

2013

# Fitness costs associated with resistance to Cry3Bb1 corn in western corn rootworm

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**Fitness costs associated with resistance to Cry3Bb1 corn in western corn  
rootworm**

by

**Amanda Marie Hoffmann**

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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2013

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## CHAPTER 1. GENERAL INTRODUCTION

In 2003 the United States Environmental Protection Agency (US EPA) approved genetically modified maize (*Zea mays* L.) that produced an insecticidal toxin, Cry3Bb1, derived from the bacterium *Bacillus thuringiensis* (Bt) in both roots and above ground tissue (EPA 2003). This Bt toxin was approved for the management of western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), and is only effective against larvae (EPA 2003). Studies from the field indicate that Bt maize for rootworm management will be “as good or better” than soil applied insecticides at protecting maize roots from larval feeding (Rice 2004). However, Cry3Bb1 toxin is not expressed at a high dose but rather a moderate dose, which means susceptible insects are able to survive exposure to the toxin (Siegfried et al. 2005). In order for Bt toxin to be effective it must be consumed by the insect where the activated Cry toxin binds to the midgut, creating pores and eventually leading to death of the insect (Schnepf et al. 1998). In 2012, 67% of maize planted in the United States was genetically engineered to produce at least one Bt toxin (Economic Research Service 2012). The wide-spread planting of Bt maize applies a large amount of selection pressure for the evolution of insect resistance.

In the United States, non-Bt host plants are planted along with Bt crops as a refuge in an effort to slow resistance development (Gould 1998). The central goal of the refuge strategy is to provide non-Bt host plants to promote survival of Bt-susceptible insects to mate with rare resistant insects (Carrière and Tabashnik 2001, Tabashnik et al. 2003). Mating of homozygous susceptible insects with homozygous

resistance insects produce progeny that are heterozygous for resistant traits (Tabashnik and Carrière 2009) and to the extent that heterozygotes have reduced fitness on Bt crops, compared to homozygous resistant individuals, resistance evolution will be delayed (Carrière and Tabashnik 2001).

Several factors may augment the efficacy of a refuge strategy including: recessive inheritance of Bt resistance, low initial frequency of resistant alleles, random mating between resistant and susceptible insects, and the presence of fitness costs of Bt resistance (Carrière et al. 2010). Delays in the evolution of resistance expected under the refuge strategy increase as the recessiveness of resistance increases and are greatest when resistance is completely recessive (Tabashnik et al. 2004). Fitness costs of resistance to Bt are expected to delay the evolution of resistance and delays becoming greater as fitness costs increase (Carrière et al. 2010). Furthermore, as the area planted to the refuge increases, delays in resistance become greater (Carrière and Tabashnik 2001). The EPA currently requires a 20% structure refuge or a 10% blended refuge for maize that produce a single Bt toxin targeting western corn rootworm, and 5% refuge, either blended or structured, for maize that produce two toxins against rootworm (EPA 2011). However, because Bt maize targeting western corn rootworm is not high dose, Tabashnik and Gould (2012) recommend an increase in refuge to 50% for a single toxin and 20% for two toxins.

A fitness cost of Bt resistance occurs when individuals with resistance alleles have lower fitness than Bt-susceptible individuals in the absence of Bt toxin (Gassmann et al. 2009a). Fitness costs delay resistance evolution by reducing the

frequency of resistant alleles in the refuge (Carrière and Tabashnik 2001, Gassmann et al. 2009a, Pittendrigh et al. 2004). Thus, resistance evolution is affected not only by the production of susceptible insects from refuges, but also by the dominance and magnitude of fitness costs present.

Fitness costs can affect a variety of life-history traits such as survival, egg viability, fecundity, developmental rate, adult longevity, and overwintering survival (Carrière et al. 2001, Bird and Akhurst 2004, Janmaat and Myers 2005). Gassmann et al. (2009a) reviewed 77 studies, spanning 18 species, for fitness costs of Bt resistance and found a mean fitness cost of 23% among life-history traits. Importantly, ecological factors, including host plant variety and the presence of entomopathogens can magnify fitness costs of Bt resistance (Gassmann et al. 2009a). A better understanding of life-history traits affected by fitness costs, the dominance of these costs, and how environmental factors influence fitness costs can be valuable for devising resistance management strategies.

The dominance of fitness costs is important because dominant costs will impose greater delays in resistance evolution (Carrière and Tabashnik 2001). When fitness costs are recessive only resistant insects have lower fitness compared to susceptible insects, but there is no difference between susceptible and heterozygous insects. Non-recessive fitness costs in the refuge reduce fitness for heterozygotes when compared to susceptible insects. Non-recessive fitness costs strongly favor a decrease in resistance allele frequency by selecting against the heterozygous insects (Bird and Akhurst 2007, Carrière and Tabashnik 2001,



Pittendrigh et al. 2004). In the review of fitness costs by Gassmann et al. (2009a), 26% of studies examining the dominance of fitness costs found non-recessive costs.

Western corn rootworm is a univoltine pest that feeds and develops almost exclusively on maize (Chiang 1973). Adults lay their eggs in the soil in late summer, and eggs hatch the following spring (Levine and Oloumi-Sadeghi 1991). Following hatch, larvae locate and begin to feed on maize roots (Moeser and Hibbard 2005). There are three larval instars and their feeding can severely injure maize plants. Feeding from larvae weakens the root structure, makes maize plants more vulnerable to disease and drought, and stability of the maize plant is greatly reduced (Moellenbeck 2001). On average, one node of root tissue lost to larval feeding can decrease yield by 17.9% (Dun et al. 2010). Adult males emerge before females and newly emerged females can mate shortly after emerging from the soil, within 12 h (Nowatzki et al. 2002, Ball 1957, Guss 1976, Hill 1975). Adult western corn rootworm feed on maize leaves, as well as silks, pollen, and young maize kernels. Billions of dollars have been lost to western corn rootworm in reduced yield and management costs (Levine and Oloumi-Sadeghi 1991).

This economically significant pest has repeatedly demonstrated an ability to develop resistance to management strategies including insecticides, crop rotation, and recently to Cry3Bb1 maize (Gray et al. 2009, Levine and Oloumi-Sadeghi 1991, Meinke et al. 1998, Gassmann et al. 2011). Resistance to pesticides such as methyl-parathion has developed in western corn rootworm (Siegfried and Mullin 1989) and has been retained for at least 20 years after this insecticide has been removed from use (Parimi et al. 2006). In Gassmann et al. (2012), the S2

population was found to be resistant to Cry3Bb1 even though that field had not been planted to Cry3Bb1 maize for 2 years, which indicates that once resistance evolves to Bt toxins it may persist in a manner similar to the aforementioned resistance to methyl-parathion or other insecticides.

Western corn rootworm have shown increased survival on Cry3Bb1 maize in the field in as few as three generations (Gassmann et al. 2011). Cry3Bb1-resistant western corn rootworm survival was three times greater than Bt-susceptible insects when reared on Cry3Bb1 Bt maize in Gassmann et al. (2011) and 11 times greater in Gassmann et al. (2012). Oswald et al. (2011) had four times greater survival of Cry3Bb1-resistant western corn rootworm than their susceptible counterpart when reared on Bt maize. Bt resistance in western corn rootworm has been found to be inherited non-recessively (Petzold-Maxwell et al. 2012, Meihls et al. 2008).

Traits conferring high levels of resistance are more likely to be costly than those conferring lower levels of resistance (Gassmann et al. 2009a). Resistance ratios ( $LC_{50}$  of the resistant strain divided by the  $LC_{50}$  of the susceptible strain) for resistant western corn rootworm selected in the laboratory and reared on diet were 22 for Cry3Bb1 (Meihls et al. 2008). When compared to lepidopteran pests such as *Ostrinia nubilalis* (Lepidoptera: Crambidae), which displayed a resistance ratio >3,000 when reared on diet with Bt toxin Cry1F (Pereira et al. 2008), these values are relatively low.

Little evidence has been found to indicate western corn rootworm experience fitness costs of Bt resistance. Oswald et al. (2012) determined Cry3Bb1-resistant western corn rootworm did not experience fitness costs in survival to adulthood,

fecundity, and egg viability when reared on non-Bt maize, and actually experienced fitness benefits for resistance in quicker development and higher fecundity. A study looking at fitness costs in western corn rootworm in the presence of two nematode species, *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* found that mortality was higher when nematodes were present, however; no fitness costs were observed (Petzold-Maxwell et al. 2012). By contrast, Meihls et al. (2012) found fitness costs in fecundity and adult male lifespan in Cry3Bb1-resistant strains of western corn rootworm in greenhouse and field experiments.

Features such as secondary metabolites, physical defenses and nutrient composition in host plants can affect a variety of life-history traits for insects (Awmack and Leather 2002). These features may affect Bt-resistant insect to a greater extent than Bt-susceptible insects and therefore, the dominance and magnitude of fitness costs may be increases by host plant species and cultivar. Selection of host plant varieties for refuges can be used to not only delay resistance by promoting the survival of susceptible insects, but also to delay the development of resistance by magnifying fitness costs. Some studies have found that as the suitability of a host plant decreases, fitness costs may increase (Janmaat and Meyers 2005, Raymond et al. 2005, Raymond et al. 2007a). By contrast, a study of *Helicoverpa armigera* (Lepidoptera: Noctuidae) reared on three hosts, cotton, pigeon pea, and sorghum, found that susceptible insects had similar fitness on all three hosts, but Cry1Ac-resistant insects experienced fitness costs that were generally greater and more dominant on cotton and sorghum (Bird and Akhurst 2007). Furthermore, secondary metabolites, such as gossypol in cotton, have been shown

to increase both the dominance and magnitude of fitness costs (Carrière et al. 2004, Williams et al. 2011). Host plant variety can not only affect fitness cost, but can also reduce the overall fitness of insects (Awmack and Leather 2002). Reduced survival for western corn rootworm among maize lines has been demonstrated on some maize lines that possess antixenosis and antibiosis towards western corn rootworm (Khishen et al. 2009).

Soil habitats have been found to support naturally occurring populations of entomopathogenic nematodes and fungi, and these pathogens can kill western corn rootworm larvae (Liu and Berry 1995, Pilz et al. 2008). Fungal conidia contact the insect cuticle, germinate, and penetrate the haemocoel where they grow, producing insecticidal compounds, which leads to death of the insect host (Lewis et al. 2006, Shah and Pell 2003). Upon death of the host, fungi produce conidia on the surface of the cuticle that then disperse (Hajek and St. Leger 1994). Infective juveniles of entomopathogenic nematodes are a motile, free living stage that enters the haemocoel of a host through natural openings, where they release symbiotic bacteria which replicate and kill the host. Nematodes feed on the bacteria and host tissue and reproduce inside the cadaver (Kaya and Gaugler 1993). Infective juvenile will leave the host when all available nutrients are depleted and searches for a new host (Grewal et al. 1994). Entomopathogenic nematodes exhibit one of three foraging types: cruisers which actively seek their host; ambush, also called sit and wait, which infect active host that they contact; and intermediate which exhibit both cruiser and ambush tendencies (Grewal et al. 1994). Entomopathogenic fungi and

nematodes have the ability to impose mortality and reproduce in insect hosts and therefore may magnify fitness costs and also may be used in biological control.

Entomopathogens can magnify fitness costs of Bt resistance in pest species. Cry1Ac-resistant *Pectinophora gossypiella* (Lepidoptera: Gelechiidea) reared on diet and exposed to *S. riobrave* experienced a recessive fitness cost affecting survival, (Gassmann et al. 2006, 2008). When reared on non-Bt cotton, Cry1Ac-resistant *P. gossypiella* suffered a recessive fitness cost when exposed to *S. riobrave* and *H. bacteriophora* imposed a fitness cost affecting only heterozygotes (Gassmann et al. 2009b). Entomopathogenic nematodes *S. riobrave*, *S. carpocapsae*, *Steinernema* sp. (ML18 strain), and *H. sonorensis* were used to determine if their presence impose fitness costs in Cry1Ac-resistant *P. gossypiella* and results indicate a dominant fitness costs in the presence of *S. riobrave*, but no other fitness costs (Hannon et al. 2010). Cry1Ac-resistant *Plutella xylostella* (Lepidoptera: Plutellidae) reared on cabbage leaves were evaluated for virus-mediated fitness costs and the virus imposed fitness costs in decreased egg fertility (Raymond et al. 2007b).

