Assessment of Variation in Susceptibility of the Fall Armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), to *Bacillus thuringiensis* Toxins

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ASSESSMENT OF VARIATION IN SUSCEPTIBILITY OF THE FALL
ARMYWORM, Spodoptera frugiperda (J.E. SMITH) (LEPIDOPTERA: NOCTUIDAE),
TO Bacillus thuringiensis TOXINS

by

Karen Ferreira da Silva

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 Blair D. Siegfried

Lincoln, Nebraska

May, 2015
ASSESSMENT OF VARIATION IN SUSCEPTIBILITY OF THE FALL ARMYWORM, *Spodoptera frugiperda* (J.E. SMITH) (LEPIDOPTERA: NOCTUIDAE), TO *Bacillus thuringiensis* TOXINS

Karen Ferreira da Silva, M.S.
University of Nebraska, 2015

Adviser: Blair D. Siegfried

The fall armyworm, *Spodoptera frugiperda*, is a polyphagous insect pest affecting multiple crops. Fall armyworm is managed with insecticides and corn hybrids expressing insecticidal proteins derived from *Bacillus thuringiensis*. The early detection of insect resistance is important for making appropriate management decisions informs IPM and IRM recommendations.

The objective of the first study was to establish baseline susceptibility of fall armyworm populations to the Cry1F Bt insecticidal protein, emphasizing collections from locations where fall armyworm overwinters in the U.S. Fall armyworm neonates were exposed to artificial diet treated with increasing Cry1F concentrations, and mortality and growth inhibition were evaluated after 7 days. Differences in Cry1F susceptibility between the most susceptible and the most tolerant field populations were 2- and 6- fold for 2012 and 2013, respectively. These results are consistent with other baseline studies of Bt toxicity in other species although reduced susceptibility in some populations may suggest resistance development.
The second study was designed to identify possible sources of variability in laboratory bioassays. Efforts to standardize the laboratory methods used in bioassays of microbial products have been part of an overall attempt to minimize variation. The objective of this second project was to determine if pre-treatment conditions contribute to variation in a *Spodoptera frugiperda* laboratory population response when exposed to Vip3Aa19 insecticidal protein. Neonates were exposed to the LC$_{70}$ of Vip3Aa19 under five pretreatment conditions: 1) larval storage time prior to exposure, 2) prior feeding on artificial diet, 3) larval storage at reduced temperature, 4) larval storage at reduced humidity, and 5) lab colony introgression with field collected individuals. Extremes of photoperiod settings used during the course of the bioassay itself were also tested. Significant effects of pre-treatment conditions were observed when neonates were fed prior to bioassay, when stored overnight at 14°C and when exposed to extreme photoperiod conditions. There was no significant difference observed in the other pretreatment conditions.
ACKNOWLEDGEMENTS

I would like to express the deepest appreciation and gratefulness to my committee chair Dr. Blair Siegfried for giving me the opportunity to obtain a degree in a Master program, placing confidence in my potential and goals for my career. Furthermore, I acknowledge him for his competence and professionalism as a mentor, and for his impressive leadership and such great personality.

I would like to thank my supervisory committee, Dr. Daniel Moellenbeck and Dr. Thomas Hunt for their help in the research and for providing an enormous collaboration towards my career goals. I also would like to recognize my unofficial committee members Dr. Andre Crespo and Dr. Frederick Walters for their input on my research and experimental design, for providing constructive criticism and mentorship.

I would like to thank Dupont Pioneer for providing Cry1F toxin and for the assistance with field collected population and Syngenta for supplying with Vip3Aa19 toxin and providing field collection from Florida. In addition, I would like to thank Bruce and Vicky Lang from Bio Costume products and Dr. Daniel Moellenbeck from DM Crop Research, for helping with the rearing process of fall armyworm and shipments, therefore for their great partnership.

I would like to acknowledge my colleagues from the UNL Insect Toxicology Lab: Adriano Pereira, Matheus Ribeiro, Dr. Neetha Nanoth; Natalie Matz and temporary workers: Matt Bond, Albina Divizinskaya, Nathalia Divizinskaya, Thais Moreira and Ethan Siegfried for their assistance in my research work, and for their great friendship. Special thanks to Bruce Koehler and
Carolina Camargo for helping with statistical analysis, Dr. John Wang for protein quantification analysis assistance, Dr. Ana Maria Vélez for providing most of the knowledge of fall armyworm biology and rearing, and Terence Spencer for the immense help in the bioassay techniques and for heartening with his adorable personality.

I would like to thank the Entomology faculty and staff members for their kindness and encouragement. Special thank to Dr. John Foster, Dr. Gary Brewer and Dr. Stephen Danielson for the great mentorship, and Jeri Cunningham, Marilyn Weidner and Marissa Kemp for the bureaucratic assistance needs and their charisma.

I would like to demonstrate my colossal gratitude to the students, exchange students and alumni from the Entomology department for granting me a great work environment, for their encouragement, patience and advices that contributed to my endurance during the coursework. Distinctively to Lia Marchi-Werle, Camila Oliveira-Hoffman, Thais Barros, Ashley Yates, Laramy Enders, Daniele Pinheiro, Jessica Putney Jurzenski, Zach Rystrom and Justin McMechan.

I also want to acknowledge my undergraduate advisor Dr. Edson L. L. Baldin, for providing the first step into the Entomology area and for his great professionalism and friendship.

I would like to show my immeasurable gratefulness to my family, especially to my parents Barbara A. A. Silva and Ronaldo M. F. da Silva for their support, boost and inspiration during my entire journey. Thank you.
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CHAPTER 1: Introduction

Fall armyworm

The fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith, 1797), is native to the tropical regions of the western hemisphere from the United States to Argentina. *Spodoptera frugiperda* is an important pest of maize (*Zea mays*) and many other crops throughout the Americas, remaining one of the most common lepidopteran pests in the United States. Fall armyworm has no diapause mechanisms and overwinters in southern Florida and Texas-Mexico where hosts are continually available and temperatures below 50°F are rare (Sparks 1979). Larvae cause damage by consuming foliage, and they are most numerous in late summer or early fall, with damage reported beginning in late July or early August, and occasionally causing severe outbreaks as early as mid-April (Flanders *et al.* 2007). They are strong fliers, and disperse long distances, moving northward into the eastern and central regions of United States and into southeastern Canada during spring and early summer migrations (Mitchell 1991, Capinera 1999).

The life cycle is completed in about 30 days during the summer, but can reach 80 to 90 days during the winter. The egg is dome shaped, and the number of eggs per mass varies considerably but is often 100 to 200. Total egg production averages about 1500 with a maximum over 2000 per female, with most production of eggs during the first to five days of life (Capinera 1999). Duration of the egg stage is only two to three days during the summer months. The larval stage has usually six instars with duration of about 14 days during the summer and 30 days during cooler weather. Pupation normally takes place in the soil at a
depth 2 to 8 cm. The pupa is reddish brown in color, and the duration is about eight to nine days during summer, but 20 to 30 days during the winter in Florida. Adults are nocturnal, and are most active during warm and humid evenings (Capinera 1999).

This species displays a very wide host range, with 80 plants recorded. In the United States, fall armyworm is responsible for substantial economic damage, primarily in grasses, including field and sweet corn, *Zea mays* L., sorghum, *Sorghum vulgare* Pers., and several turfgrass varieties (Sparks 1979, Foster 1989, Capinera 1999). Larvae of *S. frugiperda* cause damage by consuming foliage; initially on the leaf surface from one side, leaving the opposite epidermal layer intact (Capinera 1999). Larval densities are usually reduced to one to two per plant when larvae feed in close proximity to one another because of cannibalistic behavior. The larvae also will burrow into plant growing point, such as whorl and bud, destroying the growth potential of plants (Capinera 1999).

**Management of fall armyworm and field evolved resistance**

Detecting fall armyworm infestations before they cause economic damage is the key to their management. Moth populations can be sampled with black light traps and pheromone traps, the latter being more efficient. Once moths are detected it is advisable to search for eggs and larvae (Capinera 1999). If infestations are detected too late, the damage may already have occurred (Flanders et al. 2007). Other strategies have been used to manage fall armyworm including cultural practices, enhancement of natural enemies, conventional Bt
insecticides, and transgenic crops (corn and cotton) that express toxins from *Bacillus thuringiensis* (Capinera 1999, Entwistle *et al.* 1993, Knutson 2008, Siebert *et al.* 2008a, 2008b). Cultural practices employed include early planting in the southern states, use of early maturing varieties, early harvest, planting of tolerant varieties and crop rotation. Insect parasites such as wasps and flies, ground beetles, and other predators can help suppress armyworm numbers (Flanders *et al.* 2007). Diseases such as insect viruses, including nuclear polyhedrosis virus (NPV), and fungi can also be important. Conventional insecticides used against fall armyworm are primarily pyrethroids, methomyl and carbaryl (Capinera 1999 and Knutson 2008). However, chemical control strategies are inconsistent and often unsatisfactory to control *S. frugiperda* in field corn due to their movement into whorl region of the plant where insecticide sprays cannot reach. Widespread insecticide resistance has aroused the search for alternatives to conventional insecticides. Hence, insecticides derived from *Bacillus thuringiensis* (Bt) are becoming increasingly important for pest management (Entwistle *et al.* 1993) and for controlling some key pests, thereby reducing the dependence on chemical insecticide applications.

The most recent strategy to control fall armyworm is the use of Bt transgenic corn and cotton (Siebert *et al.* 2008a, 2008b). In order to induce an effective response, Bt toxins need to be ingested by the insects, solubilized and enzymatically processed by proteinases in the midgut (Schnepf *et al.* 1998).

*Bacillus thuringiensis*, a gram-positive soil bacterium, is well known for its ability to produce crystalline inclusions during sporulation that contain
insecticidal proteins called δ- endotoxins. The most common Bt toxins used against *S. frugiperda* are Cry1Ab and Cry1F expressed in cotton and Cry1F in corn. A number of studies have demonstrated that corn and cotton hybrids containing Cry1F provide better fall armyworm control than corn hybrids producing Cry1Ab or cotton varieties containing Cry1Ac alone (Buntin 2008, Stewart *et al.* 2001, Waquil *et al.* 2002). The vegetative stages of bacterial growth before the sporulation produce non-δ-endotoxins with insecticidal activities, which include the vegetative insecticidal protein Vip3A, and represent a novel insecticidal agent, with efficacy against lepidopterans such as black cutworm, fall armyworm, beet armyworm and tobacco budworm (Estruch *et al.* 1996). Vip3Aa has been shown to kill larvae of susceptible insects by a series of steps that resemble those caused by Cry proteins in their mode of action. Vip3Aa is secreted from *B. thuringiensis* cell as protoxin, which is partially processed by proteases in the larva midgut rendering the active toxin (Yu *et al.* 1997, Lee *et al.* 2003). This toxin then binds to specific receptors in the midgut membrane, which are different from those of Cry proteins (Lee *et al.* 2003, 2006, Sena *et al.* 2009, Abdelkefi-Mesrati *et al.* 2009, Liu *et al.* 2011). For conferring a different mode of action compared to Cry toxins, Vip insecticidal represent a promising alternative for insect management and improved resistance management (Lee *et al.* 2003).

