12-2019

USING ADULTS TO MONITOR RNAi SUSCEPTIBILITY OF WESTERN CORN ROOTWORM, *DIABROTICA VIRGIFERA VIRGIFERA* LECONTE, FIELD POPULATIONS

Matthew Welter
University of Nebraska - Lincoln

USING ADULTS TO MONITOR RNAi SUSCEPTIBILITY OF WESTERN CORN ROOTWORM, *DIABROTICA VIRGIFERA VIRGIFERA* LECONTE, FIELD POPULATIONS

by

Matthew Welter

A THESIS

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Master of Science

Major: Entomology

Under the Supervision of Professor Ana M. Vélez Arango and Lance J. Meinke

Lincoln, Nebraska

December, 2019

USING ADULTS TO MONITOR RNAi SUSCEPTIBILITY OF WESTERN CORN ROOTWORM, *DIABROTICA VIRGIFERA VIRGIFERA* LECONTE, FIELD POPULATIONS

Matthew Welter, M.S.

University of Nebraska, 2019

Advisors: Ana M. Vélez Arango and Lance J. Meinke

The western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte, is the most damaging corn pest in the U.S. Corn Belt, costing producers over $1 billion annually in control and damage costs. Currently, corn producers rely on three control strategies for WCR management: crop rotation, chemical insecticides, and transgenic corn expressing *Bacillus thuringiensis* Berliner (Bt) proteins. Populations of WCR have evolved resistance to all of these tactics, limiting effective control strategies for producers. RNA interference (RNAi), is the newest mode of action developed for WCR management. In July 2017, the first RNAi plant-incorporated protectant (PIP) corn product was approved for production in the United States. This product, marketed under the trade name SmartStax PRO®, will express two Bt proteins and *DvSnf7* double-stranded RNA for WCR control. Similar to current PIPs, resistance monitoring protocols must be established before adoption of this technology to delay resistance evolution.

This study characterized the variability of adult susceptibility due to age and sex. Male beetles were most tolerant to dsRNA at 2-days post-emergence, but responded uniformly to dsRNA at 10-, 20-, and 30-days post-emergence and were significantly

more susceptible than their female counterparts at 10- and 20-days post-emergence. Female adults responded uniformly for 2-, 10-, and 20-days post-emergence, but were significantly more susceptible at 30-days post-emergence. Baseline susceptibilities for U.S. Corn Belt populations of WCR were established and the potential for use of adult WCR for *DvSnf7* dsRNA susceptibility monitoring in field populations was evaluated. Overall, most field populations were uniform in their larval susceptibility to *DvSnf7* dsRNA. Adult male susceptibility was more variable compared to larvae and correlation ratios between adult males and larvae were not always consistent. Therefore, it may not be possible to use adult WCR to monitor changes in *DvSnf7* dsRNA susceptibility, especially if small shifts in susceptibility impact product performance.

Keywords: *Diabrotica*, Rootworm, RNA interference, RNAi, corn, resistance monitoring, SmartStax PRO®, *DvSnf7*

ACKNOWLEDGEMENTS

First, I would like to thank my advisors, Dr. Ana María Vélez Arango and Dr. Lance J. Meinke. You were both invaluable resources for knowledge and personal guidance throughout my graduate tenure. Thank you for the opportunity and encouragement to get involved in the department graduate club, speak at symposiums, attended conferences and explore my interests. Your influences have made me a much better researcher and prepared me for a successful career.

I would also like to thank the other members of my committee, Dr. Dan Moellenbeck and Dr. Chitvan Khajuria, for your time spent reading manuscripts, written examinations, and my thesis. Your expertise was always appreciated. Also, thank you to Dan for collecting some field populations for testing.

To the toxicology lab members, your great attitude and encouragement made the lab a great working environment. Fiesta Fridays, lab barbeques and escape rooms will be some of my favorite memories from my time at UNL.

To my family, thank you for always being there to support me and refresh me when I got the chance to make it home. You all helped make me into the person I am today. I know I will always have a place to put my education to good use as the Welter Farms IPM consultant. I love you all.

My fellow graduate students. We made a lot of great memories together that I will cherish forever. It was nice to always have someone around that I could chat with when I wanted to avoid writing. I could not have picked a better group of people to call co-workers. I wish you all luck in your future careers.

To the Reinders Family. Thank you for all of your extra help to making this project happen. Without your expert knowledge of rootworm rearing, I would not have the data sets that I do. It was always nice knowing that there was always someone down in the rootworm lab if I ever had any questions.

To Molly Darlington. You have been a great friend throughout my time in Nebraska. You have opened my mind to new ideas, I will be eternally grateful for that. I know you will get more work done in the grad room now. I look forward to seeing what Dr. Darlington discovers.

Finally, I would like to thank my girlfriend, Anna Bednarko. Thank you for all of your love and support, without it, this would not be completed. Your weekend visits always picked me up and kept me motivated, even when we had to spend our time together, transferring insects for bioassays. I am so excited to continue to grow with you, Scooter and Kleo.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

Insect species from the genus *Diabrotica* are among the most damaging insects to corn, *Zea mays* L., in the United States Corn Belt. Four species, northern corn rootworm (NCR), *Diabrotica barberi* Smith & Lawrence, southern corn rootworm (SCR), *D. undecimpunctata howardi* Barber, Mexican corn rootworm (MCR), *D. virgifera zeae* Krysan and Smith, and western corn rootworm (WCR), *D. virgifera virgifera* LeConte, have the potential to cause significant corn damage in the United States (Krysan and Smith 1987). In particular, WCR is of concern as it is the most economically damaging pest in the U.S. Corn Belt (Gray et al. 2009). Economic estimates suggest that WCR costs U.S. corn producers over one billion dollars annually in management and yield losses (Metcalf 1986, Dun et al. 2010). Larval feeding occurs in the root system of the plant, causing a reduction in plant growth and grain yield (Gray and Steffey 1998, Urías-López and Meinke 2001, Dun et al. 2010, Tinsley et al. 2013). Whereas, adults feed on pollen and ear silks, potentially reducing pollination and seed set at high population densities (Branson and Krysan 1981).

Currently, corn producers rely on three major strategies: crop rotation, insecticides, and *Bacillus thuringiensis* (Bt) protein plant-incorporated protectants (PIPs) for WCR control. However, populations of WCR have evolved resistance to all of these tactics, therefore limiting control options as resistance spreads (Meinke et al. 2009, Pereira et al. 2015, Jakka et al. 2016). RNA interference (RNAi), is a novel mode of action for this pest and in July 2017, the first RNAi PIP corn product was approved for production in the United States (US EPA 2017). In order to delay resistance to this new

control tactic, resistance monitoring protocols and baseline susceptibility levels are conditions of registration for PIPs. Current resistance monitoring for Bt is completed in the larval stage

This study aims to establish baseline susceptibilities for U.S. Corn Belt populations and evaluate the potential use of adult western corn rootworm for *DvSnf7* dsRNA susceptibility monitoring in field populations. Additionally, potential variability in adult susceptibility due to various factors such as age and sex were characterized.

1.2 Western Corn Rootworm

1.2.1 Biology and Crop Damage

The western corn rootworm is a univoltine species with an active life cycle from May through September, overwintering in the egg stage (Chiang 1973). Egg hatching begins in late May/early June and lasts 4-5 weeks (Meinke et al. 2009). Larvae progress through three larval instars while feeding on corn roots during June and July when corn is rapidly growing (Bryson et al. 1953). Adult emergence begins in July and continues into August. As a protandrous species, adult males emerge and become sexually mature before females and mating begins upon female emergence (Spencer et al. 2009). Oviposition begins in July and lasts through September, or until a killing frost (Ball 1957). Females typically lay their eggs near corn plants, in damp areas of the soil via drought cracks or earthworm holes. Ovipositional depth appears to vary; Ball (1957) determined that 80% of eggs were laid in the upper 15 cm of soil, while, Gray et al. (1992) reported that 60% of WCR eggs were found in the bottom 10 cm of 30 cm cores in dryland corn under dry conditions. Soil moisture appears to be a driving factor for oviposition depth with 46 ± 8% of eggs located in the top 15 cm of soil for dryland corn

and 93 ± 4% of eggs located in the top 15 cm of soil for irrigated corn (Weiss et al. 1983). WCR eggs develop for approximately two weeks before entering an obligate cold diapause that can range between 78 to 163 days, varying greatly by population, latitude, and even between individuals of the same population (Krysan 1982). In temperate regions, diapause is terminated midwinter and eggs remain dormant in a facultative state of chill-quiescence until the soil temperatures rise above 11°C, upon which eggs begin post diapause development (Meinke et al. 2009).

Larval feeding reduces root mass, causing physiological and physical stress to the plant. Unable to properly absorb nutrients and water, one node of feeding by WCR larvae can reduce corn yields up to 15% (Tinsley et al. 2013). Additionally, major reduction of the root system causes plants to lodge or "gooseneck", preventing much of the grain from being harvested by modern mechanized harvester systems. Adults can potentially generate economic losses by clipping silks off the ear, reducing ear pollination and feeding on corn pollen at high population densities (Ball 1957, Levine and Oloumi-Sadeghi 1991, Culy et al. 1992).

1.2.2 History of Expansion

Western corn rootworm was first identified as a pest of corn in 1909 in north-central Colorado (Gillette 1912). WCR became a significant corn pest in the 1940s when the popularity of continuous corn cultivation and introduction of irrigation supplied large habitable areas leading to rapid population growth (Meinke et al. 2009). Initial expansion across Kansas and Nebraska proceeded slowly with the pest reaching the Iowa border in 1954. Rapid expansion followed, with populations reaching Wisconsin by 1964 and Indiana by 1968 (Metcalf 1983, 1986, Meinke et al. 2009). Today, WCR is found from

Oregon to Maine in the north and from northern Georgia to Arizona in the south, with the U.S. Corn Belt states being the area of highest risk for economic damage (Murphy et al. 2014). WCR is not isolated to the U.S. It was first detected in Europe in 1992 near the Belgrade airport in Serbia. Similar to the U.S., WCR distribution expanded rapidly with 20 European countries reporting WCR presence by 2007 (Gray et al. 2009), making management of this insect internationally important. As this pest range continues to expand, demand for control will also increase.

1.2.3 Control Strategies and Resistance

One of the most effective control strategies for WCR is crop rotation (US EPA 2015). Rotation of cropland from corn to a non-host plant, such as soybeans, reduces pest pressure as oviposition occurs primarily in corn fields and larva are unable to develop on non-host plants crops the following spring (Levine and Oloumi-Sadeghi 1991, Levine et al. 2002). Rotation continues to be an effective control tactic for much of the U.S. Corn Belt, however failure of crop rotation was documented in 1987 near Piper City, Illinois (Levine and Oloumi-Sadeghi 1996). Resistant beetles exhibited a behavioral change reducing the affinity for oviposition in corn. Gravid females began laying eggs into non-corn fields that, when planted to corn the following growing season, sustained economic pest damage (Knolhoff et al. 2006). Chu et al. (2013) also identified that the gut microbiota present in the rotation-resistant strain allowed beetles to tolerate soybean anti-herbivory defenses improving their ability to utilize soybean as a diet source (Chu et al. 2013). Rotational resistance has remained relatively isolated to areas of Illinois, Indiana, Wisconsin, and northwest Iowa (Meinke et al. 2009, Dunbar and Gassmann

2013), leaving crop rotation as an effective tool for integrated pest management systems throughout much of the Corn Belt.

Throughout the mid to late 1900s, soil- and foliar-applied insecticides were the main WCR management strategy in continuous corn (Levine and Oloumi-Sadeghi 1991). Soil insecticides, typically organochlorines, organophosphates, carbamates, or pyrethroids, are applied to protect the root zone from larval feeding under low to moderate population intensities and are typically applied in-furrow or as a band over the row during planting (Levine and Oloumi-Sadeghi 1991, Gray and Steffey 1998). Foliar insecticides, typically pyrethroids, are applied to protect corn from adult silk clipping and to reduce egg laying by gravid females (Branson and Krysan 1981, Meinke 2014). Reducing the adult population and female oviposition drastically decreases WCR pressure the subsequent growing season, potentially increasing the success of other management strategies (Meinke 2014).

Insecticide resistance was first documented for WCR during 1959 in Nebraska to organochlorines (Ball and Weekman 1962a). This resistance spread rapidly, with the expanding population being uniformly resistant even in areas where organochlorines were no longer in use (Siegfried and Mullin 1989, Parimi et al. 2006). During the 1970s, organophosphate and carbamate insecticides replaced organochlorine insecticides for WCR control. Utilized as soil and aerial applied insecticides, intense selection pressure resulted in resistance evolution by the 1990s to both insecticide classes in areas with extensive use (Wright et al. 1996, Meinke et al. 1998). Currently, pyrethroids and organophosphates are the most commonly used insecticides for control of WCR. Adult control with pyrethroids requires multiple applications per year to reduce oviposition

(Pereira et al. 2015). In 2013 WCR populations collected in western Nebraska and southwestern Kansas exhibited field-evolved resistance to pyrethroids. WCR pyrethroid resistance has a limited distribution (Pereira et al. 2015), so preventive strategies should be implemented in these areas to mitigate the spread of resistance and allow for continued use of this pesticide as a control tool.

Plant incorporated protectants expressing *Bacillus thuringiensis* (Bt) proteins targeting WCR became available in 2003 when Monsanto Company released a transgenic hybrid event expressing the Cry3Bb1 protein (MON863). Since this initial introduction, three additional Bt proteins, Cry34/35Ab1 (DAS-59122-7), mCry3A (MIR 604) and eCry3.1Ab (Event 5307) have been commercialized for root protection against rootworms. Initially, hybrids expressing a single Cry protein were extensively utilized in the U.S. Corn Belt. However, to increase product durability and mitigate resistance evolution, single protein hybrids have been replaced by transgenic hybrids expressing two rootworm-active Bt proteins (Andow et al. 2016). Pyramids expressing Cry3Bb1 + Cry34/35Ab1, mCry3A + Cry34/35Ab1, mCry3A + eCry3.1Ab are currently registered for sale in the United States (US EPA 2015).

In 2009, Iowa populations collected from fields of continuous corn expressing the Cry3Bb1 protein exhibited field evolved resistance to this protein (Gassmann et al. 2011). Since the first detection in Iowa, WCR resistance to the Cry3Bb1 toxin has been confirmed in Illinois, Nebraska, and Minnesota (Wangila et al. 2015, Zukoff et al. 2016). Cross-resistance, (i.e. selection for resistance to a toxin which causes resistance to a second toxin(s)) to the mCry3A and eCry3.1Ab proteins was documented for populations resistant to Cry3Bb1, conferring resistance to three of the four commercially

available rootworm-active Bt PIPs. Cross-resistance effectively eliminates the benefit of pyramids containing a combination of these three proteins for populations with documented resistance to toxins present in the pyramids (Jakka et al. 2016, Zukoff et al. 2016). The binary protein Cry34/35Ab1 has yet to demonstrate any form of cross-resistance with the other available Bt proteins (Jakka et al. 2016, Zukoff et al. 2016). However, field evolved resistance to the Cry34/35Ab1 protein has been documented. Gassmann et al. (2016) determined that Iowa populations collected in 2013, exhibited resistance to the Cry34/35Ab1 protein. Populations in Minnesota and Nebraska have also recently demonstrated incomplete resistance to this protein (Head et al. 2017, Ludwick et al. 2017).

Western corn rootworm has demonstrated its ability to adapt to multiple management strategies including chemical, cultural, and transgenic control (Gray et al. 2009). Resistance limits options for WCR management and increases the risk of economic damage. Many of the single Bt trait hybrids and some pyramided hybrids are no longer sufficient for control of this pest. Novel modes of action combined with proper integrated pest management strategies are needed for producers to control WCR effectively.

1.3 RNA Interference

1.3.1 RNA Interference for Pest Management

RNA interference (RNAi) was discovered in 1998 when Fire et al. (1998) determined that injections of double-stranded RNA (dsRNA) into *Caenorhabditis elegans* could trigger post-transcriptional gene silencing. Since this initial discovery, RNAi has been described in multiple eukaryotic taxa and has been utilized extensively to

understand gene function (Agrawal et al. 2003). In insects, successful gene knockdown by dsRNA molecules has been documented via injection, exogenous application and oral ingestion to understand gene function. Double-stranded RNAs triggering the RNAi response against housekeeping genes, genes vital to survival, are being developed as an insect management tools (Baum et al. 2007). Genes targeting reproduction have also been successful (Niu et al. 2017)

In vivo feeding of dsRNA is the most practical way for dsRNA to be used for pest management. Coleopteran insects are highly sensitive to oral dsRNA, while sensitivity varies in Lepidopteran, Orthopteran, Dipteran, and Hemipteran insects (Baum and Roberts 2014; Christiaens and Smagge 2014; Niu et al. 2018; Zhang et al. 2017). Variability in response to oral dsRNA results from extra-oral digestion (Allen and Walker 2012), degradation of dsRNA in the gut, hemolymph, or salivary fluids (Allen and Walker 2012; Garbutt et al. 2013; Luo et al. 2013; Christiaens et al. 2014; Wynant et al 2014), limited cellular uptake of dsRNA (Shukla et al. 2016; Yoon et al. 2017), and viral interactions (Christiaens and Smagghe 2014). Understanding and overcoming factors that affect the dietary dsRNA response might allow for the development of strategies to overcome these limitations, therefore allowing for RNAi-based control strategies pest species for a variety of insect orders.

1.3.2 RNAi in WCR Management

In 2007, Baum et al. (2007) demonstrated that the use of dsRNA caused mortality and growth inhibition in WCR larvae by triggering the RNAi response. Of the 290 genes screened, 15 were identified with LC_{50} values below 10 ng dsRNA/ cm^2 in artificial diet assays. The target for *vacuolar ATPase subunit A* (*V-ATPase-A*) was transformed into

corn and provided protection from larval root feeding (Baum et al. 2007). Additional experiments determined that dsRNA targeting the WCR gene *Snf7* (*DvSnf7*) also protected corn roots from damage (Bolognesi et al. 2012, Ramaseshadri et al. 2013, Levine et al. 2015).

Snf7 belongs to the Endosomal Sorting Complex Required for Transport (ESCRT) – III complex, which sorts transmembrane proteins for lysosomal degradation. Briefly the process involves endocytosis of membrane receptor proteins for recycling or degradation, ubiquitination of the receptors as a label for degradation, transportation of ubiquitinated proteins to lysosome lumen, removal and recycling of ubiquitin, and degradation through lysosomal degradation or autophagy (Schuh and Audhya 2014). Down regulation of the *Snf7* gene disrupts the cells ability to remove ubiquitin molecules from proteins destined for degradation, resulting in stunting of larval WCR development after five days of exposure (Bolognesi et al. 2012).

Additional RNAi targets have been identified in WCR including orthologs for the *Drosophila* genes *snakeskin* (*ssk*), *mesh*, *wings up A* (*wupA*), and *Sec23*. The orthologs of *ssk* and *mesh* are vital to proper function of smooth septate junctions (SSJ) in the insect gut and have been termed *dvssj1* and *dvssj2* respectively (Hu et al. 2016). The *wupA* ortholog encodes a Troponin I protein required for muscle contraction ((Fishilevich et al. 2019). Whereas, *Sec23* encodes a component of the coat (COPII) complex that mediated ER-Golgi transport (Vélez et al. 2019). Corn plants transformed with *dvssj1*, *dvssj2*, *wupA,* or *Sec23* provided significant root protection from WCR feeding (Hu et al. 2016, Fishilevich et al. 2019, Vélez et al. 2019), highlighting the potential use of RNAi for WCR control.

