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SPATIAL VARIATION IN WESTERN CORN ROOTWORM (COLEOPTERA:
CHRYSOMELIDAE) SUSCEPTIBILITY TO *BACILLUS THURINGIENSIS* CORN
EVENTS IN NEBRASKA

by

Jordan D. Reinders

A THESIS

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The Graduate College at the University of Nebraska
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Major: Entomology

Under the Supervision of Professor Lance J. Meinke

Lincoln, Nebraska

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SPATIAL VARIATION IN WESTERN CORN ROOTWORM (COLEOPTERA:
CHRYSOMELIDAE) SUSCEPTIBILITY TO *BACILLUS THURINGIENSIS* CORN
EVENTS IN NEBRASKA

Jordan D. Reinders, M.S.

University of Nebraska, 2017

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The western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte, is an economically important pest species of field corn (*Zea mays* L.) in the U.S. Corn Belt. Yield losses and control costs exceed greater than \$1 billion annually. Over the past 10+ years, growers have adopted transgenic corn hybrids expressing rootworm-active *Bacillus thuringiensis* (Bt) proteins as a primary WCR management tactic. Field-evolved resistance to Cry3Bb1 and mCry3A proteins expressed in single-trait Bt hybrids has been confirmed in some areas of Nebraska. Growers have used different tactics as needed to prevent or mitigate resistance, resulting in a mosaic of selection pressure placed on local populations. Currently, research is needed to characterize WCR susceptibility to these Bt toxins on a local spatial scale.

Therefore, this study was conducted to characterize spatial variation in WCR susceptibility to Bt corn events in two corn-intensive production areas of Nebraska. Single-plant larval bioassays were conducted in 2016 and 2017 to characterize the susceptibility of populations from pre-established grids in Keith and Buffalo counties to single-trait Cry3Bb1 and mCry3A proteins. Bioassay results confirmed a mosaic of susceptibility to Cry3Bb1 and mCry3A existed within the two landscapes. Patterns at

several spatial scales were observed and some gene flow of resistant alleles was indirectly documented in the landscape.

A field history index, comprised of additive and weighted WCR management tactics and agronomic practices was developed in an attempt to explain variation in WCR susceptibility. Regression analyses indicated a strong relationship between past management practices and WCR survival on Cry3Bb1, indicating that localized selection pressure is a major contributing factor in determining current susceptibility. These two study areas can be viewed as model systems to draw potential inferences about the relationship of susceptibility to past Bt management histories. These retrospective case histories will inform use of current rootworm-Bt technologies and contribute to the future development of sustainable rootworm management strategies conducted within an IPM framework.

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CHAPTER 1: LITERATURE REVIEW

1.1. Introduction

The genus *Diabrotica* Chevrolat 1836 (Coleoptera: Chrysomelidae: Galerucinae) includes over 400 described species native to the Americas (Derunkov and Konstantinov 2013). Within the genus, there are three distinct species groups: *fucata*, *signifera*, and *virgifera* (Wilcox 1972). Species within *fucata* and *signifera* are found exclusively in Central and South America (Wilcox 1972, Branson and Krysan 1981, Campbell 2009); in contrast, the *virgifera* species group inhabits North and Central America (Krysan and Smith 1987, Krysan 1999, Cabrera Walsh 2003, Campbell 2009).

The North American corn rootworm (*Diabrotica* spp.) field corn (*Zea mays* L.) pest complex consists of the northern corn rootworm (*D. barberi* Smith & Lawrence), the western corn rootworm (WCR) (*D. virgifera virgifera* LeConte), the Mexican corn rootworm (*D. virgifera zae* Krysan & Smith), the southern corn rootworm (*D. undecimpunctata howardii* L.), and western spotted cucumber beetle (*D. undecimpunctata undecimpunctata* L.) (Levine and Oloumi-Sadeghi 1991, Rice 2004, Sappington et al. 2006). *Diabrotica* species are responsible for over U.S. \$1 billion annually in control costs and yield losses related to field corn (Metcalf 1986, Rice 2004, Sappington et al. 2006, Dun et al. 2010, Tinsley et al. 2013). The most economically important *Diabrotica* species within the U.S. Corn Belt is the WCR (Gray et al. 2009). Larval feeding on the root system of field corn leads to loss of vegetative and reproductive biomass (Godfrey et al. 1993), which facilitates decreased plant growth and lower grain yields (Gray and Steffey 1998, Urías-López and Meinke 2001, Dun et al. 2010, Tinsley et al. 2013). Additionally, adults feed on corn pollen and silks, which under

high population densities can cause silk clipping and an overall reduction in pollination efficiency (Branson and Krysan 1981, Prystupa et al. 1988, Levine and Oloumi-Sadeghi 1991, Darnell et al. 2000).

The WCR was first described by John LeConte in 1868. However, it did not achieve recurring pest status until the late 1920s in Nebraska and the 1940s in Kansas (Bare 1930, Tate and Bare 1946, Bryson et al. 1953). A couple factors that aided in the development of the WCR as a pest were increased use of irrigation systems and planting of continuous corn in large monocultures. These factors increased overall densities and contributed to WCR range expansion from the Rocky Mountains throughout the entire U.S. Corn Belt (Gray et al. 2009, Meinke et al. 2009). The WCR emerged as an invasive species in Europe as early as 1992, causing economic problems in cornfields in a non-native range as well (Gray et al. 2009).

The spread of the WCR across the U.S. Corn Belt and into Europe has made management of this corn pest increasingly necessary. Historically, growers have used two main strategies to manage the WCR: crop rotation and insecticides. In recent years, transgenic corn has been widely adopted by growers as a primary rootworm management tactic in continuous corn (Andow et al. 2016). However, the WCR has demonstrated the ability to adapt to crop rotation (Levine et al. 2002), insecticides (Ball and Weekman 1962, 1963; Meinke et al. 1998; Pereira et al. 2015), and transgenic crops (Gassmann 2012; Gassmann et al. 2011, 2012, 2014, 2016; Wangila et al. 2015). In Nebraska, continuous corn is a fairly common agronomic practice; therefore, resistance evolution by WCR to Bt (*Bacillus thuringiensis* Berliner) traits is a primary concern. Since single-trait

Bt corn events have been on the market for 10-14 years and greater than expected injury has occurred in a number of Cry3Bb1 and mCry3A fields, it is important to understand the variability in susceptibility of WCR populations to rootworm-active Bt traits within the Nebraska landscape to facilitate development of appropriate WCR mitigation plans and future sustainable management strategies.

1.2. Biology and Ecology

1.2.1. Life Cycle and Feeding Behavior

The WCR is a univoltine pest of corn, meaning one generation emerges per year (Ball 1957, Levine and Oloumi-Sadeghi 1991, Meinke et al. 2009). Tate and Bare (1946) described the appearance of adults as approximately ¼ inch in length, yellowish to green in color often with two black stripes on the elytra, although the amount of black present is variable. Adults often emerge in early July, feeding on corn silks and tassels (Ball 1957, Levine and Oloumi-Sadeghi 1991). The WCR is a protandrous species; males reach sexual maturity approximately 5 days after adult emergence, while females are sexually mature upon adult emergence (Spencer et al. 2009). Mating and oviposition occur from July through September, or until the first killing frost (Ball 1957). Oviposition generally occurs in cornfields, although the rotation-resistant population in the eastern Corn Belt has been documented to lay eggs in other crops (O'Neal et al. 1999; Levine et al. 2002; Rondon and Gray 2003, 2004). Females search for damp areas in the soil via drought cracks and earthworm burrows to deposit eggs, typically in close proximity to the corn plant (Kirk et al. 1968, Kirk 1979, Kirk 1981a, Kirk 1981b, Levine and Oloumi-Sadeghi 1991, Spencer et al. 2009). Ball (1957) determined that 23% of eggs were laid in the upper 5 cm of soil, 58% in the upper 10 cm, and 80% in the upper 15 cm. In contrast,

Gray et al. (1992) discovered that 60% of WCR eggs were found in the bottom 10 cm of 30 cm cores in dryland corn under dry conditions.

In a comparison of dryland versus irrigated conditions, Weiss et al. (1983) discovered deeper egg oviposition under dryland conditions. The upper 10 and 15 cm of soil contained $33 \pm 8\%$ and $46 \pm 8\%$ of WCR eggs, respectively, in dryland corn. However, the upper 10 and 15 cm of soil under irrigated conditions contained $74 \pm 7\%$ and $93 \pm 4\%$ of eggs, respectively. This indicates that soil moisture is important for oviposition of WCR eggs (Weiss et al. 1983). In Nebraska, geographical differences between the western and eastern portions of the state result in environmental variations due to differing soil types (Godfrey et al. 1995). Deeper egg oviposition favors survival from extreme temperatures; if tillage occurs and eggs are moved to the soil surface, extended cold temperatures may result in increased mortality (Gray et al. 1992, Meinke et al. 2009). Additionally, egg survival is influenced by surface residue; crop residue insulates WCR eggs near the field surface, increasing the probability of survival (Godfrey et al. 1995).

The following spring, eggs begin hatching in late May/early June in most of the U.S. Corn Belt (Levine and Oloumi-Sadeghi 1991, Meinke et al. 2009). Larval feeding occurs from June through July, usually around the same time as the most rapid growth of field corn (Bryson et al. 1953). First instar larvae are able to colonize corn roots more efficiently if eggs are deposited near the plant, leading to an increased chance of survival and root injury (Spencer et al. 2009). Respiring roots release carbon dioxide, which WCR larvae orient toward (Massimino et al. 1980, Strnad and Bergman 1987a, Spencer et al.

2009). Carbon dioxide is commonly released by many plants species; therefore, determining other volatiles released by corn and closely related grass species is important in determining host location by WCR larvae. In addition to carbon dioxide, other corn seedling volatiles such as 6-methoxy-2-benzoxazolinone (MBOA) and long-chain free fatty acids are important semiochemicals used by WCR larvae to find suitable host plants (Hibbard and Bjostad 1988, Hibbard and Bjostad 1990, Bjostad and Hibbard 1992, Hibbard et al. 1994). MBOA only occurs in corn and other grass species, allowing WCR larvae to distinguish between host and non-host plants (Bjostad and Hibbard 1992). In general, first instar larvae are more commonly found on the distal portions of the roots (2mm diameter or less), while later-instar larvae were found towards the newest nodes and stalk of the corn plant (Strnad and Bergman 1987b, Spencer et al. 2009). Larval feeding greatly reduces root mass, which can cause decreased plant growth, lodging, and yield reductions (Gray and Steffey 1998, Urías-López and Meinke 2001).

1.2.2. Larval Hosts

Field corn has been identified as a superior larval host for the WCR; increased hectares of continuous corn favor increased population densities over time (Hill & Mayo 1980, Meinke et al. 2009). Under greenhouse conditions, Clark and Hibbard (2004) discovered that larval survival to adulthood can occur on some grassy weeds commonly found within and adjacent to cornfields, such as western wheatgrass (*Pascopyrum smithii* (Rydb.) Á. Löve), fall panicum (*Panicum dichotomiflorum* Michx.), large crabgrass (*Digitaria sanguinalis* (L.) Scop.), and giant foxtail (*Setaria faberi* Herrm.). This is important when transgenic corn is grown, as Bt toxins primarily target first instar larvae. If larval development occurs on grass/weed species past the first instar, larvae may be

able to colonize transgenic roots with only a sublethal effect (Clark and Hibbard 2004, Oyediran et al. 2004, Wilson and Hibbard 2004). Development past the first instar could significantly affect survival on Bt traits and impact the rate of resistance evolution.

1.2.3. Adult Movement

Movement of WCR beetles is influenced by environmental factors such as temperature, wind speed, and precipitation (Spencer et al. 2009). In general, increased flight activity occurs during the morning and evening when temperatures are between 22 and 27°C (Witkowski et al. 1975, Spencer et al. 2009). Interfield movement can occur as a result of maturing corn; adult WCR detect volatiles from underdeveloped silks and pollinating corn, leading to intra- or interfield movement (Prystupa et al. 1988, Spencer et al. 2009). Due to phenological contrasts of adult host plants (i.e. corn), WCR are likely to redistribute within a local area as adults move toward pollinating and/or young reproductive plants when primary food source attractiveness decreases (Darnell et al. 2000, Campbell and Meinke 2006, Meinke et al. 2009). Recently mated females generally remain in natal cornfields to feed for a period of time before dispersing to different fields during high flight activity periods (Isard et al. 2004, Spencer et al. 2009). Therefore, mated females in sustained migratory flight may colonize distant fields and eventually oviposit eggs, effectively facilitating gene flow.

Tethered flight studies indicate that most flights are trivial (i.e. within/among fields in close proximity). Additionally, mean female flights are often longer than mean male flights, with most migratory flights occurring after mating but before females become gravid (Coats et al. 1986, Naranjo 1990, Stebbing et al. 2005). Coats et al. (1986)

determined that although long duration flights were uncommon, young, mated WCR females could travel up to 40 kilometers in a single day. Additionally, long-distance dispersal of WCR across the Corn Belt can be the result of storm transport (Grant and Seevers 1989, Spencer et al. 2009). Both of these modes of long-distance dispersal can greatly enhance gene flow throughout the U.S. Corn Belt (Meinke et al. 2009).

1.3. Crop Rotation as a Management Tactic

A primary rootworm management strategy throughout the eastern Corn Belt is crop rotation. The rotation of field corn with another non-host plant, such as soybeans (*Glycine max* L.), provides excellent control because oviposition takes place primarily in corn and larval development is eliminated on non-host plants (Levine and Oloumi-Sadeghi 1991, Levine et al. 2002, Clark and Hibbard 2004). Therefore, first-year cornfields often have undetectable to very low WCR populations. Crop rotation has remained an effective part of WCR management programs in some areas across the U.S. Corn Belt; however, failure of crop rotation as a management tactic starting in 1995 has made crop rotation an ineffective control tactic in some areas of the eastern Corn Belt (Levine et al. 2002).

1.3.1. Resistance to Crop Rotation

In June 1987, unexpected root injury occurred in six first-year seed-production cornfields planted to soybeans the previous year in Ford County, Illinois, near Piper City (Levine and Oloumi-Sadeghi 1996, Levine et al. 2002). One hypothesized explanation for this was an extended diapause trait, where eggs remain in diapause for more than one year; this generally occurs in northern corn rootworm populations, although research has

documented that <1% of WCR eggs are also capable of extended diapause (Levine and Oloumi-Sadeghi 1991, Levine et al. 2002). However, it was determined that this trait was not present in WCR eggs collected from adults near Piper City (Levine and Oloumi-Sadeghi 1996). Another hypothesis was that the application of permethrin (i.e. a pyrethroid insecticide) repelled gravid females to nearby soybean fields for oviposition, which turned out to be a plausible explanation (Levine and Oloumi-Sadeghi 1996).

In 1995, failure of crop rotation as a rootworm management tactic occurred in northeast Illinois and western Indiana. First-year corn plants rotated from soybeans the previous year experienced unexpected root injury. It was determined the injury resulted from a reduced affinity to corn for oviposition; WCR females were now laying enough eggs in soybean fields to cause economic injury from larval feeding the following season (Levine and Gray 1996, Levine et al. 2002, Rondon and Gray 2004). However, the greatest numbers of eggs were still oviposited within cornfields (Rondon and Gray 2004). In many areas, greater than 50% of typical yields were lost due to root injury, and high adult densities caused high levels of silk clipping, leading to pollination issues (Levine et al. 2002). It was determined that 97% of cornfields were free of grassy weeds and volunteer corn, which have been shown to increase egg-laying, larval development, and adult emergence in some fields (Shaw et al. 1978, Clark and Hibbard 2004, Wilson and Hibbard 2004). A genetic basis for the rotation-resistant behavior of the variant WCR has not been identified, but likely plays a role in this behavior (Miller et al. 2009).

As time has progressed, the rotation-resistant phenotype has slowly spread to other parts of the eastern U.S. Corn Belt, specifically parts of Wisconsin and Iowa

(Meinke et al. 2009). According to the model developed by Onstad et al. (1999), the rotation-resistant population would spread between 10 and 30 kilometers per year, depending on wind direction, wind speed, and storms. By 2004, the population was expected to reach parts of Ohio, Michigan and Wisconsin; by 2009, the model indicated it would reach portions of Iowa. However, eastward expansion of rotation resistance has stalled out due to increased landscape diversity (Onstad et al. 2003). The biology of the rotation-resistant WCR is slightly different than populations susceptible to crop rotation. Rondon and Gray (2004) discovered gravid females in corn, soybeans, oat (*Avena sativa* L.) stubble, and alfalfa (*Medicago sativa* L.) at different times throughout the growing season; WCR eggs were recovered in all four crops. Therefore, it is evident that rotation-resistant western corn rootworms have lost some fidelity to oviposition in cornfields (Knolhoff et al. 2006).

Rotation-resistant beetles exhibit periodicity, or intervals of high interfield activity between soybean and cornfield edges (Isard et al. 2000). This movement may be due to the fact that soybean tissues do not support egg development (Mabry and Spencer 2003). The decrease in ovipositional preference for corn can correlate to an increased threat of root injury the following year for corn planted after a traditional non-host crop such as soybeans or oats (Rondon and Gray 2004). Onstad et al. (2001) proposed that behavioral resistance develops when greater than 80% of the plant landscape is rotated and that selection on a single gene for adult movement facilitates the evolution of resistance to crop rotation. Crop rotation in less diverse landscapes may select for adult polyphagy due to selection for phenotypes that are able to exploit new hosts (i.e. WCR

found in a variety of crops besides soybeans and corn) (Onstad et al. 2001, Rondon and Gray 2004).

Recently, a physiological mechanism for adaptation to crop rotation by the variant WCR has been proposed. It was hypothesized that crop rotation selected for a specific type of gut microbiota that facilitated adaptation of rotation-resistant WCR populations to this management tactic (Chu et al. 2013). Results indicated that rotation-resistant WCR populations exhibit tolerance to soybean anti-herbivory defenses, specifically cysteine protease inhibitors, which function to prevent protein digestion. Antibiotic suppression of rotation-resistant WCR gut bacteria significantly reduced tolerance of soybean feeding to wildtype levels (Chu et al. 2013). Therefore, crop rotation appears to have selected for a distinctive gut microbiota in rotation-resistant WCR populations, providing a digestive advantage for the variant.

Even with the evolution of variant WCR populations that are resistant to crop rotation, much of the U.S. Corn Belt is free of beetles with this phenotype (i.e. natural wildtype tightly associated with corn). Although crop rotation is not economically or agronomically viable in some areas, crop rotation provides an effective alternative to insecticide use to reduce WCR population densities (Miller et al. 2009). The EPA's most recent Framework to Delay Corn Rootworm Resistance includes crop rotation as the first bullet point under required integrated pest management (IPM) practices to delay resistance evolution (USEPA 2016). Crop rotation should remain an integral part of any IPM program targeting the WCR.

1.4. Chemical Control as a Management Tactic

1.4.1. Soil- and Foliar-Applied Insecticides

During the mid-1900s, the use of soil-applied and/or foliar-applied insecticides was adopted as the main WCR control strategy in continuous corn (Levine and Oloumi-Sadeghi 1991, van Rozen and Ester 2010). Soil insecticides have been typically applied at planting time in-furrow or as a band over the row (Levine and Oloumi-Sadeghi 1991, van Rozen and Ester 2010). The ideal soil insecticide should persist for approximately 6-10 weeks, corresponding to the peak larval feeding period (Levine and Oloumi-Sadeghi 1991). Efficacy of the insecticide is influenced by many variables, including organic matter content, soil pH, soil type, adsorption, and chemical degradation (Levine and Oloumi-Sadeghi 1991, van Rozen and Ester 2010). It is important to note that soil insecticides do not greatly reduce larval densities and survival to the adult stage, but primarily protect the root system from feeding damage (Levine and Oloumi-Sadeghi 1991, Gray and Steffey 1998).