While transgenic plants offer many unique opportunities for the management of *S. frugiperda* populations, they also present new challenges. Perhaps the greatest ecological challenge is the potential for the rapid evolution of resistance in pest populations to the toxins. Depending on the level of the toxin
expression in the plant and the level of exposure of insect population to the crop, simulations suggest that pest populations could evolve resistance in as little as 1-2 years under worst case conditions (Gould et al. 1997, Roush and Osmond 1997). The introduction of transgenic maize lines event TC1507 (Herculex® I insect protection technology by Dow AgroSciences and Pioneer Hi-Bred International) expressing Cry1F protein provided a new opportunity to manage S. frugiperda. This product was launched in the United States and Canada in 2003, Argentina in 2005, Colombia in 2006, and Honduras and Brazil in 2009. In 2006, reports of potential resistance to TC1507 maize in Puerto Rico were received (Storer et al. 2010). Subsequent investigation confirmed that pest populations collected from several sites in Puerto Rico were largely unaffected by the Cry1F protein in bioassays, with resistance ratios likely in excess of 1000 (Storer et al. 2010). The resistance was shown to be autosomally inherited and highly recessive and the resistant insects were only moderately less sensitive than a laboratory susceptible population to Cry1Ab and Cry1Ac (Storer et al. 2010, Vélez et al. 2013). In addition to Puerto Rico, resistance of S. frugiperda to Cry1F was recently reported in Brazil and United States. In Brazil the event TC1507 maize was launched in 2008 and commercially available for the 2009/2010 crop season. Similar to Puerto Rico, most of the Brazilian agriculture is in a tropical climate, where in some regions the maize is grown in intensive system of production, which allows maize production entire year. In 2011, after reports of reduced effectiveness of this Bt maize event in some areas of Brazil, S. frugiperda neonates were collected from damaged TC1507 maize fields in western Bahia
state. Further studies indicated that this population was able to survive on Cry1F maize plants under laboratory conditions and subsequently produced normal adults (Farias et al. 2014). Significantly reduced efficacy to Cry1F maize has been seen in the southern region of United States as well, and further investigations based on F1 and F2 screens revealed the presence of Cry1F resistant alleles (Vélez et al. 2013, Huang et al. 2014). Sumerford et al. (2012) recommend that detection of resistant individuals in the field or laboratory is not equivalent to field-evolved resistance. It is necessary to determine stability, cause and geographical extent of resistance using an extensive monitoring program (EPA, 1998).

**Managing insect resistance to Bt crops**

One of the strategies for delaying insect resistance to transgenic plants recommended by the US Environmental Protection Agency (EPA) is the “High Dose/Refuge Strategy” (Gryspeirt and Grégoire 2012). It requires planting “refuge zones” composed of non-Bt plants suitable for the target pest and in close proximity to a “Bt zone” with a high concentration of Cry toxin. Moreover, Bt plants expressing a high concentration are an important condition for this strategy (Gryspeirt and Grégoire 2012). The high dose strategy assumes that the relatively large number of susceptible pests produced in refuges will mate with the few resistant homozygotes that emerge in the transgenic crop. The heterozygotes produced will likely be killed by the high dose expressed in the transgenic plants, slowing the evolution of the resistance. The most direct way to test the high-dose hypothesis is to let resistant and susceptible adults mate in the laboratory and
measure survival of their hybrid progeny on \textit{Bt} plants (Tabashnik 2008). In this manner, the EPA guidelines for a high dose specify that \textit{Bt} plants should kill at least 99.99\% of susceptible insects in the field. The refuge strategy would fail in all cases where the inheritance of the resistance is not completely recessive and the concentration of Cry protein is low (Tabashnik 2008). Two other aspects that should be considered are the initial frequency of the resistance allele. The frequency of resistant homozygotes must be low enough to make it extremely unlikely that two resistant individuals could mate, and the random mating must reflect the behavior of the insects in migrating from the refuge to any part of the \textit{Bt} crop and vice versa (Ferré \textit{et al.} 2008). An important concern for this strategy is larval movement from plant to plant, which can favor the selection of resistance if caterpillars feed on a transgenic plant and then migrate to a non-transformed plant before ingesting a lethal dose of Cry protein. Insects with low levels of resistance could escape from the selection and mate, combining resistant genes and conferring higher levels of resistance to the offspring (Cohen \textit{et al.} 2008).

Refuge size and placement are important considerations for this strategy in order to maximize random mating between potentially resistant moths from \textit{Bt} corn fields and refuge moths (Siegfried and Hellmich 2012). Seed mixtures, which consists of \textit{Bt} and non-\textit{Bt} seeds mixed in the same bag, are convenient for growers to plant and avoid size and placement concerns for maximizing insect random mating, but larvae movement from susceptible plant to transgenic and vice versa can be a potential problem (Davis and Onstad 2000).
The strategy called “Pyramided Plants” involves the expression in the same plant of more than one biochemically distinct toxins, which is becoming an important part of the Bt market and a more common resistance management strategy. According to Tabashnik (1989), for a successful implementation of this strategy, several criteria are required such as high mortality for each component of the mixture alone, low probability of high levels of cross-resistance, some of the population are untreated and the pesticides should have more or less equal persistence. Also, similar to high-dose/refuge strategy, this option requires low frequency of resistance alleles. Additional modes of action against lepidopteran pests have been developed simultaneously to Cry1Ab and Cry1Ac (Storer et al. 2012). A variety of options of pyramided plants were released in the last years that might contain a mixture of previous and novel insecticides, such as Agrisure®VipteraTM 3220 presenting Vip3A, Cry1Ab and Cry1F toxins in the same plant (Storer et al. 2012). The pyramided hybrids containing these proteins, regardless of the parental sources of genes for event being male or female, provided significant control of three major lepidopteran pests Helicoverpa. zea, Ostrinia. nubilalis and S. frugiperda (Burkness et al. 2010).

In general, combinations of multiple, complementary toxins will allow GM maize to protect against several arthropod pests and improve resistance management, delaying resistance with smaller and more acceptable refuge sizes (Roush 1998).
Insect resistance monitoring to Bt

Resistance monitoring for Bt plants has been required by the US Environmental Protection Agency (EPA) for the registration process since 1996. Companies that commercially release these products must conduct an annual resistance-monitoring program that requires field collection of insects, laboratory bioassays and reporting the results back to the agency (Shelton and Zhao 2009). This is an important engine of Insect Resistance Management programs and contains the regular assessment of target pest populations from areas where the risk of resistance evolution is high. The management of resistant pests would be easier if they could be detected before their frequencies become unmanageable (Yu 2008). A goal of resistance monitoring is to determine baseline levels of susceptibility to transgenic insecticides of target pest populations from appropriate geographical areas so that changes in the frequencies of resistance alleles can be detected and to document control failures due to resistant insects. These data provide insights into the natural variation among pest population in that geographical range and can be used to assess future shifts in susceptibility to the proteins in the transgenic crops (Caprio and Sumerford 2007, Sivasupramaniam et al. 2007). A consequence of established baseline susceptibility is that field resistance is more likely to be based on a measure of deviation from the baseline (Sumerford et al. 2012).

A useful tool for detection and documentation of insecticide resistance is the development of accurate and reliable bioassays techniques. Bioassays of insecticide toxicity involve in vivo assays of living insects involving insecticide
exposure (ffrench-Constant and Roush 1990). Such tests usually measure samples from field-populations and determine the response of the progeny to the toxin in a laboratory bioassay. Assessment of insecticide resistance has traditionally involved complete concentration-mortality tests requiring 4-5 doses of insecticide that produce 10-90% mortality. Resistance is expressed by the ratio of the LC$_{50}$ or LC$_{95}$ of the most tolerant strain divided by the most susceptible (Halliday and Burnham 1990). Decreased susceptibility is typically demonstrated as significant increase in toxin concentration killing 50% (LC$_{50}$) of the insects (Tabashnik 2008). Additional response criteria used for in vivo assays can be a complement to access the sensitivity to Bt toxins (Marçon 1999). For instance, the evaluation of larval growth inhibition assays use sublethal concentrations of proteins and are considered to be more sensitive than dose-response mortality assay (Caprio and Sumerford 2007).

An alternative to traditional dose-mortality involves tests based on diagnostic or discriminating doses or concentrations. These techniques offer more efficiency in detecting changing in susceptibility because all individuals are tested at an appropriate dose, where percentage of mortality is not informative (Halliday and Burnham 1990). However, if no information is available on the susceptibility of different phenotypes for a particular bioassay technique, complete concentration-mortality tests are still a necessary prerequisite to developing diagnostic/discriminating bioassays (Brewer and Trumble 1989). In addition, diagnostic concentration tests require fewer individuals and less time. In order to determine the best diagnostic dose, the extrapolation from work on other species
is sometimes used as an indicator of the level of resistance (Halliday and Burnham 1990). When extrapolating between laboratory and field strains, the results gathered should be carefully related, because variation in susceptibility measured in laboratory does not mean there is a resistance situation in the field (Sumerford et al. 2012). Unfortunately, the genetics and toxicology of resistance are understood for few species and the use of the diagnostic bioassays to monitor resistance might not be wise if that resistance is not well understood (Halliday and Burnham 1990).

The main pests targeted by Bt crops have been monitored for the evolution of resistance to maximize the effectiveness of the technology through time (Siegfried et al. 2007). When susceptibility to the toxin decreases, resistance-monitoring programs must be conducted in association with a realistic resistance management plan that would be implemented in case resistance development is identified (Siegfried et al. 2007).

**Bioassay variation to Bt**

Estimating the variation in susceptibility that is naturally present is prerequisite to detect biologically important changes in key pests (Siegfried et al. 2007). Among the different insecticide bioassays, the diet overlay for feeding bioassay is one of the most commonly used bioassays for resistance monitoring and consists of a sample of insecticide which has been diluted to the correct concentration and been pipetted onto the surface of solidified diet. The advantages are that it provides quick and easy application and uses significantly less toxin
(Sivasupramaniam et al. 2007). The disadvantage can be uneven distribution of the toxin on the diet surface leading to non-uniform treatment and inconsistent exposure of larvae (Siegfried et al. 2007).

