APPLICATIONS OF ECOLOGICAL AND GENETIC TOOLS TO ADVANCE FALL ARMYWORM PEST MANAGEMENT

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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December 2020

Major Subject: Entomology

ABSTRACT

The fall armyworm *(Spodoptera frugiperda*) is a highly polyphagous agricultural pest native to the Western Hemisphere. This species is comprised of two morphologically identical, but genetically distinct strains, commonly referred to as the corn strain (C-strain) and the rice strain (R-strain). These strains have a sympatric distribution and may show a slight difference in their host ranges. Although the same species, these two strains respond differently to commonly used insecticides, and thus can be considered unique agricultural pests. Implementing strain specific pest management plans is a future goal to better control this species in the field.

Here, I applied ecological and genetic tools to uncover new insights about fall armyworm populations in the United States (US) that can be used to inform pest management plans. Specifically, I had three primary objectives. First, I used population genomics to characterize the population structure of fall armyworms in the central US and identify genomic patterns of gene flow between the C- and R- strain. Second, I developed new diagnostic tools for differentiating between fall armyworm strains, and then demonstrated how these tools can be used to study strain behavior in the field. Last, I compared the nutritional ecology of fall armyworm lab colonies to field populations to determine the utility of lab colonies as models to understand the behavior and physiology of field insect populations.

In addition to providing new diagnostic tools to differentiate between fall armyworm strains, my research provides novel insights into the pre- and postzygotic

mechanisms underlying reproductive isolation between strains. Growers and researchers often assume a fall armyworm's strain based on the host plant on which an insect is feeding. However, my data indicates that both C- and R- strain individuals often occupy the same fields, highlighting the importance of using molecular diagnostic tools to differentiate between these strains. Finally, I show that laboratory colonies of fall armyworms exhibit significant differences in their physiology and behavior when compared to the wild population, and should be used with caution when drawing generalize conclusions about field derived insect populations.

DEDICATION

This dissertation is dedicated to my parents, Cathy & Rudy Tessnow, who always encouraged me to pursue my interests and who have believed in me every step of the way; my brother, Travis Tessnow, who has been an incredible support throughout my educational journey; my fiancé, Bert Foquet, who always makes me smile even when nothing seems to be going as planned; and my uncle, Richard Stevens, who helped me discover my passion for science.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisor, Dr. Greg Sword for his guidance and encouragement thought this project. His mentorship and enthusiasm were integral to my success over the past several years. I would also like to thank my committee members, Dr. Spencer Behmer, Dr. Micky Eubanks, and Dr. Christine Merlin for always providing feedback, advice, and unfailing support throughout my graduate studies.

I would like to express my gratitude to Dr. Tom Walsh for welcoming me into his lab and mentoring me as a visiting graduate student at CSIRO, and for opening my eyes to the world of molecular ecology.

I would like to thank Dr. Tyler Raszick for teaching me the basics of bioinformatics, sharing scripts, and helping me troubleshoot computationally intense analyses throughout this project. I would also like to recognize Dr. Todd Gilligan for all of his assistance and insights regarding molecular diagnostic tools.

This project would not have been possible without the help and support of Dr. Pat Porter, Eric Burkness, Danielle Sekula, Dr. Juan Luis Jurat-Fuentes, and Dr. Dominic Reisig who provided the moth samples and insects colonies necessary to complete this research. I especially want to recognize Dr. Pat Porter who not only allowed me to spend several nights in his field in Lubbock collecting moths, but also loaned me his truck so I wouldn’t get stuck driving through muddy fields.

I would like to give a special shout out to Cesar Valencia, who ordered most of the materials I needed to complete this research, and who rescued me from the field at 8am on a Sunday morning when the truck broke down.

The work in this dissertation would not have been possible without the support and encouragement of many family members and friends. I would especially like to thank Bert Foquet and Liz Walsh for their camaraderie, support, advice, and patience throughout my graduate studies.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by the dissertation committee consisting of professors Gregory Sword (committee chair), Spencer Behmer, and Micky Eubanks from the Department of Entomology and Christine Merlin from the Department of Biology.

Samples used in this research were collected by Dr. Pat Porter, Dr. Robert Bowling, and Danielle Sekula from Texas A&M AgriLife Research, Eric Burkness from the University of Minnesota, and Dr. Juan Luis Jurat-Fuentes from the University of Tennessee. Insects used in behavioral assays were provided by Dr. Dominic Reisig from North Carolina State University, or purchased from Benzon Research Inc.

All other work conducted for this dissertation was completed by the student independently.

Funding Sources

The first three years of graduate study was supported by an Excellence in Entomology fellowship from the College of Agriculture and Life Science at Texas A&M University.

This work was also made possible in part by funding from the Agricultural and Food Research Initiative - Education and Workforce Development Award (#2020-67034-31748) from the USDA-NIFA awarded to Ashley Tessnow, a USDA-APHIS Cooperative Agreement (#AP19PPQS&T00C071) between Dr. Gregory Sword and Dr.

Todd Gilligan, and the Charles R. Parencia Endowment to Gregory Sword. Its contents are solely the responsibility of the author and do not necessarily represent the official views of any of the funding agencies listed.

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1. INTRODUCTION

1.1. The fall armyworm

The fall armyworm, *Spodoptera frugiperda* J.E. Smith (Lepidoptera: Noctuidae), is a highly polyphagous caterpillar pest reportedly feeding on up to 353 host plats from 76 plant families (Montezano et al., 2018). Although native to the New World, this insect has received a lot of attention recently due to their introduction and subsequent spread across Africa, Asia, and most recently Australia (Chakroun et al., 2016; Goergen et al., 2016; Otim et al., 2018; Sharanabasappa et al., 2019; Tay et al., 2020; Zhang et al., 2019). Plant damage is caused by the destructive feeding of caterpillars, often deep within the developing whorl of its host, making it nearly impossible to target with insecticide sprays. While primarily an economic pest on corn and sorghum, the fall armyworm can also cause significant damage to cotton, Bermuda hay, rice, alfalfa, peanuts, watermelon, sweet potatoes, soybeans, and sugarcane (Luginbill, 1928). To reduce the economic impact of *S. frugiperda,* Bt corn and cotton expressing the insecticidal proteins Cry1F and Vip3Aa, derived from the bacterium *Bacillius thuringenisis,* have been deployed as the primary control method for this species. However, reports of field-evolved practical resistance to Bt toxins in multiple regions across the Western Hemisphere threaten the sustainability of this Bt technology (Chandrasena et al., 2018; Farias et al., 2014; Huang et al., 2014; Storer et al., 2010; Yang et al., 2018). Improved characterization of fall armyworm genetic population

structure and its relationship to dispersal patterns will allow us to better identify regions that are at risk of invasion by these Bt resistant insects.

Although the fall armyworm has always been present in the United States (US), extension agents and growers have noticed increasingly large outbreaks of this pest over the past several years. In August 2015, Dr. Larry Godfrey, an extension agent based out of UC Davis, noted unprecedented levels of fall armyworms on rice in California (Keatly-Garvey, 2015). In 2016, Georgia extension entomologist Dr. David Buntin described an "Armageddon-type outbreak", with reports of this pest wiping out entire fields in just a matter of days (Dowdy, 2016). In June 2018, both Dr. Pat Porter and Dr. David Kerns of Texas A&M Extension reported a 6.5- fold increase in the number of fall armyworms feeding on corn and sorghum across the state of Texas (Domel, 2018). The increased attention fall armyworms have received due to their invasion of the Eastern Hemisphere, combined with the frequent reports of uncontrollable fall armyworm outbreaks in the US, highlight a gap in our knowledge of this pest's basic biology and population structure across its native range. The primary goal of this dissertation research was to fill these gaps of knowledge and use new insights about the fall armyworm's molecular ecology and nutritional ecology to inform pest management plans.