Foliar-applied insecticides are used to reduce adult population densities to prevent silk clipping (i.e. to ensure pollination) or decrease oviposition (Pruess et al. 1974, Branson and Krysan 1981, Prystupa et al. 1988, Levine and Oloumi-Sadeghi 1991, Darnell et al. 2000). Adult control to prevent silk clipping is only necessary for a short period to allow the crop to pollinate; once pollination occurs, silk clipping will not adversely affect grain yield (Meinke 2014). Foliar insecticides require one or more appropriately timed applications. This takes extensive scouting, which can drive up the overall cost of control (Levine and Oloumi-Sadeghi 1991, van Rozen and Ester 2010). Foliar-applied insecticides were initially used as a stand-alone management tactic.

Presently, aerial applications or chemigation are used to complement other management tactics. By reducing the adult population and oviposition, the likelihood of other management tactics working better the following growing season drastically increases due to decreased WCR pressure (Meinke 2014). Instead of being used as a stand-alone management tactic, foliar-applied insecticide applications should be viewed as one tool in the toolbox of IPM to improve the beneficial properties of other management tactics (Meinke 2014). To reduce oviposition, foliar applications should occur when female beetles begin to oviposit, and the insecticide must remain efficacious against extended beetle emergence and immigrating gravid females (Levine and Oloumi-Sadeghi 1991; Meinke 1995, 2014). In addition to higher cost, foliar insecticides can also have a negative impact on non-target and beneficial organisms and residual activity can be deteriorated due to precipitation.

Soil-applied and foliar-applied insecticides are still important components of WCR management programs in field corn. However, resistance to some classes of insecticides has been documented, leading to reduced field efficacy of these products (Ball and Weekman 1962, 1963; Meinke et al. 1998; Parimi et al. 2006; Pereira et al. 2015).

1.4.2. Evolution of Insecticide Resistance

Control of western corn rootworms with soil insecticides has occurred since the late 1940s. Organochlorine insecticides (i.e. BHC, aldrin, and heptachlor) were the first chemicals used until around 1954; these early insecticides were broadcast applied in the field (Hill et al. 1948, Muma et al. 1949, Ball and Weekman 1962). In 1959, the first case

of ineffective WCR control was noted in Nebraska. Ball and Weekman (1963) determined that adults from central Nebraska required 100x more aldrin or heptachlor to kill 50% of the population than populations in eastern Nebraska. Control issues were documented where organochlorine insecticides had been used the longest (Ball and Weekman 1963). Organochlorine insecticides have not been used for WCR control in these problem areas for many years, yet some level of resistance persists in the absence of selection pressure (Siegfried and Mullin 1989, Parimi et al. 2006).

By the 1970s, carbamate and organophosphate soil insecticides had replaced organochlorine insecticides for WCR control (Meinke et al. 1998). These two classes of insecticides were used for over 20 years and provided adequate control of larval and adult corn rootworms with no indication of resistance evolution (Meinke et al. 1998). These products were applied in-furrow or as bands across the row, providing a natural refuge between rows; this may be a major contributing factor preventing resistance evolution to carbamate and organophosphate insecticides.

In contrast, foliar-applied insecticide control failures were increasing in number beginning in the early 1990s as a result of high selection pressure from broadcast applications. In some areas, growers had increased application rates and the number of applications in an attempt to increase adult corn rootworm control (Meinke et al. 1998). Results of bioassays from Meinke et al. (1998) indicated that repeated use of broadcast-applied organophosphate insecticides over many years increased selection pressure, leading to resistance evolution. In turn, increasing rates and number of applications accelerated selection for increased levels of resistance to methyl parathion and carbaryl in

some areas of Nebraska (Meinke et al. 1998). As was seen with aldrin and heptachlor, methyl parathion resistance persisted in corn rootworm populations in the absence of selection pressure (Parimi et al. 2006). These results indicate that resistance to methyl parathion is stable without selection pressure in the lab or field, and that genes conferring resistance do not pose a fitness disadvantage to the insect (Parimi et al. 2006). Because of the persistence of resistant alleles to these different insecticides, management strategies had to be changed in order to effectively manage WCR adult densities.

At the present time, pyrethroid insecticides are one of the main chemical control options for corn rootworm larvae and adults due to regulatory changes and EPA guidelines that removed harsher chemistries from the market. In areas of western Nebraska and southwestern Kansas, inadequate control with bifenthrin, a pyrethroid insecticide, has been documented in recent years (Pereira et al. 2015). The results of adult diagnostic bioassays in 2013 indicated that susceptibility to bifenthrin was reduced in WCR populations from southwestern Nebraska and southwestern Kansas in comparison to susceptible populations east of the Missouri River (Pereira et al. 2015). Therefore, resistance to bifenthrin has evolved in certain populations of WCR with a limited distribution and preventative management strategies need to be implemented to prevent the further spread of pyrethroid resistance.

The WCR has greatly adapted over time to four different classes of insecticides: organochlorines, carbamates, organophosphates, and pyrethroids. This development of resistance has led to decreased efficacy of insecticides within these classes, leading to increased root injury in field corn. With the rotation-resistant WCR adapting to crop

rotation in the eastern Corn Belt and evolution of insecticide resistance in the western Corn Belt, new management tactics were needed to prevent corn yield losses.

1.4.3. Seed Treatments

In order to control seedling pests, seed treatments are often coated on the outside of the corn seed to provide protection for seedling corn (van Rozen and Ester 2010).

Three neonicotinoids (clothianidin, thiamethoxam, and imidacloprid) are currently used at low-moderate rates on almost all corn seed to protect seeds and seedlings from

herbivory by seed-feeding insects (i.e. wireworms, grubs, seed corn maggots, etc.).

However, these rates will not significantly impact WCR populations. These insecticides were specifically chosen because of their high insecticidal potency, broad spectrum, and low mammalian toxicity (Jeschke and Nauen 2008, van Rozen and Ester 2010). High-rate neonicotinoid seed treatments, which can be used as a stand-alone rootworm tactic, are most effective with low to moderate WCR population densities; high larval densities will reduce effectiveness of seed treatments (van Rozen and Ester 2010). However, results from Tinsley et al. (2015) indicate that seed treatments will not protect corn roots against larval injury as effectively as other management tactics. Specifically, using a seed treatment as a stand-alone management tactic in place of a soil insecticide, single-toxin Bt hybrid, or dual-toxin Bt hybrid can increase root injury potential by 86, 136 and 420%, respectively (Tinsley et al. 2015).

As with other management tactics, determining potential resistance evolution to neonicotinoid seed treatments is vital to its preservation as a rootworm management practice. Magalhaes et al. (2007) conducted an experiment to determine baseline

susceptibility to the contact and systemic neonicotinoid, clothianidin. Because clothianidin is a common seed treatment, increased use could increase selection pressure and lead to field-evolved resistance to this insecticide. The results of this experiment indicated no increased levels of tolerance or resistance to clothianidin, allowing the seed treatment to maintain excellent control of WCR larval populations and provide an alternative method of chemical control (Magalhaes et al. 2007). One plausible explanation for the absence of resistance evolution to seed treatments is reduced selection pressure. Most seed treatments are only present for a portion of the larval feeding period, and larger larvae often feed on roots growing outside of the treatment zone.

1.5. Transgenic Corn as a Management Tactic

1.5.1. Development of Bt Corn

Bacillus thuringiensis Berliner (Bt) is a soil microorganism that has been used as a biopesticide for many decades. The largest class of Bt insecticidal proteins is the δ -endotoxins, parasporal crystalline inclusions that have been used in crop plants such as cotton, potatoes, rice, and corn to resist attack by insect pests (Moellenbeck et al. 2001, Ellis et al. 2002). The parasporal crystalline inclusion is solubilized in the midgut after ingestion, releasing proteins (δ -endotoxins). Midgut proteases activate these proteins, which then attack the WCR larval midgut epithelium by binding to brush border membrane vesicles (BBMV) (Gill et al. 1992). This binding causes a disruption in membrane ion transport, which ultimately leads to larval mortality via septicemia (Gill et al. 1992). For example, the production of protein crystals from Bt strain PS149B1 (Cry34/35Ab1) exhibit oral toxicity to WCR larvae, which has provided excellent

protection from corn rootworm damage in the field (Moellenbeck et al. 2001).

Specifically, PS149B1 targets the midgut epithelium tissue, where absorption of nutrients takes place. Cry toxins bind to receptors on the microvilli of the midgut epithelium, causing pores to open. Open pores change the movement of solutes across the epithelium and also increase the inflow of water (Sanahuja et al. 2011).

Because Bt products provide excellent protection of corn roots, Bt traits have been commercialized as management tactics against the corn rootworm pest complex and have been widely adopted by growers (Moellenbeck et al. 2001, Andow et al. 2016). Additionally, seed treatments are used to increase the spectrum of control against seedling pests not affected by Bt proteins. Host plant resistance in the form of Bt corn coupled with prophylactic chemical control (i.e. seed treatments) has become the norm for WCR field control in recent years (Onstad et al. 2011).

In 2003, the first commercial corn hybrid containing the rootworm-active Bt toxin Cry3Bb1 was registered and marketed (USEPA 2010a). Since 2003, three other rootworm-active Bt corn proteins have been commercially marketed for corn rootworm control: Cry34/35Ab1 in 2005, mCry3A in 2006, and eCry3.1Ab in 2012 (USEPA 2010b, 2007, 2012a). The adoption of transgenic corn hybrids by farmers has generally replaced conventional insecticides as the primary rootworm management tactic in continuous corn in the western Corn Belt. In addition, three rootworm-active Bt pyramids were registered in the United States (i.e. Cry3Bb1 and Cry34/35Ab1 [USEPA 2011], mCry3A and Cry34/35Ab1 [USEPA 2012b, 2012c], and mCry3A and eCry3.1Ab [USEPA 2013]). These pyramided traits are slowly replacing the single-trait hybrids in

the United States to slow potential resistance evolution or mitigate the occurrence of resistance to single-trait Bt products (Andow et al. 2016).

A “silver bullet mentality” for using transgenic crops has led to overuse across the U.S. Corn Belt. In some areas of Nebraska, growers have been experiencing greater than expected root injury and lodging in Cry3Bb1 fields over time, resulting from continuous planting of single-trait Cry3Bb1 corn (Wangila et al. 2015; also Gassmann et al. 2011, 2012, 2014 for similar results in Iowa; Gray 2012, 2014 and Schrader et al. 2016 for similar results in Illinois). Greater than expected injury occurs when over 50% of tested transgenic corn plants have greater than one root node destroyed by a suspected resistant WCR population (Oleson et al. 2005, Andow et al. 2016). As a result of growers planting Cry3Bb1 hybrids for consecutive years, confirmation of field-evolved resistance to Cry3Bb1 was documented. In order to mitigate resistant areas and prevent resistance evolution in currently unaffected areas, new insect resistance management (IRM) and IPM tactics must be implemented to preserve susceptibility to commercially available Bt corn hybrids (Gassmann et al. 2011, Wangila et al. 2015, Andow et al. 2016).

1.5.2. Benefits of Bt Corn

In addition to control of WCR field populations, planting Bt corn can provide numerous agronomic, environmental, and societal benefits (Rice 2004). Increased root protection, which leads to higher yields and economic increases, is the foremost agronomic benefit of planting rootworm-active Bt hybrids. Because the Bt toxin is already incorporated into the plant, growers will not have to handle health-hazardous

insecticides, and by using fewer insecticides, farm worker safety increases, labor efficiency increases, and farm waste decreases (Rice 2004).

In addition, transgenic corn can preserve insect biodiversity. Research has shown that most populations of beneficial organisms (i.e. predators) are not directly affected by Bt traits, preserving possible biological control agents within the field (Al-Deeb et al. 2001; Al-Deeb and Wilde 2003; Bhatti et al. 2005a, 2005b; Lundgren and Wiedenmann 2005; Li and Romeis 2010). Many research studies have also examined the impact of Bt corn on non-target organisms, such as honey bees (*Apis mellifera* L.) and the monarch butterfly (*Danaus plexippus* L.). Hanley et al. (2003) discovered no significant impact of transgenic pollen on honey bees; additional studies indicated Bt corn posed little or no threat to honey bee populations (Rose et al. 2007, Duan et al. 2008). Studies evaluating the impact of Bt corn pollen on monarch larval life history parameters indicated pollen posed little risk under field conditions (Sears et al. 2001, Jesse and Obrycki 2003, Anderson et al. 2004, Mattila et al. 2005). Although some research has shown that non-target species can be negatively affected by Bt corn, most studies support the idea that there are minimal risks associated with Bt corn. Because of the reduced environmental impact, transgenic corn has the ability to transform IPM efforts in the U.S. Corn Belt targeted at controlling WCR populations (Rice 2004).

1.5.3. Greenhouse and Laboratory-Selected Resistance Experiments

Because of the increased adoption of transgenic corn and the practice of using single-trait Bt hybrids in continuous corn for many consecutive years, many entomologists believed there was an increased possibility for resistance evolution.

Theoretically, a single point mutation in the gene encoding for a δ -endotoxin receptor could promote resistance evolution (Sanahuja et al. 2011). Additionally, because of failure by some growers to comply with refuge requirements, constant selection pressure is possible in transgenic fields (Gassmann et al. 2011). A few greenhouse and laboratory studies have been conducted in order to determine the response of selection to the different transgenic traits currently on the market.

Meihls et al. (2008) determined that after three generations of constant exposure to Cry3Bb1, a selected colony exhibited a 22-fold greater LC_{50} than a control colony (i.e. concentration required to kill 50% of the population). After six generations of selection, percent survival on Bt corn was 11.7-fold greater than on near-isoline (i.e. genetically equivalent hybrid without Bt toxins). In a similar experiment, Oswald et al. (2011) discovered that after 10 generations of moderate to intense selection, adult survivorship was approximately 4x greater than the control colony on Cry3Bb1 (event MON863). The results indicated that resistance can occur at an increasing rate under constant selection pressure. These results were supported by Meihls et al. (2012); their results indicated that resistance to Cry3Bb1 can result in as few as three generations.

Meihls et al. (2011) also conducted a study to select for resistance to mCry3A (event MIR604). After four, seven, and ten generations of selection, resistance ratios were 0.5, 4.3, and 15.4, respectively. In comparison to Cry3Bb1, resistance to mCry3A developed more slowly (i.e. 7-10 generations) under similar selection and bioassay procedures. After 14 generations, resistance to mCry3A was still incomplete—the selected population still developed slower on MIR604 than its near-isoline.

Similar to Cry3Bb1 and mCry3A, selection for resistance on Cry34/35Ab1 (event DAS-59122-7) was also conducted (Lefko et al. 2008). After the first generation of selection, survivors had a slight increase in injury potential and non-random mating led to a gradual increase in injury to Cry34/35Ab1. Lefko et al. (2008) concluded there is a tolerance trait that confers a low level of survival on Cry34/35Ab1, which appears to act independently of a single allele conferring complete resistance to the toxin.

Although not sold as a single-trait product, determining the potential for resistance evolution to eCry3.1Ab is an important factor in determining efficacy of pyramided products containing this event. Within 4 generations of selection, a low level of resistance to eCry3.1Ab was discovered. After 11 generations of selection, the selected colony exhibited a resistance ratio of 2.58 (Frank et al. 2013).

1.5.4. Field Measures of Mortality to Bt Corn Events

Although greenhouse and laboratory experiments provide important insight into selection for resistance to transgenic corn, field experiments must be conducted in order to determine the potential for resistance evolution under natural conditions. A few publications provide insight into field mortality and efficacy of products for both single-trait and pyramided Bt corn events.

Clark et al. (2012) determined the mortality impact of Cry3Bb1-expressing plants (event MON863) on WCR larvae in the field. Emergence as a percentage of viable eggs on MON863 ranged from 0.02% to 0.10%, significantly lower than the 1.09% to 7.14% from its near-isoline. Over a two year period (2003-2004), average mortality on MON863 was 98.10% and 98.49%. MON863 also caused an 18 day delay in time to 50%

emergence when compared to its near-isoline. Although it is evident that the toxin has a high efficacy towards susceptible WCR larvae, increased planting of Cry3Bb1 expressing hybrids (i.e. continuous corn) may increase the rate of resistance to this toxin and reduce developmental and adult emergence delays. Wangila and Meinke (2016) discovered no significant difference in development rate or mean larval weight of Cry3Bb1-resistant populations, indicating delays in development and adult emergence associated with Cry3Bb1 feeding decrease or disappear as a result of resistance evolution to the trait.

Hibbard et al. (2010) determined that MIR604 proteins resulted in a 94.88% mortality rate against WCR larvae. The delay time to 50% emergence was approximately 5.5 days, and emergence as a percentage of viable eggs ranged from 0.01% to 1.41% on MIR604 compared to 0.82% to 11.76% on its near-isoline. From these results, it can be concluded that MIR604 expressing the mCry3A protein significantly reduces rootworm damage, although the mortality rate and delay in development are lower in comparison to Cry3Bb1.

In Cry34/35Ab1 field experiments, significantly fewer adults emerged from the transgenic crop than from its near-isoline (Storer et al. 2006). Reduction in WCR adults due to Cry34/35Ab1 was between 96% and 99.4%. As with Cry3Bb1, there was a delay in time to 50% emergence. However, it was highly variable in Cry34/35Ab1-expressing hybrids, ranging from no apparent delay to a 3-6 day delay to a 7 day delay. Thus, it is evident that corn events containing the Cry34/35Ab1 toxin are highly efficacious against WCR larvae.

Pyramided Bt events were also studied to determine mortality rates and efficacy of the product against WCR larvae. Hibbard et al. (2011) conducted an experiment to determine mortality rates on mCry3A, eCry3.1Ab, and mCry3A + e Cry3.1Ab. Half as many beetles emerged from the pyramided hybrid compared to eCry3.1Ab alone and 24-fold fewer beetles than mCry3A alone. The pyramid provided excellent protection against WCR larvae, exhibiting a 99.91% mortality rate compared to 99.79% on eCry3.1Ab and 97.83% on mCry3A alone. Additionally, the delay to 50% emergence was also noted in eCry3.1Ab and the pyramided treatment (8 days and 4.6 days, respectively).

Two different studies aimed at evaluating the efficacy of SmartStax® (Cry3Bb1 + Cry34/35Ab1) against WCR larvae (Prasifka et al. 2013, Head et al. 2014). The rootworm-active components of SmartStax® include MON 88017 (Cry3Bb1) and DAS-59122-7 (Cry34/35Ab1), along with other traits targeting Lepidopteran pests. Prasifka et al. (2013) concluded that SmartStax® provided superior root protection compared with single-trait hybrids or non-transgenic corn. SmartStax®, Cry3Bb1, and Cry34/35Ab1 each provided a high level of consistency and protection against yield loss (99.8%, 98.5%, and 97.5% consistency below a 0.25 node-injury rating, respectively). Researchers concluded that multiple modes of action provide “redundant control” of larvae that survive on a single toxin; larvae must overcome two toxins instead of a single toxin, except in cases of cross-resistance between toxins. Head et al. (2014) reported similar results; they determined that significantly lower damage ratings occurred on SmartStax® than single-trait and non-transgenic control plants. A similar pattern emerged in regards to beetle emergence, with fewer adults emerging on SmartStax®

(98.2% to 99.97% reduction) in comparison to Cry34/35Ab1 (94.2% to 99.2% reduction) or Cry3Bb1 (96.2% to 99.96% reduction).

The results from Hibbard et al. (2011), Prasifka et al. (2013), and Head et al. (2014) indicate that pyramided hybrids are more efficacious than single-trait hybrids as stand-alone management tactics. Greater root protection occurs when multiple modes of action (i.e. toxins) are present to reduce larval populations. With the increased shift from single-trait to pyramided hybrids, it is possible that resistance evolution may take longer, preserving product efficacy for extended periods of time.

