaction, the seedling-mat assay was the most sensitive, requiring a sample size of nine seedling mats per treatment group. The seedling-mat assay for larval survival, single-plant assay for larval survival, and the small, single-plant assay for survival to adulthood required sample sizes of 10, 17, and 18 per treatment group, respectively. The least sensitive method was the large, single-plant assay for survival to adulthood that required a sample size of 182 (Table 5).

Regarding the power analyses for detecting a significant difference between strains on Cry3Bb1 corn, the seedling-mat assay for survival to adulthood was again the most sensitive, requiring a sample size of seven. The seedling-mat assay for larval survival, single-plant assay for larval survival, and small, single-plant assay for survival to adulthood required intermediate sample sizes of 10, 10, and 18 respectively. The large, single-plant assay for survival to adulthood required a total sample size of 56, and was again the least sensitive (Table 5).

Discussion

We evaluated Cry3Bb1 resistance in a field-derived, non diapausing strain of western corn rootworm (Hopkinton) compared non-diapausing, susceptible strain (Standard) using five plant-based bioassay methods. Bioassay results were compared by the significance of strain by hybrid interaction terms from analysis of variance and of subsequent pairwise comparisons, as well as required sample sizes per treatment group derived from power analyses. All of the
bioassays tested except for the large, single-plant assay for survival to adulthood resulted in significant strain by hybrid interactions in their ANOVA analyses of proportion survival (Table 1). However, subsequent pairwise comparisons were significant only in the seedling-mat bioassays for larval survival and survival to adulthood (Figs. 1b, e). These results suggest that seedling mat assays are the most sensitive method measuring resistance. This result was supported by the two power analyses because seedling-mat bioassays required smaller sample sizes than the single-plant bioassays to detect significant effects (Table 5). Of the two seedling mat assays tested, the assay measuring survival to adulthood required a smaller sample size to detect a significant effect than did the seedling mat assay measuring larval survival (Fig. 5).

For the proportion of third instar data of the two bioassays for larval survival, only the seedling-mat assay for larval survival resulted in a significant strain by hybrid interaction (Figure 2). None of the three survival to adulthood bioassay methods produced statistically significant results of their ANOVA analyses of adult head capsule width. These results suggest that the seedling(mat assay for larval survival was more sensitive than the single-plant assay for measuring larval development. However, the seedling-mat bioassay for survival to adulthood was not more sensitive than either of the single-plant bioassays.

The EPA requires resistance monitoring as part of insect resistance management (IRM) programs for Bt corn. Monitoring programs must include routine monitoring and analyses of populations from fields with greater than expected damage (EPA 2006). The purpose of monitoring programs is the early detection of resistance so that remedial actions can be made to delay further resistance evolution (Devos et al. 2013). Effective resistance monitoring programs should include a well-documented baseline susceptibility level to allow for the monitoring of changes in the pest’s susceptibility to Bt (EPA 2003, Devos et al. 2013), and include bioassays
that are reliable and accurate (Siegfried et al. 2007). Resistance monitoring programs are also important in that they test the effectiveness of the IRM programs currently in use and the monitoring techniques used in them, as well as providing evidence for or against the model parameters used in designing monitoring programs (EPA 2003, Glaser and Matten 2003).

Resistance monitoring studies involving bioassays are used to characterize Bt resistance in many species of pest insects. Tabashnik et al. (2002) conducted Cry1Ac resistance in a laboratory reared strain of pink bollworm (*Pectinophora gossypiella* Saunders, Lepidoptera: Gelechiidae). Diet-based bioassays for larval mortality were conducted on larvae of the resistant strain, a corresponding susceptible strain, and reciprocal crosses of the two pure strains to determine the inheritance of the Cry1Ac resistance (the relative fitness of heterozygote insects compared to resistant homozygotes (Gould 1998, Tabashnik et al. 2004)). Additional diet-based bioassays for larval growth (measured via weight) were also conducted on the progeny of backcrosses between the reciprocally crossed strains and the resistant strain to estimate the number of loci involved. The inheritance of resistance was calculated as being recessive at high concentrations of Cry1Ac. It was also determined by comparing the data from the backcrossing experiment to existing models that the Cry1Ac resistance of the resistant strain was likely the result of a single gene with at least three loci, or more than one gene. Crespo et al. (2009) conducted similar diet-based bioassays on a field-derived strain of Cry1Ab resistant European corn borer (*Ostrinia nubilalis* Hübner, Lepidoptera: Crambidae), as well as additional plant-based bioassays using pollen, silks, and whole corn plants. It was found that Cry1Ab resistance was non-recessive and likely controlled by multiple genes or loci. The resistant strain exhibited significantly increased survival when feeding on the pollen and silks of Bt corn plants compared to the susceptible strain and progeny of reciprocal crosses. Additionally, no insects of either the
resistant, susceptible, or reciprocal crosses were capable of surviving on vegetative stage plants, whereas the Cry1Ab resistant strain was capable of surviving on reproductive stage plants. Surviving resistant larvae were found feeding on pollen, silks, and parts of the corn ear. This suggested that the Cry1Ab *O. nubilalis* were able to survive by avoiding feeding on the leaves of the corn plants, which exhibited the highest concentrations of Bt toxin, and instead feeding on reproductive tissues that expressed Cry1Ab in lower concentrations.

Gassmann et al. (2011) compared the efficacies of two Bt toxins (Cry3Bb1 and Cry34/35Ab1) for the management of western corn rootworm. Plant-based bioassays for larval survival were used to measure the magnitude of Bt resistance in strain populations of western corn rootworm collected from corn fields with greater than expected damage from the pest. Bioassay results identified Cry3Bb1 resistance in populations of western corn rootworm in Iowa, which was the first report of field-evolved Bt resistance in western corn rootworm. Gassmann et al. (2014) conducted plant-based bioassays using the same methods as Gassmann et al. (2011) study, but also included the Bt toxin mCry3A in addition to Cry3Bb1 and Cry34/35Ab1. Cry3Bb1 resistance was again identified in populations of western corn rootworm collected from the field. However, additional cases of resistance to mCry3A were also identified, and cross resistance between Cry3Bb1 and mCry3a. These results indicated that western corn rootworm resistance to Bt toxins has worsened since the study published in Gassmann et al. (2011), and question the efficacy of current IRM strategies for western corn rootworm.

The results of the small and large, single-plant assays for survival to adulthood differed markedly from similar studies in the published literature. Both bioassays resulted in very low overall levels of survival and only the small, single-plant assay for survival to adulthood resulted in a significant strain by hybrid interaction term from ANOVA analysis of proportion survival.
However, subsequent pairwise comparisons were not significant. Meihls et al. (2011, 2012) conducted single-plant bioassays for survival to adulthood in the greenhouse using similar methods that resulted in greater degrees of adult recovery as well as significant strain by hybrid interactions and pairwise comparisons between colonies of western corn rootworm reared on Cry3Bb1 corn and non-Bt corn. The methods employed in the single-plant assays for survival to adulthood in this study, and those of Meihls et al. (2011, 2012) were not significantly different. However, the watering regimen used in this study may have contributed to the greatly decreased survival to adulthood in the results of each of the two greenhouse bioassays. Plants were watered until water began to seep out of the drainage holes in the bottoms of the containers in which the plants were grown. This excessive watering may have drowned out larvae on the plants, confounding the results from each of the two bioassays. Highly variable temperatures in the greenhouse may have also contributed to low adult recovery.

The proportions of larval recovery and pairwise comparisons of the seedling-mat assay for larval survival were consistent with those of Petzold-Maxwell et al. (2012). The results from the seedling-mat assay for survival to adulthood were similar to those of the control and intensely selected non-diapausing strains of western corn rootworm reported in Oswald et al. (2012). They were also similar to Hoffmann (2013) in that they resulted in significant pairwise comparisons. However, the resistant strain used in Hoffmann (2013) exhibited incomplete resistance to Cry3Bb1 (the resistant strain had significantly reduced survival on Bt corn than on non-Bt corn) whereas Hopkinton’s Cry3Bb1 resistance was complete.