Although RNAi targets that generate larval mortality are most practical for in season pest management, additional targets have been identified that could be used to manage population pressure. Parental RNAi (pRNAi), or gene knockdown in progeny resulting from female parent exposure to dsRNA, has been demonstrated in WCR (Vélez, Fishilevich, et al. 2016a). Adult ingestion of dsRNA targeting developmental genes *hunchback* (*hb*) and *brahma* (*brm*) reduced egg hatch to zero (Khajuria et al. 2015). Additionally, reproductive RNAi (rRNAi), or gene knockdown resulting in reduced insect fecundity is another target for WCR population control. Exposure of 3rd instar larvae and adults to dsRNA targeting WCR homologs of the *vitellogenin receptor* (*VgR*) and *boule* (*bol*) genes significantly reduced fecundity (Niu et al. 2017). Parental RNAi and reproductive RNAi provide additional strategies to manage pest population pressure through reduction in pest abundance the following season.

1.3.3 RNAi Mode of Action in WCR

The RNAi mechanism in rootworms is a multi-step process involving uptake of dsRNA into the insect cell, silencing of the targeted mRNA, and systemic spread of the RNAi signal from cell to cell. Although these steps are clearly defined, only the mode of action for the silencing of the target mRNA has been thoroughly described.

In *C. elegans*, SID-1 and SID-2 proteins are responsible for the uptake of extracellular dsRNA into the organism (Whangbo and Hunter 2008). "RNAi-of-RNAi" experiments have been utilized to evaluate the role of orthologs of these proteins in insect dsRNA uptake. "RNAi-of-RNAi" experiments knock down specific target genes (i.e., SID-like proteins) using RNAi then treated the insects with an additional, often lethal, dsRNA construct to determine if the pathways still function. Knockdown of the WCR

orthologs of SID-1 and SID-2 did not affect the RNAi pathway, suggesting that other mechanisms are involved in dsRNA uptake (Miyata et al. 2014, Pinheiro et al. 2018). Similar experiments indicated clathrin-dependent endocytosis as a potential uptake pathway. Down regulation of *clathrin* and *AP50* significantly reduced the knockdown of a non-lethal reporter gene, suggesting that clathrin-dependent endocytosis plays a significant role in dsRNA uptake (Saleh et al. 2006, Pinheiro et al. 2018). Additional extracellular receptors and channels have been tested; however, no single protein or receptor has been identified, suggesting that multiple receptors or proteins may be involved in dsRNA uptake (Cooper et al. 2019). Although the exact mechanism of uptake in WCR is unclear, feeding assays indicate that dsRNA must be greater than 60 bp long for uptake in WCR gut cells (Bolognesi et al. 2012).

Once inside the cell, dsRNA is cleaved by the RNaseIII-type enzyme Dicer into 21-23 base pair small interfering RNAs (siRNAs). The siRNAs then bind to a complex of proteins, the RNA Induced Silencing Complex (RISC), that uses the siRNA as a sequence-specific template for screening of mRNA in the cell. When RISC interacts with an mRNA that matches the siRNA template, the mRNA is cleaved by the Argonaut protein within RISC resulting in reduced transcript levels for that gene, therefore reducing subsequent protein production of the targeted gene (Meister and Tuschl 2004, Vélez et al. 2016). Interactions between dsRNA and the RNAi machinery across inset orders is highly conserved (Cooper et al. 2019).

The final step in the RNAi pathway is the systemic spread of the RNAi response to other cells in the organism. The RNAi systemic response has only been demonstrated in WCR by observing gene knockdown in tissues distant from the place of uptake.

Feeding assays with WCR larvae found knockdown of *DvSnf7* in the fat body of insects that were fed *DvSnf7* dsRNA, indicating spread from the gut to the fat body (Ramaseshadri et al. 2013). This is supported by the low levels of dsRNA required to generate mortality and effective knockdown throughout the insect (Bolognesi et al. 2012, Levine et al. 2015). Although systemic spread is active in WCR the exact mechanism remains unknown (Cooper et al. 2019).

1.3.4 SmartStax PRO®

In 2017, the EPA approved the first RNAi product, containing *DvSnf7* dsRNA, for production and consumption in the United States (US EPA 2017). This product will be marketed under the trade name of SmartStax PRO® (SSP) and will provide farmers with the first novel mode of action for control of WCR since the release of the Cry34/35Ab1 protein in 2005 (US EPA 2015). In multiyear field trials across multiple midwestern states, SmartStax PRO® provided excellent root protection (NIS <0.5) in areas with high WCR pressure and potential resistance to Cry3Bb1 (Head et al. 2017).

Moar et al. (2017) exposed a Cry3Bb1-resistant colony to *DvSnf7* dsRNA to evaluate the potential for cross resistance between Cry3Bb1 and *DvSnf7* dsRNA. The Cry3Bb1-resistant population exhibited a significant 2.7-fold decrease in susceptibility to *DvSnf7* dsRNA compared to the Cry3Bb1-susceptible population (Moar et al. 2017). However, this decrease in susceptibility was similar to *DvSnf7* susceptibilities generated from seven field-collected WCR populations tested in diet overlay bioassays, indicating that variations were generated by natural bioassay variation, not cross-resistance (Moar et al. 2017). Results from a greenhouse study comparing Cry3Bb1 susceptible and resistant population performance on single and pyramided Bt or dsRNA-expressing corn also

indicated a lack of cross-resistance between Cry3Bb1 and *DvSnf7* dsRNA (Moar et al. 2017). Additionally, the Cry3Bb1 resistance gene(s) is located on linkage group 8 (LG8) (Flagel et al. 2014) and the resistance gene for *DvSnf7* dsRNA is located on linkage group 4 (LG4) (Khajuria et al. 2018). These genes are located on different chromosomes, therefore supporting the lack of cross-resistance between *DvSnf7* dsRNA and Cry3Bb1 (Moar et al. 2017, Khajuria et al. 2018). Thus, *DvSnf7* dsRNA provides a new mode of action separate from Bt proteins in transgenic corn for WCR management.

1.3.5 Adult Activity

In contrast to *Bt* proteins, dsRNA generates mortality in the adult stage of WCR (Rangasamy and Siegfried 2012, Pereira et al. 2016a, Khajuria et al. 2018). Adult WCR RNAi response is rapid and persistent, with a 76% knockdown of *Lac2* 10 hours after ingestion and 86% knockdown 20 days after ingestion (Wu et al. 2018). Although tissue expression of *DvSnf7* dsRNA in pollen and silks is not sufficient enough to generate mortality in adults, (0.224 ng/g and 0.893 ng/g fresh weight respectively) (Bachman et al. 2016), it may present implications for resistance monitoring and management.

Adult WCR are known to move from field to field consuming plant tissue in each field (Spencer et al. 2009), increasing the risk that WCR will be exposed to sublethal concentrations of dsRNA at some point in their lifecycle after the release of SSP. Exposure to sublethal concentrations of a toxin can accelerate the rate of resistance evolution by adding selection pressure benefiting resistant individuals (Tabashnik et al. 2004, 2013).

1.4 Insect Resistance Management

1.4.1 Insect Resistance Evolution

Resistance is defined as “a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species” (IRAC 2019). Resistance evolves as the phenotypic result of intense genetic selection pressure on a population favorable to survival after exposure to a specific control tactic. Repeated use of the same control strategy applies continual selection pressure and will eventually generate resistance (Dover 1985, IRAC 2019). Resistance evolution drives increases in application rates until no label approved rate will effectively control the insect. A new insecticide is then substituted for the ineffective insecticide and the cycle continues. This phenomenon is commonly referred to as the pesticide treadmill. If new insecticides are not available pest populations expand rapidly and elevate the risk of damage (Dover 1985, Knight and Norton 1989). Efforts to conserve the remaining effectiveness of Bt traits and improve the durability of future of PIPs, such as RNAi, allow for long term control of pest populations without significant shifts in susceptibility. Without proper insect resistance management, resistance evolution will inevitably occur.

1.4.2 Insect Resistance Management for Plant Incorporated Protectants

The U.S. EPA requires that registrants of PIPs complete and submit an insect resistance management (IRM) plan for the major target pest(s) of the PIP before registration (US EPA 2013). Insect resistance management (IRM) is the scientific approach to managing pests over extended periods while minimizing the risk of resistance evolution to the management tactics used. In 1988, building on the strategies already used in host plant resistance, four major IRM strategies for PIPs were proposed: low dose toxin expression, temporal or localized toxin expression, mixtures of toxic and

non-toxic cultivars (refuge) and pyramiding of two or more toxins within cultivars (Gould 1988). The high dose strategy was introduced in 1991, after industry scientists demonstrated they could produce cultivars with toxin titers far greater than required to kill 100% of susceptible individuals (Perlak et al. 1991, Gould 1998).

Due to technological feasibility, practicality for marketing, and ease of agronomic implementation, low dose and temporal or localized toxin expression are not utilized in current IRM strategies for corn (Gould 1998, EPA 2017). Initial PIP IRM plans utilized the high dose strategy in combination with areas of non-toxic cultivars known as the high-dose refuge strategy (HDR). HDR utilizes two concepts. First, that plants express a high enough dose of the toxin to kill >99.99% of susceptible individuals, often 25-50 times the LD_{99}. This high dose functions to make inheritance of resistance functionally recessive even if it is not phenotypically recessive by generating similar mortality in homozygous susceptible (SS) and heterozygous genotypes (RS). Only homozygous resistant (RR) individuals would be the source of resistant alleles and drive the evolution of resistance (Gould 1998). Second, that a refuge area planted with plants that do not express the trait will generate enough susceptible individuals to interbreed with any possibly resistant individuals developing in the area expressing the trait. Random interbreeding between resistant and susceptible individuals generates heterozygous offspring that will be killed by the high dose toxin, therefore maintaining an extremely low frequency of resistant alleles provided almost exclusively by the homozygous resistant individuals (Gould 1998).

The use of the HDR strategy for PIPs relies on three assumptions for effective resistance management: (1) the initial resistant allele frequency must be low ($<10^{-3}$), (2)

resistance inheritance is recessive, and (3) mating between susceptible and resistant insects is random (Gould 1998). Violations of these assumptions will significantly decrease the effectiveness and durability of the IRM strategy (Campagne et al. 2016, Carrière et al. 2016). Retrospective analysis of the past 20 years also suggests that the presence of fitness costs and incomplete resistance contributes to delays in resistance evolution (Tabashnik et al. 2013, Carrière et al. 2015).

In recent years the use of pyramided products, plants expressing multiple toxins with unique modes of action targeting a single pest, has become a valuable strategy for resistance management (Tabashnik et al. 2013). Pyramids delay resistance evolution through "redundant killing", in which insects resistant to one toxin will die after exposure to the second toxin, and totally susceptible insects effectively "die twice" (Gould 1998, Carrière et al. 2016). Pyramided PIPs with unique modes of action would require resistance alleles at two independent gene loci for the insect to evolve resistance to the pyramid. If resistance is recessively inherited, only one of the nine potential genotypes results in double homozygote resistant insects. These double homozygotes are expected to be extremely rare (10^{-12}) at the initial PIP commercialization, therefore enhancing product durability. (Gould 1998, Tabashnik et al. 2013, Carriere et al. 2015). The benefits of pyramids are reduced significantly if constituent toxins are deployed concurrently as single toxin cultivars, resistance to one of the pyramided toxins is present, or toxins exhibit cross-resistance (Carrière et al. 2016).

Current resistance evolution has renewed emphasis on incorporating integrated pest management (IPM) strategies into IRM plans to mitigate the spread of field evolved resistance and minimize resistance risk. IPM is the development of a pest management

protocol that incorporates multiple, unique control tactics to maintain pest populations below economically significant levels while limiting negative perturbations to the remainder of the system (Stern et al. 1959, Kogan 1998). IPM aims to mitigate issues such a resistance, resurgence and secondary pests through monitoring of pest populations and only apply control tactics when populations reach economic thresholds (Stern et al. 1959, Kogan 1998). IPM combats resistance by reducing the selection pressure placed on a population through rotation of MOAs for pest control. Any pests that may be resistant to a specific control tactic should be susceptible to a tactic with a different MOA and be eliminated during the next control period when the producer rotates MOAs. Implementing control tactics with low disruption to the overall system helps to control pest populations through preservation of natural enemies for the pest that would be killed by broad-spectrum strategies such as insecticides. Integrating PIPs into IPM frameworks assists in eliminating the "silver bullet" mentality and is expected to significantly increase product durability (Martinez and Caprio 2016).

1.4.3 Refuge Configuration

The size and proximity of the refuge area is critical to ensure random mating. Refuge areas can be configured in multiple ways including seed blends, strips/rows within a field, strips around the perimeter of the field, blocks adjacent to fields, separate fields, or can be provided by naturally occurring plant species in agricultural ecosystems, known as a "natural refuge" (Onstad et al. 2018). To ensure random mating, pre-mating dispersion patterns of the target pest should be considered when selecting the appropriate refuge configurations. Separate field refuges are recommended for many Lepidopteran pests, as adults typically disperse and mate greater than a half-mile from their native field

(Bates et al. 2005, Siegfried and Hellmich 2012). Seed blends are preferred for WCR as they promote random mating for WCR as females primarily mate in their native field (Kang and Krupke 2009, Spencer et al. 2013).

Larval movement and feeding behaviors must also be considered for refuges. Corn pests are capable of interplant movement within a growing season (Ross and Ostlie 1990, Zukoff et al. 2012). In seed mixtures, non-toxic plants are randomly scattered throughout the field, creating a mosaic of toxin expression in the field and possibly in certain tissues such as corn ears due to cross-pollination. Insects may feed and develop on a non-toxic refuge plant during the early larval stages, when the insect is most susceptible to Bt, then complete development after moving to nearby Bt plants. Additionally, some insects exhibit a behavior avoidance of Bt after feeding on Bt expressing cultivars; larvae can demonstrate a decreased affinity for Bt plants and move from Bt plants to non-toxic refuge plants without ingesting sufficient toxin to generate mortality (Zukoff et al. 2012). Sublethal toxin exposure due to larval movement accelerates resistance evolution by allowing the survival of heterozygous individuals (RS) and increasing the likelihood of F1 homozygous resistant individuals (Gould 1998). Refuges as a separate field reduce the risk of larval movement between cultivars as the surrounding plants are toxic in the treated area and non-toxic in the refuge area. Cultivars that express Bt traits for multiple pests offer a unique challenge for refuge configurations as what is beneficial for one pest may compromise IRM for another pest of the same crop.

An additional concern with refuge is non-compliance or reduced compliance. Non-compliance producers do not plant adequately sized refuges, therefore reducing the

population of susceptible insects for random mating and increasing the selection pressure for resistance. To ensure grower compliance, industry has promoted the use of seed mixtures, containing both PIP and non-PIP seeds, known as integrated refuges. Integrated refuges result in 100% grower compliance and are common in the upper U.S. Corn Belt. Current integrated refuge requirements for Bt WCR PIPs are 90:10 Bt to non-Bt seed mixtures for single protein cultivars and 95:5 Bt to non-Bt seed mixtures for pyramided cultivars (US EPA 2017).

1.4.4 High Dose Refuge Strategy Success

Insect resistance management to PIPs has been particularly successful for the European corn borer (ECB), *Ostrinia nubilalis* Hübner and other insects in which one or more PIP's is a "high" dose. Since the introduction of the first PIP expressing the Cry1Ab protein in 1996, there have been no documented cases of field evolved resistance, despite the high selection pressure placed on ECB. ECB has recently evolved resistance to the Cry1F protein in Nova Scotia (Baute 2019), but no cases have been documented in the United States (Hutchison et al. 2010). This example demonstrates the ability for HDR to be a successful resistance management tool when the underlying high dose theoretical assumption is met. Additionally, this pest highlighted other factors that can potentially affect HDR success. For example, the target organism's biology and ecology can significantly affect the success of the HDR management strategy (Siegfried and Hellmich 2012). Understanding components of the organism's growth and development can help to guide recommendations for appropriate refuge size and placement. Insects that emerge and then complete a pre-mating dispersal might best be managed with a block refuge, whereas an insect that mates immediately after emergence

will benefit from a seed mixture refuge. Feeding habits can also alter the expression level required to achieve a high dose status. Given the differences in the biology between organisms, IRM plans should be designed with regard to the target insect biology and behavior. Careful deliberation should be taken when developing and recommending IRM strategies for current and future management tools (Tabashnik et al. 2013).

1.4.5 High Dose Refuge Strategy Failure

Despite the success of HDR for European corn borer, the high dose refuge strategy has failed to prevent resistance evolution due to violations in the underlying assumptions for various insect species including WCR (Gassmann et al. 2011, Tabashnik et al. 2013, Andow et al. 2016, Jakka et al. 2016). Many factors such as refuge compliance, high selection pressure, and weather events have attributed to resistance. However, a commonality to all documented resistance cases is the lack of a high dose expression of the toxin (Tabashnik and Carrière 2017). PIPs with non-high dose toxin expression do not satisfy the assumption of 99.9% mortality of heterozygous individuals allowing for rapid increases in the frequency of resistant alleles in a population. Pink bollworm (*Pectinophora gossypiella* Saunders), in India and the United States provides insight into resistance evolution when this assumption was violated. In the United States, cotton cultivars produce the Cry1Ac toxin at high dose levels and the pest has been eradicated successfully with the use of PIPs and other integrated pest management strategies (Perdue 2018). However, in India, various non-approved and F2 cultivars were used that did not express the toxin at high dose levels. Additionally, Indian farmers did not plant adequate refuge areas of Bt cotton (Stone 2004, Mohan 2018). Due to the lack of a high dose, coupled with the lack of refuge; pink bollworm resistance to Cry1Ac

occurred within six years of product introduction (Dhurua and Gujar 2011). To remediate this resistance crisis, cotton expressing both Cry1Ac and Cry2Ab was deployed in India in 2006. In 2014, monitoring indicated field-evolved resistance to pyramided cotton products expressing the Cry1Ac and Cry2Ab toxins in India (Naik et al. 2018); highlighting the importance of meeting the high dose requirement for toxin expression and the increased risk of resistance evolution for pyramided traits released sequentially.

WCR resistance to Bt traits can also be explained through violations of HDR assumptions. Expression of all the currently available Bt WCR traits is insufficient to meet the high dose requirements and field evolved resistance occurred within six years of product introduction (Meihls et al. 2008, Gassmann 2012). This is of particular importance as *DvSnf7* dsRNA is similarly not expressed at a high dose and high adoption rates are anticipated (Head et al. 2017). Characterization of resistant populations revealed that the initial frequency of resistant alleles to Cry3Bb1 was much higher than initially anticipated, therefore violating the second assumption of HDR (Onstad and Meinke 2010). Initial implementation of refuges as blocks for WCR also violated the random mating assumption because most WCR beetles mate in their emergence field (Spencer et al. 2013). These violations have resulted in resistance to multiple Bt toxins throughout the U.S. Corn Belt. Special considerations and adaptations to the HDR strategy must be taken to ensure that similar outcomes to Bt proteins do not occur with dsRNA toxins.

1.4.6 Risk of dsRNA Resistance Evolution in WCR

Similar to previous Bt PIPs, selection pressure from continuous exposure to dsRNA will eventually facilitate resistance evolution. Potential mechanisms of resistance to dsRNA are degradation of dsRNA in the gut, reduced dsRNA uptake, alteration in proteins involved in dsRNA transport or formation of the RISC complex, loss of siRNA recognition by the RISC complex, mutation of the target gene, or failure of the systemic spread of RNAi (Palli 2014, Fishilevich et al. 2016, Cooper et al. 2019). Determining which of these mechanisms confers resistance to *DvSnf7* dsRNA in WCR is crucial for establishing effective IRM strategies and ensuring product durability.