Entomopathogens can not only magnify fitness costs, but also serve as a biological control agents (Journey and Ostlie 2000). The entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* and the entomopathogenic nematodes *Heterorhabditis* species and *Steinernema* species have been found to naturally occur in maize fields (Pilz et al. 2008). Several species of entomopathogenic fungi and nematodes have been shown to infect and kill western corn rootworm (Toepfer et al. 2009, Pilz et al. 2007). Studies indicate that entomopathogenic nematodes have the ability to infect all three larval stages of

western corn rootworm and are able to significantly reduce survival and maize root injury (Kurtz et al. 2009, Toepfer et al 2008, Toepfer et al. 2010). An experiment conducted with commercially available *Steinernema* species and *H. bacteriophora* revealed that *H. bacteriophora*, *S. arenarium*, and *S. feltiae* had the highest potential for further testing as a biological control agent because these nematodes imposed the highest larval mortality in western corn rootworm, were able to propagate in the western corn rootworm larvae, and have low production costs (Toepfer et al. 2005). Pilz et al. (2007) determined that *M. anisopliae* strains are more virulent against western corn rootworm than *B. bassiana* and *B. brongniartii*, and adults were more susceptible than larvae. Two entomopathogens, *M. anisopliae* and *H. bacteriophora*, were all able to significantly reduced western corn rootworm survival and root injury (Pilz et al. 2009). Petzold-Maxwell et al. (2013) observed that entomopathogens were able to reduce injury to maize roots under certain conditions and that their presence increased overall yield. Thus, several entomopathogens have been shown to cause mortality in western corn rootworm, reduce maize root injury, and increase yield, indicating potential application as part of an integrated pest management strategy.

The refuge strategy is used to reduce the heritability of resistance by providing a habitat free from Bt toxin to produce susceptible insects to mate with the rare resistant insects that survive on the Bt crop. Delays in resistance development are affected by the production of susceptible insects from the refuge and by the presence of fitness costs. This means more effective refuges could be designed by considering how ecological factors might be used to impose larger or more dominant

fitness costs. My research objectives were 1) quantify resistance to Cry3Bb1 maize in a laboratory-selected strain of western corn rootworm, 2) determine if fitness costs are present for Bt-resistant western corn rootworm, and 3) test whether host plant variety or entomopathogens can magnify these costs. Results of this work will be beneficial in more effective strategies for integrated pest management and insecticide resistance management.

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**CHAPTER 2. FITNESS COSTS OF RESISTANCE TO CRY3BB1 MAIZE BY  
WESTERN CORN ROOTWORM: EFFECTS OF LARVAL HOST PLANT**

A paper submitted to *the Journal of Economic Entomology*

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**Abstract**

Crops producing toxins derived from the bacterium *Bacillus thuringiensis* (Bt) are widely planted to manage insect pests, including western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), a significant pest of maize. The evolution of resistance would diminish the effectiveness of this technology; however, fitness costs can delay resistance. We quantified the level of resistance and tested for fitness costs of resistance in western corn rootworm with resistance to transgenic maize (*Zea mays* L.) that produces the Bt toxin Cry3Bb1.

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Survival on Cry3Bb1 maize was more than twice as high for resistant insects versus susceptible insects. Fitness costs were measured in two experiments with non-Bt maize, the first used three commercial hybrids and the second used three inbred lines. The experiment with commercial hybrids compared resistant and susceptible strains and the experiment with maize inbreds also included heterozygous individuals and tested for non-recessive fitness costs. The only non-recessive fitness cost detected was an additive fitness cost affecting adult size. Recessive fitness costs were observed for developmental rate, female survival, and egg viability. However, fitness benefits of higher fecundity, higher male survival, and greater longevity were detected for the resistant strain in the absence of Bt maize. Including all statistically significant fitness costs and benefits, the average difference between strains, in the absence of Bt maize, was a 5.3% fitness benefit for resistant insects. These results suggest that resistance to Bt maize by western corn rootworm may not impose fitness costs, and consequently, may evolve quickly and persist once present.

**Keywords:** *Bacillus thuringiensis*, *Diabrotica virgifera virgifera*, ecological variation, refuge strategy

### Introduction

In the United States during 2012, 67% of maize (*Zea mays* L.) planted was genetically modified to produce toxins derived from the bacterium *Bacillus thuringiensis* (Bt) (Economic Research Service 2012), placing intense selection on pests to develop resistance. In 2003 genetically modified maize producing the insecticidal Bt toxin Cry3Bb1 was registered for commercial cultivation by the United

States Environmental Protection Agency (EPA 2003). Cry3Bb1 maize targets western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), which is one of the most economically significant pests of maize in the United States (Gray et al 2009). This pest has developed resistance to several pest management strategies including conventional insecticides (Meinke et al. 1998), crop rotation (Levine and Oloumi-Sadeghi 1991) and Cry3Bb1 maize (Gassmann et al. 2011).

In the United States, the refuge strategy is used to delay the evolution of Bt resistance. This strategy consists of growing non-Bt host plants in close proximity to Bt crops, to permit the survival of Bt-susceptible insects to mate with resistant insects that survive on a Bt crop (Carrière and Tabashnik 2001, Gould 1998). Several factors may enhance the efficacy of the refuge strategy to delay resistance, including recessive inheritance of Bt resistance, low initial frequency of resistant alleles and the presence of fitness costs of Bt resistance (Carrière et al. 2010).

A fitness cost of Bt resistance occurs, in the absence of Bt toxin, when Bt-resistant insects have lower fitness than Bt-susceptible insects (Gassmann et al. 2009). Fitness costs may affect a variety of life-history traits and can act to delay resistance evolution (Carrière and Tabashnik 2001, Carrière et al. 2004, Gassmann et al. 2009). Delays in resistance from fitness costs become greater as the magnitude and dominance of fitness costs increase, and ecological factors including larval host plant can affect the dominance and magnitude of fitness costs (Carrière and Tabashnik 2001, Carrière et al. 2005, Gassmann et al. 2009). Furthermore, host plants attributes such as secondary metabolites, physical defenses and nutrient



composition can affect the survival and reproduction of pest insects (Panda and Khush 1995). Thus, for some pest species, selection of host plant varieties for refuges can be used both to reduce pest populations and to delay the development of resistance by magnifying fitness costs.

In this study we quantified the level of resistance and fitness costs for a laboratory-selected strain of western corn rootworm with resistance to Cry3Bb1 maize. Resistance was quantified by rearing resistant and susceptible strains on Bt and non-Bt maize. Fitness costs were evaluated by comparing the resistant strain to the susceptible strain in the absence of Bt maize, and this was done in two experiments, the first with three non-Bt maize hybrids and the second with three non-Bt inbred lines of maize.

### **Materials and Methods**

**Insect strains.** Field-collected adult males of western corn rootworm from four locations were crossed with females from a non-diapausing strain, and two strains were generated from this single initial starting strain (Oswald et al. 2011). These two strains were 1) a susceptible strain that was not exposed to Bt toxin and 2) a resistant strain, the moderately selected strain in Oswald et al. (2011), that was fed Cry3Bb1 maize for an increasing duration over 11 generations (F0 to F10). Strains were sent to Iowa State University in the F13, and reared on maize seedlings mats of either Cry3Bb1 maize or non-Bt maize following Jackson (1986) and Oswald et al. (2011). Strains were maintained at a population size of 1200 adults per generation. The susceptible strain was reared on non-Bt maize (hybrid 34M94 DuPont Pioneer, Johnston, Iowa). During the F13, F15 and F17, larvae of

the resistant strain were selected on seedling mats of Cry3Bb1 maize (hybrid DKC 61-69, Monsanto Company, Saint Louis, Missouri). To increase the genetic similarity between strains, adults of the resistant strain were backcrossed to the susceptible strain in a 1:1 ratio during the F13 and F15. The subsequent progeny of this backcross were reared on non-Bt maize (F14 and F16). The F18 to F24 of the resistant strain were reared on non-Bt maize.

**Quantifying resistance to Cry3Bb1.** This experiment was conducted in May 2012 using the F24 for resistant and susceptible strains. Bioassays were run using seedling mats that consisted of Cry3Bb1 maize (DKC 61-69) and non-Bt maize (DKC 61-72), which is the genetic near isoline to Cry3Bb1 maize but does not produce rootworm active Bt toxin. Twelve small seedling mats were established for each combination of maize type (Bt versus non-Bt) and insect strain (resistant versus susceptible) for a total of 48 replicates. Small seedling mats were created in 0.95 L clear plastic containers (Pactiv Showcase®, Johnson Paper and Supply Co., Minneapolis, Minnesota) by combining 60 mL of water with 40 mL (~65 kernels) of maize that had been pre-soaked in deionized (DI) water for 12 h. Seeds and water were then covered with 200 g of a soil mixture that was, by volume, 40% soil collected from the top 15-20 cm of an agricultural field in Ames, Iowa, and 60% potting soil (LC1, Sun Gro Horticulture Vancouver, British Columbia). Containers were covered with fine mesh fabric and a lid with several small openings for ventilation, and then placed in a growth chamber (25°C; 16:8, L:D; 65% RH) for 7 d, after which 50 neonate larvae were placed onto each seedling mat before returning it to the growth chamber for 7 d.

After 7 d, larvae were transferred to larger seedling mats by placing each small seedling mat on top of a larger seedling mat, where it remained for the duration of the bioassay. These larger seedling mats were produced by adding 150 mL of maize, soaked in DI water for 12 h, to a plastic container (21 cm × 27 cm × 10 cm; 1642N3, Rubbermaid, Fairlawn, Ohio) and then covering the seed with 1,200 g of soil. Larger seedling mats were moistened with 400 mL of water and held in a growth chamber for 1 wk prior to transferring larvae. Each larger seedling mat contained the same maize hybrid used in the corresponding small seedling mat. Larger seedling mats with larvae were held in a growth chamber (25°C; 16:8, L:D). When insects were 21 d old, larger seedling mats were checked every 2 to 3 d for adults, until no adults were collected from any container for 2 consecutive wks.

Adults were stored in ethanol and data were collected on number of adults surviving per seedling mat, sex for each adult insect, and dry mass. Data on dry mass were collected for a random sample of up to 10 males and 10 females per seedling mat, for a total maximum sample size of 120 individuals per strain by sex by maize hybrid. In some cases, fewer adults were obtained: susceptible females on Cry3Bb1 maize = 57, susceptible males on Cry3Bb1 maize = 89, resistant females on Cry3Bb1 maize = 115, susceptible females on non-Bt maize = 118. Dry mass was determined by placing adults in a drying oven (60°C) for 48 h and then weighing them to the nearest 0.01 mg on an analytical balance (XS205 DualRange Analytical Balance, Mettler Toledo, Columbus, Ohio). Sex was determined following the procedures of Hammack and French (2007).

**Assessing fitness costs impose by commercial hybrids.** This experiment was conducted from February 2011 to August 2011, using F18 of the resistant and susceptible western corn rootworm strains. The experiment was a fully crossed design with two western corn rootworm strains (resistant and susceptible) and three maize hybrids: Blue River hybrid 36R19cn (Blue River Hybrid, Kelley, Iowa), Mycogen hybrid 2T783 (Mycogen Seeds, Indianapolis, Indiana), and DeKalb hybrid DKC 61-72. None of the maize hybrids produced Bt toxins targeting rootworm, and any seed treatments were removed following Gassmann et al. (2011).

Maize was grown in a greenhouse (29°C; 16:8, L:D) with supplemental lighting (400 W high-pressure sodium bulbs, Ruud Lighting Inc., Racine, Wisconsin). Each plant was held in a 2 L plastic pot (product number 61086, Encore Plastics Corporation, Pittsburg, Pennsylvania) with all drainage holes covered by fine mesh to prevent escape of larvae. Pots were filled with 2 L of potting medium (20% field soil by volume, 40% SB 300, and 40% LC1; Sun Gro Horticulture Canada Ltd., Vancouver, British Columbia). Beginning 2 wk after planting, plants were fertilized weekly with 250 mL of Peters Excel 15-5-15 Cal-Mag Special (Everris International, Geldermalsen, Netherlands) at 4 mg/mL. Three wk after planting, 25 neonate larvae were placed at the base of each plant. During the experiment, plants were watered and pots were kept free from weeds. Sixteen pots were used for each combination of insect strain by maize hybrid, for a total of 96 pots for the experiment.

One wk after larvae were placed in pots, the surface of the soil was covered by securing one end of a cone of fine mesh fabric around the top of each flower pot and the other end of the cone around the base of each plant. This was done to

capture all adult western corn rootworm that emerged from the soil. Pots were checked every other day for adults beginning 2 wk after larvae were added and this continued until no adults were collected for 2 consecutive wk. Adults were collected using a hand-held aspirator, anesthetized with carbon dioxide, and sex was determined following Hammack and French (2007). The total number of adults per pot was recorded.

Adults collected on the same day, and from the same combination of insect strain and maize hybrid, were placed in a small screen cage (18 cm x 18 cm x 18 cm L x H x W; Megaview Science, Taichung, Taiwan) and held in a growth chamber (25°C; 16:8, L:D, 65% RH) for 6 d, after which mated pairs (i.e., one male and one female) were established. Mated pairs of western corn rootworm adults were held in a 0.5 L clear plastic container (product number RD 16, Johnson Paper and Supply Co. Minneapolis, Minnesota) and covered with a lid that provided ventilation through an opening (diameter = 7 cm) covered with fine mesh fabric. A 1.5% agar solid was provided as a water source, and artificial diet (western corn rootworm diet, Bio-Serv, Frenchtown, New Jersey) and maize leaf were provided as food, and these were replaced every 2 d. Each mated pair was given an oviposition substrate of sieved soil (particle size < 180  $\mu\text{m}$ ) held in a Petri dish (diameter = 4 cm) that was replaced every 7 d. For each combination of insect strain by maize hybrid, 30 mated pairs were established.