While there was a significant strain by hybrid interaction in the ANOVA analysis of the proportion survival data from the single-plant assay for larval survival, subsequent pairwise comparisons were not. This differs from the results of the greenhouse bioassays for
larval survival in Meihls et al. (2011, 2012) that also used non-diapausing strains of western corn rootworm, but resulted in significant pairwise comparisons between strain by hybrid treatment groups. The results of the single-plant assay for larval survival also differ from the bioassay results of Gassmann et al. (2014) in the same manner. The bioassay method used in this study was derived from that of Gassmann et al. (2014) except for the use of non-diapausing strains of western corn rootworm, and larger plants (plants in Gassmann et al. (2014) were V5-V6 as opposed to the V7-V8 plants used here). The larger plants used in this study provided a greater root mass on which larvae could feed. However, the larger root mass also made the soil of each plant difficult to break up during placement on Berlese funnels for larval extraction. Improper breaking up of the soil resulted in the loss of eight replications of the single-plant assay for larval survival, subsequently the sample size available for statistical analysis was much smaller than intended. A larger sample size may have resulted in significant pairwise comparisons. Larger containers, such as the 3.3 L containers used in Meihls et al. (2011, 2012) may have prevented the soil from becoming root-bound. While care was taken to sufficiently break up the soil for the remaining eight replications of the bioassay, some soil clumps were still present. Larvae housed in soil clumps may have become encased due to drying on multiple sides instead of the top-down drying intended in Berlese funnel use. Encased larvae would have been unable to escape the soil mass, and subsequently would not have been collected.

The availability of space and materials are limiting factors when conducting bioassays. Space requirements for bioassays will differ depending on the size of containers used in them. When conducting bioassays in growth chambers, the cubic space available limits the number of containers which can be fit in a single chamber. The 1 L containers used in the single-plant assay for larval survival with plants cut to 20 cm high were approximately 30 cm tall, and had a
diameter of 12 cm. This gave space for 144 containers with corn plants (36 replications) on three shelves in the Percival growth chamber. The 1 L containers used in the seedling-mat assays for larval survival and survival to adulthood were 20x17x8 cm. It was therefore possible to fit 190 containers (47 full replications), stacked two high on five shelves in a growth chamber. Lastly, the 0.5 L containers in the seedling-mat assay for survival to adulthood were 8 cm high and had a diameter of 12 cm. The 0.5 L containers were grown in a 1:1 ratio with the 1 L containers, therefore 95 (23 full replications) of each sized container could be fit in the growth chamber on five shelves, stacking the 0.5 L containers on top of the 1 L containers. In the greenhouse it was possible to fit 85 of the containers used in the small, single-plant bioassay for survival to adulthood (diameter = 16.5 cm), or 48 of the containers used in the large, single-plant bioassay for survival to adulthood (diameter = 26 cm) on a 2.7 x 1 m bench. This equates to 21 and 12 replications of each respective bioassay.

All of the corn seed used in the experiments in this study was untreated. However, most commercially available corn seed is treated with insecticides or fungicides. It is possible to remove most of the seed treatment by soaking the corn in a bleach solution. Bleached seed may then be used in single-plant bioassays. However, untreated seed is required for seedling-mat bioassays due to the large number of corn seeds planted per container. Obtaining untreated seed from manufacturers may be difficult, and would subsequently limit the number of replications of a seedling-mat bioassay that can be prepared, even if there is enough space available.

Bioassay results will likely differ between different strains of western corn rootworm due to differing genetic backgrounds and rearing histories. The results presented in this study are from a single, non-diapausing strain of western corn rootworm with field-derived Cry3Bb1 resistance. Subsequently, the application of these results is limited to Hopkinton strain.
Additional data from other non-diapausing strains should be obtained in order to draw broader conclusions. The results from the five bioassays examined also will likely differ with the use of corn hybrids producing Bt toxins from different families. Cry3Bb1 and mCry3A are both in the three-domain family of cryotoxins, whereas Cry34/35Ab1 is a binary toxin that consists of two individual toxins, Cry34Ab1 and Cry35Ab1, that exhibit minimal toxicity to western corn rootworm on their own but severe toxicity when expressed together (Ellis et al. 2002). Results from bioassays with Cry34/35Ab1 are likely to be different from those involving Cry3Bb1 or mCry3A due to their differences in structure and mode of action (Bravo et al. 2013). The ability of Hopkinton strain to cause injury to Bt corn in the field is unknown. It is therefore not recommended to apply these results to a field setting.

A final limitation of the results of this study lies in their applicability to diapausing strains of western corn rootworm due to possible genetic and phenotypic differences between diapausing and non-diapausing strains of western corn rootworm. Non-diapausing strains of western corn rootworm all originate from a single parental stock (Branson 1976) into which alleles from other populations have been introgressed to create separate strains. Subsequently, all non-diapausing populations of western corn rootworm have an extensive background of rearing in the laboratory, giving potential for loss of genetic diversity (Kim et al. 2007, Lefko et al. 2008). This effect may be eliminated by the occasional introgression of new alleles into populations reared in the laboratory for extended periods (Kim et al. 2007, Li et al. 2014). Additionally, the fitness of laboratory colonies of insects may also change over time due to adaptation to laboratory settings (Rossler, 1975). Such changes may negatively affect the direct comparability of laboratory and field colonies (Li et al. 2014).
In summary, we compared five bioassays (three single-plant and two seedling-mat) for measuring resistance to Cry3Bb1 corn in a non-diapausing strain of western corn rootworm with field-evolved Cry3Bb1 resistance. The seedling-may bioassays were more sensitive at detecting a strain by hybrid interaction and detecting a significant difference between resistant and susceptible strains. Future studies should compare other Bt toxins and other strains, including diapausing strains from the field; to determine the potential utility of the bioassays studied here, as well as diet-based bioassays, for monitoring of Bt resistance.

Acknowledgements

Richard Hellmich and Nick Lauter provided comments on this manuscript. Research was supported by the Monsanto Company and by Biotechnology Risk Assessment Grant Program competitive grant no. 2012-33522-20010 from the USDA National Institute of Food and Agriculture.

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Meinke, L. J., B. D. Siegfried, R. J. Wright, and L. D. Chandler. 1998. Adult susceptibility of Nebraska western corn rootworm (Coleoptera: Chrysomelidae) populations to selected insecticides. J. Econ. Entomol. 91: 594-600.


### Tables

**Table 1: Analysis of variance for proportion of survival**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Effect</th>
<th>df</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
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<td>Single-plant assay for larval survival&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Strain</td>
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<td>Strain x Hybrid</td>
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</tbody>
</table>

<sup>a</sup> Two-way ANOVA

<sup>b</sup> Mixed model ANOVA all random factors were pooled in the model
**Table 2: Analysis of variance of proportion of third instar**

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<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
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<td>Single-plant assay for larval survival&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>1,25</td>
<td>0.1</td>
<td>0.7662</td>
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<td>Hybrid</td>
<td>1,25</td>
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<td>1.9</td>
<td>0.1756</td>
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<td>38.5</td>
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<td></td>
<td>Hybrid</td>
<td>1,56</td>
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</tr>
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<td></td>
<td>Strain x Hybrid</td>
<td>1,56</td>
<td>23.1</td>
<td>&lt;0.0001</td>
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</tbody>
</table>

<sup>a</sup> Two-way ANOVA

<sup>b</sup> Mixed model ANOVA, all random factors were pooled in the model
Table 3: Analysis of variance for adult head capsule width

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<tbody>
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<td>1,26</td>
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<td>Sex</td>
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<td>0.3618</td>
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<td>1,26</td>
<td>4.8</td>
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<td>Strain x Hybrid x Sex</td>
<td>1,26</td>
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<td>0.1282</td>
</tr>
<tr>
<td>Large, single-plant assay for survival to adulthood&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Strain</td>
<td>1,16</td>
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<td>Sex</td>
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<td>Strain x Hybrid x Sex</td>
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<sup>a</sup> Three-way ANOVA

<sup>b</sup> Mixed model ANOVA, all random factors were pooled in the model
Table 4: Adult head capsule widths from assays for survival to adulthood