Khajuria et al. (2018) collected WCR adults emerging from areas planted with transgenic corn expressing *DvSnf7* dsRNA. Field collected beetles were crossed with a non-diapausing WCR colony and exposed to *DvSnf7* dsRNA for 11 generations, generating a population with ≥ 130-fold resistant to *DvSnf7* dsRNA (Khajuria et al. 2018). *DvSnf7* resistance resulted from reduced uptake of dsRNA in gut cells. Cross-resistance to dsRNAs targeting *vATPase A*, *COPI β* (Coatomer Subunit beta), and *Mov34* (26s proteasome) suggest that resistance is not dsRNA sequence-specific, therefore dsRNA represents a single unique mode of action for WCR (Khajuria et al. 2018). *DvSnf7* dsRNA resistance was determined to be recessively inherited, located on a single locus, and autosomal (Khajuria et al. 2018). This is the first dsRNA-resistant WCR colony developed, and the results from this study will be useful in optimizing IRM plans and increasing the lifetime of RNAi technologies.

1.4.7 Current Resistance Monitoring in WCR

Current resistance monitoring for PIPs in WCR is slow, time-consuming, and expensive because Bt proteins only cause mortality in the larval stage of the pest

(Gassmann et al. 2011). Briefly, the process involves: gathering reports from producers with greater than expected damage, traveling to areas of concern, collecting adult beetles, rearing the adults and collecting eggs, incubating eggs through cold diapause, hatching the eggs, and testing larvae on single plants or artificial diet to determine if there has been a shift in susceptibility to the toxin. On-plant assays require sufficient quantities of single protein seed, adequate space to grow plants, and sufficient numbers of eggs/larvae to conduct assays. Diet based assays require sufficient numbers of eggs/larvae, adequate space for diet assays, and sufficient amounts of protein for screening. This process can take six months or longer to generate susceptibly measurements (Jackson 1986, Siegfried et al. 2005, Gassmann et al. 2011). Many producers may not be able to wait this long to make management decisions for their operation and may already purchase inputs for the next growing season before resistance data is available. Long data delays also create uncertainty in effectively diagnosing resistance as is evolves, especially during initial resistance detection.

Bt resistance has been documented throughout the U.S. Corn Belt, however widespread resistance has yet to occur (Gassmann et al. 2011, 2016, Wangila et al. 2015, Andow et al. 2016, Jakka et al. 2016). Producers may avoid the implementation of a proper mitigation plan without the confirmation of resistance in their area. These long delays allow for undetected increases in resistant alleles and could exacerbates resistance evolution for non-high dose toxins (Andow et al. 2016). Decreasing the time required to obtain accurate resistance measurements will allow for more immediate mitigation implementation. This study aims to provide this reduction in time by utilizing the adult

stage of WCR for resistance monitoring to *DvSnf7* dsRNA and providing resistance information two weeks after a field collection.

1.5 Research Justification

WCR is currently the most damaging pest of corn in the U.S. Corn Belt. Resistance has been documented for crop rotation, chemical insecticides and PIPs limiting control strategies available to producers. Double-stranded RNA is the first unique MOA PIP targeting WCR since the commercialization of the Cry34/35 toxin in 2005. Insect resistance management is crucial to maintaining the efficacy of new strategies over time and mitigating current resistance issues. In order to implement a proper IRM strategy baseline susceptibility measures must be gathered for future susceptibility comparisons. Additionally, an effective and efficient monitoring procedure should be developed to assess annual susceptibility changes, if present.

Due to the high adoption rates anticipated for this technology and EPA conditions of registration, baseline data must be collected for populations throughout the Corn Belt. Currently, there is limited baseline data for populations from some of the major corn-growing areas in the U.S., including parts of Nebraska, Illinois, Colorado, and Minnesota (Moar 2017). This project establishes larval and adult baseline susceptibilities for populations from Nebraska, South Dakota, Minnesota, and Iowa. Additionally, variations in adult susceptibility due to age and sex were characterized.

1.6 Research Objectives

This thesis focuses on the following objectives and working hypotheses:

1. Develop WCR larval and adult dsRNA susceptibility bioassays for resistance monitoring.

2. Establish a larval and adult baseline susceptibility of native U.S. Corn Belt western corn rootworm populations to *DvSnf7* dsRNA and determine a mortality correlation ratio between adult and larval life stages.

   Working hypothesis: WCR populations have similar adult and larval susceptibilities to *DvSnf7* dsRNA and have equivalent correlation ratios between populations.

3. Characterize WCR adult response to *DvSnf7* dsRNA for age and sex.

   Working hypothesis: Adult susceptibility to *DvSnf7* dsRNA is higher for males than females and increase as adults age.

Literature Cited

Agrawal, N., P. V. N. Dasaradhi, A. Mohmmed, P. Malhotra, R. K. Bhatnagar, and S. K. Mukherjee. 2003. RNA interference: biology, mechanism, and applications. Microbiol. Mol. Biol. Rev. 67: 657–685.

Allen, M. L., and W. B. Walker. 2012. Saliva of *Lygus lineolaris* digests double stranded ribonucleic acids. J. Insect Physiol. 58: 391–396.

Andow, D. A., S. G. Pueppke, A. W. Schaafsma, A. J. Gassmann, T. W. Sappington, L. J. Meinke, P. D. Mitchell, T. M. Hurley, R. L. Hellmich, and R. P. Porter. 2016. Early detection and mitigation of resistance to Bt maize by western corn rootworm (Coleoptera: Chrysomelidae). J. Econ. Entomol. 109: 1–12.

Bachman, P. M., K. M. Huizinga, P. D. Jensen, G. Mueller, J. Tan, J. P. Uffman, and S. L. Levine. 2016. Ecological risk assessment for *DvSnf7* RNA: a plant-incorporated protectant with targeted activity against western corn rootworm. Regul. Toxicol. Pharmacol. 81: 77–88.

Ball, H. J. 1957. On the biology and egg-laying habits of the western corn rootworm. J. Econ. Entomol. 50: 126–128.

Bates, S. L., J.-Z. Zhao, R. T. Roush, and A. M. Shelton. 2005. Insect resistance management in GM crops: past, present and future. Nat. Biotechnol. 23: 57–62.

Baum, J. A., T. Bogaert, W. Clinton, G. R. Heck, P. Feldmann, O. Ilagan, S. Johnson, G. Plaetinck, T. Munyikwa, M. Pleau, T. Vaughn, and J. Roberts. 2007. Control of coleopteran insect pests through RNA interference. Nat. Biotechnol. 25: 1322–1326.

Baum, J. A., and J. K. Roberts. 2014. Chapter five - progress towards RNAi-mediated insect pest management, pp. 249–295. In Dhadialla, T.S., Gill, S.S. (eds.), Adv. Insect Physiol., Insect Midgut and Insecticidal Proteins. Academic Press.

Baute, T. 2019. European Corn Borer Resistance to Bt Corn Found in Canada. Field Crop News.

Bolognesi, R., P. Ramaseshadri, J. Anderson, P. Bachman, W. Clinton, R. Flannagan, O. Ilagan, C. Lawrence, S. Levine, W. Moar, G. Mueller, J. Tan, J. Uffman, E. Wiggins, G. Heck, and G. Segers. 2012. Characterizing the mechanism of action of double-stranded RNA activity against western corn rootworm (*Diabrotica virgifera virgifera* LeConte). PLOS ONE. 7: e47534.

Branson, T. F., and J. L. Krysan. 1981. Feeding and oviposition behavior and life cycle strategies of *Diabrotica* : an evolutionary view with implications for pest management. Environ. Entomol. 10: 826–831.

Bryson, H. R., D. A. Wilbur, and C. C. Burkhardt. 1953. The western corn rootworm, *Diabrotica virgifera* Lec. In Kansas. J. Econ. Entomol. 46: 995–999.

Campagne, P., P. E. Smouse, R. Pasquet, J. Silvain, B. Le Ru, and J. Van den Berg. 2016. Impact of violated high-dose refuge assumptions on evolution of Bt resistance. Evol. Appl. 9: 596–607.

Carriere, Y., N. Crickmore, and B. E. Tabashnik. 2015. Optimizing pyramided transgenic Bt crops for sustainable pest management. Nat. Biotechnol. 33: 161–168.

Carrière, Y., J. A. Fabrick, and B. E. Tabashnik. 2016. Can pyramids and seed mixtures delay resistance to Bt crops? Trends Biotechnol. 34: 291–302.

Chiang, H. C. 1973. Bionomics of the northern and western corn rootworms. Annu. Rev. Entomol. 18: 47–72.

Christiaens, O., and G. Smagghe. 2014. The challenge of RNAi-mediated control of hemipterans. Curr. Opin. Insect Sci., Pests and resistance/Parasites/Parasitoids/Biological control/Neurosciences. 6: 15–21.

Chu, C.-C., J. L. Spencer, M. J. Curzi, J. A. Zavala, and M. J. Seufferheld. 2013. Gut bacteria facilitate adaptation to crop rotation in the western corn rootworm. Proc. Natl. Acad. Sci. 110: 11917–11922.

Cooper, A. M., K. Silver, J. Zhang, Y. Park, and K. Y. Zhu. 2019. Molecular mechanisms influencing efficiency of RNA interference in insects. Pest Manag. Sci. 75: 18–28.

Culy, M., C. Richard Edwards, and J. Ronald Cornelius. 1992. Effect of silk feeding by western corn rootworm (Coleoptera: Chrysomelidae) on yield and quality of inbred corn in seed corn production fields. J. Econ. Entomol. 85: 2440–2446.

Dhurua, S., and G. T. Gujar. 2011. Field-evolved resistance to Bt toxin Cry1Ac in the pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae), from India. Pest Manag. Sci. 67: 898–903.

Dover, M. 1985. Getting off the pesticide treadmill. Technol. Rev. 53–63.

Dun, Z., P. D. Mitchell, and M. Agosti. 2010. Estimating *Diabrotica virgifera virgifera* damage functions with field trial data: applying an unbalanced nested error component model. J. Appl. Entomol. 134: 409–419.

Dunbar, M. W., and A. J. Gassmann. 2013. Abundance and distribution of western and northern corn rootworm (*Diabrotica* spp.) and prevalence of rotation resistance in eastern Iowa. J. Econ. Entomol. 106: 168–180.

Fire, A., S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver, and C. C. Mello. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature. 391: 806–811.

Fishilevich, E., A. J. Bowling, M. L. F. Frey, P.-H. Wang, W. Lo, M. Rangasamy, S. E. Worden, H. E. Pence, P. Gandra, S. L. Whitlock, G. Schulenberg, E. Knorr, L. Tenbusch, J. R. Lutz, S. Novak, R. L. Hamm, K. D. Schnelle, A. Vilcinskas, and K. E. Narva. 2019. RNAi targeting of rootworm Troponin I transcripts confers root protection in maize. Insect Biochem. Mol. Biol. 104: 20–29.

Fishilevich, E., A. M. Vélez, N. P. Storer, H. Li, A. J. Bowling, M. Rangasamy, S. E. Worden, K. E. Narva, and B. D. Siegfried. 2016. RNAi as a management tool for the western corn rootworm, *Diabrotica virgifera virgifera*. Pest Manag. Sci. 72: 1652–1663.

Flagel, L. E., R. Bansal, R. A. Kerstetter, M. Chen, M. Carroll, R. Flannagan, T. Clark, B. S. Goldman, and A. P. Michel. 2014. Western corn rootworm (*Diabrotica virgifera virgifera*) transcriptome assembly and genomic analysis of population structure. BMC Genomics. 15: 195.

Garbutt, J. S., X. Bellés, E. H. Richards, and S. E. Reynolds. 2013. Persistence of double-stranded RNA in insect hemolymph as a potential determiner of RNA interference success: Evidence from *Manduca sexta* and *Blattella germanica*. J. Insect Physiol., Immune Interactions Between Insects and Their Natural Antagonists: a Workshop Honoring Professor Stuart E. Reynolds. 59: 171–178.

Gassmann, A. J. 2012. Field-evolved resistance to Bt maize by western corn rootworm: Predictions from the laboratory and effects in the field. J. Invertebr. Pathol., SIP Symposium on Resistance to Bt Crops. 110: 287–293.

Gassmann, A. J., J. L. Petzold-Maxwell, R. S. Keweshan, and M. W. Dunbar. 2011. Field-evolved resistance to Bt maize by western corn rootworm. PLOS ONE. 6: e22629.

Gillette, C. P. 1912. *Diabrotica virgifera* LeConte as a corn rootworm. J. Econ. Entomol. 54: 364–366.

Gould, F. 1998. Sustainability of transgenic insecticidal cultivars: integrating pest genetics and ecology. Annu. Rev. Entomol. 43: 701–726.

Gray, M. E., G. L. Hein, M. A. Boetel, and D. D. Walgenbach. 1992. Western and northern corn rootworm (Coleoptera: Chrysomelidae) egg densities at three soil depths: implications for future ecological studies. J. Kans. Entomol. Soc. 65: 354–356.

Gray, M. E., T. W. Sappington, N. J. Miller, J. Moeser, and M. O. Bohn. 2009. Adaptation and invasiveness of western corn rootworm: intensifying research on a worsening pest. Annu. Rev. Entomol. 54: 303–321.

Gray, M. E., and K. L. Steffey. 1998. Corn rootworm (Coleoptera: Chrysomelidae) larval injury and root compensation of 12 maize hybrids: an assessment of the economic injury index. J. Econ. Entomol. 91: 723–740.

Head, G. P., M. W. Carroll, S. P. Evans, D. M. Rule, A. R. Willse, T. L. Clark, N. P. Storer, R. D. Flannagan, L. W. Samuel, and L. J. Meinke. 2017. Evaluation of SmartStax and SmartStax PRO maize against western corn rootworm and northern corn rootworm: efficacy and resistance management. Pest Manag. Sci. 73: 1883–1899.

Hu, X., N. M. Richtman, J.-Z. Zhao, K. E. Duncan, X. Niu, L. A. Procyk, M. A. Oneal, B. M. Kernodle, J. P. Steimel, V. C. Crane, G. Sandahl, J. L. Ritland, R. J. Howard, J. K. Presnail, A. L. Lu, and G. Wu. 2016. Discovery of midgut genes for the RNA interference control of corn rootworm. Sci. Rep. 6: 30542.

Hutchison, W. D., E. C. Burkness, P. D. Mitchell, R. D. Moon, T. W. Leslie, S. J. Fleischer, M. Abrahamson, K. L. Hamilton, K. L. Steffey, M. E. Gray, R. L. Hellmich, L. V. Kaster, T. E. Hunt, R. J. Wright, K. Pecinovsky, T. L. Rabaey, B. R. Flood, and E. S. Raun. 2010. Areawide suppression of european corn borer with Bt maize reaps savings to non-Bt maize growers. Science. 330: 222–225.

IRAC. 2019. Resistance. IRAC. (https://www.irac-online.org/about/resistance/).

Jackson, J. J. 1986. Rearing and handling of *Diabrotica virgifera* and *Diabrotica undecimpunctata howardi*, pp. 25–47. In Methods Study Pest Diabrotica, Springer Series in Experimental Entomology. Springer, New York, NY.

Jakka, S. R. K., R. B. Shrestha, and A. J. Gassmann. 2016. Broad-spectrum resistance to *Bacillus thuringiensis* toxins by western corn rootworm (*Diabrotica virgifera virgifera*). Sci. Rep. 6: 27860.

Kang, J., and C. H. Krupke. 2009. Likelihood of multiple mating in *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae). J. Econ. Entomol. 102: 2096–2100.

Khajuria, C., S. Ivashuta, E. Wiggins, L. Flagel, W. Moar, M. Pleau, K. Miller, Y. Zhang, P. Ramaseshadri, C. Jiang, T. Hodge, P. Jensen, M. Chen, A. Gowda, B. McNulty, C. Vazquez, R. Bolognesi, J. Haas, G. Head, and T. Clark. 2018. Development and characterization of the first dsRNA-resistant insect population from western corn rootworm, *Diabrotica virgifera virgifera* LeConte. PLOS ONE. 13: e0197059.

Khajuria, C., A. M. Vélez, M. Rangasamy, H. Wang, E. Fishilevich, M. L. F. Frey, N. P. Carneiro, P. Gandra, K. E. Narva, and B. D. Siegfried. 2015. Parental RNA interference of genes involved in embryonic development of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte. Insect Biochem. Mol. Biol. 63: 54–62.

Knight, A. L., and G. W. Norton. 1989. Economics of agricultural pesticide resistance in arthropods. Annu. Rev. Entomol. 34: 293–313.

Knolhoff, L. M., D. W. Onstad, J. L. Spencer, and E. Levine. 2006. Behavioral differences between rotation-resistant and wild-type *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae). Environ. Entomol. 35: 1049–1057.

Kogan, M. 1998. Integrated pest management: historical perspectives and contemporary developments. Annu. Rev. Entomol. 43: 243–270.

Krysan, J. L. 1982. Diapause in the nearctic species of the *virgifera* group of *Diabrotica*: evidence for tropical origin and temperate adaptations. Ann. Entomol. Soc. Am. 75: 136–142.

Krysan, J. L., and R. F. Smith. 1987. Systematics of the *virgifera* species group of *Diabrotica* (Coleoptera: Chrysomelidae: Galerucinae). Entomography. 5: 375–484.

Levine, E., and H. Oloumi-Sadeghi. 1991. Management of Diabroticite rootworms in corn. Annu. Rev. Entomol. 36: 229–255.

Levine, E., and H. Oloumi-Sadeghi. 1996. Western corn rootworm (Coleoptera: Chrysomelidae) larval injury to corn grown for seed production following soybeans grown for seed production. J. Econ. Entomol. 89: 1010–1016.

Levine, E., J. L. Spencer, S. A. Isard, D. W. Onstad, and M. E. Gray. 2002. Adaptation of the western corn rootworm to crop rotation: evolution of a new strain in response to a management practice. Am. Entomol. 48: 94–107.

Levine, S. L., J. Tan, G. M. Mueller, P. M. Bachman, P. D. Jensen, and J. P. Uffman. 2015. Independent action between *DvSnf7* RNA and Cry3Bb1 protein in southern corn rootworm, *Diabrotica undecimpunctata howardi* and colorado potato beetle, *Leptinotarsa decemlineata*. PLOS ONE. 10: e0118622.

Luo, Y., X. Wang, X. Wang, D. Yu, B. Chen, and L. Kang. 2013. Differential responses of migratory locusts to systemic RNA interference via double-stranded RNA injection and feeding. Insect Mol. Biol. 22: 574–583.

Martinez, J. C., and M. A. Caprio. 2016. IPM use with the deployment of a non-high dose Bt pyramid and mitigation of resistance for western corn rootworm (*Diabrotica virgifera virgifera*). Environ. Entomol. 45: 747–761.

Meihls, L. N., M. L. Higdon, B. D. Siegfried, N. J. Miller, T. W. Sappington, M. R. Ellersieck, T. A. Spencer, and B. E. Hibbard. 2008. Increased survival of western corn rootworm on transgenic corn within three generations of on-plant greenhouse selection. Proc. Natl. Acad. Sci. 105: 19177–19182.

Meinke, L. J. 2014. Corn rootworm management in the transgenic era: adult corn rootworm suppression. (http://plantmanagementnetwork.org/edcenter/seminars/corn/AdultRootworm/presentation.html) (accessed 10 September 2019).

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

Meinke, L. J., T. W. Sappington, D. W. Onstad, T. Guillemaud, N. J. Miller, J. Komáromi, N. Levay, L. Furlan, J. Kiss, and F. Toth. 2009. Western corn rootworm (*Diabrotica virgifera virgifera* LeConte) population dynamics. Agric. For. Entomol. 11: 29–46.

Meister, G., and T. Tuschl. 2004. Mechanisms of gene silencing by double-stranded RNA. Nature. 431: 343.

Metcalf, R. L. 1983. Implications and prognosis of resistance to insecticides, pp. 703–733. In Pest Resist. Pestic. Springer, Boston, MA.

Metcalf, R. L. 1986. Methods for the study of pest *Diabrotica*, Springer Series in Experimental Entomology. Springer-Verlag, New York.