For each oviposition dish, soil was washed away using a 250  $\mu\text{m}$  sieve and eggs were counted using a microscope (MZ6, Leica Microsystems, Wetzlar, Germany). Egg viability for each mated pair was measured from a random sample of

25 eggs collected 2 to 3 wk after a pair was established. To measure viability, eggs were placed on 1.5% agar medium in Petri dish (diameter = 5 cm), held in a growth chamber (25°C; 16:8, L:D, 65% RH) and larvae counted every 2 to 3 d using a microscope until no larvae were collected for 2 consecutive wk.

For all adults in the mated pairs, adult longevity was measured. For a random sample of 16 adult males and females in each combination of insect strain by maize hybrid, head capsule width was measured from eye to eye to the nearest 0.1  $\mu\text{m}$  using a microscope and a digital camera (Moticam 2500, Meyer Instruments, Houston, Texas) with image analysis software (Motic Images Plus 2.0 ML, Motic Images Inc., British Columbia, Canada).

**Assessing fitness costs imposed by maize inbreds.** This experiment was conducted from June 2011 to November 2011. Three inbred maize lines were studied: CRW8-1, NGSDCRW-1 and PHZ51 (Nelson et al. 2008, Russell et al. 1976, Kahler et al. 1985). CRW8-1 and NGSDCRW-1 have tolerance to feeding by western corn rootworm and were used here to test effects on fitness costs of Cry3Bb1 resistance in western corn rootworm (Russell et al. 1976, Kahler et al. 1985). Resistant and susceptible strains of western corn rootworm used in this experiment were in the F20. The experiment was a fully crossed design with three genotypes of western corn rootworm (resistant, susceptible, and heterozygotes) and the three inbred maize lines (CRW8-1, NGSDCRW-1 and PHZ51). For each combination of insect genotype by maize inbred, 14 pots were established, for a total of 126 pots for the experiment.

The three insect genotypes studied here were from the same population cages used in Petzold-Maxwell et al. (2012). Newly emerged adults from the resistant and susceptible strains were collected from seedling mats every 3 to 4 h to ensure insects were virgins, because females begin to mate 12 h after emerging from the soil (Ball 1957, Guss 1976, Hill 1975). Sex of adults was determined following Hammack and French (2007) and adults were placed in population cages using a 1:1 ratio of males and females. The following four population cages were established: resistant genotype (resistant ♂ by resistant ♀), susceptible genotype (susceptible ♂ by susceptible ♀), and one cage for each of the reciprocal heterozygous crosses (resistant ♂ by susceptible ♀ and resistant ♀ by susceptible ♂). Adults were added to each cage over a period of 10 wk with the goal of maintaining a population of ca.1000 adults.

Maize inbreds were grown in the greenhouse using the same methods applied in the experiment with refuge hybrids. Twenty-four neonate larvae were placed at the base of each plant with only one insect genotype placed into each pot. For pots receiving heterozygotes, 12 larvae from each of the two heterozygous crosses were placed into a pot, for a total of 24 larvae. Adults were collected following methods described previously and placed in cages by genotype (resistant, susceptible and heterozygote) and maize inbred.

Due to a low number of adults that emerged from the soil, insects collected from each combination of insect genotype by maize inbred were pooled by week, and held in cages for 4 to 8 d before establishing mated pairs. The number of mated pairs for each combination of insect genotype by maize inbred was: CRW8-1,

susceptible = 7, resistant = 5, and heterozygotes = 8; NGSDCRW-1, susceptible = 8, resistant = 1, and heterozygotes = 6; PHZ51, susceptible = 8, resistant = 7, and heterozygotes = 6. Mated pairs were established in the same fashion as the refuge hybrid experiment, measuring the same variables with the exception of egg viability, which failed because of fungal contamination of the eggs. Adult head capsule width was measured for  $15.1 \pm 1.5$  (mean  $\pm$  S. D.) randomly sampled individuals from each combination of sex by insect genotype by maize inbred.

**Data Analysis.** All data were analyzed with a mixed-model analysis of variance (ANOVA) using PROC MIXED (SAS Enterprise Guide 4.2, SAS Institute Inc., Cary, North Carolina). When significant main effects or interactions were present, pairwise comparison were made using the PDIFF statement based on LSMEANS. Significance levels in pairwise comparisons were adjusted with a Bonferroni correction (Sokal and Rohlf 2003). Random factors were tested for significance using the log-likelihood ratio statistic (-2 RES log-likelihood) based on a one-tailed  $\chi^2$  test with one degree of freedom (Littell et al. 1996). To increase statistical power, random factors were removed from the model when not significant at  $P < 0.25$  following Quinn and Keough (2002). If higher order interactions were significant, then lower order terms were retained. To ensure normality of residuals, data were transformed as needed using log and square root functions.

For the experiment quantifying resistance to Cry3Bb1 maize, data for survival to adulthood and adult dry mass were analyzed with fixed factors for sex, strain, hybrid, and all possible interactions. Random factors in the model were 1) seedling mat nested within strain by hybrid and 2) sex crossed by seedling mat nested within



strain by hybrid. For survival to adulthood and dry mass, all possible pairwise comparisons were made among means for insect strain by maize hybrid, with a significance level of  $P < 0.008$  based on Bonferroni correction for six pairwise comparisons. Corrected survival on Bt maize for the resistant and susceptible strains was calculated following Abbott (1925). The magnitude of resistance for the resistant strain was then calculated by dividing mean corrected survival to adulthood for the resistant strain on Bt maize by the mean corrected survival to adulthood of the susceptible strain on Bt maize.

For the experiment assessing fitness costs on refuge hybrids, data on developmental rate and survival to adulthood were analyzed with the fixed factors of sex, strain, hybrid, and all possible interactions. Random factors were 1) pot nested within strain by hybrid and 2) sex crossed with pot nested within strain by hybrid. For head capsule width and longevity of adults, fixed factors were sex, strain, hybrid, and all possible interactions. Random factors were 1) mated pair nested within strain by hybrid and 2) sex crossed with mated pair nested within strain by hybrid. In the analysis of egg viability, fixed effects were strain, hybrid, and their interaction, and the random factor was mated pair nested within strain by hybrid. For fecundity, a repeated measures ANOVA was conducted based on a split-plot design, and included the fixed effects of hybrid, strain, week, and all interactions. Random factors were 1) mated pair by hybrid by strain and 2) week nested within hybrid by strain by mated pair. Fecundity of resistant and susceptible strains was compared within each of the 15 one week intervals, with a significance level set at  $P < 0.003$  based on 15 pairwise comparisons. In cases where life-history traits differed among

the three maize hybrids, all possible pairwise comparisons were made among hybrids with a significance level of  $P < 0.017$  based on three pairwise comparisons.

For the experiment assessing fitness costs on maize inbreds, larval developmental rate, survival to adulthood, and size were analyzed in the same manner as the experiment with refuge hybrids. However, data on fecundity and adult longevity from the three inbred maize genotypes were pooled due to low survival to adulthood. Thus, analysis of fecundity included the fixed factor of genotype and the random factor of mated pair nested within genotype. Fixed factors for longevity were genotype, sex, and their interaction and the random factors were 1) mated pair nested within genotype and 2) sex crossed with mated pair nested within genotype. Significant differences among genotypes were present for survival to adulthood and adult size (see Results), and linear contrasts were used to test for non-recessive costs (affecting both heterozygotes and resistant individuals) and recessive costs (present for resistant individuals only) following Gassmann et al. (2008).

When a statistically significant difference was detected between genotypes in either the experiment with maize hybrids or the experiment with maize inbreds, the magnitude of the fitness cost was calculated following Gassmann et al (2009) as  $[(F_s - F_r)/F_s] \times 100\%$ , except for developmental rate, which was calculated as  $[(F_r - F_s)/F_s] \times 100\%$ . In these calculations,  $F_s$  was the mean value of the fitness component for the susceptible genotype and  $F_r$  was the mean value for the fitness component of either the resistant or heterozygous genotype (Gassmann et al. 2009). The overall mean for statistically significant fitness costs detected among life-history

traits in both experiments was tested against zero with a t test (Sokal and Rohlf 2003).

## Results

**Quantifying resistance to Cry3Bb1 maize.** There was a significant interaction between strain and hybrid for survival to adulthood (Table 1; Fig. 1a). Survival of resistant and susceptible strains did not differ on non-Bt maize, but was significantly higher for resistant insects compared to susceptible insects on Cry3Bb1 maize (Fig. 1a). Additionally, resistant insects had lower survival on Cry3Bb1 maize than non-Bt maize (Fig. 1a). The interaction of hybrid by sex also was significant (Table 1). Although survival was significantly greater for males than females on Bt maize ( $t = 2.07$ ;  $df = 88$ ;  $P = 0.04$ ) and non-Bt ( $t = 5.18$ ;  $df = 88$ ;  $P < 0.0001$ ), this difference was more pronounced on non-Bt maize (Fig. 1a). Corrected survival of the resistant and susceptible strains on Cry3Bb1 maize was 0.66 and 0.31, respectively, which equates to 2.13 ( $0.66 \div 0.31$ ) times higher survival for the resistant strain on Cry3Bb1 maize. Both resistant and susceptible strains had significantly lower dry mass on Bt maize than non-Bt maize, and mass of resistant and susceptible strains did not differ from each other on either type of maize (Table 1; Fig. 1b). Females had significantly greater dry mass than males (Table 1; Fig. 1b).

**Assessing fitness costs imposed by commercial hybrids.** The resistant strain took significantly longer to reach adulthood ( $32.02 \pm 0.14$  d) (mean  $\pm$  S. E.) than the susceptible strain ( $31.15 \pm 0.14$  d), which represents a 2.8% reduction in fitness (Table 2; Fig. 2a). For adult longevity, the resistant strain ( $49.69 \pm 2.53$  d)

lived significantly longer than the susceptible strain ( $41.37 \pm 2.32$  d), which represents a 16.7% fitness benefit for the resistant strain (Table 2; Fig. 2b). Additionally, females took significantly longer to reach adulthood than males (Fig. 2a), lived significantly longer than males (Fig. 2b), displayed higher survival than males (Fig. 2d), and females (head capsule width =  $1225.0 \pm 8.4$   $\mu\text{m}$ ) were significantly larger than males (head capsule width =  $1173.8 \pm 8.1$   $\mu\text{m}$ ) (Table 2).

The resistant strain had a significantly lower proportion of viable eggs ( $0.83 \pm 0.03$ ) compared with the susceptible strain ( $0.92 \pm 0.01$ ), which represents a 10% fitness cost (Table 2; Fig. 2c). Resistant females produced significantly more eggs ( $538.0 \pm 38.99$ ) than susceptible females ( $412.06 \pm 43.20$ ), which represents a fitness benefit of 30.6% for the resistant strain (Table 3; Fig. 3). Additionally, there was a significant strain by week interaction with the resistant strain producing significantly more eggs than the susceptible strain during weeks two and three (Fig. 3). There were no other significant effects of insect strain.

Western corn rootworm larvae developed more quickly on hybrid DKC 61-72 ( $30.99 \pm 0.12$  d) than hybrid 2T783 ( $32.27 \pm 0.21$  d), but no difference was present between either hybrid and 36R19cn ( $31.93 \pm 0.19$  d) (Table 2; Fig. 2a). Maize hybrid also significantly affected adult head capsule width, because insects reared on hybrid DKC 61-72 were significantly larger ( $1234.9 \pm 9.2$   $\mu\text{m}$ ) than those reared on hybrid 2T783 ( $1169.8 \pm 9.4$   $\mu\text{m}$ ;  $P < 0.0001$ ) and on hybrid 36R19cn ( $1193.5 \pm 11.6$   $\mu\text{m}$ ;  $P = 0.0091$ ) (Table 2). Significantly more insects per flower pot survived on hybrid DKC 61-72 ( $8.92 \pm 0.42$ ) than on hybrids 2T783 ( $6.61 \pm 0.39$ ) and 36R19cn ( $7.16 \pm 0.44$ ) (Table 2; Fig. 2d). Thus, insects had the highest fitness on

hybrid DKC 61-72 because they were larger, developed more quickly and had higher survival.

**Assessing fitness costs imposed by maize inbreds.** Resistant adults ( $1109.4 \pm 8.5 \mu\text{m}$ ) were significantly smaller than susceptible adults ( $1158.9 \pm 7.3 \mu\text{m}$ ;  $P < 0.0001$ ), which represents a 4.3% fitness cost (Table 4). Heterozygotes ( $1133.2 \pm 8.0 \mu\text{m}$ ) were significantly smaller than susceptible insects ( $P = 0.02$ ), which represents a 2.2% reduction in fitness, and a non-recessive fitness cost. Because heterozygotes were significantly larger than resistant insects ( $P = 0.03$ ) but smaller than susceptible insects, the fitness cost affecting size was additive.