<table>
<thead>
<tr>
<th>Assay</th>
<th>Sex</th>
<th>Cry3Bb1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Non-Bt&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Cry3Bb1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Non-Bt&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small, single-plant</td>
<td>♂</td>
<td>1.17 ± 0.03</td>
<td>1.18 ± 0.01</td>
<td>1.14 ± 0.01</td>
<td>1.19 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>♀</td>
<td>1.15 ± 0.02</td>
<td>1.16 ± 0.03</td>
<td>1.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.19 ± 0.02</td>
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<tr>
<td>Large, single-plant</td>
<td>♂</td>
<td>1.17 ± 0.01</td>
<td>1.16 ± 0.03</td>
<td>1.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.14 ± 0.02</td>
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<tr>
<td></td>
<td>♀</td>
<td>1.19 ± 0.02</td>
<td>1.15 ± 0.03</td>
<td>1.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.12 ± 0.02</td>
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<tr>
<td>Seeding-mat assay</td>
<td>♂</td>
<td>1.22 ± 0.02</td>
<td>1.25 ± 0.01</td>
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<tr>
<td></td>
<td>♀</td>
<td>1.22 ± 0.02</td>
<td>1.23 ± 0.02</td>
<td>1.23 ± 0.02</td>
<td>1.24 ± 0.02</td>
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<sup>a</sup> Mean head capsule width ± standard error of the mean measured in millimeters

<sup>b</sup> Single data point
Table 5: Power analyses of required sample sizes to detect significant effects

<table>
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<tr>
<th>Assay</th>
<th>Strain x Hybrid&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Strains on Cry3Bb1&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
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<td>Single-plant assay for larval survival</td>
<td>17</td>
<td>10</td>
</tr>
<tr>
<td>Seedling-mat assay for larval survival</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Small, single-plant assay for survival to adulthood</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Large, single-plant assay for survival to adulthood</td>
<td>182</td>
<td>56</td>
</tr>
<tr>
<td>Seedling-mat assay for survival to adulthood</td>
<td>9</td>
<td>7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Required sample size per treatment group
Figures

Fig. 1: Proportion survival in bioassays. Data are presented for a) Single-plant assay for larval survival, b) Seedling-mat assay for larval survival, c) Small, single-plant assay for survival to adulthood, d) Large, single-plant assay for survival to adulthood, e) Seedling-mat assay for survival to adulthood. Bars are sample means and error bars are the standard error of the mean. Letters indicate pairwise difference among means.

Fig. 2: Proportion third instar data. Bars are simple means, error bars are the standard errors of the means. Letters are least square means pairwise comparisons. Data are presented for a) Single-plant assay for larval survival, b) Seedling-mat assay for larval survival.
Fig. 1:

a. Proportion of Larval Survival

b. Proportion of Larval Survival

c. Proportion Survival to Adulthood

d. Proportion Survival to Adulthood

e. Proportion Survival to Adulthood
Fig. 2a:

CHAPTER 3: INHERITANCE AND FITNESS COSTS OF CRY3BB1 RESISTANCE
FOR FIELD-DERIVED STRAINS OF WESTERN CORN ROOTWORM
(COLEOPTERA: CHRYSONEMELIDAE)

A paper for submission to the *Journal of Economic Entomology*

Authors to be determined

**Abstract**

The refuge strategy is central to current strategies for management of resistance to toxins derived from the bacterium *Bacillus thuringiensis* (Bt) in western corn rootworm (*Diabrotica virgifera virgifera* LeConte). A proportion of all fields planted with a transgenic, Bt hybrid of corn (*Zea mays* L) must be devoted to a non-Bt hybrid in order to preserve the survival of susceptible insects. Mating between resistant insects from Bt fields and susceptible insects from refuges produces heterozygous progeny, and the ability of heterozygous progeny to survive on a Bt crop will affect the rate of resistance evolution, with resistance developing more quickly for resistance traits with greater effective dominance. Fitness costs occur when individuals with resistance alleles have lower fitness than homozygous susceptible individuals of Bt resistance arise in the absence of Bt toxin (e.g., in refuges) and can delay the evolution of Bt resistance when non-Bt refuges are present. Here we quantify the magnitude, inheritance and fitness costs of resistance to Cry3Bb1 corn in two non-diapausing strains of western corn rootworm, Hopkinton and Cresco, which were derived from field populations that evolved resistance to Cry3Bb1 corn. Hopkinton exhibited significantly greater resistance to Cry3Bb1 than Cresco. Non-recessive inheritance (*h*) was detected in Hopkinton but not in Cresco. Fitness costs
affecting developmental rate, survival to adulthood, and fecundity were identified in Cresco; none were identified in Hopkinton. The non-recessive inheritance and paucity of fitness costs will act to facilitate the evolution of resistance to Cry3Bb1 corn in the western corn rootworm.

**Keywords:** *Bacillus thuringiensis*, Cry3Bb1, fitness costs, refuge strategy, resistance

**Introduction**

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), is one of the foremost agricultural pests of corn (*Zea mays* L) in the United States (Gray et al. 2009). Larval feeding on corn root tissue results in decreased yields, with one node of root injury resulting in an approximate yield reduction of 17% (Dun et al. 2010). The Environmental Protection Agency (EPA) defines one node of root injury as the threshold for classifying greater than expected feeding injury in a field planted with transgenic corn producing a single toxin derived from the bacterium *Bacillus thuringiensis* (Bt) (EPA 2011). Additionally, severe pruning of corn roots due to larval feeding makes the plants more susceptible to lodging, which complicates harvest. Western corn rootworm has been a challenging pest to manage because it has evolved resistance to several management practices including conventional insecticides (Meinke et al. 1998), crop rotation (Levine et al. 2002, Gray et al. 2009), and transgenic corn producing the Bt toxins Cry3Bb1 and mCry3A (Gassmann et al. 2011, Gassmann 2012, Gassmann et al. 2012b, Gassmann et al. 2014).

For corn hybrids with a single Bt trait for the management of western corn rootworm, 20% of a field must be planted as non-Bt refuge if a structured refuge (i.e. block refuge) is used, or 10% if an integrated refuge (i.e. blend of Bt and non-Bt seeds) is used (EPA 2010). Assuming a single-locus model, the purpose of this refuge is to reduce the proportion of homozygous resistant individuals, which in turn delays the evolution of resistance and prolongs the efficacy of
a Bt trait (Gould 1998, Tabashnik et al. 2003, Gassmann et al. 2011). The non-Bt refuge allows for the survival of Bt susceptible insects. Matings between resistant insects, which develop on plants from the Bt portion of the field, and susceptible insects, from the refuge, produce heterozygote offspring. To the extent that these heterozygous progeny have lower fitness on a Bt crop than the homozygous resistant parent, delays in resistance can be achieved (Gould 1998, Gassmann 2012, Tabashnik et al. 2013).

The effective dominance of resistance refers to the fitness of the heterozygote relative to the resistant homozygotes on a Bt crop given the ecological conditions present in the environment (Gould 1998, Tabashnik et al. 2004). In addition to the initial frequency of resistance alleles and refuge size, effective dominance will strongly affect the rate of resistance evolution in a population (Gould 1998, Tabashnik et al. 2008, Tabashnik et al. 2013, Gassmann et al. 2014). As the effective dominance increases, the fitness of heterozygote insects on Bt plants will more closely resemble that of resistant parent, resulting in increased survival and a subsequent increase in the rate of resistance evolution. Importantly, the effective dominance will decrease as the concentration of the toxin increases, and a resistance trait is expected to be completely recessive if a high-dose of toxin is achieved (Tabashnik et al. 2004, Taylor and Georghiou 1979, Roush and McKenzie 1987, Gould 1998). A high-dose Bt crop produces Bt toxin sufficient to kill at least 99.99% of susceptible insects (or concentrations at least 25 times greater than that required to kill a susceptible individual) (EPA 1998). As such, understanding the inheritance of resistance traits is central to the development of sound strategies for insect resistance management (IRM) (Petzold-Maxwell et al. 2012). For western corn rootworm, past studies have found that none of the currently commercialized Bt traits achieve a high-dose of toxin (Meihls et al. 2008, Gassmann 2012, Tabashnik and Gould 2012).
The capacity of refuges to delay resistance is diminished by dispersal of Bt resistant insects from Bt fields into refuges (Comins 1977). However, fitness costs of Bt resistance can act to remove resistance alleles from refuge populations (Gassmann et al. 2009). Fitness costs result from deleterious effects of Bt resistance alleles that arise in the absence of Bt toxin (Crowder and Carrière 2009, Gassmann et al. 2009, Carrière et al. 2010). As a result, the presence of fitness costs will act to delay the development of resistance to Bt crops when refuges are present, and quantifying the magnitude of fitness costs that accompany resistance traits can be used to assess the risk of resistance developing (Gassmann et al. 2008, Gassmann et al. 2012b). Fitness costs of Bt resistance have been found to affect a variety of life-history traits such as developmental rate, survival, fecundity, and male mating success (Gassmann et al. 2009, Miehls et al. 2012, Jakka et al. 2014). To date, all data on fitness costs of Bt resistance in western corn rootworm are from strains that were selected for resistance in the laboratory (Miehls et al. 2012, Oswald et al. 2012, Petzold-Maxwell et al. 2012, Hoffmann et al. 2014).

