Inheritance and fitness costs of field-derived resistance to Cry3Bb1 corn by western corn rootworm

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Inheritance and fitness costs of field-derived resistance to Cry3Bb1 corn by western corn rootworm

by

Aubrey R. Paolino

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
2016

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>LIST OF FIGURES</th>
<th>iii</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES</td>
<td>iv</td>
</tr>
</tbody>
</table>

## CHAPTER 1  INTRODUCTION .................................................................  1

Western corn rootworm biology and resistance..............................................  1  
*Bacillus thuringiensis* and insect management ...........................................  3  
Insect resistance management ........................................................................  6  
Fitness costs to Bt resistance.........................................................................  7  
Inheritance of resistance to Bt.......................................................................  10 
Focus of thesis and relevance .........................................................................  11 
References .......................................................................................................  12

## CHAPTER 2  INHERITANCE AND FITNESS COSTS OF FIELD-DERIVED RESISTANCE TO CRY3BB1 CORN BY WESTERN CORN ROOTWORM......  21

Abstract .........................................................................................................  21 
Introduction ....................................................................................................  22 
Methods ..........................................................................................................  25 
Results ............................................................................................................  34 
Discussion ......................................................................................................  38 
Acknowledgements ...........................................................................................  43 
References .......................................................................................................  43 
Tables .............................................................................................................  51 
Figures ...........................................................................................................  58

## CHAPTER 3  CONCLUSION ............................................................................  63

ACKNOWLEDGEMENTS .....................................................................................  66
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Survival to adulthood on Cry3Bb1 corn and non-Bt corn for (a) The seedling-mat bioassay with Elma (Cry3Bb1 resistant) and Susceptible (b) the seedling-mat bioassay with Monona (Cry3Bb1 resistant) and Susceptible and (c) larval survival in the single-plant bioassay with Monona and Susceptible</td>
<td>59</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Larval mortality in the diet-based bioassays for the Susceptible, Monona (Cry3Bb1 resistant), and heterozygous strains</td>
<td>60</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Comparisons of life-history data for Susceptible and Monona (Cry3Bb1 resistant) strains on non-Bt corn</td>
<td>61</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Comparisons of life-history data for Susceptible and Elma (Cry3Bb1 resistant) strains on non-Bt corn at high or low larval food availability (5 vs 10 kernels in the initial seedling mats) and in the presence and absence of competition from southern corn rootworm (SCR)</td>
<td>62</td>
</tr>
<tr>
<td>Table</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>-------</td>
<td>----------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>1</td>
<td>Relative survival of western corn rootworm and southern corn rootworm larvae</td>
<td>51</td>
</tr>
<tr>
<td>2</td>
<td>Mixed-model analysis of variance for Susceptible vs Monona and Susceptible vs Elma survival on Cry3Bb1 vs non-Bt corn</td>
<td>52</td>
</tr>
<tr>
<td>3</td>
<td>Goodness of fit and LC$_{50}$ values for diet-based bioassays with Cry3Bb1</td>
<td>53</td>
</tr>
<tr>
<td>4</td>
<td>Analysis of variance for Susceptible and Monona life-history traits</td>
<td>54</td>
</tr>
<tr>
<td>5</td>
<td>Repeated measures analysis of variance for fecundity</td>
<td>55</td>
</tr>
<tr>
<td>6</td>
<td>Mixed-model analysis of variance for Susceptible and Elma life-history traits</td>
<td>56</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION

Western Corn Rootworm Biology and Resistance

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) is a serious pest of corn (*Zea mays* L.) in the Midwestern United States (Levine and Oloumi-Sadeghi 1991). The first recorded collection of western corn rootworm in North America occurred in 1867 in Kansas (LeConte 1868) and western corn rootworm was recognized as a pest in 1909 (Gillette 1912). The distribution of western corn rootworm has since expanded east across the Great Plains, facilitated by the expansive planting of corn beginning in the 1950s (Gray et al. 2009). Western corn rootworm has also been introduced in Central and Western Europe from the United States in at least five separate introductions beginning in 1992 (Baca 1993, Ciosi et al. 2008).

Larvae are the most destructive stage of this pest. Root feeding by western corn rootworm larvae can reduce water and nutrient uptake (Kahler et al. 1985), facilitate root and stalk infection (Palmer and Kommedahl 1969), and complicate harvest by making plants more susceptible to lodging (Levine and Oloumi-Sadeghi 1991). This feeding can be quantified with the 0-3 node-injury scale, where 0 is no feeding and 3 is three pruned (defined as consumed to about 3.8cm of the stalk) nodes (Oleson et al. 2005). One node of injury is associated with a 17% loss in yield (Dun et al. 2010).

Rootworm oviposition occurs in the late summer at a depth of 10-20 cm in the field where adults are feeding and eggs undergo an obligatory diapause (Branson and Krysan 1981, Gray and Tollefson 1988). Extended periods of cold soil temperatures, in the range of -7.5° to -13° C, can cause egg mortality, but eggs can tolerate periods of soil saturation (Gustin 1981, Levine and Oloumi-Sadeghi 1991). After overwintering in the soil, the eggs
hatch in late spring, where establishment of individuals on roots is estimated to be 5-10% (Hibbard et al 2004). Soil saturation during hatch can prevent the establishment of larvae on corn roots and larval survival is lower in sandy soil compared to those with higher clay content (Levine and Oloumi-Sadeghi 1991). Rootworm larvae feed on corn roots and have three instars. Over the course of their development, larvae may move up to three plants down a row (Hibbard et al 2003). Pupation also occurs in the soil and western corn rootworm adults emerge beginning in late June or early July.

Adult males are sexually mature 5 to 7 days after emergence. Females begin to emerge later than males and are sexually mature upon emergence (Guss 1976, Branson 1987, Hammack 1995). For females, there is a 13 day prevoipositional period followed by up to 60 days of oviposition (Branson and Johnson 1973). Adults can be found in a cornfield from emergence until the first frost in the fall (Levine and Oloumi-Sadeghi 1991) and feed on corn tassels, pollen, kernels, and leaf tissue but do not often cause significant damage (Clark and Hibbard 2004). Adult males move 6-17m per day (Spencer et al 2003). Rootworm have few natural enemies. Some ants feed on rootworm larvae while ground beetles and mites can prey on eggs and larvae (Levine and Oloumi-Sadeghi 1991, Meinke et al 2009). There are also some fungi that infect all rootworm life stages and nematodes that attack rootworm larvae, pupae, and adults (Levine and Oloumi-Sadeghi 1991, Toepfer et al. 2009).

The western corn rootworm is a highly adaptable pest and populations have evolved resistance to many management strategies including certain insecticides, crop rotation, and transgenic corn that produces toxins derived from Bacillus thuringiensis (Bt). Resistance is a genetically based decrease in susceptibility to a management strategy (Tabashnik 1994) and develops in a population as a result of repeated exposure to a management practice.
Populations of western corn rootworm have evolved resistance to some organochlorine (Ball and Weekman 1963), organophosphate (Meinke et al. 1998), and carbamate (Meinke et al. 1998) insecticides used for adult management and to the pyrethroid insecticide bifenthrin that is used for larval and adult management (Pereira et al. 2015). These instances of resistance occurred after multiple years of corn production using these insecticides.

Because larvae complete their development only on the roots of corn and some other grasses (Wilson and Hibbard 2004), crop rotation to a non-host is often used as a cultural strategy to manage rootworm populations. Root injury by western corn rootworm larvae in first year rotated corn was first reported in Illinois in 1987 (Levine and Oloymi-Sadeghi 1996). This rotation resistance is the result of a loss of ovipositional fidelity to corn and developed in a county under high selection pressure for resistance to crop rotation because 87% of the land was rotated annually between corn and soybean (Levine et al. 2002, Gray et al. 2009). Rotation resistance became more widespread in Illinois and Indiana in the mid-1990s (Gray et al. 2009).

**Bacillus thuringiensis and Insect Management**

*Bacillus thuringiensis* is a Gram-positive bacterium that produces a crystalline (Cry) protein with insecticidal properties. Strains of *B. thuringiensis* have been isolated from soil, stored grains, plant material, and dead insects (Schnepf et al 1998, de Maagd et al 2001). In addition to the incorporation of Bt toxins in transgenic crops, *B. thuringiensis* and its associated Cry proteins are applied to crops as sprays and mixtures (Bravo et al 2007). Toxins have been identified that work against insects in the insect orders Coleoptera, Lepidoptera, Diptera, and Hymenoptera as well as nematodes (Bravo et al 2007).
A Bt toxin must be ingested to kill an insect. After ingestion, the toxin binds to specific receptors in the brush border membrane of the insect midgut and causes the midgut cells to lyse (Schnepf et al 1998, Gonzalez-Cabrera et al 2006). The benefits of transgenic Bt technology for pest management include target specificity, plant and yield protection, and reduced insecticide use (Rice 2003). The first crops that produced Bt toxins were commercialized in 1996 and targeted lepidopteran pests (transgenic corn producing Cry1Ab and cotton producing Cry1Ac) (Tabashnik and Carrière 2009).

As with any management strategy, the repeated use of transgenic crops places selective pressure on populations of insects to develop resistance. Resistance to crops that produce Bt toxins may occur as a result of decreased cleavage of the protein, decreased binding to the midgut epithelium, decreased pore formation, or increased digestion of the active fragment (Gould 1998). Resistance to Cry1A in many lepidopteran pests such as the pink bollworm (*Pectinophora gossypiella* Saunders), cotton bollworm (*Helicoverpa armigera* Hübner), and tobacco budworm (*Heliothis virescens* Fabricius), is the result of reduced toxin binding (Tabashnik and Carrière 2009).

Transgenic corn producing the Bt toxin Cry3Bb1 for management of rootworm was first commercialized in 2003 (EPA 2003). Corn hybrids are also currently available that produce the single Bt toxins mCry3A (EPA 2010a) and Cry34/35Ab1 (EPA 2010b). Corn producing multiple toxins that target rootworm, referred to as a pyramid, such as Cry3Bb1 with Cry34/35Ab1, mCry3A with Cry34/35Ab, and eCry3.1Ab with mCry3A toxins, are also available (EPA 2011).

Laboratory populations of western corn rootworm have demonstrated a capacity to evolve resistance to Bt corn quickly, with increased survival on corn producing the Bt toxin
Cry3Bb1 after as few as three generations of selection (Meihls et al. 2008). Field-evolved resistance by western corn rootworm to corn that produces Cry3Bb1 was first identified by Gassmann et al. (2011) from insects collected from fields in Iowa in 2009. These populations came from fields where corn had been in continuous production for at least three years and there was a positive correlation between the number of years Cry3Bb1 corn was planted and survival of these populations on Cry3Bb1 corn in bioassays. In 2014, resistance to mCry3A was detected in Iowa as well as cross-resistance between Cry3Bb1 and mCry3A (Gassmann et al. 2014). Resistance to Cry3Bb1 and mCry3A has also evolved in fields in Nebraska (Wangila et al. 2015).