1.5.5. Field-Evolved Resistance to Bt Corn Events

In 2011, eight years after the introduction of the first Bt corn event (Cry3Bb1), field-evolved resistance was detected in Iowa (Gassmann et al. 2011). Field-evolved resistance can be defined as a genetically based decrease in susceptibility of a population to a certain toxin caused by previous exposure to the toxin in a field setting (Tabashnik et al. 2009, 2014). It is possible to document field-evolved resistance by conducting bioassays on progeny of survivors on Bt corn (Sumerford et al. 2013, Tabashnik et al. 2014). Gassmann et al. (2011) used a single-plant diagnostic bioassay technique to characterize susceptibility levels of progeny of WCR populations collected from Cry3Bb1 problem fields (i.e. EPA criteria for greater than expected injury, Andow et al. 2016). Survival on Cry3Bb1 corn for larvae from problem fields was 3x greater than control populations, indicating resistance to Cry3Bb1. However, survival on Cry3Bb1 for larvae from problem fields was lower than on its near-isoline, indicating resistance was incomplete. Under constant selection pressure, these results mirror those discovered in

Meihls et al. (2008), indicating that resistance could occur in as few as three generations under constant selection pressure. It was hypothesized that resistance occurred as a result of Bt corn events being less than high-dose products and/or failure to plant a sufficient refuge (Gassmann et al. 2011). There is also some evidence that resistance to Cry3Bb1 can persist in populations after selection pressure is removed (Gassmann et al. 2012, Wangila and Meinke 2016).

Additionally, Cry34/35Ab1 efficacy was tested against the progeny from problem fields; this toxin significantly reduced rootworm survival, indicating a lack of cross-resistance between these two traits (Gassmann 2012; Gassmann et al. 2011, 2012, 2014; Wangila et al. 2015; Schrader et al. 2016; Zhao et al. 2016; Zukoff et al. 2016). However, cross-resistance between Cry3Bb1 and mCry3A was detected, as survival on both traits was similar (Gassmann et al. 2014).

Field-evolved resistance to Cry3Bb1 has also been documented in Illinois. Multiple fields with severe root damage and lodging had been reported, and these fields had been planted with continuous Cry3Bb1 corn for many years (Gray 2012). The results from Illinois bioassays were similar to those reported by Gassmann et al. (2011), indicating the presence of field-evolved resistance to Cry3Bb1 in Illinois (Schrader et al. 2016). In 2014, three additional counties in northwestern Illinois also had confirmed cases of field-evolved resistance to Cry3Bb1 (Gray 2014).

Wangila et al. (2015) reported similar results in Nebraska to those in Iowa and Illinois. Greater than expected rootworm injury occurred in fields with a history of continuous Cry3Bb1 corn production. After conducting single-plant bioassays, it was

concluded that some level of field-evolved resistance to Cry3Bb1 was present in response to selection pressure in northeast and southwestern Nebraska. Cross-resistance was also present between Cry3Bb1 and mCry3A, as corrected and proportional survival were similar on both treatments; no cross-resistance was noted between Cry3Bb1 and Cry34/35Ab1, supporting the results from Gassmann et al. (2014).

Zukoff et al. (2016) collected WCR adults from Cry3Bb1 performance-problem fields and known susceptible fields in 2011. Diet, seedling, and single-plant bioassays were conducted on neonate larvae in 2012. Additionally, a susceptible control from the diapausing colony at the USDA-ARS in Brookings, South Dakota, was used in the bioassays. Their results confirmed Cry3Bb1 resistance in multiple fields in Minnesota. Cross-resistance was exhibited among Cry3Bb1, mCry3A, and eCry3.1Ab in all instances, although varying levels were seen among populations. Importantly, cross-resistance of these three traits with Cry34/35Ab1 was not exhibited. Results from Jakka et al. (2016) support these findings, as field populations exhibited increased survival on Cry3Bb1, mCry3A, and eCry3.1Ab, but not on Cry34/35Ab1. Zukoff et al. (2016) indicated that relative survival of susceptible field sites on Cry3Bb1 and mCry3A was higher than relative survival of susceptible control populations from the USDA-ARS, although not to a significant level. This may indicate that a low level of resistant alleles has penetrated the entire landscape, although Bt trait performance does not appear to be significantly affected.

Recently, incomplete resistance to Cry34/35Ab1 from single-plant bioassays was confirmed by Gassmann et al. (2016). Populations were collected in 2013 from Iowa

fields with greater than expected injury to Cry34/35Ab1 either as a single trait (> 2 nodes of injury) or pyramided hybrid (> 1 node of injury). Results of single-plant bioassays confirmed incomplete resistance to Cry34/35Ab1, as well as resistance to Cry3Bb1 and mCry3A (Gassmann et al. 2016). In addition, Ludwick et al. (2017) reported incomplete resistance to Cry34/35Ab1 from a SmartStax® performance problem field in Minnesota. Head et al. (2017) documented incomplete resistance to Cry34/35Ab1 at a location in Cuming County, Nebraska, during 2015. Mean root damage ratings were 0.92 on plants expressing Cry34/35Ab1 and 1.69 on control plants not expressing the trait. This field site was characterized by periodic continuous use of single-trait Cry3Bb1 and Cry34/35Ab1 hybrids within a 15 year period. These are the first documented cases of field-evolved resistance to Cry34/35Ab1. With Cry3Bb1 resistance proliferating and cross-resistance among Cry3Bb1, mCry3A, and eCry3.1Ab, the reduced efficacy of Cry34/35Ab1 significantly affects future Bt management strategies.

With the presence of field-evolved resistance to Cry3Bb1 and cross-resistance to mCry3A present, the efficacy and durability of these two single-trait toxins is declining as a result of less than high-dose traits, increased selection pressure, failure to rotate Bt traits, and reduced grower compliance with IRM guidelines. Additionally, the increased survival of WCR larvae on Cry34/35Ab1 raises concerns over the future of Bt management programs, as this trait is included in most pyramided hybrids. Developing IRM plans will be vital to preventing resistance in areas where it is currently absent and for any future traits and hybrids that are commercially available to growers.

1.5.6. Fitness Costs Associated with Bt Resistance

Determining fitness costs associated with resistance to transgenic crops is important; if fitness costs are absent, it is possible the allele(s) conferring resistance may persist in a population for an extended period of time. Tabashnik et al. (2014) defined fitness costs as “a trade-off in which alleles conferring resistance to a pesticide [in this case Bt] reduce fitness in an environment lacking the pesticide [Bt toxin].” Fitness is defined as the ability of an individual in a given population being able to survive and reproduce (Tabashnik et al. 2014). Investigating whether resistance to Bt corn results in fitness costs is of vital importance for developing resistance management programs.

According to Gassmann et al. (2009), very few studies have investigated the fitness costs associated with Bt resistance by the WCR. In a literature review, they determined that fitness costs were magnified on host plants versus lab diet, indicating that researchers should evaluate fitness costs under field conditions.

In a greenhouse selection experiment for resistance to Cry3Bb1, Meihls et al. (2012) detected no fitness costs on near-isoline corn that led to decreased larval survival or size. However, it was noted that selected females exhibited lower fecundity than control females on isoline corn, indicating a potential fitness cost. Since this experiment was conducted in a greenhouse, it may not mimic field conditions; a similar study should be conducted under field conditions to support the original results. Another laboratory experiment led to the conclusion that selection for Bt resistance increased larval development rates and led to higher fecundity, which would pose a selective advantage for developing resistance to Cry3Bb1 (Oswald et al. 2012). The results from this

experiment indicated no fitness costs related to survivorship, fecundity, or viability; the fitness advantage related to larval development may be a potential contributing factor leading to cases of field-evolved resistance being discovered in Iowa, Illinois, and Nebraska (Gassmann et al. 2011; Gray et al. 2012, 2014; Wangila et al. 2015).

In contrast, Hoffmann et al. (2015) discovered a 2.8% fitness cost related to larval development rate of a resistant strain compared to a susceptible strain. Egg viability also exhibited a 9.8% fitness cost for the resistant strain. However, fitness benefits were also reported with resistance to Cry3Bb1. The resistant strain exhibited 20.1% greater longevity and 30.6% higher fecundity (supports Oswald et al. 2012) in comparison to the susceptible strain. When all life history traits were averaged, resistance to Cry3Bb1 presented the WCR with a fitness benefit of $4.8 \pm 10.0\%$. When averaging traits linked to survival and reproduction, a fitness benefit of $7.0 \pm 20.8\%$ was reported (Hoffmann et al. 2015). This data suggests that fitness costs associated with resistance to Cry3Bb1 are either absent or minimal. Because of the lack of fitness costs reported thus far, resistant alleles may increase and persist in a population, posing a potential increase in resistance evolution to transgenic corn over time (Gassmann et al. 2009). Results from Wangila and Meinke (2016) also suggest that as resistance to Cry3Bb1 increases in a population, developmental and adult emergence delays are likely to decrease compared to susceptible populations.

Ingber and Gassmann (2015) characterized inheritance and fitness costs of two resistant field strains from Iowa. The Hopkinton strain was collected from a field planted with Cry3Bb1 corn for 7 consecutive years, while the Cresco strain only experienced

selection pressure for 5 consecutive years. Results from diet and plant bioassays indicated a higher magnitude of resistance in the Hopkinton strain (i.e. complete resistance, no difference in survival on Cry3Bb1 compared to near-isoline); incomplete resistance was documented in the Cresco strain (i.e. lower survival on Cry3Bb1 compared to near-isoline). The Hopkinton strain exhibited non-recessive inheritance, while recessive inheritance was detected in the Cresco strain. Additionally, the Cresco strain exhibited fitness costs affecting larval development rate, survival to adulthood, and fecundity; no fitness costs were detected in the Hopkinton strain. Researchers theorized that the increased exposure to Cry3Bb1 resulted in complete resistance in the Hopkinton strain. However, the differences in inheritance of resistance and fitness costs may indicate multiple mechanisms of Cry3Bb1 resistance in the field. They concluded that these fitness costs may put the Cresco strain at a selective disadvantage compared to a susceptible population when developing in a refuge; additionally, the presence of fitness costs may help to alleviate the presence of resistant alleles in a refuge, reducing the rate of resistance evolution (Gassmann et al. 2009, Ingber and Gassmann 2015). The lack of fitness costs coupled with non-recessive inheritance may increase the rate of resistance evolution to Cry3Bb1 in the field; the variability of results in this study suggest that different fields will experience different rates and levels of resistance evolution to Cry3Bb1 (Ingber and Gassmann 2015).

Meihls et al. (2016) researched the fitness costs associated with removing an mCry3A-selected colony from selection after 8 generations. Their results indicated a lack of significance in overall relative survival of the removed from selection colony compared to the selected colony. Additionally, there was no significant difference

between the removal from selection colony and the selected colony in mean weight or mean head capsule width, indicating minimal (if any) fitness costs associated with mCry3A resistance (Meihls et al. 2016). Heritability values were also calculated based on larval and adult recovery data. The results indicate that mCry3A resistance is inherited non-recessively, as is evidenced by the heritability values of 0.66 for larvae and 1.03 for adults (Meihls et al. 2016). This contrasts with Cry3Bb1 heritability values, which ranged from 0.15 to 0.296 (Meihls et al. 2008, Oswald et al. 2011). In contrast, Zhao et al. (2016) determined that mCry3A resistance was autosomally inherited and incompletely recessive, with heritability values ranging from 0.23 to 0.25. Additional experiments showed that reduced mCry3A binding to midgut brush border membrane vesicles occurs in resistant individuals, the first report of reduced binding of a Cry3 protein (Zhao et al. 2016).

1.5.7. Impact of Field History on Efficacy of Bt Hybrids

Understanding the correlation between past management tactics and current susceptibility levels and beetle abundance is vital to delay resistance evolution or mitigate current resistance. In 2013 and 2014, Dunbar et al. (2016) sampled 47 cornfields with four differing management types across Iowa:

1. Recently rotated fields (planted to corn for 2 years before sampling)
2. Continuous corn (at least 7 consecutive years)
3. Past Cry3Bb1/mCry3A problem field (> 1 node of unexpected root injury previously)
4. Current Cry3Bb1/mCry3A problem field (> 1 node of unexpected root injury)

At each site, root ratings were taken and Pherocon AM yellow sticky traps were set up in transects to obtain information regarding beetle abundance. Regarding field sites, 89% of all sites were planted with Bt corn; pyramided Cry34/35Ab1 and Cry3Bb1 comprised

47%, while single-trait Cry3Bb1, Cry34/35Ab1, and mCry3A were present in 21%, 15%, and 6% of fields, respectively. Non-transgenic corn was planted in the remaining 11% of fields sampled. Soil insecticides were used in 25% of all fields sampled, and 83% of fields with soil insecticides applied also had Bt corn planted. Out of 13 past problem fields, 11 were planted with pyramided hybrids and 45% contained soil insecticides. Cry3Bb1 comprised 78% of current problem fields, while mCry3A expressing hybrids were planted in 22%. Soil insecticides were not applied in any current problem fields (Dunbar et al. 2016).

In 2013 and 2014, significantly higher root injury was documented in current problem fields compared to rotated fields, continuous cornfields, and past problem fields, with > 2.0 nodes damaged. Root ratings from rotated fields, continuous cornfields, and past problem fields did not significantly differ from one another, and very little injury was noted (Dunbar et al. 2016). In addition to significantly higher root ratings, current problem fields also exhibited a significantly higher abundance of WCR (Dunbar et al. 2016). Based on these results, it can be implied that growers with continuous cornfields applied soil insecticides to lessen rootworm pressure, and growers with past problem fields increased use of pyramided Bt hybrids and applied soil insecticides to mitigate the issue. Multiple layers of tactics in the landscape studied significantly reduced rootworm densities and associated injury compared to continuous planting of single-trait Bt hybrids. Using multiple IPM tactics to manage WCR populations would be most advantageous to growers; this allows IPM to complement IRM by increasing the modes of mortality within the landscape. Dunbar et al. (2016) indicated that fields lacking management diversity (i.e. using a single tactic versus multiple tactics) were associated with fields

exhibiting severe root injury and high WCR population densities. Therefore, using IRM within an IPM framework can help effectively reduce population densities and root injury while also preventing resistance to remaining active ingredients, Bt traits, or future technologies.

1.5.8. Assessing Field-Evolved Resistance and Delaying Further Evolution

There are numerous reasons for the rapid development of field-evolved resistance to Cry3Bb1 field corn in less than 10 years after it first became commercially available. One of the initial IRM plans implemented by the EPA was similar to the high-dose refuge strategy used for lepidopteran pests, specifically the European corn borer (*Ostrinia nubilalis* Hübner). The high-dose refuge strategy means that when a dose of the toxin is ingested, the dose is high enough to kill 99.99% of the pest population (USEPA 2002, Tabashnik and Gould 2012). This strategy assumes that if 99.99% of susceptible individuals are killed, individuals heterozygous for resistance will also be killed, slowing resistance evolution (USEPA 2002, Gould 1998). The high-dose refuge strategy makes three critical assumptions: 1) resistant alleles are rare, 2) random mating between susceptible and resistant individuals occurs, and 3) resistance is recessive (i.e. plant produces toxin at high enough dose to kill heterozygous insects) (Andow et al. 2016). Violation of any assumption significantly reduces the effectiveness of this strategy in delaying resistance to Bt toxins.

In laboratory selection experiments, resistance to single-trait Bt hybrids has been shown to develop rapidly (Lefko et al. 2008; Meihls et al. 2008, 2011, 2012; Oswald et al. 2012), which indicates that resistant alleles are relatively common. Additionally,

determining that substantial feeding and yield loss in the field are associated with a 3- to 6-fold increase in resistance indicates the potential for resistant alleles being common in WCR populations (Gassmann et al. 2011, 2012, 2014). These findings violate the first assumption listed above, posing potential problems for the high-dose refuge strategy as a stand-alone IRM strategy.

Currently, all corn rootworm products are registered as less than high-dose products (USEPA 2002). As was noted in the section on “Field Measures of Mortality on Bt Corn” above, survival on Cry3Bb1, mCry3A, and Cry34/35Ab1 were approximately 2-4% below the 99.99% requirement for the high-dose refuge strategy, providing support that these products are indeed less than high-dose (Storer et al. 2006, Hibbard et al. 2010, Clark et al. 2012). Campbell et al. (2017) investigated the doses associated with Cry3Bb1, Cry34/35Ab1, and pyramided Cry3Bb1 and Cry34/35Ab1 (SmartStax®). Results showed dose estimates of 97.4-99.3%, 98.8-99.9%, and 99.68-100%, respectively. All products exhibit a dose below 99.99% (except the maximum for SmartStax®), indicating WCR exposed to these events are not receiving a dose high enough to meet the high-dose requirement. Additionally, Ingber and Gassmann (2015) discovered a resistant WCR strain in Iowa that exhibited non-recessive inheritance; together, these findings violate the third assumption of the high-dose refuge strategy.

In theory, the refuge provides an area where rootworm larvae develop without being exposed to a Bt toxin, leading to random mating of susceptible individuals with resistant individuals, producing heterozygous offspring that exhibit lower survival on Bt corn than homozygous resistant individuals (Cullen et al. 2013). Because all rootworm-

active Bt hybrids express less than high-dose, resistance may not be functionally recessive, promoting survival of heterozygous individuals (Ingber and Gassmann 2015). Studies focusing on adult intrafield movement indicated that males move shorter distances before mating than previously thought, promoting assortative mating of resistant individuals (Kang and Krupke 2009, Andow et al. 2016). Additionally, delays in adult emergence also increase the incidence of assortative mating between resistant individuals (Storer et al. 2006, Hibbard et al. 2011, Frank et al. 2015, Andow et al. 2016). These findings indicate the assortative mating occurs frequently, violating the second assumption of the high-dose refuge strategy, greatly increasing the rate of resistance evolution to Bt corn (Deitloff et al. 2016).

Another potential factor increasing field-evolved resistance in some areas regards grower compliance with the refuge strategy (Gassmann et al. 2011). In order to combat non-compliance, the idea of an integrated refuge, or refuge-in-the-bag, was proposed and implemented for many rootworm-active Bt hybrids. Using an integrated refuge poses many benefits, but also poses some drawbacks for transgenic resistance evolution. Increased movement between Bt and non-Bt plants in close proximity to one another may increase sub-lethal exposure to the toxin, thereby increasing resistance rates (Gould 1998, Petzold-Maxwell et al. 2013). However, random mating should occur more frequently due to the close proximity of transgenic and non-transgenic plants, reducing the amount of resistant alleles in the field (Gould 1998). This strategy is effective if resistance is inherited recessively and if resistant alleles are rare in the field population (Tabashnik 1994, Deitloff et al. 2016). Petzold-Maxwell et al. (2013) discovered no difference in survival in pure stand Bt corn versus the integrated refuge of Cry34/35Ab1 and non-Bt

corn. Head et al. (2014) reported similar results from Cry3Bb1 + Cry34/35Ab1 and non-Bt corn. Using seed blends should decrease insecticide use, allow for increased adoption, and preserve natural enemies within agroecosystems (Onstad et al. 2011).

A potential drawback of seed blends is the increased difficulty of scouting and monitoring pest populations. With a pure-block refuge, early warning signs of potential problems are easy to detect; with non-Bt seeds throughout the field, scouting becomes more difficult (Onstad et al. 2011). It is also possible that an increase in seed blend planting may increase selection pressure and facilitate resistance evolution in the field (Onstad et al. 2011).

