PARTIAL CHARACTERIZATION OF BT RESISTANCE AND THE BACTERIOME OF WESTERN CORN ROOTWORM

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by

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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

CHARACTERIZING BT RESISTANCE AND THE BACTERIOME OF WESTERN CORN ROOTWORM

Presented by Dalton Ludwick, a candidate for the degree of doctor of philosophy, and hereby certify that, in their opinion, it is worthy of acceptance.

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ABSTRACT

Western corn rootworm (*Diabrotica virgifera virgifera* LeConte) has been a pest of corn (*Zea mays L.*) for more than one century. In that time, this insect has spread across the United States and has also had several introductions into Europe. Though corn growers have applied a number of control tactics over the years, including crop rotation, synthetic insecticides, and transgenic corn, this insect is persistently an issue for much of the corn growing areas in the United States and Europe. Western corn rootworm is infamous for its ability to overcome the tactics that are used against it, several times within a single decade. Transgenic corn expressing one or more protein(s) from *Bacillus thuringiensis* Berliner (Bt) have been used against this insect for more than one decade. Western corn rootworm had developed resistance to three of the four proteins (Cry3Bb1, mCry3A, and eCry3.1Ab) and had been documented before the start of this dissertation. Immediately prior to the start of this dissertation, information existed that resistance to the fourth protein (Cry34/35Ab1) may have occurred in a Minnesota population. In this dissertation, we placed insects from control colonies or the field population in containers with corn expressing the Cry34/35Ab1 protein or in containers with artificial diet covered by the protein. The results of these three experiments indicated that incomplete resistance to Cry34/35Ab1 and Cry3Bb1 had occurred. The artificial diets used in the first experiment are proprietary and owned by separate companies. Efforts in separate studies led to the formulation of an improved artificial for western corn rootworm larvae which is now available to the public. This single, artificial diet was tested for its ability to detect resistance to the different proteins currently targeting western corn rootworm. Laboratory colonies of insects selected for resistance to each protein were compared to susceptible

insects on the new diet for their ability to survive and develop. The proprietary diets were also used to make comparisons about the similarity of the diets to the new, public diet. The new diet performed similarly to proprietary diets in terms of mortality and developmental rates. Indeed, the new diet was able to detect resistance to all proteins and documented strong differences between Cry34/35Ab1-resistant insects and susceptible insects for the first time. Lastly, we investigated which bacteria were associated with this insect. Laboratory insects were raised in two geographically and microbially distinct soils on non-Bt corn. Insects were collected at different time points to understand which bacteria were present in each life stage and across all life stages. Bacteria in soil samples were also identified and compared to those found in the insect to determine which bacteria were unique to the insect. These results suggest that 16 different bacteria are found in every western corn rootworm, some of which appear to only occur in the insect. The knowledge of bacteria associated with western corn rootworm may one day help us better understand the mechanisms of Bt resistance and how to better optimize nutrition.

Chapter 1

Rootworm management: status of GM traits, insecticides and potential new tools*

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ABSTRACT

Western corn rootworm (*Diabrotica virgifera virgifera* LeConte) and northern corn rootworm (*Diabrotica barberi* (Smith and Lawrence)) are major pests of maize in the USA. These pests have been managed with a variety of tactics over the last century. Both *Diabrotica* spp. have adapted to crop rotation in different ways in certain regions of the USA as well as to some of the insecticides targeted at them. *D. v. virgifera* has adapted to more of the chemical control measures and transgenic control methods. Discussed in this review are the challenges associated with managing both species, and how current management strategies might be combined and implemented to help manage damage from these species. Also, we discuss the potential for new technologies, such as RNA interference, to be used in the future.

BACKGROUND/INTRODUCTION

Since 1909, western corn rootworm (*Diabrotica virgifera virgifera* LeConte) has been known to attack the roots of maize, *Zea mays* L. (Spencer et al. 2009; Meinke et al. 2009; Miller et al. 2009; Gassmann et al. 2009; Gillette 1912). Over the last century, the range of *D. v. virgifera* as a pest of maize has expanded beyond Fort Collins, Colorado to cover 39 states in the USA, Mexico, two provinces of Canada, three countries in Central America and 29 countries in Europe (Baca 1994; CABI 2016). There is some evidence that the pest followed the migration of maize northward from Mexico thousands of years ago (Meinke et al. 2009).

Economic losses caused by *D. v. virgifera* and *Diabrotica barberi* have been estimated between one and two billion dollars (Metcalf 1986; Mitchell 2011). These estimates include yield losses through direct and indirect routes, and include management costs. *D. v. virgifera* eggs have a developmental threshold of 11.1°C and hatch in the soil after accumulating the needed number of degree days (Wilde 1971; Levine et al. 1992), which depends somewhat on the location and temperature fluctuations. Upon hatch, neonate larvae find respiring plant roots by orienting to carbon dioxide (Strnad et al. 1986; Hibbard and Bjostad 1988; Bernklau and Bjostad 1998). Using behavioral bioassays, it is possible to distinguish hosts from nonhosts (Bernklau et al. 2015). Host recognition cues involved in this assay include monogalactosyldiacylglycerols (MGDGs) and other cues, which were recently isolated identified from corn root extracts (Bernklau and Bjostad 2005) based on the same behavioral bioassays. Long-chain free fatty acids and short-chain sugars were similarly isolated and identified as *D. v. virgifera* larval feeding stimulants

(Bernklau et al. 2013). Maize is its primary host, but larvae can develop at least partially on many grass species and can develop to the adult stage on a number of these plants (Clark and Hibbard 2004, Oyediran et al. 2004). Larvae continue to develop and consume root tissue until the insect reaches the pupal stage, at which time it creates an earthen cell. Adults will begin to emerge approximately 10 days later, with males emerging first due to protandry (Branson 1987). Overall timing of adult emergence (June–August) will vary depending on accumulated degree days (Wilde 1971; Levine et al. 1992).

Larval feeding can have an array of impacts on the maize plant's development. Root regrowth is often triggered, depending on maize genotype, by root damage (Robert et al. 2015). If root damage is minor, then the plant may be better off than had its roots not been damaged at all, especially under dry conditions (Gray and Steffey 1998; Mahmoud et al. 2016). At higher densities, the root feeding may cause severe and permanent damage to the root system. Such severe root damage can limit the ability of the plant to uptake moisture and nutrients from the soil, which then impacts yield (Kahler et al. 1985; Riedell 1990; Gavloski et al. 1992; Hou et al. 1997). Often, the ability of the plant to stand upright is impacted, so an additional yield loss component is due to unharvested grain (Spike and Tollefson 1991). Adult feeding typically does not have an impact on the plant's yield unless densities are very high prior to anthesis (Culy et al. 1992; Capinera et al. 1986).

Rearing and handling techniques for *D. v. virgifera* on a large scale have been well established for decades (Jackson 1986). Non-diapausing colonies of *D. v. virgifera* populations, allow some research to be expedited (Branson et al. 1976). Unfortunately, research with *D. barberi* is lacking because adult handling techniques have not been able to produce sufficient numbers of eggs for large experiments. Additionally, a non-

diapausing strain of *D. barberi* does not exist, hampering research in many aspects with this species. The non-diapausing trait has been documented in varying degrees (Patel and Apple 1967; Fisher et al. 1994) and may make a non-diapausing strain a real possibility.

A HISTORICAL PERSPECTIVE ON *DIABROTICA* MANAGEMENT, RESEARCH, AND RESISTANCE DEVELOPMENT

Crop rotation. *D. v. virgifera* has been a difficult pest to manage in the USA. Crop rotation, where a non-host is planted following a host, was initially recommended (Gillette 1912). This tactic was the only control tactic for the first half of the twentieth century. Management with this tactic is still by far the most effective management tactic in most areas against both species. Unfortunately, scientists in Illinois discovered a strain of *D. v. virgifera*, which had lost its fidelity to lay eggs only in the maize fields (Levine et al. 2002). Instead, *D. v. virgifera* females began to lay eggs in both maize and soybean fields in this region. The strain began near Urbana, Illinois, and has since spread to larger portions of Illinois, Indiana, and to a lesser extent in surrounding states (Gray et al. 2009).

Larval gut tissue of *D. v. virgifera* has a diverse microbial community (Dematheis 2012a). In *D. v. virgifera*, a shift in adult gut microbiota enterotype was associated with increased resistance to soybean defense compounds, and likely contributed to the development of resistance to crop rotation (Chu et al. 2013). Comparison of gut microbiota between rotation resistant *D. v. virgifera* populations and wild-type *D. v. virgifera* populations revealed shifts in the microbial community composition upon adaptation to soybean tissue diet in adult D. v. virgifera. Note that *D. v. virgifera* larvae cannot survive

on soybean tissues. Manipulation of the gut microbiota through the use of antibiotics reduced the resistance to soybean defence compounds to a level similar to that of wild-type *D. v. virgifera* (Chu et al. 2013).

Similar to *D. v. virgifera*, *D. barberi* has developed a mechanism to circumvent the effectiveness of crop rotation. 'Extended diapause' means some eggs hatch two or more winters after being laid in the soil (Krysan et al. 1986). Because larvae die if eggs hatch when corn is absent, extended diapause allows *D. barberi* to selectively adapt to local crop rotations, putting all corn at risk. Extended diapause had been a problem in parts of Minnesota, Wisconsin, South Dakota and Iowa prior to the population crashes of *D. barberi* when Bt corn targeting rootworm started to dominate the landscape. Areas formerly dominated by *D. barberi* (parts of Minnesota, Wisconsin, South Dakota and Iowa) had a drastic reversal in the dominant *Diabrotica* species, with *D. v. virgifera* becoming the predominant species. More recently, *D. barberi* populations have recovered in some of these areas as documented by the Wisconsin Department of Agriculture (Wisconsin Department of Agriculture 2012). *D. barberi* populations spiked dramatically in 2015, nearly a decade after the populations crashed. Scattered extended diapause problems have just begun to resurface in rotated corn fields in Minnesota (Ken Ostlie, personal communication). In Missouri, where extended diapause has not been documented, populations of *D. barberi* were also found in large numbers in first year corn during the 2016 growing season (Unglesbee 2016). These developments suggest changes are underway within *D. barberi* populations.

Chemical control. Near the middle of the twentieth century, soil applied insecticides became available for *Diabrotica* spp. management (Metcalf 1986).

Cyclodienes were broadcast over the entire field, thereby exposing all larvae to the pesticide. This widespread exposure likely hastened the development of resistance by *D. v. virgifera*, which was documented within just a few years (Ball and Weekman 1962). Cyclodiene resistance has persisted decades beyond the ban of this pesticide class (Parimi et al. 2006; Wang et al. 2013). Insecticides applied directly over rows of maize were referred to as banded insecticides. Two insecticide classes replaced the cyclodienes (carbamates and organophosphates) but were more expensive, so these pesticides were only applied over the row. No resistance has developed using this application method. Current theory suggests that roots outside the insecticidal zone provide a built-in 'refuge' to produce susceptible adults (Pereira et al. 2015). Refuges will be discussed below in reference to transgenic corn targeting *D. v. virgifera*.

A practice sometimes referred to as 'beetle bombing' uses foliar applications of the insecticide to prevent gravid females from laying eggs in maize fields, thus reducing the insect's impact on next year's crop (Pruess et al. 1974). Beetle bombing with organophosphates and carbamates resulted in *D. v. virgifera* adults developing resistance to insecticides within these two classes in the same Nebraska region where resistance to cyclodienes evolved (Meinke et al. 1998). This resistance also significantly impacted the larval susceptibility to organophosphates and carbamates (Wright et al. 2000).

More recently, a newer class of insecticide, pyrethroids, has been used to control *D. v. virgifera* adults. Interestingly, foliar applications of this insecticide are also used to control other pests, such as the two spotted spider mite (*Tetranychus urticae* Koch) or western bean cutworm (*Striacosta albicosta* (Smith)). While the application of the insecticide may be used to control other pests, *D. v. virgifera* adults are likely to be exposed

and experience the selection pressure. Multiple applications within a season and non-target effects have likely contributed to the development of bifenthrin resistance for *D. v. virgifera* (Pereira et al. 2015).

Biological control agents. Currently, there are several different options being explored in the biological control area, all of which are entomopathogenic organisms. Two genera of entomopathogenic fungi, *Metarhizium* and *Beauvaria*, have been investigated for their potential as a biological control agent of *D. v. virgifera* (Mulock and Chandler 2001; Meissle et al. 2009; Pilz et al. 2011; Petzold-Maxwell et al. 2012, 2013). No commercial products are available as a result of the entomopathogenic fungus work. A great deal of research has gone into studying entomopathogenic nematodes and their interactions with *D. v. virgifera* (Petzold-Maxwell 2012, 2013; Rassmann et al. 2005; Hiltpold et al. 2010, 2012). *Heterorhabditis* and *Steinernema* spp. have been the two genera of focus in recent studies involving nematode–rootworm interactions. Nematodes are available for management of *D. v. virgifera* in parts of Europe (Toepfer et al. 2014). Again, little work with these biological control agents has been done with *D. barberi*, likely due to a shortfall in available eggs for studies.

BT MAIZE, MORTALITY AND IMPLICATIONS FOR RESISTANCE MANAGEMENT

Refuges, theory and concerns of resistance development. Transgenic maize hybrids expressing crystalline proteins with insecticidal activity derived from a soil-dwelling bacterium, *Bacillus thuringiensis* Berliner (Bt), have been available since 1996

(USDA-APHIS 1995). Since organic growers used Bt to control pests, concern over the possibility of Bt resistance development were heightened more than for insecticides and contributed to the U.S. Environmental Protection Agency's (EPA) mandate for insect resistance management (IRM) plans to be in place to slow the development of Bt resistance. Products were registered with a structured refuge in hopes of slowing resistance development (EPA 1998b). The IRM plans for the first Bt crops in the USA implemented a 20% structured refuge. More recently, refuge requirements for seed blends of single events were set at 10 and 5% for pyramided products in the USA. The structured refuge strategy is optimal if toxin mortality is very high, initial frequency of alleles bestowing resistance is low, fitness costs of resistance are present, targeted insects mate randomly in the field and resistance to the Bt toxin is recessive (Gould 1998; Tabashnik and Gould 2012). The mortality caused by a Bt product is perhaps the most important because this can lead to a landscape in which resistant alleles from the Bt crop are vastly overwhelmed by susceptible alleles from the refuge.

The mathematics on why the level of toxicity is so important in the effectiveness of refuge is clarified in Table 1. If a toxin kills 90% of susceptible larvae, survivorship from a perfect 20% block refuge compared with an 80% Bt field is only 2.5:1 ((0.2 proportion of field × 1.0 survivors)/(0.1 survivors × 0.8 proportion of the field)). The minimum definition of 'high dose' in the field is that 99.99% of susceptible larvae die following exposure to a transgenic plant (EPA 1998a). The ratio of insects produced from a perfect 20% block refuge with a hypothetical efficacy of 99.99% would be 2500:1 ((0.2 proportion of field × 1.0 survivors)/(0.0001 survivors × 0.8 proportion of the field)). This ratio is considered a minimum: 'Think in terms of thousands to one or millions to one.'

Said Bruce Tabashnik during his talk at the Entomological Society of America in 2014 when referring to these ratios. The refuge program has been quite successful for some products, especially those which are truly high dose (Tabashnik et al. 2013). Even growers who do not plant Bt crops sometimes benefit from those who do plant Bt crops (Hutchison et al. 2010). The structured refuge program has been less successful in instances in which the Bt crop is not high dose (Tabashnik et al. 2013). There are some that believe that the era of the Cry toxin is ending (Porter et al. 2016).

Transgenic maize targeting *Diabrotica* spp. In 2003, the first hybrids expressing a Bt-derived insecticidal protein (Cry3Bb1, event MON863) active against *Diabrotica* spp. was registered for commercial use (EPA 2013). Over the next decade, four more proteins (Cry34Ab1/Cry35Ab1, mCry3A and eCry3.1Ab) and four additional events (DAS-59122-7, MIR604, MON88017 and 5307) were registered for commercial sale (EPA 2005, 2007, 2015). Event MON863 with an antibiotic marker was replaced by event MON88017, which also expressed Cry3Bb1, but came with resistance to glyphosate as a selectable marker. Expression of both Cry34Ab1 and Cry35Ab1 proteins are required for activity against *Diabrotica* spp. Some of the genes responsible for the expression of these Bt proteins have been stacked in maize hybrids resulting in pyramid Bt products. Pyramided products are designed to extend the life of both proteins by improving efficacy and adding multiple modes of action (Roush 1998). Maize products expressing the eCry3.1Ab protein are only sold in hybrids that also express the mCry3A simultaneously under the product name Agrisure Duracade®.

Concerns of resistance development led to laboratory selection experiments with *D. v. virgifera*. Within three generations of selection, colonies of *D. v. virgifera* developed

nearly complete resistance to Cry3Bb1 (Meihls et al. 2008). Nearly complete resistance to maize expressing mCry3A or eCry3.1Ab singly was also selected for within a few generations (Meihls et al. 2012; Frank et al. 2013). Each laboratory selection attempt for Cry3Bb1, mCry3A and eCry3.1Ab has been successful (Meihls et al. 2008, 2012; Frank et al. 2013; Oswald et al. 2011). Maize expressing Cry34/35Ab1 has been much more difficult to develop resistance to and complete resistance has not yet been achieved after more than 20 generations of selection. For example, after 10 generations of selection, survival of *D. v. virgifera* on Cry34/35Ab1-expressing maize was only 20% relative to a near-isoline (Lefko et al. 2008). Deitloff et al. (2016) evaluated refuge scenarios by selecting *D. v. virgifera* on Cry34/35Ab1 in a laboratory setting. A seed mix scenario failed to delay the development of resistance after 10 generations.

The highest published estimate for mortality was for the combination of eCry3.1Ab +mCry3A with an efficacy of 99.91% (Hibbard et al. 2011). However, this efficacy with a 5% refuge only provides a ratio of 58 susceptible insects for each adult from the Bt portion of the maize field (Table 2). Cry3Bb1 (Event MON863) registration initially required a 20% block refuge. The ratio of insects from refuge to Bt was 16.56:1, and for mCry3A, it is only 4.88:1 (Table 2). Since these ratios are not remotely close 'thousands to one or millions to one' as for truly high dose products, it is not surprising that resistance developed quite quickly to these events in the field (Gassmann et al. 2011, 2014; Zukoff et al. 2016), especially since the only instance in which the refuge concept was tested with lower dose events, it did not delay resistance when deployed in the manner currently dominating the market (Deitloff et al. 2016). As with other strategies, Bt maize has not remained as effective after its popularity as a management strategy has increased. Since the first report

of field-evolved resistance to Cry3Bb1, other states have documented *D. v. virgifera* populations with Cry3Bb1 resistance (Zukoff et al. 2016; Wangila et al. 2015; Schrader et al. 2016). Unfortunately, some level of resistance to Cry3Bb1 confers cross-resistance to both mCry3A and eCry3.1Ab (Zukoff et al. 2016). One publication has documented incomplete resistance to Cry34/35Ab1 (Gassmann et al. 2016). Single-gene products are just now beginning to be phased out of the market. Unfortunately, all current commercial transgenic products including pyramids targeting *Diabrotica* spp. also are not considered 'high-dose' so refuges are likely to do little, if anything as currently implemented (Deitloff et al. 2016).