There was a significant genotype by sex interaction for survival (Table 4; Fig. 4). No significant differences were present between susceptible females versus heterozygous females ( $P = 0.59$ ) or between susceptible males versus heterozygous males ( $P = 0.93$ ). However, compared to heterozygotes and susceptible females, resistant females had significantly lower survival ( $P = 0.02$ ), amounting to 42.9% recessive fitness cost (Fig. 4). By contrast, resistant males had significantly higher survival ( $P = 0.04$ ) than heterozygous and susceptible males, which was a 49.9% fitness benefit for resistant insects. There were no other significant effects involving either insect genotype or maize inbred. Females ( $28.44 \pm 0.41$  d) took significantly longer to reach adulthood than males ( $24.48 \pm 0.20$  d), and females ( $51.11 \pm 3.55$  d) lived significantly longer than males ( $34.45 \pm 2.85$  d) (Table 4).

Excluding fitness benefits of fecundity, survival of males to adulthood, and adult longevity, the mean recessive fitness cost experienced by western corn rootworm was  $15.0 \pm 9.4\%$  ( $(2.8 + 10 + 4.3 + 42.9) \div 4$ ). When the cases of

increased fitness for the resistant strain were included, there was a fitness benefit of  $5.3 \pm 11.4\%$  for resistant insects  $((2.8 + 10 + 4.3 + 42.9 - 30.6 - 16.7 - 49.9) \div 7)$ . The mean fitness cost did not differ significantly from zero when fitness benefits were excluded ( $t = 1.59$ ;  $df = 3$ ;  $P = 0.21$ ) or were included ( $t = 0.47$ ;  $df = 6$ ;  $P = 0.66$ ), indicating that no overall difference in fitness was present between the resistant and susceptible strains.

### Discussion

Field-evolved resistance by western corn rootworm to Cry3Bb1 maize has been documented in some fields and wide-spread resistance evolution could further diminish the effectiveness of this technology (Gassmann et al. 2011, Gassmann et al. 2012, Gassmann 2012). The presence of fitness costs associated with Cry3Bb1 resistance in western corn rootworm may help to delay the evolution of resistance (Gassmann et al. 2009). In this study, fitness costs of Bt resistance were observed for egg viability, female survival, developmental rate and adult size. However, higher fitness for the resistant strain (i.e., a fitness benefit) was found for male survival, fecundity and adult longevity. Although significant effects of maize hybrids on the fitness of western corn rootworm were present, neither maize hybrids nor maize inbreds significantly affected the magnitude or dominance of fitness costs, suggesting that the choice of refuge plants will not have a strong effect on fitness costs of Cry3Bb1 resistance in western corn rootworm.

Considering only traits for which resistant insects suffered lower fitness, the mean recessive fitness cost was 15.0%. However, when cases where resistant individuals had higher fitness were taken into account, a net increase in fitness (i.e.

a fitness benefit) of 5.3% was observed for the resistant strain. In either case, the fitness cost of Cry3Bb1 resistance observed here was less than the mean 23% fitness cost found among 77 studies reviewed in Gassmann et al. (2009). This suggests that the two times higher survival on Cry3Bb1 maize for the resistant strain studied here does not impose much, if any, fitness cost, and consequently, resistance may evolve quickly and may be able to persist within populations.

In the fitness cost experiment with inbreds, there was an additive fitness cost affecting adult size. The dominance of fitness costs is important because dominant costs will impose greater delays on resistance than recessive costs (Carrière and Tabashnik 2001). However, because overall fitness was greater for the resistant strain compared to the susceptible strain, it is difficult to predict whether a 2.2% fitness cost affecting size of heterozygotes would counter balance the selective benefit of a 5.3% increase in fitness, on non-Bt maize, for the resistant strain. Even with this additive fitness cost, it appears that the overall fitness cost experienced by Cry3Bb1-resistant western corn rootworm was very slight, if present at all.

Host-plant variety and secondary metabolites have the ability to increase fitness costs of Bt resistance for insect pests. In some cases, fitness costs have been shown to increase as suitability of a host plant decreases (Janmaat and Meyers 2005, Raymond et al. 2005, Raymond et al. 2007). Furthermore, secondary metabolites, such as gossypol in cotton, have been shown to increase both the dominance and magnitude of fitness costs of Bt resistance (Carrière et al. 2004, Williams et al. 2011). In other cases, fitness costs were found to vary among equally suitable hosts (Bird and Akhurst 2007). The host plants studied here did not

significantly affect fitness costs, even though differences in fitness of western corn rootworm were present among maize hybrids (Table 2). These results suggest the fitness costs of Cry3Bb1 resistance in western corn rootworm will not be affected by the maize variety planted in the refuge.

In addition to affecting fitness costs of Bt resistance, host-plants can affect the overall fitness of insects (Awmack and Leather 2002). This was seen here in western corn rootworm larvae fed hybrid 39R19cn and hybrid 2T783 versus hybrid DKC 61-72, with insects displaying higher fitness on hybrid DKC 61-72 (Fig. 2a, 2c; Table 2). The mechanism responsible for these differences in fitness is not known. Differences in survival for western corn rootworm among maize lines have been demonstrated and some maize lines possess antixenosis against western corn rootworm (Khishen et al. 2009, Bernklau et al 2010). Younger roots are softer, less lignified, and more suitable for larvae of western corn rootworm (Strnad and Bergman 1987), thus differences in production of new roots among hybrids may have affected fitness of western corn rootworm. Additionally, nutritional factors may contribute to differences in fitness (Mattson 1980). Planting maize hybrids that reduce survival or reproduction may be beneficial for managing western corn rootworm. However, reduced fitness experienced by western corn rootworm on refuge hybrids also may decrease the number of insects produced in refuges, thereby increasing rates at which western corn rootworm develops resistance to Bt maize (Hannon et al. 2010).

Other studies have found an absence of fitness costs of resistance to Bt maize in western corn rootworm. Oswald et al. (2012) evaluated survivorship to



adulthood, fecundity, and egg viability for five Cry3Bb1-resistant strains of western corn rootworm (including the resistant strain here) and found no evidence of fitness costs, however; fitness benefits of resistance were observed in faster developmental rate and higher fecundity. Petzold-Maxwell et al. (2012) determined that the presence of entomopathogenic nematodes did not impose fitness costs for the same Cry3Bb1-resistant strain evaluated in this study. By contrast, Meihls et al. (2012) evaluated three Cry3Bb1-resistant strains of western corn rootworm and tested for fitness costs affecting mass of larvae and adults, survival to adulthood, developmental rate, adult size, adult lifespan, fecundity, and egg viability. Fitness costs were detected in all resistant strains for fecundity and adult male lifespan (Meihls et al. 2012). Taken together, these studies and the data presented here, suggest that fitness costs do not always accompany resistance to Cry3Bb1 maize in western corn rootworm.

We observed more than two times higher survival on Cry3Bb1 maize for the resistance strain compared to the susceptible strain. Survival on Cry3Bb1 maize for western corn rootworm with field-evolved resistance was three times greater than Bt-susceptible insects in Gassmann et al. (2011) and 11 times greater in Gassmann et al. (2012). The ratio of larvae recovered on Bt maize to non-Bt maize for a laboratory-selected strain of Cry3Bb1-resistant western corn rootworm studied by Meihls et al. (2008) was 11.7 times greater. High levels of resistance are more likely to be costly than lower levels of resistance (Gassmann et al. 2009), and this may be one reason larger fitness costs were not found in this study. Strains with field-evolved resistance to Cry3Bb1 maize, which appear to be more resistant than the

strain studied here (Gassmann et al. 2011, 2012), may have greater fitness costs associated with Bt resistance than those observed in this study. Additional research will be required to answer this question.

Western corn rootworm have become resistant to conventional insecticides such as aldrin and methyl-parathion (Siegfried and Mullin 1989) and have retained this resistance for at least 20 years since these products were retired from use (Parimi et al. 2006). The S2 population in Gassmann et al. (2012) was found to be resistant to Cry3Bb1 maize even though the field from which it was collected had not been planted to Cry3Bb1 maize for 2 years, suggesting that once Bt resistance evolves, it may persist. In this experiment, the choice of maize variety did not affect fitness costs and in general fitness costs of Bt resistance were minimal. This lack of fitness costs emphasizes the importance of delaying Bt resistance in western corn rootworm through the use of integrated pest management and resistance management.

### **Acknowledgements**

Sean Bradley and Brandon Sorgatz assisted with experiments. Funding was provided by the United States Department of Agriculture through a Biotechnology Risk Assessment Grant 2009-33120-20256.

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## Figures

Fig. 1A

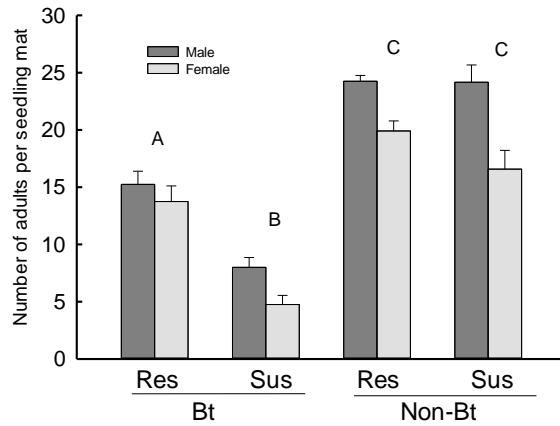
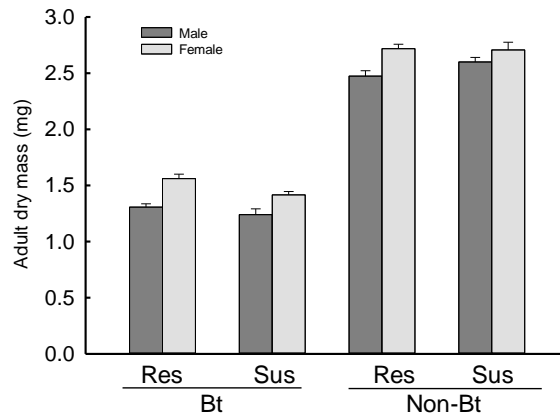


Fig. 1B



**Figure 1.** A) survival to adulthood and B) dry mass for resistant (Res) and susceptible (Sus) western corn rootworm adults reared on hybrid maize producing Cry3Bb1 and on its non-Bt near isogenic hybrid. Bars represent sample means and error bars are the standard error of the mean. Letters indicate significant differences among means for the four combinations of strain by hybrid. For A) survival to adulthood 50 neonate larvae were placed in each seedling mat.

Fig. 2A

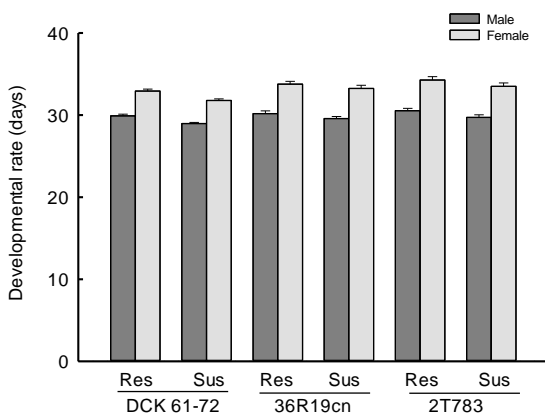


Fig. 2B

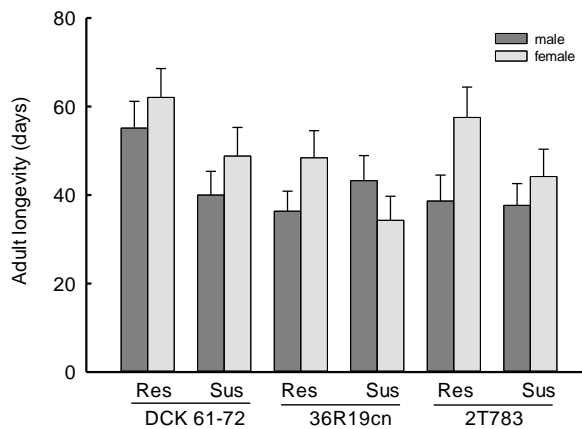


Fig. 2C

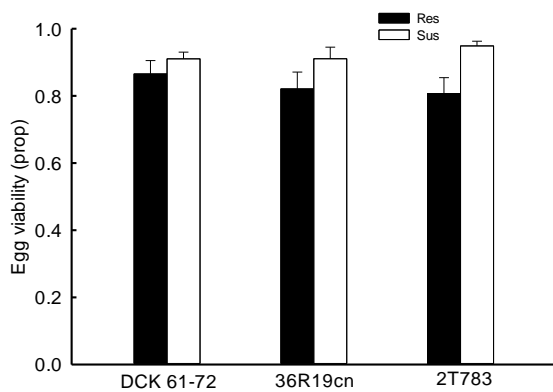
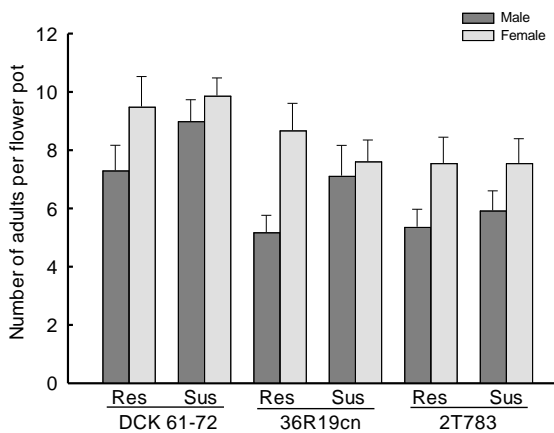