In the present study, we measured the magnitude, inheritance, and fitness costs of Cry3Bb1 resistance in two non-diapausing strains of western corn rootworm with field-evolved resistance. Two field-collected, Cry3Bb1-resistant strains of western corn rootworm were crossed with a non-diapaus­ing, Bt susceptible strain, and their progeny subsequently selected on Cry3Bb1 corn. Strains were studied using diet-based bioassays to measure the magnitude of resistance, plant-based bioassay to measure the inheritance of resistance, and growth chamber experiments to measure fitness costs of resistance. Data from these experiments will provide a better understanding of the risks of Bt resistance evolving, and the biological characteristics of the resistance.
Materials and methods

Standard strain. The Standard strain of western corn rootworm is the base, non-diapausing strain of western corn rootworm (Branson 1976). The F1 generation was obtained from the USDA-ARS North Central Agricultural Research Laboratory in Brookings, South Dakota, and brought into the laboratory in October, 2009. Throughout its rearing in the laboratory, Standard was never exposed to Bt, and was reared solely on non-Bt corn with no seed treatment.

Hopkinton strain. The Hopkinton strain of western corn rootworm is a non-diapausing strain with field-evolved resistance to Cry3Bb1 corn. On 20 August, 2010, adult male western corn rootworm (N = 53) were collected from two fields that had been planted with Cry3Bb1 corn for 7 y. Fields were P1 in Gassmann et al. (2011) and a nearby field on the same farm, which was studied in Gassmann (2012). These males were crossed with 356 virgin females from Standard. The Hopkinton strain was selected on Cry3Bb1 corn (hybrid DKC61-69, Monsanto Company, St. Louis, MO) three times (F1, F2, F5); and backcrossed to Standard three times (Parental, F1 and F4) to make Hopkinton more genetically similar to Standard.

Cresco strain. Cresco strain was also a non-diapausing strain of western corn rootworm with field-evolved resistance to Cry3Bb1 corn. Adult male western corn rootworm (N = 148) were collected from Site P5 in Gassmann et al. (2014), which had been planted continuously with Cry3Bb1 corn for 5 y. Field-collected males were crossed with 150 virgin females from the Standard strain. Cresco was selected four times on Cry3Bb1 corn (F3, F5, F7, F8) and backcrossed to Standard three times (Parental, F3, and F5).

Strain rearing. Adult insects were held in cages (30x30x30cm, MegaView Science Co. Ltd., Taichung, Taiwan) housed in an incubator (I41-LL Percival Scientific, Perry, IA) (25°C;
16/8 L/D) and provided with diet (Bio-Serv, Frenchtown, NJ), a 1.5% agar solid for water, and corn leaf tissue, all of which were replaced three times per week. Eggs were collected in an oviposition substrate of sieved soil (< 180 µm) placed in a Petri dish (diameter = 150 mm).

Larvae were reared in seedling mats grown plastic containers (volume = 1 L, C32DE; Dart Container Corporation, Mason, MI) following Jackson (1986), with 600 western corn rootworm eggs dispensed onto each seedling mat. Seedling mats were prepared with 25 mL of non-Bt corn (Pioneer 34m94), 80 mL of tap water, and 0.5 L of a soil mixture consisting of 50% sieved field soil (particle size < 1 cm) and 50% potting soil (Sunshine LC1 Professional Growing Mix, SunGro Horticulture, Vancouver, British Columbia, Canada). Mesh fabric (Chiffon; Hobby Lobby Stores, Inc. Oklahoma City, OK) covered containers to prevent larvae from escaping. After 9 d the contents of each container were transferred to fresh mats of corn seedlings held in plastic bins (volume = 5.7 L, product #1642, Sterlite Corporation, Townsend, MA) and composed of 120 mL corn seed, 0.5 L of tap water, and 3 L of soil. Fabric covers and lids prevented larvae and adults from escaping. For generations when larvae were selected on Cry3Bb1 corn, 900 eggs were used per ca. 1 L container instead of 600.

**Diet-based bioassays.** Diet bioassays involving an overlay of Cry3Bb1 toxin followed Siegfried et al. (2005), and were used to evaluate Hopkinton, Cresco, and Standard. Bioassays comparing Hopkinton and Standard were run between 6 June and 17 August, 2013 using eggs from F15 and F16 for Hopkinton and F22 and F23 of Standard. Bioassays comparing Cresco and Standard were run between 4 December, 2013 and 8 March, 2014 using F15 and F16 of Cresco and F24 and F25 of Standard. In both cases, Standard was tested alongside each resistant strain (i.e. Hopkinton and Cresco). Eggs were incubated in soil in a climate controlled room (25°C and 80% RH) until immediately before hatching. At that time, eggs were washed from soil using a
mesh sieve and tap water, and separated from remaining debris via salt floatation (Chandler et al. 1966). Eggs were then surface sterilized in a 2% bleach solution for 3 min, rinsed three times with deionized water, surface sterilized for 3 min in a 0.08% Roccal-2 solution (Pfizer Inc., New York, NY) and rinsed three times. Surface sterilized eggs were placed atop a coffee filter (Hy-Vee Inc., West Des Moines, IA) covering a 1cm thick 1.8% agar solid held in a 0.5L plastic container (RD-16 Placon Corporation, Madison, WI).

Diet in 96 well plates, concentrated Cry3Bb1 protein solution, and a Cry3Bb1 buffer solution were obtained from the Monsanto Corporation (Monsanto Company, St. Louis, MO). Diet was based on the recipe of Pleau et al. (2002). Toxin was overlaid on diet at the following concentrations: 341.6 µg/cm², 170.8 µg/cm², 85.4 µg/cm², 42.7 µg/cm², and 21.4 µg/cm². A control was run that consisted only of buffer and did not contain any Bt toxin. Twelve larvae were tested at each concentration and in the experimental control, for a total of 72 larvae per 96 well plate.