Many scientists argue that because the current Bt products are not high-dose, the refuge requirements are not adequate and need to be dramatically increased to prevent resistance evolution (Onstad and Meinke 2010, Onstad et al. 2011, Tabashnik and Gould 2012). Tabashnik and Gould (2012) recommend that the refuge requirements for single-trait Cry3Bb1 be increased to 50% (currently 20%) to prevent and mitigate further resistance evolution. With pyramided hybrids, the refuge requirement is currently 5-10%; Tabashnik and Gould (2012) argue that this is also insufficient to prevent resistance evolution, and the refuge requirement should be increased to 20% to improve the durability of these traits. This is in part due to the fact that some populations are already resistant to one of the two toxins involved in the pyramid (i.e. Cry3Bb1 resistance in SmartStax® leaves only Cry34/35Ab1 to protect corn roots, leading to increased selection pressure placed on the remaining toxin).

Collectively, these findings highlight that using the high-dose refuge strategy as a stand-alone IRM strategy is not sufficient in delaying WCR resistance to Bt corn. However, it is important to note that refuges delay resistance evolution in models, but with less than high-dose products IRM strategies should be conducted within an IPM framework. In addition to the refuge strategy, other complementary tactics, such as rotation of Bt traits, should be implemented to prevent resistance evolution. Andow et al. (2016) described the failure of the initial IRM strategy (i.e. high-dose refuge strategy) due to violation of the assumptions listed above; the entomologists involved in the article highlighted that “future plans should emphasize a more adaptive, integrated IRM and IPM approach.” As a result of efforts made by corn pest entomologists, the EPA drafted the “Framework to Delay Corn Rootworm Resistance,” which requires many IPM strategies to be used within an IRM program (USEPA 2016).

1.6. Justification for Research

The WCR (*Diabrotica virgifera virgifera* LeConte) is one of the main insect pests of field corn in the United States Corn Belt. There are numerous control tactics and strategies available to combat this pest. However, the WCR has adapted to crop rotation, four classes of insecticides, and single-trait transgenic corn events. Because of these adaptations, insect resistance management is becoming increasingly necessary to prevent future resistance evolution and mitigate areas with documented resistance. However, a multitude of factors make it increasingly difficult to track rootworm resistance and its spread in a field or across a landscape. These factors include, but are not limited to, the following:

- 1) Field-evolved resistance to some single-trait Bt hybrids (i.e. Cry3Bb1) in multiple locations has developed fairly quickly (within 3 or more generations)
- 2) Single-trait Bt hybrids have been commercialized for 10+ years, allowing for potential gene flow of resistant alleles within a landscape over time
- 3) Multiple tactics have been layered over and used to complement Bt traits (i.e. insecticides, crop rotation, trait rotation, pyramided hybrids) to manage rootworm densities, prevent/slow resistance evolution, or mitigate existing resistance
- 4) Increasing use of pyramided traits may mask the level of resistance to a single-trait hybrid that is present
- 5) The high-dose refuge strategy failed as a stand-alone IRM plan to slow evolution of resistance when less than high-dose products were deployed in the field (Andow et al. 2016)

Because of these factors, a more holistic approach to WCR resistance management is being promoted using Bt hybrids as only one tactic within a management program in order to manage rootworm densities and conduct IRM programs within an IPM framework (Andow et al. 2016, USEPA 2016).

To clarify how the layers of factors listed above may impact WCR response to Bt traits, understanding current single trait susceptibility of WCR field populations at various spatial scales in the landscape is needed. Currently, there is no data addressing spatial variability in susceptibility within a landscape in relation to where various management tactics and technologies have previously been used. This research project characterizes this relationship in several areas of Nebraska, investigating retrospective case histories to inform the use of current products and future technologies that will be deployed (i.e. SmartStax® PRO: triple pyramid of Cry3Bb1 + Cry34/35Ab1 + DvSnf7 dsRNA), as well as helping to support current efforts to develop more holistic WCR resistance management programs conducted within an IPM framework.

1.7. Research Objectives

This thesis focuses on the following main objectives and working hypotheses:

1. Characterize spatial variation in relative WCR susceptibility to Cry3Bb1 and mCry3A from Keith and Buffalo county sampling grids using the Gassmann diagnostic single-plant larval bioassay. The working hypothesis for this objective was: a mosaic of susceptibility to Cry3Bb1 and mCry3A will be documented within each sampling grid.
2. Indirectly document the presence or absence of gene flow of resistant (and/or susceptible) alleles through the landscape. The working hypothesis for this objective was: gene flow of resistant and susceptible alleles will occur in the landscape.
3. Determine the impact of past management strategies on current susceptibility levels to Cry3Bb1 by creating a field history index comprised of weighted past WCR management and agronomic practice values. The working hypothesis for this objective was: past management strategies will significantly impact current susceptibility levels to Cry3Bb1.

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CHAPTER 2: LANDSCAPE VARIATION IN WESTERN CORN ROOTWORM (COLEOPTERA: CHRYSOMELIDAE) SUSCEPTIBILITY TO CRY3 TOXINS IN NEBRAKSA

2.1. Introduction

The western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte, is one of the most serious pests of field corn (*Zea mays* L.) in the United States Corn Belt. North American *Diabrotica* species are responsible for upwards of \$1 billion annually in control costs and yield losses (Metcalf 1986, Rice 2004, Sappington et al. 2006, Dun et al. 2010). Root feeding by WCR larvae can significantly decrease plant growth and biomass by reducing water and nutrient uptake under moderate to high population densities (Godfrey et al. 1993, Gray and Steffey 1998, Urías-López and Meinke 2001, Tinsley et al. 2013). For every node of root injury (Oleson et al. 2005), a 15-17% yield reduction is possible (Dun et al. 2010, Tinsley et al. 2013). Adult feeding on corn silks under some conditions can significantly reduce pollination, also facilitating reduced grain yield (Branson and Krysan 1981, Prystupa et al. 1988, Levine and Oloumi-Sadeghi 1991).

Historically, WCR management programs included crop rotation (i.e. corn-soybean rotation) or chemical control (i.e. foliar- and soil-applied insecticides) in continuous corn. However, WCR populations have evolved resistance to crop rotation (Levine et al. 2002) and four classes of insecticides (Ball and Weekman 1962, Meinke et al. 1998, Pereira et al. 2015). In 2003, transgenic corn expressing the insecticidal protein Cry3Bb1 derived from the soil bacterium *Bacillus thuringiensis* Berliner (Bt) was commercially introduced as a WCR management tactic (USEPA 2010a, Andow et al. 2016). This was followed by the registration of Cry34/35Ab1 (2005), mCry3A (2006),

and eCry3.1Ab (2012) (USEPA 2007, 2010b, 2012a). The efficacy, increased farmer safety, and convenience of transgenic corn versus soil insecticides led to rapid adoption of rootworm Bt hybrids in the U.S. Corn Belt during the mid-2000s (Rice 2004). Widespread adoption of rootworm Bt corn led to a significant reduction in insecticide applications and became the primary management tactic in continuous corn (Moellenbeck et al. 2001, Andow et al. 2016). The pyramided Bt traits Cry3Bb1 + Cry34/35Ab1 (USEPA 2011a), mCry3A + Cry34/35Ab1, (USEPA 2012b, 2012c), mCry3A + eCry3.1Ab (USEPA 2013), and Cry3Bb1 + Cry34/35Ab1 + DvSnf7 (USEPA 2017) are the most recent rootworm-active trait combinations registered in the United States. Currently, single-trait hybrids are gradually being replaced by pyramided rootworm Bt hybrids in the marketplace.

Greater than expected injury (i.e. root injury exceeding one node) as defined by the USEPA (USEPA 2011b, Andow et al. 2016) to Cry3Bb1-expressing Bt hybrids has been documented in different areas of the Corn Belt since 2009 (Gassmann et al. 2011, 2012, 2014, 2016; Gray 2012, 2014; Wangila et al. 2015; Schrader et al. 2016; Ludwick et al. 2017). Miehls et al. (2008) determined that under constant selection pressure in the laboratory, WCR were able to evolve incomplete resistance to Cry3Bb1 in as few as three generations. A similar scenario was later observed in the field as greater than expected injury occurred in fields planted to Cry3Bb1 for three or more consecutive years (Gassmann et al. 2011, 2012, 2014; Wangila et al. 2015). Cross-resistance between Cry3Bb1 and mCry3A or eCry3.1Ab has also been well documented (Gassmann et al. 2011, 2012, 2014; Gray 2012, 2014; Wangila et al. 2015; Zukoff et al. 2016). Incomplete field-evolved resistance of WCR to Cry34/35Ab1 was first observed in 2013 from a few

Iowa locations (Gassmann et al. 2016) and later from a Minnesota population (Ludwick et al. 2017).

Preserving the durability and efficacy of rootworm-active Bt proteins is of primary concern in Nebraska agroecosystems because of the adaptability of WCR to different management tactics. To date, published studies on rootworm resistance to Bt traits have characterized WCR susceptibility primarily from fields exhibiting greater than expected injury to confirm resistance in various locations within a state (Gassmann et al. 2011, 2012, 2014, 2016; Wangila et al. 2015; Shrestha et al. 2016). However, no studies have addressed the general spatial variation in WCR susceptibility to rootworm Bt corn events at the local level (i.e. field to field variation within a county).

Within a landscape, many factors interact in an area over time to determine WCR susceptibility to rootworm Bt traits. Examples include: length of time a single-trait or pyramided traits are used, frequency that other WCR tactics are used to complement transgenic hybrids, rootworm densities in an area, field-evolved resistance to single-trait Bt hybrids, and potential gene flow of resistant alleles. High adoption of single-trait Cry3Bb1 hybrids for 10+ years has placed significant selection pressure on this toxin, leading to greater than expected injury in some areas of Nebraska (Wright and Meinke 2011, Wangila et al. 2015). Cross-resistance between Cry3Bb1 and mCry3A has also impacted the efficacy of each deployed as single rootworm traits as part of commonly used pyramided hybrids. The high efficacy of Cry34/35Ab1 when included with either Cry3Bb1 or mCry3A in a pyramided hybrid may mask field-evolved resistance to Cry3Bb1 or mCry3A, placing significant selection pressure on Cry34/35Ab1.

Collectively, these factors make it increasingly difficult to track the spread of rootworm resistance in an area over time. Therefore, understanding WCR susceptibility to Bt corn events at various spatial scales is important to preserve susceptibility to current technologies and increase understanding of Bt resistance within the landscape.

An initial objective of this research was to characterize spatial variability in susceptibility of WCR field populations to Cry3Bb1 and mCry3A in two intensive corn production areas of Nebraska. The focus was limited to Cry3Bb1 and mCry3A because WCR field-evolved resistance to these traits had been documented in Nebraska (Wangila et al. 2015, Wangila and Meinke 2016). To address this objective, adult sampling grids were established in Keith and Buffalo counties, Nebraska, and single-plant larval bioassays were conducted with progeny from adult field collections to characterize susceptibility to Cry3Bb1 and mCry3A proteins. A second objective was to indirectly document gene flow of resistant or susceptible alleles throughout the grid systems. Beetle movement is an understudied area of WCR biology; therefore, indirectly documenting gene flow would provide insight into understanding the spread of Bt resistance within the landscape. The final objective was to characterize the relationship between past WCR management practices and current WCR susceptibility levels as retrospective real-world case histories to inform the use of current rootworm Bt products and future technologies that will be deployed (i.e. SmartStax® PRO: triple pyramid of Cry3Bb1 + Cry34/35Ab1 + DvSnf7 dsRNA). This was accomplished by collecting and incorporating field histories into correlation and regression analyses to aid in understanding the relationship between past WCR management practices and current WCR susceptibility levels to Cry3Bb1 within both sampling grids. Our hypotheses were as follows: 1) a spatial mosaic of WCR

susceptibility to Cry3Bb1 and mCry3A would be documented within each sampling grid; 2) gene flow of resistant alleles would be evident in both sampling grids; and 3) localized selection pressure and individual field WCR management programs would significantly impact current WCR susceptibility levels.

2.2. Materials and Methods

2.2.1. Sampling Grid System

In 2015, initial sampling grids were established in Keith and Buffalo counties, Nebraska (Fig. 2.1). These counties are two high-yielding, intensive corn production areas of the state. A high proportion of fields within both grids were planted to continuous corn under center pivot irrigation, often producing moderate to high rootworm population densities. Individual fields were systematically selected in 2015 (i.e. 3-8 km apart) to characterize susceptibility across a defined area of each county. Most sites had been planted to corn for at least two years but some sites were selected because Cry3Bb1 or mCry3A expressing Bt hybrids had not been used. Additional field sites were collected in each defined sampling grid to increase the total sample size per grid and to characterize susceptibility on a smaller scale within each grid.

The Keith County grid, located south of Paxton, contained 18 total field sites in a 10 by 20 km (6 by 12 mi) area, with sampling sites 2-8 km (1-5 mi) apart (Fig. 2.2A). The majority of the Keith County grid was characterized by continuous corn planted to Bt-expressing hybrids as single-trait or pyramided products. Cry3Bb1 and mCry3A expressing hybrids were commonly planted for multiple years in many fields in the northern half of the grid, although the southern half contained five fields in which neither

trait had been planted since 2010 (Table 2.1). Previous research confirmed the presence of field-evolved resistance to Cry3Bb1 in areas of Keith County (Wangila et al. 2015).

The Buffalo County grid, located between Gibbon and Kearney, consisted of 16 total field sites in an 11 by 13 km (7 by 8 mi) area; sampling sites were 2-5 km (1-3 mi) apart (Fig 2.2B). Field histories indicated that use of multiple IPM tactics and non-Bt hybrids was more common in the western portion of the grid (Table 2.2). In contrast, the eastern portion of the Buffalo County grid was characterized by continuous Cry3Bb1 use, and greater than expected injury was reported in the northeast portion of the grid in 2013. Similar to the Keith County grid, four fields were included in which Cry3Bb1 or mCry3A expressing hybrids had not been planted since 2010. In both counties, sites not planted to Cry3Bb1 or mCry3A expressing hybrids served as field control sites and survival on Bt toxins in these fields was useful to help determine the potential for gene flow to spread resistant alleles within the landscape.

2.2.2. Adult Collections

Beetles were initially collected from 8 different field sites in Buffalo County (collected 6 August 2015) and 9 different field sites in Keith County (collected 24-25 August 2015). Nine additional field sites were sampled in Keith County (collected 3-4 August 2016) and 8 additional field sites were sampled in Buffalo County (collected 2 September 2016) the following year (Fig. 2.2, Tables 2.1, 2.2). A minimum of 50 gravid females (range: 50-250) were collected per site to obtain an adequate subset of the natural variation present. In conjunction with beetle collection, lateral flow strips (Envirologix Inc., Portland, ME) were used to determine or confirm the presence of Bt traits in each field during both years of sampling. Adults were brought to the laboratory at the

University of Nebraska-Lincoln and maintained by site in plexiglass cages (each 28 cm³). WCR diet consisted of milk-stage sweet corn; food was replaced every 3-4 days and cages were regularly changed to maintain optimal beetle health. A small dish (150mL, 13 cm diameter) of moistened, pre-sifted autoclaved soil was placed in each cage to provide an ovipositional site for gravid females. After oviposition, soil dishes were washed through a U.S.A. Standard Testing Sieve No. 60 (Thermo Fisher Scientific, Waltham, MA) for separation of eggs and soil. Eggs were placed into Petri dishes (Thermo Fisher Scientific, Waltham, MA) containing moistened (ca. 30% by weight), autoclaved, pre-sifted soil. Petri dishes were sealed with Parafilm M (Bemis Company, Inc., Neenah, WI) and were held at 25°C for 1 month, 10°C for 1 month, and 7°C for approximately 4-5 months to allow obligatory diapause development to occur and terminate (Fisher 1989) prior to use in bioassays (Wangila and Meinke 2016).

Four diapausing WCR populations collected before commercialization of Bt corn hybrids in 2003 were used as controls in laboratory bioassays (no prior exposure to Bt corn). Populations were collected from Butler County, Nebraska (1990), Potter County, South Dakota (1995), Finney County, Kansas (2000), and Centre County, Pennsylvania (2000). These populations were maintained at the USDA North Central Agricultural Research Laboratory in Brookings, South Dakota. The same four populations were used as controls in bioassays during 2016 and 2017.

A WCR field population from the Eastern Nebraska Research and Extension Center, Ithaca, Nebraska, was also used as a field control in bioassays. Large areas of continuous corn without rootworm Bt traits were annually planted at ENREC so a large

refuge was maintained at the site. Hybrids expressing rootworm-active traits were only periodically planted in small-plot research trials.

2.2.3. Single-Plant Larval Bioassays

Single-plant larval bioassays following the procedure described by Gassmann et al. (2011) and adapted by Wangila et al. (2015) were used to document spatial variation in susceptibility to Cry3Bb1 and mCry3A in both sampling grids. Bioassays were conducted at the University of Nebraska-Lincoln in spring and summer of 2016 and 2017. Control populations were assayed at various time points throughout the period Keith and Buffalo county bioassays took place. During both years, Cry3Bb1 (Stone 6021VT3) and mCry3A (Syngenta N68B-3000GT) expressing hybrids and their non-transgenic near-isolines (Stone 6021RR2 and Syngenta N68B-GT, respectively), hereafter termed isoline, were used in bioassays. A neonicotinoid seed treatment was applied to all seeds at 0.25 mg (AI) per kernel and was removed prior to the bioassay using the procedure described in Gassmann et al. (2011).

Corn plants were grown in the greenhouse in a 1:1 mixture of Sun Gro[®] Metro-Mix[®] 900 Grower Mix and Sun Gro[®] Sunshine[®] LC1 Grower Mix (Sun Gro Horticulture, Inc., Agawam, MA) following the methods outlined in Wangila et al. (2015). Moistened soil was placed in 32 ounce clear plastic containers (Johnson Paper & Supply Co., Minneapolis, MN), and water was applied in an equal volume per pot as needed using a 60mL catheter-tipped syringe (Covidien LLC, Mansfield, MA). One seed was planted per pot, and 12 pots of each hybrid were organized in a randomized complete block design. Supplemental lighting (400W 208 volt Jasonod light bulbs, P. L. Light Systems,

Beamsville, ON, Canada) was provided as needed to reach a 14:10 (L:D) h cycle.

Average daily low and high temperatures ranged from 19.9-33.2°C and 18.7-30.8°C in 2016 and 2017, respectively. MiracleGro® Water Soluble All Purpose Plant Food (The Scott's Company LLC, Pacific Junction, IA) was applied at the V2 growth stage at a ratio of 4mg: 1mL of water; 100mL was applied to each plant.

Bioassays were conducted with V4 to V5 growth stage plants (Abendroth et al. 2011). Each plant was infested with 12 neonate larvae (i.e. less than 24h after hatch). A small hole was dug to expose root tissue, and each larva was individually placed on the roots with a soft hair brush size 20/0. Roots were re-covered with soil after infestation. Plant foliage was cut 20cm above the soil prior to placement in growth chambers (Percival Scientific Inc., Perry, IA) at 24°C with a 14:10 (L:D) h photoperiod. Preliminary bioassays and results from Wangila et al. (2015) determined that under these growth chamber conditions, terminating feeding on day 17 would ensure larvae did not reach the pre-pupal stage. Plants were organized in a randomized complete block design in growth chambers to account for variability in temperature and lighting. To facilitate larval development, 50mL of water was not applied until 3 days post-infestation and applied every other day thereafter to maintain corn vigor and protein expression.

After 17 days, the remaining above-ground plant mass was cut off at the soil line before the root mass was placed into a Berlese funnel (40W, 120V bulbs – Philips Lighting Company, Worcester, MA) for larval recovery. Root masses were gently separated to increase light and heat penetration to maximize larval recovery. Clear glass jars (Solo Cup Company, Lake Forest, IL) containing 70% ethyl alcohol were attached to

each funnel. After four days, jars containing larval survivors were brought to the laboratory and counted.