1.2. Population genetics of the fall armyworm in the US

One of the most notable characteristics of the fall armyworm is its extreme mobility. *Spodoptera frugiperda* moths are strong fliers estimated to travel an average of 500km

each generation on prevailing winds, with individual moths reportedly traveling up to 1,600km (Johnson, 1987). Additionally, this species does not undergo diapause. In the US, native populations are only known to overwinter in south Florida and south Texas. This creates two geographically distinct source populations of fall armyworms in the US that can be differentiated by their overwintering locations. At the start of each spring, moths being a northward expansion across multiple generations ultimately making their way into Canada by the end of the season (Sparks, 1979). Prior to molecular tools, the dispersal patterns of this species were described by monitoring the time when fall armyworms arrived at different locations across the US (Luginbill, 1928), and by coordinating pheromone baited moth trap captures with meteorological data (Mitchell et al., 2017; Westbrook and Sparks, 1986). Although these methods allowed researchers to characterize the spread of fall armyworms throughout the country, there was not enough resolution to map the source of the fall armyworms to their overwintering location.

With the rise of molecular tools, researchers were increasingly interested in mapping the overwintering origin of fall armyworm populations that dispersed throughout the US. These efforts led to the characterization of two unique genetically-distinct fall armyworm strains by Pashley (1986). These strains were originally identified using allozyme makers and named for the plant on which they were originally discovered, corn and rice. These strains are described in detail below. Despite collecting fall armyworms from 18 locations across the Western Hemisphere, Pashley did not uncover any additional population structure within these strains that could be associated with sampling location (Pashley, 1986). Because of the high mobility of this pest, some

genetic admixture is likely to occur between geographically distant populations, challenging efforts to identify population structure. Although higher resolution molecular markers became available, later studies using amplified length polymorphism (AFLPs) also did not detect genetic differentiation between populations, concluding that all fall armyworm moths spread from southern Argentina to Canada comprise a single panmictic population (Belay et al., 2012; Clark et al., 2007). Importantly, neither of these studies accounted for genetic variation due to strain, which could have confounded their results.

With respect to the genetic differentiation between the two overwintering populations, two polymorphic sites within the mitochondrial cytochrome c oxidase subunit I (COI) gene have been identified that result in four distinct haplotypes of C-strain moths (Nagoshi et al., 2008). The ratio of these haplotypes differs between the Texas and Florida geographic populations and has remained constant over time (Nagoshi and Meagher, 2008). As a result, if a subset of the population is collected and screened in the northern US, these individuals can potentially be mapped back to either the Texas or Florida overwintering sites based on the relative proportion of each haplotype that is present in the sample. This indirect approach indicates that genetic differentiation exists between these two geographically distinct populations of fall armyworms. However, because a ratio must be calculated, a large sample of C-strain moths must be collected in order to confidently determine their origin. Additionally, if a small subset of a source population is introduced into a new range, but by chance has a different ratio of haplotypes than their population of origin due to bottleneck effects, one cannot be

certain of their site of origin. Nevertheless, these differences in haplotype ratios are the only evidence of geographic population structure to date in fall armyworms. They have since been used to map the dispersal patterns of moths (Nagoshi et al., 2012), test migratory model simulations (Westbrook et al., 2016), and inform pest management practices.

1.3. Fall armyworm host strains

Spodoptera frugiperda is comprised of two morphologically identical, but genetically distinct, strains that were initially named for the host plant on which they were discovered, corn and rice. The corn strain, or C-strain, has generally been associated with large grasses such as corn and sorghum, whereas the rice strain, or R-strain, is more commonly associated with smaller grasses such as Bermuda grass, turf grass, millet, and pasture grasses (Pashley, 1986; Pashley, 1988a). It is thought these host strains diverged approximately 2 mya, before the domestication of cereal crops in the New World (Kergoat et al., 2012; Arias et al., 2019).

When pheromone-baited moth traps are placed in corn and sorghum fields, individuals of both strains are typically recovered, indicating these host strains have a sympatric distribution and some overlap in host use (Nagoshi and Meagher, 2004; Meagher and Nagoshi, 2013). When these traps are placed in and around pastureland, predominantly R-strain individuals are recovered, potentially suggesting asymmetric host use between strains where the C-strain is slightly more specialized than the R-strain (Groot et al., 2010). Behavioral studies have not recovered consistent differences in host

plant preference or performance between strains (Groot et al., 2010; Meagher et al., 2004; Pashley et al., 1995; Pashley, 1988b). Together, this overlap in host use and lack of clear host plant preference or fitness advantage, suggests that differences in host plant use are unlikely to be the primary factor maintaining genetic differentiation between strains. Because these strains remain genetically distinct, there are two other hypothesized reproductive isolation mechanisms: differences in the female pheromone composition (Groot et al., 2008; Lima and McNeil, 2009), and allochronic differences in mating times whereby the C-strain mates early in the night while R-strain mates late in the night (Pashley et al., 1992; Schöfl et al., 2009; Hänniger et al., 2017).

Although, C- and R- strain fall armyworms are considered the same species and have a sympatric distribution, they are also inherently different agricultural pests known to respond differently to common insecticides (Adamczyk et al., 1997). As such, strain specific management strategies guided by knowledge of their differences can help reduce the acres of crop lost to this species. In order to control these two pests in the field, it is imperative that we are able to differentiate between strains to determine the best treatment options. No morphological differences have been identified between strains, therefore rapid, cost-effective, molecular diagnostic assays are essential.

1.4. Molecular markers for differentiating host strains

Although these host strains are morphologically identical, they can be distinguished using molecular markers including allozymes (Pashley, 1986), AFLPs (Prowell et al., 2004), RFLPs within the mtDNA (Levy et al., 2002; Nagoshi et al., 2006), and a polymorphism in the Triose phosphate isomerase (*Tpi)* gene (Nagoshi, 2010). Though

these markers have been extensively used to determine host strain, none can reliably differentiate pure strain individuals from their hybrids. Both allozymes and AFLPs are dominant markers and therefore cannot detect hybrid heterozygotes. Likewise, because mtDNA is maternally inherited, it is similar to a dominant marker in that it cannot detect hybrid heterozygotes. The *Tpi* gene is often considered the most accurate indicator of host strain in the literature (Nagoshi, 2012). However, because it is located on the sex chromosome, *Tpi* is able to detect interstrain hybrids in males who have two copies of the Z-chromosome, but not in females who are hemizygous. Therefore, if a mix of male and female caterpillars is collected in the field, it is not possible to determine the relative proportion of interstrain hybrids.

Both laboratory and field studies have indicated a potential for interstrain hybridization, with multiple genetic markers inconsistently assigning host strain in 11-24% of individuals collected in the field (Nagoshi 2010; Nagoshi et al., 2006; Prowell, 1998). Although several laboratory studies have suggested mating incompatibilities between strains (Dumas et al., 2015; Kost et al., 2016; Quisenberry, 1991; Whitford et al., 1988), the direction of these incompatibilities is highly variable between studies. Due to a lack of genetic markers capable of differentiating strain and their hybrids, very few studies have been able to explore the prevalence and distribution of interstrain hybrids. The only study to date that has addressed questions about the ecology of interstrain hybrids using mismatches between multiple genetic markers concluded that hybrid individuals have host plant distributions than differ from both parental strains (Nagoshi et al., 2006; Nagoshi and Meagher, 2008). The development of rapid diagnostic assays

capable of differentiating strains and detecting hybrids will help managers and growers know which type of pest is present in their fields so they can manage them accordingly. Additionally, these tools will facilitate further exploration into the distribution, ecology and behavior the two strains and their hybrids across the Western Hemisphere.

To date, studies on the population structure and host strain distribution of *S. frugiperda* have been limited by the resolution of molecular markers, and the availability of diagnostic tools. A major objective of this dissertation is to use a population genomic approach to identify thousands of high-resolution SNP markers that can be used to characterize the population structure of *S. frugiperda* across various locations in the central US, and differentiate between strains. This will allow us to identify patterns of dispersal and interstrain hybridization of fall armyworm moths in the central US.

1.5. Reevaluating Bt resistance monitoring programs

One of the primary methods for controlling fall armyworms on corn is through the use of Bt transgenic crops expressing Cry1F and Vip3A toxins. Since their introduction in 1996, the acreage of corn expressing Cry and Vip insecticidal proteins has increased to 82% (USDA ERS, 2019). Although, this widespread adoption of genetically modified crops has transformed pest management by allowing growers to specifically target lepidopteran pests, without causing harm to most off target species (Comas et al., 2014; Mendelsohn et al., 2003; Nicolia et al., 2014; Tabashnik and Carrière, 2017), it has led the increased selection pressure on the target pest populations to overcome these insecticidal proteins, allowing for the rapid evolution of resistance (Tabashnik et al., 2003a).