In commodity treatment experiments, misunderstandings by regulatory agencies can occur because of failure to define natural variation of the exotic pest in response to a treatment used for purposes of exclusion (Robertson et al. 1994). These variations result from numerical differences in response that is detected each time a bioassay is repeated with one genetic strain, either within a single generation or >1 generation (Robertson et al. 1994). Natural variation is relevant to other types of practical problems. In repetitive tests with insect colonies maintained to test pesticide efficacy, failure to define natural variation for a population can cause concern about chemical efficacy in population surveys (Robertson and Stock 1985). Many baseline studies of susceptibility to Bt proteins for organisms that are not highly susceptible report great variation among populations in LC50 values. Bernardi et al. (2014) conducted studies of baseline susceptibility and monitoring of Brazilian populations of S. frugiperda to the Vip3Aa20 insecticidal protein. The LC50 ranged from 92.38 to 611.65 ng Vip3Aa20/cm² (6.6 fold variation) for 16 populations collected in different regions of the country. Marçon et al. (1999) also tested populations of Ostrinia nubilalis to Cry1 Ab and they found significant differences (P <0.05) in susceptibility among some of the populations tested, and differences between the most susceptible and most tolerant populations were 4- and 6-fold at the LC50, and LC95 respectively.
Different laboratories rely on different standard methods of mortality bioassays and the variations in bioassays are not very well understood. These susceptibility variation concerns above could be extended to other sources that might cause variation in mortality bioassay response.

Crespo et al. (2008) reported that standardization of toxin preparations derived from *Bacillus thuringiensis* (Berliner) used in laboratory bioassays is critical for accurately assessing possible changes in the susceptibility of field populations of target pests. They reported that SDS-PAGE/densitometry may improve data consistency in monitoring efforts to identify changes in insect susceptibility when compared to the other quantification methods of Cry toxins.

Chakroun et al. (2012) tested mortality of *S. frugiperda* after 7 and 10 days exposure to Vip3A toxin in different preparations and activation conditions on insecticidal activity. The LC$_{50}$ for “functional mortality”, or the number of dead larvae plus larvae arrested at L1 evaluation, measured at 7 days was significant lower than the mortality. The LC$_{50}$ value of trypsin-activated samples was approximately 9-fold lower in functional mortality than for mortality. Scoring mortality after 10 days had a marked effect on the LC$_{50}$ values, which were considerably lower than those at 7 days and with narrower fiducial limits.

When testing the susceptibility in different days of the cycle of diamondback moth Robertson et al. (1995) observed a toxicity ratio of LC$_{50}$ was 3.7, with the highest LC$_{50}$ value of 0.66 mg ml$^{-1}$. The highest upper 95% CL of a toxicity ratio was 10.2, which they interpreted to be the upper limit of natural
variation. On the basis of the ratio tests, only six of the LC$_{50}$ were not significantly different from the lowest value.

The diet used on the bioassays can also affect the responses. The effects of raw or heat-denatured soybean flour in an artificial diet on the detection of Cry1Ac resistance in *Helicoverpa armigera* were examined (Gunning and Moores 2009). Resistant neonate larvae reared on denatured soybean flour diet showed resistance factors of 7980 and 16,901 at the LC$_{50}$ and LC$_{99}$ levels, respectively. By comparison, resistance could not be detected in neonate larvae reared on raw flour diet. Third instar larvae reared on denatured flour diet showed resistance factors of 322 and 21,190 at the LC$_{50}$ and LC$_{99}$ levels. Resistance was not detected in third instar larvae reared on raw flour diet. There was 68% survival of resistant neonate larvae on Bollgard II® cotton leaf feeding assays, compared to 100% mortality in a susceptible strain. The authors concluded that detection of Cry1Ac resistance in *H. armigera* from Australia can be masked if an artificial diet gives chronic exposure to potent, protease inhibitors present in raw soy flour (Gunning and Moores 2009).

Besides the natural variation, variation in susceptibility due to other laboratory conditions is critical to control or standardize all conditions. Understanding the accuracy and reproducibility of a bioassay method is essential to its downstream interpretation.
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Research objectives

The launching of new technologies in insect resistance transgenic crops has increased concern of field evolved resistance in target pests. The fall armyworm (FAW), Spodoptera frugiperda, is a tropical insect pest in corn, mainly in subtropical and tropical regions of United States, where it overwinters. Despite the documentation of resistance of FAW to Cry1F toxin and indications of decrease of susceptibility in southern areas of US territory, the Cry1F Bt protein is still largely commercialized and efficacious. The first objective of this research was to establish a U.S. FAW baseline susceptibility to Cry1F Bacillus thuringiensis insecticidal protein and determining the inter and intra population variation in FAW susceptibility to Cry1F, emphasizing collections from geographic locations where FAW overwinters in the U.S., and locations exhibiting different migration origins, and hosts.

Along with the determination of baseline susceptibility to FAW through mortality bioassay studies, variation in susceptibility in insect monitoring bioassays have been observed in different laboratories using different proteins and across different orders of insects, which can influence the interpretation of those results. Therefore, the second objective of this research was to detect possible sources of variation in susceptibility estimates by examining pre-treatment laboratory conditions which will expose neonates to different treatments prior to the bioassay and that can contribute to variations in bioassay response.
CHAPTER 2: Susceptibility of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) field populations to the Cry1F *Bacillus thuringiensis* insecticidal protein.

**Introduction**

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith), is one of the most important lepidopteran pests in the United States. It is native to the tropical regions of the western hemisphere from the United States to Argentina and is an important pest of corn (*Zea mays* L.) and many other crops throughout its distribution (Sparks 1979). The fall armyworm is a migratory pest and does not diapause (Luginbill 1928). Because it does not survive prolonged freezing, annual infestations affecting most of North America result from migrants that fly north from southern Texas and Florida where winter temperatures are mild and host plants are continuously available (Nagoshi *et al.* 2012). This species displays a very wide host range but prefers grasses, including corn, sorghum (*Sorghum vulgare* Pers.), and several turf grass varieties (Sparks 1979, Capinera 1999).

Transgenic corn and cotton (*Gossypium hirsutum* L.) that express genes from *Bacillus thuringiensis* Berliner (Bt) encoding insecticidal proteins to control specific target pests have been widely deployed in the United States and globally since 1996 (Storer *et al.* 2010). The introduction of the transgenic maize event TC1507 (Herculex® I insect protection technology developed jointly by Dow AgroSciences and Dupont Pioneer), which expresses Cry1F protein, has provided a new opportunity to manage *S. frugiperda* populations. This product was
launched in the United States and Canada in 2003, Argentina in 2005, Colombia in 2006, and Honduras and Brazil in 2009. In 2006, potential resistance to TC1507 maize in Puerto Rico was first reported and populations collected from several sites were largely unaffected by the Cry1F protein in bioassays, with resistance ratios in excess of 1,000 (Storer et al. 2010). Resistance to Cry1F was shown to be autosomally inherited and highly recessive (Storer et al. 2010, Vélez et al. 2013).

In addition to Puerto Rico, resistance of *S. frugiperda* to Cry1F has been recently reported in Brazil (Farias et al. 2014). The event TC1507 maize was launched in Brazil in 2008 and commercially available for the 2009/2010 growing season. Similar to Puerto Rico, most Brazilian agriculture occurs in a tropical or subtropical climate, allowing maize production year round. In 2011, *S. frugiperda* neonates were collected from damaged TC1507 corn fields in western Bahia after reports of reduced effectiveness of this trait. Results of this study showed that this population was able to survive on Cry1F maize plants under laboratory conditions and subsequently produced normal adults (Farias et al. 2014).

High resistance ratios and the presence of Cry1F resistant alleles have also been reported in some populations from the Southern U.S. (Vélez et al. 2013, Huang et al. 2014). Huang et al. (2014) reported significantly reduced efficacy of Cry1F maize in fields from Florida, Louisiana and North Carolina where some of the field populations collected from non-Bt corn and from unexpectedly damaged Bt corn plants exhibited approximately 85-fold resistance. Further investigations based on F₁ and F₂ screens revealed the presence of Cry1F resistant alleles among
populations from Florida, Louisiana and Texas (Vélez et al. 2013, Huang et al. 2014). Additionally, recent findings suggest that fall armyworm populations from Puerto Rico have significant genetic exchange with populations in Florida and that there are migratory patterns involving substantial genetic exchange with the U.S. continental regions (Nagoshi et al. 2010, Nagoshi et al. 2012). Such genetic exchange with Puerto Rico may result in the introduction of resistance alleles into Florida.

The possibility of resistance development in fall armyworm highlights the need for effective resistance monitoring programs that allow early detection of resistance and implementation of appropriate management decisions (Dennehey 1987). The first step in such programs involves establishing baseline susceptibility among geographically distinct populations (Marçon et al. 1999). The objectives of this study were to establish baseline susceptibility of U.S. *S. frugiperda* populations to the Cry1F Bt insecticidal protein, and to determine the inter- population variation in Cry1F susceptibility, emphasizing collections from areas where fall armyworm overwinters, and that have been reported to have different origins and hosts (Nagoshi et al. 2012).

**Materials and methods**

*Field collections and rearing*

Eleven field collected populations of fall armyworm were obtained from cooperators across the southern of the U.S. (Table 1). Populations were collected from either non-Bt corn or other grass species in 2012 and 2013 and from
overwintering areas in Florida and Texas. Additionally, a migratory fall armyworm population was collected in Iowa. Field collections were delivered overnight to Custom Bio-Products, Maxwell, IA, or DM Crop Research, Polk City, IA, where the collections were maintained until egg mass production. The eggs were delivered overnight and used as a source of 1st instars for bioassay at the University of Nebraska Insect Toxicology Lab (Lincoln, NE). A susceptible strain was purchased from BioServ (Frenchtown, NJ) which has been reared continuously since November 1997 with regular screenings to monitor for any changes in insecticide susceptibility. Adults were placed in 31 x 23 cm hermit crab cages (Florida Marine Research, Sarasota, FL) with adult diet placed in a cotton pad inside of the bottom of a 100 x 15 mm petri dish (Fisherbrand, Waltham, MA) and replenished daily. Adult diet consisted of stale beer, containing ascorbic acid (1.5 mg/ml) propionic acid (2.1 µl/ml) and aureomycin (0.5 mg/ml) (Vélez et al. 2013). Adults were held in an environmental chamber with a photoperiod of 14L: 10D at 27±1 °C and relative humidity of 75±10% during photophase and at 22.5±1 °C and relative humidity of 60±10% during scotophase. Adults were allowed to mate and eggs were oviposited on wax paper that surrounded the cage.