2.2.4. Field History Index

Field histories dating back to 2010 were collected in 2015 and 2016 to evaluate the potential impact of past management practices on current WCR susceptibility levels to Cry3 toxins (Tables 2.1, 2.2). The exceptions were two field sites in Buffalo County where field histories were unavailable. To test the hypothesis that management tactics applied at the local level (i.e. individual field and nearby fields) significantly influence susceptibility, a field history index was created from weighted past WCR management variables and agronomic practices and assigned to individual fields in each grid (Table 2.3). The rating system was created to quantify the potential level of selection pressure placed on a population by the past management tactics deployed within individual fields. The overall hypothesis was that as index values increase, WCR susceptibility to Cry3Bb1 or mCry3A will decrease. Values used to create the field history index were additive and based on current knowledge of the agronomic system in Nebraska and the potential effects of management practices on WCR susceptibility levels to Cry3 toxins gleaned from published literature.

The first step when assigning an index value to a specific field was to determine the Bt traits planted from 2010 to collection year and assign hybrid values per year (Table 2.3). A value of 1.0 was assigned for single-trait Cry3-expressing hybrids and a value of 0.5 was assigned for pyramided hybrids containing Cry3 toxins per year. These values were weighted because evolution of resistance often occurs faster with single-trait versus pyramided hybrids (Onstad and Meinke 2010). Cry3Bb1 and mCry3A were treated

equally because of cross-resistance between them (Gassmann et al. 2014, Wangila et al. 2015, Zukoff et al. 2016). Popcorn, conventional hybrids, and single-trait Cry34/35Ab1 were assigned a value of 0.1. Although selection pressure for Cry3Bb1 or mCry3A toxins is absent when non-rootworm Bt hybrids or Cry34/35Ab1 expressing hybrids are planted, resistant alleles present from prior selection should be maintained in WCR densities produced (Wangila and Meinke 2016). Popcorn is usually planted after rotation from a non-WCR host in Nebraska (L. J. Meinke, personal observation), so no selection pressure would be applied and few WCR would be produced. In addition to yearly hybrid values, fields planted to Cry3-expressing hybrids for 3 consecutive years were assigned 3 additional points to account for a potential susceptibility shift. This was based on multiple field reports of field-evolved resistance to Cry3Bb1 after planting Cry3Bb1 expressing hybrids for 3 or more consecutive years (Gassmann et al. 2011, Wangila et al. 2015). An additional value of 1.0 or 0.5 was assigned for each consecutive year a single-trait or pyramided Cry3 hybrid was planted after the initial 3 years of selection, respectively.

The rotation of field corn with a non-host plant, such as soybeans (*Glycine max* L.) or pinto beans (*Phaseolus vulgaris* L.), is an effective WCR management tactic in the western Corn Belt because oviposition takes place primarily in corn and eggs overwinter in the soil until the following growing season (Meinke et al. 2009, Spencer et al. 2009). When crop rotation occurred after 2010, all index values previously assigned were excluded from the total index summary; values were only assigned to years following rotation. Crop rotation “resets” the field—first-year cornfields often have undetectable to very low WCR populations, eliminating most resistant alleles within the field.

The impact of potential beetle movement from surrounding fields was also taken into account in the field history index (Table 2.3). The area effect category of the field history index is based on incomplete knowledge of all fields, but recolonization of first-year cornfields routinely occurs (Godfrey and Turpin 1983). The potential of WCR exhibiting trivial or migratory flight may facilitate gene flow among fields (Godfrey and Turpin 1983, Prystupa et al. 1988, Isard et al. 2004, Spencer et al. 2009). Fields within one mile of a potential resistant population were assigned a value of 1.0, while fields within one mile of a potential susceptible population were deducted a value of 1.0. If multiple resistant populations and one known susceptible population occurred within one mile, a value of 0.5 was added; if multiple susceptible populations and one resistant population occurred within one mile, a value of 0.5 was deducted.

2.2.5. Data Analysis

Proportional survival bioassay data were fit using a beta distribution and analyzed using a two-way analysis of variance (ANOVA; PROC GLIMMIX in SAS 9.4) to compare mean proportional survival between each Bt and its associated isoline hybrid. A mean lab control value from the four lab control populations was used in the analyses as preliminary analyses indicated no significant differences in proportional survival on Cry3Bb1 or mCry3A expressing hybrids occurred among lab control populations within bioassay years (Cry3Bb1: 2016: $F=0.66$; $df=3, 44$; $P=0.5785$; 2017: $F=0.38$; $df=3, 44$; $P=0.7652$; mCry3A: 2016: $F=0.86$; $df=3, 44$; $P=0.4693$; 2017: $F=1.43$; $df=3, 44$; $P=0.2461$; Appendix 1). When analyzing proportion or percentage data, the response is a continuous variable with a minimum value of 0 and a maximum value of 1. In biological datasets, data with a restricted range from 0-1 are often fit to a beta distribution (Ferrari

and Cribari-Neto 2004; Stroup 2013, 2015). Fixed factors included population (field site), corn hybrid (Bt or respective isoline), and the interaction between population and corn hybrid. The LSMEANS statement was used to compare two different means using a t-test at $P=0.05$ significance level to identify significant treatment differences. The SLICEDIFF option in PROC GLIMMIX was used to determine significant differences in mean survivorship between isoline and Bt hybrids within a population and to compare survival on a given Bt hybrid among populations. Tukey's adjustment for multiple comparisons was used to control type I error.

Variation in susceptibility on a broad spatial scale (i.e. across groups of fields within the grid) was also evaluated in each grid. Fields in the Keith County grid were divided into north versus south areas and the Buffalo County grid into east versus west areas based on trends obtained from past trait use histories compiled for each grid. Greater use of Cry3Bb1 and mCry3A expressing hybrids occurred in the northern area of Keith grid and eastern half of the Buffalo grid. A one-way analysis of variance (ANOVA) using PROC GLIMMIX (SAS 9.4) was conducted to determine geographic differences in susceptibility between categories in each grid. Proportional survival data for Cry3Bb1 and mCry3A expressing hybrids were used in these analyses; analyses for both traits were conducted separately, and the data were fit using a beta distribution. Geographic location (i.e. area) was included in the model as a fixed factor. The LSMEANS statement was used to identify the mean and standard error of each area of the sampling grid, and the PDIFF option was used to determine significant differences between locations.

To evaluate local variation in susceptibility on Cry3Bb1 and mCry3A and create geographical maps documenting this variation, pooled 2016-2017 survivorship data was analyzed using a one-way analysis of variance (ANOVA; PROC GLIMMIX in SAS 9.4) and fit using a beta distribution. Data from the isoline hybrids and controls were excluded. Analyses for Cry3Bb1 and mCry3A were conducted separately for each county grid. Population was included in the model as a fixed factor. The LSMEANS statement with the PDIFF option was used to identify significant differences in survivorship among populations. The LINES option presented results of pairwise comparisons from the LSMEANS statement to display significant and non-significant differences in survivorship among all populations in each grid. Tukey's adjustment for multiple comparisons was used to control type I error.

A simple linear regression analysis of field history index values on proportional survival was conducted to quantify the relationship between past management and current susceptibility levels on Cry3Bb1 using PROC GLIMMIX (SAS 9.4). Because the data are proportions in a limited range from 0-1, the data were subsequently fit using a beta distribution. Datasets from the Keith and Buffalo county grids were analyzed separately. Initial analyses indicated no significant differences in survivorship among years in Keith ($F=0.001$; $df= 1, 16$; $P=0.9862$) and Buffalo counties ($F=0.75$; $df=1, 11$; $P=0.4065$); therefore, data from 2016 and 2017 were combined into a single dataset for regression analysis. A correlation analysis using PROC CORR (SAS 9.4) was also conducted to determine the strength of the relationship between field history index value and proportional survival. Kendall's tau coefficient was used due to the non-parametric nature of the dataset and because the data does not satisfy the assumptions required when

using the Pearson correlation coefficient. Additionally, the data does not exhibit a monotonic relationship required to use Spearman's rank correlation coefficient.

A scoring analysis was used to predict survivorship values from 2017 bioassays based on survivorship and field history index data from 2016 bioassays. Pooled Keith and Buffalo county bioassay data were categorized by year to use 2016 data to test on 2017 data. A dataset containing only 2016 values was created, and a model was fit to this data using a simple linear regression analysis (PROC GLIMMIX; SAS 9.4). The STORE statement was used to save the context and results of the analysis. PROC PLM was used to score the 2017 dataset using the previous model fit to the 2016 dataset. Predicted survivorship values were calculated based on the model produced by the 2016 dataset. Actual versus predicted survivorship was plotted using PROC GPLOT, and PROC CORR was used to determine the strength of relationship between the predicted and actual survivorship values.

Results provided in the text were fit using a beta distribution and transformed back to the data scale using the ILINK option in the LSMEANS statement. Figures present data on the logit scale (basis of beta distribution) with corresponding proportional survival on the secondary y-axis as a reference.

2.3. Results

2.3.1. Survivorship on Transgenic and Non-Transgenic Hybrids

Keith County Grid Cry3Bb1 Bioassays. A significant interaction between population (field site) and hybrid occurred for populations assayed on Cry3Bb1 and its non-Bt near-isoline in 2016 ($F=7.40$; $df=10, 314$; $P<0.0001$) and 2017 ($F=13.92$; $df=11,$

336; $P < 0.0001$) (Fig. 2.3). A significant difference in mean survivorship on Cry3Bb1 was documented among populations in both years. Mean survivorship on Cry3Bb1 ranged from 9-47% and 4-68% in 2016 and 2017, respectively. Mean survival of one population was not significantly different than the lab control each year. In 2016 bioassays, mean survival of 6 of 9 populations was not significantly different than mean survival of the field control, while only one field exhibited this characteristic in 2017 bioassays (Fig. 2.3). Within populations, mean survival on the isoline was significantly greater than survival on Cry3Bb1 in 5 of 9 populations in 2016, and 9 of 10 populations in 2017 (Fig. 2.3). Mean survival between hybrids was not significantly different in the remaining 2016 and 2017 populations.

Keith County Grid mCry3A Bioassays. The results of mCry3A bioassays followed a pattern similar to that observed in Cry3Bb1 bioassays. A significant interaction occurred between population and hybrid for populations assayed on mCry3A and its isoline in 2016 ($F=19.82$; $df=10, 306$; $P < 0.0001$) and 2017 ($F=15.55$; $df=11, 336$; $P < 0.0001$) (Fig. 2.4). Significant differences in mean survivorship occurred among populations, with survival levels ranging from 22-67% in 2016 and 6-58% in 2017. In 2016, all populations exhibited significantly greater survival on mCry3A compared to the lab control (Fig. 2.4). A similar trend was observed in 2017 except for one population that was not significantly different than the lab control. Mean survival of 2 of 9 populations was not significantly different from the field control in 2016 bioassays, while mean survival of 4 of 10 populations was not significantly different from or significantly lower than the field control in 2017 bioassays (Fig. 2.4). Within populations, mean survival on the isoline compared to Cry3Bb1 was not significantly different in 5 of 9

populations in 2016 bioassays; two populations also exhibited significantly greater survival on mCry3A compared to the isoline (Fig. 2.4). Significant differences in mean survival between mCry3A and its isoline were detected in 6 of 10 populations in 2017 bioassays.

Buffalo County Grid Cry3Bb1 Bioassays. A significant interaction between population and hybrid was documented in populations assayed in 2016 ($F=13.32$; $df=9, 284$; $P<0.0001$) and 2017 ($F=10.00$; $df=9, 284$; $P<0.0001$) (Fig. 2.5). The general pattern detected among populations in Buffalo County was consistent with the results from Keith County. Mean survivorship ranged from 8-38% in 2016 and 11-40% in 2017. Fifteen of sixteen populations assayed exhibited significantly higher survival on Cry3Bb1 compared to the lab control (Fig 2.5). Compared to the field control, 4 of 8 populations from 2016 and 1 of 8 populations from 2017 exhibited similar survival on Cry3Bb1 (Fig. 2.5). Within populations, mean survival on the isoline was significantly greater than survival on Cry3Bb1 in 4 of 8 populations assayed in 2016 and all populations assayed in 2017 (Fig. 2.5).

Buffalo County Grid mCry3A Bioassays. A significant interaction between population and hybrid occurred for populations assayed on mCry3A and its isoline in 2016 ($F=9.69$; $df=9, 284$; $P<0.0001$) and 2017 ($F=16.57$; $df=9, 284$; $P<0.0001$) (Fig 2.6). Mean survivorship ranged from 14-45% in 2016 and 26-46% in 2017. All populations exhibited significantly greater survival on mCry3A compared to the lab control in 2016 and 2017 assays. In 2016 assays, only 1 of 8 populations exhibited significantly greater survival on mCry3A compared to the field control; in 2017, this occurred in 3 of 8

populations (Fig. 2.6). Within populations, mean survival was significantly greater on isoline than mCry3A in 5 of 8 populations and 6 of 8 populations in 2016 and 2017, respectively (Fig 2.6).

2.3.2. Geographic Variation in Cry3 Susceptibility

Keith County Grid, large scale variation. The main effect of geographic location was highly significant for both Cry3Bb1 ($F=74.42$; $df=1, 226$; $P<0.0001$) and mCry3A ($F=84.14$; $df=1, 226$; $P<0.0001$) susceptibility within the Keith County grid. Survivorship on Cry3Bb1 in nine field sites on the south side of the grid was significantly lower (0.160 ± 0.02) than eight field sites on the north side of the grid (0.394 ± 0.02) (Fig. 2.7). A similar trend was documented with mCry3A susceptibility (0.264 ± 0.02 versus 0.541 ± 0.02 , respectively) (Fig. 2.8).

Buffalo County Grid, large scale variation. As in the Keith County grid, the main effect geographic location significantly affected survivorship on both Cry3Bb1 ($F=9.29$; $df=1, 182$; $P=0.0026$) and mCry3A ($F=9.17$; $df=1, 182$; $P=0.0028$). Seven fields located on the west side of the grid exhibited lower survivorship on Cry3Bb1 (0.184 ± 0.02) and mCry3A (0.269 ± 0.02) compared to nine fields on the east side of the grid (0.260 ± 0.02 and 0.350 ± 0.02 on Cry3Bb1 and mCry3A, respectively) (Figs. 2.9, 2.10).

Keith County Grid, small scale variation. The main effect of population on mean survivorship was significant for both Cry3Bb1 ($F=10.96$; $df=18, 209$; $P<0.0001$) and mCry3A ($F=13.79$; $df=18, 209$; $P<0.0001$) (Figs. 2.7, 2.8).

Buffalo County Grid, small scale variation. As in Keith County, the main effect of population on mean survivorship was significant for both Cry3Bb1 ($F=6.24$; $df=15, 168$; $P<0.0001$) and mCry3A ($F=4.01$; $df=15, 168$; $P<0.0001$) (Figs. 2.9, 2.10).

2.3.3. Regression Analyses of Field History Index Values on Proportional Survival

Keith County Grid. A simple linear regression of field history index values on proportional survival revealed the independent variable history had a significant effect on proportional survival ($F=11.90$; $df=1, 17$; $P=0.0031$) (Fig. 2.11A). The estimated regression equation based on the data was $(-1.5580 + 0.1209 \cdot \text{field history index value})$. Results from the correlation analysis indicated a moderate positive relationship between field history index value and proportional survival ($r=0.547$, $P=0.0011$).

Buffalo County Grid. Results from the simple linear regression indicated the independent variable history had a significant effect on proportional survival ($F=5.83$; $df=1, 12$; $P=0.0326$) (Fig. 2.11B). The estimated regression equation based on the data was $(-1.3989 + 0.01129 \cdot \text{field history index value})$. Results from the correlation analysis indicated a weak positive relationship between field history index value and proportional survival ($r=0.322$, $P=0.1113$).

2.3.4. Predicting 2017 Survivorship Based on 2016 Survivorship Data

Linear regression analysis of the 2016 dataset produced the linear model $(-1.2414 + 0.05161 \cdot \text{field history index value})$ used to predict 2017 survivorship values (Figure 2.12A). Predicted survivorship values were calculated and plotted against actual survivorship values from 2017 field sites in Keith and Buffalo counties (Fig. 2.12B). The estimated regression equation from the predicted versus actual survivorship values was

($0.2133 + 0.2023 \times \text{field history index value}$). Correlation analyses indicated a strong positive relationship between predicted and actual survivorship values ($r=0.777$, $P=0.0001$).

2.4. Discussion

The frequency of individuals surviving Cry3Bb1 and mCry3A exposure within Keith and Buffalo county grids consistently ranged from low to very high within years. Incomplete resistance was documented in many populations, as survival on the isoline was significantly greater than survival on the transgenic hybrid (Carrière and Tabashnik 2001, Gassmann et al. 2009, Carrière et al. 2010, Meihls et al. 2012, Tabashnik et al. 2014) (Figs. 2.3, 2.4, 2.5, 2.6). A few WCR populations also exhibited complete resistance, or equivalent survival on the isoline and rootworm Bt hybrid (Gassmann et al. 2016). When viewed spatially, proportional survival data document a mosaic of WCR susceptibility to both Cry3Bb1 and mCry3A toxins within the landscape. This pattern was observed for both toxins in 2016 and 2017 bioassays in each grid (Figs. 2.7, 2.8, 2.9, 2.10).

Large scale spatial trends were evident in different sections of each grid. The northern portion of the Keith County grid (i.e. north of Road 50) was dominated by continuous corn containing Cry3Bb1 or mCry3A expressing proteins. The southern portion of this grid was characterized by some continuous corn containing Cry3 toxins, but many fields had never been planted to rootworm-active Bt hybrids. North-south differences in WCR susceptibility were apparent, as survival on the transgenic hybrid was approximately 2.5x and 2.0x greater on Cry3Bb1 and mCry3A, respectively,

compared to fields in the southern portion of the grid (Figs. 2.7, 2.8). A similar spatial trend in trait use and susceptibility was also exhibited when moving east to west within the Buffalo County grid. Reduced susceptibility was documented in the eastern portion of the grid, as larval survival was approximately 1.4x greater on Cry3Bb1 (Fig. 2.9) and 1.3x greater on mCry3A (Fig. 2.10) compared to larval survival in the western portion of the grid. Large-scale differences in survival in each grid likely occurred as a result of high market penetration of Cry3Bb1 or mCry3A hybrids over time, facilitating evolution of multiple point sources of resistance through these areas (i.e. patchy neighborhood resistance problem).

Spatial variability in WCR susceptibility to Cry3 toxins was also evident on a smaller scale within each grid. For example, within the Keith County grid, a high proportion of individuals from field 16 survived exposure to Cry3Bb1 (Fig. 2.3B). Hybrids expressing Cry3Bb1 and mCry3A were frequently planted in this field (Table 2.1), placing continued selection pressure on this WCR population. In contrast, Cry3Bb1 survivorship of WCR larvae from the surrounding fields (i.e. fields 3, 11, and 12) was significantly lower compared to field 16. A similar scenario was evident in Buffalo County when comparing field 15 (high WCR Cry3Bb1 survival, mCry3A history) to fields 8, 12, and 14 (lower WCR survival on Cry3Bb1) (Figs. 2.5, 2.9). Thus in both grids, the susceptibility of WCR populations to either Cry3Bb1 or mCry3A in close proximity could be very different.

A number of factors probably contributed to the observed spatial mosaic of WCR susceptibility. WCR larvae from most sites without previous trait exposure exhibited

significantly greater survivorship on Cry3Bb1 (Keith County fields 1, 2, 10, and 11; Buffalo County fields 1, 2, 3, and 5) (Figs. 2.3, 2.5; Tables 2.1, 2.2) and mCry3A (Keith County field 1 (2016)) (Fig. 2.4) expressing hybrids than the lab control. This increased survivorship in fields planted with non-rootworm Bt hybrids or single-trait Cry34/35Ab1 hybrids (Tables 2.1, 2.2) indirectly indicates some gene flow of resistant alleles into these sites occurred as a result of beetle movement. However, local field to field variation (i.e. small-scale) in WCR susceptibility observed in this study indicates large-scale beetle movement did not cause resistant alleles to inundate each grid (i.e. create uniform resistance levels over the landscape). This result and the fact that WCR from two fields without trait history (Keith County fields 1 (2016) and 3) (Fig. 2.3) exhibited susceptibility levels that were not significantly different than lab controls collectively suggest that individual fields can be managed within the landscape to slow the evolution of resistance.