Bt technology is foundational to current agricultural pest management practices in major cropping systems. To ensure the sustainability of these transgenic technologies, the US EPA has mandated resistance-monitoring programs be put into place for all Bt toxins being expressed. Current resistant monitoring strategies rely on two major assumptions, 1) the susceptibility phenotype is invariable across environments (i.e., constitutively-expressed or often improperly referred to as 'fixed') (Deans et al., 2016; Deans et al., 2017), and 2) the underlying physiology of all insects within a species (regardless of if they are from a laboratory colony or wild field population), is inherently the same. Since accurate measures of insect susceptibility to Bt toxins are key to implementing and evaluating insect resistance management plans (IRMs), it is imperative we test these assumptions to ensure our monitoring assays are accurate in reporting insect susceptibility.

1.6. Research Objectives

The goal of this dissertation was to use ecological and genetic tools to uncover new insights about fall armyworm populations in the US that can be used to inform pest management plans. Specifically, I had three primary objectives. First, I set out to characterize the population structure of fall armyworms in the central US and identify genomic patterns of gene flow and hybridization between the C- and R- strains. Second, I developed new diagnostic tools for differentiating between fall armyworm strains, and then demonstrated how these tools can be used to study strain behavior in the field. Last, I determined the extent to which nutritional ecology and domestication impact *S. frugiperda's* response to the Bt toxin Cry1F.

2. GENOMIC PATTERNS OF GENE FLOW AND STRAIN DIVERGENCE IN THE FALL ARMYWORM, *SPODOPTERA FRUGIPERDA* (J.E. SMITH)

2.1. Introduction

Understanding speciation and the origin of biodiversity is fundamental to the field of evolutionary biology. Most definitions of ecological speciation require the development of reproductive isolation between populations as a result of disruptive or divergent selection (Mayr, 1942; Seehausen et al., 2014). This process is not instantaneous, and the strength of reproductive isolation can vary depending on the time since divergence and the magnitude of the selection pressure. This creates a speciation continuum with various degrees of hybridization and gene flow occurring between incipient species.

The most widely accepted scenario for disruptive selection is when two populations become spatially separated, and thus experience different selection pressures as a result of their geographic isolation (Coyne, 1992). However, disruptive selection can also act on sympatric populations. For example, the availability of a novel host plant can drive divergence leading to genetically isolated host strains and eventually host-associated species as was seen in *Rhagoletis pomonella* (Feder et al., 1988; Filchak et al., 2000; Rice, 1984). Additionally, two divergent populations may arise due to sexual selection resulting in assortative mating (Turner and Burrows, 1995), a phenomenon which is thought to have contributed to the high diversity of cichlids in East African Lakes (Seehausen and Van Alphen, 1999). As a final example, divergent selection can act on phenology resulting in populations or species that mate at different times of the

day or in different seasons as has been documented in *Acropora* coral and the pine processionary moth, respectively (Fukami et al., 2003; Santos et al., 2011; Santos et al., 2007). Because speciation takes many generations and is nearly impossible to study in real-time for multicellular organisms, researchers rely on populations that are undergoing various stages of speciation to gain insights into this process.

When diverging populations or incipient species are morphologically identical, observing the process of sympatric speciation is reliant on molecular data. Sequencing and genomic analyses have extensive application for studying population relatedness and the speciation process. For example, several studies have found that sex chromosomes tend to accumulate more nonsynomous mutations than the autosomes indicating they play a disproportionate role in the speciation process, a phenomenon now referred to as the large X or fast Z effect (Charlesworth et al., 1987; Mank et al., 2007). Sequencing data has also led to the identification of genomic signatures indicating population divergence (Seehausen et al., 2014), the detection of patterns of introgression or selection (Alexander et al., 2009; Patterson et al., 2012), and the characterization of genes involved in reproductive isolation (Slate, 2005). Thus, analyzing genomic data of populations that are in the process of diverging can give us a glimpse into the origin of species and biodiversity.

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), is a moth species native to the Western Hemisphere. In the US, this insect only overwinters in south Texas and south Florida, and these locations serve as the source for fall armyworms reinvading the northern US and Canada each year (Sparks,

1979). No evidence of a return migration has been found for this species. Thus, at the end of the season, individuals occurring north of these overwintering sites die and their genetic variation is lost (Nagoshi & Meagher, 2008). Importantly, this species is comprised of two morphologically identical but genetically distinct sympatric strains that have been previously described as 'host-associated'. These strains were originally described by Pashley (1986) and named for the crops on which they were discovered, corn and rice (Pashley, 1986). Although this species is highly polyphagous feeding on up to 353 host plats from 76 plant families (Montezano et al., 2018), the larvae of the corn-strain, or C-strain, are described as primarily being associated with corn and sorghum, whereas the rice-strain, or R-strain, are more commonly associated with pasture grasses, Bermuda grass, and rice. However, the host ranges of these two strains largely overlap, with evidence of asymmetric host use between strains (Groot et al., 2010). So, although the C-strain is more commonly associated with corn and sorghum, the R-strain can also be found feeding on these hosts. It is uncommon, however, for the C-strain to be found feeding on smaller grasses such as pasture grasses and turf (Machado et al., 2008; Nagoshi, 2010). Several studies have used behavioral assays to assess whether the strains show a strong preference or fitness benefit when fed on different host plants, but no consistent differences have been found (Meagher et al., 2004; Pashley et al., 1995; Pashley, 1988b). Despite being defined as 'host-associated' – which implies that the divergence is due to differential selection on host use – the empirical evidence that host plant differences are driving divergence is limited. Considering this, several studies have

suggested that it may be more appropriate to refer to these strains as incipient species or genetic forms (Juárez et al., 2014; Kergoat et al., 2012).

Although the two fall armyworm strains can be consistently identified using genetic markers, hybridization has been reported in both the lab and field. Field studies have suggested that 16-24% of moths collected show inconsistencies between multiple strain specific genetic markers, likely as a result of interstrain hybridization (Nagoshi 2012; Prowell et al., 2004). In these field assessments, the majority of putative hybrids have the maternally inherited mitochondrial markers from the R-strain, indicating a directional mating bias (Nagoshi et al., 2006; Nagoshi, 2010; Prowell et al., 2004). In the lab, single pair matings have been conducted in both directions with evidence of reduced fertility amongst interstrain hybrids (Dumas et al., 2015; Kost et al., 2016). Still, these genetic markers have not become homogenous between the two strains, suggesting these strains remain genetically distinct despite occasional gene flow.

This species complex provides a unique opportunity to study speciation in action. In this study, we used genomic data to better elucidate the patterns of divergence and gene flow between *S. frugiperda* strains. We collected moths from five locations across the central US and used high-resolution SNP markers to identify genomic patterns of divergence between the two fall armyworm strains. Specifically, we assessed the extent to which these strains are reproductively isolated in the central US and identified patterns of divergence across the genome.

2.2. Methods

2.2.1. Insect collections

S. frugiperda moths were collected using universal moth traps baited with Scentry PSU 2-component lures (Scentry Biologicals, Billings, MT) and containing Hercon Vaportape. Each trap was placed in or around corn and sorghum fields at five locations across the central US. Multiple sampling times were selected throughout the year that roughly corresponded to seasons when moths were present at each location (Table 2.1). During each sampling period, traps were checked daily until a minimum of 24 moths were captured. At sites in the Lower Rio Grande Valley, larvae were occasionally collected by hand from nearby host plants. All sampled insects were immediately preserved in 95% ethanol and stored at 4°C until shipment to Texas A&M University in College Station, TX. Upon arrival, all specimens were stored at -80°C until DNA extraction. In total, 426 moths were sequenced across the two years.

Table 2.1 Collection location and date for all sequenced fall armyworm samples. The numbers of individuals from each collection is given both as the number per predetermined strain mitochondrial haplotype (R- or C-) and the number of total individuals from each collection (R- + C-).