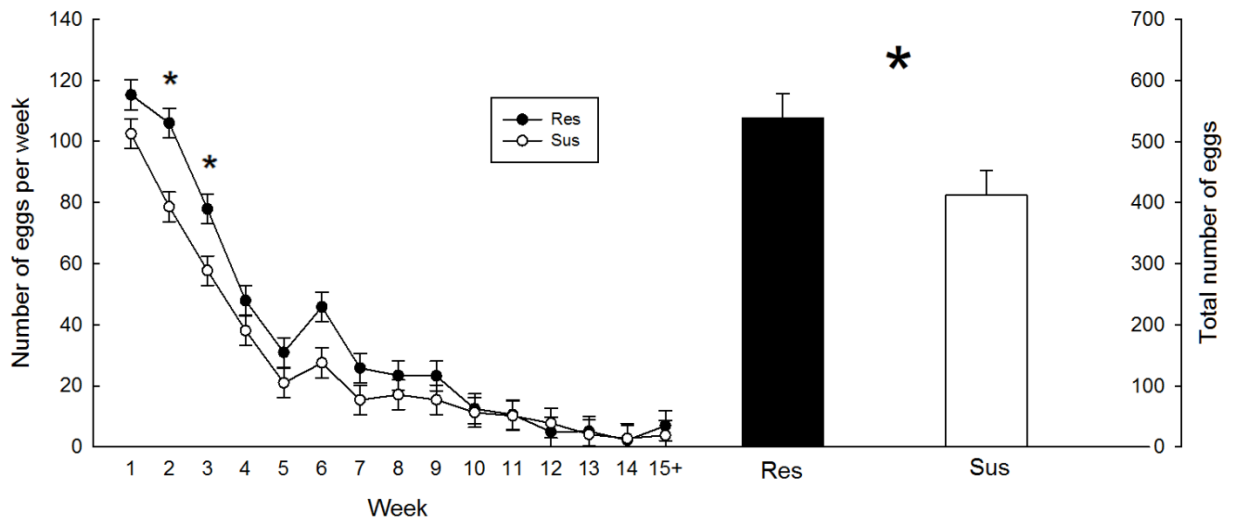
Fig. 2D



**Figure 2.** A) developmental rate, B) longevity, C) egg viability and D) survival to adulthood for resistant (Res) and susceptible (Sus) western corn rootworm reared on maize hybrids lacking a rootworm active Bt toxin (DCK 61-72, 2T783, and 36R19cn). Bars are sample means and error bars are the standard error of the mean. For D) survival to adulthood 25 neonate larvae were placed in each pot.

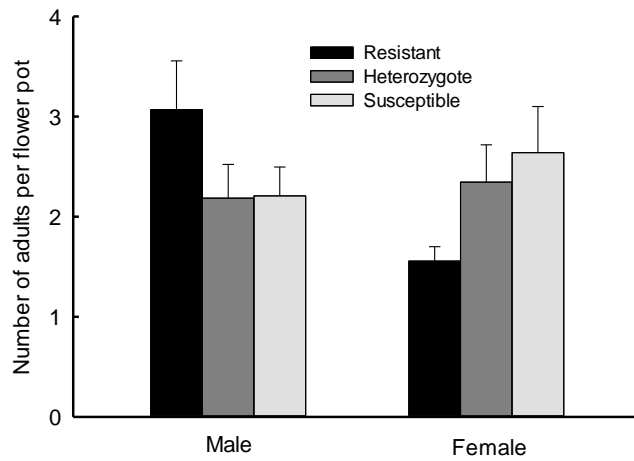


Fig. 3



**Figure 3.** Fecundity of Bt-resistant (Res) and Bt-susceptible (Sus) western corn rootworm reared on maize hybrids lacking rootworm active Bt toxin. Circles represent mean number of eggs produced per female per week. Mean lifetime fecundity of resistant and susceptible western corn rootworm is indicated by the bar height. Error bars are the standard error of the mean. An asterisk indicates a significant difference between strains.

Fig. 4



**Figure 4.** Survival to adulthood for Bt-resistant, Bt-susceptible and heterozygous western corn rootworm reared on non-Bt maize inbreds. Twenty-four neonate larvae were placed in each flower pot. Bars are sample means and error bars are the standard error of the mean.

## Tables

**Table 1:** Mixed model analysis of variance for survival and adult mass of resistant and susceptible strains reared on Cry3Bb1 and non-Bt maize.

	Effect	df <sup>a</sup>	F value	P value
Survival <sup>b</sup>	Strain	1,88	36.61	<.0001
	Hybrid	1,88	176.38	<.0001
	Sex	1,88	26.29	<.0001
	Hybrid*Strain	1,88	15.59	0.0002
	Hybrid*Sex	1,88	4.86	0.03
	Strain*Sex	1,88	2.37	0.13
	Hybrid*Strain*Sex	1,88	0.21	0.65
	Dry Mass <sup>c</sup>	Strain	1,36	0.26
Hybrid		1,36	1515.46	<.0001
Sex		1,36	36.14	<.0001
Hybrid*Strain		1,36	2.83	0.10
Hybrid*Sex		1,36	0.40	0.53
Strain*Sex		1,36	2.98	0.09
Hybrid*Strain*Sex		1,36	0.47	0.50

<sup>a</sup> numerator, denominator degrees of freedom

<sup>b</sup> All random factors were pooled in the model.

<sup>c</sup> All random factors remained in the model.

**Table 2:** Mixed model analysis of variance for life history-traits examined in the experiment assessing fitness costs of Bt resistance on commercially available refuge hybrids.

	Effect	df <sup>a</sup>	F value	P value	
Developmental rate <sup>b</sup>	Strain	1,90	6.42	0.01	
	Hybrid	2,90	4.42	0.01	
	Sex	1,90	232.14	<.0001	
	Hybrid*Strain	2,90	0.09	0.91	
	Hybrid*Sex	2,90	1.35	0.26	
	Strain*Sex	1,90	0.01	0.94	
	Hybrid*Strain*Sex	2,90	0.07	0.93	
	Survival <sup>c</sup>	Strain	1,180	1.58	0.21
Hybrid		2,180	9.04	0.0002	
Sex		1,180	15.25	0.0001	
Hybrid*Strain		2,180	0.24	0.78	
Hybrid*Sex		2,180	0.1	0.91	
Strain*Sex		1,180	3.06	0.08	
Hybrid*Strain*Sex		2,180	0.6	0.55	
Adult head capsule width <sup>c</sup>		Strain	1,180	0.56	0.45
	Hybrid	2,180	11.46	<.0001	
	Sex	1,180	20.77	<.0001	
	Hybrid*Strain	2,180	0.51	0.60	
	Hybrid*sex	2,180	0.28	0.76	
	Strain*Sex	1,180	0.05	0.82	
	Hybrid*Strain*Sex	2,180	0.80	0.45	
	Longevity <sup>d</sup>	Strain	1,174	5.45	0.02
Hybrid		2,174	2.29	0.10	
Sex		1,174	4.44	0.04	
Hybrid*Strain		2,174	0.32	0.73	
Hybrid*Sex		2,174	1.12	0.33	
Strain*Sex		1,174	3.38	0.08	
Egg viability <sup>c</sup>		Strain	1,119	8.37	0.004
		Hybrid	2,119	0.16	0.86
	Strain*Hybrid	2,119	0.88	0.42	
	Hybrid*Strain*Sex	2,174	1.11	0.33	

<sup>a</sup> numerator, denominator degrees of freedom

<sup>b</sup> All random factors remained in the model.

<sup>c</sup> All random factors were pooled in the model.

<sup>d</sup> Random factor included in the model was mated pair nested within strain by hybrid.

**Table 3:** Repeated measures analysis of variance for fecundity of females reared on commercially available refuge hybrids.

Effect	df <sup>a</sup>	F value	P value
Strain	1,174	4.67	0.03
Hybrid	2,174	2.50	0.08
Hybrid*Strain	2,174	0.44	0.64
Week	14,2436	127.68	<.0001
Hybrid*Week	28,2436	1.11	0.31
Strain*Week	14,2436	2.16	0.007
Hybrid*Strain*Week	28,2436	1.41	0.08

<sup>a</sup> numerator, denominator degrees of freedom

**Table 4:** Mixed model analysis of variance for life history-traits examined in the experiment assessing fitness costs of resistance for insects reared on inbred maize lines.

	Effect	df <sup>a</sup>	F value	P value
Developmental rate <sup>b</sup>	Genotype	2,49	0.64	0.53
	Inbred	2,49	2.34	0.11
	Sex	1,49	62.04	<.0001
	Inbred* Genotype	4,49	2.2	0.08
	Inbred*Sex	2,49	2.54	0.09
	Genotype *Sex	2,49	2.05	0.14
	Inbred* Genotype *Sex	4,49	0.37	0.83
	Survival <sup>c</sup>	Inbred	2,49	2.85
	Genotype	2,49	0.11	0.90
	Sex	1,49	1.21	0.28
	Inbred* Genotype	4,49	0.35	0.84
	Inbred*Sex	2,49	0.64	0.53
	Genotype *Sex	2,49	6.32	0.004
	Strain* Genotype *Sex	4,49	1.8	0.14
Adult head capsule width <sup>d</sup>	Genotype	2,254	10.17	<.0001
	Inbred	2,254	2.50	0.08
	Sex	1,254	0.87	0.35
	Inbred* Genotype	4,254	0.81	0.52
	Inbred*Sex	2,254	0.04	0.96
	Genotype *Sex	2,254	1.33	0.26
	Inbred* Genotype *Sex	4,254	1.30	0.27
	Longevity <sup>d</sup>	Genotype	2,254	2.21
Sex		1,106	10.25	0.002
Genotype *Sex		2,106	0.45	0.64
Fecundity <sup>d</sup>	Genotype	2,53	1.06	0.35

<sup>a</sup> numerator, denominator degrees of freedom

<sup>b</sup> All random factors remained in the model.

<sup>c</sup> Random factor included in the model was pot nested within inbred by strain.

<sup>d</sup> All random factors were pooled in the model.

**CHAPTER 3. EFFECTS OF ENTOMOPATHOGENS ON MORTALITY OF WESTERN  
CORN ROOTWORM AND FITNESS COSTS OF RESISTANCE TO CRY3BB1 MAIZE**

A paper submitted to *the Journal of Economic Entomology*

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**Abstract**

Fitness costs can delay pest resistance to crops that produce insecticidal toxins derived from the bacterium *Bacillus thuringiensis* (Bt) and past research has found that entomopathogens impose fitness costs of Bt resistance. Additionally, entomopathogens can be used for integrated pest management by providing biological control of pests. The western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), is a major pest of maize and is currently managed by planting of Bt maize. We tested whether entomopathogenic nematodes and fungi increased mortality of western corn rootworm and whether these entomopathogens increased fitness costs of resistance to Cry3Bb1 maize. We exposed western corn rootworm larvae to two species of nematodes,

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*Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae) and *Steinernema feltiae* Filipjev (Rhabditida: Steinernematidae), and to two species of fungi, *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae) (strain GHA) and *Metarhizium brunneum* (Metschnikoff) Sorokin (Hypocreales: Clavicipitaceae) (strain F52) in two assay types: seedling mat and small cup. Larval mortality increased with the concentration of *H. bacteriophora* and *S. feltiae* in the small cup assay, and mortality from entomopathogens was significantly greater than zero for all entomopathogens in both assays, with the exception of *S. feltiae* and *B. bassiana* in the seedling mat assay. However, no fitness costs were observed in either assay type for any entomopathogen. The increased mortality of western corn rootworm larvae caused by these entomopathogens supports their potential use in biological control; however, the lack of fitness costs suggests that entomopathogens will not delay the evolution of Bt resistance in western corn rootworm.

**Key words:** biological control, *Diabrotica virgifera virgifera*, fungi, nematodes, refuge strategy

### Introduction

Western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), is one of the most economically significant pests of maize in the United States (Gray et al. 2009). This pest has repeatedly developed resistance to management strategies including insecticides, crop rotation, and recently to the insecticidal toxin Cry3Bb1, which is derived from the bacterium *Bacillus thuringiensis* (Bt) and is produced by transgenic maize (Levine and Oloumi-Sadeghi 1991, Meinke et al. 1998, Gassmann et al. 2011). In 2003 the United States Environmental



Protection Agency registered genetically modified maize (*Zea mays* L.) that produces Cry3Bb1 for the management of western corn rootworm (EPA 2010). In 2012, 67% of maize planted in the United States produced at least one Bt toxin and this widespread planting places intense selection on pest populations to develop resistance (Economic Research Service 2012).

In the United States and elsewhere, the refuge strategy is used to delay the development of resistance to Bt crops (Gould 1998). The refuge strategy uses non-Bt host plants to allow the survival of Bt-susceptible insects to mate with Bt-resistant insects. To the extent that heterozygous progeny have lower fitness on a Bt crop than their homozygous resistant parents, resistance is expected to be delayed, with delays becoming greater as the genetic dominance of resistance decreases (Tabashnik et al. 2004). Additionally, as the amount of refuge increases, delays in resistance become greater (Carrière and Tabashnik 2001). Furthermore, fitness costs will delay the evolution of resistance and these delays become greater as fitness costs become larger (Carrière et al. 2010).