Miyata, K., P. Ramaseshadri, Y. Zhang, G. Segers, R. Bolognesi, and Y. Tomoyasu. 2014. Establishing an *in vivo* assay system to identify components involved in environmental RNA interference in the western corn rootworm. PLOS ONE. 9: e101661.

Moar, W., C. Khajuria, M. Pleau, O. Ilagan, M. Chen, C. Jiang, P. Price, B. McNulty, T. Clark, and G. Head. 2017. Cry3Bb1-resistant western corn rootworm, *Diabrotica virgifera virgifera* (LeConte) does not exhibit cross-resistance to *DvSnf7* dsRNA. PLOS ONE. 12: e0169175.

Mohan, K. S. 2018. Refuge-in-bag for Bt cotton. Curr Sci. 726–727.

Murphy, A. F., S. I. Rondon, C. H. Wohleb, and S. Hines. 2014. Western corn rootworm in eastern Oregon, Idaho, and eastern Washington.

Naik, V. C., S. Kumbhare, S. Kranthi, U. Satija, and K. R. Kranthi. 2018. Field-evolved resistance of pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae), to transgenic *Bacillus thuringiensis* (Bt) cotton expressing crystal 1Ac (Cry1Ac) and Cry2Ab in India. Pest Manag. Sci. 74: 2544–2554.

Niu, J., C. N. T. Taning, O. Christiaens, G. Smagghe, and J.-J. Wang. 2018. Chapter one - rethink RNAi in insect pest control: challenges and perspectives, pp. 1–17. In Smagghe, G. (ed.), Adv. Insect Physiol., Crop Protection. Academic Press.

Niu, X., A. Kassa, X. Hu, J. Robeson, M. McMahon, N. M. Richtman, J. P. Steimel, B. M. Kernodle, V. C. Crane, G. Sandahl, J. L. Ritland, J. K. Presnail, A. L. Lu, and G. Wu. 2017. Control of western corn rootworm (*Diabrotica virgifera virgifera*) reproduction through plant-mediated RNA Interference. Sci. Rep. 7: 12591.

Onstad, D. W., A. L. B. Crespo, Z. Pan, P. R. Crain, S. D. Thompson, C. D. Pilcher, and A. Sethi. 2018. Blended refuge and insect resistance management for insecticidal corn. Environ. Entomol. 47: 210–219.

Palli, S. R. 2014. RNA interference in colorado potato beetle: steps toward development of dsRNA as a commercial insecticide. Curr. Opin. Insect Sci., Pests and resistance/Parasites/Parasitoids/Biological control/Neurosciences. 6: 1–8.

Perdue, S. 2018. Eradication of pink bollworm.

Pereira, A. E., N. P. Carneiro, and B. D. Siegfried. 2016. Comparative susceptibility of southern and western corn rootworm adults and larvae to *vATPase-A* and *Snf7* dsRNAs. J. RNA Genomics. 12: 528–535.

Pereira, A. E., H. Wang, S. N. Zukoff, L. J. Meinke, B. W. French, and B. D. Siegfried. 2015. Evidence of field-evolved resistance to bifenthrin in western corn rootworm (*Diabrotica virgifera virgifera* LeConte) populations in western Nebraska and Kansas. PLOS ONE. 10: e0142299.

Perlak, F. J., R. L. Fuchs, D. A. Dean, S. L. McPherson, and D. A. Fischhoff. 1991. Modification of the coding sequence enhances plant expression of insect control protein genes. Proc. Natl. Acad. Sci. 88: 3324–3328.

Pinheiro, D. H., A. M. Vélez, E. Fishilevich, H. Wang, N. P. Carneiro, A. Valencia-Jiménez, F. H. Valicente, K. E. Narva, and B. D. Siegfried. 2018. Clathrin-dependent endocytosis is associated with RNAi response in the western corn rootworm, *Diabrotica virgifera virgifera* LeConte. PLoS ONE. 13.

Ramaseshadri, P., G. Segers, R. Flannagan, E. Wiggins, W. Clinton, O. Ilagan, B. McNulty, T. Clark, and R. Bolognesi. 2013. Physiological and cellular responses caused by RNAi- mediated suppression of *Snf7* Orthologue in western corn rootworm (*Diabrotica virgifera virgifera*) Larvae. PLOS ONE. 8: e54270.

Rangasamy, M., and B. D. Siegfried. 2012. Validation of RNA interference in western corn rootworm *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) adults. Pest Manag. Sci. 68: 587–591.

Ross, S. E., and K. R. Ostlie. 1990. Dispersal and survival of early instars of european corn borer (Lepidoptera: Pyralidae) in Field Corn. J. Econ. Entomol. 83: 831–836.

Saleh, M.-C., R. P. van Rij, A. Hekele, A. Gillis, E. Foley, P. H. O'Farrell, and R. Andino. 2006. The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing. Nat. Cell Biol. 8: 793.

Schuh, A. L., and A. Audhya. 2014. The ESCRT machinery: From the plasma membrane to endosomes and back again. Crit. Rev. Biochem. Mol. Biol. 49: 242–261.

Shukla, J. N., M. Kalsi, A. Sethi, K. E. Narva, E. Fishilevich, S. Singh, K. Mogilicherla, and S. R. Palli. 2016. Reduced stability and intracellular transport of dsRNA contribute to poor RNAi response in lepidopteran insects. RNA Biol. 13: 656–669.

Siegfried, B. D., and R. L. Hellmich. 2012. Understanding successful resistance management. GM Crops Food. 3: 184–193.

Siegfried, B. D., T. T. Vaughn, and T. Spencer. 2005. Baseline susceptibility of western corn rootworm (Coleoptera: Crysomelidae) to Cry3Bb1 *Bacillus thuringiensis* Toxin. J. Econ. Entomol. 98: 1320–1324.

Spencer, J. L., B. E. Hibbard, J. Moeser, and D. W. Onstad. 2009. Behaviour and ecology of the western corn rootworm (*Diabrotica virgifera virgifera* LeConte). Agric. For. Entomol. 11: 9–27.

Spencer, J., D. Onstad, C. Krupke, S. Hughson, Z. Pan, B. Stanley, and L. Flexner. 2013. Isolated females and limited males: evolution of insect resistance in structured landscapes. Entomol. Exp. Appl. 146: 38–49.

Stern, V. M., R. F. Smith, and K. S. Hagen. 1959. The integration of chemical and biological control of the spotted alfalfa aphid; the integrated control concept. Higardia. 81–101.

Stone, G. D. 2004. Biotechnology and the Political Ecology of Information in India. Hum. Organ. 63: 127–140.

Tabashnik, B. E., T. Brévault, and Y. Carrière. 2013. Insect resistance to Bt crops: lessons from the first billion acres. Nat. Biotechnol. 31: 510–521.

Tabashnik, B. E., and Y. Carrière. 2017. Surge in insect resistance to transgenic crops and prospects for sustainability. Nat. Biotechnol. 35: 926–935.

Tabashnik, B. E., F. Gould, and Y. Carrière. 2004. Delaying evolution of insect resistance to transgenic crops by decreasing dominance and heritability. J. Evol. Biol. 17: 904–912.

Tinsley, N. A., R. E. Estes, and M. E. Gray. 2013. Validation of a nested error component model to estimate damage caused by corn rootworm larvae. J. Appl. Entomol. 137: 161–169.

Urías-López, M. A., and L. J. Meinke. 2001. Influence of western corn rootworm (Coleoptera: Chrysomelidae) larval injury on yield of different types of maize. J. Econ. Entomol. 94: 106–111.

US EPA, O. 2013. Summary of the federal insecticide, fungicide, and rodenticide act. US EPA. (https://www.epa.gov/laws-regulations/summary-federal-insecticide-fungicide-and-rodenticide-act).

US EPA, O. 2015a. Framework to delay corn rootworm resistance. US EPA. (https://www.epa.gov/regulation-biotechnology-under-tsca-and-fifra/framework-delay-corn-rootworm-resistance).

US EPA, O. 2015b. Current and previously registered section 3 plant-incorporated protectant (PIP) registrations. US EPA. (https://www.epa.gov/ingredients-used-pesticide-products/current-and-previously-registered-section-3-plant-incorporated).

US EPA, O. 2017. EPA registers innovative tool to control corn rootworm. US EPA. (https://www.epa.gov/pesticide-registration/epa-registers-innovative-tool-control-corn-rootworm).

Vélez, A. M., C. Khajuria, H. Wang, K. E. Narva, and B. D. Siegfried. 2016. Knockdown of RNA interference pathway genes in western corn rootworms (*Diabrotica virgifera virgifera* Le Conte) demonstrates a possible mechanism of resistance to lethal dsRNA. PLOS ONE. 11: e0157520.

Wangila, D. S., A. J. Gassmann, J. L. Petzold-Maxwell, B. W. French, and L. J. Meinke. 2015. Susceptibility of Nebraska western corn rootworm (Coleoptera: Chrysomelidae) populations to Bt corn events. J. Econ. Entomol. 108: 742–751.

Weiss, M. J., Z. B. Mayo, and J. P. Newton. 1983. Influence of irrigation practices on the spatial distribution of corn rootworm (Coleoptera: Chrysomelidae) eggs in the soil. Environ. Entomol. 12: 1293–1295.

Whangbo, J. S., and C. P. Hunter. 2008. Environmental RNA interference. Trends Genet. 24: 297–305.

Wu, K., C. E. Taylor, E. Fishilevich, K. E. Narva, and B. D. Siegfried. 2018. Rapid and persistent RNAi response in western corn rootworm adults. Pestic. Biochem. Physiol. 150: 66–70.

Wynant, N., D. Santos, and J. Vanden Broeck. 2014. Chapter five - biological mechanisms determining the success of RNA interference in insects, pp. 139–167. In Jeon, K.W. (ed.), Int. Rev. Cell Mol. Biol. Academic Press.

Yoon, J.-S., D. Gurusamy, and S. R. Palli. 2017. Accumulation of dsRNA in endosomes contributes to inefficient RNA interference in the fall armyworm, Spodoptera frugiperda. Insect Biochem. Mol. Biol. 90: 53–60.

Zhang, J., S. A. Khan, D. G. Heckel, and R. Bock. 2017. Next-generation insect-resistant plants: RNAi-mediated crop protection. Trends Biotechnol. 35: 871–882.

Zukoff, S. N., W. C. Bailey, M. R. Ellersieck, and B. E. Hibbard. 2012. Western corn rootworm larval movement in SmartStax seed blend scenarios. J. Econ. Entomol. 105: 1248–1260.

CHAPTER 2: CHARACTERIZATION OF WESTERN CORN ROOTWORM ADULT SUSCEPTIBILITY TO *DVSNF7* DOUBLE STRANDED RNA FOR AGE AND SEX

2.1 Introduction

The western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte, is a significant agricultural pest in the United States Corn Belt costing producers over $1 billion annually in yield losses and management costs (Dun et al. 2010). Larvae feed on corn roots, which reduces nutrient and water uptake (Gray and Steffey 1998, Urías-López and Meinke 2001, Tinsley et al. 2013). One node of root injury can reduce yield by up to 15% (Dun et al. 2010, Tinsley et al. 2013). Additionally, plant lodging due to heavy root feeding can make mechanized harvest ineffective. Adult feeding on corn silks can reduce pollination, further reducing yield (Branson and Krysan 1981, Levine and Oloumi-Sadeghi 1991). Producers utilize multiple tactics to control WCR; primarily crop rotation, chemical insecticides, and plant-incorporated protectants. Repeated use of the same control strategy allowed the WCR to evolve resistance to all of these strategies (Ball and Weekman 1962b, Meinke et al. 1998, Levine et al. 2002, Gassmann et al. 2011, Pereira et al. 2015), highlighting the need for novel modes of action for this pest and proper mitigation of current and future resistance issues.

Double-stranded RNA (dsRNA) is a novel mode of action for the control of WCR (Baum et al. 2007). In 2017, the first RNA insect control product was approved for sale in the U.S. (US EPA 2017). SmartStax PRO® (SSP) hybrids contain the MON 87411 transformation event that encodes for two Bt proteins (Cry3Bb1 and Cry34/35Ab1) and a

DvSnf7 RNA, making it the first product available with three unique modes of action for control of WCR. DsRNA is anticipated to be released as a plant-incorporated protectant (PIP), therefore annual resistance monitoring must be conducted (EPA 2013). DsRNA generates mortality in the adult WCR (Rangasamy and Siegfried 2012, Pereira et al. 2016), potentially allowing adult susceptibility to dsRNA to be used as an indicator for resistance evolution of WCR to dsRNA.

Insect susceptibility to insecticides often varies between adult ages and sexes (Rathman et al. 1992, Bouvier et al. 2002, Mbepera et al. 2017). Previous experiments demonstrated that female *Diglyphus begini* (Ashmead) were more tolerant than males to methomyl, oxamyl, fenvalerate, and permethrin (Rathman et al. 1992). Other experiments have also demonstrated increased tolerance in females (Abdelrahmen 1973, Scott & Rutz 1988), with size being a significant factor for increased tolerance. Variations in susceptibility for different aged insects have also been documented (Bouvier et al. 2002, Erasmus et al. 2016, Mbepera et al. 2017). Typically, as immature insects age, tolerance to a toxin may increase. The codling moth, *Cydia pomonella*, was found to be most resistant to teflubenzuron in the late instars (Bouvier 2002). Additionally, the level of larval survival of the maize stalk borer, *Busseola fusca,* when introduced to Bt plant tissue was increased as larvae became older (Erasmus et al. 2016). Conversely, as adult insects age, tolerance to a toxin may decrease. As adult *Anopheles arabiensis* age increased, susceptibility to lambda-cyhalothrin also increased (Mbepera et al. 2017).

Although susceptibility variations are well documented, these experiments were conducted with chemical insecticides and Bt PIPs. Adult susceptibility differences for

dsRNA technologies have yet to be evaluated in WCR. If beetles with different age and sex characteristics have different susceptibilities, sexing and age synchronizing of field populations must occur for accurate susceptibility monitoring. To assess the potential variation in adult WCR susceptibility to *DvSnf7* dsRNA, adult WCR of different ages and sexes were subjected to a concentration-response bioassay to generate lethal concentration (LC) values. These data will assess the potential for utilizing adult WCR for resistance monitoring to dsRNA.

2.2 Materials and Methods

2.2.1 *DvSnf7* dsRNA

Bayer CropScience synthesized *DvSnf7* RNA for this experiment according to Urquhart et al (2015) (Bayer CropScience, Chesterfield, MO, USA). This 968-mer *DvSnf7* RNA is produced in MON 87411 containing hybrids and contains a 240-mer dsRNA region and a 488-nucleotide single-strand RNA region including a hairpin and 3' and 5' untranslated regions. Cellular uptake of a 240-mer dsRNA occurs after degradation of the 488-nucleotide single strand region in the insect gut (Urquhart et al 2015).

2.2.2 Adult Rearing

Adult WCR were purchased from Crop Characteristics, Inc. one day post-emergence (Farmington, MN). Insects were maintained in 28cm x 28cm x 28cm plexiglass cages and fed milk stage sweet corn ear tissue (kernel, cob, and silks). Sweet corn was replaced every 3-4 days and beetles were moved weekly to new cages to maintain optimal health. Subsets of the population were removed, separated by sex, and then subjected to the bioassay 2-days, 10-days, 20-days, and 30-days post-emergence.

2.2.3 Adult Bioassay

Once the beetles reached the target age, ten beetles per treatment were placed into a 70 mL plastic container (Baby Yummy, MJSteps, Zurich, Switzerland). An 8 mm diameter artificial diet plug, produced according to Khajuria et al. (2015), was placed in each container. Briefly, the diet was made by microwaving water and agar (3% w/v) then adding a diet mix (48% w/v), glycerol (5.6% v/v) and a mold inhibitor (0.22% v/v). The diet mix consisted of soy flour, milled wheat germ, casein, alphacel (fiber), fructose, brewer's yeast, vitamix, Wesson salt mix, and cholesterol. A detailed recipe for the diet can be found **Appendix 1**. The diet was mixed thoroughly then poured into Petri dishes and allowed to cool to room temperature. Diet pellets were surface treated with 10 µl of *DvSnf7* RNA solution (Bayer CropScience, Chesterfield, MO, USA) with the corresponding concentrations of 0 ng/cm^2, 18.75 ng/cm^2, 37.5 ng/cm^2, 75 ng/cm^2, 150 ng/cm^2, 300 ng/cm^2, 600 ng/cm^2, and 3600 ng/cm^2. Dilutions of *DvSnf7* RNA solution were completed using RNAse free microcentrifuge tubes and UltraPure™ Distilled Water (Invitrogen, Waltham, Massachusetts, USA). Dilutions were stored at -20°C between treatments. Beetles were transferred every other day to new containers with fresh, treated diet for five transfers. Beetles were then transferred to new containers with fresh, untreated diet for two additional transfers for a total of 14 days. Mortality was recorded daily for the 14 days. Plates were held in a growth chamber at 25 ±1 °C, relative humidity >80%, and 12:12 L:D photoperiod.

2.2.4 Data Analysis

All bioassays were analyzed with a probit regression (Finney 1971) using PoloPlus-PC software (LeOra Software LCC 1987) to generate LC_{50} values with their

corresponding 95% confidence intervals (CIs), regression slopes, and Pearson goodness-of-fit chi-square values ($\chi 2$). A ratio test between population LC_{50} values was used to determine statistical susceptibility differences between populations (Robertson et al. 2007).

2.3 Results

DvSnf7 RNA LC_{50} values estimated for adult WCR ranged from 100.9 ng/cm^2 (Male, 20-day) to 623.8 ng/cm^2 (Female, 10-day) (**Table 2.1**). Overall, females were 2.0-fold less susceptible than males when analyzed across all ages (**Figure 2.1)**. When comparing the susceptibilities of both sexes at different ages, WCR susceptibility was significantly higher for the 30-day age group than any other age group (**Figure 2.2**).

Male and female susceptibilities were not significantly different from each other for the 2-day and 30-day ages. However, susceptibilities between the sexes were significantly different. for the 10-day and 20-day ages with females being more tolerant to dsRNA than males (**Figure 2.3**). Females were 3.8 and 5.3-fold more tolerant than their male counterparts at the 10-day and 20-day age groups, respectively. Female WCR susceptibility was lowest in the 10-day age group, which was 4.1-fold more tolerant than the most susceptible age group of 30-days. Male WCR susceptibility was lowest in the 2-day age group, which was 3.7-fold more tolerant than the most susceptible age group of 20-days. (**Table 2.2**). Male susceptibility was not significantly different at the 10-, 20- and 30-day ages, while female susceptibility was not significantly different at the 2-, 10- and 20-day ages (**Figure 2.3**).

2.4 Discussion

The results of this study showed that there were significant differences in the susceptibility to *DvSnf7* RNA between sexes and age groups within the same sex in WCR adults. This is the first report demonstrating that the response to dsRNA can vary between insect ages and sexes in adult insects. Previous studies have observed differences between life stages (i.e., nymphs/larvae and adults), but not between sexes (Guo et al. 2015, Pereira 2016). Differences in susceptibilities between sexes could be attributed to physiological differences between males and females, size differences between sexes, or egg production in females. Guo et al. (2015) documented differential RNAi activity between larval stages of *Leptinotarsa decemlineata* potential resulting from differential resting expression levels of core RNAi genes between larval stages. Differential expression levels between male and female may be contributing to susceptibility variations.