For field collected populations, pupae were placed in 30 x 32 x 61 cm wired cages (Custom Bio-Products, LLC, Maxwell, IA) and allowed to emerge with diet placed in a cotton pad inside of the bottom of a 2 oz portion container (Dart Brand, Iowa-Des Moines Supply, Inc., Des Moines, IA). Adult diet consisted of stale beer and was replaced every other day. Adults were allowed to mate and lay eggs on wax paper. Eggs were harvested daily and placed in 1 qt.
food storage bags (Glad Brand) with moistened filter paper and held at 10°C until shipping. Larvae were reared on multispecies lepidopteran diet (Southland Products, Lake Village, Arkansas). For colony increase, 2 neonate larvae were placed in each of up to 300 - 1 oz. translucent polystyrene soufflé portion cups (Iowa-Des Moines Supply, Inc., Des Moines, IA) with 7 ml of diet to minimize cannibalism. Pupation occurred within the cups. Pupae were transferred twice weekly to mating cages for adult emergence and egg production. Adults were held in an environmental chamber with a photoperiod of 15L: 9D at 30±1°C and relative humidity of 70±10% during photophase and at 20±1°C and relative humidity of 60±10% during scotophase. And larvae were held in 24h scotophase at 26±1°C and relative humidity of 65±10%. Eggs were harvested daily and neonates obtained from field-collected parents were considered the F_1 generation and were used in most bioassays. In populations with lower production of eggs, neonates from the F_2 and F_3 generations were also used in some bioassays.

*Bt toxins*

The Cry1F used in diet bioassays was expressed in BtG8 cells grown in CYS2 media with tetracycline for 6 days at 30°C. Cells were harvested by centrifugation and the pellets were washed 5 times with 0.5 M NaCl and twice with water. Washed pellets were stored at -20°C. Pellets were lysed with 50mM sodium carbonate pH 11.7, containing 10mM DTT overnight at 4°C. Aliquots of ~1.6mg and 40mg were flash frozen in liquid nitrogen and then lyophilized.

Toxin preparations were quantified by gel electrophoresis and densitometry (Crespo *et al.* 2008) and adjusted to 0.8mg/ml based on the 60-65
kDa peptides observed after sodium dodecyl sulfate-polyacrylamide gel electrophoresis and compared to a standard curve prepared with a bovine serum albumin standard (>95% purity). The quantified preparations were stored at -80°C.

**Bioassays**

Bioassays were performed based on the methodology described in Marçon *et al.* (1999) in 128-well bioassay trays (CD International, Pitman, New Jersey). One ml of European corn borer wheat germ-based diet (Lewis and Lynch 1969) was dispensed into each well and allowed to solidify. Seven concentrations of the toxin were used for LC_{50} determinations. Dilutions were made in 0.1% Triton-X 100 non-ionic detergent to obtain uniform spreading on the diet surface. Each well was surface treated by applying 30 µl of 0.1% Triton-X 100. The negative control wells were treated with 30 µl of 0.1% Triton-X 100 (Vélez *et al.* 2013).

The treated wells were allowed to dry and one randomly selected neonate (unfed and <12 hours after hatching) was transferred to each well using a fine camel hair paint brush. The wells were covered with vented lids (BIO-CV-16, C-D International), and trays were held at 27°C, 24 h scotophase, and 80% RH. Mortality and group larval weights were recorded 7 days after infestation. Larvae that had not grown beyond first instar and weighed ≤0.1mg were considered dead. Therefore severe growth inhibition and death were considered as mortality. In each experiment bioassays were ideally replicated six times for each strain depending on availability of neonates, with 16 larvae per concentration (total of 96 larvae per concentration).
Statistical analysis

Concentration-mortality data was analyzed using probit analysis (Finney 1971) and POLO-PC (LeOra Software 1987) to estimate LC$_{50}$’s and LC$_{90}$’s with their respective 95% confidence intervals, slopes and standard errors.

Sensitivity ratios were calculated using the concentration-response statistics based on mortality, by the ratio of LC$_{50}$ of the field population and susceptible strains. These values were considered significant if the 95% confidence limit (CL) of the ratio did not include 1.0 (Wheeler et al. 2006). The confidence intervals for each ratio were calculated based on the intercepts and slopes of two probit lines and estimates of their variance-covariance matrixes (Robertson and Priesler 2007). Larval weights were transformed to percentage growth inhibition relative to the controls, and these data were analyzed by nonlinear regression (PRONLIN, SAS 9.4) fitted to a probit model (2003-2012 SAS Institute Inc., Cary, NC, USA).

Results

Mortality assays

Results of Cry1F bioassays for fall armyworm field populations collected in 2012 are presented in Table 2. LC$_{50}$ values and the respective confidence intervals ranged from 8.32 (6.86-9.95) (Muleshoe, TX) to 14.53 (11.48-18.12) ng cm$^{-2}$ (Lubbock, TX) in 2012. The LC$_{50}$ of the susceptible laboratory population in 2012 was 2.89 (2.39-2.45) ng cm$^{-2}$. For 2013 collections the LC$_{50}$ values ranged from 3.61 (2.73-4.65) (Bradenton, FL) to 22.11(13.02-36.84) ng cm$^{-2}$ (Palm
Beach, FL), while the LC$_{50}$ of the susceptible laboratory population was similar to the results of 2012, 2.79 (2.39-3.26) ng cm$^{-2}$. Differences in Cry1F susceptibility between the most susceptible and the most tolerant field populations were 2- and 6-fold for 2012 and 2013, respectively. The slopes of the concentration- mortality regressions were similar between field collected populations in both years, but slightly higher in laboratory colonies.

The sensitivity ratios indicated that most of the field collected populations tested in this study exhibited a significant reduced susceptibility Cry1F relative to the laboratory populations tested. The highest LC$_{50}$ ratio in both years of the study was for the Palm Beach Co., FL population, 7.64 (5.93-9.85). In contrast, the only field population tested that was not significantly different from the laboratory population [LC$_{50}$ ratio, 1.25 (0.97-1.61)] was Bradenton, FL collected in 2013.

_Growth inhibition assays_

Results regarding growth inhibition of _S. frugiperda_ treated with Cry1F are presented in Figs. 1 and 2. EC$_{50}$ and the respective confidence interval values ranged from 0.10 (0.07-0.14) (Muleshoe, TX) to 0.48 (0.37-0.60) ng.cm$^{-2}$ (Lubbock, TX), while the EC$_{50}$ of the susceptible laboratory population was 0.33(0.32-0.34) ng.cm$^{-2}$. EC$_{50}$ values ranged from 0.10 (0.08-0.12) (Polk County, IA) to 0.29 (0.23-0.34) ng.cm$^{-2}$ (Palm Beach, FL) in 2013 studies, while in the susceptible laboratory population was 0.41(0.40-0.42) ng.cm$^{-2}$. The range of variation in susceptibility indicated by growth inhibition between the most susceptible and the most tolerant populations was approximately 5- and 3-fold for the 2012 and 2013 studies, respectively. The pooled data for mortality and growth
inhibition for each year illustrates that growth inhibition provides a more sensitive estimate of susceptibility than mortality for *S. frugiperda* field populations (Fig. 3), indicating that larvae are responding to concentrations of Cry1F that do not necessarily cause mortality.

**Discussion**

Differences in susceptibility were observed among *S. frugiperda* populations exposed to Cry1F toxin and ranged from 2- and 6-fold among field populations. Baseline studies for *B. thuringiensis* susceptibility involving target species, especially *S. frugiperda*, are generally lacking (Luttrell *et al.* 1999), although similar variation (between 3- to 8-fold) has been observed in other lepidopteran species (Marçon *et al.* 1999, Stone and Sims 1993, Blanco *et al.* 2008). Estimates of lethal concentrations (LC’s) and effective concentrations (EC’s) based on growth inhibition exhibited similar variation although in general the EC$_{50}$ based on growth inhibition was generally lower than the LC$_{50}$ indicating that Cry1F causes sublethal effects to growth and development in *S. frugiperda*. Interestingly, the EC$_{50}$ values were slightly higher for the laboratory populations relative to the field populations in contrast to the LC$_{50}$ data where the laboratory populations were consistently the most susceptible of all populations tested. These results might suggest possible adaptation to long term rearing on artificial diet allowing increased larval growth even in the presence of the Cry1F toxin.

Previous studies that have specifically measured *S. frugiperda* susceptibility to Cry1F have shown high rates of survival to Cry1F expressing
corn hybrids in the field and relatively high frequency of resistance alleles in certain populations from Florida (Vélez et al. 2013, Huang et al. 2014). In addition to Florida, resistant alleles have been detected in Louisiana and Texas (Vélez et al. 2013, Huang et al. 2014) although at lower frequencies. The higher frequency of resistance alleles observed in Florida is limited to certain counties (Vélez et al. 2013) and may suggest that there is local selection from exposure to Cry1F expressing hybrids. These results are consistent with the reduced susceptibility of the Palm Beach, Florida populations bioassayed in 2013 which was the least susceptible of all populations assayed. In contrast to the higher LC\textsubscript{50} in this population, our results did not show any general pattern of reduced susceptibility in other populations. The reduced susceptibility of the Palm Beach population may be the result of increased use of Cry1F corn and increased selective pressures in localized areas since other populations assayed from Florida did not suggest a difference from the overall baseline.

Farias et al. (2014) reported field-evolved resistance of fall armyworm to Cry1F in populations from Brazil with resistance ratios >5000-fold in diet overlay bioassays. However, comparisons of the frequency distribution of haplotypes using polymorphism in the mitochondrial CO I gene reveled that corn-strain populations from Brazil identified as being resistant to Cry1F were different from corn-strain populations found in Florida (Nagoshi et al. 2007). Moreover, Florida populations are more closely related to populations from Puerto Rico (Nagoshi et al. 2010). Therefore, the results of the present study provide additional support for increasing tolerance in certain populations of \textit{S. frugiperda} because of substantial
gene exchanges with Caribbean island populations that in combination with localized selection pressure may result in increased tolerance of some populations. However, in general the overall variation of LC and EC estimates observed among overwintering populations suggests that most populations are still susceptible to Cry1F. In addition, the susceptibility of the one migratory population from Iowa was similar in susceptibility to the other populations sampled.