A fitness cost of Bt resistance occurs, in the absence of Bt toxin, when individuals with resistance alleles have lower fitness than Bt-susceptible individuals (Gassmann et al. 2009a). Fitness costs can delay resistance evolution by reducing the frequency of resistance alleles present in the refuge (Carrière and Tabashnik 2001, Gassmann et al. 2009a). Thus, the rate of resistance evolution is affected not only by production of susceptible insects from the refuge, but also by the presence of fitness costs in refuges. Importantly, ecological factors, including

entomopathogens, can magnify fitness costs of Bt resistance (Gassmann et al. 2006, Hannon et al. 2010, Raymond et al. 2007).

Several species of entomopathogenic fungi and nematodes have been shown to infect and kill western corn rootworm larvae and have potential for use in biological control (Toepfer et al. 2009, Pilz et al. 2007, Meyling and Eilenberg 2007). Furthermore, entomopathogenic nematodes and fungi have been found to occur naturally in maize fields (Pilz et al. 2008). Fungal conidia contact the insect cuticle, germinate, and subsequently penetrate the haemocoel where they grow and produce insecticidal compounds, leading to death of the host (Lewis et al. 2006, Shah and Pell 2003). Following death of the host, conidia are subsequently produced on the cuticle and then disperse to infect additional insects (Hajek and St. Leger 1994). Infective juveniles of entomopathogenic nematodes are a motile, free living stage that enter the haemocoel through natural openings and release symbiotic bacteria, which kill the hosts within 24 to 48 h. Nematodes then feed and reproduce inside the cadaver, and a subsequent generation of infective juveniles disperses into the environment (Kaya and Gaugler 1993, Grewal et al. 1994).

This study examined whether entomopathogenic nematodes and fungi cause mortality of larvae of western corn rootworm and whether these entomopathogens can magnify fitness costs of resistance to Cry3Bb1 maize. These results are relevant both to the application of entomopathogens in biological control of western corn rootworm and to insecticide resistance management (IRM) for Bt crops. By testing which ecological factors magnify fitness costs of Bt resistance, it may be possible to design non-Bt refuges that enhance fitness costs, thereby more

effectively delaying Bt resistance (Carrière and Tabashnik 2001, Pittendrigh et al. 2004).

### **Materials and Methods**

**Insect strains.** Field-collected adult males of western corn rootworm from four locations (Hamilton County, Ohio; Moody/Lake County, South Dakota; Phillips County, Colorado; and Will County, Illinois) were crossed with females from a non-diapause strain at the North Central Agricultural Research Laboratory (NCARL) (Oswald et al. 2011). From this cross two strains were developed: 1) a susceptible strain not exposed to Bt toxin and 2) a resistant strain, the moderately selected strain in Oswald et al. (2011), that was fed Cry3Bb1 maize for increasing durations over 11 generations ( $F_0$  to  $F_{10}$ ). Strains were sent from NCARL to Iowa State University at the  $F_{13}$ , where they were reared on maize seedling mats following the methods of Jackson (1986) and Oswald et al. (2011), and maintained at a population size of >1200 adults. The  $F_{13}$ ,  $F_{15}$ , and  $F_{17}$  of the resistant strain were reared on Cry3Bb1 maize (hybrid DKC 61-69, Monsanto Company, Saint Louis, Missouri) and the susceptible strain was reared on non-Bt maize (hybrid 34M94 DuPont Pioneer, Johnston, Iowa). The susceptible strain was maintained concurrently with but independently from the resistant strain. To increase genetic similarity between strains, the resistant strain was backcrossed to the susceptible strain at a 1:1 ratio during the  $F_{13}$  and  $F_{15}$ . During the  $F_{14}$ ,  $F_{16}$ , and  $F_{18}$  through  $F_{24}$ , the resistant and susceptible strains were reared on non-Bt maize (hybrid 34M94). In the  $F_{24}$ , survival to adulthood on seedling mats of Cry3Bb1 maize was more than twice as high for the resistant strain compared to the susceptible strain (Hoffmann 2013).

**Entomopathogens.** Effects of two entomopathogenic nematodes and two entomopathogenic fungi were studied. The entomopathogenic nematodes *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae) and *Steinernema feltiae* Filipjev (Rhabditida: Steinernematidae) were received from Becker-Underwood (Ames, Iowa) and reared in *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae following Kaya and Stock (1997). Infective juvenile nematodes were used in experiments within 2 wk of emerging from *G. mellonella* cadavers. The entomopathogenic fungi *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae) (strain GHA) and *Metarhizium brunneum* (Metschnikoff) Sorokin (Hypocreales: Clavicipitaceae) (strain F52) were received from the USDA-ARS, Northern Plains Agricultural Research Laboratory, and stored at 6°C until they were used in experiments.

**Seedling mat assay.** This experiment was conducted during February and March of 2012 using the F<sub>22</sub> of the resistant and susceptible strains. For each entomopathogen, a separate experiment was conducted using a fully crossed block design with two insect strains (resistant and susceptible) and four concentrations of entomopathogen. For each of the four species of entomopathogen, eight blocks were run. Both the resistant and susceptible strains were tested in each block using one seedling mat for each of four entomopathogen concentrations and three control seedling mats, for a total of 14 seedling mats per block. Control seedling mats were not treated with entomopathogens but were otherwise identical to seedling mats with entomopathogens. Thus, for each of the four entomopathogen species, 112

seedling mats were tested, and for the entire experiment, a total of 448 seedling mats were tested.

Seedling mats were made using 40 mL of maize seed (~ 65 kernels of hybrid 34M94) that was presoaked for 12 h in deionized (DI) water and then placed in a 0.95 L container (Pactiv Showcase<sup>®</sup>, Johnson Paper and Supply Company, Minneapolis, Minnesota) with a lid that contained six holes (diameter = 1 cm) for ventilation. Seeds were covered with a moist paper towel (23504, Georgia Pacific, Atlanta, Georgia), and placed in a growth chamber (25°C; 16:8, L:D; 65% RH) for 3 to 4 d. Seeds were then covered with 150 g (dry mass) of soil that was a mixture of 40%, by volume, of field-collected soil and 60% Sunshine Sun Gro LC1 potting soil mix (Sun Gro Horticulture Canada Ltd., Vancouver, British Columbia). Before being placed in containers, the soil was moistened with either 30 mL of entomopathogen solution, or in the case of control mats, 30 mL of a control solution that lacked entomopathogens.

Concentrations of live infective juvenile nematodes were measured with a microscope (MZ6, Leica Microsystems, Wetzlar, Germany) and a Sedgewick-Rafter counting cell (Pyser-SGI, Edenbridge, Kent, United Kingdom). Nematode solutions were made using DI water and added to soil to achieve four concentrations of nematodes within the soil, 50, 100, 150, and 200 nematodes per g of dry soil. Concentrations were selected based on the results of Petzold-Maxwell et al. (2012a). Each seedling mat with nematodes received 30 mL of nematode solution and each control seedling mat received 30 mL of DI water. Thirty mL of liquid

moistened the soil to 25% water holding capacity. Solutions were incorporated into soil by hand and the moistened soil was then placed on top of the seedling mats.

Solutions of fungal entomopathogens were made by combining conidia with an autoclaved solution of 0.10% sorbitan monooleate (Tween 80<sup>®</sup> Acros Organics, Morris Plains, New Jersey). Concentration of conidia was determined using a hemocytometer (3520, Fisher Scientific, Waltham, Massachusetts) and microscope (Eclipse E200, Nikon, Melville, New York) with viability measured 24 h prior to application, following Goettel and Inglis (1997). Four concentrations of conidia were tested:  $1.0 \times 10^4$ ,  $1.0 \times 10^5$ ,  $1.0 \times 10^6$ , and  $1.0 \times 10^7$  viable conidia per g of dry soil. These concentrations were selected based on the results of Petzold-Maxwell et al. (2012b). Soil for seedling mats received either 30 mL of solution with fungal conidia, or in the case of control mats, 30 mL of 0.10% sorbitan monooleate solution that did not contain fungal conidia. Each solution was mixed into the soil by hand before soil was placed on top of and underneath maize seeds. This modification from the seedling mat assay with nematodes was made to increase contact of the soil with maize roots.

For all assays, seedling mats with soil were returned to a growth chamber and allowed to grow for an additional 3 to 4 d, after which time 25 neonate larvae (< 1 d old) of the appropriate strain of western corn rootworm were placed on the surface of the soil with a fine-hair paintbrush. Fine mesh fabric (25 x 25 cm, 194811 Poly Chiffon, Hobby Lobby Stores, Inc., Oklahoma City, Oklahoma) covered the underside of the plastic lid to prevent larvae from escaping. Containers were placed in a growth chamber (25°C; 16:8, L:D; 65% RH) for 10 d, with seedling mats

receiving 30 mL of DI water 7 d after neonates were added. After 10 d, seedling mat, soil and larvae from each bioassay container were placed individually on Berlese funnels for 3 d to extract live larvae into vials containing 85% ethanol. The total number of larvae extracted per bioassay container was counted.

**Small cup assay.** This experiment was conducted at the same time as the seedling mat assay. Each of the four species of entomopathogens was tested in a separate set of assays. For each species of entomopathogen, a fully crossed block design was used with two insect strains (susceptible and resistant) and four concentrations of entomopathogen. For each species of entomopathogen, eight blocks were run. Within a block, both the resistant and susceptible strains were tested in two containers for each of four concentrations and in four control containers, for a total of 24 containers per block. For each species of entomopathogen, a total of 192 bioassay containers were evaluated, and in the entire experiment, a total of 768 bioassay containers were evaluated.

Bioassays used 44 mL containers with lids (Translucent Plastic Souffle Cup, Solo Cup Company, Highland Park, Illinois). Maize seed was the same as in the seedling mat assay. Seed was soaked for 12 h then placed on moistened paper towels for 3 d, after which time there was ca. 2 cm of a radical root per seed. Three germinated seedlings were placed at the bottom of each bioassay container and covered with soil that contained pathogens, or in the case of control containers, lacked pathogens. Solutions of nematodes and fungi were prepared in the same manner as the seedling mat assay. For each bioassay container, 30 g of sieved field soil (<600 $\mu$ m) were combined with 4.5 mL of either the appropriate

entomopathogen solution or a control solution that lacked pathogens, and then placed on top of the maize seedlings. Adding 4.5 mL of liquid moistened the soil to 25% of water holding capacity. Finally, six neonate larvae from either the resistant or susceptible strain were placed on the surface of the soil in each bioassay container. Small holes in the lid provided ventilation and mesh fabric under the lid prevented larvae from escaping. Containers were placed in a single layer between two plastic trays (CT1216, Carlisle Foodservice Products, Oklahoma City, Oklahoma) that were lined with moistened paper towels. Plastic trays containing cups were then placed inside a large plastic bag (Hefty EasyFlaps® 13 Gallon Tall Kitchen Bags, Reynolds Consumer Products, Lake Forest, Illinois) to retain humidity and provide a dark environment. Trays were placed inside a growth chamber (25°C; 65% RH) for 10 d. Soil in each container received 1 mL of DI water at day 7. On day 10, soil with seedlings and larvae were placed on Berlese funnels for 3 d to extract live larvae into a vial with 85% ethanol. The total number of larvae extracted in each bioassay container was recorded.

**Data Analysis.** Analyses were conducted using SAS Enterprise Guide 4.2 (SAS Enterprise Guide 4.2, SAS Institute Inc., Cary, North Carolina). For each bioassay, data on larval mortality in the experimental controls was compared between resistant and susceptible strains with an analysis of variance (ANOVA) using the MIXED procedure. Larval mortality in the presence of entomopathogens was first adjusted for control mortality using Abbott's correction (Abbott 1925), and then analyzed separately for each combination of entomopathogen species and assay type using a mixed model ANOVA and analysis of covariance (ANCOVA)



based on the MIXED procedure, following Hannon et al. (2010). Additionally, a *t*-test was used to test whether corrected mortality was significantly greater than zero (PROC TTEST).

For corrected mortality in each combination of entomopathogen species and assay type, an ANOVA was used first to test whether a difference in the regression slope of rootworm mortality onto entomopathogen concentration was present between strains. Fixed factors in this ANOVA were the continuous variable of entomopathogen concentration, the categorical variable of insect strain, and their interaction. If the slopes did not differ, data were then analyzed within an ANCOVA that included the categorical variable of insect strain and the continuous covariate of entomopathogen concentration. In both the ANCOVA and ANOVA, block and its interactions with fixed factors were coded as random factors and were tested for significance with a log-likelihood ratio statistic (-2 RES log likelihood in PROC MIXED) based on a one-tailed  $\chi^2$  test with one degree of freedom (Littell et al. 1996). If a random factor was not significant at  $P < 0.25$ , it was removed from the model to increase the statistical power (Quinn and Keough 2002). Lower order terms were retained if their higher order interactions were significant. Data were log transformed to ensure normality of the residuals.