Adult WCR males emerge and become sexually mature within the first 5-7 days (Guss 1976). After sexual maturation, males progress through limited physiological changes; therefore, similar susceptibilities may be expected. Female WCR emerges sexually mature (Hammack 1995), but multiple physiological changes occur after mating lasting through egg production. Approximately 6-10 days post-mating, female beetles become gravid and are noticeably larger than male beetles of the same age. Larger insects might require higher amounts of dsRNA to generate mortality. A study performed in WCR larvae showed the spread of the RNAi response by detecting a reduction of mRNA molecules in gut and fat body using microscopy, evidencing a systemic RNAi response. However, secondary siRNA production was not detected, suggesting that siRNA production is restricted to the processing of the initial dose of

dsRNA (Li et al. 2018). Therefore, if rootworms are transporting dsRNA/siRNA to cells around the organism to generate a systemic RNAi response instead of creating additional secondary small interfering RNAs (siRNA), larger females will require a higher concentration of *DvSnf7* RNA.

Furthermore, gravid females are likely investing most of their energy into producing viable eggs (Schwenke et al. 2016), which may shift resources from other organismal functions, such as the immune function, potentially reducing the RNAi response (Vogel et al. 2019). Adult WCR females export RNAs into eggs as they develop and experiments treating adults *via* orally ingested dsRNA, reduced transcript levels in eggs collected from treated adults (Vélez et al. 2016b). The knockdown of egg RNAs may be due to the systemic RNAi response from the mother being communicated to the eggs or might be due to egg exposure to dsRNAs received from RNA imports from the mother. If adult females are sending dsRNAs to eggs, more dsRNA may be required to generate a response in the females. Females in this study had an ovipositional period for about 30 days, and at this point, reached a post-reproductive state. These beetles may no longer possess the characteristics that caused the decrease in susceptibility (i.e., larger size and egg production) and therefore responded similarly to males for the 30-day age after *DvSnf7* RNA exposure. Further studies aiming to identify the cause of the reduced susceptibility in gravid females will help to better understand differences in susceptibility between different ages and sexes of WCR adults. Experiments testing size sorted beetles, unmated females, or females mated with sterilized males may all provide insights into what characteristics are affecting female susceptibility.

Based on the results reported in this study, monitoring adult field populations for resistance to dsRNA is most practical with males. After reaching 10 days of age, males responded similarly to the toxin from 10-days to 30-days post-emergence, suggesting low variability in response to *DvSnf7* RNA throughout the insect lifespan. Lower variability will be preferred for monitoring programs since changes in susceptibility will suggest shifts in the population response. Bioassays conducted with male beetles will have the least variability and require lower amounts of RNA to screen compared to females. RNA is relatively expensive to manufacture and using less will reduce the resources needed for resistance monitoring.

Furthermore, the results obtained in this study may impact future dsRNA control tactics. If the tactic is designed to control adults, alterations in concentration or application timing may be needed to achieve the desired level of control. The biology and reproductive habits of each targeted insect must be considered; however, some general conclusions can be suggested. Increased concentrations may be required if females are of more concern than males. Application schedules may also be shifted to apply dsRNA during the time when insects are most susceptible to dsRNA or to ensure that adequate levels of dsRNA are applied to insects based on their age. Additional research should be completed to determine which factors affect dsRNA susceptibility and if differences between sexes exist for other insects.

2.5 References Cited

Abdelrahmen, I. 1973. Toxicity of malathion to the natural enemies of California red scale, Aonidiella aurantii (Mask.). Aust. J. Agric. Res. 24: 119-133.

Ball, H. J., and G. T. Weekman. 1962. Insecticide resistance in the adult western corn rootworm in Nebraska. J. Econ. Entomol. 55: 439–441.

Baum, J. A., T. Bogaert, W. Clinton, G. R. Heck, P. Feldmann, O. Ilagan, S. Johnson, G. Plaetinck, T. Munyikwa, M. Pleau, T. Vaughn, and J. Roberts. 2007. Control of coleopteran insect pests through RNA interference. Nat. Biotechnol. 25: 1322–1326.

Bouvier, J.-C., T. Boivin, D. Beslay, and B. Sauphanor. 2002. Age-dependent response to insecticides and enzymatic variation in susceptible and resistant codling moth larvae. Arch. Insect Biochem. Physiol. 51: 55–66.

Branson, T. F., and J. L. Krysan. 1981. Feeding and oviposition behavior and life cycle strategies of *Diabrotica*: An evolutionary view with implications for pest management. Environ. Entomol. 10: 826–831.

Dun, Z., P. D. Mitchell, and M. Agosti. 2010. Estimating *Diabrotica virgifera virgifera* damage functions with field trial data: applying an unbalanced nested error component model. J. Appl. Entomol. 134: 409–419.

Erasmus, A., J. Marais, and J. Van den Berg. 2016. Movement and survival of *Busseola fusca* (Lepidoptera: Noctuidae) larvae within maize plantings with different ratios of non- Bt and Bt seed. Pest. Manag. Sci. 72: 2287–2294.

Finney, D. J. 1971. Probit Analysis, 3rd ed. Cambridge University Press, 32 E. 57th St., New York, Ny 10022.

Gassmann, A. J. 2012. Field-evolved resistance to Bt maize by western corn rootworm: Predictions from the laboratory and effects in the field. J. Invertebr. Pathol., SIP Symposium on Resistance to Bt Crops. 110: 287–293.

Gassmann, A. J., J. L. Petzold-Maxwell, R. S. Keweshan, and M. W. Dunbar. 2011. Field-evolved resistance to Bt maize by western corn rootworm. PLOS ONE. 6: e22629.

Gray, M. E., and K. L. Steffey. 1998. Corn rootworm (Coleoptera: Chrysomelidae) larval injury and root compensation of 12 maize hybrids: an assessment of the economic injury index. J. Econ. Entomol. 91: 723–740.

Guo, W.C., Fu, K.Y., Yang, S., Li, X.X., and Li, G.Q. 2015. Instar-dependent systemic RNA interference response in *Leptinotarsa decemlineata* larvae. *Pesticide Biochemistry and Physiology* 123**,** 64-73.

Guss, P. L. 1976. The sex pheromone of the western corn rootworm (*Diabrotica virgifera*). Environ. Entomol. 5: 5.

Hammack, L. 1995. Calling behavior in female western corn rootworm beetles (Coleoptera: Chrysomelidae). Ann. Entomol. Soc. Am. 88: 562–569.

Khajuria, C., A. M. Vélez, M. Rangasamy, H. Wang, E. Fishilevich, M. L. F. Frey, N. P. Carneiro, P. Gandra, K. E. Narva, and B. D. Siegfried. 2015. Parental RNA interference of genes involved in embryonic development of the western

corn rootworm, *Diabrotica virgifera virgifera* LeConte. Insect Biochem. Mol. Biol. 63: 54–62.

LeOra Software. 1987. POLO-PC. A user's guide to probit and logit analysis. Berkeley, CA.

Levine, E., and H. Oloumi-Sadeghi. 1991. Management of Diabroticite rootworms in corn. Annu. Rev. Entomol. 36: 229–255.

Levine, E., J. L. Spencer, S. A. Isard, D. W. Onstad, and M. E. Gray. 2002. Adaptation of the western corn rootworm to crop rotation: evolution of a new strain in response to a management practice. Am. Entomol. 48: 94–107.

Li, H., A. J. Bowling, P. Gandra, M. Rangasamy, H. E. Pence, R. E. McEwan, C. Khajuria, B. D. Siegfried, and K. E. Narva. 2018. Systemic RNAi in western corn rootworm, *Diabrotica virgifera virgifera*, does not involve transitive pathways.

Mbepera, S., G. Nkwengulila, R. Peter, E. A. Mausa, A. M. Mahande, M. Coetzee, and E. J. Kweka. 2017. The influence of age on insecticide susceptibility of *Anopheles arabiensis* during dry and rainy seasons in rice irrigation schemes of Northern Tanzania. Malar. J. 16: 364.

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

Pereira, A. E., N. P. Carneiro, and B. D. Siegfried. 2016. Comparative susceptibility of southern and western corn rootworm adults and larvae to *vATPase-A* and *Snf7* dsRNAs. J. RNA Genomics. 12: 528–535.

Pereira, A. E., H. Wang, S. N. Zukoff, L. J. Meinke, B. W. French, and B. D. Siegfried. 2015. Evidence of field-evolved resistance to bifenthrin in western corn rootworm (*Diabrotica virgifera virgifera* LeConte) populations in western Nebraska and Kansas. PLOS ONE. 10: e0142299.

Rangasamy, M., and B. D. Siegfried. 2012. Validation of RNA interference in western corn rootworm *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) adults. Pest Manag. Sci. 68: 587–591.

Rathman, R. J., M. W. Johnson, J. A. Rosenheim, B. E. Tabashnik, and M. Purcell. 1992. Sexual differences in insecticide susceptibility and synergism with piperonyl butoxide in the leafminer parasitoid *Diglyphus begini* (Hymenoptera: Eulophidae). J. Econ. Entomol. 85: 15–20.

Robertson, J. L., R. M. Russell, H. K. Preisler, and N. E. Savin. 2007. Bioassays with Arthropods, Second Edition. ed. CRC Press, Boca Raton, FL.

Schwenke, R. A., B. P. Lazzaro, and M. F. Wolfner. 2016. Reproduction–immunity trade-offs in insects. Annu. Rev. Entomol. 61: 239–256.

Scott, J. G. & D. A. Rutz. 1988. Comparative toxicities of seven insecticides to house flies (Diptera: Muscidae) and Urolepis rufipes (Ashmead) (Hymenoptera: Pteromalidae). J. Econ. Entomol. 81: 804-807.

Tinsley, N. A., R. E. Estes, and M. E. Gray. 2013. Validation of a nested error component model to estimate damage caused by corn rootworm larvae. J. Appl. Entomol. 137: 161–169.

Urías-López, M. A., and L. J. Meinke. 2001. Influence of western corn rootworm (Coleoptera: Chrysomelidae) larval injury on yield of different types of maize. J. Econ. Entomol. 94: 106–111.

Urquhart, W., G. M. Mueller, S. Carleton, Z. Song, T. Perez, J. P. Uffman, P. D. Jensen, S. L. Levine, and J. Ward. 2015. A novel method of demonstrating the molecular and functional equivalence between *in vitro* and plant-produced double-stranded RNA. Regul. Toxicol. Pharmacol. 73: 607–612.

US EPA, O. 2013. Summary of the federal insecticide, fungicide, and rodenticide act. US EPA. (https://www.epa.gov/laws-regulations/summary-federal-insecticide-fungicide-and-rodenticide-act).

Vélez, A. M., E. Fishilevich, N. Matz, N. P. Storer, K. E. Narva, and B. D. Siegfried. 2016. Parameters for successful parental RNAi as an insect pest management tool in western corn rootworm, *Diabrotica virgifera virgifera*. Genes. 8.

Vogel, E., D. Santos, L. Mingels, T.-W. Verdonckt, and J. V. Broeck. 2019. RNA Interference in Insects: Protecting Beneficials and Controlling Pests. Front. Physiol. 9.

Tables and Figures

Table 2.1 LC_{50} values (±95% CI) of adult western corn rootworm males and females to *DvSnf7* dsRNA at different ages, with their respective slope and $\chi 2$. Mortality was recorded after 14 days. Only bioassays with <20% control mortality were included.

Sex	Age	N [1]	LC_{50} (95% C.I.) [2]	Slope ± SE	$\chi 2$
Male	2 Day	420	374.4 (147.9 – 633.6)	1.434 ± 0.248	1.26
	10 Day	420	164.6 (96.7 – 259.5)	1.226 ± 0.178	0.87
	20 Day	420	100.9 (46.7 – 175.9)	1.087 ± 0.182	0.87
	30 Day	420	152.3 (27.5 – 421.2))	1.070 ± 0.268	1.05
Female	2 Day	420	450.1 (278.0 – 679.1)	1.651 ± 0.195	1.60
	10 Day	420	623.8 (398.7 – 894.8)	1.209 ± 0.275	2.28
	20 Day	420	538.6 (319.6 – 921.8)	0.970 ± 0.135	0.96
	30 Day	420	155.8 (89.2 – 245.6)	1.091 ± 0.149	0.85

[1] Number of insects evaluated in the concentration response assay
[2] ng/cm^2 *DvSnf7* dsRNA

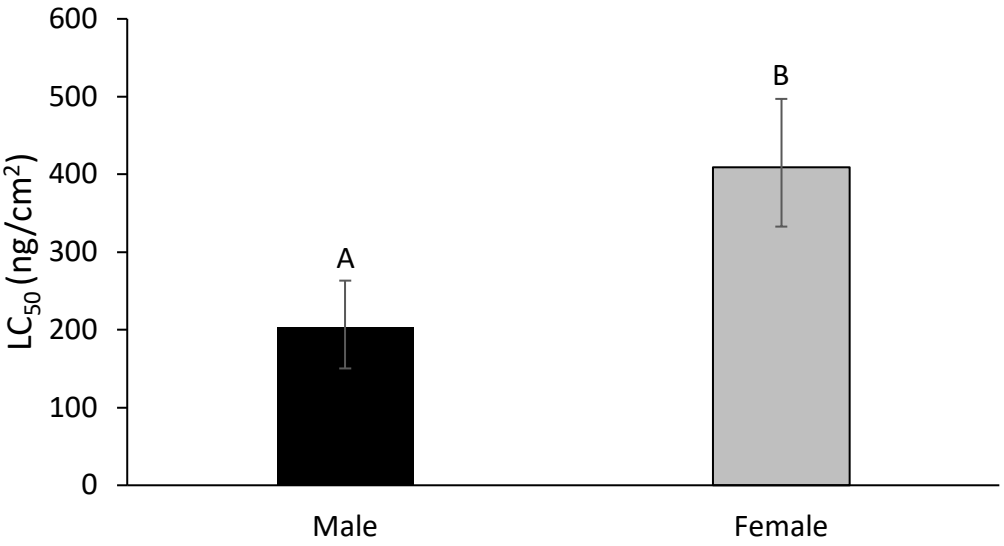

Figure 2.1 LC_{50} values (±95% CI) of male and female adult western corn rootworm fed *DvSnf7* dsRNA treated artificial diet across all age. Mortality was recorded after 14 days. Error bars represent the 95% confidence intervals for LC_{50} values. Values with different letters indicate significant differences (P=0.05).

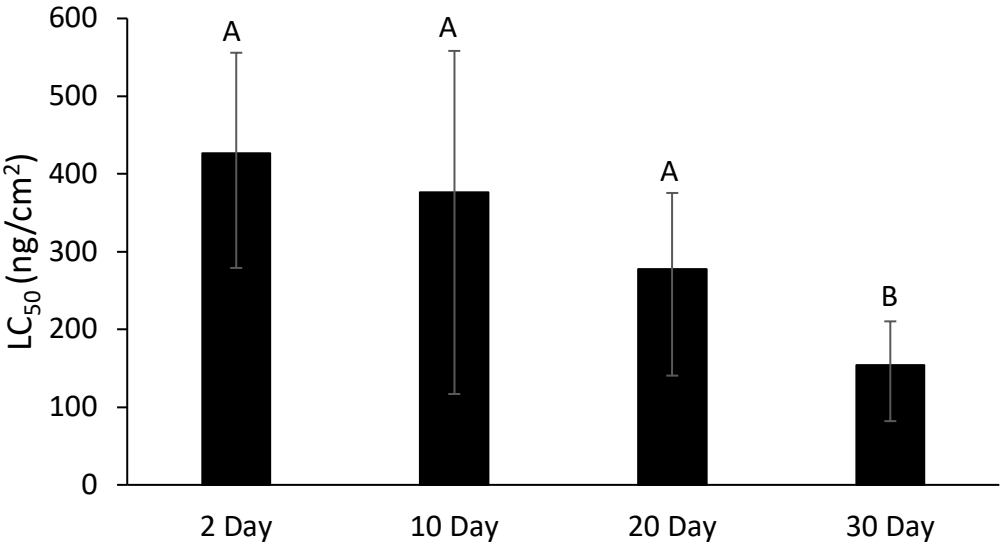

Figure 2.2 LC_{50} values (±95% CI) of mixed sex adult western corn rootworm fed *DvSnf7* dsRNA treated artificial diet at various ages: 2-day, 10-day, 20-day and 30-day post emergence. Mortality was recorded after 14 days. Error bars represent the 95% confidence intervals for LC_{50} values. Values with different letters indicate significant differences (P=0.05).

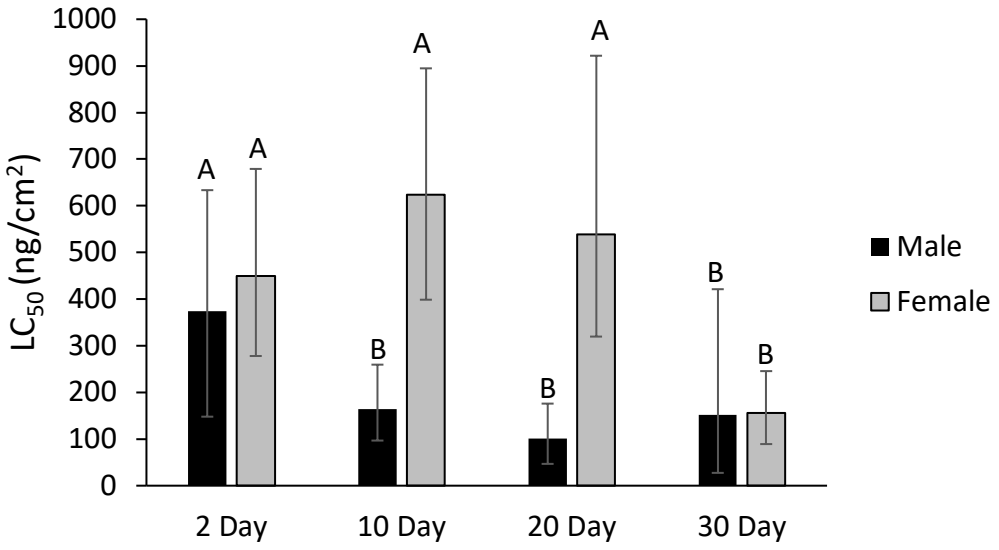

Figure 2.3 LC_{50} values (±95% CI) of male and female adult western corn rootworm fed *DvSnf7* dsRNA treated artificial diet at different ages: 2-day, 10-day, 20-day and 30-day post emergence. Mortality was recorded after 14 days. Error bars represent the 95% confidence intervals for LC_{50} values. Values with different letters indicate significant differences (P=0.05).

CHAPTER 3 ESTABLISHMENT OF LARVAL AND ADULT BASELINE SUSCEPTIBILITIES OF NATIVE U.S. CORN BELT WESTERN CORN ROOTWORM POPULATIONS TO *DVSNF7* DSRNA

3.1 Introduction

The western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte, is currently the most damaging corn pest in the United States Corn Belt costing producers over $1 billion annually in yield losses and management costs (Metcalf 1987, Dun et al. 2010). WCR primarily generates damage through larval feeding on the root mass of corn, reducing water and nutrient uptake (Branson et al. 1977). One node of feeding may result in up to a 17% yield reduction (Dun et al. 2010, Tinsley et al. 2013). Reductions in root mass may also lead to plant lodging making mechanized harvest ineffective. At high population levels, adult feeding on corn reproductive tissues can also reduce yield (Branson and Krysan 1981, Levine and Oloumi-Sadeghi 1991).

WCR has evolved resistance to chemical insecticides, crop rotation, and plant-incorporated *Bacillus thuringiensis* Berliner (Bt) proteins (Ball and Weekman 1962b, Meinke et al. 1998, Levine et al. 2002, Gassmann et al. 2011, Pereira et al. 2015), highlighting the need for new management strategies. Plant incorporated RNA has been developed for control of this pest and approved for use in the U.S. (US EPA 2017). This dsRNA is a component of the MON 87411 transformation event and will be deployed in a pyramid with two Bt proteins (Cry3Bb1 and Cry34/35Ab1). MON 87411 hybrids will be marketed under the trade name SmartStax PRO® (SSP). SSP provided excellent root protection (NIS <0.5) even in areas with high WCR pressure and potential resistance to Cry3Bb1 (Head et al. 2017).