			# Sequenced		
Location	**Date**	**GPS Coordinates**	**R-**	**C-**	**Total**
Lower Rio Grande Valley, TX	March 13-15, 2017	26.1556, -97.9618 &	6	16	22
	November 16, 2017	26.1556, -97.9618	12	0	12
	May 10-11, 2018	26.0924, -97.8814 &	7	15	22
	July 12-13, 2018	26.1556, -97.9618	22	1	23
	December 11-12, 2018	26.1556, -97.9618	16	2	18
Corpus Christi, TX	April 18-20, 2017	27.7827, -97.5621	20	1	21
	September 28-30, 2017	27.7827, -97.5621	16	3	19
	May 12-13, 2018	27.7827, -97.5621	8	10	18
	July 10-11, 2018	27.7827, -97.5621	2	12	14
	October 7-8, 2018	27.7827, -97.5621	1	12	13
College Station, TX	May 25-26, 2017	30.6206, -96.3617	13	10	23
	July 6-7, 2017	30.6206, -96.3617	0	16	16
	October 23-27, 2017	30.6206, -96.3617	12	0	12
	May 16-18, 2018	30.6206, -96.3617	11	7	18
	June 28-29, 2018	30.6206, -96.3617	5	10	15
	October 19-24, 2018	30.6206, -96.3617	12	0	12
Lubbock, TX	May 24-31, 2017	33.6912, -101.8259	0	15	15
	June 21-27, 2017	33.6912, -101.8259	7	16	23
	September 21, 2017	33.6912, -101.8259	9	13	22
	May 2, 2018	33.6912, -101.8259	0	12	12
	June 12, 2018	33.6912, -101.8259	1	11	12
	September 13, 2018	33.6912, -101.8259	5	13	18
Rosemount, MN	September 12-14, 2017	44.7069, -93.1068	8	18	26
	August 21. 2018	44.7069, -93.1068	0	20	20

2.2.2. DNA extraction

Prior to DNA extraction, the thorax was isolated from each specimen and surface sterilized in 95% ethanol. Tissues were tapped dry, placed individually in 2ml Eppendorf

tubes, and then frozen in liquid nitrogen. Sterilized plastic pestles were used to macerate the frozen thorax tissue. The Qiagen Gentra Puregene Tissue Kit was used to extract DNA following the manufacturer's protocol. The concentration of each DNA sample was measured on a NanoDrop spectrophotometer and all samples were diluted to a concentration of 50ng/ul.

2.2.3. Strain haplotype determination

After DNA extraction, strains were assigned using two known RFLPs in the *Cytochrome C Oxidase subunit I* (*COI*) mitochondrial gene (Levy et al., 2002; Nagoshi et al., 2006). Briefly, the primer pair *JM-76/JM-77* was used to amplify a 568bp fragment of *COI* (Levy et al., 2002). Then 4ul of the PCR product was added to both 2.5ul of *SacI* (New England BioLabs) and 2.5ul of *MspI* (New England BioLabs) diluted to their optimal working concentrations. Reactions were incubated at 37°C for 1 hour, and the products were run on a 1.8% agarose gel. The amplified C-strain mtDNA is cut once by *MspI*, and not by *SacI* while the R-strain mtDNA shows the reciprocal pattern (Nagoshi et al., 2006). Based on the cut patterns of both restriction enzymes, each individual was assigned as having either a C-strain or an R-strain mitochondrial haplotype. After haplotype determination, DNA was stored at -20°C until sequencing.

2.2.4. DNA sequencing, SNP calling and filtering

DNA samples were sent to Texas A&M AgriLife Genomics and Bioinformatics Services (TxGen) for quality control, library preparation, and double digest restriction-site associated DNA sequencing (ddRADseq) (Peterson et al., 2012). Prior to library prep, DNA was purified using the Agencourt AMPure XP purification system. Libraries were

prepared by digesting the total genomic DNA with *MseI* and *EcoRI* restriction enzymes, and 300bp to 500bp fragments were size selected for sequencing. Each fragment was ligated to standard Illumina adapters, sequencing primers, and multiplexing indexes. All sequencing was conducted on the Illumina NovaSeq 6000 to yield 150bp paired end reads. Sequence cluster identification, quality prefiltering, base calling and uncertainty assessment was then conducted using Illumina's NCS 1.0.2 and RFV1.0.2 software with default parameter settings.

TxGen provided the demultiplexed raw sequences and FastQC v.0.11.7 reports. FastQC reports were reviewed to ensure suitable quality, and then sequences were uploaded into the Texas A&M High Performance Research Computing 'Ada' cluster for bioinformatic analyses. All sequences are now available through the NCBI Sequence Read Archive.

On average, 1.83 million 150bp reads were obtained in each individual ddRAD library. This translated to an average of 275MB of sequence data per sample before filtering.

FastQ Screen v.0.14.0 with the BWA aligner was used to align raw reads to both the C-strain and R-strain published *S. frugiperda* genomes (Gouin et al., 2017). Sequences that did not match uniquely to one or both genomes were removed to clear the remaining sequences of all potential contaminant DNA (e.g. bacteria, pathogens, etc.). Forward and reverse reads were then matched together using the repair function in BBMAP v.3.8.08 (Chaisson and Tesler, 2012). After filtering out contaminant DNA and

DNA that matched multiple locations in the *S. frugiperda* genome, an average of 42.5% of the initial raw reads were retained for SNP calling.

Genomic loci that contained SNPs were identified using the dDocent v.2.2.16 SNP-calling pipeline (Puritz et al., 2014). In brief, dDocent removed low quality bases using Trimmomatic, and then mapped reads to the Liu et al., (2019) published chromosome map for *S. frugiperda* using BWA. The program FreeBayes then identified genomic loci containing SNPs and indels, and these variants were concatenated into a single VCF file. Our initial VCF file contained 441,437 variants.

Variants were filtered using VCFtools v.0.1.16 (Danecek et al., 2011). Specifically, all indels were removed and the remaining SNPs were filtered for a minimum PHRED score of 30. Only SNPs that were present in all individuals at a minimum of 3x coverage were kept in the final dataset. Finally, the dDocent_filters script (https://github.com/jpuritz/dDocent/blob/master/scripts/dDocent_filters) was run to complete SNP filtering. After filtering, the VCF file was manually examined and 236 SNPs did not map to a specific chromosome but rather to an 'unplaced_scaffold.' These unmapped SNPs were removed, leaving 5,439 mapped SNPs in the final dataset.

2.2.5. Analysis of molecular variance

The VCF file was uploaded into RStudio v.3.6.1 using R/vcfR v 1.9.0 (Knaus and Grünwald, 2017). To determine which collection factors (sampling year, location, season, and strain) significantly impacted the population structure of *S. frugiperda*, we carried out an analysis of molecular variance (AMOVA) using R/poppr v.2.8.3 (Kamvar

et al., 2014). We used a Monte Carlo test with 1000 random permutations to determine the statistical significance of each factor in the AMOVA.

2.2.6. Testing for population structure and strain admixture

To examine the population structure within fall armyworm samples, the VCF file was converted to a biallelic .bed (Plink Binary Biallelic Genotype Table) file and then to Eigenstrat format using PLINK v.1.07 (Purcell et al., 2007) and EIGENSOFT v.7.2.1 (Price et al., 2006), respectively. We then used the *smartpca* function within EIGENSOFT 7.2.1 to conduct a smart principal component analysis that identified and removed outliers in the dataset caused by population stratification (Patterson et al., 2006; Price et al., 2006). This program calculated Tracy-Widom statistics to determine the number of significant eigenvalues, or principal components, within the PCA. The PCA results were then plotted using R/ggplot2 (Wickham, 2016). Because the smartPCA revealed two distinct SNP based population clusters that corresponded to host strains, putative hybrids were identified as individuals that either did not fit neatly into the R-strain or C-strain SNP clusters, or individuals that had a mismatch between their mtDNA and SNP-based strain assignments.

To determine if the putative hybrids showed significant evidence of interstrain admixture, outgroup *f3* statistics were calculated using the 3-populations test function (qp3Pop) in AdmixTools v.5.0 (Patterson et al., 2012). In this test, pure-strain individuals from the R-strain and the C-strain were defined as the ancestral populations, and each putative hybrid individual was uniquely assessed for admixture using the

model *f3*(C-strain, R-strain; putative hybrid individual). Only individuals that had significantly negative *f3* values were considered true hybrids.

The program ADMIXTURE 1.3.0 was run using default parameters to determine the probability that individual moths were assigned to one or more genetically distinct groupings (Alexander and Lange, 2011). The K-values input ranged from 1 to 15, and the optimal value of K was determined as the run that resulted in the lowest cross validation (CV) error. The browser based program CLUMPAK was used to visualize the population assignment of all individuals (Kopelman et al., 2015).