The current study provides important information of the status of susceptibility of fall armyworm to Cry1F toxin in migratory populations to northern regions of the United States.

Annual resistance monitoring programs for target pest species using laboratory bioassays are an important component of the insect resistance management (IRM) programs (Shelton and Zhao 2009). The regular assessment of susceptibility of target pest population from areas where the risk of resistance evolution is high should allow resistance to be detected before resistance frequencies become unmanageable (Yu 2008). Additionally, it is ponderous to establish a baseline susceptibility study to integrate monitoring studies, as a quick and effective way to access the genetic variability of target insects, in order to understand migratory patterns, preferred hosts and other factors associated with geographically distinct landscapes.
Acknowledgements

We thank UNL toxicology laboratory for the bioassays assistance. Bruce Lang, Vicky Lang and Daniel Moellenbeck for rearing field collection populations and the cooperation with shipments. The several cooperators in the south of U.S. for coordinating the field population collections. Dupont Pioneer for providing the Cry1F protein material.
References


Nagoshi, R. N., Meagher, R. L., and D. A. Jenkins. 2010. Puerto Rico fall armyworm has only limited interactions with those from Brazil or Texas but could have substantial exchanges with Florida populations. J. Econ. Entomol. 103: 360-367.


**Tables**

*Table 1.* Source description of *Spodoptera frugiperda* populations used to establish baseline susceptibility to Cry1F from *B. thuringiensis*.

<table>
<thead>
<tr>
<th>Location</th>
<th>Year of Collection</th>
<th>Generation</th>
<th>Month of Collection</th>
<th>Host Plant</th>
<th>Initial larvae number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bradenton, FL</td>
<td>2012</td>
<td>1</td>
<td>July</td>
<td>Sweet corn</td>
<td>340</td>
</tr>
<tr>
<td>Lubbock, TX</td>
<td>2012</td>
<td>1&amp;2</td>
<td>June/July</td>
<td>Corn</td>
<td>309</td>
</tr>
<tr>
<td>Muleshoe, TX</td>
<td>2012</td>
<td>1</td>
<td>August</td>
<td>Sorghum</td>
<td>260</td>
</tr>
<tr>
<td>Altoona, FL</td>
<td>2012</td>
<td>1</td>
<td>August</td>
<td>Corn</td>
<td>258</td>
</tr>
<tr>
<td>Bradenton II, FL</td>
<td>2012</td>
<td>1</td>
<td>October</td>
<td>Sweet corn</td>
<td>300</td>
</tr>
<tr>
<td>Colhoun, TX</td>
<td>2012</td>
<td>3</td>
<td>September</td>
<td>Bermuda grass</td>
<td>375</td>
</tr>
<tr>
<td>Bradenton, FL</td>
<td>2013</td>
<td>1&amp;2</td>
<td>May</td>
<td>Sweet corn</td>
<td>300</td>
</tr>
<tr>
<td>Palm Beach, FL</td>
<td>2013</td>
<td>1</td>
<td>May</td>
<td>Corn</td>
<td>300</td>
</tr>
<tr>
<td>Cameron, TX</td>
<td>2013</td>
<td>1</td>
<td>May/June</td>
<td>Sweet corn</td>
<td>300</td>
</tr>
<tr>
<td>Lubbock, TX</td>
<td>2013</td>
<td>1</td>
<td>September</td>
<td>Corn</td>
<td>300</td>
</tr>
<tr>
<td>Polk County, IA</td>
<td>2013</td>
<td>1</td>
<td>September</td>
<td>Corn</td>
<td>500</td>
</tr>
</tbody>
</table>
Table 2. Probit analysis of mortality and sensitivity ratios of Spodoptera frugiperda neonates exposed to the Cry1F protein from B. thuringiensis.

<table>
<thead>
<tr>
<th>Location</th>
<th>Year of collection</th>
<th>N</th>
<th>Slope ± SE</th>
<th>LC50 (95% CI)</th>
<th>LC90 (95% CI)</th>
<th>$\chi^2$</th>
<th>df</th>
<th>RR50</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bradenton, FL</td>
<td>2012</td>
<td>767</td>
<td>1.68 ± 0.1</td>
<td>13.04 (10.84-15.69)</td>
<td>75.46 (57.95-104.04)</td>
<td>2.18</td>
<td>5</td>
<td>4.51*</td>
<td>3.39-5.98</td>
</tr>
<tr>
<td>Lubbock, TX</td>
<td>2012</td>
<td>1,275</td>
<td>2.076 ± 0.13</td>
<td>14.53 (11.48-18.12)</td>
<td>60.22 (45.37-87.54)</td>
<td>7.4681</td>
<td>5</td>
<td>5.02*</td>
<td>3.89-6.49</td>
</tr>
<tr>
<td>Muleshoe, TX</td>
<td>2012</td>
<td>767</td>
<td>2.173 ± 0.18</td>
<td>8.32 (6.86-9.95)</td>
<td>32.35 (25.72-43.33)</td>
<td>1.602</td>
<td>5</td>
<td>2.88*</td>
<td>2.17-3.82</td>
</tr>
<tr>
<td>Altoona, FL</td>
<td>2012</td>
<td>510</td>
<td>1.832 ± 0.15</td>
<td>10.1 (6.77-14.9)</td>
<td>50.56 (31.33-105.03)</td>
<td>8.8014</td>
<td>5</td>
<td>3.49*</td>
<td>2.56-4.74</td>
</tr>
<tr>
<td>Bradenton II, FL</td>
<td>2012</td>
<td>763</td>
<td>1.51 ± 0.12</td>
<td>13.08(10.35-16.27)</td>
<td>92.03 (68.53-133.26)</td>
<td>4.966</td>
<td>5</td>
<td>4.52*</td>
<td>3.31-6.17</td>
</tr>
<tr>
<td>Colhoun, TX</td>
<td>2012</td>
<td>767</td>
<td>1.375 ± 0.09</td>
<td>10.23 (8.11-12.78)</td>
<td>60.22 (45.37-87.54)</td>
<td>1.999</td>
<td>5</td>
<td>3.54*</td>
<td>2.59-4.84</td>
</tr>
<tr>
<td>UNL</td>
<td>2012</td>
<td>768</td>
<td>2.25±0.2</td>
<td>2.89 (2.39-2.45)</td>
<td>10.72 (8.50-14.51)</td>
<td>0.068</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bradenton, FL</td>
<td>2013</td>
<td>1,518</td>
<td>1.946±0.11</td>
<td>3.61 (2.73-4.65)</td>
<td>16.46 (12.02-25.26)</td>
<td>10.287</td>
<td>5</td>
<td>1.25</td>
<td>0.97-1.61</td>
</tr>
<tr>
<td>Palm Beach, FL</td>
<td>2013</td>
<td>1,572</td>
<td>1.39±0.06</td>
<td>22.11 (13.02-36.84)</td>
<td>183.83 (96-535.96)</td>
<td>38.455</td>
<td>5</td>
<td>7.64*</td>
<td>5.93-9.85</td>
</tr>
<tr>
<td>Cameron, TX</td>
<td>2013</td>
<td>509</td>
<td>2.249±0.23</td>
<td>13.86 (11.03-17.13)</td>
<td>51.5 (39.26-74.28)</td>
<td>2.438</td>
<td>5</td>
<td>4.79*</td>
<td>3.53-6.50</td>
</tr>
<tr>
<td>Lubbock, TX</td>
<td>2013</td>
<td>1,020</td>
<td>2.11±0.13</td>
<td>5.19 (4.14-6.47)</td>
<td>20.99 (15.74-30.73)</td>
<td>6.5758</td>
<td>5</td>
<td>1.79*</td>
<td>1.39-2.33</td>
</tr>
<tr>
<td>Polk County, IA</td>
<td>2013</td>
<td>764</td>
<td>1.47±0.09</td>
<td>6.97 (5.07-9.53)</td>
<td>51.74 (33.84-91.86)</td>
<td>7.0789</td>
<td>5</td>
<td>2.41*</td>
<td>1.79-3.23</td>
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<tr>
<td>UNL</td>
<td>2013</td>
<td>767</td>
<td>2.436 ± 0.18</td>
<td>2.79 (2.39-3.26)</td>
<td>9.37 (7.57-12.25)</td>
<td>2.365</td>
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*aResistant ratios calculated based on LC50 of field population relative to laboratory.
*LC values are significantly different from lab population.
Figure 1. EC$_{50}$’s estimated by nonlinear regression of growth inhibition fitted to a probit model and the 95% confidence intervals $S. frugiperda$ neonates collected in 2012 and exposed to the Cry1F toxin.
Figure 2. EC50’s estimated by nonlinear regression of growth inhibition fitted to a probit model and the 95% confidence intervals for S. frugiperda neonates collected in 2013 and exposed to the Cry1F toxin.
Figure 3. Mean percent of mortality and mean of growth inhibition responses of *S. frugiperda* for 2012 and 2013 field collected populations exposed to Cry1F toxin.
CHAPTER 3: Impact of *Spodoptera frugiperda* neonate pretreatment conditions on insecticidal protein activity and laboratory bioassay variation

**Introduction**

While standardized bioassays provide a reliable method to determine insecticidal activity of a test material, including those which contain insecticidal proteins, the susceptibility of laboratory-reared insect larvae can vary. This variation in susceptibility can arise from differing geographical sources of the insect, different testing laboratories, or even through time for the same laboratory and insect source (Robertson et al. 1995, Marçon et al. 1999, Gaspers et al. 2011, Bernardi et al. 2014). Furthermore, bioassay variability from different labs using similar methodologies has been observed across different orders of insects and for various proteins tested. An understanding of this natural variation in susceptibility, or that which is inherent to the bioassay methods employed, is a prerequisite to detecting biologically important differences (Siegfried et al. 2007).

Within a given controlled artificial diet bioassay system, a number of factors have been identified which may influence bioassay response, such as source and type of diet (Blanco et al. 2009), different Bt insecticidal protein preparations and quantification methods (Crespo et al. 2008), and selection of different time points after exposure to assess the final mortality (Chakroun et al. 2012). An additional factor, the innate heterogeneity across individual insects tested, is suggested by the fact that bioassay response variation may routinely range from 3- to 6-fold, or even 12-fold for lab-reared population comparisons.
(using the same methodology), and may be greater across field-derived population comparisons (Siegfried et al. 2007, Bird and Ackhurst 2007). Among different field populations of the same species, the variability in response to the same protein can even be extremely high, on the order of 10 to 100-fold or more (Stone and Sims 1993, Ali and Luttrell 2011). Other factors that may influence bioassay results have not been as thoroughly investigated, however, such as larval pretreatment conditions, which are often not well defined or controlled. It is likely that these pretreatment conditions might also contribute to subsequent variability in susceptibility determinations for an assay system involving a particular protein and pest insect species.