While the Bt proteins expressed by transgenic maize hybrids do add protection against *Diabrotica* spp., limited studies have shown that the efficacy is less for *D. barberi* versus *D. v. virgifera* (Table 2). Given Bt's reduced efficacy against *D. barberi*, there were early concerns that populations of *D. barberi* might develop resistance to Bt before *D. v. virgifera*. This scenario did not manifest; instead, *D. barberi* populations crashed during the time frame when Bt corn targeting *Diabrotica* spp. became widely adopted. *D. v. virgifera* populations later blossomed from development of Bt resistance. Why has Bt resistance not yet developed in *D. barberi*? It is tempting to infer reduced capacity for resistance, but a more likely explanation may lie in their resistance to crop rotation via extended diapause (Krysan et al. 1986). The extended diapause biotype predominates in the geographical region mentioned above. Thus, only one *D. barberi* generation every 2 years would delay resistance to Bt corn at least two fold. *D. v. virgifera* resistance to Bt took six years to develop (2003–2009). Given this length of time with *D. v. virgifera*, signs

of *D. barberi* Bt resistance could be expected to occur soon, assuming similar mechanism of resistance and gene frequencies are present in both species.

Microbes and their implications for Bt resistance. Microbes have been documented to influence the susceptibility of lepidopteran insects targeted by Bt plants. Gut microbiota actually appear to be required for Bt susceptibility in lepidopteran pests (Caccia et al. 2016; Broderick et al. 2006; Paramasiva 2014a, 2014b; Visweshwar et al. 2015). Gut microbes also play a role in crop rotation resistance in *D. v. virgifera*, but the role of gut microbiota in Bt resistance and susceptibility is unknown (Dematheis et al. 2012a). Feeding of *D. v. virgifera* larvae on corn root tissue was shown to affect root rhizosphere microbiota composition, indicating complex, multitrophic interactions (Dematheis et al. 2012b).

Changing guidelines for resistance management. Previously, registrants were required to conduct annual, random sampling programs to monitor susceptibility as a condition of registration (EPA 2003, 2005, 2007, 2015). In order to comply, registrants collected both random populations and targeted populations (fields with greater than expected damage) when possible (i.e. if notified before adults died, resources available for collection, etc.), and eggs produced were collected. These eggs were then overwintered, allowed to hatch and then tested in diet toxicity assays and possibly plant assays. When resistance developed to Cry3Bb1 and mCry3A, the US EPA convened a Scientific Advisory Panel (SAP) to discuss changes to resistance monitoring programs. After considering the recommendations of the SAP, the EPA altered the compliance requirements for registrants. Registrants are no longer required to conduct random sampling; instead, they are required to collect adults from fields with performance issues,

when possible, to test the offspring. Furthermore, registrants are now encouraged to conduct plant assays instead of diet toxicity assays (EPA 2016).

Registrants and academic researchers can use a variety of assays to test *D. v. virgifera* populations for Bt resistance and product efficacy characterizations. Researchers tend to use just one assay to make characterizations (Gassmann et al. 2014; Wangila et al. 2015). There are at least three different plant assays that could be used, each with the capability to estimate the survival rate and developmental parameters (Zukoff et al. 2016). Developmental parameters are extremely valuable to characterize Bt resistance, but have not always been used. The first plant assay, a seedling assay, uses many maize seeds (Bt and non-Bt) and eggs or larvae in a relatively small, plastic container with a few dozen germinated seeds. The second and third assays, a single plant and greenhouse pot assay, are not much different. The single plant assay uses neonate larvae (<24 h old) on a V5 maize plant (Bt or non-Bt). Larvae are left to feed for 17 days before being extracted and data collected (Gassmann et al. 2011; Gassmann et al. 2016). Greenhouse pot assays may use larvae or eggs but still uses V5 maize plants like the single plant assay. While each assay is likely capable of detecting resistance, there may be one assay or one variable that is best able to discriminate between susceptible and resistant populations and this could also be toxin specific. Further research is needed to clarify optimal assays.

As discussed above, resistance monitoring programs by registrants were previously conducted using diet-toxicity assays. Data generated and submitted to the EPA for different proteins have not been comparable due to different proprietary artificial diet formulations used by each of the major companies. Since these diets are proprietary, academic researchers must obtain special permission to access to them. This issue may have

influenced the EPA towards a shift away from diet toxicity assays in resistance monitoring programs. If a single, public and easy-to-use artificial diet can be generated for this purpose, then some of the problems will be addressed. Data from resistance monitoring programs could be compared for the different Bt proteins. Secondly, a public diet would allow academic researchers to conduct these assays with their own toxins or other toxins for which they are able to access.

Future concerns and efforts. Due to the very adaptive nature of *Diabrotica* spp., we propose areas of research that could be further improved upon or investigated for applications to rootworm management. Soil insecticides often do not significantly control *D. v. virgifera* population levels (Gray et al. 1992). When feeding stimulants were added to thiamethoxam, the level of the toxin needed to kill 50% of the larvae was reduced by more than 100 000-fold (Bernklau et al. 2011). This demonstrates that understanding the chemical ecology of this pest can improve management strategies. Repellant properties of methyl anthranilate from corn root extract have been documented (Bernklau et al. 2016). Perhaps by placing methyl anthranilate in furrow with non-Bt maize plants, larvae could be pushed to plants with insecticides or transgenic maize as part of a push-pull strategy analogous to other similar strategies utilized in other systems.

Earlier work on host location cues, specifically CO2 (Strnad et al. 1986; Hibbard and Bjostad 1988; Bernklau and Bjostad 1998), is now being utilized in experiments with an attract-and-kill strategy (Schumann et al. 2014). Through use of CO2-emitting capsules, larvae were attracted to maize roots treated with the insecticide tethfluthrin. Similarly, use of a repellant may create a similar effect as the attract-and-kill strategy. As more research

is conducted with *Diabrotica* spp. ecology and chemical ecology, additional management strategies may become available in the future.

Molecular biology, like chemical ecology, continues to give insight into finding additional management strategies. By understanding how cells work, different processes can be manipulated to control *D. v. virgifera*. Baum et al. (2007) was the first to discuss the potential of RNA interference (RNAi) for the control of *D. v. virgifera*. Expression of specific double-stranded RNA fragments by plants elicits a defense mechanism where cells no longer transcribe targeted genes into proteins. Depending on how many redundancies are present in the insect genome, the insect may begin to deteriorate or even die if the protein plays a crucial role in the survival of the insect. Monsanto Company was the first to announce a maize product, which utilizes RNAi technology. This product, announced as SmartStax Pro, will express two Bt events (MON88017 and DAS-59122-7) targeting *Diabrotica* spp. and RNAi technology with one target gene (DvSnf7). SmartStax Pro is registered for commercial use by the US EPA, but awaiting import approval in China. Recently, at least two other companies have announced plans to use RNAi in a product. DuPont Pioneer announced two *Diabrotica* spp. target genes that will be expressed simultaneously by maize plants. Syngenta has announced a product where dsRNA is applied as a soil treatment rather than through a transgenic plant.

Lastly, one recently published scientific breakthrough may help to overcome Bt resistance (Badran et al. 2016). Phage-assisted continuous evolution takes advantage of naturally occurring processes to expedite Bt toxin evolution. These evolved Bt toxins have a high binding affinity for new receptors on the midgut tissue. This technique allowed the

researchers to improve the efficacy of the Cry1Ac by 335-fold, even against Cry1Ac resistant insects. This tool might be effective for overcoming Bt resistance.

CONCLUDING REMARKS

Diabrotica spp. have a long history of adapting to management practices. Some practices have remained effective for several decades, while others begin to lose efficacy within just a few generations of selection. Until high dose transgenic maize hybrids targeting *Diabrotica* spp. are created, current refuge strategies are likely inadequate to significantly delay resistance development by these pests (Tables 1, 2). Multiple management tactics should be employed by growers, industry and regulatory agencies, when possible, to combat the adaptive nature of these pests (Andow et al. 2016). While some new products are nearing the market, it is clear that *Diabrotica* spp. will continue to adapt. Continued research on all aspects of *Diabrotica* spp. is needed if maize growers are to have permanent success against *D. v. virgifera* and *D. barberi*. This needs to include adaptive IRM approaches and pro-active, integrated IRM-pest management strategies (Andow et al. 2016).

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Table 1. Effects of various hypothetical efficacies with a 20 percent block refuge on the total number of insects produced from refuge plants versus Bt plants.

Hypothetical Efficacy (%)	Ratio of insects from refuge versus transgenic[1]
90	2.5:1
99	25:1
99.9	250:1
99.99	2,500:1
99.999	25,000:1

[1]Ratio calculated through use of this formula: (1 × percent refuge size)/(corrected survival × percent Bt size)

Table 2. Efficacies of Bt products with current events targeting *Diabrotica* spp. and the effect they have on the number of insects produced from refuge plants versus Bt plants.

Protein in Current or Former Commercial Events (Scenario)[1]	*D. v. virgifera* Efficacy (%)[2]	Calculated Ratio[3]	*D. barberi* Efficacy (%)[2]	Calculated Ratio[3]
Cry3Bb1 (Field)[5]	98.49	16.56:1	86.39	1.84:1
Cry34/35Ab1 (Field)[7, 8]	97.3	9.26:1	78.94	1.19:1
mCry3A (Field)[8]	94.88	4.88:1	86.68	1.88:1
eCry3.1Ab (Field)[9, 10, 11]	99.79	119.05:1	95.92	6.13:1
Cry3Bb1 + Cry34/35Ab1, pure (Field)[12]	99.14	6.12:1	97.71	2.30:1
mCry3A + eCry3.1Ab, pure (Field)	99.9[11]	58.48:1	96.26[13]	1.41:1

[1]Refuge size assumed to be 20 percent block refuge for single toxin products and 5 percent for pyramided toxin products sold as refuge-in-bag; Refuge-in-bag products have a smaller requirement than block refuges for single toxin products
[2]Highest efficacy data used
[3]Ratio calculated through use of this formula: (1 × percent refuge size)/(corrected survival × percent Bt size)
[4]Data from Meihls et al. (2008)
[5]Data from Clark et al. (2012)
[6]Data from Binning et al. (2010)
[7]*D. v. virgifera* data from Storer et al. (2006), *D. barberi* data from Table 7 of Head et al. (2014)
[8]Data from Hibbard et al. (2010)
[9]Commercial hybrids express mCry3A and eCry3.1Ab proteins
[10]This treatment is not commercially available but was used to evaluate the likelihood of resistance development
[11]Data from Hibbard et al. (2011)
[12]Data from Tables 4 and 8 of Head et al. (2014)
[13]Data from Frank et al. (2015)

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Chapter 2

Minnesota field population of western corn rootworm (Coleoptera: Chrysomelidae) shows incomplete resistance to Cry34/Cry35Ab1 and Cry3Bb1*

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ABSTRACT

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), is commonly managed with transgenic corn (*Zea mays* L.) expressing insecticidal proteins from the bacteria *Bacillus thuringiensis* Berliner (Bt) in the United States of America. Colonies of this pest have been selected in the laboratory on each commercially available transformation event; some field populations are also resistant. In the current study, progeny of a western corn rootworm population collected from a Minnesota corn field planted to SmartStax® corn were evaluated for resistance to corn hybrids expressing Cry3Bb1 (event MON88017) or Cry34/35Ab1 (event DAS-59122-7) and to the individual constituent proteins in diet-overlay bioassays. Results from all of these assays suggest that this population has resistance to Cry3Bb1 and has incomplete resistance to Cry34/35Ab1. In diet toxicity assays, larvae of the MN population had resistance ratios of 4.71 and >13.22 for Cry34/35Ab1 and Cry3Bb1 proteins, respectively, compared with the control colonies. In all on-plant assays, the relative survival of the MN population on the DAS-59122-7 and MON88017 hybrids was significantly greater than the control colonies. Larvae of the MN population had inhibited development when reared on DAS-59122-7 compared with larvae reared on the non-Bt hybrid, indicating resistance was incomplete. Overall, these results document resistance to Cry3Bb1 and an incomplete resistance to Cry34/35Ab1 in a SmartStax® performance problem field.

BACKGROUND/INTRODUCTION

At the end of the 20th century, the United States Environmental Protection Agency (EPA) approved the commercialization of crops expressing Cry toxins derived from *Bacillus thuringiensis* (Bt). The first corn (*Zea mays* L.) product expressing a Bt toxin active against lepidopteran pests of corn was registered in 1995 (USDA-APHIS 1995) and became commercially available in 1996. Farmers have quickly adopted transgenic crops expressing Bt protein since commercialization in 1996. Currently, Bt corn is planted on 81% of acres for the benefit of enhanced protection from herbivores (UDSA-NASS 2015). Area-wide suppression of some pests, such as European corn borer, *Ostrinia nubilalis* (Hübner), has been reported following widespread adoption with both those planting Bt and those not growing Bt corn benefiting from several $billion in cumulative savings (Hutchison et al. 2010). In 2003, the first corn product with Bt toxins (event MON863, Cry3Bb1) active against corn rootworms was approved for commercial sale (EPA 2003). Over the next decade, three more proteins, including Cry34Ab1/Cry35Ab1 (hereafter Cry34/35Ab1, event DAS-59122-7), mCry3A (event MIR604), and eCry3.1Ab (event 5307) were commercialized for management of corn rootworms (EPA 2005, 2007, 2015).

Three Bt proteins (Cry3Bb1, mCry3A, and Cry34/35Ab1) targeting western corn rootworm (*Diabrotica virgifera virgifera* LeConte) are expressed singly in corn hybrids. Some examples of single-traits products are: YieldGard™ VT Triple Pro (Cry3Bb1, event MON88017), Agrisure™ RW or 3111 (mCry3A), and Herculex RW™ (Cry34/35Ab1). Hybrids expressing multiple proteins targeting the same pest in a single plant, referred to as pyramids, are also available commercially and some of the pyramid products are: SmartStax® (Cry3Bb1 + Cry34/35Ab1), Agrisure® 3122 (mCry3A + Cry34/35Ab1) and

Optimum® Intrasect Xtreme (Cry34/35Ab1 + mCry3A). The most recent Bt protein, eCry3.1Ab (event 5307), was approved for commercialization in 2013, and is sold only as a pyramid (mCry3A + eCry3.1Ab) (EPA 2015) under the product name, Agrisure Duracade®.

Pyramided products, like those mentioned above, can significantly delay resistance by having more than one mode of action, but the occurrence of cross-resistance between incorporated proteins decreases the efficacy of the pyramid (Roush 1998). Resistance is defined as a genetically based shift in overall susceptibility (e.g. survival, development, etc.) as a result of a population's exposure to a toxin (National Research Council 1986; Tabashnik 1994). In more practical terms, resistance means a population exposed to a toxin has greater fitness or survival on the toxin than a population or colony that has never been exposed to the toxin. The presence of a single-trait product within the dispersal distance of a pyramided product was suggested to decrease the longevity of both products in modeling studies (Zhao et al. 2005; Onstad and Meinke 2010).

The potential durability of each Bt protein targeting western corn rootworm was previously evaluated under laboratory conditions. Laboratory colonies of western corn rootworm were selected for resistance to single Bt proteins (Lefko et al. 2008; Meihls et al. 2008, 2011, 2012; Oswald et al. 2012; Frank et al. 2013). Some wild populations have also rapidly developed resistance to corn expressing the Cry3Bb1 protein (Gassmann et al. 2011). Planting cornfields with hybrids expressing Cry3Bb1 for three or more consecutive years was correlated with resistance development. Since the initial documentation of field-evolved resistance to Cry3Bb1 in Iowa (Gassmann et al. 2011), resistance has also been reported for populations in Nebraska, Minnesota, and Illinois (Wangila et al. 2015; Zukoff

et al. 2016; Schrader et al. 2016). Cross-resistance between Cry3Bb1 and mCry3A has been documented previously (Gassmann et al. 2014) and more recently between Cry3Bb1, mCry3A, and eCry3.1Ab (Zukoff et al. 2016). The documented resistance of wild western rootworm populations to Cry3Bb1 (Gassmann et al. 2011), mCry3A (Gassmann et al. 2014), and their cross-resistance with eCry3.1Ab (Zukoff et al. 2016) increases selection pressure on the second protein in any pyramid containing any of the proteins (Zhao et al. 2005; Onstad and Meinke 2010).

Against this backdrop of relatively widespread field resistance to Cry3Bb1 and mCry3A, field performance issues have been infrequent with corn expressing Cry34/35Ab1 and laboratory development of Cry34/35Ab1 resistance has been slower. In multiple laboratory studies to select for Cry34/35Ab1 resistance, complete resistance (i.e. survival on Bt is equivalent to isoline) was not attained (Lefko et al. 2008; Deitloff et al. 2015) even after more than 20 generations of selection (Stephen Thompson, DuPont Pioneer, personal communication). Contrary to what was observed with Cry34/35Ab1 selection attempts, nearly complete resistance to the other three Bt proteins (Cry3Bb1, mCry3A, eCry3.1Ab) under laboratory selection was obtained within six generations or fewer (Meihls et al. 2008, 2011; Frank et al. 2013). Survival on corn expressing Cry34/35Ab1 was only ~20% of the near-isoline survival even after 10 generations of selection (Lefko et al. 2008), which likely contributes to delaying resistance in the field.

The EPA mandates that fields with single or pyramided events that experience nodal injury ratings (Oleson et al. 2005) greater than 1.0 for a single protein and 0.5 for pyramided proteins be investigated by product registrants for performance issues (Andow et al. 2016). Due to these requirements, a field in Brown County, MN reported greater-

than-expected damage on SmartStax®, and triggered the interest of researchers. Field-evolved Cry34/35Ab1 had not been documented until very recently (Gassmann et al. 2016). This study's objective was to characterize the western corn rootworm population for resistance or susceptibility to one or both proteins expressed by SmartStax® corn hybrids.

RESULTS

Diet Toxicity Assay

Cry34/35Ab1. Based on confidence intervals, the LC_{50} and EC_{50} values were significantly greater for the MN field population than those of the diapausing control colony (Table 3). The LC_{50} value of the MN field population was 4.71 times greater than the control colony. The EC_{50} value of the MN field population was 3.78 times greater than the diapausing control colony.

Cry3Bb1. When confidence intervals were compared with the non-diapausing control colony, the MN field population had significantly greater LC_{50} and EC_{50} values. Resistance ratios of the MN field population and MON88017-selected population were both >13.22 (Table 3). Relative potency (i.e. the EC_{50} of the selected population divided by the EC_{50} for the control colony) of the MN field and MON88017-selected sources were 3.07 and >15.42, respectively.

Seedling Assays

Relative larval recovery (Bt/non-Bt) for DAS-59122-7. Survival of the MN field population on DAS-59122-7 relative to the non-Bt survival (0.68±0.09) was significantly greater than the non-diapausing (0.17±0.09) and diapausing control (0.27±0.12) colonies, but not significantly different from the MON88017-selected colony (0.46±0.10) (Fig. 1A).

Relative survival of the MON88017-selected laboratory colony (0.46±0.10) on DAS-59122-7 was also significantly greater than that of its paired non-diapausing control colony (0.17±0.09).