In addition to western corn rootworm, populations of corn earworm (Helicoverpa zea Boddie), maize stalk borer (Busseola fusca Fuller), pink bollworm, and fall armyworm (Spodoptera frugiperda Smith) have evolved resistance in response to field exposure to Bt toxins (Tabashnik et al. 2013). Resistance to Cry1Ac corn was first reported in the United States for corn earworm in 2006 based on evidence from diet-incorporated bioassays (Ali et al. 2006, Tabashnik et al. 2008). Resistance to Bt corn producing Cry1Ab was reported for maize stalk borer in South Africa in 2007 for insects collected in 2006 and tested in an on-plant experiment (van Rensburg 2007). Dhurua and Gujar (2011) found resistance to Cry1Ac cotton in pink bollworm populations that were collected from the field in India in 2008 and subjected to diet-incorporated bioassays. In the United States, pink bollworm remains susceptible to Cry1Ac cotton, likely due to compliance with refuge requirements and cotton that produces a high dose of the Bt toxin (Tabashnik et al. 2012). Resistance to Cry1F maize was reported for fall armyworm in Puerto Rico in 2010 using diet overlay
bioassays with individuals collected in 2007 and 2008 (Storer et al. 2010) and resistance has also developed in Brazil (Farias et al. 2014).

**Insect Resistance Management**

Insect resistance management (IRM) plans are implemented to prevent or delay the evolution of resistance to insecticides in insect populations. A number of strategies have been theorized, including planting refuges of non-Bt hosts in proximity to the Bt crop, pyramiding multiple toxins in a single plant, using crops that express a high dose of toxin, using plants that produce a low dose of toxin in conjunction with natural enemies, or planting crops with differential expression of toxin over time or throughout the plant (Gould 1998). For rootworm IRM, the refuge strategy and the planting of corn that produces pyramids of multiple rootworm active Bt toxins with a refuge have been used to delay the evolution of resistance to Bt.

When planting a corn hybrid that produces a single Bt toxin targeting rootworm, it is required that 20% of a field is planted to non-Bt corn in a block refuge or 10% if a blended refuge is planted (EPA 2010c). The non-Bt refuge serves as a source of susceptible individuals that mate with resistant insects and produce heterozygous offspring. This reduces the number of homozygous resistant individuals in a field (Gould 1998). Nonrandom mating, for example, a lack of movement between the refuge and the Bt portion of the field or temporal asynchrony between the development of resistant and susceptible insects, will decrease the effectiveness of a refuge (Gould 1998).

Pyramiding of multiple Bt toxins also can be referred to as redundant killing because individuals that are resistant to one toxin will be killed by the other toxin (Gould 1998). An important consideration of this strategy is that the toxins must be distinct enough that
there is a low likelihood of cross-resistance (Gould 1998). For rootworm management, the
use of pyramided toxins is combined with the refuge strategy. A 5% block or blended refuge
is required when planting a hybrid that produces two toxins targeting rootworm (EPA 2010c).
Inheritance of resistance and the presence of fitness costs can impact the success of
an IRM strategy. The greatest delay in the development of resistance is expected when the
inheritance of resistance is recessive and fitness costs are associated with resistance
(Gassmann 2012, Tabashnik et al. 2013).

**Fitness Costs to Bt Resistance**

A fitness cost occurs, in the absence of Bt, when individuals with resistance alleles
have reduced fitness compared to susceptible individuals (Gassmann et al. 2009). Fitness
costs function to remove resistance alleles from the non-Bt refuge and delay the evolution of
2013). The trade-off between resistance to Bt and fitness in the absence of Bt manifests
when the trait that confers resistance also has a negative effect on the fitness of the insect
when not exposed to Bt. For example, the resistance trait may negatively affect food
assimilation, increase metabolism, cause greater gut permeability to phytochemicals, or
change the dynamics of tri-trophic interactions (Gassmann et al. 2006, Tabashnik and
Carrière 2009).

The dominance of fitness costs impacts the degree to which resistance evolution is
delayed, with greater delays when the costs are non-recessive, meaning that heterozygote
fitness is reduced compared to susceptible insects in the absence of Bt toxins (Carrière and
Tabashnik 2001). Costs can vary depending on ecological conditions including host plant
type (Carrière et al. 2005), competition (Raymond et al. 2005), and the presence of
entomopathogens (Gassmann et al. 2006, Raymond et al 2007). One way that fitness costs can be detected is by evaluating fitness components such as survival, size, and developmental rate of a resistant population in the absence of Bt compared to a susceptible population. Alternatively, the stability of resistance in the absence of Bt can be quantified over time. Using this method, a decrease in resistance over time indicates a fitness cost that selects against resistance in the absence of Bt toxins (Gassmann et al. 2009). In a review of fitness costs, Gassmann et al. (2009) found fitness costs were detected in 34% of experiments that tested one or more individual fitness components, as has been done for western corn rootworm, and fitness costs were detected in 62% of experiments that tested for declines in resistance over multiple generations in the absence of Bt.

Past studies comparing life-history characteristics using populations of western corn rootworm with laboratory-selected and field-evolved Bt resistance have found variation in the presence and magnitude of fitness costs. Meihls et al. (2012) conducted greenhouse, field, and laboratory experiments on non-Bt corn with western corn rootworm strains that were selected in the greenhouse on Cry3Bb1 corn. The fitness components of larval survival, survival to adulthood, development time, size, fecundity, egg viability, and longevity were tested. The Cry3Bb1-selected colonies had lower fecundity and a shorter average male lifespan compared to unselected colonies. Oswald et al. (2012) conducted laboratory experiment that also used laboratory-selected colonies of Cry3Bb1-resistant western corn rootworm and measured the fitness components of survival, fecundity, and egg viability. The authors found increased fecundity and development rate for resistant individuals compared to susceptible individuals on non-Bt corn. The presence of nematodes and fungi did not induce fitness costs in two laboratory experiments measuring larval survival of strains of western
corn rootworm with laboratory-selected resistance to Cry3Bb1 corn (Petzold-Maxwell et al. 2012, Hoffmann et al. 2014). Ingber and Gassmann (2015) found variation in fitness costs between two strains of western corn rootworm with field-evolved resistance to Cry3Bb1 corn in a laboratory experiment. The fitness components measured were survival to adulthood, development time, size, fecundity, egg viability, and longevity. No fitness costs were detected in one of the resistant strains but for the other resistant strain, there were fitness costs of increased time to development, decreased survival to adulthood, and decreased fecundity.

Fitness costs are also associated with some of the lepidopteran species that have evolved resistance to Bt crops in the field. Fitness costs are associated with corn earworm resistance to Cry1Ac cotton. Anilkumar et al. (2008), using laboratory-selected strains, found fitness costs including increased larval mortality and decreased larval size. For strains of pink bollworm with laboratory-selected resistance to Cry1Ac cotton, Carrière et al. (2001) found a fitness cost of reduced survival for two of three resistant strains compared to two susceptible strains in the absence of Bt. Fitness costs for pink bollworm are also affected by the presence of entomopathogenic nematodes (Gassmann et al. 2012b). A fitness cost of longer larval development time was detected in a strain of fall armyworm with field-evolved resistance to Cry1F maize in a laboratory experiment (Jakka et al. 2014). However, resistance was stable for this strain after 12 generations, suggesting that the delay in development did not impose a significant fitness cost in this strain. Kruger et al. (2014) found no fitness cost associated with field-evolved resistance of maize stalk borer to Cry1Ab corn among the fitness components of longevity, fecundity, fertility, larval mass and survival, and sex ratio.
Inheritance of Resistance to Bt

Another factor that affects the success of the refuge strategy to delay resistance is the extent to which the offspring from resistant and susceptible matings can survive on a Bt crop. Resistance is expected to develop fastest when inheritance is dominant, meaning that the survival of heterozygous individuals on Bt plants is equivalent to homozygous resistant insects (Gould 1998, Tabashnik et al. 2008). At high doses, when the concentration of toxin is 25 times greater than required to kill a susceptible individual or a dose that kills 99.99% of susceptible individuals, resistance is effectively recessive because nearly all heterozygous and homozygous susceptible insects are killed by the Bt toxin (Gould 1998, Tabashnik et al. 2013). Corn hybrids that produce Bt toxins for rootworm management do not produce a high dose of Bt toxin (Siegfried et al. 2005, Storer et al. 2006, Meihls et al. 2008, Binning et al 2010), so resistance is not expected to be functionally recessive.

Studies of western corn rootworm strains with laboratory-selected and field-evolved resistance to Cry3Bb1 have found non-recessive inheritance of resistance (Meihls et al. 2008, Petzold-Maxwell et al. 2012, and Ingber and Gassmann 2015), which means that delays in the evolution of resistance associated with the refuge strategy will be less than if inheritance were recessive. Dominance of resistance can be calculated using survival on Bt corn with the equation: \( h = \frac{\text{heterozygote} - \text{susceptible}}{\text{resistant} - \text{susceptible}} \), where \( h = 0 \) is recessive, \( h = 0.5 \) is additive, and \( h = 1 \) is dominant inheritance (Liu and Tabashnik 1997). Using laboratory-selected resistant western corn rootworm in a greenhouse experiment, Meihls et al. (2008) calculated inheritance values of \( h = 0.285 \) for larval survival and \( h = 0.296 \) for adult survival. Petzold-Maxwell et al. (2012), also using western corn rootworm strains with laboratory-selected resistance to Cry3Bb1 corn, found sex linkage in a laboratory
experiment. Resistant females × resistant males had an inheritance value of $h = 0.19$ but susceptible females × resistant males had an inheritance value of $h = 1.22$ because there was higher survival on Cry3Bb1 than resistant females × resistant males. Ingber and Gassmann (2015), using two western corn rootworm strains with field-evolved resistance to Cry3Bb1 corn, found one strain with an inheritance value of $h = 0.37$. Another strain had an inheritance value of $h = 0.27$ but survival on Cry3Bb1 corn was not significantly different than a susceptible strain in a laboratory experiment. This suggests that there is variation in the inheritance of Cry3Bb1 resistance in strains with field-evolved resistance.