Understanding the inherent variation and identifying the factors that contribute to insect bioassay variation are therefore critical to obtain accurate, reproducible datasets for measuring insecticidal protein activity against target or non-target arthropods. These datasets are fundamental to other studies needed in support of risk assessment to consider the likelihood that crops containing the transgenic insecticidal protein might harm the environment or human health (Craig et al. 2008, Romeis et al. 2008). Additionally, registrants of Bt plant-incorporated protectants must provide an insect resistance management plan to the EPA (USEPA 1998, USEPA 2001), with a component of this plan to include resistance monitoring for targeted pests. This monitoring has an ongoing need for accurate and reproducible measurements of activity using insect bioassay methods. Detecting shifts in target species susceptibility to Bt insecticidal proteins through bioassay-based monitoring programs is a valuable tool to evaluate the

The present study was designed to examine selected potential sources of laboratory bioassay variation. The objective was to determine if differential control of certain pretreatment conditions (which may routinely vary after hatching) could influence the susceptibility of Spodoptera frugiperda laboratory populations to Vip3Aa19 insecticidal protein when using a standardized artificial diet bioassay method.

**Material and methods**

*Strains and insect rearing*

A susceptible *S. frugiperda* colony, identified as SUS, was purchased from BioServ (Frenchtown, NJ) and reared continuously (methods described in Vélez et al. 2013) for approximately 2 years at the UNL toxicology laboratory in the absence of selective pressure by any insecticidal agent. A second strain, identified as K-SUS, was generated from the SUS colony by randomly selecting 300 larvae from the SUS colony and then continuously rearing this as a new colony (in isolation from the SUS colony) using the same rearing conditions.

Adult rearing techniques for *S. frugiperda* described by Perkins (1979) and adapted by Vélez et al. (2013) were used, with at least 200 adults mating randomly in each generation. Adults were placed in 31x 23 cm wired hermit crab cages (Florida Marine Research, Sarasota, FL) with adult diet placed on a cotton pad inside of the bottom of a 100 x 15 mm petri dish (Fisherbrand, Waltham,
MA) and replenished daily. Adult diet consisted of stale beer, containing ascorbic acid (1.5 mg/ml), propionic acid (2.1 µl/ml), and aureomycin (0.5 mg/ml). Adults were held in an environmental chamber with a photoperiod of 14 h: 10 h (L:D) at 27±1 °C and 75±10% RH in light, and 22.5±1 °C and 60±10% RH in the dark. Adults were allowed to mate and oviposit on wax paper lining the inside of the cage. Eggs were harvested daily and placed in 100 x 15 mm Petri dishes with moistened filter paper until hatching.

Neonates were placed on shredded multispecies lepidopteran diet (BioServ, Frenchtown, NJ), allowed to grow to the third instar, and transferred into individual rearing cups containing the same diet. Approximately 300 third instars were individually transferred into 1 oz. translucent polystyrene soufflé portion cups (Solo Cup Company, Lake Forest, IL) with 4.5 ml of diet to minimize cannibalism. Pupation and adult emergence occurred in the cups. Larvae and pupae were maintained in an environmental chamber at 26±1 °C, with a photoperiod of 24 h L and 60±10% RH. Emerging adults were transferred to mating cages daily.

Neonates used in the bioassays were obtained from routine larval collections that consisted of a daily harvest of eggs that were visually homogeneous in color and egg mass size, and collected during the peak of oviposition, (3 to 5 days after initial egg production; Vélez et al. 2014). The collected eggs were stored in Petri dishes with moistened filter paper in environmental chambers at 14 °C, 24 h L and 44±2% RH for approximately 3 days. First instar S. frugiperda that hatched within a 4 hour period were used for
all experimental conditions tested. Both strains were bioassayed over 17 generations using standardized artificial diet bioassay methods described below to estimate variation over time.

**Insecticidal protein**

The Vip3Aa19 insecticidal protein derived from an *Escherichia coli* expression system was provided by Syngenta Crop Protection, LLC (Research Triangle Park, NC) as lyophilized, purified protein which was stored at -80°C. The protein (86.5% purity) was aliquotted and preweighed, so that all Vip3Aa19 protein dilutions could be made on the same day as bioassay initiation. The purified protein was solubilized in 0.25x phosphate buffered saline (PBS) by a gentle agitation technique until completely dissolved, then briefly centrifuged at low speed (5000 rpm for 5 sec). Dilutions were prepared in 0.25x PBS to obtain the desired concentrations prior to bioassay.

**Bioassays**

Artificial diet bioassays were performed based on the methods described by Marçon *et al.* (1999), in 128-well bioassay trays (each well 16 mm diameter, 16 mm high. CD International, Pitman, NJ). One ml of wheat germ-based multispecies lepidopteran diet (Lewis and Lynch, 1969) was dispensed into each well and allowed to solidify.

Each well was treated by applying 50 μl of the appropriate concentration of Vip3Aa19 solution. The negative control consisted of wells treated with 50 μl of 0.25x PBS buffer. The treatments were dried onto the diet surface by stacking
the trays onto an orbital shaker and using a low rotation speed to insure uniform coverage of the treatments over the diet.

One *S. frugiperda* neonate (<4 hour after hatching) was transferred into each well using a fine camel hair paint brush. Wells were covered with vented lids (BIO-CV-16, C-D International), and trays were held in an incubator at 27°C, 24 h scotophase, and 60±10% RH. Mortality was recorded 7 days after infestation and larvae that were unable to respond to a gentle probe technique were considered dead. In each experiment, bioassays were replicated three to four times for each strain, with 16 larvae per each treatment or control tested.

To establish the variation in LC$_{50}$ estimates for Vip3Aa19 over multiple generations, diet bioassays were performed using seven concentrations to generate dose-responses for both laboratory colonies of *S. frugiperda* larvae. These analyses were conducted over 17 generations for each colony.

To determine the effect of different larval pretreatment conditions, bioassays were performed with a single concentration of Vip3Aa19 that corresponded to the estimated LC$_{70}$ (lethal concentration that causes 70% mortality) against the laboratory *S. frugiperda* larvae. This concentration was approximated for both lab colonies, based on the estimate in the bioassay for the first generation K-SUS colony (immediately after isolation from the parental SUS colony).

**Pretreatment conditions**

Routine laboratory bioassays to determine larval susceptibility to a given test material involve larval maintenance conditions which may vary *in advance of*
any exposure to insecticidal agents (=“pretreatment condition”). To determine the impact of selected pretreatment conditions on the susceptibility to Vip3Aa19 protein, each condition was examined independently with the standardized bioassay methods at a concentration that approximated the LC$_{70}$ dose as described above. The following five pretreatment conditions were examined: 1) larval storage time prior to exposure; 2) prior feeding on control artificial diet; 3) larval storage at reduced temperature prior to exposure; 4) larval storage at reduced humidity prior to exposure; and 5) colony perturbation following introgression with field-collected individuals. In addition to these pretreatment conditions, one additional bioassay condition (condition 6) was examined that involves extremes of photoperiod settings used during the course of the bioassay itself.

1) Impact of larval storage time prior to exposure. To assess the impact of larval storage time prior to exposure to insecticidal protein, larvae (within 0-4 h after hatch) were distributed among Petri dishes containing moistened filter paper and kept for four different time periods in the absence of food. To establish the pretreatment time periods, larvae were then either transferred directly to bioassay trays or held for an additional 2, 6 or 12 h in the Petri dishes on moistened filter paper prior to the start of the bioassays. Mortality was determined after 7 days exposure to the estimated LC$_{70}$ concentration of Vip3Aa19 as described above. The procedure was repeated three times for each colony, with a total of 336 insects tested for each treatment.

2) Impact of prior feeding on artificial diet. To determine the impact of prior feeding, larvae (within 0-4 h after hatch) were transferred to individual wells
of artificial diet trays (one larva per well) where they were allowed to feed for 2, 6 or 12 h. After the respective pretreatment holding times, the larvae were transferred to bioassay trays for subsequent exposure to the estimated LC$_{70}$ concentration of Vip3Aa19, and mortality was assessed as described above. Control bioassays consisted of neonates that did not feed on diet prior to being assayed. This procedure was repeated four times for each colony, with a total of approximately 448 insects tested in each treatment.

3) **Impact of larval storage at reduced temperature.** To assess the impact of storage at reduced temperature, larvae (within 0-4 h after hatch) were either transferred directly to bioassay trays (= a control of no storage pretreatment) or stored for 12 h at 14°C, 24 h L and 44±2% RH without food and then transferred to bioassay trays. Larvae were exposed to the estimated LC$_{70}$ concentration of Vip3Aa19 and mortality was assessed as described above. The study was repeated four times for each colony, with approximately 448 insects tested in each treatment.

4) **Impact of storage under reduced humidity.** To examine the impact of storage at high humidity (= routine condition with moistened filter paper in a Petri dish sealed with Parafilm®) or at reduced humidity, larvae (within 0-4 h after hatch) were exposed to the standard condition, or a low humidity environment which was created in a desiccator. For the low humidity environment, the larvae were stored in a Petri dish which was covered with an 80-mesh screen and suspended over a saturated potassium acetate solution (Greenspan 1977) in the bottom of the desiccator. Relative humidity was measured using data loggers and
sensors (model HOBO UX100; Onset Computer Corporation, Bourne, Massachusetts). The relative humidity was approximately 90% for the high, and 15% for the low RH environment. After 3 hours of either high or low humidity pretreatment condition, the larvae were transferred to bioassay trays for subsequent exposure to the estimated LC\textsubscript{70} dose of Vip3Aa19. Mortality was assessed as described above. The study was repeated four times for each colony, with a total of approximately 448 insects tested in each treatment.

5) Impact of lab colony introgression with field collected individuals. To assess the impact of colony introgression with field collected individuals on subsequent Vip3Aa19 susceptibility, a temporary colony of *S. frugiperda* was established with larvae collected from Winter Beach, Indian River Co., Florida. To establish the colony, 600 larvae collected from fields planted to conventional non-Bt corn were shipped overnight to the University of Nebraska, and reared on artificial diet until pupation. From the field collected individuals, a total of 120 male and 140 female pupae were sexed and separated from the field collected colony to be crossed with laboratory susceptible strain K-SUS individuals. The F\textsubscript{1} progeny from this cross were reared as described previously, but kept isolated from K-SUS to obtain F\textsubscript{2} and F\textsubscript{3} progenies. Mortality was determined after exposure to the estimated LC\textsubscript{70} dose of Vip3Aa19.