Relative larval weight (Bt/non-Bt) for DAS-59122-7. The relative weights of two insect sources (MN field population and the diapausing control colony) were significantly greater than the relative weight of the non-diapausing control colony (Fig. 1B). Relative weights of larvae from the MN field population were significantly more than the relative weights of the MON88017-selected colony following exposure to DAS-59122-7 relative to its paired non-Bt hybrid. The MON88017-selected colony was intermediate in relative weight, and not significantly different from the diapausing control or non-diapausing control colonies. The relative weight of the MON88017-selected colony on DAS-59122-7 was only 0.18±0.04 relative to weight of the same colony on the paired non-Bt hybrid (Fig. 1B).

Relative larval head capsule width (Bt/non-Bt) for DAS-59122-7. There was a significant effect of insect source on the average relative head capsule width for larvae reared on DAS-59122-7 versus its paired non-Bt hybrid. Larvae of the MN field population had significantly larger relative head capsule widths on DAS-59122-7 versus its paired non-Bt hybrid than larvae of the diapausing control, non-diapausing control, or MON88017-selected colonies (Fig. 1C). Larvae of the MON88017-selected colony had head capsule widths significantly larger than those of the paired non-diapausing control colony, though they did not differ significantly from those of the diapausing control colony. The non-diapausing control colony had the smallest relative head capsule widths overall.

Relative larval recovery (Bt/near-isoline) for MON88017. The relative survival of the MON88017-selected laboratory colony on MON88017 versus its near-isoline was largest (1.11±0.20) and was significantly greater than the diapausing control colony (0.09±0.04) (Fig. 1D; Table 4). The relative survival of the MN field population was intermediate between the MON88017-selected and the diapausing control colonies. The MN field population, which had been exposed to Cry3Bb1-expressing corn for four years previously, had significantly greater relative survival on Cry3Bb1-expressing seedlings (0.43±0.11) than the diapausing control colony (0.09±0.04) (Fig. 1D). However, this level of relative survival for the MN field population did not achieve the high level observed with the MON88017-selected colony (selected on Cry3Bb1-expressing corn for >35 generations).

Relative larval weight (Bt/near-isoline) for MON88017. When reared on MON88017 seedlings, larvae of the MN field population and the MON88017-selected colony weighed significantly more than those of the diapausing control colony relative to weight on near-isoline plants (Fig. 1E). Despite the low relative survival of the MN field population on Cry3Bb1-expressing seedlings versus the MON88017-selected colony, the MN field larvae that did survive had relative larval weights (0.75±0.08) similar to those of the MON88017-selected colony (Fig. 1E).

Relative larval head capsule width (Bt/near-isoline) for MON88017. There were no significant differences between the head capsule width of the MN field population and MON88017-selected colony (Fig. 1F; Table 4).

Greenhouse Pot Assay

Relative larval recovery. Larval recovery was significantly impacted by the main effects of insect source, corn type, and trial (Table 4). The relative survival of the MN field population was significantly greater (0.63±0.12; 0.5±0.15) than the diapausing control colony (0.25±0.05; 0.08±0.04) on DAS-59122-7 and MON88017, respectively (Fig. 2A). Additionally, the survival of both populations was significantly greater on DAS-59122-7 than MON88017.

Relative larval weight. Relative larval weight was significantly impacted by the main effects of corn type and trial (Table 4). When averaged across insect sources, relative larval weight on DAS-59122-7 was lower than relative larval weight on MON88017 (Fig. 2B).

Relative head capsule width. Relative head capsule width was affected by the main effects of insect source, corn type, trial, and the insect source × corn type interaction (Table 4). The relative head capsule width was greater for the MN field population than the diapausing control colony on MON88017, but not DAS-59122-7 (Fig. 2C).

DISCUSSION

Complete resistance to Cry34/35Ab1 has not been reported in the refereed literature. Even after 10 generations of selection in the laboratory, colony fitness (survival to adulthood in this instance) of two selected colonies on DAS-59122-7 was only ~20% of the same colonies on near-isoline corn (Lefko et al. 2008). There also was no difference between selected and control populations in diet assays (Nowatzki et al. 2008) despite between 15- and 58-fold increases in survival (Lefko et al. 2008). In contrast, nearly complete resistance to Cry3Bb1, mCry3A, and eCry3.1Ab was reported within just a few generations of laboratory selection every time it was tried (Meihls et al. 2008, 2011, 2012;

Oswald et al. 2011; Frank et al. 2013) and when data were available, these had significant differences in diet assays also. Until very recently (Gassmann et al. 2016), field populations collected from corn expressing Cry34/35Ab1 had not been shown to demonstrate either incomplete or complete resistance. Now, two populations of western corn rootworm (Gassmann et al. 2016 and this study) located more than 160 km from one another have developed incomplete resistance to Cry34/35Ab1. This suggests that resistance to Cry34/35Ab1 is developing independently in multiple rootworm populations as has occurred with resistance to Cry3Bb1 (Gassmann et al. 2011; Wangila et al. 2015; Zukoff et al. 2016). As laboratory selection experiments have shown Cry34/35Ab1 resistance to take many generations of selection (Lefko et al. 2008; Deitloff et al. 2016), the level of resistance to Cry34/35Ab1 found in the MN field population is not close to full resistance. Instead, we present evidence that progeny of adults collected from the SmartStax® corn field in Minnesota show evidence of incomplete resistance to Cry34/35Ab1-expressing corn in multiple bioassays.

The MN field population had significantly greater LC_{50} and EC_{50} values in the Cry34/35Ab1 and Cry3Bb1 diet toxicity assay when compared with the control colonies (Table 3). Although the MN field population's Cry34/35Ab1 diet toxicity data fall within the range of data collected from field populations in the past (EPA 2014), these data were significantly greater than those of the diapausing control colony. Historical data for western corn rootworm populations exposed to Cry34/35Ab1 in diet toxicity assays is highly variable. This variability could be due to changes in the length of assays as well as differences in baseline susceptibility between western corn rootworm populations (EPA 2014), or even how the LC_{50} and EC_{50} values were calculated. Even selected populations

with greater than a 15-fold increase in survivorship did not show significantly elevated LC_{50} values (Nowatzki et al. 2008), so it could also be that the resistance mechanism to Cry34/35Ab1 cannot be detected in diet assays. Plant-based bioassay data from this study (Figs. 1A, 1C, and 2A; Table 3) also suggest this population had both resistance to Cry3Bb1 and incomplete resistance to Cry34/35Ab1. Since resistance to both toxins in SmartStax® was present at some level, this may have been the cause for the high damage to SmartStax® corn in the Minnesota field where adults were collected. This damage may have been prevented by following best management practices (Andow et al. 2016) for western corn rootworm, such as rotating to soybean or another non-host, rotating modes of action, or planting corn expressing multiple proteins (EPA 2016), as was done to this field following this discovery.

Baseline survival data are available for western corn rootworm larvae exposed to DAS-59122-7; Binning et al. (2010) observed 33% survivorship on DAS-59122-7 relative to near-isoline survivorship after 17 d for two previously unexposed populations. Survivorship of the MN population was 2.1- and 1.9-fold greater in seedling and greenhouse pot assays, respectively, when compared to the Binning et al. (2010) data. Earlier data for an unexposed colony fed Cry3Bb1-expressing corn (Meihls et al. 2008) documented a relative survival of 0.25. When we compared the MN survival data to the previous relative survival rate of a control colony on Cry3Bb1-expressing corn, we observed 1.7- and 2.0-fold increases in rates of relative survival in the seedling and greenhouse pot assays, respectively.

The Cry3Bb1 LC_{50} and EC_{50} values of larvae from the MN field population were at least 18.6- and 12-fold greater than the greatest baseline LC_{50} and EC_{50} values recorded

for different Nebraska field populations of western corn rootworm prior to the commercialization of Cry3Bb1 (Siegfried et al. 2005). For Cry3Bb1 diet toxicity studies, the MON88017-selected and MN populations were too resistant for accurate calculation of LC_{50} values in our study because the highest dose provided by Monsanto, 170.8 µg/cm^2, produced less than 50% mortality in the population. In future work, higher doses of Cry3Bb1 will need to be included when performing diet toxicity testing on potentially resistant populations if accurate data are to be collected.

The population collected from the Minnesota SmartStax® field included some teneral adults, which means these recently emerged insects were most certainly from this field. However, there is no way to determine what percentage of adults collected had emerged from this field versus neighboring fields. This field was consistently planted later than neighboring fields, and movement of adults from nearby fields into late pollinating fields can be significant (Naranjo 1994). Some of the data indicate that the MN field population was resistant to both Cry3Bb1 and Cry34/35Ab1 (Figs. 1A, 1C-F, and 2A; Table 3), but also demonstrate resistance was not complete (Figs. 1A-F, and 2A-C). Due to the reduced development on DAS-59122-7 hybrid compared to the non-Bt hybrid, incomplete resistance is the only logical conclusion. This experiment also emphasizes the need to use multiple techniques to evaluate potential resistance(s) in populations (Zukoff et al. 2016).

The field where the MN field population was collected had been planted to Cry3Bb1-expressing corn from 2009-2012 and appears to have put a significant strain on the durability of the pyramided product, SmartStax®. This observation supports previous modeling studies (Zhao et al. 2005; Onstad and Meinke 2010). As we are not certain of

the origin(s) of all collected insects, we offer some possible causes for the failure of the SmartStax® product which include: 1) a large CryBb1-resistant population overwhelmed the Cry34/35Ab1 toxin, 2) an influx of adults of a Cry34/35Ab1-resistant population from another field, such as a Herculex field on the same farm also had performance issues in 2013 or several fields within 25 km SSW of this site had unusual amounts of feeding on Herculex and SmartStax® hybrids in 2012, or 3) two separate populations of western corn rootworm resistant to Cry3Bb1 or Cry34/35Ab1 could be present in the same field.

These results indicate the early stages of resistance development (i.e. incomplete resistance) as larvae showed some developmental delays on corn expressing Cry34/35Ab1 (Rudeen and Gassmann 2013; Shrestha et al. 2016) or Cry3Bb1 when compared to the respective isolines. When the SmartStax® field damage is coupled with these data, these points indicate the MN field population was significantly less susceptible to corn expressing Cry34/35Ab1 than other populations tested in this study or other studies (Gassmann et al. 2011, 2016; Shrestha et al. 2016; Wangila et al. 2015). Insects were only evaluated on corn expressing Cry34/35Ab1 or Cry3Bb1 separately, so we cannot draw conclusions about what these data mean for a pyramided product. We present the second case of a shift in susceptibility by western corn rootworm to corn expressing Cry34/35Ab1.

Cry34/35Ab1-expressing corn has been on the market since 2006 (EPA 2005). Field failures have been much more common with the other single gene products targeting rootworm than corn expressing Cry34/35Ab1. However, as this product ages, and gains market share as a pyramid with other genes, selection continues to increase. This is especially the case in areas with resistance to Cry3Bb1, mCry3A, and/or eCry3.1Ab (Zhou et al. 2005; Zukoff et al. 2016). Industry, regulatory agencies, and growers need to be

mindful that shifts in susceptibility to toxins become more likely as selection increases (Porter et al. 2012). As shifts in susceptibility begin to occur, such as documented here, best management practices must be implemented quickly to prevent further development and spread of rootworm genotypes with increased tolerance to Cry34/35Ab1 (Andow et al. 2016). Since no fully independent, new modes of action have been registered for commercial use in transgenic corn against rootworms as since 2006, preserving Cry34/35Ab1 for as long as possible is vital for rootworm management for the foreseeable future.

MATERIAL AND METHODS

Field History

In 2013, a grower in Brown County, MN, reported greater-than-expected damage to SmartStax® corn, which expresses both Cry34/35Ab1 and Cry3Bb1 proteins. In each of the previous 4 yr, the field was planted to corn expressing Cry3Bb1. We investigated the field, confirmed Bt expression of both Cry3Bb1 and Cry34Ab1 proteins through lateral flow strip testing (Envirologix, Portland, ME), sampled roots to verify corn rootworm injury, and confirmed more than one node of injury per SmartStax® plant. After the greater-than-expected damage was documented, adult insects were collected and eggs were subsequently obtained. Following this incidence of greater-than-expected damage, a best management practice was implemented (rotation to soybean, Andow et al. 2016) to prevent further survival of this population.

Insect Sources

Minnesota field population. Western corn rootworm adults were collected from the field on 25 September 2013. Adults recovered from this field were delivered to French Agricultural Research in Lamberton, MN, on the day of collection where they were allowed to mate, if needed, and oviposit. Plastic containers (8 cm wide × 5 cm deep) with unsieved field soil from the area were provided as an oviposition substrate and resulting eggs were overwintered at 7-8 °C after adjustment periods of 2 wk each at 24 °C and 15 °C. Oviposition dishes with eggs were provided to the USDA-ARS Plant Genetics Research Unit in Columbia, MO on 28 May 2014. Insects from this population will, hereafter, be referred to as "MN field".

Methods for flotation of western corn rootworm eggs from soil were modified from Števo and Cagaáň (2012) for our purposes. Briefly, a 2M $MgSO_4$ solution was prepared by mixing 493 g of Epsom salts ($MgSO_4 \cdot 7H_2O$) (Vi-Jon Inc., Smyrna, Tennessee, USA) per liter of water. The Epsom salt solution was then placed in a 2 liter graduated cylinder and 0.47 liter of soil was carefully added. Extracted eggs were collected with a transfer pipette and placed into a 0.15% agar solution for suspension. Eggs were gently stirred to create a homogenous solution. Five 1 ml samples were counted to estimate the number of eggs per ml, allowing for extrapolation to the entire solution.

Control colonies. Control western corn rootworm colonies originated from the USDA-ARS laboratory in Brookings, SD, and included both the primary diapausing colony (Jackson 1986) and the primary non-diapausing colony (Branson 1976). Both colonies were collected prior to the release of transgenic crops. Insects from the primary diapausing colony will, hereafter, be referred to as "diapausing control". Insects from the primary non-diapausing colony will, hereafter, be referred to as "non-diapausing control".

A shipment of the non-diapausing control eggs was received from the USDA-ARS laboratory in Brookings, SD, and the colony was maintained for multiple generations at the USDA-ARS laboratory in Columbia, MO. Eggs of the diapausing control were shipped from the USDA-ARS laboratory in Brookings, SD, and removed from cold storage as needed for experiments.

MON88017-selected colony. This colony was developed in 2013 by mixing three MON88017-selected colonies from Meihls et al. (2008, 2012) to reduce labor and maintenance costs. Insects of this colony will, hereafter, be referred to as "MON88017-selected". This non-diapausing colony from the USDA-ARS laboratory in Columbia, MO had been reared on a Cry3Bb1-expressing corn hybrid, DKC 61-88 (Monsanto Company, St. Louis, Missouri, USA) and its non-Bt near-isoline corn hybrid, DKC 61-79 (Monsanto Company) as described in Meihls et al. (2012) and more recently reared continuously on Cry3Bb1-expressing corn for more than 35 generations. A near-isoline corn hybrid shares similar genetics to the Bt corn hybrid but does not contain the Bt gene.

Rearing conditions. Non-diapausing insects were reared under the following conditions at the Columbia, MO, site. Western corn rootworm adults were reared in cages (30 cm^3, Megaview Science Co., Ltd., Taichung, Taiwan) with a photoperiod of 14:10 (L:D) h at 25 °C. Adults were fed isoline leaf tissue, zucchini slices, adult artificial diet (Frontier Agricultural Sciences, Newark, Delaware, USA), and water. Petri dishes containing 70 mesh sieved autoclaved field soil served as an oviposition site for females and were replaced on a weekly basis. Eggs were recovered from oviposition plates by washing through a 60 mesh sieve.

Diet Toxicity Assay

Diet toxicity assays were conducted in June 2014 by Custom Bio-Products in Maxwell, IA, with methods similar to Siegfried et al. (2005). The Cry34Ab1 and Cry35Ab1 proteins were provided by Dow AgroSciences. The Cry3Bb1 protein was provided by Monsanto Company. Briefly, neonate larvae of the non-diapausing control, MON88017-selected, and MN field sources were exposed to increasing concentrations of Cry3Bb1 protein overlaid on artificial diet (0-170.80 μg/cm^2) for 5 d. Neonate larvae of the diapausing control and MN field sources were exposed to increasing concentrations of both Cry34Ab1 and Cry35Ab1 proteins overlaid on artificial diet (0-60 μg/cm^2) for 5 d. Testing with Cry3Bb1 and Cry34/35Ab1 were conducted separately at a similar time. Larval survival and weight data were recorded upon completion.

Seedling Assays

For the assay, a two-way factorial (western corn rootworm source × corn type) arrangement was used in a randomized complete block design for the seedling assay with DAS-59122-7 corn (DuPont Pioneer, variety 33Y76) and its paired non-Bt hybrid (DuPont Pioneer, variety 34H31). The same experimental design was used for a separate seedling assay with MON88017 corn and its near-isoline. All corn seeds used in this study were untreated, but were still washed with a 10% bleach solution before being dried and used in assays.

Eggs were placed in a 0.15% agar solution and gently stirred to achieve uniform egg density. The number of eggs per ml were counted a minimum of three times, averaged, and then dispensed at the targeted rate into the bottom of a plastic container (15 × 10 cm GladWare®, The Glad Products Company, Oakland, California, USA). Different rates were used due to limited egg availability of some insect sources. The number of eggs per

container for each insect source was as follows: MON88017-selected (250 eggs), diapausing control (312 eggs), non-diapausing control (250 eggs), and MN field (200 eggs). Based on hatch rates and hatch delays from subsamples taken earlier, eggs of the MN field population and diapausing control colony were incubated at 23 °C on 7 August 2014, and 11 August 2014, respectively, in 2 cm of growth medium and 20 ml of tap water per plastic container. Growth medium for all experiments was mixed by volume at a 2:1 ratio of soil:Pro-Mix BX potting medium (Premier Horticulture Inc., Quakertown, Pennsylvania, USA) and then autoclaved. Pre-incubation was done to achieve a more synchronized hatch at a similar corn stage of all insect sources before adding seed. For the MON88017-selected and non-diapausing control colonies, eggs, tap water, and 2 cm of growth medium were added to plastic containers on 15 August 2014.

Corn seed (13 ml, ~50 kernels) were spread across the growth medium surface (eggs at bottom) and covered with approximately 4 cm of growth medium followed by 80 ml of tap water on 15 August 2014. Containers that had seed from DAS-59122-7 or its paired non-Bt hybrid were then placed into the model LIFLY-VIEW incubator (Sheldon Manufacturing, Inc., Cornelius, Oregon, USA) at 25 °C. Containers that were planted with MON88017 or its near-isoline were placed in a Conviron (Model E15, Controlled Environments, Inc., Pembina, North Dakota, USA) set to run at 25 °C. The two seedling assays were run simultaneously in separate growth chambers due to space limitations. Two subsamples of 50-100 eggs from each insect source were placed onto moistened filter paper in Petri dishes and then placed in each growth chamber to observe the start of hatch. Similar time to first hatch was observed for each insect source after the planned pre-incubation of the MN field and diapausing control insect sources described above.