2.2.7. Analyzing genomic patterns of strain divergence (Z-chromosome vs. autosomes)

Using the smartPCA and admixture results, each individual was assigned to the R-strain or the C-strain based on their SNP groupings. Individuals with significant evidence of admixture from the *f3* test were removed from this analysis. To determine the level of divergence between strains at each SNP locus, a fixation index, or F_{st} value, was calculated for every mapped SNP using R v.3.6.2/genepop (Rousset, 2008). A Manhattan plot visualizing the chromosome position of each SNP and its associated F_{st} values was created using R v.3.6.2/qqman (Turner, 2018).

SNPs with F_{st} values >0.7, or that appeared as outliers on the Manhattan plot, were identified and mapped back to the published chromosome map (Liu et al., 2019) using Geneious v.11.0.2. After mapping, a 501bp DNA fragment that included the SNP and 250bps up and downstream of the variant was extracted from the genome. In several cases, divergent SNPs were in close proximity to one another and were grouped

together. In these case, a DNA segment 250bp upstream of the most 5' SNP and downstream of the most 3' SNP was extracted. Each DNA sequence was then uploaded and searched in the NCBI insect nucleotide database (taxid:6960) using megablast to identify any similar, previously characterized, nucleotide sequences.

The VCF file containing all mapped SNPs was then split into two new VCFs. The first included only SNPs that matched to chromosome 1 – the Z-chromosome – and the second contained SNPs that matched to chromosomes 2-31 – the autosomes. Global F_{st} values were calculated in R v.3.6.2/genepop (Rousset, 2008) using all mapped SNPs in the genome (Z-chromosome + autosomes), just SNPs on the Z-chromosome, and just SNPs on the autosomes.

Both the Z-chromosome and the autosome VCF files were then converted to PHYLIP file formats using PGDSpider (Lischer and Excoffier, 2012). Maximum likelihood phylogenetic trees were then constructed using RAxML v.2.1.1.3 implementing a generalized model for sequence evolution (GTRCAT) (Stamatakis, 2006; Stamatakis, 2014). Statistical support was assessed through rapid bootstrapping using the autoMRE setting within RAxML. This resulted in 360 rapid bootstrap searches for the autosome tree and 460 for the Z-chromosome tree. Equal-angle unrooted phylogenetic trees were plotted using R v. 3.6.2 /ggtree (Yu et al., 2017).

2.3. Results

2.3.1. Factors that contribute to genetic variation

Prior to sequencing, we determined the relative proportion of each collection from a given location and time that was comprised of the R-strain and C-strain individuals using mtDNA haplotypes. Although traps were placed in and around corn and sorghum fields which are typically considered C-strain host plants (Pashley, 1988a), we found that most locations contained a mix of both C- and R-strain haplotypes. Unexpectedly, several collections, especially during the fall season, were solely comprised of individuals with R-strain haplotypes (Figure 2.1). Because we had collected a representative sample of both host strains during most collection times, a mix of individuals comprising both host strains were sequenced (Table 2.1).

Although there were several potential sources of genetic structure in our dataset (year, location, season, and strain), our analysis of molecular variance (AMOVA) revealed predetermined host strain haplotype was the only factor that significantly accounted for genetic variability in the data (Table 2.2, ϕ=0.09, $p<.001$). The variables year, location, and season were not significant ($p<0.05$).

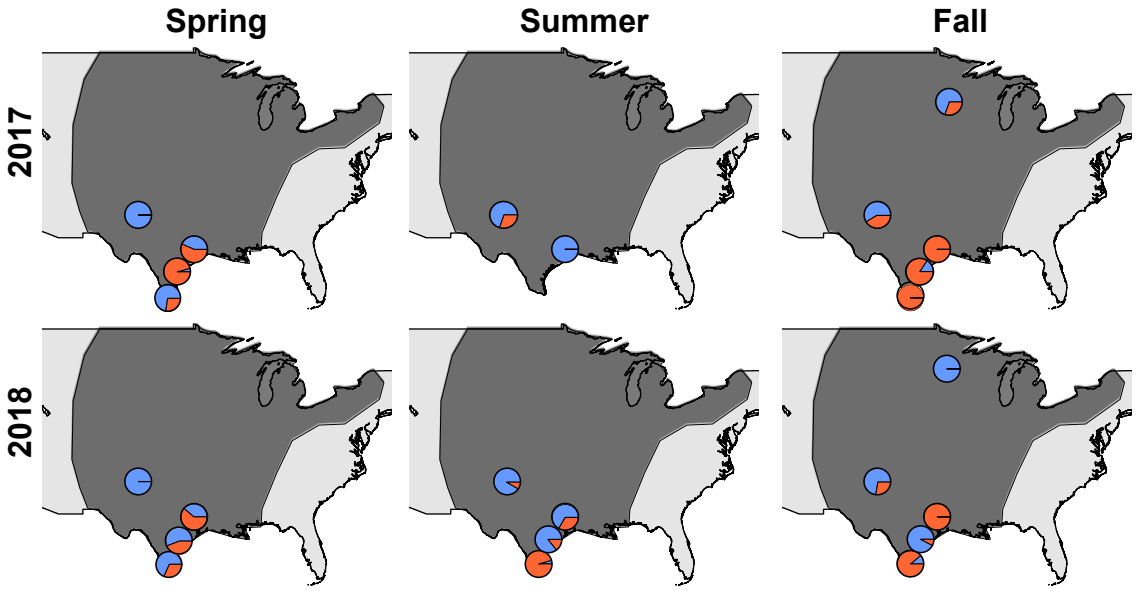

Figure 2.1 Proportion of individuals with C-strain (blue) or R-strain (orange) mtDNA profiles at each collection location and season. The dark grey overlay illustrates the proposed central US flyway for fall armyworm moths (inferred from Westbrook et al., 2016).

Table 2.2 Sources of genetic variation between all fall armyworm samples determined by an analysis of molecular variance (AMOVA). The factors assessed include collection year, sampling location, sampling season, and host strain. Host strain was the only collection factor that contributed significantly to the population structure of *S. frugiperda*.

Source of Variance	df	% Variance	ϕ-statistic	p-value
Between Years	1	-0.44	-0.004	0.618
Within Year between Locations	8	0.98	0.01	0.209
Within Locations between Seasons	14	-2.06	-0.021	0.788
Within Season between Strains	16	9.17	0.09	<0.001***
Between Samples	386	5.97	0.065	<0.001***
Within Samples	426	86.40	0.136	<0.001***

The major effect of host strain haplotype on genetic structure was further supported by a smartPCA conducted on the SNP dataset. In this analysis, the data clustered into two distinct groupings along PC1 that roughly corresponded with the predetermined mitochondrial haplotypes (Figure 2.2a). Other principal components did not identify any additional population groupings. When conducting the SmartPCA, 33 samples were removed as outliers due to cryptic relationships (genetically too similar within collections). Roughly half of these outliers were from the spring collections conducted in the Lower Rio Grande Valley, in which caterpillars were collected. Since, it is reasonable that some sibs or half sibs were collected when sampling caterpillars from the same fields, we continued our analysis with only the remaining 393 unrelated individuals.

In addition to conducting a PCA, Tracy-Widom statistics were calculated to evaluate the statistical significance of each eigenvalue or principal component (Patterson et al., 2006). We found that only the first principal component was statistically significant (TW=405.962, $p<0.0001$), while all other PCs had p-values greater than 0.05 (Table A-1). Together, this indicates that the only factor contributing to genetic diversity in our data was host strain. Finally, as part of the smartPCA, an ANOVA was run using each of the first 10 eigenvectors to determine if the population assignments significantly explained the genetic differentiation across the first 10 principal components. Here, we assigned individuals to one of two populations (R- or C-) based on their mtDNA haplotypes. The ANOVA stats were then summed across eigenvectors, giving a chi-

square distribution with the df=10. Significant genetic differentiation in the dataset could be explained by strain assignment (X^2= 471.194, p<0.0001).

Because multiple lines of evidence indicated that *S. frugiperda* population structure across the central US was explained entirely by host strain, further analyses focused on genetic differentiation and admixture between host strains.