The prediction that resistance will develop faster when inheritance is non-recessive is also supported in other species with resistance to Bt crops. In general, resistance is less common when it is recessively inherited (Tabashnik et al. 2013). Resistance is non-recessive for corn earworm (Burd et al. 2000), maize stalk borer (Van Rensburg 1999), pink bollworm in India (Nair et al. 2016), and fall armyworm (Storer et al. 2010) and they are not exposed to a high dose of toxin in the field. These species have all evolved resistance to Bt toxins as a result of repeated exposure in the field. By contrast, tobacco budworm, a closely related species to corn earworm, does experience a high dose of Cry1Ac and has yet to evolve resistance in the field (Lutrell et al. 1999, Tabashnik et al. 2008). Likewise, European corn borer (Ostrinia nubilalis Hübner) and southwestern corn borer (Diatraea grandiosella Dyar) are exposed to a high dose of Cry1Ab on corn and populations remain susceptible to the toxin (Huang et al. 2011).

Focus of Thesis and Relevance

This thesis quantifies the inheritance of resistance and fitness costs in two strains of western corn rootworm with field-evolved resistance to the Bt toxin Cry3Bb1. The two
strains were collected from fields in Iowa with more than one node of root injury to Cry3Bb1 corn. Elma experienced two selections on Cry3Bb1 corn in the field and Monona experienced four field selections. These two strains were also shown to be resistant to Cry3Bb1 in plant-based bioassays (Gassmann et al. 2012, Gassmann et al. 2014). Field-collected adults were crossed with a non-diapausing Bt-susceptible strain of western corn rootworm and selected on Cry3Bb1 corn to facilitate their use in experiments and allow for comparison with the susceptible strain. The inheritance of resistance to Cry3Bb1 was investigated by crossing resistant and susceptible western corn rootworm and evaluating the survival of their progeny on Bt using diet-based assays and experiments with whole plants and seedling mats. Laboratory and greenhouse experiments were conducted to determine if fitness costs were associated with resistance. This research will give insight into the dynamics of the evolution of Bt resistance for western corn rootworm and the persistence of resistance in the field.

References


(EPA) Environmental Protection Agency. 2010b. Biopesticides registration action document: *Bacillus thuringiensis* Cry34Ab1 and Cry35Ab1 proteins and the genetic material necessary for their production (PHP17662 T-DNA) in event DAS-59122-7 corn (OECD Unique Identifier: DAS-59122-7) (http://www3.epa.gov/pesticides/chem_search/reg_actions/pip/mcry3a-brad.pdf) [Accessed 14 February 2016].


Environmental Entomology 5:219–223.


LeConte, J.L. 1868. New Coleoptera collected on the survey for the extension of the Union


field-relevant resistance to the Bacillus thuringiensis protein Cry1Ac in Pectinophora gossypiella (Lepidoptera: Gelechiidae) collected from India. Pest Management Science 72:558-565.


**2015.** Susceptibility of Nebraska western corn rootworm (Coleoptera: Chrysomelidae) populations to Bt corn events. *Journal of Economic Entomology* 108:742–751.

CHAPTER 2: INHERITANCE AND FITNESS COSTS OF FIELD-DERIVED RESISTANCE TO CRY3BB1 CORN BY WESTERN CORN ROOTWORM

A paper for submission to the Journal of Economic Entomology

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Abstract

The western corn rootworm (Diabrotica virgifera virgifera LeConte) is an economically important pest of corn. One strategy used to manage western corn rootworm is the planting of transgenic corn that produces one or more Cry toxins derived from Bacillus thuringiensis (Bt). Refuges of non-Bt corn function to delay the development of resistance and the greatest delay in resistance is expected when the inheritance of resistance is recessive and there are associated fitness costs. We characterized the inheritance of resistance of two strains of western corn rootworm with field-derived resistance to the Bt toxin Cry3Bb1 (Elma and Monona) and tested for fitness costs of resistance. Plant-based and diet-based bioassays found that inheritance of resistance was non-recessive. In a greenhouse experiment in which larvae were reared on whole corn plants in field soil, no fitness costs of resistance were detected for Monona. In a laboratory experiment with Elma, in which larvae experienced intraspecific and interspecific competition for food, a fitness cost of delayed larval development was identified, however, no other fitness costs were found. These results highlight the potential for rapid evolution of resistance to Cry3Bb1 corn by western corn rootworm, and will aid in the development of resistance management strategies for this pest.

Keywords: Bacillus thuringiensis, Bt corn, Diabrotica virgifera virgifera, resistance management, refuge strategy
Introduction

The western corn rootworm (*Diabrotica virgifera virgifera* LeConte) is a serious pest of corn in the United States (Gray et al. 2009). Rootworm larvae feed on the roots of corn, reducing yield and making plants more susceptible to lodging, which can complicate harvest (Koehler et al. 1985, Dunn et al. 2010). Pruning of one node of roots from larval rootworm feeding is associated with a 17% loss in yield (Dun et al. 2010). Rootworm management has been complicated by the evolution of resistance to several management strategies, including organochloride, organophosphate, carbamate, and pyrethroid insecticides (Ball and Weekman 1963, Meinke et al. 1998, Pereira et al. 2015), crop rotation (Levine et al. 2002, Gray et al. 2009), and corn that produces insecticidal toxins from *Bacillus thuringiensis* (Bt) (Gassmann et al. 2011, Gassmann 2012, Gassmann et al. 2014, Wangila et al. 2015).

Transgenic crops that produce Bt toxins are used in the management of many agricultural pests. Corn producing the Bt toxin Cry3Bb1 was first commercialized for management of larval rootworm in 2003 (EPA 2003). The planting of Bt corn places selective pressure on populations to develop resistance, and laboratory studies have demonstrated the capacity of rootworm populations to evolve Bt resistance quickly (Meihls et al. 2008, Deitloff et al. 2015). Populations of western corn rootworm that evolved resistance to Bt corn as a result of field exposure were first collected in 2009 from fields in Iowa with severe root injury to Cry3Bb1 corn (Gassmann et al. 2011). Other instances of field-evolved resistance to Cry3Bb1, as well as cross-resistance between Cry3Bb1 corn and mCry3A corn have since been identified (Gassmann et al. 2012, 2014, Wangila et al. 2015).

The refuge strategy, in which a portion of the field is planted to a non-Bt host, is one approach to manage the development of resistance to Bt crops. For a corn hybrid expressing
a single Bt trait for western corn rootworm, 20% of a field must be planted to non-Bt corn for a spatially segregated (i.e., block) refuge and 10% of the field must be non-Bt corn if an integrated (i.e., blended) refuge is planted (EPA 2010). The non-Bt portion of the field, or refuge, serves as a source of susceptible individuals that may mate with resistant insects, thereby producing heterozygous offspring and reducing the number of homozygous resistant individuals (Gould 1998). The delay in resistance achieved by the refuge strategy is expected to be greatest when the inheritance of resistance to Bt is recessive and there are associated fitness costs (Gassmann 2012, Tabashnik et al. 2013).

Fitness costs occur, in the absence of Bt, when individuals with one or more resistance alleles have lower fitness compared to susceptible individuals (Gassmann et al 2009). Fitness costs remove resistance alleles from the refuge, thereby delaying the evolution of resistance (Gould 1998, Crowder and Carrière 2009, Gassmann et al. 2009, Tabashnik et al. 2013). Ecological variables such as host plants (Carrière et al. 2005), the presence of entomopathogens (Gassmann et al. 2006, Raymond et al. 2007), and competition (Raymond et al. 2005) can influence the magnitude of fitness costs. Fitness costs of resistance have been investigated in rootworm populations with laboratory-selected resistance (Meihls et al. 2012, Oswald et al. 2012, Petzold-Maxwell et al. 2012, Hoffmann et al. 2014) and field-derived resistance (Ingber and Gassmann 2015), with variation found in the presence and magnitude of costs among populations. Further investigations of fitness costs and the effect of ecological variables on fitness costs will provide a better understanding of the extent to which fitness costs may be associated with Bt resistance in western corn rootworm populations.
Another factor determining the effectiveness of the refuge strategy to delay resistance is the inheritance of resistance traits, in particular, the effective dominance of resistance. The effective dominance of resistance is the degree to which the survival of heterozygous resistant insects on a Bt crop resembles that of homozygous resistant insects (Gould 1998, Tabashnik et al. 2008). At a high dose of Bt toxin, which can be defined as either a concentration of Bt toxin 25 times greater than is required to kill a susceptible individual or that which kills 99.99% of susceptible individuals, nearly all heterozygous and homozygous susceptible insects are killed by a Bt crop and resistance is effectively recessive (Gould 1998, Tabashnik et al. 2013). Corn hybrids currently available for rootworm management do not produce a high dose of Bt toxin (Gassmann 2012, Andow et al. 2016), so resistance is expected to be inherited as a non-recessive trait. Understanding the ability of heterozygous resistant individuals to survive in the Bt portion of the field is important for predicting the ability of the refuge strategy to delay the evolution of resistance.

Our study quantified the inheritance and fitness costs of resistance to the Bt toxin Cry3Bb1 in two strains of western corn rootworm with field-derived resistance (Monona and Elma). Heterozygous crosses were established between the resistant strains and a susceptible strain to assess inheritance of resistance using a variety of bioassays including single-plant assays, seedling mat assays and diet-based assays. We also tested for fitness costs of resistance under differing ecological conditions. One experiment, conducted in a greenhouse, tested for fitness costs when larvae were reared on corn plants grown in field soil, and a second experiment, conducted in a growth chamber, examined the effect of competition on fitness costs. The data from these experiments will add to the current
knowledge about Bt resistance by western corn rootworm and will aid in improving resistance management for this pest.

**Methods**

**Rootworm Strains.** In total three strains of western corn rootworm and one strain of southern corn rootworm (*Diabrotica undecimpunctata howardi* Barber) were studied in these experiments. The Susceptible strain is a non-diapausing strain of western corn rootworm that was brought into laboratory culture in the mid-1960s (Branson 1976, Kim et al. 2007) and was never exposed to Bt corn. Insects were acquired from the USDA-ARS North Central Agricultural Research Laboratory in Brookings, South Dakota to establish the Susceptible strain at Iowa State University in October 2009 (F₁). This research used F₂₈ to F₃₅ of Standard.

The Monona and Elma strains are non-diapausing strains of western corn rootworm with field-evolved resistance to Cry3Bb1 corn. In August 2011, adult male western corn rootworm were collected from field S5 in Gassmann et al. (2012) to establish Monona and field P2 in Gassmann et al. (2014) to establish Elma. Two hundred field-collected adult males were collected to initiate Monona and 142 field-collected adult males were collected to initiate Elma. To generate each strain, field-collected males were crossed with 150 virgin females from Susceptible. Monona was subsequently selected on Cry3Bb1 corn and backcrossed with Susceptible at a 1:1 ratio twice (F₆ and F₈) and selected on Cry3Bb1 corn without backcrossing four more times (F₁₀, F₁₁, F₁₄, and F₁₅). Elma was selected on Cry3Bb1 corn and backcrossed with Susceptible at a 1:1 ratio twice (F₄ and F₇) and selected on Cry3Bb1 corn without backcrossing twice (F₁₀ and F₁₁). In all other generations, the strains were reared on non-Bt corn (Pioneer 34M94, DuPont Pioneer Johnston, IA). Our assays and
experiments used F_{20} to F_{26} of Monona and F_{18} to F_{20} of Elma. The adult population size was maintained at ca. 2500 adults for the three western corn rootworm strains, and all corn seed did not contain any type of pesticidal seed treatment.