The small-scale survival data coupled with known trait histories do provide some examples where possible local movement and associated gene flow can be inferred to have affected WCR susceptibility in individual fields. Cry3Bb1 and mCry3A expressing hybrids were planted in Keith County field 6 consecutively from 2010 to 2016. However, the relative susceptibility of WCR to Cry3Bb1 in the field 6 population was greater than expected based on trait history. This suggests that gene flow of susceptible alleles from surrounding fields (field 2 to the north, south unknown, Fig. 2.3A) may have slowed the evolution of resistance in field 6. Field 2, and two adjacent pivots (not sampled) located one mile north of field 6, had never been planted with Cry3Bb1 or mCry3A expressing hybrids.

WCR are able to recolonize fields quickly after crop rotation or adult management strategies are implemented if a source population is nearby (Pruess et al. 1974, Godfrey and Turpin 1983, Meinke et al. 2009). Therefore, beetle movement into first-year cornfields could have two significant impacts: 1) in areas with field-evolved resistance, migration could significantly negate the impact of crop rotation to slow or mitigate resistance evolution; or 2) migration of susceptible individuals into a neighboring population could reduce the proportion of resistant alleles present, slowing resistance evolution. Recolonization after crop rotation was documented in the Buffalo County grid, specifically in field 4. This field was rotated to soybeans in 2011 and 2014 in an attempt to mitigate resistance to Cry3Bb1. However, continuous Cry3Bb1 use in the area directly adjacent to but outside the sampled grid area allowed recolonization to occur quickly. Many individuals from field 4 survived exposure to Cry3Bb1 in 2016 laboratory bioassays, indicating a high frequency of resistant alleles were present in the immigrant population (Fig 2.5A). The survival rate in field 4 was not expected based on recent rootworm management history.

Rootworm densities in each field may have contributed to the small-scale differences in WCR susceptibility observed in some parts of each grid. Within the Nebraska agroecosystem studied, large monocultures of continuous corn under center pivot irrigation facilitate the buildup of WCR densities over time, potentially affecting gene flow in a positive or negative way. Large densities of a resistant population in close proximity to large densities of a relatively susceptible population may make it difficult for gene flow from either field to significantly alter the resistance allele frequency in each field. Immigrant gene flow may get swamped out by a large resident allele frequency. A

possible example of this could have been Keith County field 16 versus fields 3 and 11, which were within 1.5 miles of each other. Field 16 was highly resistant to Cry3Bb1 and had high WCR densities. In contrast, WCR were ineffectively managed with insecticides in fields 3 and 11, leading to large annual WCR densities but had not been planted with Cry3Bb1 or mCry3A expressing hybrids. Because much WCR dispersal is trivial (Coats et al. 1986, Naranjo 1990, Isard et al. 2004, Stebbing et al. 2005) and many WCR probably remain in their natal field (Meinke et al. 2009, Spencer et al. 2009) in large continuous cornfields, large densities may reinforce/retain the level of selection pressure that is present in each field.

Cross-resistance was documented between Cry3Bb1 and mCry3A in all WCR populations. However, the ratio of cross-resistance between the two toxins was not always 1:1. Variation in cross-resistance has been previously documented in recent literature (Gassmann et al. 2016, Zukoff et al. 2016). Gassmann et al. (2016) proposed that multiple mechanisms of resistance to Cry3Bb1 potentially exist, and only some confer cross-resistance to mCry3A. Therefore, it is possible that uneven cross-resistance may have also contributed to significant variation in susceptibility to Cry3Bb1 and mCry3A.

Regression analyses of field history index values on proportional survival indicated a significant effect of field history in determining current susceptibility levels to Cry3Bb1 in the landscape. Using 2016 survivorship data as a model to predict 2017 survivorship resulted in a significant linear regression explaining approximately 60% of the variation in predicted values (Fig. 2.12B). A strong positive correlation between

actual and predicted survivorship values reinforced the importance of field history and suggests that the field history index concept may be a useful way to predict current susceptibility levels in the field.

Management practices in Buffalo County grid fields contrast greatly with many Keith County grid fields. More IPM practices such as use of crop rotation, Bt trait rotation, and use of non-rootworm Bt hybrids were used in portions of the Buffalo County grid than recorded for many Keith County fields. As a result, few history index values exceeding 4.0 were assigned to Buffalo County grid fields (Table 2.2). In order to strengthen the field history index concept as a potential predictive model, sensitivity analyses and further testing to determine optimum values for each specific criteria are needed. Potential limiting factors in these analyses included unequal distribution of field history index values (need more high and low index value fields), natural variation within an individual WCR population, potential variation in gene flow among fields, and a relatively low number of sampling sites per grid. Adding variables such as WCR population density and use of insecticides as management tactics may also improve the field history index to explain greater variation in survivorship.

In summary, this study documents significant variation in WCR susceptibility to Cry3Bb1 and mCry3A on different spatial scales within two different sampling grids in Nebraska counties. At the local level, results indicate that cornfields in close proximity may support WCR populations with very different susceptibility levels. Therefore, hypothesis 1 is accepted. Comparison of WCR susceptibility in fields without Cry3Bb1 or mCry3A history to fields with trait histories or lab controls enable inferences to be

made that suggest gene flow of resistant or susceptible alleles occurred in each grid. Therefore, hypothesis 2 is accepted. The impact of gene flow on local susceptibility appeared to vary with individual fields/populations. Based on susceptibility and field history analyses, management tactics and associated selection pressure appear to be key drivers of susceptibility in these grids, supporting the third hypothesis. With refinement, the field history index concept may serve as a potential tool to predict susceptibility in areas of the western Corn Belt. Individual fields and larger areas in this study can be viewed as retrospective case histories that can inform development of best management practices to delay or mitigate resistance evolution. Results from this study support current recommendations to slow the evolution of resistance or mitigate existing resistance by using a multi-tactic approach to manage WCR densities within an IPM framework (Andow et al. 2016, USEPA 2016).

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Table 2.1. Yearly trait history of field sites and total index value of collected populations in Keith County in 2015 and 2016.

| Year Collected | Field | Trait History | | | | | | | Index ⁺ | |
|----------------|-------------|---------------|-----------|-----------|-----------|-----------|-------------|-----------|--------------------|-------|
| | | 2016 | 2015 | 2014 | 2013 | 2012 | 2011 | 2010 | | |
| 2015 | 1 | X | No traits | No traits | No traits | No traits | No traits | No traits | No traits | 0.6 |
| | 2 | X | No traits | No traits | No traits | No traits | No traits | No traits | No traits | 1.6 |
| | 3 | X | No traits | No traits | No traits | No traits | No traits | No traits | No traits | 0.1 |
| | 4 | X | mCry3A* | mCry3A | Soybeans | Soybeans | mCry3A | mCry3A | NA | 1 |
| | 5 | X | mCry3A | mCry3A | Popcorn | Popcorn | NA | NA | NA | 2.1 |
| | 6 | X | Cry3Bb1* | mCry3A* | Cry3Bb1 | Cry3Bb1 | Cry3Bb1 | Cry3Bb1 | Cry3Bb1 | 9 |
| | 7 | X | Cry3BB1 | Cry3Bb1 | Cry3Bb1* | Cry3Bb1* | Cry34/35Ab1 | Popcorn | Soybeans | 6.7 |
| | 8 | X | Cry3Bb1 | mCry3A | Cry3Bb1 | Cry3Bb1 | mCry3A | No traits | Popcorn | 9.2 |
| | 9 | X | mCry3A | mCry3A | mCry3A | mCry3A | NA | NA | NA | 6 |
| 2016 | 1 | No traits | No traits | No traits | No traits | No traits | No traits | No traits | No traits | 0.7 |
| | 2 | Cry34/35Ab1 | No traits | No traits | No traits | No traits | No traits | No traits | No traits | 1.7 |
| | 10 | Cry34/35Ab1 | No traits | No traits | No traits | No traits | No traits | No traits | No traits | 1.2 |
| | 11 | No traits | No traits | No traits | No traits | No traits | No traits | No traits | No traits | 0.2 |
| | 12 | mCry3A* | Cry3Bb1* | Cry3Bb1 | Cry3Bb1* | Cry3Bb1* | Popcorn | Soybeans | mCry3A | 6.6 |
| | 13 | Cry3Bb1* | Cry3Bb1* | Cry3Bb1 | Cry3Bb1 | Cry3Bb1 | Cry3Bb1 | Cry3Bb1 | Cry3Bb1 | 10.75 |
| | 14 | mCry3A | Cry3Bb1* | Cry3Bb1* | mCry3A | No traits | No traits | Cry3Bb1 | Cry3Bb1 | 9.6 |
| | 15 | mCry3A | No traits | mCry3A* | Cry3Bb1* | Cry3Bb1* | Cry3Bb1 | Cry3Bb1* | Cry3Bb1 | 8.6 |
| 16 | Cry34/35Ab1 | Cry3Bb1* | Cry3Bb1 | Cry3Bb1* | Cry3Bb1* | Cry3Bb1 | Cry3Bb1 | mCry3A | 10.6 | |
| 17 | Cry3Bb1* | Cry3Bb1* | Cry3Bb1* | Cry3Bb1 | Cry3Bb1 | Cry3Bb1 | No traits | Cry3Bb1 | 9.6 | |

* indicates trait was part of pyramided hybrid also containing Cry34/35Ab1

No traits includes conventional corn or non-rootworm Bt hybrids

NA indicates field history not available for specified year

+ Criteria for total field history index values are available in Table 2.3.

Table 2.2. Yearly trait history of field sites and total index value of collected populations in Buffalo County in 2015 and 2016.

| Year Collected | Field | Trait History | | | | | | | | | Index ⁺ |
|----------------|-------|---------------|-------------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------|--------------------|
| | | 2016 | 2015 | 2014 | 2013 | 2012 | 2011 | 2010 | | | |
| 2015 | 1 | X | No traits | No traits | No traits | No traits | No traits | No traits | No traits | No traits | 0.6 |
| | 2 | X | No traits | No traits | No traits | Soybeans | Cry34/35Ab1 | No traits | No traits | No traits | 0.2 |
| | 3 | X | No traits | Cry34/35Ab1 | No traits | No traits | No traits | Soybeans | NA | NA | 0.4 |
| | 4 | X | No traits | Soybeans | Cry3Bb1 | Cry3Bb1 | No traits | Soybeans | Cry3Bb1 | Cry3Bb1 | 1.1 |
| | 5 | X | No traits | No traits | No traits | No traits | Soybeans | Soybeans | No traits | No traits | 0.3 |
| | 6 | X | mCry3A | mCry3A | mCry3A | No traits | No traits | Soybeans | No traits | Soybeans | 2.2 |
| | 7 | X | NA | NA | NA | NA | NA | NA | NA | NA | X |
| | 8 | X | NA | NA | NA | NA | NA | NA | NA | NA | X |
| 2016 | 9 | mCry3A | No traits | No traits | No traits | No traits | No traits | No traits | No traits | No traits | 1.6 |
| | 10 | mCry3A | No traits | Soybeans | Soybeans | Cry3Bb1 | No traits | No traits | NA | NA | 1.1 |
| | 11 | mCry3A | mCry3A | No traits | No traits | Soybeans | No traits | No traits | No traits | No traits | 2.1 |
| | 12 | Cry3Bb1* | No traits | mCry3A | mCry3A | Soybeans | Cry3Bb1 | Cry3Bb1 | No traits | No traits | 2.6 |
| | 13 | mCry3A | mCry3A | No traits | Soybeans | Soybeans | Cry3Bb1 | Cry3Bb1 | NA | NA | 2.1 |
| | 14 | Cry3Bb1* | Cry3Bb1* | No traits | No traits | No traits | No traits | No traits | No traits | No traits | 1.5 |
| | 15 | mCry3A | Cry3Bb1*, mCry3A* | mCry3A | mCry3A | mCry3A | No traits | No traits | Cry3Bb1 | Cry3Bb1 | 9.6 |
| | 16 | mCry3A | No traits | No traits | No traits | No traits | No traits | No traits | Cry3Bb1 | Cry3Bb1 | 3.4 |

* indicates trait was part of pyramided hybrid also containing Cry34/35Ab1
 No traits includes conventional corn or non-rootworm Bt hybrids
 NA indicates field history not available for specified year
 + Criteria for total field history index values are available in Table 2.3.

Table 2.3. Field history index values and the criteria used to assign values to individual fields.

| Category | Index Value* | Criteria |
|---|--------------|--|
| Hybrid Values (assigned yearly) | 0.1 | Popcom, conventional hybrid, or single-trait Cry34/35Ab1 hybrid planted |
| | 0.5 | Pyramided hybrid containing Cry3Bb1 or mCry3A planted |
| | 1 | Single-trait hybrid containing Cry3Bb1 or mCry3A planted |
| Selection Values (assigned as necessary) | 3 | Three consecutive years of a single-trait or pyramided hybrid containing Cry3Bb1 or mCry3A |
| | 0.5 | Pyramided hybrid containing Cry3Bb1 or mCry3A planted after 3 consecutive years (yearly) |
| | 1 | Single-trait hybrid containing Cry3Bb1 or mCry3A planted after 3 consecutive years (yearly) |
| Crop Rotation | RESET | When crop rotation occurred after 2010, all previous history values were excluded (i.e. reset to 0). Values were assigned the year(s) following rotation. |
| Area Effect | 0.5 | Multiple resistant populations and one susceptible population within 1 mile |
| | 1 | Resistant population(s) within 1 mile |
| | -0.5 | Multiple susceptible populations and one resistant population within 1 mile |
| | -1 | Susceptible population(s) within 1 mile |

*All values per site were additive to obtain an overall index value used in statistical analyses.

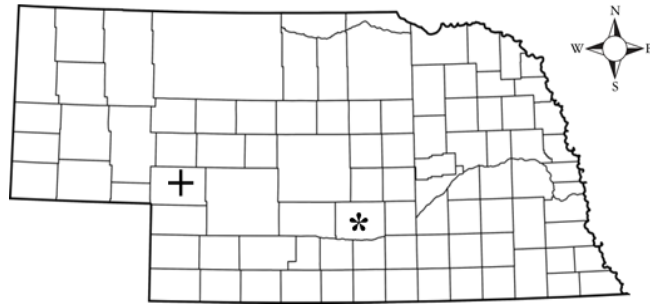


Figure 2.1. County map of the state of Nebraska. Sampling grids were established in Keith County in western Nebraska (indicated by +) and Buffalo County in central Nebraska (indicated by *). Field sites were sampled in both counties in 2015 and 2016.



Figure 2.2. Spatial distribution of sampling sites and adult collections in A) Keith County: sites 1 through 9 were collected in 2015, and sites 10-17 were collected in 2016; and B) Buffalo County: sites 1 through 8 were collected in 2015, and sites 9-16 were collected in 2016.

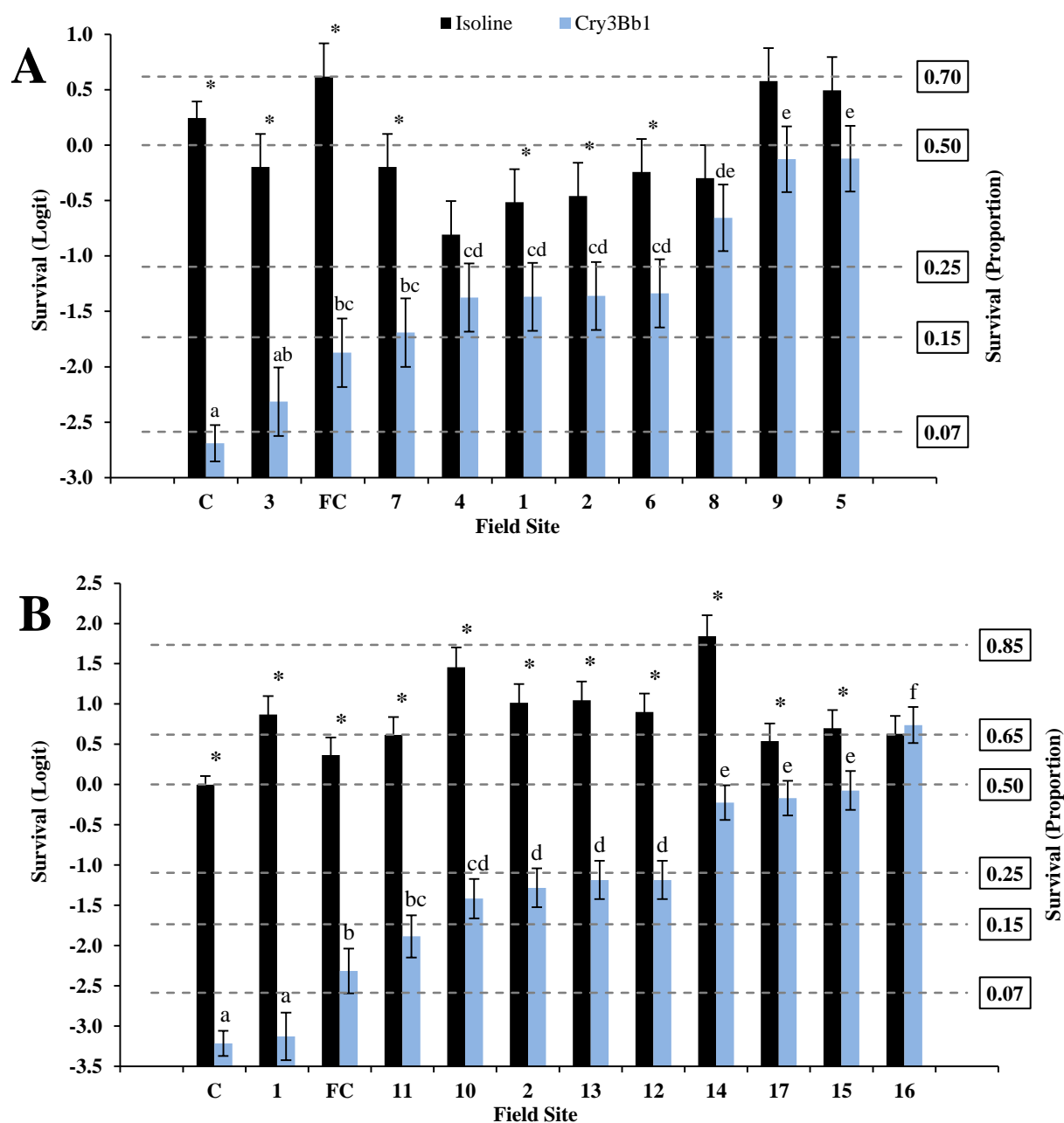


Figure 2.3. Logit of mean (\pm SE) larval proportional survival on Stone 6021RR2 (non-transgenic) and Stone 6021VT3 (Cry3Bb1) from Keith County field sites collected in (A) 2015 and (B) 2016. “C” refers to a composite of four laboratory control populations never exposed to rootworm-active Bt traits. “FC” indicates the field control collected from UNL-ENREC rarely exposed to rootworm-active Bt traits. Bars with an asterisk indicate significantly higher survival on Stone 6021RR2 compared to Stone 6021VT3. Cry3Bb1 means (blue bars) with the same letter are not significantly different (two-way ANOVA, $P > 0.05$; LSMEANS option).