One neonate was placed in each well and plates were covered with adhesive plate sleeves (Thermo Fisher Scientific Inc., Waltham, MA). The plates with larvae were held for 5 d in a climate controlled room (25°C and 80% RH) and then checked for survival. Surviving larvae were defined as those that were visibly moving or would do so when prodded with a pin. A criterion of at least 75% survival in the experimental control was used to determine if a bioassay was successful. In the experiment comparing Hopkinton and Standard, six of six repetitions conducted for Hopkinton were successful, and six of 12 for Standard were successful. For the experiment comparing Cresco and Standard, six of 17 trials for Cresco were successful, and six of 18 trials with Standard were successful.
**Inheritance of resistance.** Reciprocal crosses were conducted following Petzold-Maxwell et al. (2012). Virgin adults from Hopkinton and Standard strain were collected, sexed following Hammack and French (2007), and reciprocally crossed (Hopkinton ♀ x Standard ♂ and Standard ♀ x Hopkinton ♂) in a 1:1 ratio. Virgin collections were conducted every 3-4 h to ensure that collected adults were unmated, and the first collection each day was disposed. Each cross was placed in a separate cage (18x18x18cm, MegaView Science Co. Ltd., Taichung, Taiwan). These two crosses, along with a subsample of the Hopkinton and Standard strains, resulted in four treatment groups. All treatment group were maintained at similar population sizes (N = ca. 275) and in the same size cages. Adults were maintained and eggs collected following strain rearing procedures. Insects were collected between 17 September, 2012 and 21 January, 2013 from generations F10 to F12 of Hopkinton and F18 to F19 of Standard. The same approach was taken with Cresco and Standard, in which insects were collected between 30 October, 2013 and 10 January, 2014 from Generations F12 to F13 of Cresco and F24 to F25 of Standard (N = ca. 110 for each of the four treatments). Survival to adulthood was measured using seedlings mats of either Cry3Bb1 corn (DKC 6169 or DKC 6262) or its non-Bt near isoline (DKC 6172 or DKC 6261). Seedling mates were prepared in 0.5 L containers by combining 15 mL of corn seeds, 50mL of tap water, and 200 mL of a 50/50 mixture of potting soil (Sunshine LC1 Professional Growing Mix) and field soil. Seedling mats were allowed to grow for 7 d in an incubator (25°C, 60% RH, 16/8 L/D), after which 15 neonate larvae (less than 1 d old) were added. After 12 d, each 0.5 L seedling mat with larvae was transferred to 1 L seedling mats that were prepared in the same manner as in the standard rearing procedure. A 0.5 L seedling mat was always transferred to a 1 L seedling mat of the same hybrid. A block consisted of two replications of each of the four strains by two hybrids for a total of 8 seedling mats per block.
The experiment with Hopkinton occurred from 22 October, 2012 to 24 April, 2013, and included seven blocks, although half of one block was lost due to growth of mold in bioassay containers. The experiment with Cresco took place from 27 November, 2013 through 7 March, 2014, and consisted of eight blocks, although one block was again lost due to mold.

Seedling mats were checked three times per week for adults beginning 28 days after larvae were added. All adults present were counted, placed in 85% ethanol, with sex determined at a later date. Seedling mates were checked until 14 consecutive days passed with no adult emergence from a block.

**Fitness costs.** Fitness costs were tested in two experiments. The first was conducted between 18 January and 27 June, 2013 used the F13 of Hopkinton and F20 of Standard. The second experiment was conducted between 14 August, 2013 and 18 March, 2014 and used the F11 of Cresco and the F23 of Standard. In both experiments, larvae were reared on non-Bt corn (Pioneer 34m94) in the same manner as the method for measuring inheritance, with 15 larvae placed on each 0.5 L seedling mat. Sixteen seedling mats were prepared each for strains in each experiment. The date of adult emergence was recorded for each individual. Adult insects were placed in cages (18x18x18cm) with one cage for all individuals from each individual seedling mat. Each cage received western corn rootworm diet and 1.5% agar solid. Oviposition dishes were prepared in the same manner as in the rearing procedure and were replaced every 7 d. Seedling mats were checked three times per week for adults beginning 28 days after larvae were initially placed on the 0.5 L seedling mats, and the first oviposition dishes were placed in cages 18 d after the first adults were collected for the experiment with Hopkinton and Standard, and 15 d after the first adults were collected for the experiment with Cresco and Standard. Eggs were washed from soil and separated from debris using the same method as in the diet-based bioassay,
stored in 85% ethanol, and later counted. For 25 eggs sampled during the second week eggs were collected from a cage, egg viability was measured by placing eggs on 1.5% agar solid held in a Petri dish (diameter = 50 mm). Petri dishes were checked three times per week and all neonates were counted and removed. Cages were checked three times per week for any dead adults, and data were recorded on sex, date of death and head capsule width, which was measured with a dissecting microscope (Leica MZ6; Leica Microsystems GmH, Wetzlar, Germany) fitted with a digital camera attachment (Moticam 2500 5.0Mp; Meyer Instruments, Houston, Texas) and accompanying image analysis software (Motic Images Plus 2.0; Motic Images, Inc., Richmond, British Columbia, Canada).

**Data analysis.** All data were analyzed with SAS 9.3 (SAS Institute Inc., Cary, NC). Data on corrected larval mortality (Abbott 1925) from diet-based bioassays were used to calculate $LC_{50}$ values, associated 95% Fiducial limits, and goodness of fit based on Pearson $\chi^2$ (PROC PROBIT). $LC_{50}$ values for corrected larval mortality of Hopkinton and Cresco, and of Standard from the two times it was tested were statistically compared to each other by overlap of their 95% Fiducial limits. Resistance ratios were calculated by dividing the $LC_{50}$ of the resistant strain (either Hopkinton or Cresco) by the corresponding $LC_{50}$ value from Standard.

Data on survival to adulthood from the experiments measuring inheritance of resistance were analyzed with mixed-model analysis of variance (ANOVA) (PROC MIXED). The significance of random effects were tested with a log-likelihood statistic ($-2 RES \log \text{Likelihood}$ in SAS) based on single-tailed $\chi^2$ tests with one degree of freedom (Littell et al. 2006). Random effects that were not significant at $P = 0.25$ were removed from the model to increase the overall statistical power (Littell et al. 2006). Non-significant lower order terms were retained in the models if their higher order interactions were significant. When significant main effects or
interactions were present for fixed effects, pairwise comparisons were made based on least squares means (PDIIFF option in PROC MIXED) with the experimentwise error rate calculated based on a Bonferroni correction. Data were analyzed separately for the experiment comparing Hopkinton and Standard, and the experiment comparing Cresco and Standard. Data were first analyzed only for reciprocal crosses (e.g., Hopkinton ♀ x Standard ♂ and Standard ♀ x Hopkinton ♂) to test for maternal effects. Fixed effects were strain, hybrid, and their interaction; block and all of its interactions with fixed effects were included as random effects. For both experiments, the reciprocal crosses did not differ (P > 0.5) and were pooled into one treatment defined as “heterozygote.” The full data sets were analyzed with mixed-model ANOVA. The fixed effects included in both models were strain, hybrid, and their interaction; random effects were block and all interactions of block with fixed effects. Because a significant interaction was present between strain and hybrid in both Hopkinton and Cresco, pairwise comparisons were made among strains (resistant, susceptible and heterozygote) within a hybrid (Cry3Bb1 and non-Bt) and between hybrids for each strain, with a significant level of P < 0.0056 based on nine pairwise comparisons.

Resistance ratios for on-plant survival were calculated by dividing the survival of the resistant strain (Hopkinton or Cresco) on Cry3Bb1 corn by the corresponding value for Standard. Inheritance (h) of resistance was calculated using the proportion of survival of the strains on Cry3Bb1 corn (h = [heterozygote – susceptible] / [resistant – susceptible]) following Liu and Tabashnik (1997), with 0 = recessive, 1 = dominant, and 0.5 = additive.

Data on fitness costs were analyzed separately for experiments with Hopkinton and Cresco using mixed-model ANOVA (PROC MIXED). In analyses of development rate, proportion survival, and egg viability the fixed effect was strain. Fixed effects in the analyses of
head capsule width and adult lifespan were strain, sex, and their interaction. Fecundity was analyzed with a repeated-measures ANOVA based on a split-plot design (Littell et al. 2006), with strain as the whole plot effect and week and its interaction with strain as split-plot effects. Random effects in each analysis were block, and all interactions between block and fixed effects. There was a significant interaction between strain and week in the analysis of Cresco fecundity. Subsequent pairwise comparisons were made between strains during each week, with a significance level of 0.0042 based on 12 pairwise comparisons.

**Results**

**Diet-based bioassays.** The LC$_{50}$ value derived from the probit analysis of corrected larval mortality for Hopkinton was $153.48 \mu g/cm^2$ (95% Fiducial Limits (FL): 104.33 \mu g/cm$^2$ and 227.84 \mu g/cm$^2$) (Fig. 1). For Standard, which was tested at the same time as Hopkinton, the LC$_{50}$ was $11.74 \mu g/cm^2$ (95% FL: 4.48 to 19.11 \mu g/cm$^2$), yielding a resistance ratio of 13.01 (Fig 1; Table 1). The LC$_{50}$ for Cresco was $44.23 \mu g/cm^2$ (95% FL: 25.97 to 63.54 \mu g/cm$^2$) (Fig. 2). When Standard was tested at the same time as Cresco it was found to have an LC$_{50}$ of $3.55 \mu g/cm^2$ (95% FL: 0.01 \mu g/cm$^2$ and 12.18 \mu g/cm$^2$), yielding a resistance ratio of 12.46.