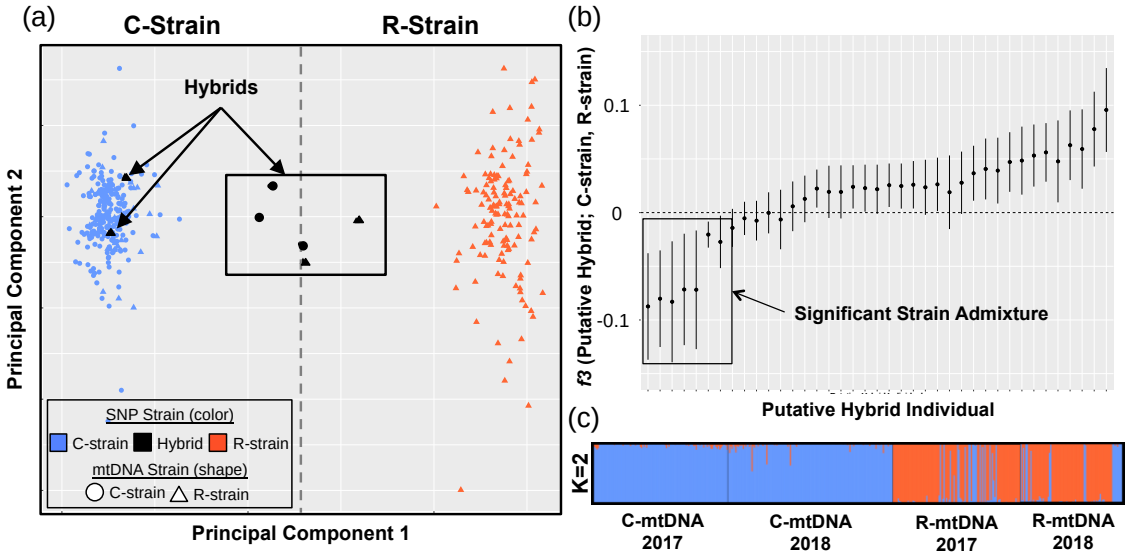

Figure 2.2 SNP data indicates clear genetic differentiation between host strains, however, some admixture is evident. (a) smartPCA results neatly split C-strain (blue) and R-strain (orange) individuals into two clusters along principal component 1. Five individuals fall in between the two clusters, and thirty-six individuals have the R-strain mtDNA but are grouped in the C-strain SNP cluster. Individuals that are marked in black exhibit significant strain admixture (*f3*<0). (b) Outgroup *f3* statistics plotted for each putative hybrid individual. Individuals with a mean *f3*<0 show significant strain admixture. (c) ADMIXTURE plot (K=2) for all individuals split according to their mtDNA and collection year. Each bar illustrates the probability of assignment to one of two genetically distinct groups.

2.3.2. Admixture between strains

Although the two clusters in the PCA could largely be explained by strain mtDNA haplotypes, there were 34 individuals that showed a mismatch between the mtDNA haplotypes and their SNP cluster assignment. 100% of these mismatched individuals contained R-strain mtDNA, but clustered within the C-strain SNP cluster. Additionally, two individuals with R-strain mtDNA and three individuals with C-strain mtDNA mapped directly in between the two SNP clusters on the smartPCA (Figure 2.2a). This assemblage of 39 individuals comprised of mismatches and those that did not group were classified as putative hybrids.

f3 statistics were calculated to determine if these putative hybrids exhibited significant admixture between the two strain source populations (Table A-2). In this analysis, *f3* values significantly lower than zero indicate significant admixture between two source populations. We found that all five individuals that did not neatly group with either SNP cluster exhibited significant admixture between the two host strains. Additionally, two individuals that grouped with the C-strain SNP cluster but carried the R-strain mtDNA (mismatches), exhibited significant strain admixture (Figure 2.2b). These seven individuals will henceforth be referred to as the hybrids and are indicated as such on Figure 2.2a.

ADMIXTURE analysis was then conducted to calculate the probability of individuals assigned to one or more (K) genotypic groups. The lowest cross-validation error (CV) occurred when K=2 or with two genotypic groups. K=3 or higher did not show any additional population resolution. These two genotypes largely corresponded

with the predetermined strain haplotypes (Figure 2.2c). Slightly more admixture was detected amongst individuals with the R-strain mtDNA compared to those with C-strain mtDNA.

2.3.3. Genomic patterns of strain divergence

To determine the level of divergence between strains at every SNP locus, we calculated the fixation index, or F_{st} values, for each of the 5,439 high quality mapped SNPs. We then visualized patterns of divergence across the genome by plotting the F_{st} for every SNP across chromosomes using a Manhattan plot (Figure 2.3a). Since the Manhattan plot indicated that the majority of divergent SNPs, those with F_{st} values closest to 1, were located on the Z-chromosome, we plotted a frequency distribution of F_{st} values for both the autosomes and Z-chromosomes (Figure 2.3b). For both distributions, the majority of F_{st} values are located near 0, however on the Z-chromosome there is a second peak in F_{st} values around 0.8. This may indicate a barrier to gene flow exists on this chromosome.

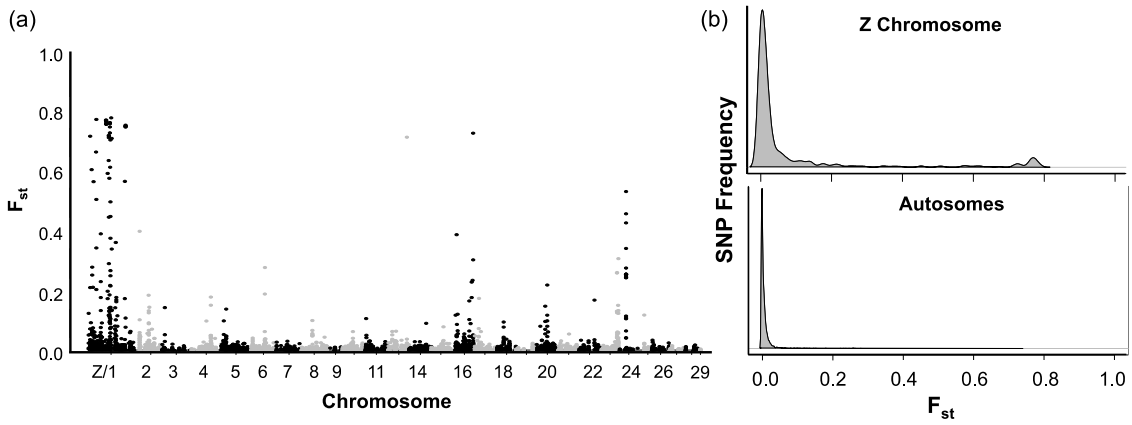

Figure 2.3 (a) Manhattan plot illustrating locus specific F_{st} values differentiating fall armyworm host strains for every SNP identified. Here, fall armyworm host strains were defined as individuals that grouped in either the C-strain or R-strain SNP cluster as seen in Figure 2.2a. (b) Frequency distribution of F_{st} values across the Z-chromosome and the autosomes.

We then calculated global F_{st} values across all 5,439 SNPs in the genome and found that there was moderate genetic differentiation between the C- and R-strains (F_{st}= 0.108). When we separated the Z-chromosome and the autosomes we found significant evidence of genetic differentiation between strains on the Z-chromosome (F_{st}=0.401), and very little differentiation across the autosomes (F_{st}=0.031). This is the first evidence of a fast Z-effect in *S. frugiperda* (Table 2.3). We further characterized SNPs with F_{st} values >0.7, and found that most of the highly divergent SNPs that mapped to coding regions of the genome resulted in synonymous mutations. However, one group of SNPs potentially disrupts the function of a suppressor of cytokine signaling gene located on the Z-chromosome (Table A-4). Since, ddRADseq is designed to randomly identify neutral SNP markers across the genome, SNPs with high F_{st} values may be linked to

genomic regions that show high levels of strain divergence, even if these SNPs do not cause changes to the protein coding sequence.

Table 2.3 Global F-statistics calculated for all SNPs across the genome, just SNPs located on the autosomes (chromosomes 2-31), and just SNPs located on the Z-chromosome.

	F_{is}	F_{st}	F_{it}
Whole Genome	0.081	0.108	0.180
Autosomes	0.065	0.031	0.094
Z-Chromosome	0.185	0.401	0.511

Maximum likelihood phylogenies generated using either the autosomes alone or the Z-chromosome data alone group individuals within a strain closer to each other than to individuals of the opposite strain. These groupings are much better supported in the Z-chromosome tree compared to the autosome tree as evidenced by longer branch lengths and higher bootstrap values on the branch differentiating the two host strains (Figure 2.4). Both autosomes and Z-chromosomes indicate more diversity amongst individuals of the C-strain compared to individuals of the R-strain.