For each entomopathogen, *t*-tests were conducted following Sokal and Rohlf (2003) to test whether 1) average corrected mortality for larvae in the small cup assay differed from average corrected mortality in the seedling mat assay, and 2) whether average corrected mortality differed between species of nematode and fungi in the small cup assay.

## Results

In the seedling mat assay, larval mortality in the experimental controls, which lacked entomopathogens, did not differ significantly between western corn rootworm strains (Table 1). No significant difference in mortality between strains was found for *H. bacteriophora* (Fig. 1a), *S. feltiae* (Fig. 1b), or *M. brunneum* (Fig. 1c), indicating an absence of fitness costs (Table 2). In the presence of *B. bassiana* (Fig. 1d) larval mortality was significantly lower for the resistant strain at the two lower concentrations when compared to the susceptible strain, again indicating an absence of fitness costs. Corrected mortality of western corn rootworm was significantly greater than zero in the presence of either *H. bacteriophora* or *M. brunneum* but not when challenged with *S. feltiae* or *B. bassiana* (Table 3).

In the small cup assay, larval mortality in experimental controls did not differ significantly between strains (Table 1). Additionally, for all four entomopathogens, there was no significant difference between strains in corrected mortality, indicating a lack of fitness costs (Fig. 2; Table 4). For both species of nematodes, there was a significant effect of concentration, with larval mortality increasing as concentration increased (Figs. 2a and 2b; Table 4). Corrected mortality for western corn rootworm was significantly greater than zero in the presence of all four pathogens (Table 3).

Significantly greater mortality was imposed on western corn rootworm in the small cup assay than in the seedling mat assay for *H. bacteriophora* ( $t = 3.09$ ,  $df = 190$ ,  $P = 0.002$ ), *S. feltiae* ( $t = 11.18$ ,  $df = 190$ ,  $P < 0.0001$ ), and *B. bassiana* ( $t = 2.38$ ,  $df = 190$ ,  $P = 0.02$ ), but not for *M. brunneum* ( $t = 0.17$ ,  $df = 190$ ,  $P = 0.86$ ). In the small cup assay, *H. bacteriophora* imposed significantly greater mortality than

either *B. bassiana* ( $t = 7.4$ ,  $df = 254$ ,  $P < 0.0001$ ) or *M. brunneum* ( $t = 7.5$ ,  $df = 254$ ,  $P < 0.0001$ ), and *S. feltiae* imposed significantly greater mortality than either *B. bassiana* ( $t = 9.9$ ,  $df = 254$ ,  $P < 0.0001$ ) or *M. brunneum* ( $t = 9.7$ ,  $df = 254$ ,  $P < 0.0001$ ).

## Discussion

Fitness costs can delay the evolution of Bt resistance, and the presence of entomopathogens can magnify fitness costs (Gassmann et al. 2009a). The entomopathogenic nematodes and fungi tested in this study did not increase larval mortality for the Bt-resistant strain when compared to the susceptible strain (Table 2 and 4), indicating that fitness costs of Bt resistance were not imposed by these entomopathogens. However, increased mortality of western corn rootworm larvae caused by entomopathogens was detected in six of the eight experiments conducted in this study (Table 3). These results parallel the work of Petzold-Maxwell et al. (2012a), which also found that entomopathogens did not increase fitness costs of resistance to Cry3Bb1 maize for western corn rootworm. Although significantly higher mortality was observed for the susceptible strain compared to resistant strain at some concentration of conidia in the seedling mat assay with *B. bassiana*, mortality in this assay was highly variable and did not differ significantly from zero (Fig. 1d; Table 3). Furthermore, no difference was observed between strains in the small cup assay with *B. bassiana* (Fig 2d). Higher concentrations of *B. bassiana* would likely need to be used in the seedling mat assay to provide a more complete test of the potential effects of *B. bassiana* on differences in fitness between resistant and susceptible strains.

Effects of entomopathogens on fitness costs of Bt resistance have been tested for pest species other than western corn rootworm. The entomopathogenic nematodes *S. riobrave* and *H. bacteriophora* imposed the fitness cost of higher mortality for Cry1Ac-resistant *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) (Gassmann et al. 2006, 2008, 2009b), although fitness costs were absent in the presence of *S. carpocapsae*, *Steinernema* sp. (ML18 strain), and *H. sonorensis* (Hannon et al. 2010). Additionally, Cry1Ac-resistant *Plutella xylostella* L. (Lepidoptera: Plutellidae) exposed to an insect virus (Vcal MNPV) experienced a fitness cost of decreased egg viability (Raymond et al. 2007). Although some entomopathogens may increase fitness costs of Bt resistance for some insect species, pathogen-mediated costs appear not to be present in some cases.

Corrected mortality of western corn rootworm was significantly greater than zero when treated with any of the four pathogens in the small cup assay, and when treated with *H. bacteriophora* and *M. brunneum* in the seedling mat assay (Table 3). For *H. bacteriophora*, *S. feltiae*, and *B. bassiana* significantly higher larval mortality was imposed in the small cup assay than in the seedling mat assay. This may be due to soil surrounding individual seedlings in the small cup assay, which would increase contact of larvae with pathogens. By contrast, in the seedling mat assay, larvae could move within a mat of maize roots and not contact with the soil. Thus, the small cup assay may more closely resemble field conditions where individual nodal maize roots are surrounded by soil. Additionally, entomopathogenic nematodes exhibit different foraging strategies, with *H. bacteriophora*, a cruiser, actively searching for a host, whereas *S. feltiae*, an intermediate forager, exhibits

both cruiser and ambush strategies (Grewal et al. 1994). The more active foraging of *H. bacteriophora* compared to *S. feltiae* may account for significant mortality imposed by *H. bacteriophora* but not *S. feltiae* in the seedling mat assay.

Data from the small cup assay support the use of entomopathogenic nematodes and fungi as one component of an integrated pest management (IPM) strategy by providing biological control of western corn rootworm. In a field study with western corn rootworm, Toepfer et al. (2008) found that *H. bacteriophora* and *H. megidis* imposed ca. 70% mortality and *S. feltiae* imposed 32% mortality when applied at  $3.4 \times 10^9$  nematodes per ha. (Toepfer et al. 2008). In the small cup assay, *H. bacteriophora* and *S. feltiae* were applied at an average rate of  $4.7 \times 10^9$  nematodes per ha and imposed an average of 39% and 46% mortality, respectively (Table 3). *Metarhizium anisopliae* imposed 31% mortality against western corn rootworm when applied in the field at a range of  $4 \times 10^{13}$  to  $7 \times 10^{13}$  conidia per ha (Pilz et al. 2009). In the small cup assay reported here, *M. brunneum* and *B. bassiana* were applied at an average rate of  $3.5 \times 10^{12}$  conidia per ha and imposed an average of 9% and 11% mortality, respectively (Table 3). At the application rates studied in the small cup assay, *H. bacteriophora* and *S. feltiae* imposed significantly higher mortality than *M. brunneum* and *B. bassiana*, suggesting that nematodes may be more effective biological control agents for western corn rootworm larvae. However, additional ecological complexities can arise in the field, including semiochemical-based recruitment of nematodes to injured maize roots (Rasmann et al. 2005) and colonization of the rhizosphere by entomopathogenic fungi (Bruck

2010). Such complexities are likely not captured by the short-duration laboratory bioassays conducted in this study.

The IPM benefit of entomopathogens for management of rootworm has been found in field studies evaluating crop yield and larval feeding injury. Field trials indicate that entomopathogens, both fungi and nematodes, have the ability to decrease injury to maize roots, and in some cases, increase yield (Journey and Ostlie 2000, Toepfer et al. 2010, Petzold-Maxwell et al. 2013, Krueger and Roberts 1997). For example, a combination of two entomopathogenic nematodes, *H. bacteriophora* and *S. carpocapsae* Weiser, and one fungus, *M. brunneum*, significantly increased maize yield for Both Bt and non-Bt maize (Petzold-Maxwell et al. 2013). Furthermore, these pathogens reduced root injury to Bt maize when western corn rootworm abundance in the field was high, and to non-Bt maize when rootworm abundance was low (Petzold-Maxwell et al. 2013). In a field study, *H. bacteriophora* significantly reduced root injury from feeding by larvae of western corn rootworm by 25 to 79% (Toepfer et al. 2010). Thus, use of entomopathogens in conjunction with other management tactics, such as crop rotation or Bt maize, may help to augment management of western corn rootworm and preserve yield.

Current data suggest that few fitness costs may accompany Cry3Bb1 resistance in western corn rootworm. Both the results of this study and the results of Petzold-Maxwell et al. (2012a), found an absence of fitness costs in the presence of entomopathogens. In a study of five Cry3Bb1-resistant western corn rootworm strains, (including the strain used here), Oswald et al. (2012) did not detect fitness costs for survival, fecundity, and egg viability, but did detect increased fitness for

resistant strains through faster developmental rate and higher fecundity. By contrast, Meihls et al. (2012) found evidence of fitness costs affecting fecundity and longevity among three Cry3Bb1-resistant strains of western corn rootworm in greenhouse and field studies. In the absence of fitness costs, resistance will evolve more rapidly and will persist once selected (Carrière and Tabashnik 2001). In the field, Cry3Bb1 resistance has been detected in western corn rootworm populations after as few as three pest generations (Gassmann et al. 2011) and has been found to persist after fields were rotated away from Cry3Bb1 maize (Gassmann et al. 2012). The increased risk of resistance associated with a lack of fitness costs highlights the need for sound IPM for western corn rootworm, and the data presented here illustrate the potential use of entomopathogens as one component of an IPM strategy.

### **Acknowledgements**

Sean Bradley assisted with experiments. Funding was provided by the United States Department of Agriculture through a Biotechnology Risk Assessment Grant 2009-33120-20256.