In two experiments, southern corn rootworm (SCR) were used in addition to western corn rootworm. This strain was generated in October 2013 from 381 adult SCR adults that were collected from the Sustainable Agriculture Garden at Iowa State University. All generations were reared on non-Bt corn and maintained at a population size of ca. 900 adults. SCR F_{6}, F_{7}, F_{14}, and F_{16} were used in our experiments.

**Strain Rearing.** Adult insects were kept in cages (18 × 18 × 18 cm, MegaView Science Co. Ltd., Taichung, Taiwan) in an incubator (Percival Scientific, Perry, IA; 25°C 16:8 [L:D] h photoperiod). Food provided was a complete adult diet (western corn rootworm adult diet, product # F9768B-M, Bio-Serv, Frenchtown, NJ) and corn leaf tissue, and the water source was a 1.5% agar solid. A petri dish (150 mm diameter) of moistened sieved field soil (<180µm) was used as an oviposition substrate and was replaced two times per week. Larvae were reared on mats of corn seedlings following the methods of Jackson (1986) and Ingber and Gassmann (2015). Adult insects were collected from seedling mats and placed into cages.

**Quantifying Inheritance of Resistance to Cry3Bb1.** Reciprocal crosses were established separately, but in an identical manner between Elma and Susceptible and between Monona and Susceptible following Petzold-Maxwell et al. (2012). First, all adults were collected and discarded from seedling mats to remove any adults that may have mated, then virgin adults were collected every 2 to 3 h to ensure that the adults had not mated. Adults were held separately in Petri dishes and sex of each insect was determined following
Hammack and French (2007). Virgin adults were then placed in one of four cages: Susceptible♀ × Susceptible♂, Susceptible♀ × Resistant♂, Resistant♀ × Susceptible♂, and Resistant♀ × Resistant♂. Crosses between Susceptible and Elma were established between 18 July and 26 September 2014 using F_{28} and F_{29} of Susceptible and F_{18} and F_{19} of Elma with cages maintained at an average population size of 109 ± 34 adults (Mean ± SD). Crosses between Susceptible and Monona were established between 10 December 2014 and 9 October 2015 using F_{31} to F_{35} of Susceptible and F_{20} to F_{26} of Monona and maintained at an average population size of 133 ± 41 adults.

*Seedling-Mat Bioassay.* The seedling mat bioassays were conducted between 21 August and 21 November 2014 using the Susceptible and Elma crosses and 21 February and 9 December 2015 using the Susceptible and Monona crosses. Assays followed Ingber and Gassmann (2015). Briefly, seedling mats of either Cry3Bb1 corn or the non-Bt near isoline were grown in 0.5 L plastic containers (RD-16 Placon Corporation, Madison, WI) for 7 d in an incubator (photoperiod 16:8 [L:D] h), after which time 25 neonate larvae (<24 h old) were placed on the corn root tissue from one of the four strains established with the reciprocal crosses. After 1 wk, the seedling mat, soil and larvae from 0.5 L containers were transferred to a corresponding 1 L plastic tray (C32DE; Dart Container Corporation, Mason, MI) that was made using same corn hybrid as was used for 0.5 L containers. After 1 wk, trays were checked for adult emergence three times per week and this continued until no adults were collected from a replicate for 14 d. A replicate consisted of a one non-Bt seedling mat and one Cry3Bb1 seedling mat for each of the four strains tested. The experiment with the Elma and Susceptible consisted of nine blocks with two replicates per
block and the experiment with the Monona and Susceptible consisted of 12 blocks with two replicates per block.

*Single-Plant Bioassay*. This experiment was conducted from 29 January to 11 November 2015 with the crosses established between Susceptible and Monona. An initial single-plant bioassay was conducted following the methods of Gassmann et al. (2011), but due to low recovery of the non-diapausing strains, the assay was modified to use older corn plants, with more root tissue that more closely resembled seedling mats on which non-diapausing strains were reared. The experiment consisted of 12 blocks with each block containing two non-Bt and two Cry3Bb1 corn plants per strain established by the reciprocal crosses. Corn plants were grown singly in 1 L plastic containers (Placon#22373; Placon Corporation, Madison, WI) filled with 750 mL of potting soil. Containers received 300 mL water just before seeds were planted (depth = 5 cm). Plants were given 100 mL of water three times per week and were fertilized weekly beginning 2 wks after planting (4mg/mL Peters Excel 15-5-15 Cal-Mag Special; Everris NA Inc., Dublin, OH). When plants had reached V6 to V8, they were trimmed to a height of 20 cm and 12 neonate larvae (< 24 h old) were placed on the base of each plant. Containers were placed in an incubator (24°C, 65% RH, 16/8 L/D) and watered as needed. After 14 d, the aboveground plant material was removed and contents of the container (soil, roots and larvae) were placed on a Berlese funnel for 4 d to extract larvae.

*Diet-Based Bioassay*. Diet-based bioassays were conducted between 14 March and 28 November 2015 and followed Siegfried et al. (2005) using the Susceptible and Monona strains, and their reciprocal crosses. Eggs were incubated in soil (26.7°C, 67% RH, 0/24h L/D) until hatching began. Soil was then washed from the eggs, and any remaining debris
separated by salt flotation (Chandler et al. 1966). Eggs were then surface sterilized in a 2% bleach solution followed by a 0.085 Lysol solution, after which they were placed on a moistened coffee filter placed on top of a 1.8% agar solid that was held in 0.5 L container. Monsanto Corporation (St. Louis, MO) provided 96 well plates with diet (Siegfried et al. 2005, Ingber and Gassmann 2015), a solution of Cry3Bb1 toxin, and a buffer solution. Toxin was overlaid on the diet at six concentrations, which varied by strain due to anticipated differences in susceptibility to Cry3Bb1 among strains. The concentrations tested were: Susceptible = 85.40, 42.70, 21.40, 10.70, 5.40, µg Cry3Bb1/cm², and a control with only buffer and no toxin; heterozygous = 170.80, 85.40, 42.70, 21.40, 10.70 µg/cm², and a control; Monona = 341.60, 170.80, 85.40, 42.70, 21.40 µg/cm² and a control. Each bioassay plate consisted of 12 larvae per concentration, for a total of 72 larvae per plate. One neonate larva was placed in each well and then covered with an adhesive cover and held in a chamber for 5 d. After five days, the plates were checked for survival (defined as showing movement when prodded). For each plate, survival of at least eight larvae in control wells was used as the threshold for a successful plate. Six out of 12 plates were successful for Susceptible, four out of 12 plates were successful for Susceptible ♀ × Monona ♂, five out of 13 plates were successful for Monona ♀ × Susceptible ♂, and five out of 16 plates were successful for Monona.

**Greenhouse Experiment Testing for Fitness Costs.** This experiment used the F₂₁ of Monona and F₃₂ of Susceptible and occurred from 2 January to 12 June 2015. Non-Bt corn with no seed treatment was grown in a greenhouse to the V5-V8 stage, at which time 25 neonate larvae (<24h old) were placed on the roots of each plant. Pots were covered with chiffon fabric secured around the outside of the pot with rubber bands and tied around the
stalk with a twist tie. A replicate consisted of one plant with larvae, and 16 replicates each were established for the Monona and Susceptible strains.

Adult insects were collected three times per week beginning 3 wk after larvae were added to pots until there were six consecutive days without emergence. Adults were sexed and placed into cages with one cage for all individuals that emerged from the same pot. Non-Bt corn plants (08T91CMV, Blue River Hybrids, Ames, IA) were also grown in the greenhouse to serve as a food source for adult rootworm. Cages received chopped corn ear, silk, and leaves from these plants as well as 1.5% agar solid as a source of water, and both were changed three times per wk. Each cage contained a petri dish with moistened sieved field soil for oviposition, which was changed once per week. Cages were checked three times per wk for dead adults, which were removed and stored in 85% ethanol. Adult beetles were later sexed and their head capsules measured according to the methods of Ingber and Gassmann (2015). Egg viability was quantified at 2, 4, and 6 wks after a cage was established by placing 25 eggs on a 1.5% agar solid and checking for hatch 5 d per wk until there were no newly hatched larvae on three consecutive days.

**Competition Experiment Testing for Fitness Costs.** This experiment was conducted between 12 August and 26 November 2014 and used F19 and F20 of Elma, F29 and F30 of Susceptible, and F6 and F7 of (SCR). The experiment was a fully crossed design with three factors: food availability, presence or absence of SCR as a competing species, and strain of western corn rootworm (Susceptible or Elma). Seedling mats were prepared in 0.5 L plastic containers with either low or high food availability achieved by adding either five or 10 kernels of non-Bt corn, 60 mL of DI water, and 200 mL of a 50% field-collected soil and 50% potting soil mixture. After 1 wk, seedling mats received 25 neonate larvae from
either Elma or Standard. At that time, half of the seedling mats also received 25 neonate SCR larvae. After 7 d, small seedling mats were transferred to larger seedling mats that consisted of either 10 or 20 kernels per tray of non-Bt corn (corresponding to low or high food availability, respectively), 60 mL of DI water, and 500 mL of soil, all of which was placed in a 1 L plastic tray. Larger seedling mats were allowed to grow for 7 d before smaller seedling mats were transferred. Adult western corn rootworm were collected and separated into cages following the same methods as the greenhouse experiment. For each combination of strain × food availability × presence or absence of SCR, there was one replication per block and a total of 10 blocks.

Data were collected and adults maintained as in the greenhouse experiment, with the exception of how food was provided. In this experiment, we simulated the reduced food availability that adult rootworm experience as corn matures in the field. For four weeks, each cage received adult rootworm diet, non-Bt corn leaf, and a 1.5% agar solid changed three times per week. Then, for the next two weeks, agar was always present but adult diet was only provided for 1d per wk. After that, cages received only agar and corn leaf.