As dsRNA will be released as a plant incorporated protectant (PIP), annual resistance monitoring must be conducted (US EPA 2013). Current WCR resistance monitoring programs utilize the larval life stage and can take upwards of 6 months to receive results (Gassmann et al. 2011), in part due to the lack of adult mortality with Bt proteins. However, dsRNA generates mortality in adult WCR (Rangasamy and Siegfried 2012, Pereira et al. 2016), potentially allowing adult susceptibility to dsRNA to be used as an indicator for resistance evolution of WCR to dsRNA. For this to be practical for monitoring, there must be a consistent susceptibility ratio between the larval and adult life stages because *DvSnf7* RNA targets the larval stage.

To date, there is limited information available about the susceptibilities of U.S. Corn Belt populations of WCR to *DvSnf7* double stranded RNA (dsRNA). Insects from different geographic areas (Moar et al. 2017) and within localized areas (Reinders et al. 2018) can vary in susceptibility to a toxin. Without establishment of baseline susceptibilities, it will be impossible to detect future susceptibility shifts in populations. To fill knowledge gaps, baseline susceptibility measures for larval and adult WCR were established for populations from major U.S. corn growing states: Nebraska, Iowa, Minnesota and South Dakota. These data will be utilized in future susceptibility comparisons for plant incorporated dsRNA products.

3.2 Materials and Methods

3.2.1 *DvSnf7* dsRNA

Bayer CropScience was synthesized *DvSnf7* RNA for this experiment according to Urquhart et al (2015) (Bayer CropScience, Chesterfield, MO, USA). This 968-mer *DvSnf7* RNA is produced in MON 87411 containing hybrids. This 968-mer contains a

240-mer dsRNA region and a 488-nucleotide single-strand RNA region including a hairpin and 3' and 5' untranslated regions. Cellular uptake of a 240-mer dsRNA occurs after degradation of the 488-nucleotide single strand region in the insect gut (Urquhart et al 2015).

3.2.2 Insect Populations

Control populations were purchased from Crop Characteristics, Inc. (Farmington, MN) or provided by Bayer CropScience (Chesterfield, MO, USA). The Bayer control population was collected from the field and crossed with a non-diapausing laboratory colony maintained by Bayer CropScience. An RNAi resistant population was developed (Khajuria et al. 2018) and provided by Bayer CropScience (Chesterfield, MO, USA). A field evolved Cry3Bb1 resistant population was collected and maintained by the University of Nebraska-Lincoln (Lincoln, NE, USA). Field populations were collected in the summer of 2017 and 2018, with 500-2000 beetles collected from each site. In 2017, insects were collected from Buffalo Co. NE, Polk Co. NE, and Dixon Co. NE. In 2018, insects were collected from Brookings Co. SD, Brown Co. MN, Dakota Co. MN, Floyd Co. IA, Scott Co. IA, Colfax Co. NE, and Stanton Co. NE (**Figure 3.1**).

3.2.3 Egg Collection

General lab rearing procedures were followed as described in Wangila et al. (2015). Briefly, insects were maintained in 28cm x 28cm x 28cm plexiglass cages and fed milk stage sweet corn ear tissue (kernel, cob, and silks). Sweet corn was replaced every 3-4 days and beetles were moved weekly to new cages to maintain optimal beetle health. A dish of moistened, No. 60 sieved and autoclaved soil was placed in each cage as an ovipositional site for gravid females (Jackson 1986). After each week of

oviposition, soil dishes were washed through a U.S.A. Standard Testing Sieve No. 60 (Thermo Fisher Scientific, Waltham, MA) to separate eggs from the soil. Eggs were placed into Petri dishes (Thermo Fisher Scientific, Waltham, MA) containing moistened (ca. 30% by weight), autoclaved, sifted soil. Petri dishes were sealed with Parafilm M (Bemis Company, Inc., Neenah, WI) and were held at 25°C for 1 month, 10°C for 1 month, and 7°C for approximately 4-5 months to allow obligatory diapause development to occur and terminate (Fisher 1989) prior to use in bioassays.

3.2.4 Egg Sterilization

Eggs were surface-sterilized one day prior to hatch to reduce contamination of artificial diet bioassays and held in sterilized containers until neonate eclosion. Eggs were washed from soil using a No. 60 mesh sieve (Thermo Fisher Scientific, Waltham, MA) to separate the eggs from the soil. Eggs were surface sterilized using a technique described by Pleau et al. (2002), briefly eggs were soaked in undiluted Lysol® (Reckitt Benckiser, Slough, United Kingdom) for 3 minutes followed by a triple rinse with autoclaved nanopure water. Eggs were soaked for an additional 3 minutes in buffered zinc formalin (Sigma Aldrich, St. Louis, MO) and triple rinsed with autoclaved nanopure water. Using a modified transfer pipette, eggs were moved to a coffee filter (8–12 cup size) and placed inside a 0.5-liter plastic deli container (Solo, Lincolnshire, IL) with five #000 insect pinholes in the lid. Containers were held in a growth chamber at 25 ±1 °C, relative humidity >80%, and 0:24 L:D photoperiod until eclosion (<24 hrs after egg was). Additional details on egg sterilization are provided in **Appendix II**.

3.2.5 Larval Bioassays

96-well diet plates were obtained from Bayer CropScience (Chesterfield, MO, USA) prefilled with diet produced according to a proprietary recipe (Moar et al. 2017). All work for larval bioassays was completed in an ultraviolet (UV) sterilized laminar flow hood (Esco Technologies, Inc., Horsham, PA, USA). Diet wells were surface treated with 20 µl of *DvSnf7* RNA solution with the corresponding to concentrations (0 ng/cm^2, 0.16 ng/cm^2, 0.8 ng/cm^2, 1 ng/cm^2, 2 ng/cm^2, 4 ng/cm^2, 20 ng/cm^2, and 100 ng/cm^2). The 0 ng/cm^2 consisted of UltraPure™ Distilled Water (Invitrogen, Waltham, Massachusetts, USA). Dilutions of *DvSnf7* RNA solution were completed using RNAse free microcentrifuge tubes and UltraPure™ Distilled Water (Invitrogen, Waltham, Massachusetts, USA). The treated diet was left uncovered for ~30 min to dry. A single neonate was transferred to each well using a sterilized 000 paint brush. Plates were sealed with a UV sterilized silicon adhesive film plate seal (VWR, Radnor, PA) with 1 hole per well punctured using #000 insect pin. Plates were held in a growth chamber at 25 ±1 °C, relative humidity >80%, and 24 h dark for 14 days with mortality recorded daily. Additional details are provided in Appendix III. Plates with <20% control mortality were used for data analysis. Contaminated wells were not included in the data analysis. Three replicates of 16 larvae per concentration were completed for each population.

3.2.6 Population Rearing

Eggs collected from the 2017 and 2018 populations were reared to adulthood for susceptibility screening. 150 neonate larvae were placed in a 1-liter deli container (Dart Container Corporation, Mason, MI) with sprouted VE-V1 stage non-transgenic corn. After 7-8 days, deli trays were transferred to a 5.7-liter shoe box (Sterilite Corporation,

Townsend, MA). A third of the shoe box was filled with the contents of the deli tray and larvae, a third of the box was additional VE-V1 corn seedlings and the remaining third was filled with soaked corn seeds mixed with soil substrate. Corn was trimmed weekly to reduce occurrences of mold and mite infestation. Shoe boxes were then placed in a 60 cm x 60 cm x 60 cm nylon mesh rearing cage (MegaView Science Co. Ltd., Taiwan) and adults were collected daily. Adults were maintained as previously described.

3.2.7 Adult Bioassays

Ten adults, 10-days post emergence, were placed into a 70 mL container (Baby Yummy, MJSteps, Zurich, Switzerland). One 8mm diameter diet plug, produced according to Khajuria et al. (2015), was placed in each container. Diet pellets were surface treated with 10 µl of *DvSnf7* dsRNA with the corresponding concentration (0 ng/cm^2, 18.75 ng/cm^2, 37.5 ng/cm^2, 75 ng/cm^2, 150 ng/cm^2, 300 ng/cm^2, 600 ng/cm^2, and 3600 ng/cm^2). Based on results from previous experiments, male beetles were tested with the full concentration range and females were only tested at the 0 ng/cm^2, 600 ng/cm^2, and 3600 ng/cm^2. The 0 ng/cm^2 consisted of UltraPure™ Distilled Water (Invitrogen, Waltham, Massachusetts, USA). Dilutions of *DvSnf7* RNA solution were completed using RNAse free microcentrifuge tubes and UltraPure™ Distilled Water (Invitrogen, Waltham, Massachusetts, USA). Dilutions were stored at -20°C between treatments. Beetles were transferred every other day to new containers with fresh, treated diet for five transfers for a total of ten days. After the five transfers with treated diet, beetles were transferred to new containers with fresh, untreated diet for two additional transfers. Mortality was recorded daily for 14-days. Plates were held in a growth chamber at 25 ±1 °C, relative humidity >80%, and 12:12 L:D photoperiod. Three

replicates of 10 adults per concentration were completed for each population. Additional details are provided in **Appendix IV**. Plates with <20% control mortality were used for data analysis.

3.2.8 Data Analysis

All bioassays were analyzed with a probit regression (Finney 1971) using PoloPlus-PC Software (LeOra Software LCC 1987) to generate LC_{50}s with their corresponding 95% confidence intervals (CIs), regression slopes, and Pearson goodness-of-fit chi-square values ($\chi 2$). PoloPlus-PC generated ratios between population LC_{50}s were used to determine statistical susceptibility differences between populations (Robertson et al. 2007).

3.3 Results

DvSnf7 RNA LC_{50}s estimated for WCR larvae ranged from 0.807 ng/cm^2 (Bayer non-diapausing population) to 5.701 ng/cm^2 (Colfax Co. NE) (**Table 3.1**). Overall, most field-collected populations responded similarly to *DvSnf7* RNA, with 8 of 14 populations mean LC_{50s} within the 1–2.5 ng/cm^2 range (**Figure 3.2**). A field-collected, laboratory-maintained Cry3Bb1-resistant population also responded similarly to other field populations (**Figure 3.2**). The RNAi resistant colony exhibited a 10.95% corrected mortality at the highest concentration tested (100 ng/cm^2). The Crop Characteristics LC_{50} was 1.07 ng/cm^2 and not statistically different from the Bayer non-diapausing, Brown Co. MN, Floyd Co. IA, Buffalo Co. NE, and Polk Co. NE populations.

Adult male WCR dsRNA LC_{50} estimates were highly variable and ranged from 105.0 ng/cm^2 (Floyd Co. IA) to >3600 ng/cm^2 (Scott Co. IA) (**Table 3.2**). Susceptibility in adult males was more variable than observed for the larval stage, with 4 of the 11

populations mean LC_{50s} within the 250-475 ng/cm^2 range (**Figure 3.3**)The most tolerant population was the Scott Co. IA population (**Table 3.2**), as only an 48.95% corrected mortality was observed at the highest tested concentration. The RNAi resistant colony exhibited a 0% corrected mortality at the highest concentration tested (3600 ng/cm^2). The Crop Characteristics LC_{50} was 164.6 ng/cm^2 and not statistically different from the Dakota Co. MN, Brown Co. MN, Floyd Co. IA, Stanton Co. NE, and Colfax Co. NE populations.

Average adult female WCR percent survival at the 3600 ng/cm^2 concentration ranged from 25% (Floyd Co. IA) to 98% (Cry3Bb1 Resistant Colony) (**Figure 3.4**). The RNAi resistant colony exhibited 100% survival. The Crop Characteristics population exhibited 13% survival.

Susceptibility ratios between adult males and larvae ranged from 1:30.05 (Colfax Co. NE) to >1:1590 (Scott Co. IA) (**Table 3.3**). The Crop Characteristic ratio was 1:153.73. The Colfax Co. NE, Floyd Co. IA, and Stanton Co. NE ratios were lower than the lower 95% CI for the Crop Characteristics population and the Bayer non-diapausing, Scott Co. IA, and RNAi resistant population were higher than the upper 95% CI for the Crop Characteristics population (**Table 3.3**).

3.4 Discussion

DvSnf7 RNA products have not yet been used for commercial corn production; therefore, little to no selection pressure has occurred in the field to date. Larval susceptibility for most field populations collected in 2017 and 2018 were similar, corroborating the lack of selection pressure. However, the Colfax Co. NE and Brookings Co. SD populations were significantly more tolerant to *DvSnf7* RNA compared to the

other field populations (**Figure 3.2**). The range of responses to *DvSnf7* dsRNA is probably indicative of natural variations found in the field. Moar et al. (2017) observed variations in *DvSnf7* dsRNA susceptibility ranging from 4.07 ng/cm^2 to 27.75 ng/cm^2 for field populations collected in 2012. Baseline susceptibility to the Cry3Bb1 protein also varied between geographically distinct populations (Siegfried et al. 2005), supporting natural variation in susceptibility of field populations to toxins. Additionally, the Cry3Bb1-resistant population responded similarly to most field populations, confirming the lack of cross resistance between RNA and the Cry3Bb1 protein (Moar et al. 2017). Therefore, SmartStax PRO® should be considered a "true pyramid" with three unique modes of action against WCR. Previously, the dsRNA resistant line reported an LC_{50} of >500 ng/cm^2 (Khajuria et al. 2018). The dsRNA resistant line, used as a positive control in this experiment, showed only 10.95% corrected mortality at the highest tested concentration (100 ng/cm^2), confirming that this assay was able to detect resistance in a WCR populations.

Tabashnik and Carrière (2017) suggest weak cross resistance between Cry3Bb1 and *DvSnf7* RNA; however, comparisons of populations from unrelated strains exhibited similar susceptibility to Cry3Bb1 and *DvSnf7* RNA, indicating a lack of cross resistance between these toxins (Moar et al. 2017). The results from this study support the lack of cross-resistance between Cry3Bb1 and *DvSnf7* RNA, as the Cry3Bb1-resistant population did not respond significantly different compared to eight of the ten field populations tested. Additionally, four of the tested field populations did not respond statistically different to known susceptible lab populations. Three of these field populations (Buffalo Co. NE, Polk, NE and Brown Co. MN) were collected from areas where product failures

of Cry3 producing hybrid have been documented, further supporting the lack of cross-resistance between Cry3 proteins and *DvSnf7* RNA.

Adult male susceptibility of field populations to *DvSnf7* RNA yielded more variable results compared to larvae. Populations that were not significantly different in the adult stage responded significantly different in the larval stage (**Figure 3.2, Figure 3.3**). For, example, the Colfax Co. NE population showed a lack of correlation between the life stages as it was the most tolerant in the larval stage (**Table 3.1**) yet adult males were more susceptible than four populations (**Table 3.2**). Adult females from field-collected populations were more tolerant to RNA than their male counterparts, confirming the results from the Crop Characteristics population explained in **Chapter 2**. The majority of field population females did not reach >50% mortality at 3600 ng/cm^2; therefore, percent survival was used to compare these populations (**Figure 3.4**). For females, there was also little correlation between larval susceptibility and female survival as the most tolerant larval populations did not have the highest mean survival for females.

Multiple factors that may be influencing the differences seen between WCR larval and adult susceptibilities to *DvSnf7* RNA. Differences in gut microbiota between the stages and populations may contribute to differences in the RNAi responses at each stage (Chu et al. 2014). In this study, the eggs and hatching containers were thoroughly sterilized to reduce contamination in larval diet plates, reducing the potential for neonates to acquire gut microbes from the soil before exposure to *DvSnf7* RNA. In contrast, adults are maintained in non-sterile conditions allowing them to acquire unique microbiota. Differences in the microbiome could affect susceptibility due to the production of dsRNA-degrading enzymes or regulate gene expression of the host (Kim et al. 2016,

Kunte et al. 2019), leading to increased variability in dsRNA susceptibility in adults. Gene expression differences between the stages may also contribute to variability. Expression levels for genes involved in dsRNA uptake and processing are variable throughout the lifecycle of WCR (Davis-Vogel et al. 2018). Differences in expression of dsRNAses in the gut and/or of adults and larvae may also be present, and degradation in the gut of dsRNA by dsRNases is known to significantly impacts the RNAi response in other insects (Spit et al. 2017, Guan et al. 2018). Furthermore, previous exposure to viruses has been linked to alterations in the RNAi response in Lepidoptera (Wu et al. 2009). Different populations may have different loads of naturally occurring viruses, potentially contributing to variations in dsRNA susceptibility.

As *DvSnf7* RNA will be implemented as a plant-incorporated protectant, the EPA requires resistance monitoring (US EPA 2013). Using the adult WCR for *DvSnf7* RNA monitoring would be ideal since it will reduce costs and allow for implementation of resistance mitigation strategies in a timely manner, However, in order to use the adult stage for resistance monitoring, susceptibility ratios between the larval and adult stage must be consistent since the larval stage is the targeted stage. Susceptibility ratios for field populations comparing adult males to larvae were variable. Six of the eleven tested populations had susceptibility ratios that were contained within the 95% confidence intervals of each other (**Table 3.3**). The remaining five populations had susceptibility ratios that were both higher (Bayer Susceptible, Scott Co. IA) and lower (Floyd Co. IA, Colfax Co. NE, Stanton Co. NE) than the Crop Characteristics population ratio.

Due to the lack of consistent susceptibility ratios, it is not recommended to utilize the adult stage for resistance monitoring if small changes in susceptibility significantly

affect product performance in the field. Since *DvSnf7* will be released as a pyramided hybrid, resistance to *DvSnf7* on its own may not affect the performance of SmartStax PRO®. However, the Bt in the pyramid (Cry3Bb1 and Cry34/35Ab1) have been previously released as single trait hybrids and cases of resistance have been documented (Gassmann et al. 2011, 2016; Ludwick et al. 2017), so in areas where resistance to these proteins is confirmed, the efficacy of the pyramid may be compromised. Resistance to one or more Bt proteins may result in increased selection pressure placed on *DvSnf7* RNA, potentially facilitating resistance evolution; however, more research should be completed to understand how these three traits interact to generate mortality in WCR. Due to the increasing prevalence of areas with resistance to one or more of these toxins, resistance monitoring with the larval stage is recommended to ensure early detection of resistance evolution and proper implementation of resistance mitigation to enhance product durability.

3.5 References Cited

Ball, H. J., and G. T. Weekman. 1962. Insecticide resistance in the adult western corn rootworm in Nebraska. J. Econ. Entomol. 55: 439–441

Branson, T. F., P. L. Guss, and J. J. Jackson. 1977. Mating frequency of the western corn rootworm. Ann. Entomol. Soc. Am. 70: 506–508.

Branson, T. F., and J. L. Krysan. 1981. Feeding and oviposition behavior and life cycle strategies of *Diabrotica* : an evolutionary view with implications for pest management. Environ. Entomol. 10: 826–831.

Chu, C.-C., W. Sun, J. L. Spencer, B. R. Pittendrigh, and M. J. Seufferheld. 2014. Differential effects of RNAi treatments on field populations of the western corn rootworm. Pestic. Biochem. Physiol. 110: 1–6.

Coates, B. S., A. P. Alves, H. Wang, K. K. O. Walden, B. W. French, N. J. Miller, C. A. Abel, H. M. Robertson, T. W. Sappington, and B. D. Siegfried. 2012. Distribution of genes and repetitive elements in the *Diabrotica virgifera virgifera* genome estimated using BAC sequencing. J. Biomed. Biotechnol. 2012: 1–9.

Davis-Vogel, C., B. Van Allen, J. L. Van Hemert, A. Sethi, M. E. Nelson, and D. G. Sashital. 2018. Identification and comparison of key RNA interference machinery from western corn rootworm, fall armyworm, and southern green stink bug. PLoS ONE. 13.