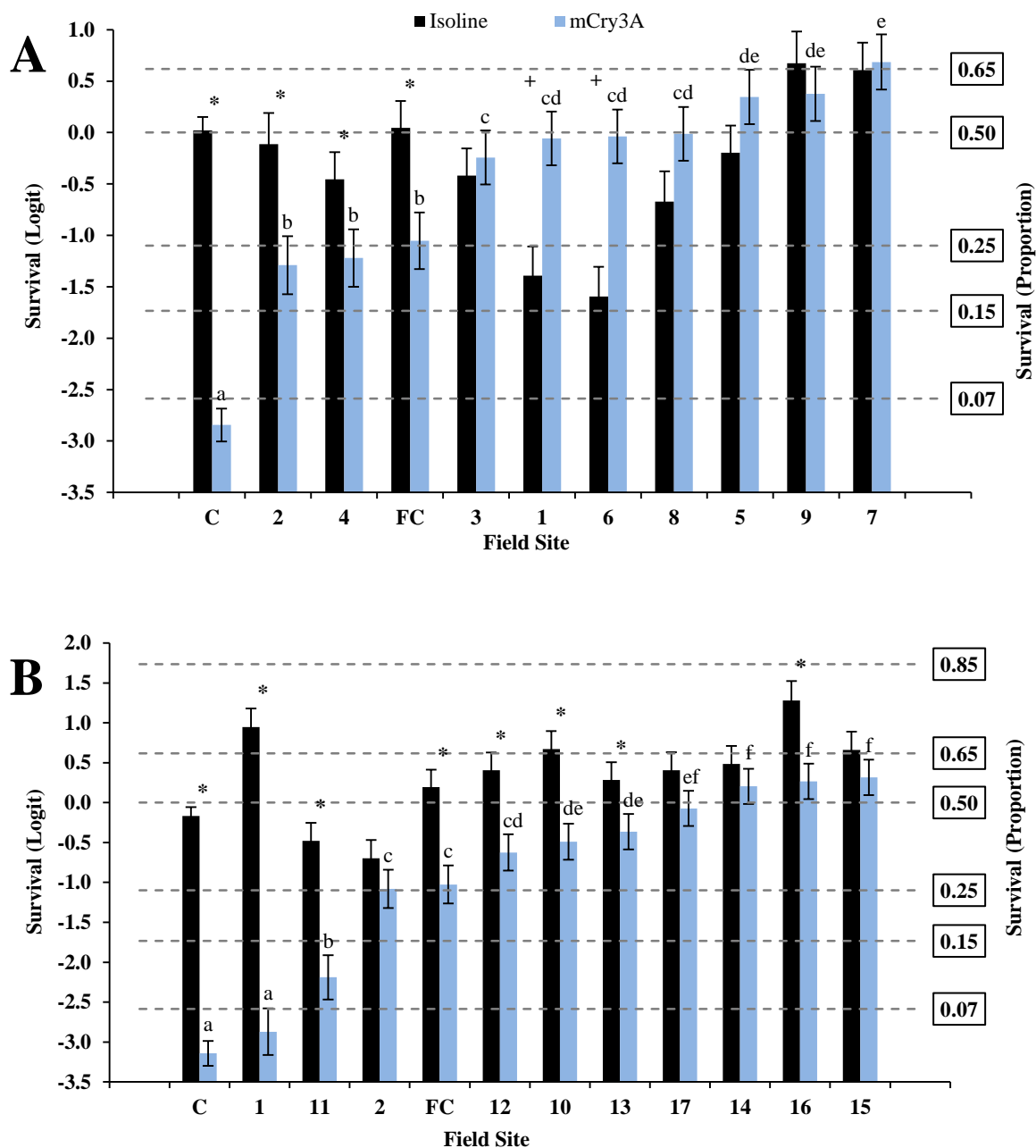


Figure 2.4. Logit of mean (\pm SE) larval proportional survival on Syngenta N68B-GT (non-transgenic) and Syngenta N68B-3000GT (mCry3A) from Keith County field sites collected in (A) 2015 and (B) 2016. “C” refers to a composite of four laboratory control populations never exposed to rootworm-active Bt traits. “FC” indicates the field control collected from UNL-ENREC rarely exposed to rootworm-active Bt traits. Bars with an asterisk indicate significantly higher survival on Syngenta N68B-GT compared to Syngenta N68B-3000GT. Bars with a plus indicate significantly higher survival on Syngenta N68B-3000GT compared to Syngenta N68B-GT. The mCry3A means (blue bars) with the same letter are not significantly different (two-way ANOVA, $P > 0.05$; LSMEANS option).

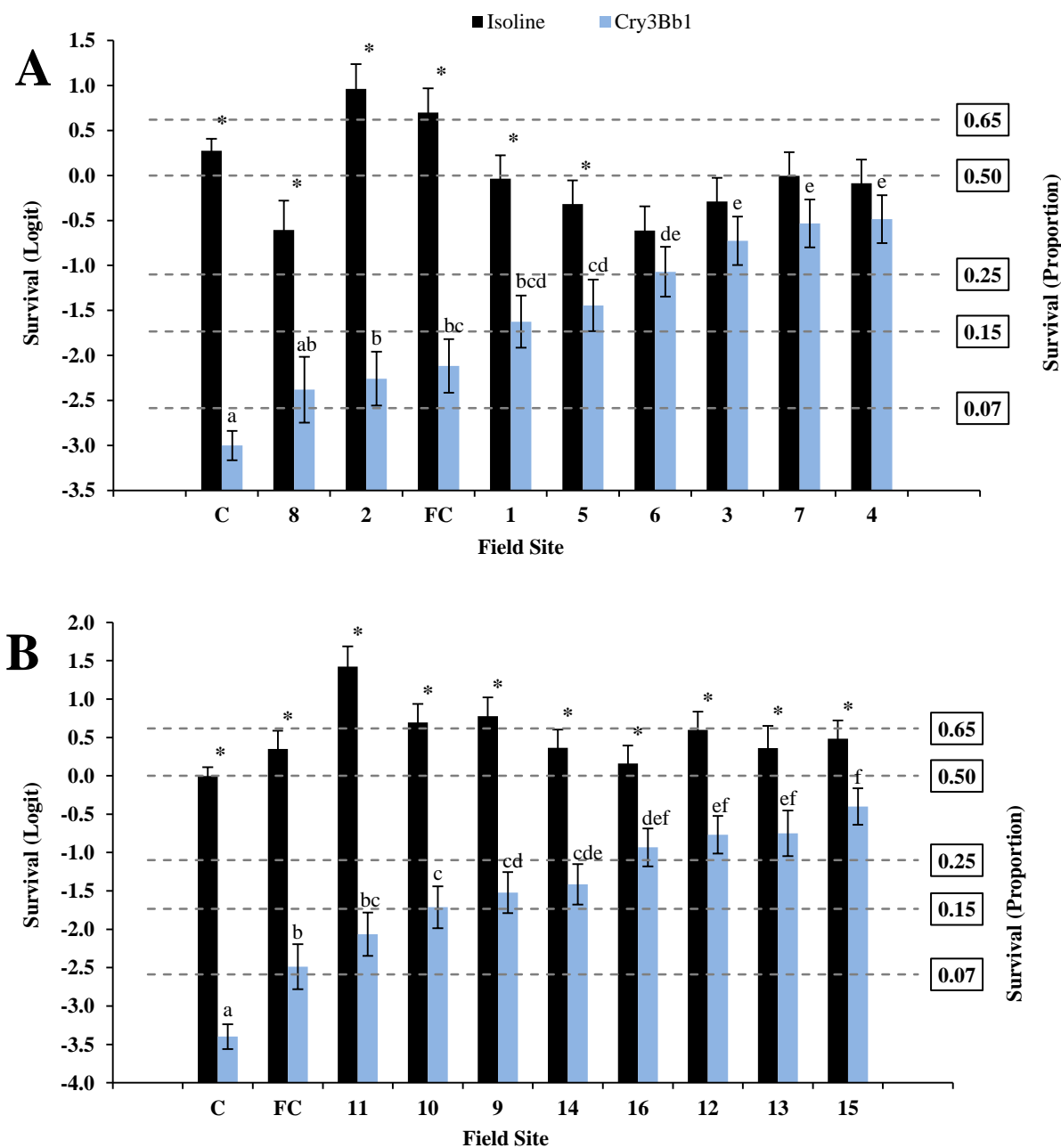


Figure 2.5. Logit of mean (\pm SE) larval proportional survival on Stone 6021RR2 (non-transgenic) and Stone 6021VT3 (Cry3Bb1) from Buffalo County field sites collected in (A) 2015 and (B) 2016. “C” refers to a composite of four laboratory control populations never exposed to rootworm-active Bt traits. “FC” indicates the field control collected from UNL-ENREC rarely exposed to rootworm-active Bt traits. Bars with an asterisk indicate significantly higher survival on Stone 6021RR2 compared to Stone 6021VT3. Cry3Bb1 means (blue bars) with the same letter are not significantly different (two-way ANOVA, $P > 0.05$; LSMEANS option).

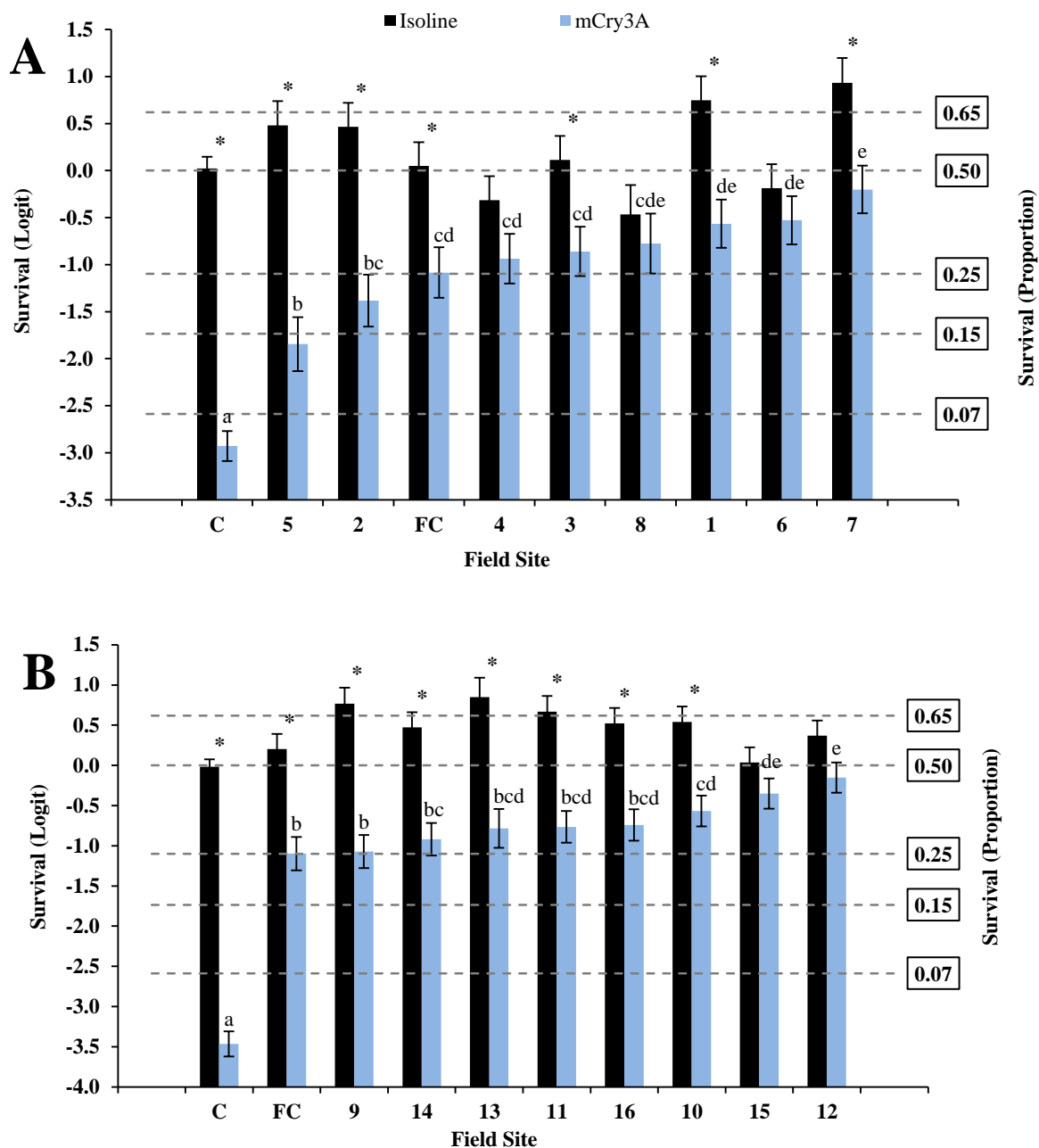


Figure 2.6. Logit of mean (\pm SE) larval proportional survival on Syngenta N68B-GT (non-transgenic) and Syngenta N68B-3000GT (mCry3A) from Keith County field sites collected in (A) 2015 and (B) 2016. “C” refers to a composite of four laboratory control populations never exposed to rootworm-active Bt traits. “FC” indicates the field control collected from UNL-ENREC rarely exposed to rootworm-active Bt traits. Bars with an asterisk indicate significantly higher survival on Syngenta N68B-GT compared to Syngenta N68B-3000GT. The mCry3A means (blue bars) with the same letter are not significantly different (two-way ANOVA, $P > 0.05$; LSMEANS option).

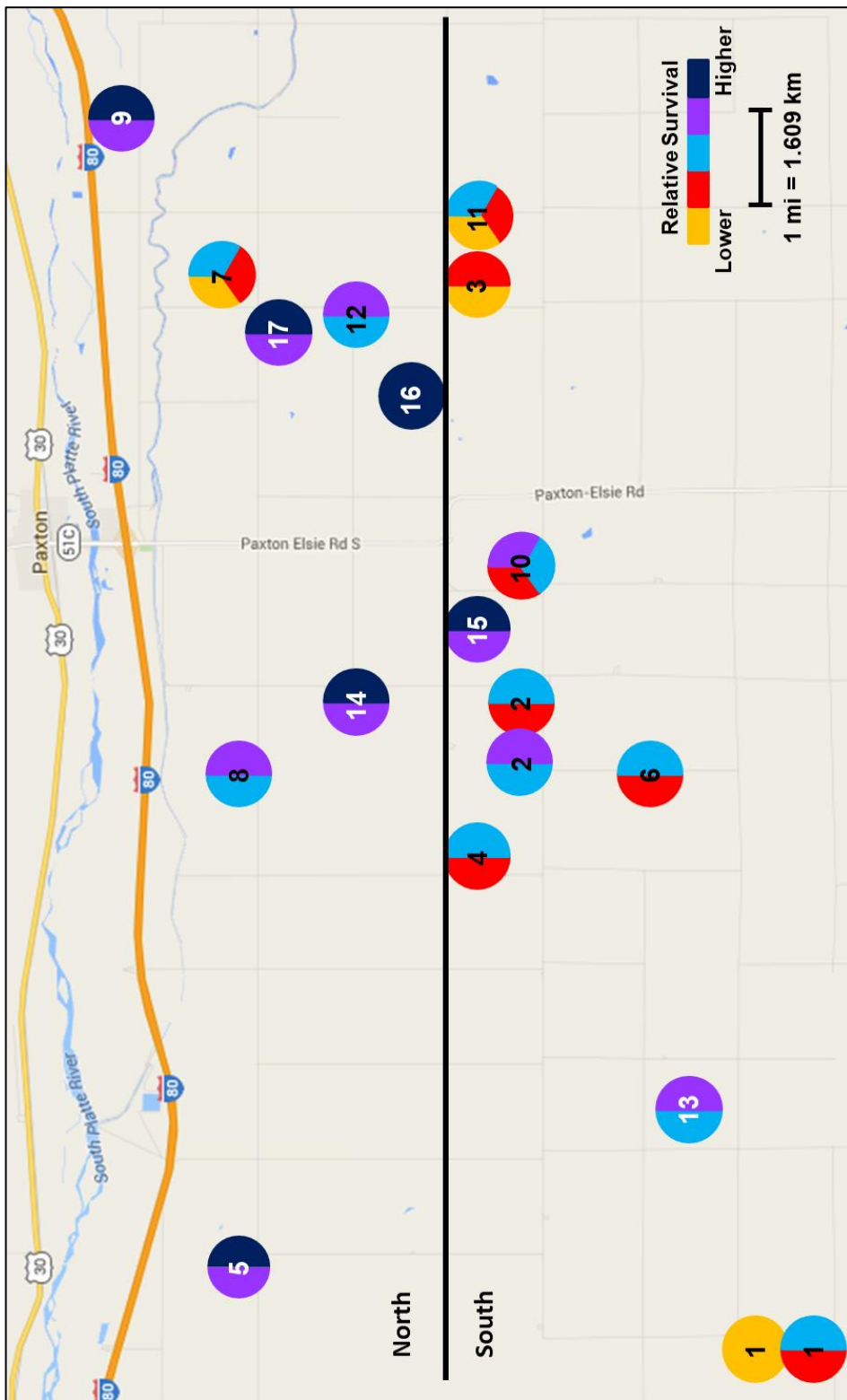


Figure 2.7. Spatial distribution of sampling sites in Keith County and relative survival of WCR larvae on Stone 6021VT3 (Cry3Bb1) in bioassays. Sites 1 through 9 were collected in 2015, and sites 10-17 were collected in 2016. Sites 1 and 2 were collected during both years. Proportional survival ranged from 0.04 to 0.66. Circles containing the same color(s) are not significantly different (one-way ANOVA, $P > 0.05$; LSMEANS option).

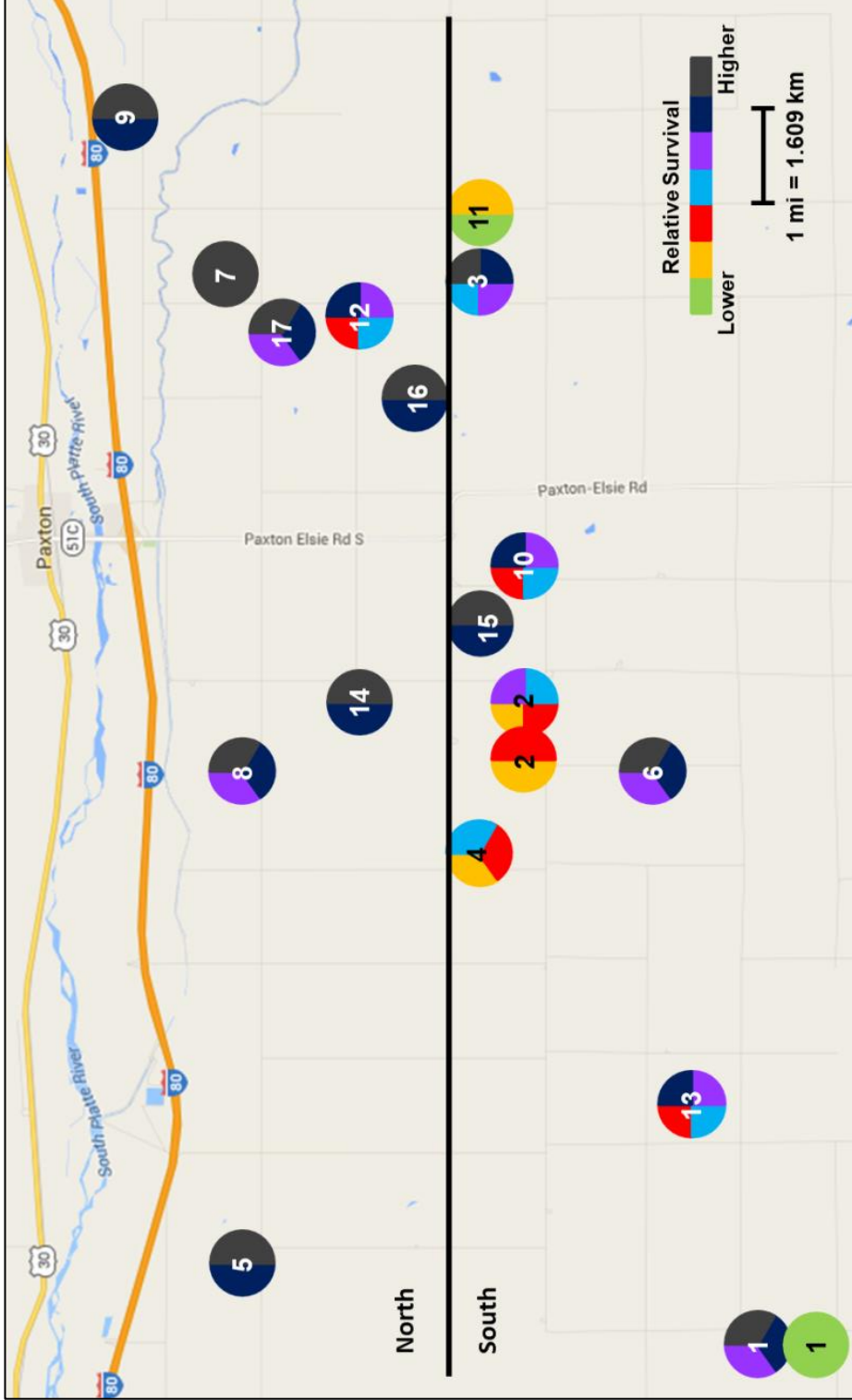


Figure 2.8. Spatial distribution of sampling sites in Keith County and relative survival of WCR larvae on N68B-3000GT (mCry3A) in bioassays. Sites 1 through 9 were collected in 2015, and sites 10-17 were collected in 2016. Sites 1 and 2 were collected during both years. Proportional survival ranged from 0.05 to 0.69. Circles containing the same color(s) are not significantly different (one-way ANOVA, $P > 0.05$; LSMEANS option).