**Interaction of Western and Southern Corn Rootworm.** This experiment was conducted between 24 July 2015 and 6 January 2016 to determine the potential for larval predation by western and southern corn rootworm and used F29 to F31 of Susceptible and F14 to F16 of SCR. Larvae were obtained from seedling mats and placed onto moistened filter paper in 65 mm petri dishes with either two or four pieces of non-Bt corn root (2.5 cm in length). These dishes were sealed with Parafilm M (Bemis, Oshkosh, WI) to prevent the larvae from escaping. Eight to 12 dishes were established for each of 10 larval treatments that tested various combinations of western corn rootworm larvae and SCR larvae (Table 1).
After 2 d, the number of live, dead, and missing larvae was recorded. Western corn rootworm and SCR larvae were distinguished morphologically based on Mendoza and Peters (1964).

**Data analysis.** All data were analyzed with SAS 9.3 (SAS Institute Inc., Cary, NC). For the seedling-mat and single-plant bioassays, data were analyzed with a mixed-model analysis of variance (ANOVA) (PROC MIXED). Fixed effects were strain, hybrid, and the interaction of strain and hybrid. Random effects were block and all interactions with fixed effects. For these and all analyses using mixed-model ANOVA, the significance of random effects was tested with a log-likelihood statistic (-2 RES Log Likelihood) based on a one-tailed $\chi^2$ test with one degree of freedom (Littell et al. 2006). A random effect was included in the model if it was significant at P < 0.25 or if higher order interactions including the effect were significant.

For the seedling-mat and single-plant bioassays, pairwise comparisons were first made between the two heterozygous crosses using the CONTRAST statement with a p value of 0.05 to determine if the strains could be combined. Corrected survival on Cry3Bb1 corn in these bioassays was calculated as the complement of corrected mortality based on Abbott (1925). Resistance ratios were the quotient of corrected survival on Cry3B1 corn for the resistant strain divided by Susceptible. Dominance of resistance ($h$) was calculated based on phenotype using corrected survival on Cry3Bb1 corn with the equation: $h = (\text{heterozygote} - \text{susceptible}) / (\text{resistant} - \text{susceptible})$, where 0 = recessive, 1 = dominant, and 0.5 = additive inheritance (Liu and Tabashnik 1997).

For the diet-based bioassay, corrected larval mortality for each plate was calculated based on Abbott’s correction (Abbott 1925). Data were analyzed with a probit analysis,
which determined LC\textsubscript{50} values, 95% fiducial limits, and goodness-of-fit based on Pearson χ\textsuperscript{2} (PROC PROBIT).

For the fitness costs experiment with plants grown in the greenhouse, data on proportion survival to adulthood and egg viability were compared between strains with a model I ANOVA (PROC GLM). In the analysis of development rate, head capsule width, and adult lifespan, a mixed-model ANOVA was used. Fixed effects were strain, sex, and the interaction of strain and sex, and the random effect was cage × strain × sex, which was the mean square error term for the analysis. Egg production was analyzed with repeated measures ANOVA based on a split-plot design. Fixed effects were strain, week and week × strain and the random effects of cage nested within strain and week × cage (strain), which were the mean square error terms for the analysis.

For the experiment measuring the effect of competition on fitness costs, data were analyzed with a mixed-model ANOVA. The analysis of data on proportion survival to adulthood and egg viability used the fixed effects of strain, number of kernels, SCR (present vs. absent), and all interactions. The fixed effects of strain, number of kernels, SCR, sex, and all interactions were used in the analysis of development rate, head capsule width, and adult lifespan. Random effects were block and all interactions of block with fixed effects. Egg production was analyzed by repeated measures ANOVA with the fixed effects of strain, week, SCR, kernels and all interactions among these factors. Random effects were block all its interactions with fixed effects. Cage (strain × kernels × SCR presence × block) and week × cage (strain × kernels × SCR presence × block) were additional random effects and were not pooled regardless of significance because they would serve as mean square error terms if other random effects were pooled.
The interaction of western and southern corn rootworm was analyzed with a model I ANOVA. Of the 2,020 larvae used in this experiment, 91 were missing and 57 were dead after 2 d. The proportion missing data were transformed with the arcsine of the square root function. Data on the proportion of western corn rootworm and SCR larvae missing were analyzed separately and factors included the number of roots, treatment (see Table 1), and the interaction between these factors. Planned pairwise comparisons were made between controls (second or third instar western corn rootworm only or second or third instar SCR only) and the other treatments if that treatment contained the same instar and species of larvae.

Results

Quantifying inheritance of resistance to Cry3Bb1 For the seedling-mat bioassay with Elma, there was a significant interaction between strain and corn hybrid for survival to adulthood (df = 3,125; F = 27.22; P <0.001) (Table 2; Fig. 1a). Survival of the heterozygous strains was similar on non-Bt corn (0.86 ± 0.023 and 0.84 ± 0.022; mean ± SE; df = 1,125; F = 0.35; P = 0.5543) but different on Cry3Bb1 corn (0.44 ± 0.035 and 0.35 ± 0.050; df = 1,125; F = 4.87; P = 0.0291). Consequently, the two heterozygous strains were not pooled. The four genotypes (resistant, susceptible and the two heterozygous strains) had equivalent survival on non-Bt corn (P > 0.15) but differed in their survival on Bt corn. Survival on Cry3Bb1 corn was greatest for Elma and there was no difference in survival on non-Bt corn compared to Cry3Bb1 for this strain (df = 1,125; F = 2.39; P = 0.1246), suggesting complete resistance (Fig. 1a). Survival of the Susceptible♀ × Elma♂ and Elma♀ × Susceptible♂ strains were significantly greater than Susceptible on Cry3Bb1 corn (Susceptible♀ × Elma♂: df = 1,125; F = 17.84; P < 0.0001; Elma♀ × Susceptible♂: df = 1,125; F = 4.09; P = 0.0452),
indicating non-recessive inheritance for these strains. Both strains had lower survival on Cry3Bb1 corn compared to Elma (Susceptible♀ × Elma♂: df = 1,125; F = 61.19; P < 0.0001; Elma♀ × Susceptible♂: df = 1,125; F = 96.57; P < 0.0001), indicating that resistance was not dominant. The corrected survival to adulthood on Cry3Bb1 corn was 0.93 (0.76 ± 0.82) for Elma, 0.52 (0.45 ± 0.86) for the Susceptible♀ × Elma♂ strain, 0.43 (0.36 ± 0.84) for the Elma♀ × Susceptible♂ strain, and 0.35 (0.29 ± 0.81) for Susceptible. This yielded a resistance ratio for Elma of 2.74 (0.93 ÷ 0.35) and inheritance values of 0.29 based on the Susceptible♀ × Elma♂ strain and 0.14 based on the Elma♀ × Susceptible♂ strain.

For the seedling-mat bioassay with Monona, there was a significant interaction between rootworm strain and corn hybrid (df = 3, 81; F = 9.69; P < 0.0001). However, no difference in the survival of the two heterozygous strains on non-Bt (0.65 ± 0.072 and 0.64 ± 0.111; df = 1,81; F = 0.01; P = 0.9258) or Cry3Bb1 corn (0.38 ± 0.062 and 0.35 ± 0.094; df = 1,81; F = 0.29; P = 0.5927) was detected so strains were pooled into a single heterozygous strain for all analyses. Using the single heterozygous strain, there was a significant interaction between strain and corn hybrid (Table 2; Fig. 1b). There was no difference among the three strains on non-Bt corn (p > 0.15) but the genotypes differed in survival on Cry3Bb1 corn. Monona had the highest survival on Cry3Bb1 corn and survival was equivalent between the Bt and non-Bt hybrids (df = 1,13; F = 0.45; P = 0.5163), indicating complete resistance. Survival on Cry3Bb1 corn was lowest for Susceptible and significantly lower compared to survival on non-Bt corn (df = 1,13; F = 50.55; P < 0.0001). Survival of heterozygous on Cry3Bb1 was significantly greater than Susceptible (df = 1,13; F = 6.42; P = 0.0249), indicating non-recessive inheritance of resistance, but significantly lower than Monona (df = 1,13; F = 13.76; P = 0.0026) indicating that resistance was not dominant.
Corrected survival to adulthood on Cry3Bb1 corn was 0.91 (0.62 ÷ 0.68) for Monona, 0.52 (0.33 ÷ 0.63) for Heterozygous, and 0.20 (0.14 ÷ 0.70) for Susceptible. The resistance ratio for Monona is 4.55 (0.91 ÷ 0.20) and the inheritance is 0.45.

With the single-plant bioassay using Monona, there was a significant interaction between strain and corn hybrid (df = 3,107; F = 3.03; P = 0.0325). Because there was no difference in larval survival between the two heterozygous strains on non-Bt (0.56 ± 0.050 and 0.63 ± 0.068; df = 1,107; F = 0.58; P = 0.4476) or Cry3Bb1 corn (0.41 ± 0.076 and 0.44 ± 0.065; df = 1,107; F = 0.07; P = 0.7978), they were combined into one heterozygous strain for all analyses. There was a significant interaction between strain and hybrid when the heterozygous strains were combined (Table 1; Fig. 1c) with equivalent survival of the strains on non-Bt corn (P > 0.15 in all cases), indicating no difference in vigor. Survival of Monona on Cry3Bb1 corn was not significantly different compared to survival on non-Bt corn (df = 1,135; F = 1.30; P = 0.2571), indicating complete resistance. On Cry3Bb1 corn, survival was significantly different between Susceptible and heterozygotes (df = 1,135; F = 12.52; P = 0.0006), indicating non-recessive inheritance of resistance. Survival of the heterozygous strain on Cry3Bb1 corn was not significantly different compared to Monona (df = 1, 135; F = 0.02; P = 0.8930), indicating that resistance was dominant. Corrected larval survival was 0.83 (0.43 ÷ 0.52) for Monona, 0.70 (0.44 ÷ 0.63) for heterozygous, and 0.34 (0.21 ÷ 0.61) for Susceptible. This produced a resistance ratio for Monona of 2.44 (0.83 ÷ 0.34) and inheritance of 0.73.

In the diet-based assay with Monona (Table 3; Fig. 2) the heterozygous strains were once again pooled. We were able to calculate LC_{50} values and 95% fiducial limits for the Susceptible strain and heterozygous strains but not for Monona, because mortality never
exceeded 50% (Table 3; Fig. 2). There was a significant difference, as evidenced by non-overlapping 95% fiducial limits, between the LC50 values for the susceptible and heterozygous genotypes (Table 3), indicating non-recessive inheritance of resistance.

**Greenhouse assessment of fitness costs.** In the experiment with Monona and Susceptible, strain and its interaction with sex were not significant for any of the variables measured (Table 4; Fig. 3). This suggests an absence of fitness costs of Cry3Bb1 resistance in Monona. There was a significant effect of sex on development rate with males emerging before females (Table 4; Fig. 3d). There was also a significant effect of week on fecundity with egg production decreasing with time (Fig. 3f).