Hopkinton had a significantly greater LC$_{50}$ value than Cresco, but the LC$_{50}$ values for Standard did not differ between the two times it was tested (Table 1), suggesting that Hopkinton is more resistant to Cry3Bb1 than Cresco.

**Inheritance of resistance.** A significant interaction was present between strain and hybrid for the experiment with Hopkinton (df = 2,11, F = 11.5, P = <0.0001). Survival to adulthood for Hopkinton did not differ between Cry3Bb1 corn and non-Bt corn (Fig. 2a), suggesting complete resistance to Cry3Bb1 corn. While none of the stains differed for survival on non-Bt corn, survival on Cry3Bb1 corn was greatest for Hopkinton, intermediate for
heterozygotes, and lowest for Standard (Fig. 2a). The inheritance of resistance \((h)\) for the Hopkinton strain was 0.37. A significant interaction was also present between strain and hybrid for the experiment with Cresco (\(df = 2,82 \, F = 3.3 \, P = 0.0424\)). Similar to the results for Hopkinton, none of the strains’ survival to adulthood differed on non-Bt corn. However, survival to adulthood for Cresco was significantly lower on Cry3Bb1 corn than on non-Bt corn (Fig. 2b), suggesting that Cresco strain’s Cry3Bb1 resistance is incomplete. Additionally, there was no significant difference between the survival of heterozygotes and Standard. This haploinsufficiency suggests that the inheritance of resistance for Cresco is recessive. When calculated, the inheritance of resistance \((h)\) for Cresco was 0.27. Resistance ratios based on on-plant survival for Hopkinton and Cresco were 5.37 and 3.43, respectively.

**Fitness costs.** For the experiment with Hopkinton, there was a significant interaction between sex and strain for adult lifespan (Table 2), however, subsequent pairwise comparisons were not significant (Fig. 3f). There were no other significant effects of strain or strain by sex interactions for any life-history traits for Hopkinton (Table 2, Fig. 3). Female insects had overall larger head capsule widths than males (Table 2), and there was a significant difference in egg production over time, with more eggs produced early compared to later in life (Table 4).

For the experiment with Cresco, we found significantly later emergence of adults for Cresco compared to Standard (Table 3; Fig. 4a). Cresco also exhibited significantly lower survival to adulthood than Standard (Table 3; Fig. 4b). There was a significant interaction between week and strain for egg production (Table 4), with Standard producing significantly more eggs than Cresco in weeks 1, 2, and 3 (Fig. 4e). This indicates the presence of fitness costs for resistance to Cry3Bb1 corn affecting developmental rate, survival to adulthood and fecundity.
Discussion

Data on the characterization of Bt resistance are vital to the development of effective IRM policies. In this study we measured the magnitude, inheritance, and fitness costs of Cry3Bb1 resistance in two non-diapausing strains of western corn rootworm with field-evolved resistance (Hopkinton and Cresco) compared to a non-diapausing, susceptible strain (Standard). The magnitude of resistance was measured using diet-based and plant-based bioassays, reciprocal crosses between the resistant strains and Standard followed by plant-based bioassays were used to measure inheritance of resistance, and growth chamber experiments were used to measure life history characteristics of resistant strains compared to Standard on non-Bt corn in order to identify fitness costs.

Hopkinton and Cresco strains were both collected from corn fields in Iowa planted continuously with Cry3Bb1 corn (7 y for Hopkinton and 5 y for Cresco). Although field histories of the sites were similar, these fields were separated by ca. 135 km. While western corn rootworm are capable of long-range dispersal covering many kilometers (Coats et al. 1986, Naranjo 1990, Spencer et al. 2009), only a small proportion do so (Naranjo 1990). It is therefore likely that Hopkinton and Cresco strains developed Cry3Bb1 resistance independently. Some notable differences between these strains were the significantly higher level of resistance to Cry3Bb1 protein for Hopkinton compared to Cresco (Table 1; Fig. 1), complete resistance to Cry3Bb1 corn for Hopkinton and incomplete resistance for Cresco (Fig. 2), and the presence of fitness costs of resistance for Cresco (Fig. 4; Tables 3, and 4).

The resistance ratios calculated for the two strains from LC$_{50}$ values, 13.01 for Hopkinton and 12.46 for Cresco, were similar to the previously calculated values for western corn rootworm with resistance to Cry3Bb1 corn. Meihls et al. (2008) reported a resistance ratio of 22 for diet-
based bioassays. Additionally, resistance ratios derived from the survival to adulthood on Cry3Bb1 corn plants, 5.37 for Hopkinton and 3.43 for Cresco, were similar to data from plant-based bioassays reported in Gassmann et al. (2014).

Bt resistance ratios for western corn rootworm are typically smaller than those of Bt-resistant, lepidopteran pests (Gassmann et al. 2014). Previous studies have reported resistance ratios from diet-based bioassays of >3,000 for Cry1F-resistant European corn borer (*Ostrinia nubilalis* Hübner, Lepidoptera: Crambidae) (Pereira et al. 2008), >500 in Cry1Ac-resistant pink bollworm (*Pectinophora gossypiella* Saunders, Lepidoptera: Gelechiidae) (Tabashnik et al. 2005), and >300 in Cry1F resistant fall armyworm (*Spodoptera frugiperda* Smith, Lepidoptera: Noctuidae) (Storer et al. 2010). Each insect strain was capable of surviving on Bt plant hybrids or tissue. Cry1F targeting *S. frugiperda* and *O. nubilalis* were not produced in high-dose concentrations by their respective plant hybrids; however, Cry1Ac targeting *P. Gossypiella* was. High-dose concentrations may promote larger resistance ratios, requiring greater magnitudes of resistance to evolve in order to cause injury to Bt crops (Gassmann et al. 2014). Biological differences between western corn rootworm and lepidopteran pests, and differing modes of action between Cry1 and Cry3 Bt proteins may also explain the differences in resistance ratio magnitudes (Gassmann et al. 2014).

Previous studies have determined that none of commercially available Bt traits for the management of western corn rootworm produce toxins in sufficient concentrations to achieve a high-dose (Meihls et al. 2008, Gassmann 2012, Tabashnik and Gould 2012). Evidence of non-recessive inheritance was found for Hopkinton, but not Cresco, and the inheritance (h) of resistance for Hopkinton was 0.37, which is between additive (0.5) and recessive (0). In other studies of Cry3Bb1 resistant strains Meihls et al. (2008) reported *h* values were 0.285 for larvae
and 0.296 for survival to adulthood in greenhouse experiments, and Petzold-Maxwell et al. (2012) who calculated $h$ values of 0.19 and 1.22 for larval survival. Bt resistance is dose-dependent (Tabashnik et al. 2004); the established lack of high-dose in Bt events for the management of western corn rootworm promotes non-recessive inheritance. Resistance is expected to develop more quickly as the effective dominance of inheritance increases (Tabashnik et al. 2004). As inheritance becomes more dominant the fitness of heterozygotes on Bt plants will more closely resemble that of resistant homozygotes, increasing survival on Bt plants and heightening the risk of resistance evolution.

Fitness costs of decreased development rate, survival to adulthood, and fecundity were detected for Cresco. Increased development times in Cresco strain could result in increased larval predation (Häggström and Larsson 1995, Benry and Denno 1997). Fitness costs, such as those in Cresco, may place the resistant strain at a selective disadvantage compared to a susceptible strain when living and developing in a non-Bt refuge. As such, these fitness costs may remove resistance alleles from the breeding population in the refuge, thereby reducing the rate of resistance evolution (Crowder and Carrière 2009, Gassmann et al. 2009, Carrière et al. 2010).