Containers with corn seedlings were removed from the incubator 14 d after the first larva was observed in hatch plates held in the same growth chamber. Aboveground plant tissue was cut and the growth medium with root tissue was placed into Tullgren funnels with a 60-W incandescent light bulb for 2 d. Larvae were collected in 473 ml jars containing approximately 2.5 cm of water with jars inspected daily. Larvae were then transferred to scintillation vials containing 95% ethanol after 1 d and 2 d. Counts of each sample were done under a dissecting microscope, head capsule width measurements were made using an ocular micrometer, and larvae were weighed after drying in an oven (Thelco model 16, GCA/Precision Scientific Co., Chicago, Illinois, USA). For samples that contained more than 10 larvae, a subsample of 10 larvae was taken and the head capsule widths of larvae were measured using an ocular micrometer. If samples contained fewer than 10 larvae, then the head capsule widths of all larvae were measured. Weights were recorded after desiccation in an oven (scale model AB135-S FACT, Mettler Toledo Inc., Columbus, Ohio, USA).

Greenhouse Pot Assay

Two trials with five replications each were set up as a two-way factorial (western corn rootworm insect source × corn type) arrangement in a randomized complete block design. These trials were planted 8 d apart and infested with eggs 7 d apart. Both trials contained only the diapausing control and MN field sources. In each replication within each trial, the same four corn hybrids described above were used: MON88017 and its near-isoline, and DAS-59122-7 and its paired non-Bt hybrid. Larvae from these insect sources were exposed to all four corn hybrids in each replication of both trials.

Clay pots (Model 100043015, Home Depot, Columbia, Missouri, USA) filled with growth medium (as described above) were watered until saturation prior to the planting of seeds. Pots contained 3.3 liter of growth medium identical to that used in the seedling assays. Two corn seeds were planted per pot equidistant from the center at a depth of ~2.5 cm. A 114-µm stainless steel mesh screen (TWP Inc., Berkeley, California) was glued over the pot drainage hole to prevent larval escape. Following coleoptile emergence, the entirety of the smaller seedling was removed to achieve one corn plant per pot.Pots were watered to saturation as needed throughout the entirety of the experiment. Once plants reached the V3-V4 growth stages (Ritchie et al. 1992), a small hole was bored 1-2 cm from the base of the plant to a depth of ~2.5 cm and used as the infestation site. All pots were infested with ~80 eggs of one of the two insect sources. Extra pots not used in the replications were infested with ~80 eggs from the diapausing control colony to be checked for larval development.

To monitor start of hatch, small subsamples of western corn rootworm eggs were placed in Petri dishes as described previously. Two or more Petri dishes were placed in the greenhouse with the pots, and two or more were placed in a Percival at 25 °C for each insect source. Petri dishes were evaluated daily to determine the start of hatch. Again, similar time to first hatch was observed for each insect source. By monitoring the extra pots from each planting date, we were able to estimate when larvae were at or close to the third instar. Once third instars were observed for a planting date, aboveground biomass was cut 5-7.6 cm from the growth medium surface. Growth medium with root tissue was treated the same as above in the seedling assays with one difference. Due to the volume of growth medium and tissue in pots, each sample was kept in a Tullgren funnel for 4 d. Each funnel's

jar was checked on day 2 and 4. Larvae were collected and processed using the same methods as the seedling assay.

Statistical Analysis

Diet Toxicity Assays. All data were analyzed using SAS® software version 9.4 (SAS 2013). Prior to analysis, protein concentrations were converted to a log scale (logdose=log(conc+.01))/log(3) for Cry34/35Ab1 and logdose=log2(conc+.01) for Cry3Bb1. These log scale conversions were done due to the tripling and doubling of non-zero doses for Cry34/35Ab1 and Cry3Bb1, respectively. LC_{50} (i.e. concentration required to produce mortality of 50% of the larvae) on artificial diet bioassays was determined using a probit analysis (PROC PROBIT). Larval weight was analyzed using a nonlinear regression (PROC NLIN). Average larval weight was calculated per replicate by dividing total larval weight by initial number of larvae (essentially giving dead larvae a weight of 0 as in Siegfried et al. (2005)). Average larval weight was then used to determine the EC_{50} (i.e. concentration required to produce a 50% weight reduction of the larvae). The resistance ratios were calculated by dividing the LC_{50} of the selected insect source by the control insect source. Relative potency values were calculated by dividing the EC_{50} of the selected source by the control insect source. For both analyses, only replicates with control mortality <20% were considered.

Seedling Assays. Data from DAS-59122-7 and MON88017 seedling assays were analyzed separately. All variables were first converted to relative values based on performance on Bt corn relative to performance on non-Bt corn (i.e., recovery from Bt for each replication/overall average recovery from the paired non-Bt hybrid for that insect source, producing a relative survival value), rank transformed as outlined by Conover and

Iman (1981), and then analyzed using a generalized linear mixed model (PROC GLIMMIX). The data were not normally distributed and common transformations did not result in homogeneity of variance, thus rank transformations were necessary to meet the assumptions of the analysis (Snedecor and Cochran 1989). All variables (relative survival, relative weight, and relative head capsule width) were run as separate analyses. Larval data were analyzed as a randomized complete block, two-way factorial (four western corn rootworm sources × two corn types for DAS-59122-7, three western corn rootworm sources × two corn types for MON88017). Analysis of the MON88017 relative head capsule width data excluded the diapausing control due to a low number of replications. The model contained the main effect of insect source. Replications were included as random variables.

Greenhouse Pot Assay. Larval data were analyzed as a randomized complete block two-way factorial (two western corn rootworm sources × four corn types) using a generalized linear mixed model. Again, all variables were first converted to relative values based on performance on Bt corn relative to performance on non-Bt corn. All larval data were first rank transformed (Conover and Iman 1981) and then analyzed with a Poisson distribution (dist=poisson option in PROC GLIMMIX). Again, the data were not normally distributed and common transformations did not result in homogeneity of variance, so rank transformations were necessary. All variables (larval count, weight, and head capsule width) were run as separate analyses. The model contained the main effects of insect source, corn type, trial, and all possible interactions. Replications within trial were included as random variables.

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Table 3. LC_{50} and EC_{50} calculations for larvae exposed to Cry34/35Ab1 and Cry3Bb1 for five days in a diet overlay bioassay

Protein	Colony	Number of Replications	LC_{50} µg/cm^2 (95% C.I.)	Resistance Ratio[a]	EC_{50} µg/cm^2 (95% C.I)	Relative Potency[b]
Cry34/35Ab1						
	Diapause Cont.	7	0.48 (0.28 – 0.77)	.	0.68 (0.47 – 1.00)	.
	Minnesota	7	2.26 (1.14 – 4.25)	4.71	2.58 (1.99 – 3.33)	3.78
Cry3Bb1						
	Non-Diapause Cont.	5	12.92 (3.08 – 50.84)	.	11.08 (4.28 – 28.70)	.
	Minnesota	7	>170.80	>13.22	34.07 (0.00 – 495,182.22)	3.07
	MON88017-selected	6	>170.80	>13.22	>170.80	>15.42

[a]Resistance ratios were calculated by dividing the LC_{50} of the selected population by the control population.

[b]Relative potency values were calculated by dividing the EC_{50} of the selected population by the control population.

Table 4. Analysis of variance for variables in all experiments.

Assay Type	Variable	Effect	Num DF	Den DF	*F* Value	Pr>*F*
Seedling DAS-59122-7	Survival	insect source	3	31	7.3	0.0008
	Dry Weight	insect source	3	21	7.09	0.0018
	Head Capsule Width	insect source	3	27	7.58	0.0008
Seedling MON88017	Survival	insect source	2	8	30.44	0.0002
	Dry Weight	insect source	2	7	9.37	0.0105
	Head Capsule Width	insect source	1	4	0.52	0.5113
Greenhouse Pot	Survival	trial	1	8	6.00	0.0399
		insect source	1	24	24.02	<0.0001
		corn type	1	24	8.39	0.0079
		trial × insect source	1	24	0.00	1.0000
		trial × corn type	1	24	0.86	0.3626
		corn type × insect source	1	24	0.67	0.4221
		trial × corn type × insect source	1	24	1.32	0.2611
	Dry Weight	trial	1	8	9.02	0.0170
		insect source	1	17	0.72	0.4093
		corn type	1	17	14.67	0.0013
		trial × insect source	1	17	0.13	0.7217
		trial × corn type	1	17	0.44	0.5164
		corn type × insect source	1	17	0.87	0.3647
		trial × corn type × insect source	1	17	2.10	0.1652
	Head Capsule Width	trial	1	8	19.14	0.0024
		insect source	1	19	7.49	0.0131
		corn type	1	19	19.96	0.0003
		trial × insect source	1	19	0.00	0.9957
		trial × corn type	1	19	1.52	0.2324
		corn type × insect source	1	19	6.52	0.0194
		trial × corn type × insect source	1	19	0.01	0.9236

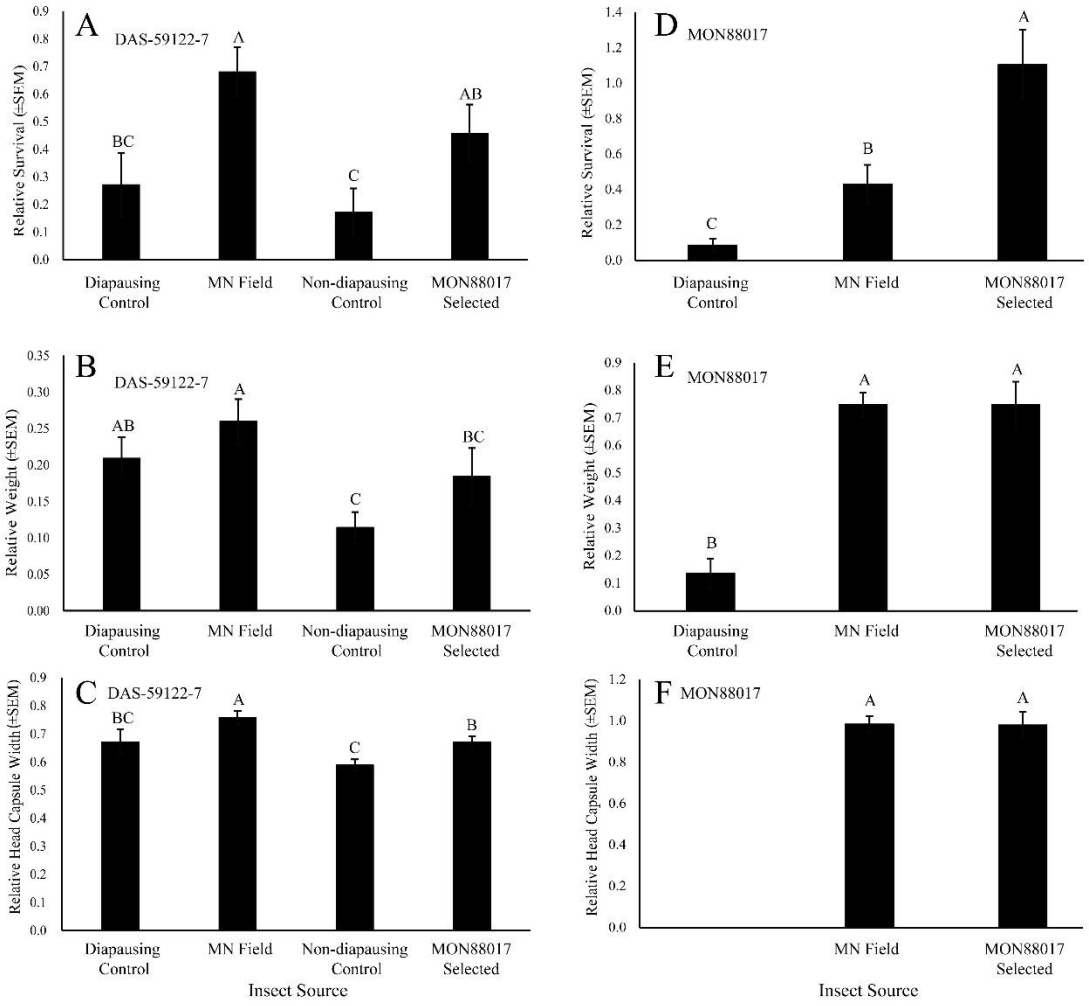

Figure 1. Results of seedling assay on DAS-59122-7 (A-C) and seedling assay on MON88017 (D-F). (A & D) Relative survival (Bt data/overall average of non-Bt data) of larvae on DAS-59122-7 and MON88017. **(B & E)** Relative average weight of recovered larvae on DAS-59122-7 and MON88017. **(C & F)** Relative head capsule width of recovered larvae on DAS-59122-7 and MON88017. Uppercase letters indicate significant differences between populations using rank transformed data. Bar heights represent the means of the treatment and error bars represent standard error of the mean.

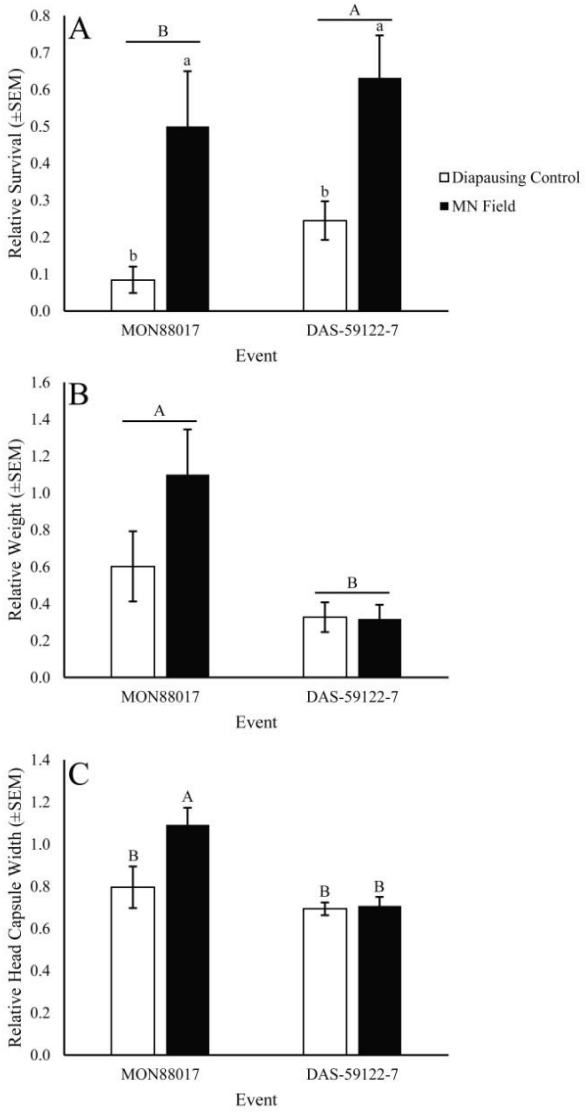

Figure 2. Results of greenhouse pot assay. (A) Relative survival (Bt data/overall average of non-Bt data) of larvae on DAS-59122-7 and MON88017. **(B)** Relative dry weight of recovered larvae. Letters over lines indicate the main effect of corn. **(C)** Relative head capsule width of recovered larvae. Uppercase letters indicate significant differences between corn types within populations. Lowercase letters indicate significant differences between populations within corn type. All data were analyzed following a rank transformation. Bar heights represent the means of the treatment.

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Chapter 3

A new artificial diet for western corn rootworm larvae is compatible with and detects resistance to all current Bt toxins*

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ABSTRACT

Insect resistance to transgenic crops is a growing concern for farmers, regulatory agencies, the seed industry, and researchers. Since 2009, instances of field-evolved Bt resistance or cross resistance have been documented for each of the four Bt proteins available for western corn rootworm (WCR) a major insect pest. To characterize resistance, WCR populations causing unexpected damage to Bt corn are evaluated in plant and/or diet toxicity assays. Currently, it is not possible to make direct comparisons of data from different Bt proteins due to differing proprietary artificial diets. Our group has developed a new, publicly available diet (WCRMO-1) with improved nutrition for WCR larvae. For the current manuscript, we tested the compatibility of all Bt proteins currently marketed for WCR on the WCRMO-1 diet and specific proprietary diets corresponding to each toxin using a susceptible colony of WCR. We also tested WCR colonies selected for resistance to each protein to assess the ability of the diet toxicity assay to detect Bt resistance. The WCRMO-1 diet is compatible with each of the proteins and can differentiate resistant colonies from susceptible colonies for each protein. Our diet allows researchers to monitor resistance without the confounding nutritional differences present between diets.

BACKGROUND/INTRODUCTION

Western corn rootworm (*Diabrotica virgifera virgifera* LeConte, WCR) has been a challenge for maize (*Zea mays* L.) farmers throughout much of the United States of America (USA) for decades. In 1909, this pest was discovered to attack maize roots in Colorado (Gillette 1912). Since then, it has expanded its geographic distribution to most of the maize growing regions of North America. Multiple introductions of WCR to Europe have increased its global importance (Miller et al. 2005). More than 30 years ago, it was estimated that the species caused over $1 billion (USD) in economic losses stemming from yield loss and control costs (Metcalf 1986).

Early attempts at managing WCR in the USA focused solely on crop rotation to a non-host such as soybean (*Glycine max* (L.) Merrill) or sorghum (Gillette 1912). When they became available, management tactics included the application of insecticides for larval or adult management (Muma et al. 1949; Pruess 1974). Recently, biotechnology has allowed farmers to grow maize which expresses one or more proteins from *Bacillus thuringiensis* Berliner (Bt). Except for banded applications of soil insecticides, each of these options has failed in one or more regions found within the distribution of this pest (Ball and Weekman 1962; Gassmann et al. 2011, 2016; Jakka et al. 2016; Levine et al. 2002; Ludwick et al. 2017; Meinke et al. 1998; Pereira et al. 2015; Zukoff et al. 2016).

When the first Bt events targeting lepidopterans were registered, insect resistance management (IRM) programs were required. Some believe these programs are especially important for products expressing toxins at less than high-dose or species with lesser susceptibility to the proteins (Tabashnik and Gould 2012), since the likelihood of survival increases when products express toxins at less than high-dose, or when some targeted

species are less susceptible to the toxins. Recently, biotechnology has allowed farmers to grow maize that expresses one or more proteins from *Bacillus thuringiensis* Berliner (Bt) for WCR control. However, modeling efforts and laboratory selection studies for current proteins targeting WCR suggested products would lose efficacy within a decade of first use (Deitloff et al. 2016; Frank et al. 2013; Kang et al. 2014; Meihls et al. 2008, 2012; Onstad 2006), and field data support this (Gassmann et al. 2011; Ludwick et al. 2017; Zukoff et al. 2016). Bt maize products targeting WCR have not yet met the high-dose criterion (Ludwick and Hibbard 2016), and this likely is a primary reason that the refuge strategy designed to delay resistance has been ineffective with this pest (Tabashnik and Carrière 2017).