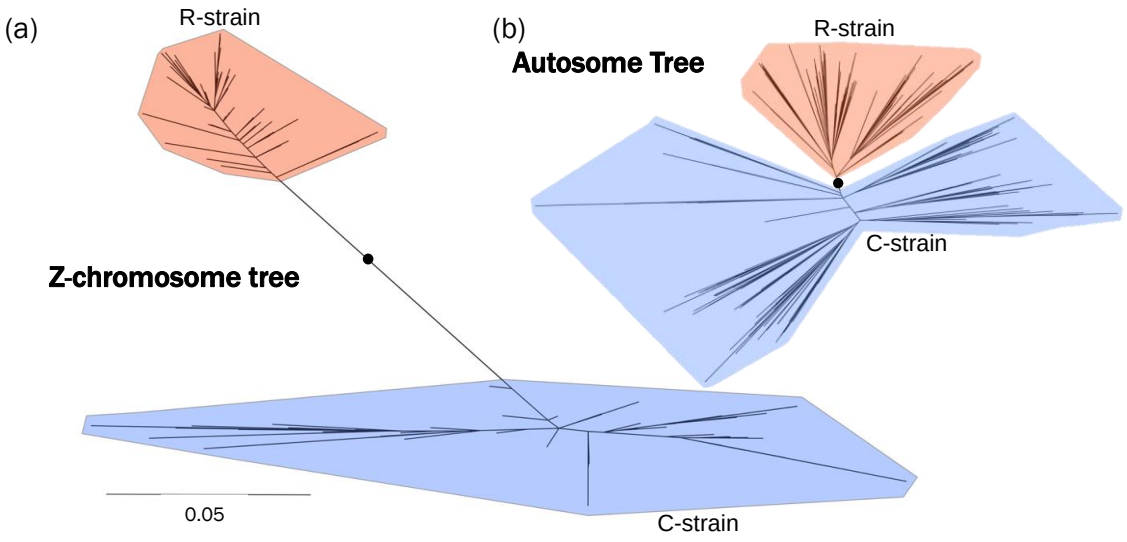

Figure 2.4 Scaled ML equal-angle phylogenetic trees created using SNPs located on (a) the Z-chromosome or (b) the autosomes. The scale bar represents number of mutations per site. Individuals that were assigned to the C-strain SNP group are highlighted in blue whilst those assigned to the R-strain are represented in orange. Bootstrap values for the long-branch dividing the C- and R-strain clusters (marked with a black circle) are 76 on the Z-chromosome tree and 37 on the autosome tree.

2.4. Discussion

We present evidence for two genetically distinct populations of fall armyworms in the central US that have previously been described as host-associated strains (Pashley, 1986). Despite collecting in fields dominated by C-strain host plants, our trap captures generally comprised both R-strain and C-strain individuals. This is consistent with previously reported trap captures across the US (Meagher & Nagoshi, 2004; Nagoshi & Meagher, 2004a).

Although these two strains are genetically distinct, they have a sympatric distribution and overlapping host ranges. While both strains can be found feeding in corn and sorghum fields, the C-strain is rarely found on smaller pasture grasses and may have

a slightly reduced host range compared to the R-strain (Machado et al., 2008; Nagoshi et al., 2007; Prowell et al., 2004). Other factors that have been implicated in the genetic divergence of these strains include allochronic differences in mating time (Hänniger et al., 2017; Pashley et al., 1992; Schöfl et al., 2009) and unique pheromone compositions (Groot et al., 2008; Lima & McNeil, 2009), which may facilitate reproductive isolation. Given that behavioral studies have failed to consistently find differences in host plant preference and performance between the strains (Groot et al., 2010; Meagher et al., 2004; Pashley et al., 1995; Pashley, 1988b). In this study, we address three primary questions about *S. frugiperda* populations structure and divergence: (1) What is the degree of reproductive isolation between these two strains/populations?; (2) To what extent does divergence occur across the genome in light of ongoing gene flow?; and (3) What are these two genetically distinct groups if not 'host associated' strains?

2.4.1. Host strain reproductive isolation

Using *f3* tests, we identified seven samples that exhibited significant interstrain admixture. These samples included five putative F1 hybrids that mapped neatly in the middle of the two host strains on the PCA, and two individuals that had a mismatch between their mtDNA strain haplotype and their SNP strain genotype. This is strong evidence that hybridization occurs between the two strains in the field. Since both R-strain and C-strain mtDNA was recovered amongst the putative F1 hybrids, we conclude that in the field, fall armyworm females of both the C-strain and the R-strain occasionally mate with males of the opposite strain. Since two mismatch individuals also showed significant signs of admixture, but appeared to be closer related to the C-strain

than the R-strain, these individuals are predicted to be the offspring of a hybrid female of R-strain maternal origin backcrossed to a C-strain male.

Thirty-two additional individuals showed a mismatch between the maternally inherited mtDNA strain marker and their SNP genotype. Although these individuals were not significantly admixed according to our *f3* test, we suspect they are the result of past hybridization events followed by several generations of backcrossing. Overtime, the signal of admixture has been reduced and can no longer be detected using *f3* statistics, however, the mismatch between the mtDNA and SNP genotype is still evident. Interestingly, 100% of moths that have a mismatch between their maternally inherited mtDNA and their SNP genotype have the R-strain mtDNA markers. Previous studies that defined hybrids as individuals with a mismatch between the mtDNA and nuclear strain markers also found the majority of putative hybrids collected in the US have R-strain maternal origin (Nagoshi & Meagher, 2003; Nagoshi, 2010; Nagoshi et al., 2017; Prowell et al., 2004). This indicates that hybrid females with R-strain maternal origin are successfully mating with C-strain males, however hybrids with C-strain maternal origin are not backcrossing to the R-strain. This is strong evidence that a unidirectional barrier to reproduction exists, limiting hybridization between these strains in the field.

Although this pattern of unidirectional introgression has been routinely recovered in field data, laboratory assays have been less consistent. Some studies have found a unidirectional mating bias where R-strain females are able to mate and produce offspring with C-strain males, but the reverse is not true (Pashley & Martin, 1987), whilst other laboratory assays successfully conducted reciprocal crosses of both strains (Quisenberry,

1991; Whitford et al., 1988a) and have even found that hybrid females of C-strain maternal origin are more fertile than hybrids of R-strain maternal origin (Kost et al., 2016). Still another study successfully conducted reciprocal interstrain crosses, but found that F1 hybrids of C-strain maternal origin had a drastic reduction in fitness, while F1 hybrids with R-strain maternal origin had only a minor fitness cost (Dumas et al., 2015). This last study by Dumas et al. (2015) is most consistent with our field data. Although it is not clear why variable results have been found in laboratory mating assays, these studies have consistently reported behavioral and/or physiological barriers that limit hybridization between strains, serve as barriers to gene flow, and can reinforce strain identity.

Although we detected hybridization between strains, mitochondrial markers have been used as reliable strain indicators across multiple regions of the Western Hemisphere for the past several decades (Juárez et al., 2014; Levy et al., 2002; Nagoshi et al., 2006; Nagoshi et al., 2007). If gene flow occurs between strains and causes a mismatch between the mtDNA and SNP genotype, then these markers would be expected to homogenize across strains and become less reliable overtime. Since this has not been observed, we hypothesize that the combination of selection against unfit hybrids, a one way migration that removes all admixture occurring north of the overwintering site, and genetic drift caused by large population size fluctuations at the overwintering site (Nagoshi & Meagher, 2004b), play a role in maintaining the genetic integrity of these strains despite interstrain gene flow.