Dun, Z., P. D. Mitchell, and M. Agosti. 2010. Estimating *Diabrotica virgifera virgifera* damage functions with field trial data: applying an unbalanced nested error component model. J. Appl. Entomol. 134: 409–419.

Finney, D. J. 1971. Probit analysis, 3rd ed. Cambridge University Press, 32 E. 57th St., New York, Ny 10022.

Jackson, J.J., 1986. Rearing and handling of Diabrotica virgifera and Diabrotica undecimpunctata howardi. In: Krysan, J.L., Miller, T.A. (Eds.), Methods for the Study of Pest Diabrotica. Springer-Verlag, New York, pp. 25e47.

Gassmann, A. J., J. L. Petzold-Maxwell, R. S. Keweshan, and M. W. Dunbar. 2011. Field-evolved resistance to Bt maize by western corn rootworm. PLOS ONE. 6: e22629.

Gassmann, A. J., R. B. Shrestha, S. R. K. Jakka, M. W. Dunbar, E. H. Clifton, A. R. Paolino, D. A. Ingber, B. W. French, K. E. Masloski, J. W. Dounda, S. Clair, and C. R. 2016. Evidence of resistance to Cry34/35Ab1 corn by western corn rootworm (Coleoptera: Chrysomelidae): root injury in the field and larval survival in plant-based bioassays. J. Econ. Entomol. 109: 1872–1880.

Green, T. R., H. Kipka, O. David, and G. S. McMaster. 2018. Where is the USA Corn Belt, and how is it changing? Sci. Total Environ. 618: 1613–1618.

Guan, R.-B., H.-C. Li, Y.-J. Fan, S.-R. Hu, O. Christiaens, G. Smagghe, and X.-X. Miao. 2018. A nuclease specific to lepidopteran insects suppresses RNAi. J. Biol. Chem. 293: 6011–6021.

Hill, R. E., and Z. B. Mayo. 1980. Distribution and abundance of corn rootworm species as influenced by topography and crop rotation in eastern Nebraska. Environ. Entomol. 9: 122-127.

Khajuria, C., S. Ivashuta, E. Wiggins, L. Flagel, W. Moar, M. Pleau, K. Miller, Y. Zhang, P. Ramaseshadri, C. Jiang, T. Hodge, P. Jensen, M. Chen, A. Gowda,

B. McNulty, C. Vazquez, R. Bolognesi, J. Haas, G. Head, and T. Clark. 2018. Development and characterization of the first dsRNA-resistant insect population from western corn rootworm, *Diabrotica virgifera virgifera* LeConte. PLOS ONE. 13: e0197059.

Khajuria, C., A. M. Vélez, M. Rangasamy, H. Wang, E. Fishilevich, M. L. F. Frey, N. P. Carneiro, P. Gandra, K. E. Narva, and B. D. Siegfried. 2015. Parental RNA interference of genes involved in embryonic development of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte. Insect Biochem. Mol. Biol. 63: 54–62.

Kim, D., M. W. Thairu, and A. K. Hansen. 2016. Novel Insights into Insect-Microbe Interactions—Role of Epigenomics and Small RNAs. Front. Plant Sci. 7.

Kunte, N., E. McGraw, S. Bell, D. Held, and L. Avila. 2019. Prospects, challenges and current status of RNAi through insect feeding. Pest Management Science.

LeOra Software. 1987. POLO-PC. A user's guide to probit and logit analysis. Berkeley, CA.

Levine, E., and H. Oloumi-Sadeghi. 1991. Management of Diabroticite rootworms in corn. Annu. Rev. Entomol. 36: 229–255.

Levine, E., J. L. Spencer, S. A. Isard, D. W. Onstad, and M. E. Gray. 2002. Adaptation of the western corn rootworm to crop rotation: evolution of a new strain in response to a management practice. Am. Entomol. 48: 94–107.

Ludwick, D. C., L. N. Meihls, K. R. Ostlie, B. D. Potter, L. French, and B. E. Hibbard. 2017. Minnesota field population of western corn rootworm (Coleoptera: Chrysomelidae) shows incomplete resistance to Cry34Ab1/Cry35Ab1 and Cry3Bb1. J. Appl. Entomol. 141: 28–40.

Meinke, L. J., B. D. Siegfried, R. J. Wright, and L. D. Chandler. 1998. Adult susceptibility of Nebraska western corn rootworm (Coleoptera: Chrysomelidae) Populations to Selected Insecticides. J. Econ. Entomol. 91: 594–600.

Meinke, L. J., T. W. Sappington, D. W. Onstad, T. Guillemaud, N. J. Miller, J. Komáromi, N. Levay, L. Furlan, J. Kiss, and F. Toth. 2009. Western corn rootworm (Diabrotica virgifera virgifera LeConte) population dynamics. Agr. Forest Entomol. 11: 29-46.

Moar, W., C. Khajuria, M. Pleau, O. Ilagan, M. Chen, C. Jiang, P. Price, B. McNulty, T. Clark, and G. Head. 2017. Cry3Bb1-resistant western corn rootworm, *Diabrotica virgifera virgifera* (LeConte) does not exhibit cross-resistance to *DvSnf7* dsRNA. PLOS ONE. 12: e0169175.

Pereira, A. E., N. P. Carneiro, and B. D. Siegfried. 2016. Comparative susceptibility of southern and western corn rootworm adults and larvae to *vATPase-A* and *Snf7* dsRNAs. J. RNA Genomics. 12: 528–535.

Pereira, A. E., H. Wang, S. N. Zukoff, L. J. Meinke, B. W. French, and B. D. Siegfried. 2015. Evidence of field-evolved resistance to bifenthrin in western corn rootworm (*Diabrotica virgifera virgifera* LeConte) populations in western Nebraska and Kansas. PLOS ONE. 10: e0142299.

Pleau, M. J., J. E. Huesing, G. P. Head, and D. J. Feir. 2002. Development of an artificial diet for the western corn rootworm. Entomologia Experimentalis et Applicata. 105: 1–11.

Quarles, W. 2017. IPM for the western corn rootworm. 24.

Rangasamy, M., and B. D. Siegfried. 2012. Validation of RNA interference in western corn rootworm *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) adults. Pest Manag. Sci. 68: 587–591.

Reinders, J. D., B. D. Hitt, W. W. Stroup, B. W. French, and L. J. Meinke. 2018. Spatial variation in western corn rootworm (Coleoptera: Chrysomelidae) susceptibility to Cry3 toxins in Nebraska. PLOS ONE. 13: e0208266.

Robertson, J. L., R. M. Russell, H. K. Preisler, and N. E. Savin. 2007. Bioassays with Arthropods, Second Edition. ed. CRC Press, Boca Raton, FL.

Siegfried, B. D., T. T. Vaughn, and T. Spencer. 2005. Baseline susceptibility of western corn rootworm (Coleoptera: Crysomelidae) to Cry3Bb1 *Bacillus thuringiensis* toxin. Journal of Economic Entomology. 98: 1320–1324.

Spit, J., Philips, A., Wynant, N., Santos, D., Plaetinck, G., and Vanden Broeck, J. (2017). Knockdown of nuclease activity in the gut enhances RNAi efficiency in the Colorado potato beetle, *Leptinotarsa decemlineata*, but not in the desert locust, *Schistocerca gregaria*. *Insect Biochem Mol Biol* 81**,** 103-116.

Swevers, L., J. Vanden Broeck, and G. Smagghe. 2013. The possible impact of persistent virus infection on the function of the RNAi machinery in insects: a hypothesis. Front. Physiol. 4.

Tabashnik, B. E., and Y. Carrière. 2017. Surge in insect resistance to transgenic crops and prospects for sustainability. Nat. Biotechnol. 35: 926–935.

Tinsley, N. A., R. E. Estes, and M. E. Gray. 2013. Validation of a nested error component model to estimate damage caused by corn rootworm larvae. J. Appl. Entomol. 137: 161–169.

Urquhart, W., G. M. Mueller, S. Carleton, Z. Song, T. Perez, J. P. Uffman, P. D. Jensen, S. L. Levine, and J. Ward. 2015. A novel method of demonstrating the molecular and functional equivalence between *in vitro* and plant-produced double-stranded RNA. Regul. Toxicol. Pharmacol. 73: 607–612.

US EPA, O. 2017. Insect Resistance Management for Bt Plant-Incorporated Protectants. US EPA. (https://www.epa.gov/regulation-biotechnology-under-tsca-and-fifra/insect-resistance-management-bt-plant-incorporated).

Wangila, D. S., A. J. Gassmann, J. L. Petzold-Maxwell, B. W. French, and L. J. Meinke. 2015. Susceptibility of Nebraska western corn rootworm (Coleoptera: Chrysomelidae) populations to Bt corn events. J. Econ. Entomol. 108: 742-751.

Wu, P., M. Li, X. Wang, P. Zhao, X. Wang, T. Liu, G. Qin, and X. Guo. 2009. Differentially expressed genes in the midgut of Silkworm infected with cytoplasmic polyhedrosis virus. Afr. J. Biotechnol. 8.

Tables and Figures

Table 3.1 Larval susceptibilities of WCR populations collected in 2017 and 2018 to *DvSnf7* dsRNA. Mortality was recorded after 14 days. Only plates with <20% contamination and control mortality <20% were included.

Year	Population	N [1]	LC_{50} (95% C.I.) [2]	Slope ± SE	$\chi 2$	Statistics[3]
Control	Crop Characteristics	330	1.07 (0.71 – 1.50)	1.99 ± 0.26	5.05	F
	Bayer non-diapausing[4]	320	0.81 (0.33 – 1.48)	1.11 ± 0.21	3.62	F
2017	Buffalo Co. NE	204	1.68 (1.02 – 2.63)	1.06 ± 0.14	5.50	DEF
	Polk Co. NE	200	1.97 (1.04 – 3.68)	1.48 ± 0.20	7.21	EF
	Thurston Co. NE	377	2.23 (1.47 – 3.46)	1.54 ± 0.16	7.42	CD
2018	Brookings Co. SD	396	3.71 (2.71 – 5.14)	1.14 ± 0.11	1.86	AB
	Brown Co. MN	405	1.51 (0.80 – 2.63)	1.40 ± 0.13	13.41	EF
	Dakota Co. MN	383	1.94 (1.14 – 3.31)	1.24 ± 0.12	9.56	DE
	Floyd Co. IA	334	1.56 (1.00 – 2.38)	1.29 ± 0.13	5.88	DEF
	Scott Co. IA	383	2.26 (1.49 – 3.51)	1.46 ± 0.15	7.45	CD
	Colfax Co. NE	367	5.70 (3.80 – 9.10)	1.37 ± 0.13	5.94	A
	Stanton Co. NE	330	3.39 (2.05 – 5.85)	1.08 ± 0.12	5.42	BC
Resistant	Cry3Bb1[5]	336	2.23 (1.04 – 4.99)	1.60 ± 0.23	10.49	CDE
	RNAi6	145	-	-	-	-

[1] Number of insects evaluated in the concentration response assay
[2] ng/cm^2 *DvSnf7* dsRNA
[3] Values with the different letters statistically different (P=0.05)
[4] Bayer non-diapausing population was collected from the field and crossed with a non-diapausing colony maintained by Bayer CropScience
[5] Cry3Bb1 resistant population is a field evolved colony collected and maintained by the University of Nebraska-Lincoln
[6] RNAi resistant population was developed and provided by Bayer CropScience

Table 3.2 Adult male WCR susceptibility to *DvSnf7* dsRNA for field populations collected in 2017 and 2018. Mortality was recorded after 14 days. Control mortality was <20%.

Year	Population	N [1]	LC_{50} (95% C.I.) [2]	Slope ± SE	$\chi 2$	Statistics[3]
Control	Crop Characteristics	420	164.6 (96.7 – 259.5)	1.23 ± 0.18	0.87	CD
	Bayer non-diapausing[4]	240	566.8 (286.2 – 919.1)	0.87 ± 0.20	0.23	A
2017	Thurston Co. NE	420	473.9 (309.7 – 729.4)	1.33 ± 0.20	4.81	AB
2018	Brookings Co. SD	420	713.7 (400.2 – 1208.1)	1.05 ± 0.19	3.25	A
	Brown Co. MN	420	305.7 (93.1 – 628.5)	1.34 ± 0.20	19.78	ABC
	Dakota Co. MN	420	274.1 (102.0 – 538.0)	1.11 ± 0.13	28.95	BCD
	Floyd Co. IA	420	105.0 (27.2 – 237.4)	0.97 ± 0.17	5.14	D
	Scott Co. IA	420	>3600	-	-	-
	Colfax Co. NE	420	171.3 (115.7 – 238.4)	1.50 ± 0.19	3.83	CD
	Stanton Co. NE	420	281.8 (153.3 – 482.8)	1.22 ± 0.15	5.34	BC
Resistant	Cry3Bb1[5]	420	523.3 (309.9 – 920.2)	1.96 ± 0.242	11.41	A
	RNAi[6]	120	>3600	-	-	-

[1] Number of insects evaluated in the concentration response assay
[2] ng/cm2 DvSnf7 dsRNA
[3] Values with the same letters not statistically different (P>0.05)
[4] Bayer non-diapausing population was collected from the field and crossed with a non-diapausing colony maintained by Bayer CropScience
[5] Cry3Bb1 resistant population is a field evolved colony collected and maintained by the University of Nebraska-Lincoln
[6] RNAi resistant population was developed and provided by Bayer CropScience

Table 3.3 Susceptibility ratios between adult males and larvae of western corn rootworm to *DvSnf7* dsRNA. Susceptibility ratios were generated by PoloPlus-PC.

Population	Larvae: Adult Male LC_{50} Ratio	95% Confidence Interval
Crop Characteristics Susceptible	153.73	88.50-267.07
Dakota Co. MN	141.46	83.51-239.64
Brookings Co SD	192.49	103.74-357.19
Brown Co. MN	202.88	105.40-390.52
Thurston Co. NE	212.68	130.04-347.85
Cry3Bb1 Resistant	234.93	152.46-362.13
Colfax Co. NE	30.05	18.86-47.89
Floyd Co. IA	67.19	30.86-146.30
Stanton Co. IA	83.22	47.92-144.51
Bayer non-diapausing	702.12	294.38-1674.61
Scott Co. IA	>1590	-
RNAi Resistant	>3600	-

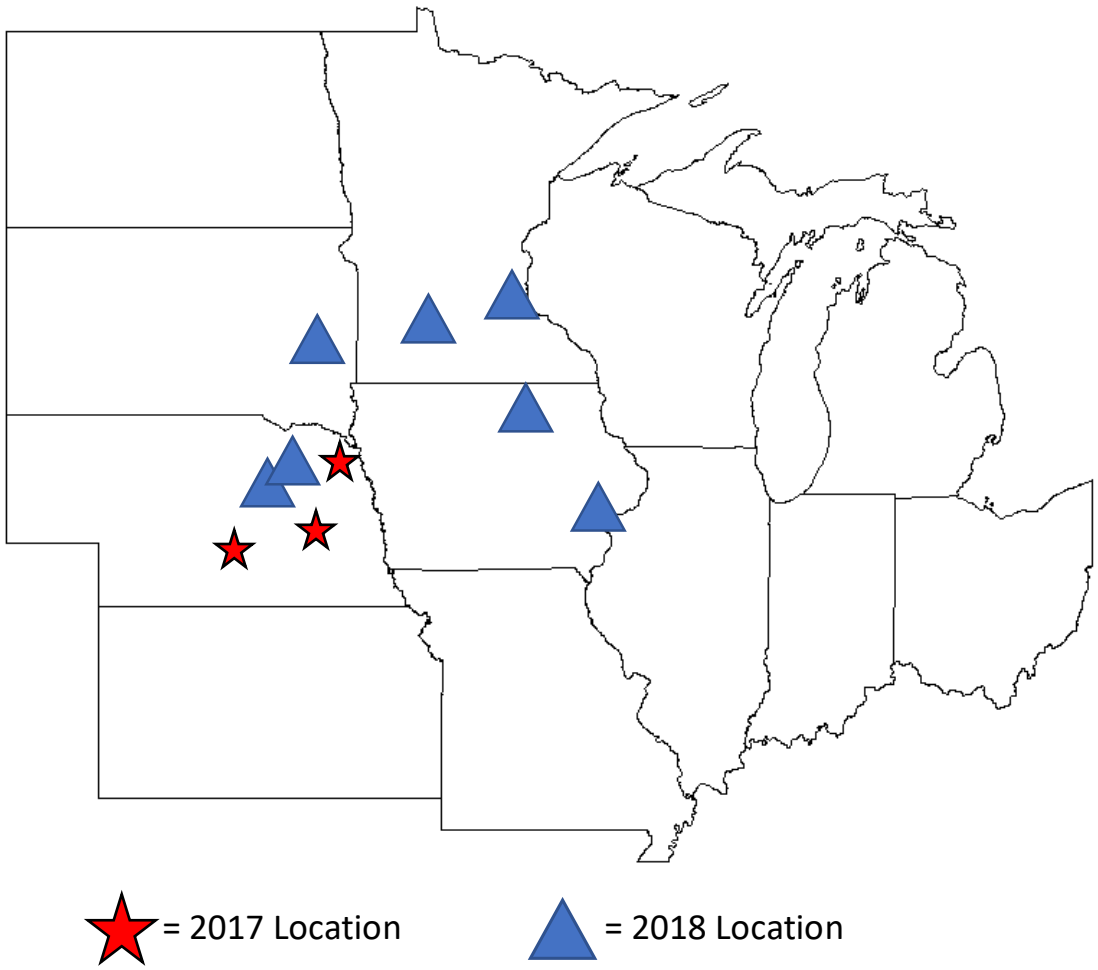

Figure 3.1 Population map for WCR field collections during the 2017 and 2018 corn growing seasons. 500-2000 beetles collected from each site and populations were maintained in the laboratory after collection.