Resistance monitoring efforts to determine whether a shift in the susceptibility of randomly sampled populations was required as part of the registration process and to date has used proprietary artificial diets developed by product registrants (EPA 2013a-c). While this effort meets the requirement for IRM programs, there have been issues with the diet toxicity assays. In addition to nutritional differences between diets, high levels of contamination often occurred. Changes in assay methodology and diet formulations have also occurred over the years for some Bt proteins, likely creating additional variability in the data. Some academic researchers have tended to use on-plant assays as opposed to proprietary diets and purified Bt proteins, which require special agreements with the owners. This barrier can be overcome with relationships between industry and academia, but often takes considerable time and negotiation. As a result, several studies have evaluated Bt resistance with on-plant assays only (Deitloff et al. 2016; Gassmann et al. 2011, 2014, 2016; Jakka et al. 2016; Tinsley et al. 2015; Wangila et al. 2015), and some

with both on-plant and diet toxicity assays (Frank et al. 2013; Ingber and Gassmann 2015; Lefko et al. 2008; Ludwick et al. 2017; Meihls et al. 2008, 2011; Zukoff et al. 2016). Following initial reports of field-evolved Bt resistance using on-plant assays, the Environmental Protection Agency (EPA) proposed on-plant assays be used as a replacement for diet toxicity assays (EPA 2014) and the proposed changes for resistance monitoring have been implemented (EPA 2016). Instead of diet toxicity assays, registrants will be required to use on-plant assays to determine shifts in susceptibility, though they may continue to conduct diet toxicity assays in conjunction with on-plant assays.

Efforts have been underway to improve WCR diets and the first new publicly available diet was recently published (Huynh et al. 2017). Referred hereafter as "WCRMO-1", this diet has improved characteristics compared to the only other publicly-available diet (Pleau et al. 2002) and all the proprietary diets used in the current study (Hibbard/Coudron labs unpublished data). Efforts are continuing to further improve the WCRMO-1 diet for weight gain and developmental rate. An optimum diet for WCR should match maize for survival, weight, and developmental rate parameters. If these parameters can be optimized, or can no longer be improved, then we believe a single diet should be used as a universal diet in all public diet toxicity assays. As a step toward this longer-term goal, we compared the WCRMO-1 diet to the appropriate proprietary diets for compatibility with Bt proteins. We also tested susceptible and resistant WCR colonies on the WCRMO-1 diet to evaluate the ability of the diet to detect differences in susceptibility to Bt proteins in these two phenotypes.

RESULTS

Lethal effects. The WCRMO-1 diet was tested with each Bt protein alongside the proprietary diet of the corresponding registrant using a Bt-susceptible colony (Brookings-ND, Table 5). Average mortality for the buffer dose (control) on WCRMO-1 was less than 10 percent for all but one instance (Fig. 3d). The average mortality for the buffer dose on proprietary diet C was between 12 and 13 percent (Figs. 3b and 3c), while the buffer dose for proprietary diets corresponding to Cry34/35Ab1 (proprietary diet A) and Cry3Bb1 (proprietary diet B) toxins was less than five percent (Figs. 3a and 3d). Based on overlapping confidence intervals between concentrations required to kill 50 percent of insects (LC_{50}), WCRMO-1 provided similar data as the proprietary diet for each protein except for one (Table 6). WCRMO-1 produced a significantly lower LC_{50} value for eCry3.1Ab compared to the corresponding proprietary diet, labelled as "proprietary diet C" (Table 6). This means less eCry3.1Ab was required on WCRMO-1 to kill half of the susceptible colony tested when compared to proprietary diet C.

When Bt-selected colonies were evaluated on WCRMO-1, significant differences in LC_{50} values were found for each Bt protein between the Brookings-ND colony and the resistant colonies (Table 6). This was the case for each selected colony on each of the four Bt toxins evaluated. Overall, resistant colonies showed minimal mortality in response to toxins. For Cry3Bb1, mCry3A, and eCry3.1, all resistant colonies had maximum mortality under 14 percent regardless of the dose (Figs. 3f-h). Only one of the DAS-59122-selected colonies reached maximum mortality 50% while the other was 41% following exposure to the highest dose Cry34/35Ab1 toxins (Fig. 3e).

Sublethal effects. Dry weight was the only parameter where a significant difference was observed between susceptible larvae fed Bt toxin on WCRMO-1 versus a proprietary diet. For eCry3.1Ab, the concentration required to cause a 50 percent reduction in dry weight (EC_{50}) for the Brookings-ND colony fed WCRMO-1 diet was significantly greater than when fed proprietary diet C (Table 6). No other significant differences were found in EC_{50} values between the WCRMO-1 diet and the respective proprietary diet for any of the other toxins (Table 6). Both DAS-59122-7-selected colonies had similar EC_{50} values compared to the Brookings-ND colony when exposed to the Cry34/35Ab1 proteins on the WCRMO-1 diet (Table 6). Both MON88017-selected colonies had significantly greater EC_{50} values for Cry3Bb1 protein on the WCRMO-1 when compared to the susceptible colony (Table 6). The Brookings-ND colony had a significantly lower EC_{50} value for eCry3.1Ab protein on the WCRMO-1 diet compared to the 5307-S colony (Table 2). Lastly, the MIR604-S colony had a significantly greater EC_{50} value for mCry3A protein when compared to the susceptible colony on the WCRMO-1 diet (Table 6).

There were large differences in susceptible WCR larval dry weight on the buffer dose between the WCRMO-1 and proprietary diet C (Figs. 4b and 4c). For both toxins, the insects reared on proprietary diet C weighed one-fifth as much as insects reared on WCRMO-1 after 10 d (Figs. 4b and 4c). Insects from Brookings-ND exposed to the buffer dose of Cry34/35Ab1 weighed more on WCRMO-1 than those reared on proprietary diet A. All but one colony (DAS-59122-7-S1) had greater dry weight than the Brookings-ND insects on the buffer dose (Figs. 4e-h). Additionally, some colonies had greater dry weight at the lowest Bt dose than for the buffer (Figs. 4a, 4d-f).

Molting was significantly inhibited for all susceptible colonies on all Bt proteins (Figs. 6a-d). The dose that causes 50 percent molting inhibition (MIC_{50}) was significantly greater for all the Cry3-selected colonies than the Brookings-ND colony (Table 6). The MIC_{50} value was not significantly different for the two Cry34/35Ab1-selected colonies and the Brookings-ND colony (Table 6).

DISCUSSION

Here, we demonstrate for the first time that a single artificial diet can be used to detect resistance with all Bt proteins currently targeting WCR. Previously, different proprietary proteins were evaluated on the proprietary diet from the company that produced the protein. Use of a single diet allows for direct comparisons of toxicity between proteins without the confounding effects of nutritional differences. In general, performance of susceptible WCR was similar between the proprietary diets and the WCRMO-1 diet (Table 6, Figs. 3-5). We also documented that differences between WCR colonies selected for resistance to each Bt protein and a control WCR population can be detected in diet toxicity assays with the WCRMO-1 diet (Table 6, Figs. 3-5).

This study adds to the limited number of studies which have documented a significant difference in LC_{50} values between colonies or populations in Cry34/35Ab1 diet toxicity assays (Ludwick et al. 2017; Zukoff et al. 2016). Resistance ratios in the previous studies were relatively minor compared to the more than 40-fold difference in LC_{50} values of the two DAS-59122-7-selected populations and the control population evaluated here (Table 6). Interestingly, a colony originally described by Lefko et al. (2008) and Nowatzki et al. (2008) showed no difference in LC_{50} values when compared to a control colony after being selected for more than 30 generations on DAS-59122-7

maize (which produces the Cry34/35Ab1 toxin) using proprietary diet A (pers. communication from Stephen Thompson, DuPont Pioneer). Using the WCRMO-1 diet, significant differences in LC_{50} values between both DAS-59122-7-selected colonies and control colonies were documented with less than 20 generations of selection (Table 6, Figs. 3-5). The difference in LC_{50} values did not translate to significant differences in EC_{50} values; however, the DAS-59122-7-S2 colony did have a significantly greater MIC_{50} value than the control WCR colony (Table 6). These results suggest a possibility for more sensitive screening of Cry34/35Ab1 proteins with WCRMO-1. However, there is also a chance that the difference in LC_{50} values detected on the WCRMO-1 diet is unique to the DAS-59122-7-selected colonies in this study. Additional comparisons of proprietary diet A and WCRMO-1 diets are needed to determine whether the basis of this detection is the result of nutritional or genetic factors, or a combination of the two factors.

Although we do not have the formulations for the proprietary diets tested here, life history parameters indicate that nutritive qualities varied among the diets. We previously found significant differences in molting rate, weight gain, and survival among the proprietary and WCRMO-1 diets (unpublished data). Research with lepidopteran insects has shown that the toxicity of Bt proteins can vary when protein to carbohydrate ratios are manipulated (Deans et al. 2017; Orpet et al. 2015). While lepidopteran and coleopteran insects are quite different, we may be able to draw parallels to this study. One proprietary artificial diet appears to lack significant nutritive qualities and/or attractiveness as a food source. Insects on proprietary diet C with buffer alone for eCry3.1Ab and mCry3A proteins weighed less than 18 percent of insects reared on the

WCRMO-1 diet with buffer alone (Figs. 4b and 4c). Such a difference in nutritive qualities could explain the significant difference in dry weight observed between larvae fed WCRMO-1 or the proprietary diet C. However, this difference in weight gain between diets did not cause a significant difference in LC_{50} for the mCry3A protein. Under conditions where an insect ingests food at a normal rate (i.e. rate on maize), then the LC_{50} is representative of the amount needed to kill targeted insects in the field. However, when a diet is less attractive, then the LC_{50} will likely shift towards a greater value as less diet is ingested.

Larvae were capable of molting in the 10-day time period used for these experiments, unlike previously reported assays with WCR which were terminated at five or six days depending on the protein. Previously reported assays had shorter time frames primarily due to contamination (EPA 2016). Longer assays for slower acting products (e.g. dsRNA) are possible using techniques described here and in Huynh et al. (2017). Consequently, we were able use molting as a measure of resistance, similar to studies with *Ostrinia nubilalis* (Hübner) (Thieme et al. 2017). Here, exuviae were clearly visible through the sealing film and provided a non-invasive determination of sublethal effects that could be recorded over time, whereas dry weight could only be collected at the end of each experiment. Recording molting data as a measure of resistance may allow for additional studies where researchers may look at genetic factors, microbes, or other variables. This approach may provide a more sensitive measure for detecting resistance in populations where no differences in LC_{50} or EC_{50} exist. For example, the DAS-59122-7-S2 colony had a significantly greater MIC_{50} value when compared to the Brookings-ND colony while the DAS-59122-7-S1 showed no significant difference (Table 6). This was

the only difference noted between the two DAS-59122-7 selected colonies. Future studies should include these data, when possible. Before molting data can be collected, a diet must have adequate nutrition to allow first instar larvae to molt into the second instar. Proprietary diet C did not produce second instar larvae, even on diet with buffer alone (Figs. 5b and 5c). All other diets produced second instar larvae on diet with buffer alone (Figs. 5a and 5d).

Nutritional improvements of an artificial diet (Huynh et al. 2017) and validation of compatibility with all current Bt proteins documented here, support the utility of the diet toxicity assay in resistance monitoring efforts. We recommend a standardized approach to how these diet toxicity assays are conducted to reduce data variability in IRM programs. Some of this variability is likely due to the alterations of some diets over the years for various reasons. Additionally, the number of days for observation varies between proteins complicating comparisons of Bt proteins. These factors likely explain some of the variability that has been reported within diet toxicity assays over the years. Shifts in susceptibility may be masked by these factors. Comparisons between current and future products could help to evaluate novel modes of action and physiological effects of protein exposure.

WCR has evaded management tactics for more than a century. Some populations of this species have developed resistance to crop rotation (Levine et al. 2002), broadcast insecticides (Ball and Weekman 1962; Meinke et al. 1998; Pereira et al. 2015), and transgenic maize expressing Bt proteins (Gassmann et al. 2011, 2014, 2016; Ludwick et al. 2017; Wangila et al. 2015; Zukoff et al. 2016)). Even with the advent of biotechnology, WCR continues to be a pest of maize. Current and future products

targeting the species will require IRM programs to ensure product viability and longevity. Both plant and diet assays have value in detecting resistance (Zukoff et al. 2016).

MATERIALS AND METHODS

Artificial Diet and Bt Protein. Lyophilized Cry34/35Ab1 proteins and the corresponding proprietary diet were provided by Dow AgroSciences. Cry3Bb1 protein was provided in solution by Monsanto Company in addition to their proprietary diet. The lyophilized mCry3A and eCry3.1Ab proteins and their proprietary diet were provided by Syngenta Biotechnology. The WCRMO-1 diet was produced by the USDA-ARS Biological Control of Insects Research Laboratory (BCIRL) in Columbia, MO (Huynh et al. 2017). Buffers to suspend the protein in solution were prepared at BCIRL (Table 5). The sodium citrate buffer was stored at 4 ˚C immediately after preparation. The sodium carbonate bicarbonate buffer was stored at room temperature in a container which blocked all light.

Insects. Eggs from the non-diapausing, susceptible colony, hereafter known as Brookings-ND, were provided by the USDA-ARS laboratory in Brookings, SD. This colony is derived from a field population collected prior to the release of transgenic crops (Branson 1976), so this colony should be susceptible to all Bt proteins active against WCR.

Several resistant colonies were used in this study, all of which have been described in other studies. Two MON88017-selected colonies, MON88017-S1 and MON88017-S2, exhibited resistance to Cry3Bb1-expressing plants (event MON88017) and protein in Zukoff et al. (2016). MON88017-S1 was derived from the Canby population while MON88017-S2 was derived from Hills population in Zukoff et al. (2016). Both MON88017-selected colonies were reciprocally crossed with adults from the Brookings-

ND colony and then reared on maize containing event MON88017 for eleven generations post Zukoff et al. (2016) at the time Cry3Bb1 diet toxicity assays were conducted.

Two DAS-59122-7-selected colonies, hereafter known as DAS-59122-7-S1 and DAS-59122-7-S2, were also used in this study. The DAS-59122-7-S1 colony was derived from a field population described in Ludwick et al. (2017). The field population had been exposed to SmartStax, a maize product which expresses both Cry34/35Ab1 (event DAS-59122-7) and Cry3Bb1 proteins. DAS-59122-7-S1 had incomplete resistance to both Cry3Bb1 and Cry34/35Ab1 proteins (Ludwick et al. 2017). Eggs from this population were reared on non-Bt corn and resulting adults were then reciprocally crossed with the Brookings-ND colony to produce a non-diapausing DAS-59122-7-S1 colony. The DAS-59122-7-S2 colony was started from pooled eggs of three field populations reared on non-Bt maize. The resulting adults were reciprocally crossed with Brookings-ND at differing points earlier and then selected on maize containing event DAS-59122-7 similarly as in Deitloff et al. (2016). The DAS-59122-7-S1 and DAS-59122-7-S2 colonies were selected on DAS-59122-7 plants for six and 18 generations under laboratory/greenhouse conditions, respectively, at the time Cry34/35Ab1 diet toxicity assays occurred.

Two additional colonies with resistance to mCry3A (Gassmann et al. 2011) and eCry3.1Ab (Frank et al. 2013) were also tested. The colony selected on eCry3.1Ab-expressing corn will hereafter be known as 5307-S. We tested this strain after 35 generations of selection on maize containing event 5307 or an earlier event also expressing eCry3.1Ab. The MIR604-selected colony will hereafter be known as MIR604-S. The MIR604-S colony was evaluated after more than 50 generations of selection on MIR604 maize which expresses mCry3A protein.

Equipment Sterilization. The WCRMO-1 diet was prepared in new 96-well immunoassay plates (product #3370, Corning Inc., Corning, NY) using sterile techniques described in Huynh et al. (2017) All proprietary diets were prepared in 96-well immunoassay plates by the registrants. For shipment, proprietary diets were placed in sterile packaging and shipped to the BCIRL in styrofoam containers with ice packs. Diets were stored in a 4 ˚C refrigerator and infested within two weeks of arrival. Lids for diet were only removed inside a UV-sterilized biosafety cabinet. Paintbrushes, insect pins, deli containers, coffee filters, and beakers were all sterilized by UV lights in a biosafety cabinet prior to use. A spray bottle with autoclaved DI water was used to keep the coffee filter and neonate larvae moist.

Egg Sterilization. The methods used to surface sterilize eggs were modified from descriptions by Pleau et al. (2002). Briefly, eggs from each colony were incubated in Petri dishes with 70 mesh sieved soil within an incubator at 25˚C in complete darkness until hatch started. Once approximately 10 percent of the eggs hatched, each dish was washed through a 60 mesh sieve. Remaining eggs were submerged in a beaker with water where debris (soil, fungus, hatched egg shells) floated to the water's surface and were then decanted. As much excess water as possible was poured off before the eggs were submerged in 20 mL of disinfectant (undiluted Lysol®) for three minutes. The disinfectant was then decanted and the eggs were triple rinsed with distilled water. Approximately 20 mL of 10 percent buffered zinc formalin (10 percent formaldehyde) covered the eggs for three minutes. Again, a triple rinse with distilled water was used to remove any chemical residues. Eggs were then dispensed onto a UV-sterilized coffee filter with a 1.5 ml transfer pipette. The coffee filter was then placed in a UV-sterilized 16 oz. Solo® deli container

with holes (#0 insect pin) punched in the lid to allow for air exchange. Incubation, typically less than three days, occurred until enough eggs hatched for infestation. If sufficient numbers of larvae did not hatch, then the coffee filter with eggs were transferred to a recently UV-sterilized deli container each additional day and placed back in the incubator. Contamination tends to increase when eggs are used more than three days after sterilization (personal observation). The deli container with larvae on its wall was placed in a sterilized biosafety cabinet. Neonate larvae were then transferred to diet using sterile equipment to prevent any possible contamination. Sealing film (Excel scientific, Inc., Thermalseal RTS^{RM}, TSS-RTQ-100) was placed over the entire plate to prevent escape. A single hole (#0 insect pin) was poked into the sealing film of each infested well for diffusion of oxygen.

Diet Toxicity Assays. All diet toxicity assays were conducted for a length of 10 days. Lyophilized proteins were dissolved in solution and then a serial dilution was made (Table 1). These solutions were then overlaid on artificial diet and allowed to dry before larvae were placed on the diet. Each row (12 wells) per plate received one dose and one colony, thereby constituting a single replication. Plates with more than 25 percent mortality on the control (buffer dose, 0 µg/cm^2) were excluded from the study. The number of replications included in this study varied between diets and proteins (Table 2). Survival and molting were recorded prior to the collection of larvae. Surviving larvae were placed in ethanol for each dose and plate and then dried in an oven at 50˚C for one week (Blue M Therm Dry Bacteriological Incubator, Model #602752). After drying, larvae were weighed on a digital microbalance (Sartorius^{TM} Cubis^{TM}, 6.6S), which weighed samples to one thousandth of a milligram.

Statistical Analyses. Mortality was calculated by dividing the number of dead larvae by the initial number of larvae infested per dose. An average mortality value of the buffer dose was calculated. Then, mortality at each subsequent dose was divided by the average mortality of the buffer dose and multiplied by 100 (Abbott 1925). These mortality percentages were analyzed with a probit analysis to generate LC_{50} and 95 percent confidence intervals using SAS software version 9.2 (SAS Institute). Values were considered significantly different when 95 percent confidence intervals did not overlap.

Dry weight was calculated by dividing the total weight per dose by the initial number of larvae infested, effectively giving all dead larvae a weight of 0. The average dry weight per larva recovered from the buffer dose was then averaged across replications to generate a mean value. The dry weight of larvae recovered from each dose was then divided by the buffer's mean dry weight and multiplied by 100 to generate a percentage relative to the buffer dose. These data were then analyzed with a nonlinear probit analysis described by Marçon et al. (1999) in SAS. This analysis calculated the EC_{50} and 95 percent confidence intervals.