Few fitness costs of Cry3Bb1 resistance have been identified in some, but not all, past studies of western corn rootworm, although these studies used strains with lab-selected resistance. Petzold-Maxwell et al. (2012) and Hoffmann et al. (2014) found a lack of fitness costs in the presence of entomopathogenic nematodes and fungi. Oswald et al. (2012) did not detect any fitness costs affecting survival, fecundity, and egg viability in five, laboratory-selected strains. Meihls et al. (2012) conducted in-field, greenhouse, and laboratory experiments with three resistant strains and measured 10 life-history characteristics. A fitness cost of reduced
fecundity was identified in one strain, costs in reduced male lifespan were also found in each of the three strains (Meihls et al. 2012). Overall, it may be inferred that there are few fitness costs associated with Cry3Bb1 resistance in western corn rootworm. The relatively small resistance ratios of Cry3Bb1 resistance in western corn rootworm may explain the low prevalence of fitness costs in their populations. Gassmann et al. (2009) found a positive relationship between the magnitude of resistance and the magnitude of associated fitness costs, suggesting that resistance to Cry3Bb1 corn, which has a resistance ratio of less than 25 (as measured in diet-based bioassays) may typically impose minimal fitness costs.

Non-recessive inheritance and minimal fitness costs accompanying resistance have likely facilitated the rapid evolution of Bt resistance in western corn rootworm. In cases where a Bt corn is not high-dose against a target pest, strategies to delay resistance could include increase size of refuges, pyramiding of multiple Bt toxins, and use of Bt crops in the context of an integrated pest management approach (Gassmann et al. 2011, Tabashnik et al. 2013, Gassmann et al. 2014). Implementing practices such as these may aid in preservation of the longevity of currently available Bt corn hybrids.

Acknowledgements

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Table 1: Goodness of fit, LC$_{50}$, and resistance ratios for diet-based bioassays

<table>
<thead>
<tr>
<th>Strain</th>
<th>df</th>
<th>$\chi^2$</th>
<th>P</th>
<th>LC$_{50}^a$</th>
<th>95% FL$^a$</th>
<th>RR$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hopkinton</td>
<td>28</td>
<td>25.0</td>
<td>0.63</td>
<td>153.48</td>
<td>104.33 to 227.84</td>
<td>13.01</td>
</tr>
<tr>
<td>Standard</td>
<td>28</td>
<td>36.2</td>
<td>0.14</td>
<td>11.74</td>
<td>4.48 to 19.11</td>
<td></td>
</tr>
<tr>
<td>Cresco</td>
<td>28</td>
<td>36.1</td>
<td>0.14</td>
<td>44.23</td>
<td>25.97 to 63.54</td>
<td>12.46</td>
</tr>
<tr>
<td>Standard</td>
<td>28</td>
<td>41.0</td>
<td>0.05</td>
<td>3.55</td>
<td>0.01 to 12.18</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Measured in µg/cm$^2$

$^b$ Resistance Ratio derived from the LC$_{50}$ of the resistant strain (Hopkinton or Cresco) divided by the LC$_{50}$ of Standard strain.
Table 2: Mixed-model analysis of variance for comparison of life-history traits of Hopkinton and Standard.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Effect</th>
<th>df</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Development rate&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Strain</td>
<td>1,30</td>
<td>0.1</td>
<td>0.8357</td>
</tr>
<tr>
<td>Proportion survival&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Strain</td>
<td>1,30</td>
<td>0.9</td>
<td>0.3454</td>
</tr>
<tr>
<td>Head capsule width&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Strain</td>
<td>1,15</td>
<td>1.3</td>
<td>0.2784</td>
</tr>
<tr>
<td></td>
<td>Sex</td>
<td>1,30</td>
<td>31.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Strain x Sex</td>
<td>1,30</td>
<td>0.5</td>
<td>0.4799</td>
</tr>
<tr>
<td>Egg viability&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Strain</td>
<td>1.29</td>
<td>3.1</td>
<td>0.0879</td>
</tr>
<tr>
<td>Adult lifespan&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Strain</td>
<td>1,15</td>
<td>0.4</td>
<td>0.5655</td>
</tr>
<tr>
<td></td>
<td>Sex</td>
<td>1,299</td>
<td>3.4</td>
<td>0.0671</td>
</tr>
<tr>
<td></td>
<td>Strain x Sex</td>
<td>1,299</td>
<td>13.0</td>
<td>0.0004</td>
</tr>
</tbody>
</table>

<sup>a</sup> All random effects were pooled in the model.

<sup>b</sup> Random effects included in the model were Block (df = 1, χ² = 1.2, P = 0.1367), Strain x Block (df = 1, χ² = 7.8, P = 0.0026).

<sup>c</sup> Random effects included in the model were Block (df = 1, χ² = 0.0, P = 1.0000), Strain x Block (df = 1, χ² = 14.9, P < 0.0001).
Table 3: Mixed-model analysis of variance for comparison of life-history traits of Cresco and Standard.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Effect</th>
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<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Development rate(^a)</td>
<td>Strain</td>
<td>1.30</td>
<td>8.3</td>
<td>0.0072</td>
</tr>
<tr>
<td>Proportion survival(^a)</td>
<td>Strain</td>
<td>1.30</td>
<td>6.3</td>
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</tr>
<tr>
<td>Head capsule width(^a)</td>
<td>Strain</td>
<td>1.60</td>
<td>0.7</td>
<td>0.2784</td>
</tr>
<tr>
<td></td>
<td>Sex</td>
<td>1.60</td>
<td>3.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Strain x Sex</td>
<td>1.60</td>
<td>0.1</td>
<td>0.4799</td>
</tr>
<tr>
<td>Egg viability(^a)</td>
<td>Strain</td>
<td>1.25</td>
<td>0.1</td>
<td>0.0879</td>
</tr>
<tr>
<td>Adult lifespan(^b)</td>
<td>Strain</td>
<td>1.21</td>
<td>2.0</td>
<td>0.1764</td>
</tr>
<tr>
<td></td>
<td>Sex</td>
<td>1.17</td>
<td>1.8</td>
<td>0.1946</td>
</tr>
<tr>
<td></td>
<td>Strain x Sex</td>
<td>1.19</td>
<td>0.2</td>
<td>0.6465</td>
</tr>
</tbody>
</table>

\(^a\) All random effects were pooled in the model.

\(^b\) Random effects included in the model were Block (df = 1, \( \chi^2 = 5.6, P = 0.0090 \)), Strain x Block (df = 1, \( \chi^2 = 0.7, P = 0.2014 \)), Sex x Block (df = 1, \( \chi^2 = 1.0, P = 0.1587 \)), and Strain x Sex x Block (df = 1, \( \chi^2 = 17.1, P < 0.0001 \)).
Table 4: Repeated measures analysis of variance for fecundity

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Effect</th>
<th>df</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hopkinton vs. Standard</td>
<td>Strain</td>
<td>1,15</td>
<td>0.5</td>
<td>0.4910</td>
</tr>
<tr>
<td></td>
<td>Week</td>
<td>12,180</td>
<td>70.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Strain x Week</td>
<td>12,180</td>
<td>0.8</td>
<td>0.6502</td>
</tr>
<tr>
<td>Cresco vs. Standard</td>
<td>Strain</td>
<td>1,15</td>
<td>4.4</td>
<td>0.0537</td>
</tr>
<tr>
<td></td>
<td>Week</td>
<td>12,165</td>
<td>36.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Strain x Week</td>
<td>12,165</td>
<td>2.3</td>
<td>0.0062</td>
</tr>
</tbody>
</table>

^a Random effects included in the model were Block (df = 1, $\chi^2 = 1219.2$, P <0.0001), Strain x Block (df = 1, $\chi^2 = 0.9$, P = 0.1715), Week x Block (df = 1, $\chi^2 = 0.3$, P = 0.2919), and Strain x Week x Block (df = 1, $\chi^2 = 44.0$, P = <0.0001).

^b Random effects included in the model were Block (df = 1, $\chi^2 = 929.4$, P < 0.0001), Strain x Block (df = 1, $\chi^2 = 22.3$, P < 0.0001), Week x Block (df = 1, $\chi^2 = 2.6$, P = 0.0534), and Strain x Week x Block (df = 1, $\chi^2 = 11.1$, P = 0.0004).
**Figures**

**Fig. 1:** Probit curves of corrected mortality data from diet-based bioassays. Points represent means and error bars are the standard error of the means. The curve plots the probits and the horizontal line denotes the LC$_{50}$. Data are presented for a) Hopkinton vs. Standard and b) Cresco vs. Standard.