**Effect of competition on fitness costs.** Survival to adulthood was affected both by food availability and presence of SCR, indicating an effect of competition on survival. Proportion survival to adulthood was greatest in seedling mats with high food availability and no SCR (Fig. 4b). However, for survival there was not a significant effect of strain or interaction of strain with other factors, indicating that a fitness cost affecting survival was not present (Table 6; Fig 4b). There was a significant effect of strain on development rate (Table 6). On average, Elma emerged about 1.49 d later than Susceptible, indicating a fitness cost of resistance (Fig. 4a). Neither strain nor any interaction with strain were significant for size, adult lifespan, fecundity or egg viability, indicating that no fitness costs were associated with these life-history components (Table 5; Fig. 4). There was a significant effect of week and an interaction between week and number of kernels for fecundity. Initially egg production was greater from insects with 10 vs. 5 kernels (week 3: 5 kernels = 272 ± 155 eggs per cage; 10 kernels = 1073 ± 118) but this difference decreased over time (week 5: 5 kernels = 30 ± 156; 10 kernels = 246 ±121).
In the larval predation experiment, there was no significant interaction of treatment with number of roots for proportion surviving western corn rootworm (df = 6,131; F = 0.46, P = 0.8380). There was also no effect of treatment (df = 6,131; F = 2.14; P = 0.0530). There was no significant treatment × number of roots interaction for proportion surviving SCR (df = 6,116, F = 0.8, P = 0.5685), but there was a significant effect of treatment (df = 6,116, F = 2.88, P = 0.0119). There were significantly fewer surviving SCR larvae in the treatment with five second instar western corn rootworm larvae and five second instar SCR larvae compared to the control of 10 second instar SCR larvae (Table 1; df = 1,116, F = 4.99; P = 0.0275) and in the treatment with 5 second and 5 third instar SCR larvae compared to the treatment with 10 third instar SCR larvae (df = 1, 116; F = 4.17; P = 0.0435). Overall, there was some evidence of larval predation, particularly of SCR larvae, but the proportion of surviving larvae was never below 85% and was typically greater than 90% (Table 1).

**Discussion**

Our study investigated the inheritance of resistance and associated fitness costs for two strains of western corn rootworm with field-evolved resistance to Cry3Bb1 corn. For these strains, resistance was non-recessive and minimal fitness costs were detected. Past studies have also documented non-recessive inheritance of Bt resistance for western corn rootworm (Meihls et al. 2008, Petzold-Maxwell et al. 2012, and Ingber and Gassmann 2015) while the presence of fitness costs has varied among strains and experiments (Petzold-Maxwell 2012, Oswald et al. 2012, Hoffmann et al. 2014, Ingber and Gassmann 2015). These findings, and those of other studies, suggest that field-evolved resistance to Cry3Bb1 corn by western corn rootworm was likely facilitated by non-recessive inheritance of
resistance traits and similar fitness between resistant and susceptible insects in refuges (Gassmann 2012, Tabashnik et al. 2013).

Fitness costs of Bt resistance function to remove resistance alleles from a population in the absence of Bt toxins. When fitness costs are minimal, resistance alleles may accumulate in the refuge population because of selection within Bt fields and subsequent dispersal into refuge populations (Gould 1998). By contrast, when fitness costs are present, the selection against resistance alleles in the refuge can delay or reverse the evolution of resistance in a population (Carrière and Tabashnik 2001). This would occur if such a cost removes resistance alleles in the refuge to a greater extent than the alleles are selected for in the Bt portion of the field (Gassmann et al. 2009). In our experiments, no fitness costs were detected for Monona (Fig. 3) in a greenhouse experiment, but there was a fitness cost of increased time to development for Elma in our competition experiment (Fig. 4a). Ingber and Gassmann (2015) also identified a fitness cost of delayed larval development for one strain (Cresco) of western corn rootworm with field-evolved Cry3Bb1 resistance. Conversely, Oswald et al. (2012) identified no fitness costs of resistance to Cry3Bb1 in laboratory-selected resistant western corn rootworm strains and found that resistant lines had an increased the rate of larval development compared to unselected strains. Results with other strains of Cry3Bb1-resistant western corn rootworm have ranged from finding no fitness costs associated with resistance (Petzold-Maxwell et al. 2012, Hoffmann et al. 2014, Ingber and Gassmann 2015) to costs that affected survival (Ingber and Gassmann 2015) and fecundity (Meihls et al. 2012, Ingber and Gassmann 2015).

Compared to western corn rootworm, more research with fitness costs of Bt resistance has been conducted on lepidopteran pests, especially the diamondback moth
(Plutella xylostella Linnaeus) and the pink bollworm (Pectinophora gossypiella Saunders) (Gassmann et al. 2009). Fitness costs have been observed for both of these species. Carrière et al. (2001) found an average of 51.5% reduction in survival of Bt resistant pink bollworm on non-Bt cotton compared to susceptible strains. Likewise, a fitness cost of reduced survival and a decrease in resistance over multiple generations in the absence of Bt toxins were associated with resistance to Cry1Ac in the diamondback moth (Tabashnik et al. 1994). A review of studies by Gassmann et al. (2009) found fitness costs in 34% of experiments that tested fitness components and costs in 62% of experiments that tested for declines in resistance over multiple generations. Future studies that test for a decline in resistance over time in strains of western corn rootworm with field-derived resistance should be conducted to better understand fitness costs of Bt resistance in the western corn rootworm.

Resistance is expected to develop faster as the effective dominance of resistance, i.e., the survival of heterozygous individuals on Bt plants compared to homozygous resistant insects, increases (Gould 1998, Tabashnik et al. 2008). We found non-recessive inheritance of resistance for both the Elma and Monona strains with our plant-based assays (Fig. 1). Others have also identified non-recessive inheritance in Cry3Bb1-resistant strains (Meihls et al. 2008, Petzold-Maxwell 2012, Oswald et al. 2012, Hoffmann et al. 2014, Ingber and Gassmann 2015), suggesting that non-recessive inheritance is common for western corn rootworm. In the diet-based bioassay, the LC50 values of the Susceptible and heterozygous strains were within the range found by Siegfried et al. (2005) for laboratory and field strains and there was a significant difference in LC50 values between the Susceptible strain and heterozygous resistant individuals (Table 3). Again, this suggests non-recessive inheritance of resistance. The relationship between dominance and dose likely affects the effective
dominance of Cry3Bb1 resistance for western corn rootworm. When insects are not exposed to a high dose of toxin, as is the case with Cry3Bb1 and western corn rootworm, the effective dominance of resistance increases, and resistance is expected to evolve more quickly (Gould 1995, Tabashnik et al. 2004, Tabashnik and Carrière 2009).

Evidence from field-evolved resistance in other insect species supports the predictions of the effect of dominance on the rate of resistance development. In other species targeted by Bt crops, resistance is less common for cases where resistance is recessively inherited (Tabashnik et al. 2013). For example, the corn earworm (Helicoverpa zea Boddie) has non-recessive inheritance (Burd et al. 2000) and evolved resistance to Cry1Ac cotton faster compared to the closely-related tobacco budworm (Heliothis virescens Fabricius) (Lutrell et al. 1999, Tabashnik et al. 2008). In addition to western corn rootworm and corn earworm, two other species with non-recessive inheritance, the maize stalk borer (Busseola fusca Fuller) (Van Rensburg 1999) and fall armyworm (Spodoptera frugiperda Smith) (Storer et al. 2010) developed resistance and on Bt crops that failed to produce a high dose of toxin (Tabashnik et al. 2013).

There were differences in the magnitude of resistance and inheritance between the two strains, with Elma having a resistance ratio of 2.74 and Monona having a resistance ratio of 4.55 in the seedling-mat bioassays. This, along with differing resistance ratios in other western corn rootworm strains with field-evolved resistance (Ingber and Gassmann 2015) may have resulted from differences in the intensity of selection each strain experienced in either the field or the laboratory. There were also differences among our three assay types used to measure inheritance. While results from the two plant-based bioassays with Monona both indicate non-recessive inheritance, heterozygotes in the seedling-mat bioassay differed
from the resistant strain for survival on Cry3Bb1 corn and had a calculated inheritance value of 0.45 (Fig. 1b), but by contrast, there was no difference between heterozygous and resistant genotypes for survival on Cry3Bb1 corn in single plant bioassays with an inheritance value of 0.75 (Fig. 1c). This difference between these two bioassays may be related to exposure to Cry3Bb1, with higher exposure in the seedling mat assay than the single-plant assay. This hypothesis is supported by higher corrected survival of the susceptible strain on Bt corn in the single-plant experiment (0.34) compared to the seedling mat experiment (0.20). Another possibility is that additional mortality of the heterozygotes on Cry3Bb1 corn occurred in later larval instars or during pupation, a phenomenon that the single-plant bioassay, which measured larval survival, would have missed. In general, the proportion survival of susceptible insects on V5 to V6 Bt corn plants is 0.00 to 0.04 (Gassmann et al. 2014), which is lower than survival for V6 to V8 corn plants used in this study. However, due to low survival of the non-diapausing strains of studied here on V5 to V6 plants, it was not possible to use the same assay that is applied to evaluate field populations.

Our findings of non-recessive inheritance and a lack of major fitness costs in western corn rootworm strains with field-derived resistance to Cry3Bb1 suggests that the refuge strategy alone is likely insufficient to delay resistance development. This highlights the need for more diversified management of western corn rootworm through an integrated pest management approach including rotation among management strategies. The use of diverse approaches such as pyramiding of multiple Bt toxins, use of soil-applied insecticide with corn lacking rootworm-active Bt toxins, and crop rotation may help to delay the evolution of resistance to current and future Bt traits for management of western corn rootworm.
Acknowledgements

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References


**Gassmann, A. J. 2012.** Field-evolved resistance to Bt maize by western corn rootworm: predictions from the laboratory and effects in the field. J. Invertebr. Pathol. 110:287-293

**Gassmann, A. J., S.P Stock, Y. Carrière, and B. E. Tabashnik. 2006.** Effect of entomopathogenic nematodes on the fitness cost of resistance to Bt toxin Cry1Ac in pink bollworm (Lepidoptera: Gelechiidae). J. of Econ. Entomol. 99:920-926


Luttrell, R., G. L. Wan, and K. Knighten. 1999. Variation in susceptibility of noctuid (Lepidoptera) larvae attacking cotton and soybean to purified endotoxin proteins and commercial formulations of *Bacillus thuringiensis*. J. Econ. Entomol. 92: 21-32.


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.