Molting was calculated by dividing the number of molted individuals per dose by the initial number of larvae infested and multiplying by 100 to obtain a percentage. The average molting rate for each colony on the buffer dose was calculated. Each subsequent dose was divided by the buffer dose's average molting rate for the corresponding colony (Abbott 1925). These molting rates were then analyzed in SAS with a probit analysis to generate MIC_{50} values and 95 percent confidence intervals. MIC_{50} values between colonies on the same protein were considered significantly different when confidence intervals did not overlap.

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Table 5. Buffers, doses, and proteins used with each colony.

				Dose (µg/cm^2)							
Protein	**Diet**	**Colony**	**Protein Buffer**	**Dose 1**	**Dose 2**	**Dose 3**	**Dose 4**	**Dose 5**	**Dose 6**	**Dose 7**	**Dose 8**
Cry34/35Ab1	Proprietary Diet A	Brookings-ND	10 mM Sodium Citrate pH 3.5	0.00	0.08	0.24	0.74	2.22	6.67	20.00	60.00
	WCRMO-1	Brookings-ND		0.00	0.09	0.28	0.85	2.55	7.65	22.94	68.82
		DAS-59122-7-S1 (6 gen.)		0.00	0.09	0.28	0.85	2.55	7.65	22.94	68.82
		DAS-59122-7-S2 (18 gen.)		0.00	0.09	0.28	0.85	2.55	7.65	22.94	68.82
Cry3Bb1	Proprietary Diet B	Brookings-ND	10 mM Sodium Carbonate Bicarbonate pH 10	0.00	22.19	44.29	88.57	177.14	.	.	.
	WCRMO-1	Brookings-ND		0.00	24.54	48.97	97.95	195.89	.	.	.
		MON88017-S1 (11 gen.)		0.00	24.54	48.97	97.95	195.89	391.79	.	.
		MON88017-S2 (11 gen.)		0.00	24.54	48.97	97.95	195.89	391.79	.	.
mCry3A	Proprietary Diet C	Brookings-ND		0.00	0.34	1.03	3.10	9.29	27.87	.	.
	WCRMO-1	Brookings-ND		0.00	0.34	1.03	3.10	9.29	27.87	.	.
		MIR604-S (50+ gen.)		0.00	0.30	0.90	2.70	8.10	24.30	.	.
eCry3.1Ab	Proprietary Diet C	Brookings-ND		0.00	0.34	1.03	3.10	9.29	27.87	.	.
	WCRMO-1	Brookings-ND		0.00	0.34	1.03	3.10	9.29	27.87	.	.
		5307-S (35 gen.)		0.00	0.30	0.90	2.70	8.10	24.30	.	.

Table 6. Concentrations (µg/cm^2) to kill 50 percent (LC_{50}), cause 50 percent weight inhibition (EC_{50} values), and cause 50 percent molt inhibition (MIC_{50}) with 95 percent confidence intervals for corresponding colony, diet, and Bt protein treatments. Non-overlapping confidence intervals indicate significant differences. Confidence intervals could not be calculated for some of the estimates.

Bt Protein	Diet	Colony	Reps	LC_{50} (95% C.I.)	EC_{50} (95% C.I.)	MIC_{50} (95% C.I.)
Cry34/35Ab1	Proprietary Diet A	Brookings-ND	3	6.28 (2.60-22.64)	2.01 (0.33-12.05)	0.96 (0.33-2.85)
	WCRMO-1	Brookings-ND	4	1.60 (0.83-3.15)	1.31 (0.15-11.26)	0.92 (0.27-3.35)
		DAS-59122-7-S1 (6 gen.)	5	>68.82	5.99 (3.37-9.13)	3.48 (0.59-18.51)
		DAS-59122-7-S2 (18 gen.)	4	>68.82	3.09 (1.38-4.94)	10.96 (4.4-25.3)
Cry3Bb1	Proprietary Diet B	Brookings-ND	4	9.11 (2.15-24.69)	12.41 (1.00-87.62)	2.19
	WCRMO-1	Brookings-ND	3	3.36 (0.35-15.17)	10.20 (0.32-88.80)	0.48
		MON88017-S1 (11 gen.)	5	>391.8	409.23 (250.35-1092.77)	>391.8
		MON88017-S2 (11 gen.)	4	>391.8	614.77 (333.64-2311.32)	>391.8
eCry3.1Ab	Proprietary Diet C	Brookings-ND	3	12.74 (5.10-61.54)	4.02 (1.89-9.20)	N/A
	WCRMO-1	Brookings-ND	4	1.75 (0.93-3.57)	20.09	0.35 (0.02-2.44)
		MIR604-S (35 gen.)	5	>24.3	>24.3	>24.3
mCry3A	Proprietary Diet C	Brookings-ND	5	3.71 (1.93-8.08)	3.13 (1.80-5.42)	N/A
	WCRMO-1	Brookings-ND	6	6.39 (2.82-20.39)	1.23 (0.14-9.7)	0.5 (0.12-1.48)
		MIR604-S (50+ gen.)	5	>24.3	>24.3	>24.3

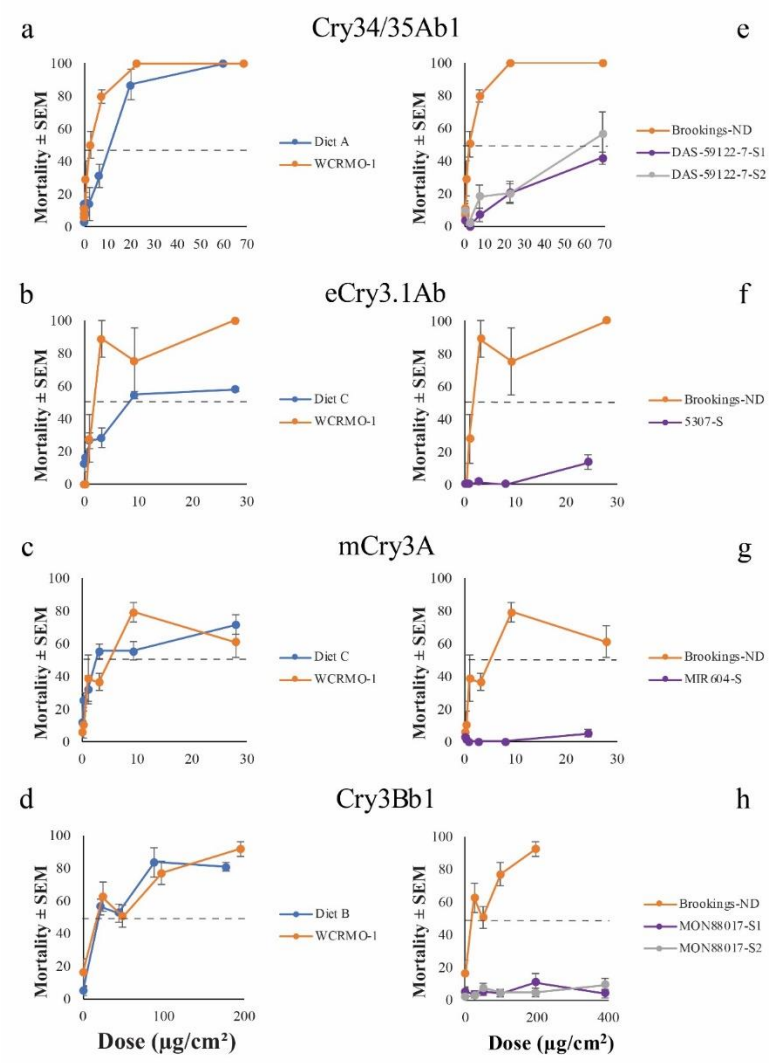

Figure 3. Impact of Bt Protein on Mortality. Percent mortality of Brookings-ND (a-d) with exposure to Bt proteins on proprietary and WCRMO-1 diets, and mortality of Brookings-ND and selected colonies (e-h) with exposure to Bt proteins on the WCRMO-1 diet. Mortality was calculated as the number of larvae that died following exposure to Bt protein(s) divided by the initial number infested. Bars represent the standard error of the mean (SEM). The dashed line represents 50 percent mortality.

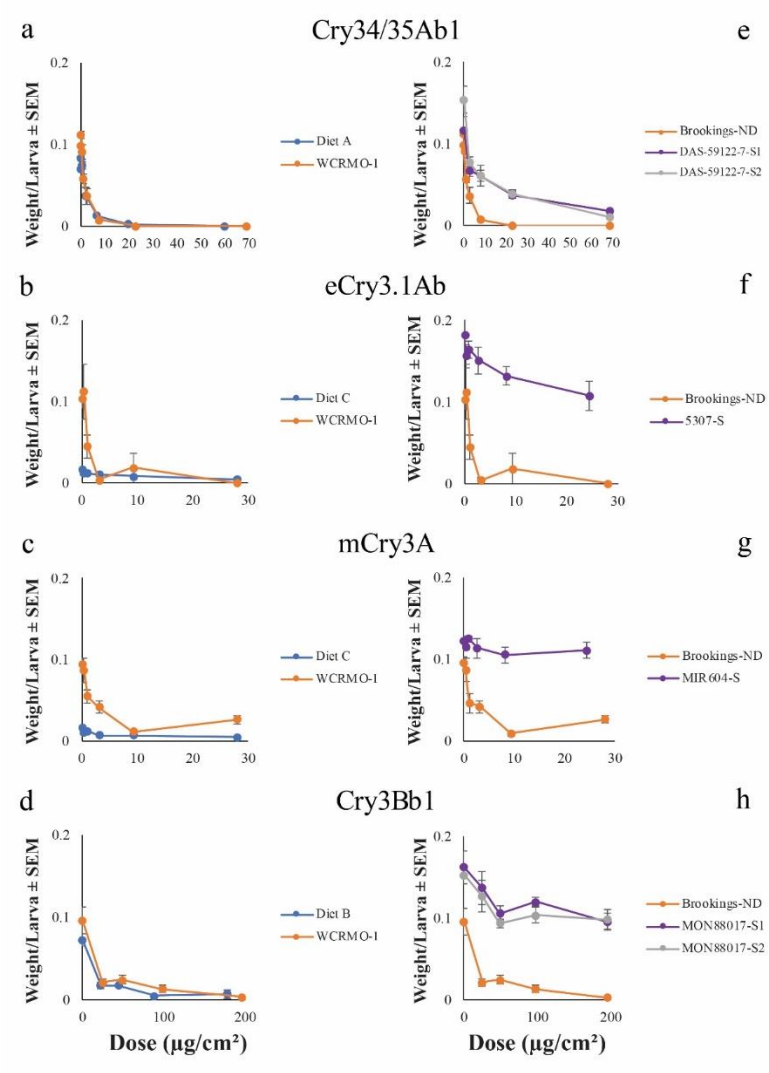

Figure 4. Impact of Bt Protein on Dry Weight. Dry weight per larva (mg) of Brookings-ND (a-d) with exposure to Bt proteins on proprietary and WCRMO-1 diets, and dry weight per larva of Brookings-ND and selected colonies (e-h) with exposure to Bt proteins on the WCRMO-1 diet. Dry weight per larva was calculated as the dry weight of larvae recovered following exposure to Bt protein(s) divided by the initial number infested. Bars represent the standard error of the mean (SEM).

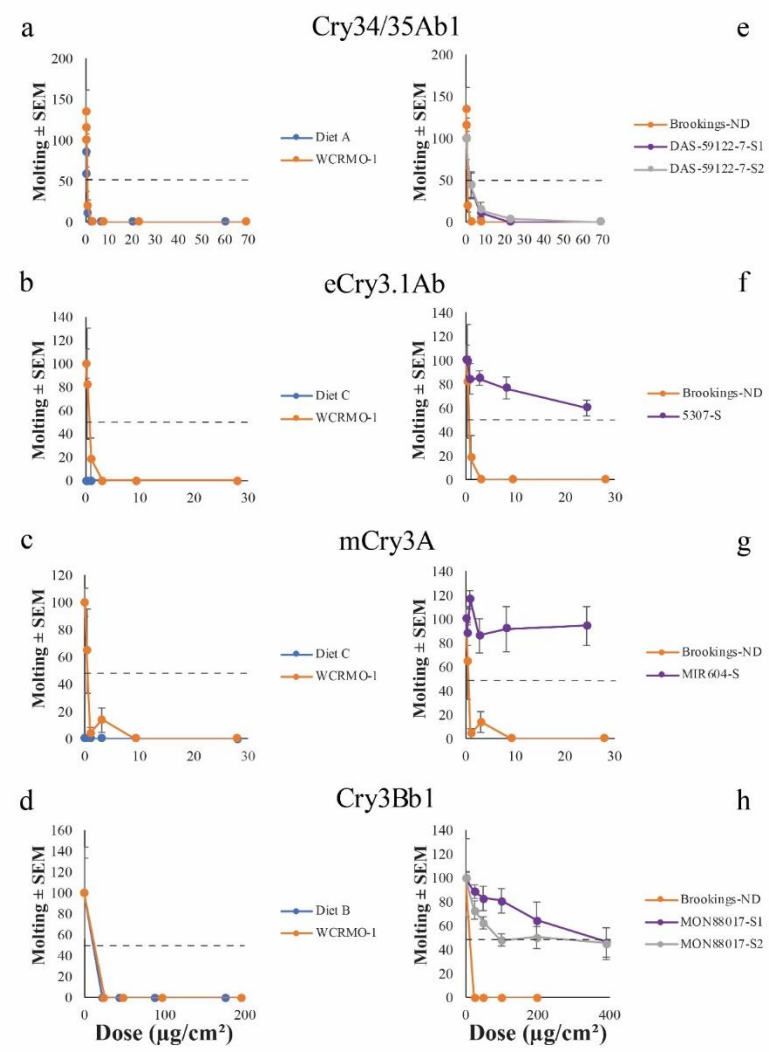

Figure 5. Impact of Bt Protein on Molting. Molting of Brookings-ND (a-d) with exposure to Bt proteins on proprietary and WCRMO-1 diets, and molting of Brookings-ND and selected colonies (e-h) with exposure to Bt proteins on the WCRMO-1 diet. First, the molting rate on the buffer was calculated by dividing the number of larvae which molted by the initial number of larvae. This established a baseline response of the insect to the diet and buffer. All values for each colony, including the buffer dose (Dose 1) for the same colony, were then divided by the buffer dose value to establish relationships relative to the buffer dose. Each of the resulting values were then multiplied by 100. Bars represent the standard error of the mean (SEM).

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Chapter 4

Survey of bacteria associated with western corn rootworm life stages reveals no difference between insects reared in different soils

ABSTRACT

Western corn rootworm (*Diabrotica virgifera virgifera* LeConte) is a serious pest of maize (*Zea mays* L.) in North America and parts of Europe. With most of its life cycle spent in the soil feeding on maize root tissues, this insect is likely to encounter and interact with a wide range of soil and root rhizosphere microbes. Our knowledge of the role of microbes play in insect pests, pest management, and plant health remains incomplete. An important component of an effective pest management strategy is to know which microorganisms are present that could play a role in life history or management. For this study, insects were reared in either autoclaved soil or soil from a geographically separate location that had not been autoclaved. Insects were sampled at each life stage to determine the possible core bacteriome. Additionally, soil was sampled at each life stage and resulting bacteria were identified to determine the contribution of soil to the rootworm bacteriome, if any. We analyzed the V4 hypervariable region of bacterial 16S rRNA genes with Illumina MiSeq to survey the different species of bacteria associated with the insect and the soils. Significant differences in bacterial communities were noted between soil and insects with principal coordinate analysis. No significant differences in community composition existed between the insects reared on soils which did or did not undergo the autoclave process, or between non-diapausing and diapausing insects. The bacteria present in immature stages and in male and female adults revealed a possible core bacteriome of approximately 16 operational taxonomic units. This research may give insights into how resistance to Bt develops, improved nutrition in artificial rearing systems, and new management strategies.

BACKGROUND/INTRODUCTION

Several studies have evaluated the microbial communities associated with lepidopteran pests and other insects that attack food crops (Caccia et al. 2016; Dhammi et al. 2016; Hadapad et al. 2016; Snyman et al. 2016). Interestingly, shifts in community composition or absence of bacteria can reduce the effectiveness of widely adopted management tactics such as crop rotation or maize expressing *Bacillus thuringiensis* Berliner (Bt) proteins. However, few studies have been conducted to document microbiomes within beetles attacking crops.

The western corn rootworm (*Diabrotica virgifera virgifera* LeConte, WCR) is a chrysomelid beetle whose larvae cause damage by feeding on maize root systems. While native to North America, this pest was introduced multiple times to Europe over 20 years ago (Baca 1994, Miller et al. 2005). Most recent estimates indicate this pest causes two billion dollars (USD) in yield loss and control costs worldwide annually (Metcalf 1986; Mitchell 2011), and any regions growing maize should monitor for the presence or arrival of this species. Since its discovery as a pest of maize, the primary control tactic has been crop rotation (Gillette 1912). Recently, transgenic maize hybrids expressing insecticidal proteins from Bt have been used to reduce root damage and economic losses. However, both of these control strategies have instances of failure in the United States of America (Gassmann et al. 2011, 2014, 2016; Levine et al. 2002; Ludwick et al 2017; Zukoff et al. 2016).

Rootworm larvae (WCR and *D. barberi* Smith & Lawrence) burrow through the soil searching for maize root tissues, and then through surrounding maize roots while feeding on root tissue. Thus, larvae of these species are exposed to many species of bacteria

and fungi in the soil and rhizosphere. The diversity of bacteria encountered is reflected on larval surfaces and digestive tracts. The microbiomes of larvae and later life stages may be assembled from bacterial and fungal species present during larval development in soil.

Insect gut microbiomes are known to influence many aspects of insect growth, nutrition, reproduction, Bt resistance, and pathogen resistance (Broderick et al. 2006; Caccia et al. 2016; Douglas 1998; Dematheis et al. 2012a, 2012; Paramasiva 2014a, 2014b; Visweshwar et al. 2015). Gut microbiota have been shown to affect the response of insects to Bt proteins in Lepidoptera (Broderick et al. 2006; Paramasiva et al. 2014a, 2014b; Visweshwar et al. 2015) and in mosquitoes (Patil et al. 2015), but this has not been investigated for Coleoptera. In the Old World bollworm (*Helicoverpa armigera* Hübner) the manipulation of the larval gut microbiota with antibiotics resulted in reduced susceptibility to a commercial formulation of Bt, as well as the purified δ-endotoxins Cry1Ab and Cry1Ac (Paramasiva et al. 2014a). In general, the use of antibiotics to manipulate lepidopteran gut microbiota resulted in reduced mortality due to Bt proteins. Selection experiments with *H. armigera* on transgenic plants were also conducted in addition to manipulation of gut microbiota with antibiotics (Visweshwar et al. 2015). When antibiotics were included, susceptibility to Bt was not altered, even with increasing generations of selection. However, selection in the absence of antibiotics (gut microbiota unaltered) resulted in a nearly 30% increase in larval survival by the F3 generation (Visweshwar et al. 2015). Thus, resistance to Bt by *H. armigera* developed only when gut microbiota were present. In fact, the reduction in susceptibility to Bt with the addition of antibiotics was greater than the reduction of susceptibility to Bt due to three generations of selection when gut microbiota were present. Gut microbiota were also required for

susceptibility of the gypsy moth, *Lymantria dispar* (L.), to Bt proteins (Broderick et al. 2006).