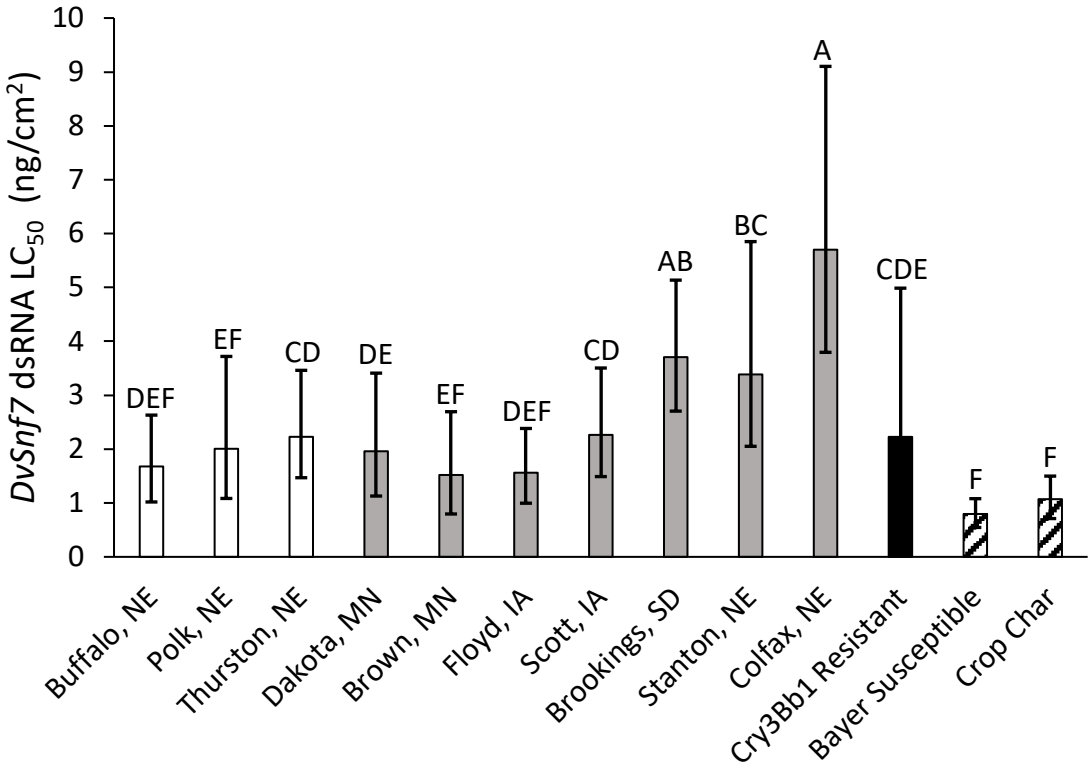

Figure 3.2 LC_{50} values (±95% CI) of larval WCR fed *DvSnf7* RNA treated artificial diet. Mortality was recorded after 14 days. Error bars represent the 95% confidence intervals for LC_{50} values. Values with different letters indicate significant differences among populations (P=0.05)

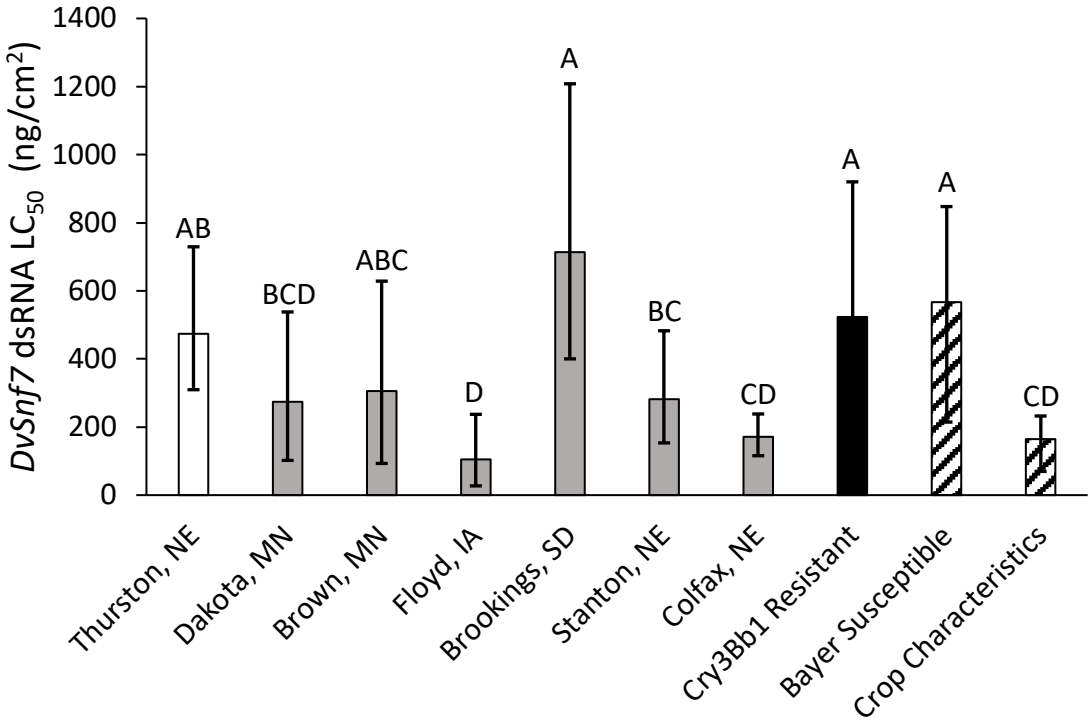

Figure 3.3 LC_{50} values (±95% CI) of adult WCR males fed *DvSnf7* dsRNA treated artificial diet. Mortality was recorded after 14 days. Error bars represent the 95% confidence intervals for LC_{50} values. Values with different letters indicate significant differences among populations (P=0.05)

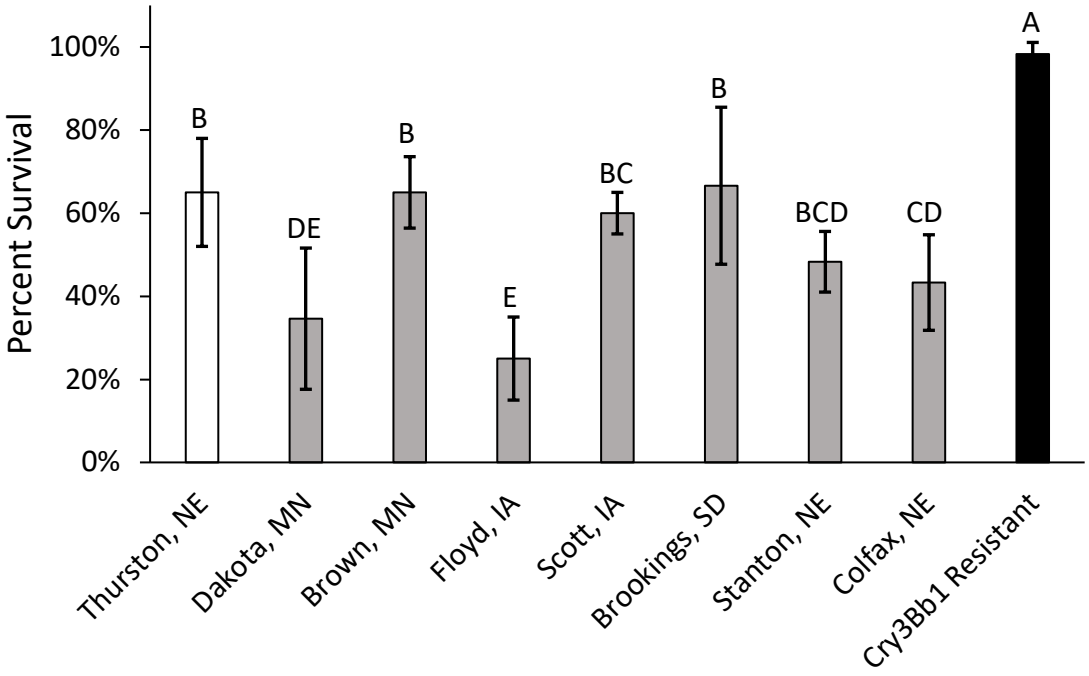

Figure 3.4 Percent survival (±SE) of adult WCR females fed *DvSnf7* dsRNA treated artificial diet at 3600 ng/cm^2 concentration. Mortality was recorded after 14 days. Control survival was >80%. Error bars represent the standard error of the mean survival values. Means with different letters indicate significant differences among populations (P=0.05)

CONCLUSIONS

WCR is currently the most damaging pest of corn in the United States Corn Belt and possesses the ability to adapt to a wide variety of control tactics. As the levels of resistance to available control strategies continue to increase, the risk of significant loss for producers also increases. RNA interference offers a novel tool to control WCR and must be properly implemented into IPM systems to ensure product durability and continued control of this pest. Proper education of producers is a must to discourage reliance on this control strategy in their WCR management programs. Producer education will be even more important if additional dsRNA products are released for control of WCR, as resistance to one dsRNA results in resistance to other dsRNA molecules (Khajuria et al. 2018). Ending the pesticide treadmill for WCR should be a major goal for producers as new technologies are released. In areas where resistance to Cry3Bb1 or Cry34/35Ab1 has been reported, small shifts in susceptibility to *DvSnf7* RNA may result in reduced product performance and accelerate the evolution of resistance if the pyramid is continuously used to control WCR.

Since dsRNA will be deployed as a transgenic hybrid, resistance monitoring should be conducted with on-plant assays of larvae with hybrid expressing only one components of the pyramid. Monitoring on-plant provides conditions that most accurately represent the exposure the pest will encounter in the field. If susceptibility shifts are detected, further investigation can be completed to determine if these susceptibility shifts will impact SmartStax PRO® performance.

This study demonstrates that a variety of WCR populations from the Corn Belt are susceptible to this upcoming technology and relatively uniform in susceptibly in the

larval stage, partially supporting the working hypothesis that WCR populations have similar larval susceptibilities to *DvSnf7* dsRNA. Adult susceptibility to dsRNA was highly variable between different sexes and ages. Females were less susceptible than males for the 10-and 20-day post-emergence age groups and overall susceptibility increased with insect age, supporting the working hypothesis that adult susceptibility to *DvSnf7* dsRNA is higher for males than females and increase as adults age. Developers of upcoming dsRNA pest control strategies should consider these differences in other insects where the adult stage is targeted. Adult susceptibility was more variable for field populations than larval susceptibility and susceptibility to dsRNA did not always positively correlate between life stages, which did not support the working hypothesis that WCR populations have similar adult susceptibilities to *DvSnf7* dsRNA and have equivalent correlation ratios between populations. Screening adult populations for *DvSnf7* RNA susceptibility may not be sensitive enough to detect small shifts in susceptibility, so if possible, monitoring with the larval stage is recommended as lower amounts of dsRNA are needed to generate mortality and the larval response was more uniform across populations. Although *DvSnf7* RNA is not intended for adult control, activity in the adult stage could present unique challenges for resistance evolution and population dynamics after the release of this product. Overall, dsRNA is a much-needed advancement for control of WCR and integration of this technology into IPM programs will delay resistance evolution, allowing producers to control this pest in sustainable and effective ways.

APPENDIX I: ARTIFICIAL DIET RECIPE FOR WCR ADULTS

Ingredients	Quantity= 1 plate	Quantity = 2 plates	Quantity = 3 plates
1. Agar	0.365 g	0.73 g	1.09 g
2. ***Diet mix***	6 g	12 g	18 g
3. Water (dd)	12.5 ml	20 ml	37.5 ml
4. Glycerol*	0.7 ml	1.4 ml	2.1 ml
5. ***Mold inhibitor***	27.5 µl	55 µl	82.5 µl

INSTRUCTIONS:

1. Add water and agar to beaker
2. Microwave beaker until water starts boiling
3. Stir and bring to boil 3 times
4. Add diet mix, glycerol, and mold inhibitor to the beaker
5. Mix thoroughly and pour mixture into petri dishes
6. Allow to cool, seal with parafilm and place upside down in 4° C refrigerator for up to one week

Diet Mix

Ingredients	Quantity (Grams)
Soy flour	113
Milled wheat germ	113
Casein	144.6
Alphacel (fiber)	169.5
Fructose	300
Brewers yeast	45.15
Vitamix	11.3
Salt mix	11.3
Cholesterol	2.3

INSTRUCTIONS:
1. Mix thoroughly
2. Store at 4°C

Mold Inhibitor

Ingredients	Quantity (ml)
Water	30
Propionic acid	47
Phosphoric acid	5

INSTRUCTIONS:
1. Combine and vortex together
2. Store at room temperature

APPENDIX II: EGG WASH AND STERILIZATION PROTOCOL

Do not sterilize eggs until a significant number of larvae are seen hatching (~ 50 larvae per petri dish). Egg sterilization should be performed just before a large number begin hatching.

Egg Surface Sterilization (performed 24 hours before intended use of larvae)

1. Clean the laminar flow hood with >70% Ethanol and allow to UV sterilize for ~10 minutes.
2. Empty egg and soil mixture into a 60-mesh sieve. (*Diabrotica* spp. eggs are too large to fit through a 60-mesh sieve, so no eggs can possibly escape during rinsing). Use a gentle water stream to break up clumped soil and eggs. Make sure water is slightly warm. Once eggs are separated and soil clumps are broken up, use the water stream to collect eggs on one side of the sieve. Transfer eggs into a 100 mL beaker with a disposable plastic pipette. Make sure there is ~30 mL of water in the beaker. Swirl gently and allow the eggs to settle for ~30 seconds. Healthy eggs will sink to the bottom of the beaker debris and hatched eggs will float to the top. Pour off the excess water/debris into a 600 mL waste beaker (you want just enough water leftover to cover the eggs). Repeat adding water, swirling, and decanting 3 times.
3. Add 10 - 20 mL of undiluted Lysol into the egg beaker. Aim to have add at least the same volume as the eggs. So, for ~10 ml of eggs, add ~10 mL of Lysol. Swirl beaker to ensure all eggs are exposed. After 3 minutes, decant the 10 - 20 mL supernatant to the same 600 mL waste beaker.
4. Add 10 - 20 mL of autoclaved water into egg beaker. Swirl beaker to ensure all eggs are exposed. Remove 10 - 20 mL supernatant to 600 mL collection container (no waiting period, add water and then immediately remove water). Repeat this step 3 times.
5. Add 10 - 20 mL formalin (buffered zinc formalin) into egg beaker. Swirl beaker to ensure all eggs are exposed. After 3 minutes, remove 10 - 20 ml supernatant to 600 mL waste beaker.
6. Add 10 - 20 ml distilled water into egg beaker. Swirl beaker to ensure all eggs are exposed. Remove the 10 - 20 ml supernatant to 600 mL waste beaker (no waiting period, add water and then immediately remove water). Repeat this step 3 times. Then add about 10 mL of autoclaved water
7. Pull eggs into 1 ml disposable pipettor, with ~1 cm of the tip cut off, and dispense onto a coffee pot filte. Use water to disperse eggs evenly across the bottom of the filter. Place 3-4 paper towels underneath the coffee filter to absorb excess water. Place filter inside 16 oz plastic deli containers with #000 insect pinholes in the lid.
8. Label deli container with date and population. Place container in small growth chamber at 25°C, >80% RH, and 24 hr dark.
9. Empty contents of 600 mL waste beaker by straining it through a coffee filter, ensuring that all eggs and other material are removed from the beaker. Freeze coffee filter for 72 hrs in -4°C. After freezing, autoclave waste material for 30 min at 15 psi and 121°C, place in biohazard safety bag after autoclaving.

10. Use sterilized eggs in the next 1-2 days. After this, the larval contamination increases significantly.
11. Any unused eggs and disposable tools must be frozen for 72 hrs at -20C. Coffee filters with eggs and tools will then be autoclaved for 30 min at 15 psi and 121 C. Discard autoclaved material in biohazard safety bag. Wipe down laminar flow hood with >70% Ethanol when finished.

APPENDIX III: LARVAL BIOASSAY PROTOCOL

1. Clean hood with >70% Ethanol and allow it to dry.
2. Gather selected tools and place in Laminar flow hood with UV sterilization light on for 15 min before beginning transfer
    a. Cleaned or new hatching deli cup
    b. Plate seals, 000 camel hairs paint brush
    c. 000 insect pin
    d. 2 microcentrifuge tubes filled with 95% Ethanol
    e. 1 microcentrifuge tube filled with Autoclaved water
    f. Scalpel
    g. Autoclaved water in a mister bottle
    h. Desktop fans and fan stands
    i. 100 μL pipette and tips
    j. Kim wipes
    k. Box for holding deli cup at angle
3. After sterilization, turn off UV light. Turn on laminar flow fans and open hood. Retrieve larval diet plates and dsRNA dilutions. Use the 15 min UV sterilization to thaw and mix the dsRNA dilutions.
4. Surface treat the diet plates with 20 μL of dsRNA solution per well. Always start from the lowest concentration and work your way to the highest concentration.
5. After all plates are treated, turn on desktop fans on their stands to blow directly onto the plates to dry off excess water. Run fans for ~30 minutes or until the diet appears matte instead of glossy.
6. When dry, lightly place a plate seal on top of the diet plates and use a scalpel to cut the seal into strips two columns wide. This is to allow for the larva to be placed in the plate without any risk of escape during transfers.
7. Retrieve the larval hatching deli cup from the small growth chamber and place it in the laminar flow hood. NEVER OPEN THE HATCHING CONTAINERS OUTSIDE OF THE LAMINAR FLOW HOOD, THIS WILL CREATE CONTAMINATION!
8. Open the deli cup with the larvae and transfer the coffee filter with the remaining eggs into the clean deli cup. Place the lid on this cup.
9. Place the deli cup with neonate larva into the box, so it is tilted towards the glass for better viewing.
10. Take the 000 camel-hair paint brush and dip it in the 95% ethanol microcentrifuge tube, wipe dry on Kimwipe, dip a second time in the 2nd microcentrifuge tube with 95% ethanol, wipe dry on Kimwipe, dip brush lastly into the microcentrifuge tube filled with autoclaved water. Dab lightly on Kimwipe to remove excess moisture, however you will still want the brush to be moist. REPEAT THIS WASHING BETWEEN EACH DOSE OF YOUR DILUTIONS OR IF THE BRUSH TOUCHES ANYTHING OTHER THAN LARVA AND THE DIET.
11. Peel up the plate seal and place one larva per well to fill the plate. Avoid placing gloves on adhesive area that covers the wells. Press and seal well the plate seal after each concentration. Spray the hatching deli cup regularly with autoclaved water from a mister bottle to prevent static electricity build up on the larva.

12. Poke a hole into each well with a #000 insect pin to allow for air exchange.
13. Wipe down laminar flow hood with >70% ethanol to ensure that any possible escaped larvae are killed. WCR larvae dry out and die if not in a high humidity area (70%>) so no larvae should be able to survive.
14. Use a microscope to ensure that all larva transferred are alive and well. Record this on the mortality sheet as day 1. Note any larva that are dead after transfer.
15. Place plate into 24 hr Dark growth chamber at 25°C and 80% RH.
16. Record mortality daily by inspecting the plate under the microscope. Place a space heater on the side of the scope, blowing over the plates, to ensure you can see into the wells as the seals get a lot of condensation since the lab is not as hot or as humid as the chamber. NEVER OPEN THE SEAL ON THE PLATE UNTIL THE LAST DAY OF THE EXPERIMENT. THIS WILL CONTAMINATE THE WELLS.
17. Run experiment for 15 days. At the end of the experiment, remove any alive larva from each concentration and place them in dose specific microcentrifuge tubes to be weighed and estimate growth inhibition. Remember to record the number of alive insects for each of the treatments.

APPENDIX IV: ADULT TRANSFER/BIOASSAY PROTOCOL

1. Make diet according to diet protocol the day before or on the day of first transfer. (Diet can be used for up to 1 week, stored in refrigerator upside down and sealed with parafilm) Label diet with name, date, etc.
2. Gather the proper number of plates (Baby Yummy, MJSteps, Zurich, Switzerland) and diet cutting tools (8mm diameter cork borer, tweezer, and pushing rod). Wipe down with 95% ethanol and allow to dry
3. Wipe down bench area with >70% ethanol followed by RNAZap
4. Get dilutions out of -20C freezer and thaw on ice. Vortex and spin centrifuge tubes.
5. Clean a 10 µL pipette with 95% ethanol and RNAZap. Place on top of tip box so that the stem of the pipette is not touching anything.
6. Using the 8mm cork borer cut and place 1 diet pellet into each cup.
7. Treat the diet pellets with 10 µL of the respective concentration needed, changing the tip after treating each pellet. After treating the last pellet at each dose, take a pipette tip and ensure the entire pellet is treated with solution. Allow solution to dry on the diet pellet ~10 min
8. Place adult beetles into 4°C cooler for ~4 min to slow movement and reduce stress. Transfer 10 insects into each well and close each container.
9. Label the plates and place them in a growth chamber set at 25°C, 80% RH and a 12:12 L:D.
10. Remove diet and dead beetles from containers and discard in an autoclavable bag.
11. Fill sink with ~1/2 inch of water, use a brush to clean out the bottom of containers, and drain the dirty water. Fill sink so that all containers are submerged and add 3 capfuls of bleach.
12. Soak overnight. Drain water and TRIPLE RINSE THE CONTAINERS! BLEACH WILL DEGRADE THE DSRNA AND RUIN THE EXPERIMENT.
13. Wipe down workspace with >70% Ethanol after finishing.
14. Record mortality every day.
15. Transfer insects every other day using the above protocol to a new set of plates and treated diet pellets. Make new diet as needed.
16. On days 11 and 13 of the experiment complete the transfer as listed above, except treat all diet pellets with 10 µL of water instead of their respective dsRNA concentration.
17. Continue to monitor and transfer insects for the entirety of the experiment (15 days).