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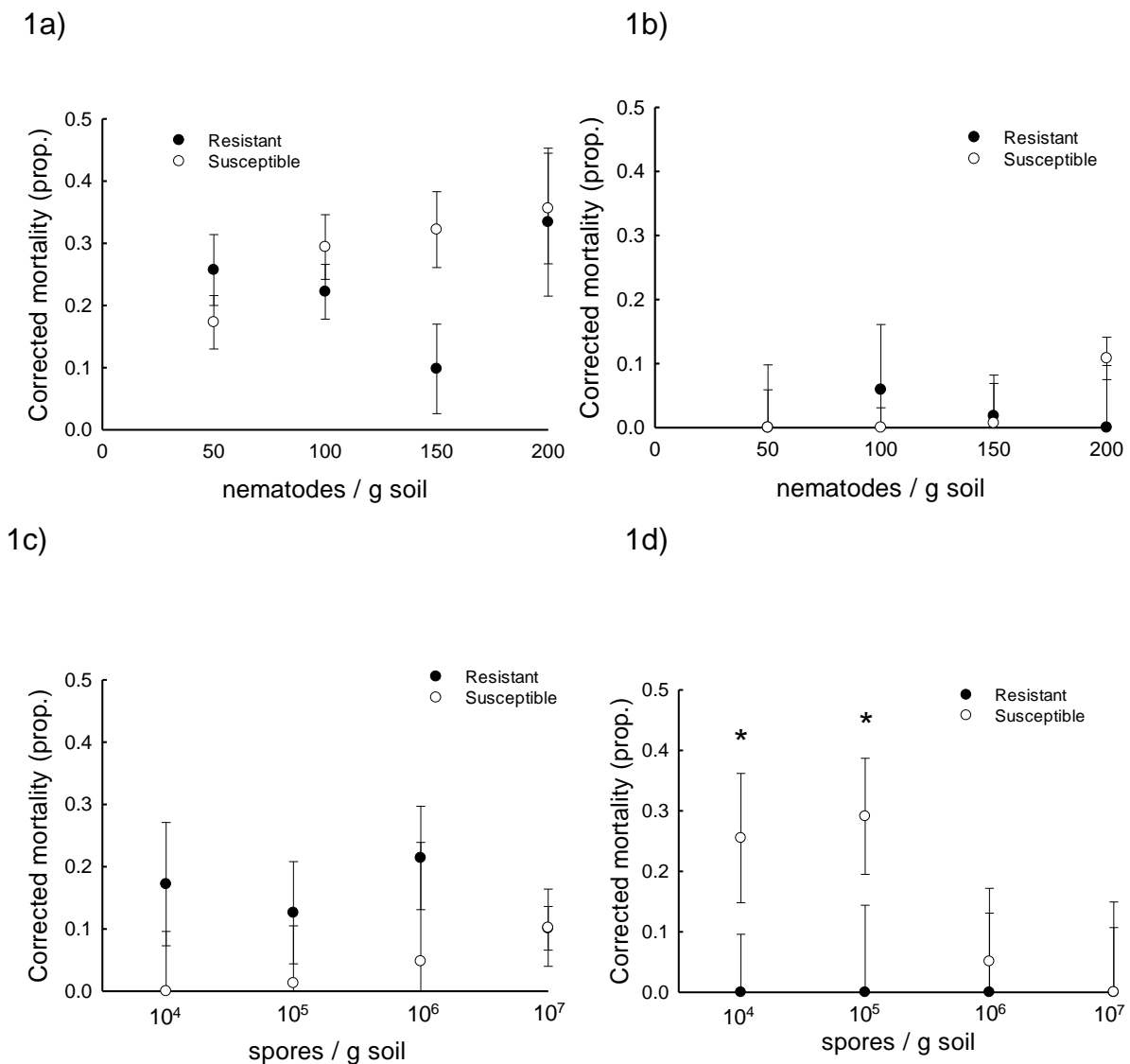
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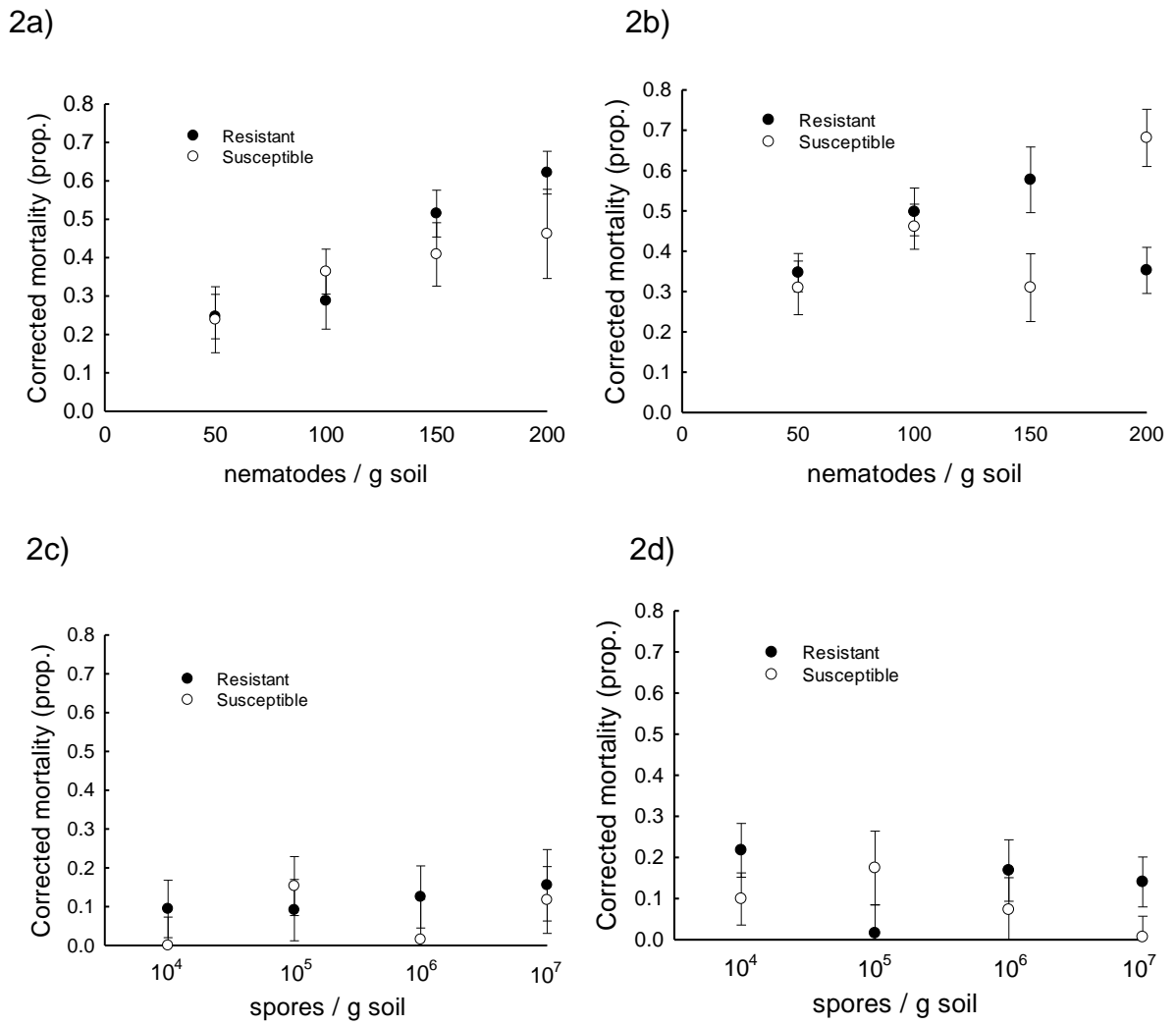
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**Figure 1:** Corrected mortality (mean  $\pm$  SE) for western corn rootworm in the seedling mat assay when exposed to entomopathogenic nematodes A) *H. bacteriophora* and B) *S. feltiae* and entomopathogenic fungi C) *M. brunneum* and D) *B. bassiana*. An asterisk indicates significant difference between strains within a concentration.



**Figure 2:** Corrected mortality (mean  $\pm$  SE) for western corn rootworm in the small cup assay when exposed to entomopathogenic nematodes A) *H. bacteriophora* and B) *S. feltiae* and entomopathogenic fungi C) *M. brunneum* and D) *B. bassiana*.

## Tables

**Table 1:** Percent larval mortality in the control containers that did not receive entomopathogens for the seedling mat assay and small cup assay, and accompanying analysis of variance comparing larval mortality between strains

Assay type	Larval mortality		df	F	P
	Resistant Strain	Susceptible Strain			
<u>Seedling mat assay</u>					
<i>H. bacteriophora</i>	18%	17%	1,7	0.1	0.76
<i>S. feltiae</i>	18%	17%	1,7	0.04	0.84
<i>M. brunneum</i>	21%	25%	1,7	1.76	0.23
<i>B. bassiana</i>	38%	39%	1,7	0	0.98
<u>Small cup assay</u>					
<i>H. bacteriophora</i>	19%	20%	1,7	0.08	0.78
<i>S. feltiae</i>	9%	15%	1,7	1.57	0.25
<i>M. brunneum</i>	32%	22%	1,7	4.52	0.07
<i>B. bassiana</i>	13%	13%	1,7	0	1.000

**Table 2:** Analysis of covariance and analysis of variance for larval mortality from four species of entomopathogens in the seedling mat assay

Effect	df	F	P
<i>H. bacteriophora</i> <sup>a,b</sup>			
Strain	1, 47	1.58	0.21
Concentration	1,7	2.16	0.18
<i>S. feltiae</i> <sup>c,d</sup>			
Strain	1,7	0.03	0.87
Concentration	1,47	0.71	0.40
<i>M. brunneum</i> <sup>e,d</sup>			
Strain	1,7	1.09	0.33
Concentration	1,47	1.79	0.19
<i>B. bassiana</i> <sup>f,g</sup>			
Strain	1,7	21.52	0.002
Concentration	1,7	1.46	0.27
Strain * Concentration	1,7	5.89	0.04

<sup>a</sup> Strain × concentration was not significant ( $F = 1.17$ ;  $df = 1,7$ ;  $P = 0.31$ ) and was removed from the model to allow data to be analyzed with an ANCOVA.

<sup>b</sup> Random factors included in the model were block and block × concentration.

<sup>c</sup> Strain × concentration was not significant ( $F = 1.67$ ;  $df = 1,7$ ;  $P = 0.24$ ) and was removed from the model to allow data to be analyzed with an ANCOVA.

<sup>d</sup> Random factors included in the model were block and block × strain.

<sup>e</sup> Strain × concentration was not significant ( $F = 2.70$ ;  $df = 1,7$ ;  $P = 0.14$ ) and was removed from the model to allow data to be analyzed with an ANCOVA.

<sup>f</sup> Because of the significant strain × concentration interaction, data were analyzed with an ANOVA.

<sup>g</sup> Random factors included in the model were block, block × strain, block × concentration, and block × strain × concentration.

**Table 3:** Corrected larval mortality imposed by entomopathogens in the seedling mat and small cup assays

Assay type	Mean	Standard Error	$t^a$	df	$P$
<u>Seedling mat assay</u>					
<i>H. bacteriophora</i>	0.26	0.03	9.92	63	<.0001
<i>S. feltiae</i>	0.02	0.03	0.70	63	0.49
<i>M. brunneum</i>	0.10	0.04	2.71	63	0.01
<i>B. bassiana</i>	-0.003	0.05	0.06	63	0.95
<u>Small cup assay</u>					
<i>H. bacteriophora</i>	0.39	0.03	13.75	127	<.0001
<i>S. feltiae</i>	0.46	0.03	18.27	127	<.0001
<i>M. brunneum</i>	0.09	0.03	2.96	127	0.004
<i>B. bassiana</i>	0.11	0.02	4.49	127	<.0001

<sup>a</sup> The null hypothesis was that the mean equals zero.



**Table 4:** Analysis of covariance for larval mortality from each of four entomopathogen species in the small cup assay

Effect		df	F	P
<i>H. bacteriophora</i> <sup>a,b</sup>				
	Strain	1,7	0.48	0.51
	Concentration	1,111	23.88	< .0001
<i>S. feltiae</i> <sup>c,d</sup>				
	Strain	1,118	1.11	0.29
	Concentration	1,118	14.89	0.0002
<i>M. brunneum</i> <sup>e,f</sup>				
	Strain	1,111	1.07	0.30
	Concentration	1,7	1.11	0.33
<i>B. bassiana</i> <sup>g,b</sup>				
	Strain	1,7	0.93	0.37
	Concentration	1,111	1.34	0.25

<sup>a</sup> Strain × concentration was not significant ( $F = 1.82$ ;  $df = 1,7$ ;  $P = 0.22$ ) and was removed from the model to allow data to be analyzed with an ANCOVA.

<sup>b</sup> Random factors included in the model were block and block × strain.

<sup>c</sup> Strain × concentration was not significant ( $F = 0.62$ ;  $df = 1,7$ ;  $P = 0.46$ ) and was removed from the model to allow data to be analyzed with an ANCOVA.

<sup>d</sup> Random factor included in the model was block.

<sup>e</sup> Strain × concentration was not significant ( $F = 0.10$ ;  $df = 1,7$ ;  $P = 0.76$ ) and was removed from the model to allow data to be analyzed with an ANCOVA.

<sup>f</sup> Random factors included in the model were block and block × concentration.

<sup>g</sup> Strain × concentration was not significant ( $F = 0.58$ ;  $df = 1,7$ ;  $P = 0.47$ ) and was removed from the model to allow data to be analyzed with an ANCOVA.

#### Chapter 4. GENERAL CONCLUSIONS

In a laboratory-selected strain of western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) with resistance to Cry3Bb1 maize, corrected survival of the resistant and susceptible strains on Cry3Bb1 Bt maize were 0.66 and 0.31, respectively, which is equal to 2.13 times higher survival for the resistant strain on Bt maize. Resistant insects had significantly lower survival and mass on Bt maize compared with non-Bt maize, indicating that resistance was incomplete.

Fitness costs were observed in a variety of life-history traits. Fitness costs observed for Cry3Bb1-resistant western corn rootworm included a 2.8% increase in developmental rate, and reduction of a 5.1% for adult size, 42.9% for female survival, and 10% for egg viability. Additionally adult size of heterozygotes was 2.6% longer than for susceptible insects, indicating a non-recessive cost. However, fitness benefits of higher fecundity by 30.6%, male survival by 49.9%, and greater longevity by 16.7% also were detected for the resistant strain. The mean recessive fitness cost experienced by western corn rootworm without the fitness benefits was 15.2%. However, when the fitness benefits were taken into account the mean value was a 5.2% increase in fitness for the resistant insects.

Host plant variety did affect the overall fitness of this pest. When reared on hybrid DCK 61-72, insects developed more quickly than those on hybrid 2T873 and had higher survival and were larger on hybrid DCK 61-72 than on hybrid 36R19cn and hybrid 2T783. Insects performed the best on hybrid DCK 61-72 when compared to those reared on hybrid 36R19cn and hybrid 2T783. These results indicate that

fitness costs experienced by Cry3Bb1-resistant western corn rootworm are unaffected by the maize variety planted in the refuge, however; the overall fitness of western corn rootworm may be reduced by certain maize hybrids.

Entomopathogens imposed no fitness costs to western corn rootworm with resistance to Cry3Bb1 maize. In the small cup assay, all four entomopathogens imposed significant larval mortality, and larval mortality increased with concentration for *Heterorhabditis bacteriophora* and *Steinernema feltiae*. In the seedling mat assay, *H. bacteriophora* and *Metarhizium brunneum* did not increase larval mortality. *Heterorhabditis bacteriophora*, *S. feltiae*, and *B. bassiana* imposed higher mortality in the small cup assay, but no difference between the assays was detected for *M. brunneum*. Entomopathogenic nematodes imposed greater mortality than the entomopathogenic fungi in the small cup assay. Results indicate that the presence of entomopathogens does not affect fitness costs, but does impose mortality, which suggests there is potential for these pathogens to be used in biological control.

The results of these experiments suggest that fitness costs are absent, or very small, for western corn rootworm with resistance to Cry3Bb1 maize. Thus, the two fold increase in Bt-resistance found in this study may persist within the population of western corn rootworm population. Furthermore, the maize variety planted in the refuge and the presence of entomopathogens likely will not affect fitness costs of Cry3Bb1 resistance for western corn rootworm.