Larval gut tissue of WCR has a diverse microbial community (Dematheis et al. 2012a; Chu et al. 2013). In WCR, a shift in gut microbiota enterotype was associated with increased resistance to soybean defense compounds, which may have contributed to the development of resistance to crop rotation (Chu et al. 2013). Comparison of gut microbiota between rotation-resistant WCR populations and wild-type WCR populations revealed shifts in the microbial community composition. Manipulation of WCR gut microbiota with antibiotics reduced the resistance to soybean defensive compounds to a level similar to that of wild- type WCR (Chu et al. 2013).

The contribution of gut microbiota to nutrition, physiology, and Bt resistance in WCR is unknown (Dematheis et al. 2012a). Feeding of larval WCR on maize root tissue was shown to affect root rhizosphere microbiota composition, indicating a complex, multitrophic interaction (Dematheis et al. 2012b). Since gut microbiota play a role in Bt susceptibility in lepidopteran pests and a role in crop rotation resistance in WCR, it is reasonable to hypothesize that the microbiota of WCR can affect how larvae respond to Bt toxins expressed in maize. Consequently, a better understanding of which microbes are associated with WCR and how the insects acquire the bacteriome is needed. In this study, we compared the microbial composition of WCR grown in two different soil environments, at each developmental stage, alongside the soil from which the various life stages were collected and show that WCR larvae can carry a core bacteriome from the egg to adult stage.

RESULTS AND DISCUSSION

To better understand the role(s) of bacteria associated with WCR, we conducted the first survey of the WCR bacteriome across all life stages and corresponding soil samples. After following several different protocols, we found one protocol extracted sufficient DNA. Insects from a non-diapausing laboratory colony, often used as a proxy for diapausing, wild-type insects, were reared to each life stage in an autoclaved soil from Columbia. All life stages and corresponding soil samples were collected and processed to extract and purify DNA. The V4 region of the 16S ribosomal gene was amplified and sequenced to putatively identify bacteria.

Uncertain whether differences existed between diapausing and non-diapausing insects, we reared insects from a diapausing laboratory colony in autoclaved soil from the same origin under the same conditions. Once the identities of the bacteria were determined, we compared the bacteriomes between the two colonies. Permutational multivariate analyses of variance (PERMANOVA) with Bray-Curtis and Jaccard indices were conducted to determine whether significant differences existed. No significant differences were detected between these colonies with the Bray-Curtis index (p=0.10; F=1.90). Non-diapausing insects appear to retain a similar bacteriome despite hundreds of generations of laboratory selection. However, PERMANOVA with a Jaccard index revealed significant differences in bacterial communities between diapausing and non-diapausing insects (p=0.0001; F=2.90). Insects from both groups appear to share many dominant taxa while rarer species appear to be isolated to individual groups.

WCR occurs across a large region in many different soils throughout the United States of America and has also colonized a new area, Europe, as well. Without access to

insects from different regions, we set forth to determine the effect the soil from different regions may have on the bacteriome. Soil was collected from a different geographic region to survey the soil bacterial background from which insects emerged. Over 2,200 unique operational taxonomic units (OTUs) were putatively identified in soil and insect samples from both colonies and soils. The results show that earlier life stages reared in differing soils contained a significantly different assemblage of bacterial species. However, as the insects matured, those differences declined and all life stages of the insects converged to a similar bacterial microbiome. Out of all samples, 16 OTUs were found in every sample regardless of the colony or rearing soil. We believe these 16 OTUs comprise a core bacteriome for WCR.

Sequencing of WCR and soil samples resulted in a mean (± SEM) of 66,759 (± 3,895) and 72,868 (± 5,308) reads per sample, respectively. To account for the potential influence of differential coverage on downstream analyses, data were randomly subsampled to a uniform depth of 10,000 reads per sample and all subsequent analyses were performed on this rarefied dataset.

Annotated to the taxonomic level of class, the WCR samples were dominated by *Alphaproteobacteria* and *Gammaproteobacteria*, with lower and inconsistent relative abundance of *Actinobacteria*, *Cytophaga*, *Sphingobacteria*, *Betaproteobacteria*, and in the case of surface-sterilized eggs, *Flavobacteriia* and *Deltaproteobacteria* (Fig. 6A). Soil samples demonstrated a seemingly more complex composition comprising a greater number of classes and more even distribution (Fig. 6B).

Microbial richness and α-diversity are two metrics of polymicrobial communities often correlated with the health of an ecosystem, be it environmental or host-associated.

Richness simply denotes the overall number of detected phylotypes in a sample, whereas α-diversity denotes both the richness and evenness of distribution of phylotypes in a sample and can be calculated many different ways. In both cases, the underlying assumption is that increased numbers of different taxa and more even distributions of those taxa are representative of ecosystems fostering cross-feeding and syntrophic relationships among microbes. In contrast, low richness or asymmetrical distributions might represent an environment with high selective pressures or the presence of dominant taxa in a competitive environment.

Analysis of richness and α-diversity in WCR and the soil in which they were maintained revealed several interesting trends. To first determine whether sample site influenced the richness or α-diversity of WCR microbiota, a two-way ANOVA was performed with sample site (i.e., Columbia or Higginsville) and life-stage as fixed variables. Significant main effects of WCR life-stage were detected in richness ($p < 0.001$, $F = 8.14$) and α-diversity using both the Shannon ($p = 0.011$, $F = 3.48$) and Simpson ($p < 0.001$, $F = 5.78$) indices. No differences were detected between sample sites in richness or α-diversity of WCR-associated microbiota ($p = 0.338$, 0.072, and 0.244, respectively). Of note however, similar testing of the soil communities at each site revealed significant site-dependent differences in richness and α-diversity ($p < 0.001$ for all three metrics, $F = 38.52$, 197.64, and 25.04, respectively). No life-stage-dependent differences in microbial richness were detected between soil plots, although α-diversity within soil did significantly vary between life-stage ($p = 0.030$, $F = 2.88$ and $p < 0.001$, $F = 5.53$ for Shannon and Simpson indices, respectively).

Collectively, we interpret these data as evidence that the environment has limited effect on the relative uniformity and richness of the WCR bacteriome. This hypothesis is supported by the nearly log-fold difference in richness between soil and rootworm samples. The fact that no site-dependent differences were detected in the rootworms themselves, despite the stark differences in the microbial richness of their respective environments, stands in contrast to the life-stage-dependent differences in richness observed in the rootworms only and not in the soil samples.

Considering WCR samples from the two sites collectively, there was a general trend toward increasing richness in each successive life-stage from egg to pupa followed by a precipitous decline during the pupal molt to adulthood (Fig. 7A). Pairwise comparisons of richness between life-stages detected significantly decreased richness of adult WCR relative to several earlier life-stages. Interestingly, an inverse trend was observed in the richness of soil samples across life-stages (Fig. 7B). In contrast, α-diversity, as assessed via the Simpson index, was higher in sterilized eggs relative to other life-stages while adult rootworms were much lower (Fig. 9A**)**, likely reflecting the increasingly skewed microbial community structure as the rootworms develop. No life-stage-dependent differences were detected in soil α-diversity (Fig. 9B).

In order to provide a more comprehensive comparison of the microbial communities present in each sample, incorporating not just the number but also the identities of shared and unique taxa, principal coordinate analysis (PCoA) and PERMANOVA were performed to visualize and statistically test for differences in community structure, respectively. In both methods, the similarity of any given pair of samples can be determined several different ways. To ensure that any differences detected

were robust and to determine the nature of detected differences, we compared samples using both the Bray-Curtis and Jaccard similarity indices. While the Jaccard index is relatively unweighted and determines sample similarity based on the shared presence or absence of taxa, the Bray-Curtis index is weighted to also incorporate similarity in the relative abundance of any shared taxa.

Regardless of the index used, robust compositional differences were detected between all groups with the exception of the WCR samples collected at the two sample sites, again suggesting selection for a specific bacterial community within the rootworms. Specifically, testing for differences using the Bray-Curtis distances detected significant compositional differences between all pairwise comparisons except between WCR samples from the two sample sites (Table 5). Accordingly, PCoA demonstrated a clear separation of soil and WCR samples along PC1 (38.1% of the total variation in the dataset), complete separation of soil communities from the two sample sites along PC2, and partial overlap between WCR communities (Fig. 8). Testing based on the Jaccard index found significant differences between all pairwise comparisons. Ordination resulted in a similar pattern and the F value generated from the comparison of WCR collected from the two sites was extremely low relative to the other comparisons, despite having the highest total number of samples included in the comparison (Table 6). Collectively, these data complement the analyses of richness and α-diversity in supporting the hypothesis that WCR select for a limited subset of host-associated microbes, largely irrespective of their environment.

Annotated to the level of operational taxonomic unit (i.e., the best taxonomic resolution afforded by the 16S rRNA amplicons), the microbial composition of the adult

WCR was incredibly sparse. Of the 474 operational taxonomic units (OTUs) detected in anywhere from 1 of 18 (5.6%) to 15 of 18 (83.3%) of the adults rootworms, the mean relative abundance was uniformly below 0.3% (Fig. 10). Conversely, the 13 OTUs detected in 16 or greater of the 18 adult rootworms were present at a mean relative abundance of greater than 1.5%. Notably, 95.4% of the microbial DNA recovered from adult rootworms was annotated to three OTUs: *Wolbachia* sp. (85.5 ± 24.0% in 18 of 18 adults), unclassified family *Enterobacteriaceae* (6.2 ± 13.0% in 16 of 18 adults), and *Acinetobacter* sp. (4.7 ± 11.6% in 17 of 18 adults).

A survey across all life stages identified 1,126 OTUs. Specifically, 16 OTUs were present in every life stage regardless of soil type and diapausing status (Table 7). No significant differences existed between non-diapausing and diapausing WCR reared in autoclaved soil (Fig. 8). Adults had significantly fewer OTUs than neonate, second and third instar larvae, and pupae (Table 5, Fig. 7). Similarly, adults had significantly less diversity than eggs or third instar larvae. Neonates had significantly less diversity than unhatched eggs (Fig. 7). Additionally, the insect microbial community differed significantly from the soils in which they were reared (Table 5, Fig. 8).

Exploratory studies documenting the bacterial communities in different organisms may lead to new insights as to the role(s) they may fill or even new management tactics. Our study documented more than 1,100 OTUs present throughout the WCR life cycle. Of these OTUs, 16 were found in every life stage of insects. Furthermore, some of these bacteria were never found in the soil suggesting vertical transmission (i.e. parent to progeny) of bacteria is the most likely mechanism for at least some of the WCR microbiome (Table 9).

Many OTUs were discovered in the sterilized eggs of diapausing insects. However, we cannot be certain whether these bacteria were alive inside the egg or dead on the surface of the egg shell. Given the sculpturing of the chorion, it is possible dead bacteria remained on the surface served as a source of non-viable DNA (Dematheis et al. 2012b; Krysan 1986). The protocol we used does not discern between live and dead bacteria. If the bacteria were alive, then it is possible the eggs serve as a source of bacteria which colonize the neonatal gut. There is evidence that some of the bacteria are passed from parents to offspring (Table 7), but we cannot be certain without additional studies. Future experiments should extract rRNA and generate cDNA before sequencing the resulting strands. This method would reduce the likelihood of dead bacterial sequences entering the analysis as RNA degrades rapidly while DNA can persist for many years.

We infer that some of these bacteria may be endosymbionts of WCR as particular OTUs never appeared outside of insect samples (Table 9). However, we used laboratory colonies to make inferences about wild-type populations. In theory, the differences between wild-type populations and laboratory colonies should be minimal. In reality, we simply do not know. The geographic distribution of this insect encompasses most of the United States of America and parts of Europe. The soils across these regions are also diverse as are the management tactics employed by farmers. It stands to reason the microbial communities are different within and between fields. Future studies will need to include more samples, samples from different locations across the Corn Belt and other regions, and wild-type specimens to validate or invalidate the findings of this research.

WCR continues to evolve and adapt to the different management tactics that maize growers are implementing now. Future technologies for pest control, including RNA

interference, are still years away from field implementation. New tools and knowledge are needed to combat this pest. This study documents the plethora of bacteria encountered by WCR in different soils and identifies a small core bacterial microbiome retained by WCR. Clearly, there is much to learn about the functions of these different bacteria with regards to WCR.

MATERIALS AND METHODS

Insect rearing. Eggs from non-diapausing and diapausing colonies of WCR were obtained from the Agricultural Research Service of the United States Department of Agriculture (USDA-ARS). The non-diapausing colony had been derived from the primary non-diapausing colony held at Brookings, SD (Branson 1976). The diapausing colony eggs were from the primary diapausing colony (Jackson 1986) also held at Brookings, SD, and remained in cold storage until needed.

For the non-diapausing colony maintained in Columbia, MO, adults of both sexes were placed in cages (30 cm^3, Megaview Science Co., Ltd., Taichung, Taiwan) with a photoperiod of 14:10 (L:D) h at 25 °C. Adults were supplied with corn leaf tissue, slices of zucchini, an agar gel to serve as a water source, and an artificial diet for adult rearing (Frontier Agricultural Sciences, Newark, DE). Petri dishes with 70 mesh sieved field soil served as an oviposition site for females. The oviposition site was moistened throughout the week and replaced weekly. The eggs in the Petri dish were separated from the soil by washing through a 60 mesh sieve. The eggs were then divided and placed in two plastic containers (15 × 10 cm, GladWare®, The Glad Products Company, Oakland, CA) with 70 mesh sieved field soil. The plastic containers were covered with lids and placed on the bottom racks of a Percival incubator set to run at 25°C.

Seedling Mats

Non-diapausing insects. Fifteen seedling mats were planted in March 2016. Each seedling mat contained approximately 15 g of maize seed (Monsanto Company, variety DKC 61-79), 6 cm of autoclaved growth medium, and 80 ml of tap water in a 15 × 10 cm plastic container. The growth medium consisted of a mixture of field soil:Pro-Mix BX potting medium (Premier Horticulture Inc., Quakertown, PA) at a 2:1 ratio (v/v) prior to being autoclaved. Seedling mats were allowed to germinate, and coleoptiles emerged through the soil surface prior to infestation.

Seedling mat containers were placed on the top rack of the same Percival incubator in which eggs were incubated. Six time points were established as points of interest for this study, and were as follows: 0 d (neonate larvae), 5 d, 10 d, 15 d, 22 d, and adult emergence. Three replicates were used for this survey of non-diapausing insects. Randomization of time points occurred within each replicate. All containers, except for the 0 d time point, received 25 neonate larvae. For the 0 d time point, 10 neonate larvae were collected into 1.5ml microcentrifuge tubes (USA Scientific) and then stored at -80°C (So-Low, Environmental Equipment, Cincinnati, OH).

Diapausing insects. A total of five replications were conducted for this survey of diapausing insects. During this survey, two different growth media were used. The first growth medium remained the same as the non-diapausing insect survey, while the second growth media was soil collected from Higginsville, MO, in July 2016. This soil was not autoclaved and remained enclosed in a metal container until use in October 2016. In addition to the time points listed previously, two types of eggs were sampled: eggs washed

from sieved soil, and eggs washed from sieved soil that were then surface sterilized (Pleau et al. 2002).

Once the desired time point was reached, the seedling mats were processed in the same manner as Meihls et al. (2008). For the 5, 10, 15, and 22 d time points, all aboveground plant material was removed from the container. Next, the soil and root tissue were placed into a Berlese funnel with an attached jar. The jar with a moist filter paper at the bottom was used to collect the larvae. Specimens of each age were transferred from the jar to a microcentrifuge tube at least once every three hours throughout a typical work day. This tube was then immediately placed into the -80°C freezer for storage until DNA extraction occurred. A new tube was used for each collection time and sample to prevent additional freezing and thawing. Soil was also collected from the bottom of the seedling mat for larval specimens.

For the adult emergence time point in the non-diapausing insect survey, we planted a fresh seedling mat in a larger container (33 × 19 cm, Sterilite Corporation, Birmingham, AL) and allowed the maize to grow for one week prior to infestation. The first and smaller seedling mat had plant tissue removed before being inverted onto the second and larger seedling mat. After one week, the larger seedling mat was covered with a mesh screen to prevent escape of emerging adults. No secondary container was used for the diapausing insect survey, but mesh screens were used to keep the adults from escaping the container. Adult emergence containers were checked daily, and adults from each container on a given day were placed into microcentrifuge tubes. Soil was collected from the soil surface where adults must pass to emerge through the soil.

DNA Extraction and Quantitation. Whole insects (1-8 larvae/treatment; 1-2 pupae/treatment; a single adult/treatment) were pooled, and DNA extracted using accepted methods (Chen et al. 2010). The samples were extracted using PowerFecal® DNA Isolation Kit (MO BIO Laboratories, Inc. Catalog No. 12830-50) following the manufacturer's protocol (https://mobio.com/media/wysiwyg/pdfs/protocols/12830.pdf) with the following modifications: one sterile 0.5 cm diameter stainless steel ball bearing was added to the Dry Bead Tube for each adult and soil sample prior to shaking; shaking time was reduced to 5 minutes for adults and 3 minutes for all other samples. DNA quality and concentration was determined for each sample by Nanodrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE) and stored at −80˚C.

Library construction and sequencing. All PCR and sequencing was performed at the University of Missouri DNA Core. DNA concentration was determined fluorometrically (Qubit 2.0, Life Technologies) prior to analysis. Based on results of fluorometry, all samples were normalized to a standard concentration for PCR amplification. Bacterial 16S rRNA amplicons were generated via amplification of the V4 hypervariable region of the 16S rRNA gene using single-indexed universal primers (U515F/806R) flanked by Illumina standard adapter sequences and the following parameters: 98 ˚C$^{(3:00)}$+[98˚C$^{(0:15)}$+50˚C$^{(0:30)}$+72˚C$^{(0:30)}$] × 25 cycles +72˚C$^{(7:00)}$. Amplicons were then pooled for sequencing using the Illumina MiSeq platform and V2 chemistry with 2×250 bp paired-end reads, as previously described (Ericsson et al. 2015).

Informatics analysis. All informatics analyses were performed as previously described (Hart et al. 2015), at the University of Missouri Informatics Research Core Facility. Input is typically for 2x350 bp reads from one of the two MiSeq machines in the

DNA Core. The read pairs are joined into contigs by the program FLASH (http://bioinformatics.oxfordjournals.org/content/27/21/2957.long) (Magoc et al. 2011), and culled if found to be short after trimming for a base quality less than 31, and those that are not joined, or are too long or short after contig formation, leaving those that are 275 to 300 nts. Cutadapt (http://journal.embnet.org/index.php/embnetjournal/article/view/200/479) was used to find and trim the primers from the 5' and the 3' ends, culling those contigs lacking both primers. Contigs with the expected number of errors greater than 0.5 were removed by Usearch (http://drive5.com/index.htm), and the remainder were trimmed to length 248. The contig read ids were modified so that samples could be followed throughout by using the Qiime script split_libraries_fastq.py. All samples were then pooled into one fasta file and metrics for all samples collated into one table. Contigs were clustered *de novo* into an OTU table using the uparse (http://drive5.com/uparse/) algorithm. *De novo* and reference-based chimera detection and removal was performed using Qiime v1.8 (Kuczynski et al. 2012) software, and remaining contiguous sequences were assigned to operational taxonomic units (OTUs) via *de novo* OTU clustering and a criterion of 97% nucleotide identity. Annotation of selected OTUs was performed using BLAST (Altschul et al. 1997) against the Silva database (https://www.arb-silva.de/) (DeSantis et al. 2006) of 16S rRNA sequences and taxonomy. Principal coordinate analysis and PERMANOVA testing were performed using ¼ root-transformed and non-transformed OTU relative abundance data, respectively, using Past 3.16 (https://folk.uio.no/ohammer/past/) (Hammer et al. 2016). Richness and α-diversity metrics were determined in Past 3.16 using Qiime-generated otu_biom.table files.