Table 1: Relative survival of western corn rootworm (WCR) and SCR larvae

<table>
<thead>
<tr>
<th>Treatment</th>
<th>WCR(^a)</th>
<th>SCR(^a)</th>
<th>Roots</th>
<th>N(^b)</th>
<th>Proportion WCR Survival (±SE)</th>
<th>Proportion SCR Survival (±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 small</td>
<td>-</td>
<td></td>
<td>2</td>
<td>12</td>
<td>0.9985 (±0.0038)</td>
<td>-</td>
</tr>
<tr>
<td>10 large</td>
<td>-</td>
<td></td>
<td>2</td>
<td>12</td>
<td>0.9803 (±0.0038)</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>10 small</td>
<td></td>
<td>2</td>
<td>9</td>
<td>-</td>
<td>0.8550 (±0.0075)</td>
</tr>
<tr>
<td>-</td>
<td>10 large</td>
<td></td>
<td>2</td>
<td>12</td>
<td>-</td>
<td>0.9652 (±0.0056)</td>
</tr>
<tr>
<td>5 small + 5 large</td>
<td>-</td>
<td></td>
<td>2</td>
<td>12</td>
<td>0.9971 (±0.0038)</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>5 small + 5 large</td>
<td></td>
<td>2</td>
<td>8</td>
<td>-</td>
<td>0.9201 (±0.0084)</td>
</tr>
<tr>
<td>-</td>
<td>5 small</td>
<td></td>
<td>2</td>
<td>8</td>
<td>0.9966 (±0.0057)</td>
<td>0.9495 (±0.0084)</td>
</tr>
<tr>
<td>5 small</td>
<td>5 small</td>
<td></td>
<td>4</td>
<td>10</td>
<td>0.9914 (±0.0046)</td>
<td>0.9137 (±0.0067)</td>
</tr>
<tr>
<td>5 small</td>
<td>5 large</td>
<td></td>
<td>2</td>
<td>8</td>
<td>0.9700 (±0.0057)</td>
<td>0.9966 (±0.0084)</td>
</tr>
<tr>
<td>5 large</td>
<td>5 small</td>
<td></td>
<td>4</td>
<td>8</td>
<td>0.9866 (±0.0057)</td>
<td>0.9866 (±0.0084)</td>
</tr>
<tr>
<td>5 large</td>
<td>5 large</td>
<td></td>
<td>2</td>
<td>12</td>
<td>0.9718 (±0.0041)</td>
<td>0.9961 (±0.0061)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>12</td>
<td>0.9295 (±0.0038)</td>
<td>0.9909 (±0.0056)</td>
</tr>
</tbody>
</table>

\(^a\) small refers to 2\(^{nd}\) instar larvae and large refers to 3\(^{rd}\) instar larvae

\(^b\) number of replicates
Table 2: Mixed-model analyses of variance for Susceptible vs Monona and Susceptible vs Elma survival on Cry3Bb1 vs non-Bt corn

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Effect</th>
<th>df</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elma seedling mat</td>
<td>Strain</td>
<td>3,125</td>
<td>26.22</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Hybrid</td>
<td>1,125</td>
<td>355.68</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Strain × Hybrid</td>
<td>3,125</td>
<td>27.22</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Monona seedling mat</td>
<td>Strain</td>
<td>2,13</td>
<td>6.98</td>
<td>0.0087</td>
</tr>
<tr>
<td></td>
<td>Hybrid</td>
<td>1,11</td>
<td>41.05</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Strain × Hybrid</td>
<td>2,13</td>
<td>10.32</td>
<td>0.0021</td>
</tr>
<tr>
<td>Monona single plant</td>
<td>Strain</td>
<td>2,135</td>
<td>3.72</td>
<td>0.0268</td>
</tr>
<tr>
<td></td>
<td>Hybrid</td>
<td>1,135</td>
<td>30.14</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Strain × Hybrid</td>
<td>2,135</td>
<td>4.11</td>
<td>0.0185</td>
</tr>
</tbody>
</table>

aRandom effects included in the model were block (df = 1, \( \chi^2 = 2.2 \), P = 0.1380), block × hybrid (df = 1, \( \chi^2 = 0.1 \), P = 0.7518), block × strain (df = 1, \( \chi^2 = 0.1 \), P = 0.7518), and block × hybrid × strain (df = 1, \( \chi^2 = 2.2 \), P = 0.1380)
bThe random effect of block (df = 1, \( \chi^2 = 6.9 \), P = 0.0086) was included in the model
cSee Fig. 1
dRefers to the Susceptible, resistant, and heterozygous strains
Table 3: Goodness of fit and LC$_{50}$ values for diet-based bioassays with Cry3Bb1

<table>
<thead>
<tr>
<th>Strain</th>
<th>df</th>
<th>$\chi^2$</th>
<th>P</th>
<th>LC$_{50}^a$ (95% FL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible</td>
<td>3</td>
<td>1.57</td>
<td>0.6653</td>
<td>6.09 (2.22 to 10.01)</td>
</tr>
<tr>
<td>Heterozygous</td>
<td>3</td>
<td>1.01</td>
<td>0.3892</td>
<td>26.90 (14.09 to 41.37)</td>
</tr>
<tr>
<td>Monona</td>
<td>3</td>
<td>2.63</td>
<td>0.0480</td>
<td>&gt;341.60</td>
</tr>
</tbody>
</table>

$^a$Measured in $\mu$g/cm$^2$
### Table 4: Analysis of variance for Susceptible and Monona life-history traits

<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(^a)</td>
<td>Strain(^b)</td>
<td>1,58</td>
<td>0.03</td>
<td>0.8651</td>
</tr>
<tr>
<td></td>
<td>Sex</td>
<td>1,58</td>
<td>11.14</td>
<td>0.0015</td>
</tr>
<tr>
<td></td>
<td>Strain × Sex</td>
<td>1,58</td>
<td>0.03</td>
<td>0.8537</td>
</tr>
<tr>
<td>Survival</td>
<td>Strain</td>
<td>1,30</td>
<td>0.18</td>
<td>0.6713</td>
</tr>
<tr>
<td>Size(^c)</td>
<td>Strain</td>
<td>1,58</td>
<td>0.69</td>
<td>0.6913</td>
</tr>
<tr>
<td></td>
<td>Sex</td>
<td>1,58</td>
<td>0.02</td>
<td>0.9018</td>
</tr>
<tr>
<td></td>
<td>Strain × Sex</td>
<td>1,58</td>
<td>0.25</td>
<td>0.6186</td>
</tr>
<tr>
<td>Adult lifespan(^d)</td>
<td>Strain</td>
<td>1,58</td>
<td>0.52</td>
<td>0.4746</td>
</tr>
<tr>
<td></td>
<td>Sex</td>
<td>1,58</td>
<td>3.02</td>
<td>0.0877</td>
</tr>
<tr>
<td></td>
<td>Strain × Sex</td>
<td>1,58</td>
<td>0.70</td>
<td>0.4059</td>
</tr>
<tr>
<td>Egg viability</td>
<td>Strain</td>
<td>1,30</td>
<td>0.71</td>
<td>0.4072</td>
</tr>
</tbody>
</table>

\(^a\)The random effect of cage × strain × sex (df = 1, \(\chi^2 = 103.1, P < 0.0001\)) was included in the model

\(^b\)Strains were Susceptible or Monona

\(^c\)The random effect of cage × strain × sex (df = 1, \(\chi^2 = 4.7, P = 0.0302\)) was included in the model

\(^d\)The random effect of cage × strain × sex (df = 1, \(\chi^2 = 19.2, P < 0.0001\)) was included in the model
Table 5: 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>Susceptible vs Monon(^a)</td>
<td>Strain</td>
<td>1,30</td>
<td>0.42</td>
<td>0.5212</td>
</tr>
<tr>
<td></td>
<td>Week</td>
<td>8,227</td>
<td>37.03</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Strain x Week</td>
<td>8,227</td>
<td>1.09</td>
<td>0.3720</td>
</tr>
<tr>
<td>Susceptible vs Elma(^b)</td>
<td>Strain</td>
<td>1,2</td>
<td>0.01</td>
<td>0.9273</td>
</tr>
<tr>
<td></td>
<td>No. kernels</td>
<td>1,2</td>
<td>3.05</td>
<td>0.2228</td>
</tr>
<tr>
<td></td>
<td>SCR</td>
<td>1,2</td>
<td>0.86</td>
<td>0.4522</td>
</tr>
<tr>
<td></td>
<td>Strain x No. kernels</td>
<td>1,2</td>
<td>1.51</td>
<td>0.3437</td>
</tr>
<tr>
<td></td>
<td>Strain x SCR</td>
<td>1,4</td>
<td>0.06</td>
<td>0.8260</td>
</tr>
<tr>
<td></td>
<td>No. kernels x SCR</td>
<td>1,2</td>
<td>0.36</td>
<td>0.6084</td>
</tr>
<tr>
<td></td>
<td>Strain x No. kernels x SCR</td>
<td>1,4</td>
<td>0.04</td>
<td>0.8564</td>
</tr>
<tr>
<td></td>
<td>Week</td>
<td>6,169</td>
<td>14.54</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Strain x Week</td>
<td>6,169</td>
<td>0.79</td>
<td>0.5797</td>
</tr>
<tr>
<td></td>
<td>No. kernels x Week</td>
<td>6,169</td>
<td>4.86</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>SCR presence x Week</td>
<td>6,169</td>
<td>0.56</td>
<td>0.7636</td>
</tr>
<tr>
<td></td>
<td>Strain x No. kernels x Week</td>
<td>5,169</td>
<td>0.79</td>
<td>0.5594</td>
</tr>
<tr>
<td></td>
<td>Strain x SCR x Week</td>
<td>6,169</td>
<td>0.66</td>
<td>0.6856</td>
</tr>
<tr>
<td></td>
<td>No. kernels x SCR x Week</td>
<td>5,169</td>
<td>0.59</td>
<td>0.7074</td>
</tr>
<tr>
<td></td>
<td>Strain x No. kernels x SCR x Week</td>
<td>5,169</td>
<td>0.02</td>
<td>0.9998</td>
</tr>
</tbody>
</table>

\(^a\)The random effects of cage (strain) (df = 1, \(\chi^2 = 72.2\), P < 0.0001) and week × cage (strain) (df = 1, \(\chi^2 = 0\), P = 1) were included in the model.