6) Impact of photoperiod during bioassay. To examine the potential impact of differing extremes of photoperiod settings on larval susceptibility during the course of the bioassay, larvae (within 0-4 h after hatch) were transferred to bioassay trays and stored under two different photoperiods, either
24 h continuous scotophase, or 24 h continuous photophase for seven days. Incubators were maintained at the same standard conditions (27°C, and 60±10% RH) for conducting the bioassay. Mortality was recorded after exposure to the estimated LC$_{70}$ dose of Vip3Aa19 as described above. The study was repeated three times for each colony, with a total of 335 insects tested in each treatment.

**Statistical analysis**

To estimate the lethal concentrations (LC$_{50}$ and LC$_{70}$) and the fiducial limits for the Vip3Aa19 bioassays over multiple generations of the lab colonies, or following introgression of a lab colony with field collected individuals, the concentration mortality data were analyzed by probit analysis (Finney 1971) using POLO-PC (LeOra Software 1987).

Data analyses for respective pretreatment conditions 1 through 4 and for the bioassay condition of differential photoperiod (condition 6) were performed as randomized complete blocks, with each block as a temporal replicate for the respective experiments. The distribution of block effects was normally and independently distributed. The percentage of mortality was transformed to mean percent mortality with respective standard errors and analyzed as a binomial distribution arranged in a factorial treatment design (interaction between pretreatment conditions and strains) and performed in PROC GLIMMIX of SAS (version 9.4; SAS Institute; Cary, NC). Values from the interactions and from least-squared means of the treatments with P-values less than 0.05 were considered statistically significant.
Results

Bioassays were conducted to determine the susceptibility of *S. frugiperda* to Vip3Aa19 insecticidal protein throughout 17 generations of continuous rearing. Even though standardized bioassay methodology was used, considerable variation in the calculated LC$_{50}$ values for both laboratory colonies (Table 1 and Figure 1) were found. The LC$_{50}$ values (and 95% CL) varied approximately 6.6-fold for the SUS strain, and ranged from 8.7 (6.9-10.4) ng cm$^{-2}$ to 54.3 (46.8-60.9) ng cm$^{-2}$. The LC$_{50}$ values (and 95% CL) for the K-SUS strain were similar in magnitude to those of the SUS strain, but varied slightly more (8.8-fold overall), and ranged from 11.6 (9.8-13.3) ng cm$^{-2}$ to 102.2 (72.8-129.1) ng cm$^{-2}$.

The estimated LC$_{70}$ value for the first generation of K-SUS was 31.5 (25.4-41.4) ng cm$^{-2}$ and this concentration was used to test the five pretreatment conditions.

*Condition 1: Impact of storage time prior to Vip3A exposure*

The larval storage time (without feeding) before Vip3Aa19 exposure did not significantly affect subsequent mortality at the tested concentration (P>0.05) (Fig.2). The control with no additional holding time had a similar mean percent mortality of 67±11.2% or 60.3±12.0%, for the SUS or K-SUS colony, respectively. Although the mean percent mortality showed some variation for each colony across the different time points up to 12 h, no significant trends were found for either, and therefore, this condition also did not cause significant interaction between factors (hours and colony, P>0.05).
Condition 2: Impact of prior feeding

Prior feeding of larvae significantly reduced the subsequent mortality resulting after Vip3Aa19 exposure. The mean percent mortality was lower for each treatment where prior feeding on artificial diet had occurred compared to control larvae which did not experience prior feeding (Fig. 3). A similar overall trend was observed for SUS and K-SUS colonies, where longer periods of pretreatment feeding significantly decreased the susceptibility of *S. frugiperda* (P<0.05) to Vip3Aa19. The net decrease in mortality over the 12 h was similar for each colony with about a 27% reduction (45.2±8.8% to 17.4±5.4%, or 51.6±8.9% to 25.3±6.8%, for the SUS, or K-SUS colony, respectively). No statistically significant colony by treatment interactions were observed (P>0.05).

Condition 3: Impact of larval storage at reduced temperature

Storage of *S. frugiperda* larvae overnight at reduced temperature (14°C) showed different results for each colony, somewhat complicating the interpretation of the impact of this pretreatment. While the SUS colony demonstrated similar mortality for both conditions (*i.e.*, larvae used within 0-4 h after hatch as compared to those which had experienced the additional 12 h pretreatment), the K-SUS colony exhibited significant increased mortality with the 12 h pretreatment (Fig. 4). The mean percent mortality for the K-SUS colony increased from 45.6 ± 3.6% to 73.0 ±3%. This change was significant for the K-SUS colony (P<0.05), and there was a significant interaction between factors (time and colonies, P<0.05), confirming the observation that one colony was affected by the pretreatment condition, while the other was unaffected.
Condition 4: Impact of storage under reduced humidity

The differential exposure of larvae to different pretreatment conditions of humidity (Fig. 5) did not have a significant impact on resultant mortality and there was no interaction between colonies (P>0.05) for this pretreatment. Overall mean percent mortalities were similar within each colony tested, irrespective of the high or low RH pretreatment, at 49.6± 6.8% and 47.5±6.8% for SUS, respectively, and 45.2±6.7% and 38.5±6.5% for K-SUS, respectively.

Condition 5: Impact of lab colony introgression with field collected individuals

The LC50 values for the field collected colony were similar to the K-SUS laboratory susceptible colony, with estimates (and 95% fiducial limits) of 24.3 (14.1-33.8) ng cm⁻² and 28.1 (22.2-34.6) ng cm⁻² for the field and laboratory colonies, respectively (Table 2). The introgressed colony exhibited increased tolerance to Vip3Aa19 relative to the two parental colonies at the first generation after crossing; with the estimated LC50 values (and 95% fiducial limits) of 117.2 (98.3-146.4) ng cm⁻² (Table 2). Bioassay of the 2nd and 3rd generations of the introgressed colony, however, showed an increase in susceptibility to Vip3Aa19 compared to the 1st generation tested, with LC50 values (and 95% fiducial limits) of 15.6 (13-18.2) ng cm⁻² and 32.9 (22.6-44.6) ng cm⁻², for F2 and F3 progeny, respectively. The 2nd and 3rd generations of the introgressed colony also demonstrated LC50 values similar to the K-SUS parental strain (Table 2).

Condition 6: Impact of photoperiod during bioassay

The presence or absence of light during bioassay of Vip3Aa19 significantly affected S. frugiperda larval mortality (Fig. 6). Mean percent
mortality for the 24 h scotophase treatment was 83.6±2.8% compared to 56.3±4.3% for 24 h photophase in the K-SUS colony. Similar results were seen with the SUS colony, where mean percent mortality decreased from 46.8±4.4% to 21.3±3.2% for 24 h scotophase compared to 24 h photophase, respectively. Although both strains showed similar response to the presence or absence of light (with net decrease of approximately 26% under 24h photophase), there were significant differences in susceptibility between strains for this treatment (P<0.05).

Discussion

Establishing the bioactivity via laboratory bioassay methods is critically important for discovery efforts to uncover new candidate insecticidal agents, but is also vital to support product development needs. The latter needs routinely include: 1) a description of efficacy and degree of activity toward the potential spectrum of target arthropods, 2) establishing the activity of a representative insecticidal protein test substance which may be used for expanded toxicological and environmental safety testing (Raybould et al. 2013), including an assessment of any activity toward representative non-target arthropods (Burns and Raybould 2014), and 3) support for a resistance management plan which often requires extensive laboratory bioassay testing over time. High confidence in bioassay results is very important for decision making during product development and registration, but can be challenging to achieve in the face of bioassay system variability. Intra-population variation in response to chemical and microbial
insecticides is clearly a common phenomenon when any bioassay is repeated (Robertson et al. 1995, Siegfried and Spencer 2012).

The present study was designed to examine selected potential sources of laboratory bioassay variation. A more specific objective was to determine if differential control of certain pretreatment conditions (which may routinely vary after hatching) could influence the susceptibility of *S. frugiperda* laboratory populations to Vip3Aa19 insecticidal protein when using a standardized artificial diet bioassay method. The vegetative insecticidal protein, Vip3A, from *Bacillus thuringiensis* has great value to agriculture due to its broad spectrum of activity against lepidopteran pests and unique mode of action compared to the insecticidal Cry proteins derived from *B. thuringiensis* (Lee et al. 2003).

The fall armyworm is native to the tropical regions of the western hemisphere from the United States to Argentina and is an important pest of maize and many other crops throughout the Americas. A number of studies have reported the insecticidal activity of Vip3A against *S. frugiperda* and have documented its utility as a novel Bt technology, and as stacking protein with other Cry proteins to delay the development of resistance (Lee et al. 2003, Kurtz et al. 2007, Burkness et al. 2010, Chakroun et al. 2012, Farias et al. 2014).

The present study identified pretreatment conditions that can significantly affect susceptibility of *S. frugiperda* larvae to Vip3Aa19, as well as other conditions which have no apparent effect. In addition, one condition was differentially controlled during the course of the bioassay and demonstrated to have a significant impact on resultant mortality.
Bioassays conducted throughout 17 generations of continuous rearing, showed considerable variation in calculated LC50 values for Vip3Aa19 for both laboratory colonies, with an overall range of approximately 6.6-fold difference for the SUS strain, and 8.8-fold difference for the K-SUS strain. Such variation is not uncommon, as noted previously, and reinforces the need to conduct treatment comparisons side-by-side in a given standardized bioassay system to draw the best conclusions about any real differences which may exist. Making comparisons across experiments over time or among laboratories would not be recommended, except for the purpose of establishing an overall expected range which one might encounter for a given insecticidal protein: larval test organism bioassay system. It remains plausible that inherent variability in larval susceptibility can arise from the innate heterogeneity of the individuals which are selected and tested, even from a laboratory colony which is tested in a standardized way. An example of this inherent variation was observed by Vélez et al. (2014), where S. frugiperda eggs laid during the peak of oviposition exhibited increased larval fitness. Similarly, variation in susceptibility to Bt proteins has been hypothesized as due to differences in genotype and nutritional status of the egg, for both Lymantria dispar dispar and Ostrinia nubilalis (Rossiter et al. 1990, Marçon et al. 1999).