Statistical analysis. Differences in raw and binned OTU richness were tested via ANOVA using SigmaPlot 12.3 (Systat Software Inc., San Jose, CA); *p* values less than 0.05 were considered significant. Differences in the overall composition of the different regions were tested via two- and one-way PERMANOVA of ranked Bray-Curtis or Jaccard distances using the open access Past 3.16 software package (Quast et al. 2013), downloaded on April 2, 2016.

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Table 7. Results of PERMANOVA testing for differences in β-diversity between western corn rootworm (WCR) and soil samples collected from two different sites, based on the Bray-Curtis distance. *p* values and F values are shown in the upper right and lower left portions of the table, respectively.

	p values	Soil		WCR from "X" soil	
F values		Columbia	Higginsville	Columbia	Higginsville
Soil	Columbia		0.0001	0.0001	0.0001
	Higginsville	27.62		0.0001	0.0001
WCR from "X" soil	Columbia	57.08	104.5		0.1498
	Higginsville	38.43	119.7	1.657	

Table 8. Results of PERMANOVA testing for differences in β-diversity between western corn rootworm (WCR) and soil samples collected from two different sites, based on the Jaccard distance. *p* values and F values are shown in the upper right and lower left portions of the table, respectively.

	p values	Soil		WCR	
F values		Columbia	Higginsville	Columbia	Higginsville
Soil	Columbia		0.0001	0.0001	0.0001
	Higginsville	24.93		0.0001	0.0001
WCR	Columbia	19.62	23.66		0.0001
	Higginsville	18.56	18.6	3.972	

Table 9. Unique operational taxonomic units (OTUs) found in all insect samples regardless of soil origin.

OTUs	Taxonomic Rank	Present in egg soil?	First found in soil[1]
Ruminococcaceae	Family	Yes	Egg
Lachnospiraceae	Family	Yes	Egg
Bacteroidales S24-7	Group	Yes	Egg
Wolbachia (*Delia antiqua*)	Genus	No	Neonate
Tsukamurella sp.	Genus	No	Never
Gordonia sp.	Genus	Yes	Egg
Oscillibacter sp.	Genus	Yes	Egg
Microbacterium sp.	Genus	Yes	Egg
Bacillus megaterium	Species	No	Never
Geobacillus toebii	Species	Yes	Egg
Klebsiella sp. Z1	Species	Yes	Egg
Mycobacterium fortuitum	Species	No	Never
Streptomyces rectiviolaceus	Species	No	Never
Lachnospiraceae NK4A136	Species	Yes	Egg
Pseudomonas sp. FSGRN7	Species	No	Never
Pseudonocardia sp. YIM 68245	Species	No	Never

[1]A particular OTU was found in one or more soil samples corresponding to the life stage denoted

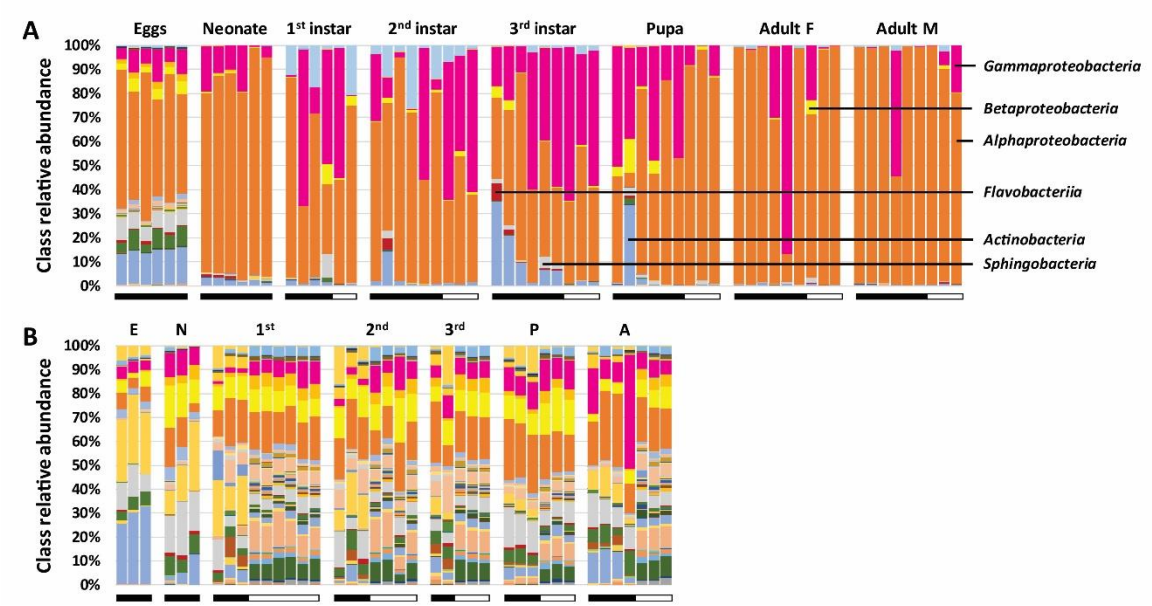

Figure 6. Stacked bar charts showing relative abundances of microbial classes detect in corn rootworms at different life stages (A) and in soil from which rootworm samples were collected (B). Horizontal bars below the vertical bars indicate original of soil; black bars = Columbia, MO, white bars = Higginsville, MO.

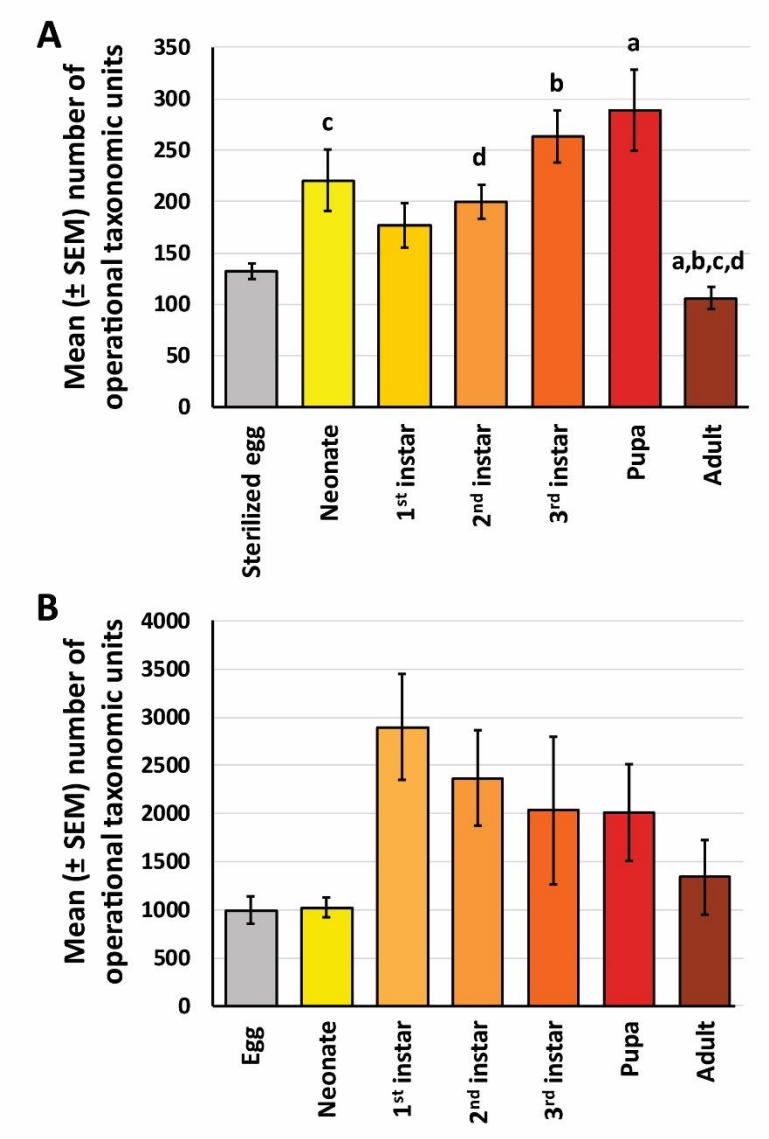

Figure 7. Main effect of life stage on microbial richness in western corn rootworm (**A,** $p<0.001$), or the soil from which WCR samples were collected (**B**, $p = 0.040$). Significant pairwise differences are indicated by like letters (Kruskal-Wallis one-way ANOVA on ranks with Dunn's *post hoc*).

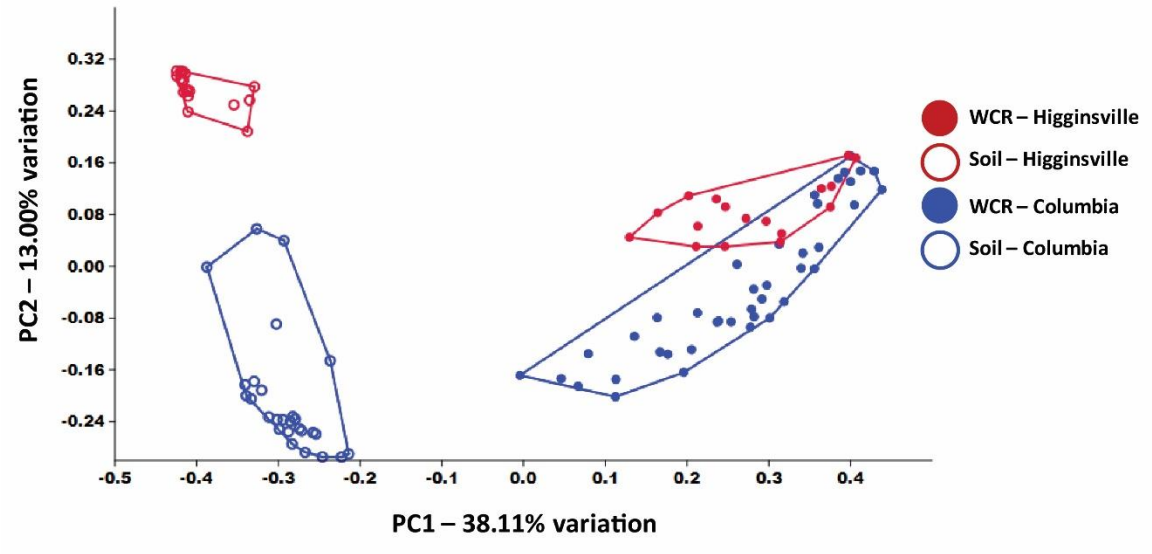

Figure 8. Principal coordinate analysis based on Bray-Curtis similarity between microbial communities detected in western corn rootworm (WCR) at various life stages and soil samples collected from two different sites.

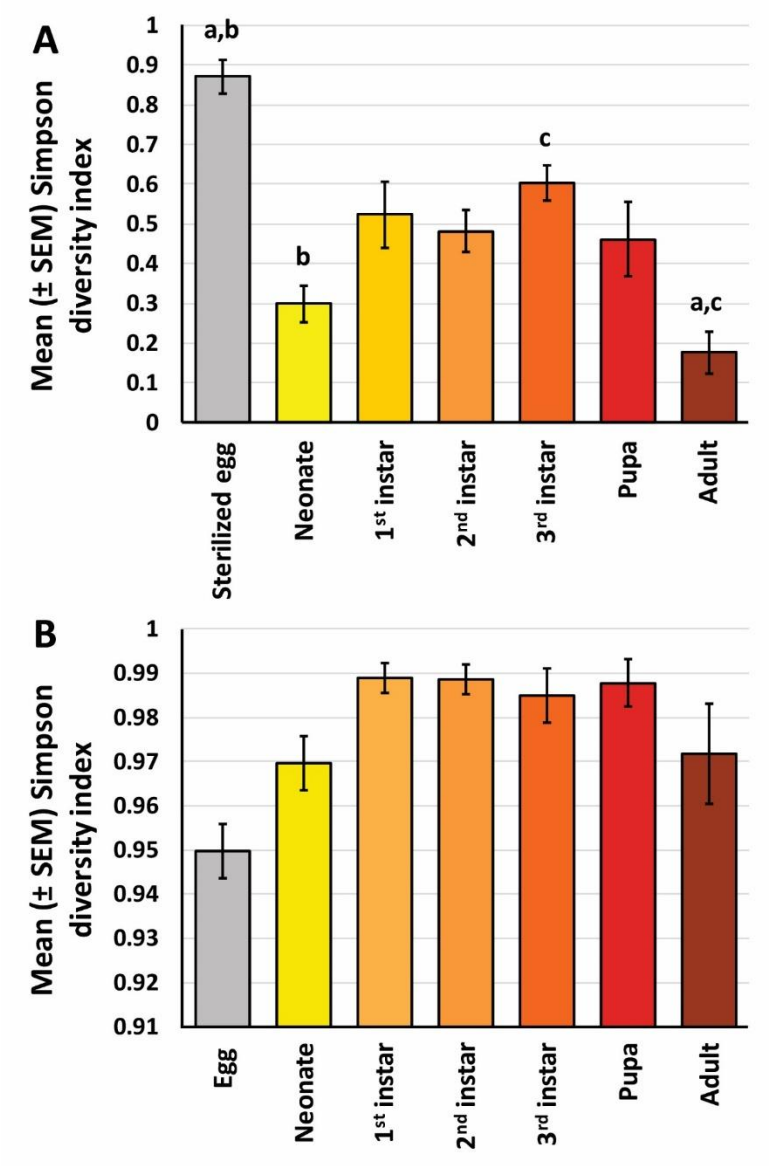

Figure 9. Main effect of life stage on microbiota α-diversity in western corn rootworms (**A,** $p < 0.001$), or the soil from which the WCR samples were collected (**B,** $p = 0.040$). Significant pairwise differences indicated like letters (Kruskal-Wallis one-way ANOVA on ranks with Dunn's *post hoc*).

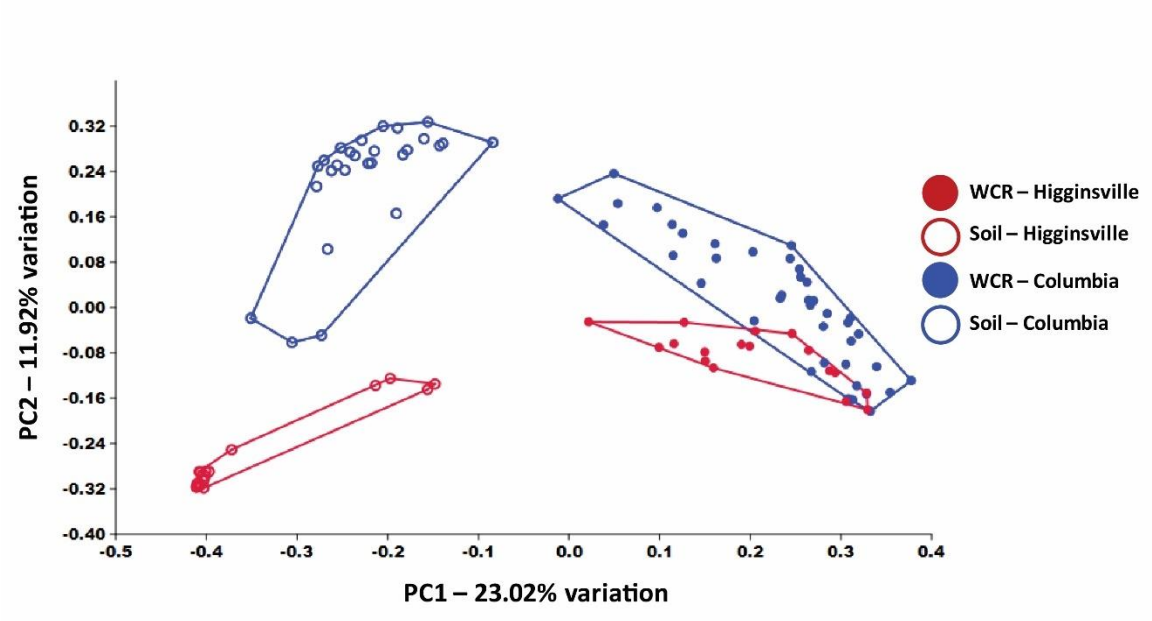

Figure 10. Principal coordinate analysis based on Jaccard similarity between microbial communities detected in western corn rootworms (WCR) at various life stages and soil samples collected from two different sites.

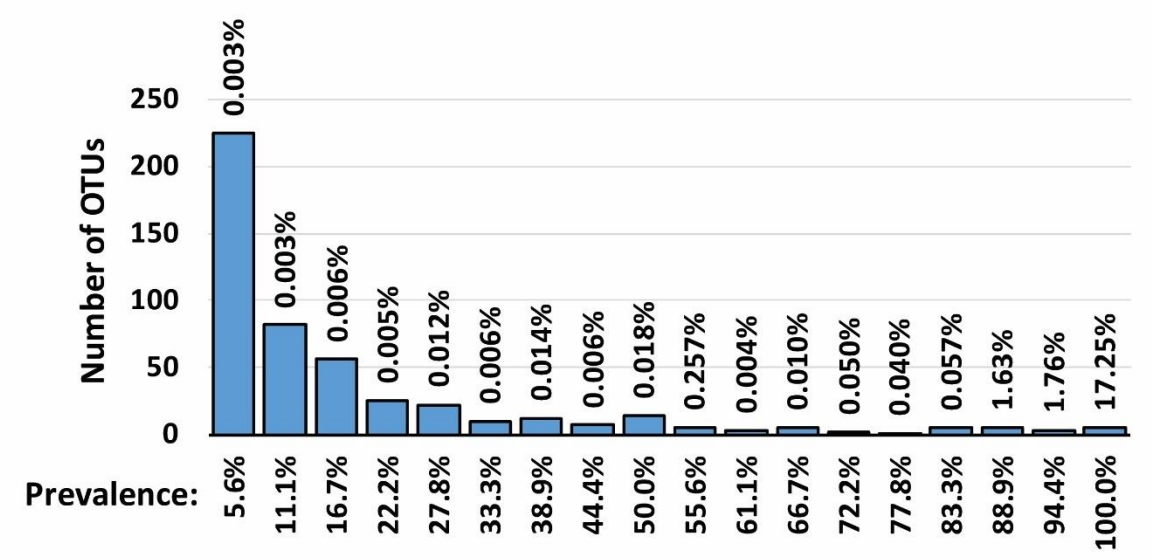

Figure 11. Number and mean relative abundance (above bars) of operational taxonomic units (OTUs) detected at increasing prevalence in adult western corn rootworm samples.

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VITA

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