\(^b\)Random effects included in the model were cage (strain × no. kernels × SCR × block) (df = 1, \(\chi^2 = 1.6\), P = 0.2059), week × cage (strain × no. kernels × SCR × block) (df = 1, \(\chi^2 = 6.3\), P = 0.0121), block (df = 1, \(\chi^2 = 2.7\), P = 0.1003), block × strain (df = 1, \(\chi^2 = 3\), P = 0.0833), block × no. kernels (df = 1, \(\chi^2 = 3.8\), P = 0.0513), block × SCR (df = 1, \(\chi^2 = 2.9\), P = 0.0886), block × strain × no. kernels (df = 1, \(\chi^2 = 5.6\), P = 0.018), block × SCR × no. kernels (df = 1, \(\chi^2 = 4\), P = 0.0455)
Table 6: Mixed-model analysis of variance for Susceptible and Elma life-history traits

<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(^a)</td>
<td>Strain(^b)</td>
<td>1.69</td>
<td>8.66</td>
<td>0.0044</td>
</tr>
<tr>
<td></td>
<td>No. kernels</td>
<td>1.9</td>
<td>11.95</td>
<td>0.0072</td>
</tr>
<tr>
<td></td>
<td>Sex</td>
<td>1.69</td>
<td>16.87</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>SCR</td>
<td>1.69</td>
<td>0.75</td>
<td>0.3890</td>
</tr>
<tr>
<td></td>
<td>Strain × No. kernels</td>
<td>1.69</td>
<td>3.00</td>
<td>0.0878</td>
</tr>
<tr>
<td></td>
<td>Strain × Sex</td>
<td>1.69</td>
<td>2.90</td>
<td>0.0929</td>
</tr>
<tr>
<td></td>
<td>Strain × SCR</td>
<td>1.69</td>
<td>2.96</td>
<td>0.0900</td>
</tr>
<tr>
<td></td>
<td>No. kernels × Sex</td>
<td>1.69</td>
<td>8.17</td>
<td>0.0056</td>
</tr>
<tr>
<td></td>
<td>No. kernels × SCR</td>
<td>1.69</td>
<td>1.25</td>
<td>0.2667</td>
</tr>
<tr>
<td></td>
<td>Sex × SCR</td>
<td>1.69</td>
<td>4.62</td>
<td>0.0352</td>
</tr>
<tr>
<td></td>
<td>Strain × No. kernels × Sex</td>
<td>1.69</td>
<td>0.63</td>
<td>0.4306</td>
</tr>
<tr>
<td></td>
<td>Strain × No. kernels × SCR</td>
<td>1.69</td>
<td>0.13</td>
<td>0.7151</td>
</tr>
<tr>
<td></td>
<td>Strain × Sex × SCR</td>
<td>1.69</td>
<td>0.29</td>
<td>0.5944</td>
</tr>
<tr>
<td></td>
<td>No. kernels × Sex × SCR</td>
<td>1.69</td>
<td>0.21</td>
<td>0.6479</td>
</tr>
<tr>
<td></td>
<td>Strain × No. kernels × Sex × SCR</td>
<td>1.69</td>
<td>1.92</td>
<td>0.1698</td>
</tr>
<tr>
<td>Survival(^c)</td>
<td>Strain</td>
<td>1.50</td>
<td>0.54</td>
<td>0.4644</td>
</tr>
<tr>
<td></td>
<td>No. kernels</td>
<td>1.50</td>
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- Random effects included in the model were block (df = 1, $\chi^2 = 1.3$, $P = 0.2542$) and block × no. kernels (df = 1, $\chi^2 = 2.6$, $P = 0.1069$)
- Strains were Susceptible or Elma
- The random effect of block (df = 1, $\chi^2 = 17.2$, $P < 0.0001$) was included in the model
- Random effects included in the model were block (df = 1, $\chi^2 = 0.7$, $P = 0.4028$), block × strain (df = 1, $\chi^2 = 0$, $P = 1.000$), block × SCR (df = 1, $\chi^2 = 0.1$, $P = 0.7518$), and block × strain × SCR (df = 1, $\chi^2 = 2.5$, $P = 0.1138$)
- Random effects included in the model were block (df = 1, $\chi^2 = 0$, $P = 1.000$), block × strain (df = 1, $\chi^2 = 0$, $P = 1.000$), block × no. kernel (df = 1, $\chi^2 = 0.9$, $P = 0.3428$), block × SCR (df = 1, $\chi^2 = 0$, $P = 1.000$), and block × strain × SCR (df = 1, $\chi^2 = 4.2$, $P = 0.0404$)
- Random effects included in the model were block (df = 1, $\chi^2 = 0.1$, $P = 0.7518$), block × strain (df = 1, $\chi^2 = 0$, $P = 1.0000$), block × no. kernels (df = 1, $\chi^2 = 0$, $P = 1.0000$), block × strain × no. kernels (df = 1, $\chi^2 = 1.7$, $P = 0.1923$)
- The strain × no. kernels × SCR interaction could not be calculated because Elma cages with 5 kernels and SCR did not lay enough eggs to test egg viability
Figures

Fig. 1. Survival to adulthood on Cry3Bb1 corn and non-Bt corn for (a) the seedling-mat bioassay with Elma (Cry3Bb1 resistant) and Susceptible, (b) the seedling-mat bioassay with Monona (Cry3Bb1 resistant) and Susceptible, and (c) larval survival in the single-plant bioassay with Monona and Susceptible. Bar heights represent sample means and error bars are the standard error of the mean.

Fig. 2. Larval mortality in diet-based bioassays for the Susceptible, Monona (Cry3Bb1 resistant), and heterozygous strains. Data were adjusted for mortality with Abbott’s correction. Points represent means, error bars are the standard error of the mean, and the curve is the plot of the probit analysis.

Fig. 3. Comparisons of life-history data for Susceptible and Monona (Cry3Bb1 resistant) strains on non-Bt corn. Bar heights represent sample means and error bars are the standard error of the mean. Data are presented for (a) developmental rate, (b) proportion survival to adulthood, (c) adult size, (d) egg viability, (e) adult lifespan, and (f) fecundity.

Fig. 4. Comparisons of life-history data for Susceptible and Elma (Cry3Bb1 resistant) strains on non-Bt corn at high or low larval food availability (5 vs 10 kernels in the initial seedling mats) and in the presence and absence of competition from southern corn rootworm (SCR). Bar heights represent sample means and error bars are the standard error of the mean. Data are presented for (a) developmental rate, (b) proportion survival to adulthood, (c) adult size, (d) egg viability, (e) adult lifespan, and (f) fecundity.
Fig. 1

(a) Survival to adulthood (Proportion)

(b) Survival to adulthood (Proportion)

(c) Larval survival (Proportion)
Fig. 2

Corrected Mortality vs. Concentration of Cry3Bb1
Fig. 3

(a) Date of Emergence

(b) Proportion Survival to Adulthood

(c) Head Capsule Width (mm)

(d) Proportion Viable Eggs

(e) Adult Lifespan (Days)

(f) Eggs per Cage per Week vs. Total Eggs per Cage
Fig. 4

(a) Date of Emergence

(b) Proportion Survival to Adulthood

(c) Head Capsule Width (mm)

(d) Proportion Viable Eggs

(e) Adult Lifespan (Days)

(f) Eggs per Cage per Week vs. Total Eggs per Cage
CHAPTER 3: CONCLUSION

The western corn rootworm is a serious and highly adaptable pest of corn. Populations have evolved resistance to a number of management strategies, the most recent of which is resistance to transgenic corn that produces Bt toxins. The evolution of resistance to Bt toxins threatens the future utility of this technology. One of the main insect resistance management strategies used to delay the development of resistance is the refuge strategy. Models predict that the greatest delays in resistance evolution occur when the inheritance of resistance is recessive, meaning that heterozygous resistant individuals are killed in the Bt portion of the field, and there are fitness costs of resistance which serve to remove resistance alleles from the non-Bt refuge. Our results suggest that western corn rootworm resistance to the Bt toxin Cry3Bb1 is not recessively inherited and we identified minimal fitness costs of resistance.

Inheritance of resistance was measured for the two resistant strains (Elma and Monona) in plant-based and diet-based bioassays. Reciprocal crosses were established between the resistant strains and a western corn rootworm strain that had never been exposed to Cry3Bb1 (Susceptible). The progeny from these crossed were used in bioassays. A seedling-mat bioassay measuring survival to adulthood was conducted with the Elma and Monona strains and resistance was found to be complete, meaning that survival of the resistant strains on Cry3bBb1 corn was equivalent to survival on non-Bt corn, and non-recessive for both. The resistance ratios were 2.74 for Elma and 4.55 for Monona, suggesting that Monona is more resistant compared to Elma. This may be related to past exposure to Bt corn. Monona had four field selections followed by six laboratory selections on Cry3Bb1 while Elma experienced two field and four laboratory selections. Monona was used in two
additional assays which provided similar, but not identical, results. In a single-plant bioassay measuring larval survival, resistance was again complete and non-recessive, but the calculated inheritance value was greater than that found with the seedling-mat assay (0.45 and 0.75). These inheritance values were calculated using corrected survival on Cry3Bb1 corn in the equation $h = (\text{heterozygote} - \text{susceptible}) / (\text{resistant} - \text{susceptible})$, where 0 = recessive, 1 = dominant, and 0.5 = additive inheritance. In a diet-based bioassay measuring larval survival, the LC$_{50}$ values for the heterozygous individuals differed significantly from Susceptible. Differences among these bioassays may relate to the outcome measured (larval survival vs survival to adulthood) or to the dose experienced by the larvae. Non-recessive inheritance will allow heterozygous resistant individuals to survive on Bt corn and allow resistance alleles to persist in the population.

Fitness cost experiments were conducted in the greenhouse and laboratory with the Susceptible, Elma, and Monona strains. No fitness costs were detected for Monona in a greenhouse experiment that measured survival to adulthood and the additional fitness components of development time, size, lifespan, and fecundity. An experiment measuring the effect of competition on the same fitness components using the Elma strain was conducted in the laboratory using seedling mats. In this experiment, there was no effect of competition on the presence of fitness costs. A single fitness cost was detected in the form of increased development time. The extent to which a 1.49 day delay in development will remove resistance alleles from the refuge could be limited due to the relatively extended period over which adult rootworm emerge into a cornfield, but longer larval development could lead to increased mortality from predators or pathogens in the soil.
Bt crops are considered high dose when the concentration of toxin produced is 25 times greater than required to kill a susceptible individual or a dose that kills 99.99% of susceptible individuals is produced. In these situations, nearly all heterozygous and homozygous susceptible individuals are killed by the toxin so resistance is functionally recessive. Corn hybrids for rootworm control do not produce a high dose of Bt toxin and our results are consistent with expectations for the evolution of resistance when a pest is not exposed to a high dose of Bt toxin. These findings underscore the need for rootworm IRM beyond the refuge strategy. Multiple tactics such as crop rotation, the planting of non-Bt corn with soil-applied insecticide, and planting pyramids of multiple Bt toxins that target western corn rootworm must be used to maintain the effectiveness of available Bt toxins.
ACKNOWLEDGEMENTS

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