Data from the present study indicate that there is no significant difference in susceptibility to Vip3Aa19 between S. frugiperda larvae that are held as much as an additional 12 hours (plus the 0 to 4 h collection time after hatching) before being exposed to Vip3Aa19 compared to larvae that are exposed within 0 to 4 h of hatching. These results confirm that there can be some flexibility in conducting
bioassays with insects that hatch asynchronously without affecting the outcome. Similarly, exposure to Vip3Aa19 following different pretreatment extremes of relative humidity indicated no significant effect on *S. frugiperda* larval susceptibility. It may be routine to have eggs contained in Petri dishes with moistened filter paper until hatching and subsequent transfer to bioassays (Marçon *et al.* 1999, Vélez *et al.* 2013, Farias *et al.* 2014); however, the relative humidity could theoretically vary during the time frame that the eggs hatch and larvae are then held before transferring to bioassays. As larval pretreatment holding time during bioassay preparation or larval transfer may be extended, neonates may be exposed to more extreme environmental conditions like decreased relative humidity. Our data suggest that exposure to a change in relative humidity (for at least up to 3 h) may not affect subsequent larval susceptibility.

In contrast to larval pretreatment storage time and differential relative humidity status, other pretreatment conditions do significantly affect larval susceptibility to insecticidal protein. In particular, prior feeding on artificial diet and, potentially, overnight storage at a reduced temperature, can have a significant impact on response to Vip3Aa19. Larvae that were previously fed with artificial diet up to 12 hours were significantly less susceptible to Vip3Aa19 than those that were unfed prior to the bioassay. This result likely reflects that even a short period of growth on the control diet can alter the actual status of the larvae which then go into the bioassay. Change in susceptibility to insecticidal protein based on the stage of larval development has been previously reported (Huang *et al.* 1999), but our data suggest that this could be manifested even before approaching a larval
molt. Therefore, it is advisable to avoid this pretreatment condition of differential prefeeding in the interest of reduced bioassay variability. Additionally, our data suggest that maintaining hatched neonates at a reduced temperature overnight could potentially affect the subsequent susceptibility of larvae to insecticidal protein. Overall results therefore indicate that maintaining hatched larvae in a hydrated condition (for up to 12 h, if necessary, but in the absence of artificial diet), and without overnight storage at reduced temperatures, should increase the consistency of bioassay results.

Infusion of wild type individuals into an established lab colony population is a common practice to increase the genetic diversity which can be lost compared to field populations (Chambers 1977, Leppla and Ashley 1989). This may be essential to have the laboratory colony more accurately reflect an anticipated response for the field population (e.g., to facilitate discovery efforts). This practice, however, may introduce further variation in the bioassay system, which can be detrimental from the standpoint of using insect bioassay as a reproducible test organism system (e.g., in support of product development needs). Our data suggest that such infusion of field collected individuals into the laboratory colony could be an important factor to consider when seeking to reduce bioassay variability. Early in the introgression of field individuals with the laboratory colony, large differences in susceptibility to Vip3Aa19 resulted. This could possibly have been due to hybrid vigor, as the original two parental colony susceptibilities to Vip3Aa19 were not that different. Within two generations however, the introgressed colony was not different from the parental colony in
susceptibility to Vip3Aa19, suggesting that if colony introgression is practiced routinely, bioassays should be delayed for at least 2-3 generations of random mating. After this delay, the susceptibility may then be expected to fall within the range of variability which was previously established for the bioassay system.

An additional factor that significantly affected susceptibility of *S. frugiperda* to Vip3Aa19 was the presence or absence of light during the course of the bioassays. This observation was true for both lab colonies tested, with a net decrease of approximately 26% mortality under 24 h photophase compared to the 24 h scotophase condition. It is possible that the presence or absence of light affects larval feeding behavior, resulting in a different ingestion of insecticidal protein from the treated artificial diets. However, even if this occurred, this interpretation is somewhat complicated as insecticidal proteins also commonly have a feeding cessation and gut paralysis effect. It would also be interesting to see how a more balanced light/dark cycle, mimicking the natural setting, would compare in terms of resultant impact on susceptibility. These results indicate that maintaining a standardized condition of lighting during a bioassay is important to obtain consistent results.

Our findings regarding the pretreatment conditions tested in this study substantiated the hypothesis that control of such conditions can impact the outcome of the bioassay. While these findings are specific for the conditions tested, and for the Vip3Aa19 insecticidal protein and neonate *S. frugiperda* larval bioassay system, they are likely to extend to other insect bioassay systems.
Controlling possible sources of variation in susceptibility testing and use of standardized laboratory bioassay methodologies will better provide accurate results to satisfy product discovery through registration needs; including resistance monitoring programs that utilize a variety of bioassay techniques. Obtaining accurate insect pest bioassay response data is vitally important and routinely required to support safety assessment of insecticidal protein trait products.

Finally, from a practical standpoint, understanding the inherent variability in a given bioassay system and which pretreatment factors may (or may not) impact the variability can be of great benefit on a day-to-day basis in the bioassay lab. For example, our data indicate that the time of selecting *S. frugiperda* larvae for setting up a bioassay with Vip3Aa19 can be more loosely controlled up to 12 h after hatch, as long as the other standardized bioassay factors are observed, and the selected larvae have not fed on artificial diet.

**Acknowledgements**

We thank the protein production team at the Syngenta Jealott’s Hill research station for the purified Vip3Aa protein. We acknowledge Clark Lovelady, from insect control team at Syngenta’s research center in Vero Beach, Florida, for collecting fall armyworm field population. We also thank Carolina Camargo (Department of Entomology, UNL) and Dr. Walter Stroup (Department of Statistics, UNL) for the statistical analysis assistance.
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## Tables

**Table 1.** *LC*$_{50}$ *estimates for two laboratory susceptible strains of S. frugiperda larvae exposed to Vip3Aa19 insecticidal protein when tested over multiple generations.*

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<th>Gen</th>
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<td><em><em>LC</em>$_{50}$ (95% CL)</em>*</td>
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<td>NA$^b$</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>380</td>
<td>11.9(10-13.8)</td>
<td>4.25</td>
</tr>
<tr>
<td>3</td>
<td>767$^c$</td>
<td>8.7(6.9-10.4)</td>
<td>2.7</td>
</tr>
<tr>
<td>4</td>
<td>381</td>
<td>12.6(10.3-15)</td>
<td>3.43</td>
</tr>
<tr>
<td>5</td>
<td>384</td>
<td>20.7(17.5-24)</td>
<td>4.36</td>
</tr>
<tr>
<td>6</td>
<td>508</td>
<td>36.2(27.4-48.2)</td>
<td>3.32</td>
</tr>
<tr>
<td>7</td>
<td>512</td>
<td>~80$^d$</td>
<td>NC</td>
</tr>
<tr>
<td>8</td>
<td>507</td>
<td>17.5(11.1-23.7)</td>
<td>2.95</td>
</tr>
<tr>
<td>9</td>
<td>512</td>
<td>28.8(18.8-45.5)</td>
<td>2.59</td>
</tr>
<tr>
<td>10</td>
<td>511</td>
<td>38.3(29.8-47.6)</td>
<td>3.08</td>
</tr>
<tr>
<td>11</td>
<td>511</td>
<td>23.2(20.51-26.13)</td>
<td>4.34</td>
</tr>
<tr>
<td>12</td>
<td>510</td>
<td>26.7(20.8-32.6)</td>
<td>3.31</td>
</tr>
<tr>
<td>13</td>
<td>511</td>
<td>23.8(21-26.8)</td>
<td>3.97</td>
</tr>
<tr>
<td>14</td>
<td>512</td>
<td>51.2(38.8-66.8)</td>
<td>3.38</td>
</tr>
<tr>
<td>15</td>
<td>510</td>
<td>27.5(11.2-42.9)</td>
<td>2.98</td>
</tr>
<tr>
<td>16</td>
<td>500</td>
<td>30.8(22.8-39.3)</td>
<td>3.4</td>
</tr>
<tr>
<td>17</td>
<td>511</td>
<td>54.3(46.8-60.9)</td>
<td>5.02</td>
</tr>
</tbody>
</table>

---

$a$ Nanograms of Vip3Aa19/cm$^2$ of diet.

$b$ Data not available (NA) for this generation, as no bioassay was conducted.

$c$ Six replicates used at this generation testing.

d $LC_{50}$ value was not calculated (NC) by probit analysis and estimated based on 50% observed mortality.

$e$ Eight replicates used at this generation testing.
Table 2. Effect of introgression on *S. frugiperda* laboratory colony susceptibility to Vip3Aa19 insecticidal protein.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Generation</th>
<th>Number of insects tested</th>
<th>LC₅₀ (95% CL)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Winter Beach</td>
<td>1</td>
<td>448</td>
<td>24.3 (14.1-33.8)</td>
</tr>
<tr>
<td>K-SUS</td>
<td>14</td>
<td>1017</td>
<td>28.1 (22.2-34.6)</td>
</tr>
<tr>
<td>Infused</td>
<td>1</td>
<td>1016</td>
<td>117.2 (98.3-146.4)</td>
</tr>
<tr>
<td>K-SUS</td>
<td>15</td>
<td>448</td>
<td>11.6 (9.8-13.3)</td>
</tr>
<tr>
<td>Infused</td>
<td>2</td>
<td>441</td>
<td>15.6 (13-18.2)</td>
</tr>
<tr>
<td>K-SUS</td>
<td>16</td>
<td>448</td>
<td>33.0 (27.8-38.5)</td>
</tr>
<tr>
<td>Infused</td>
<td>3</td>
<td>448</td>
<td>32.9 (22.6-44.6)</td>
</tr>
</tbody>
</table>

aNanograms of Vip3Aa19/cm² of diet.
**Figure 1.** Variation in susceptibility of *S. frugiperda* larvae to Vip3Aa19 insecticidal protein for two laboratory colonies over multiple generations. Dashed line between points indicates LC50 not available.
**Figure 2.** Susceptibility of *S. frugiperda* larvae to Vip3Aa19 insecticidal protein after extended holding time pretreatment.
Figure 3. Susceptibility of *S. frugiperda* larvae to Vip3Aa19 insecticidal protein after prior feeding on artificial diet.
Figure 4. Susceptibility of *S. frugiperda* larvae to Vip3Aa19 insecticidal protein following overnight storage at 14°C. Means with different letters are significantly different (LS-Means p<0.05) over time (A or B), or between strains (a or b).
Figure 5. Susceptibility of *S. frugiperda* larvae to Vip3Aa19 insecticidal protein following extreme differences in relative humidity pretreatment. Means with same letters are not significantly different (LS-Means p>0.05).
**Figure 6.** Susceptibility of *S. frugiperda* larvae to Vip3Aa19 insecticidal protein with light present or absent during the bioassay. Means with different letters are significantly different (LS-Means p<0.05) over treatment (A or B), or between strains (a or b).