**Fig. 2:** Proportion survival on Cry3Bb1 corn and non-Bt corn for resistant, susceptible and heterozygous strains. Bars heights represent sample means and error bars are the standard error of the means. Capital letters denote pairwise comparisons among strains within each hybrid of corn and lowercase letters provide pairwise comparisons of each strain between each hybrid of corn. Data are presented for a) Hopkinton, Standard and heterozygotes and b) Cresco, Standard and heterozygotes.

**Fig. 3:** Comparisons of life history data for Hopkinton and Standard. Bars heights represent sample means and error bars are the standard errors of the means. Asterisks indicate significant pairwise comparisons among strains. Data are presented for a) Development rate, b) Proportion survival to adulthood, c) Adult head capsule width, d) Egg viability, e) Mean egg production per population cage per week on the left Y axis (points are sample means and error bars are the standard errors of the means) and total mean egg production per population cage on the Right Y axis, and f) Total adult lifespan.

**Fig. 4:** Comparisons of life history data for Cresco and Standard. Bars heights represent sample means and error bars are the standard errors of the means. Asterisks indicate significant pairwise comparisons. Data are presented for a) Development rate, b) Proportion survival to adulthood, c) Adult head capsule width, d) Egg viability, e) Mean egg production per population cage per week on the left Y axis (points are sample means and error bars are the standard errors of the means) and total mean egg production per population cage on the Right Y axis, and f) Total adult lifespan.
Fig. 1a:

![Graph showing corrected mortality against concentration of Cry3Bb1 for Hopkinton Standard.]

Fig. 1b:

![Graph showing corrected mortality against concentration of Cry3Bb1 for Cresco Standard.]

Fig. 2a:

Fig. 2b:
Fig. 3:

a. Date of Emergence

b. Proportion Survived to Adulthood

c. Head Capsule Width (m)

d. Proportion Viable Eggs

e. Eggs per Cage per Week vs. Adult Lifespan (Days)

f. Adult Lifespan (Days)
Fig. 4:

a. Date of Emergence

b. Proportion Survival to Adulthood

c. Head Capsule Width (m)

d. Proportion Viable Eggs

e. Eggs per Cage per Week

f. Adult Lifespan (Days)
CHAPTER 4: CONCLUSION

Bt plant hybrids have been an extremely useful tool for protecting crop yields. However, effective Insect Resistance Management (IRM) strategies and the resistance monitoring programs contained within are paramount to the longevity of Bt for the management of western corn rootworm and other insect pest species. Reports Cry3Bb1 resistance in western corn rootworm began only six years after its initial commercialization. The reassessment of current IRM models for western corn rootworm may aid in further delaying the onset of resistance in more recently commercialized hybrids of Bt corn. The identification of a sensitive, repeatable bioassay approach will aid in the laboratory assessment of Bt resistance in western corn rootworm. Additionally, data on the magnitude, inheritance, and fitness costs of Cry3Bb1 resistance in field-derived, non-diapausing strains of the insect may provide evidence for the inadequacies of current IRM models for the management of western corn rootworm, and stress the need for reassessment.

The introgression of alleles from field-evolved, resistant strains of western corn rootworm into a non-diapausing strain to produce Bt resistant, non-diapausiing strains has become common practice in laboratory assessments of Bt resistance in western corn rootworm. A lack of a standardized bioassay method, as well as possible phenotypic differences between non-diapausing strains reared in the laboratory and field-collected, diapausing strains limits the effectiveness of many assessments of Bt resistance in western corn rootworm in laboratory settings. The seedling mat assay for survival to adulthood developed as part of this Master’s thesis was extremely effective in measuring Cry3Bb1 resistance in a non-diapausing strain of western corn rootworm with field-evolved resistance (Hopkinton).
More effective resistance monitoring techniques will provide more reliable data for the assessment of IRM policies for western corn rootworm. We used the seedling mat bioassay for survival to adulthood to measure the magnitude and inheritance of Cry3Bb1 resistance in two non-diapausing strains of western corn rootworm with field-evolved resistance (Hopkinton and Cresco). We were able to identify non-recessive inheritance of resistance in Hopkinton strain, as well as three fitness costs in Cresco strain, in addition to quantifying the magnitude of each strain’s resistance using additional diet-based bioassays.

The Cry3Bb1 resistance of Hopkinton and Cresco differed from each other in several ways. Hopkinton exhibited complete resistance, non-recessive inheritance, and no corresponding fitness costs. Whereas, the Cr3Bb1 resistance of Cresco was incomplete, is likely inherited in a recessive manner, and was associated with three fitness costs. While it is possible that the two strains each possess different genetic bases of resistance, it is also possible that these differences stem from the amount of selection each strain received in the field prior to collection. Hopkinton was collected from fields that had been planted continuously with Cry3Bb1 corn for seven years; whereas, Cresco was collected from a field planted continuously for five years. Considering that Cry3Bb1 resistance can develop in populations of western corn rootworm over relatively short timeframes, the extra two years of field-selection that Hopkinton experienced may account for the differences between it and Cresco.

The characteristics of Cry3Bb1 resistance identified for Cresco indicate that current IRM policies for western corn rootworm on Bt corn would likely be effective for the management of Bt resistance in Cresco. Incomplete resistance and recessive inheritance would result in a high degree of mortality in insects developing on Cry3Bb1 corn, and three fitness costs would remove resistance alleles from the refuge as well. However, this is not the case for Hopkinton.
Complete resistance, non-recessive inheritance, and no fitness costs indicate that the current IRM policies would likely do little for the management of Bt resistance in a western corn rootworm strain such as Hopkinton. Reassessment of IRM policies may aid in further delaying the development of resistance. Larger refuge proportions would promote the production of a greater proportion of heterozygote progeny that have decreased fitness on Cry3Bb1 corn compared to resistant homozygotes, as well as amplifying the prevalence of any existing fitness costs. The inclusion of crop rotation schema would severely affect established populations by interrupting their breeding cycle. Lastly, rotation between corn hybrids that produce different Bt toxins, such as Cry34/35Ab1 will decrease habituation of western corn rootworm populations to a single toxin.

A limitation of the studies performed as part of this Master’s thesis is the use of solely non-diapausing strains of western corn rootworm with field-evolved Cry3Bb1 resistance that were reared in the laboratory. Subsequently, the results presented are not appropriate for drawing conclusions about diapausing strains collected in the field. A second limitation lies in the collection of development rate data in the fitness costs studies. Adult western corn rootworm were not sexed upon emergence, thus the development rate data is pooled over both sexes. Separate data for male and female insects emerging from seedling-mats would have been more informative, as the biology of male and female western corn rootworm differ somewhat. Development rate data separated by sex would subsequently lead to more reliable data on adult lifespan, which were calculated by subtracting the mean date of emergence of both sexes from the death date of each individual, sexed adult. Future research recommended by these studies include the side-by side assessment of multiple bioassay methods in measuring Bt resistance in both diapausing and non-diapausing strains of western corn rootworm in attempt to
identify methods that are most effective for assessing each type of strain. In addition, further assessment of Bt resistance in non-diapausning strains of western corn rootworm with field-evolved resistance will provide valuable evidence for the assessment of current IRM policies for the pest.

The study of Bt resistance in western corn rootworm provides valuable data on Bt resistance to transgenic hybrids that do not produce Bt toxins in high-dose concentrations. A lack of high-dose promotes the evolution of low levels of Bt resistance that are generally associated with non-recessive inheritance and few fitness costs. This subsequently promotes the rapid evolution of Bt resistance in pest populations. However, many of the assumptions of western corn rootworm IRM policies are based on high-dose models, limiting their applicability. Data from western corn rootworm resistance monitoring studies may subsequently be useful for the development of IRM strategies for other pests which have developed Bt resistance to non-high-dose plant hybrids such as Cry1F resistance in fall armyworm (Spodoptera frugiperda Smith, Lepidoptera: Noctuidae) that has invaded the Eastern United States in past several years.

The western corn rootworm has been a persistent pest of corn in the United States. The long-term efficacy of transgenic hybrids of corn is paramount to protecting crop yields in the face of a rapidly increasing world population. The data presented here will be invaluable for increasing the effectiveness of resistance monitoring in non-diapausning strains of western corn rootworm, as well as providing evidence for the efficacy of current IRM policies.
ACKNOWLEDGEMENTS

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