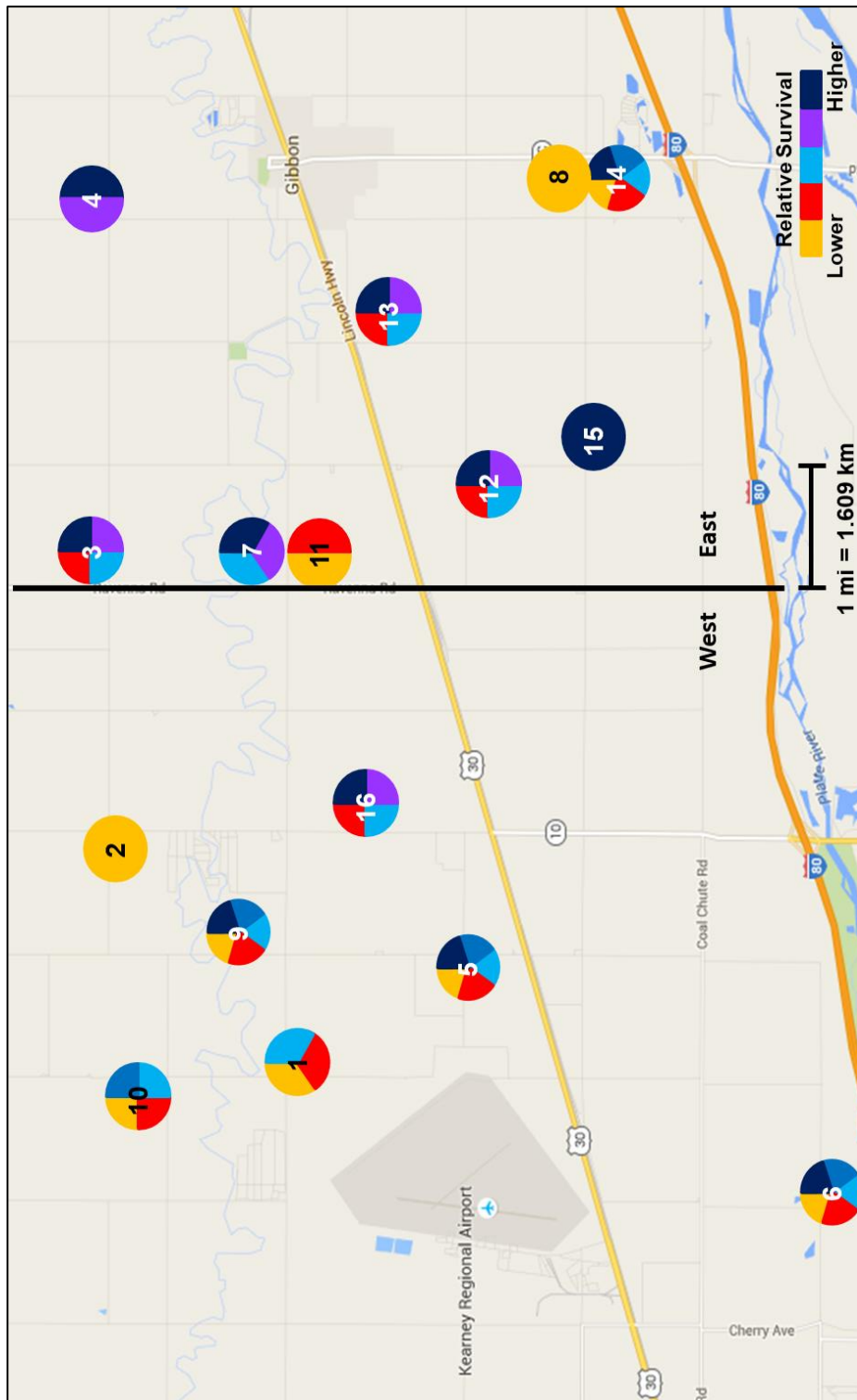


Figure 2.9. Spatial distribution of sampling sites in Buffalo County and relative survival of WCR larvae on Stone 6021VT3 (Cry3Bb1) in bioassays. Sites 1 through 8 were collected in 2015, and sites 9-16 were collected in 2016. Proportional survival ranged from 0.07 to 0.40. Circles containing the same color(s) are not significantly different (one-way ANOVA, $P > 0.05$; LSMEANS option).

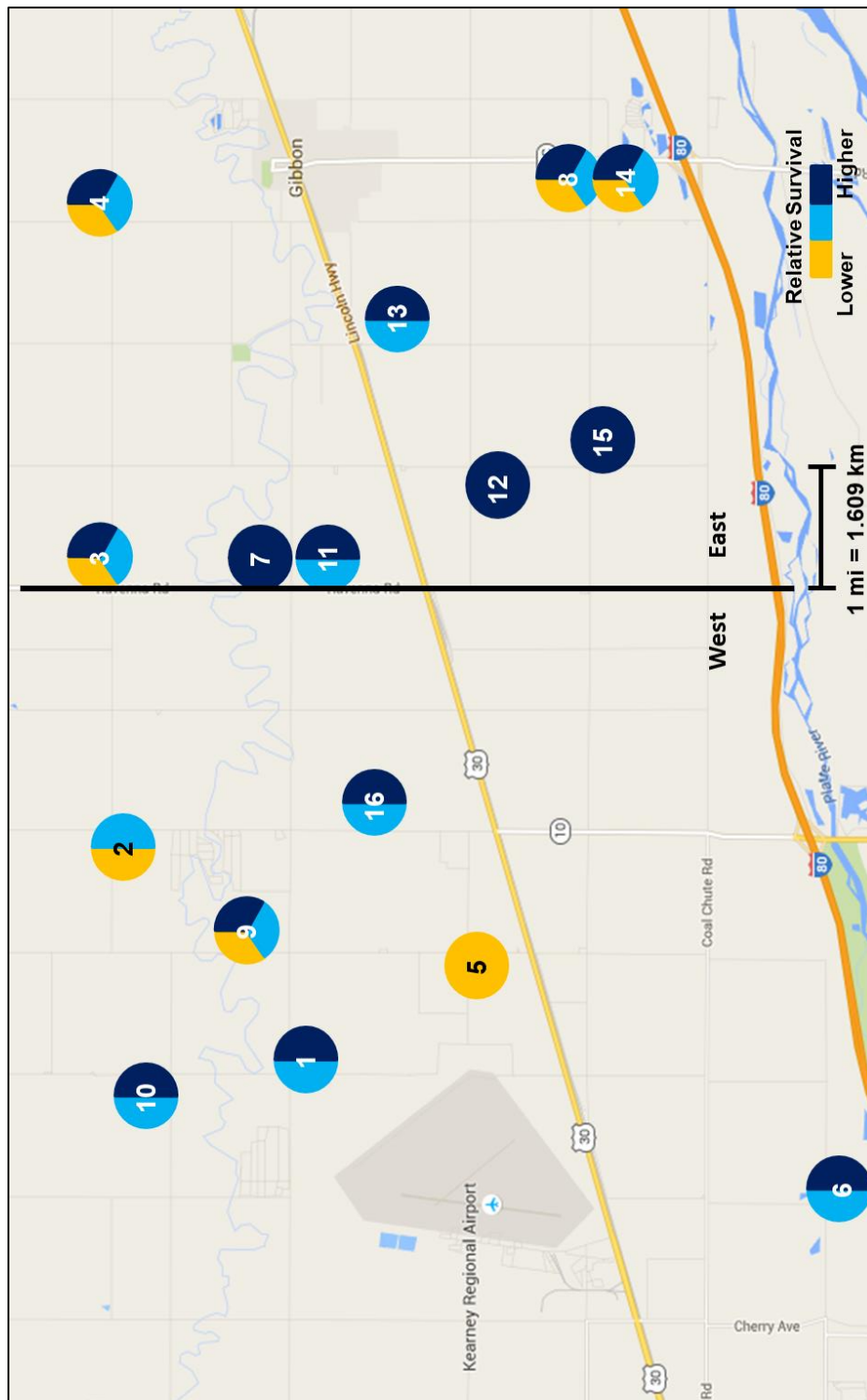


Figure 2.10. Spatial distribution of sampling sites in Keith County and relative survival of WCR larvae on Stone 6021VT3 (Cry3Bb1) in bioassays. Sites 1 through 8 were collected in 2015, and sites 9-16 were collected in 2016. Proportional survival ranged from 0.11 to 0.46. Circles containing the same color(s) are not significantly different (one-way ANOVA, $P > 0.05$; LSMEANS option).

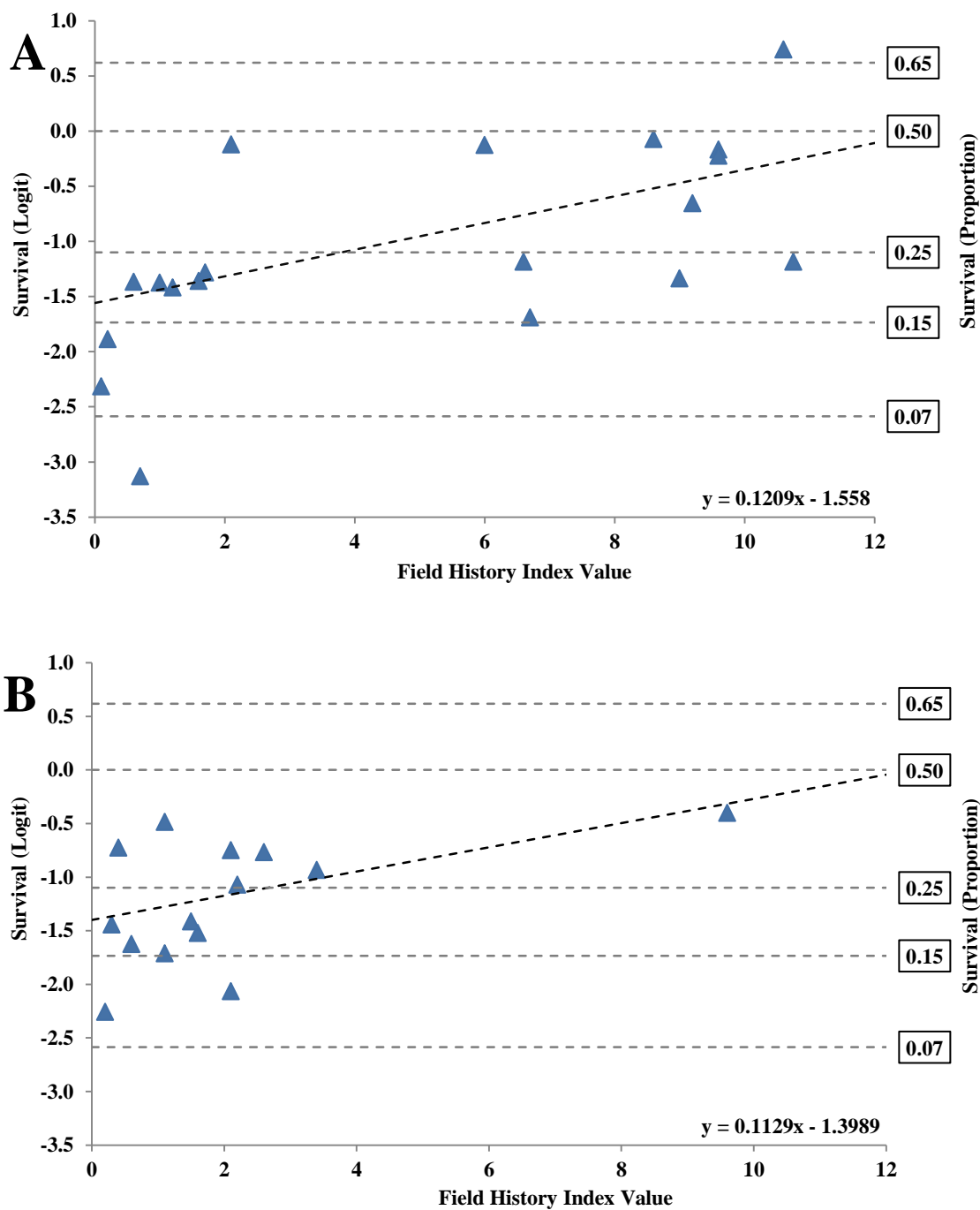


Figure 2.11. Linear regression of field history index value on logit survival in (A) Keith County and (B) Buffalo County. The estimated regression equations are presented.

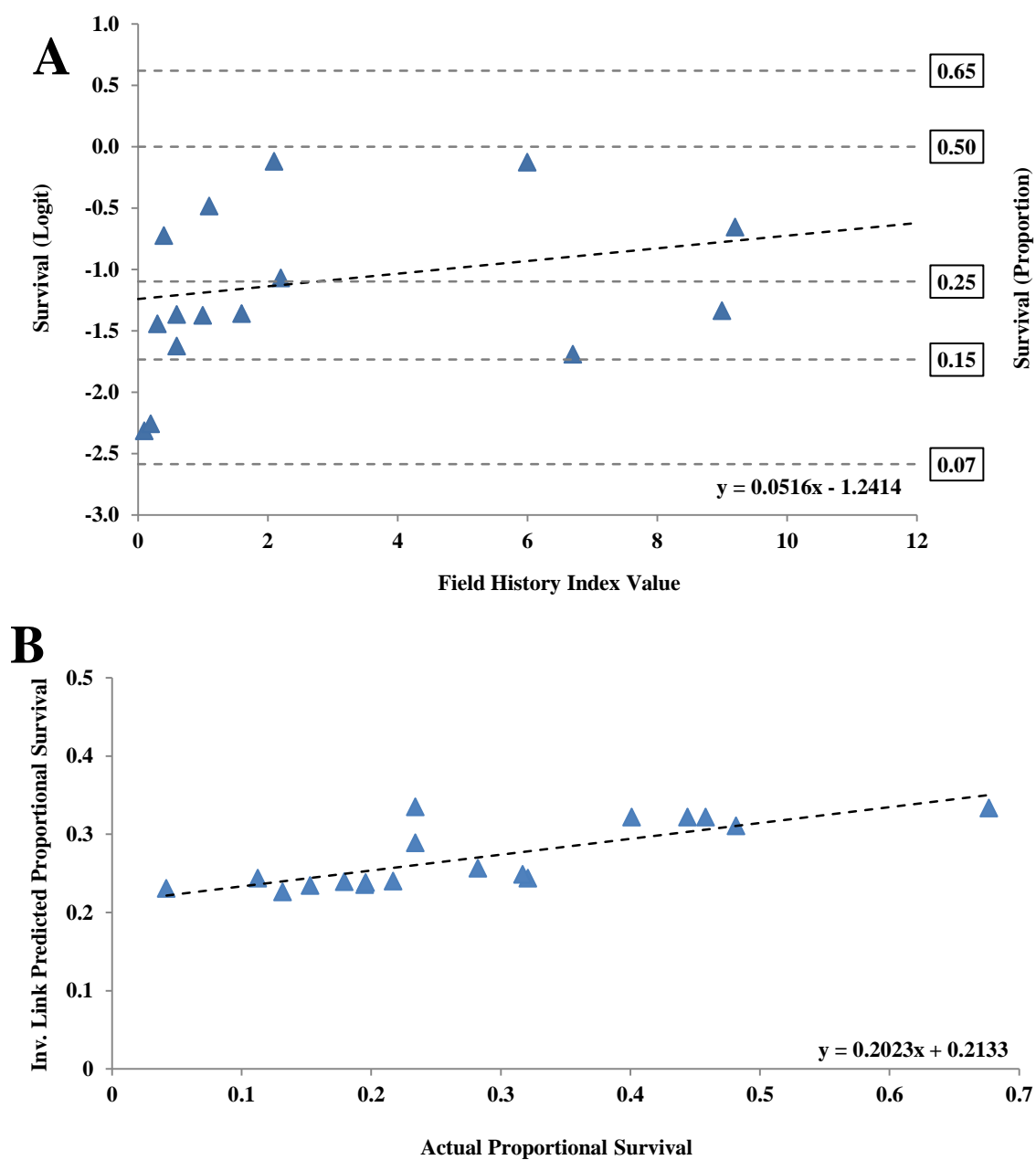


Figure 2.12. Predictive linear regression model using field history index values and proportional survival bioassay data from the (A) 2016 model to predict (B) 2017 survivorship values based on the 2016 model. The estimated regression equations are presented.

APPENDIX I: MEAN SURVIVORSHIP OF LABORATORY CONTROL POPULATIONS ON CRY3BB1 AND MCry3A EXPRESSING HYBRIDS IN LABORATORY BIOASSAYS

Table 1. Mean proportional survival (\pm SE) of laboratory control populations on A) Cry3Bb1 in 2016 bioassays, B) Cry3Bb1 in 2017 bioassays, C) mCry3A in 2016 bioassays, and D) mCry3A in 2017 bioassays. Within traits and years, no significant differences in mean survival among populations were documented (one-way ANOVA, $P > 0.05$; LSMEANS option).

| A | Population | Mean Proportional Survival (\pm SE) |
|----------|-------------------|---|
| | Kansas (KS) | 0.025 \pm 0.01 |
| | Penn I (PA) | 0.020 \pm 0.009 |
| | Whitlock (SD) | 0.028 \pm 0.01 |
| | Linwood (NE) | 0.035 \pm 0.01 |

| B | Population | Mean Proportional Survival (\pm SE) |
|----------|-------------------|---|
| | Kansas (KS) | 0.013 \pm 0.005 |
| | Penn I (PA) | 0.015 \pm 0.006 |
| | Whitlock (SD) | 0.015 \pm 0.006 |
| | Linwood (NE) | 0.019 \pm 0.007 |

| C | Population | Mean Proportional Survival (\pm SE) |
|----------|-------------------|---|
| | Kansas (KS) | 0.027 \pm 0.01 |
| | Penn I (PA) | 0.038 \pm 0.01 |
| | Whitlock (SD) | 0.050 \pm 0.02 |
| | Linwood (NE) | 0.043 \pm 0.02 |

| D | Population | Mean Proportional Survival (\pm SE) |
|----------|-------------------|---|
| | Kansas (KS) | 0.033 \pm 0.01 |
| | Penn I (PA) | 0.043 \pm 0.01 |
| | Whitlock (SD) | 0.024 \pm 0.009 |
| | Linwood (NE) | 0.051 \pm 0.02 |