2.4.2. Patterns of host strain genomic difference

In our data, we found 5,439 SNPs that were distributed across the entire fall armyworm genome. We then calculated the fixation index (F_{st}) for every SNP in the genome to determine which SNPs showed the highest signals of divergence between the R-strain and C-strain. Although there may be some genetic differentiation between strains on chromosomes 12, 16, and 24, it is evident from our data that the majority of diagnostic SNPs, or SNPs with F_{st} values nearing 1, are located on the Z-chromosome. This indicates that the sex chromosome is playing a disproportionate role in strain divergence compared to the autosomes. This phenomenon is known as the 'fast Z-effect' in species where the female is the heterogametic sex (Mank et al., 2007). We then constructed maximum likelihood phylogenies using SNP data from just the Z-chromosomes or just the autosomes, and found additional support for the fast Z-effect. Here, the Z-chromosome showed a much larger signal of divergence between host strains, relative to the autosomes. This idea that the majority of strain divergence can be found on the Z-chromosome likely explains why virtually all the markers that have been found to differentiate these strains can be mapped to this chromosome (Nagoshi, 2010; Prowell, 1998). Both the autosome and Z-chromosomes phylogenies tended to show more genetic diversity amongst C-strain genotypes than R-strain genotypes.

When two sympatric populations initially split, divergent selection likely acts upon autosomal genes because they have high genetic variation (Orr and Betancourt, 2001) and are equally exposed in both males and females (Qvarnström and Bailey, 2009). However, after this initial divergence, both theoretical and empirical evidence has

indicated sex chromosomes play a disproportionate role in speciation (Qvarnström and Bailey, 2009). When divergence causes individuals to selectively mate within their population, mutations will begin to accumulate separately between the two populations. Since novel recessive mutations that occur on the sex chromosomes will always be expressed in the heterogametic sex, selection acts faster on the sex chromosomes than the autosomes to both remove deleterious mutations or fix beneficial mutations (Charlesworth et al., 1987). Additionally, since the effective population size (N_e) of the sex chromosome is lower that the autosomes, mutations on the Z-chromosome that are neutral or slightly deleterious have a higher probability of becoming fixed by chance (i.e. genetic drift) than mutations on the autosomes (Mank et al., 2007). This results in higher observed levels of divergence on the sex chromosomes in populations in the process of speciation. The fast Z-effect that we see in fall armyworms may indicate these two strains are actually incipient species in the process of divergence.

2.4.3. Are these genetically distinct groups host associated?

In this study, we collected moths at five locations across the central US, at multiple time points throughout the year over the course of two years. However, when we analyzed our data for population structure, strain was the only factor that significantly contributed to genetic diversity within our population. Factors such as location, season, and year collected did not provide any additional population structure, indicating that a single panmictic population of each strain occurs in the central US. This is consistent with the dispersal trajectory of this species (Nagoshi et al., 2009; Nagoshi et al., 2008; Westbrook et al., 2016). Fall armyworms are known to overwinter in south Texas and south Florida,

and travel north each spring in a generational stepwise northward expansion. Because all traps were placed in the central US, far west of the Florida population, all our collected moths are expected to descend from the south Texas overwintering population (Westbrook et al., 2016).

Interestingly, all moths collected were found amongst C-strain host plants, yet 43% of our samples were made up of R-strain individuals. Since previous behavioral studies have indicated inconsistent patterns of host plant preference or performance (Meagher et al., 2004; Pashley et al., 1995; Pashley, 1988b), and many trap captures, including those reported in this study, collect a mix of both strains (Meagher & Nagoshi, 2004, 2013; Nagoshi & Meagher, 2004a), the term 'host-associated' does not seem appropriate for these two genetically distinct biotypes. This evidence suggests that host plant selection is not the intrinsic driving factor in strain divergence, but rather is more likely an extrinsic byproduct of the speciation process. Thus, continuing to refer to these strains as host associated arguably misrepresents their ecology and the process underlying their ongoing divergence in the field.

An allochronic difference in mating time is the most consistent reproductive isolating mechanism that has been found diverging fall armyworm strains (Hänniger et al., 2017; Pashley et al., 1992; Schöfl et al., 2009), and we propose that this may be the driving factor of strain isolation. There are two different scenarios that could have led to the initial divergence of two allochronic strains approximately 2mya (Kergoat et al., 2012). First, two populations of *S. frugiperda* may have become geographically isolated, resulting in either a bottleneck effect or directional selection on nocturnal activity

pushing one population to have an activity peak early in the night (current C-strain) whilst the other population peaked later in the night (current R-strain). Alternatively, in the absence of a geographic barrier, disruptive selection on nocturnal activity may have occurred in sympatry diverging the two strains, potentially as a response to nocturnal predation. Allochronic separation as an alternative hypothesis for strain divergence is further tested in Chapter 3.

3. DEVELOPING NOVEL DIAGNOSTIC TOOLS TO TEST HYPOTHESES ABOUT THE BEHAVIOR OF C- AND R- STRAIN FALL ARMYWORM MOTHS IN THE FIELD

3.1. Introduction

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith), is a highly mobile, polyphagous agricultural pest native to the Western Hemisphere. Because this species does not diapause, in the US they only survive the winter in the southern tips of Florida (eastern population) and Texas (central population), annually dispersing north from these overwintering sites as temperatures rise in the spring (Luginbill, 1928; Nagoshi and Meagher, 2008; Sparks, 1979; Westbrook et al., 2016). Across both overwintering populations, the fall armyworm as a species is comprised of two morphologically identical but genetically distinct strains that were originally described by Pashley (1986). These strains have been described as host associated, and were originally named for the host plants on which they were discovered, the corn strain or C-strain and the rice strain or R-stain (Pashley, 1986). C-strain fall armyworms have primarily been associated with large grasses such as corn and sorghum, whilst the R-strain is primarily associated with smaller grasses such as Bermuda hay, pasture grasses, and millet (Meagher and Gallo-Meagher, 2003; Nagoshi and Meagher, 2008; Pashley, 1986). Although these two strains are the same species, they have been shown to differ in their susceptibility to several insecticides including carbamates, organophosphates, pyrethroids, and Bt transgenic crops, making each strain a unique agricultural pest (Adamczyk et al., 1997).

Although these strains have been considered host associated, their host ranges have significant overlap with pheromone baited moth traps near a given crop regularly recovering a mix of both strains (Chapter 2, Meagher and Nagoshi, 2004; Meagher and Nagoshi, 2013; Nagoshi and Meagher, 2004a). It has been suggested that the corn strain may have a slightly more limited host range than the R-strain, being less frequently found on smaller grasses such as Bermuda hay (Groot et al., 2010; Machado et al., 2008; Meagher and Nagoshi, 2004; Nagoshi et al., 2006; Nagoshi et al., 2007). However, there has been no consistent evidence for differential preference or fitness benefits between strains when fed on different host plants in behavioral studies (Meagher et al., 2004; Pashley, 1988b; Pashley et al., 1995; Whitford et al., 1988).

While hybridization occasionally occurs between strains, they do remain genetically distinct, indicating barriers to gene flow must exist (Chapter 2). With such significant overlap in host range, habitat isolation due to host use is likely a weak prezygotic isolating mechanism between these strains. A more consistent reproductive isolation mechanism that has been observed using behavioral mating assays in the lab is allochronic differences in nightly activity (Hänniger et al., 2017; Pashley et al., 1992; Schöfl et al., 2009). In these laboratory assays, the C-strain was actively mating in the first 6 hours after the onset of the scotophase, whereas the R-strain was active between 6-10hrs after the onset of the scotophase (Pashley et al., 1992). This difference in mating time is heritable and has been mapped to strain specific polymorphisms in the circadian rhythm modulator gene, *vrille* (Hänniger et al., 2017). Field studies on the behavior and ecology of these strains have been rare due to challenges differentiating between strains.

Being able to rapidly and accurately differentiate between strains is imperative for both understanding the basic behavior and ecology of each strain in the field, and implementing strain specific management plans.

Currently, there are two genetic markers that are primarily used to differentiate between fall armyworm strains. The first consists of polymorphisms in the *Cytochrome C Oxidase I* (*COI*) mitochondrial gene, which can be assessed using restriction fragment length polymorphisms (RFLPs) (Levy et al., 2002; Nagoshi et al., 2006). Since mitochondrial DNA is maternally inherited, this method is incapable of detecting hybrid heterozygotes (Nagoshi, 2012). The second maker used to differentiate strains relies on polymorphisms in the *Triose phosphate isomerase gene* (*Tpi*) located on the Z-sex chromosome (Nagoshi, 2010a). Ten strain specific polymorphisms in the *Tpi* gene have been identified that can be assessed either through amplicon sequencing, or RFLP analysis (Nagoshi, 2012, 2010a). Because *Tpi* is a sex-linked gene, this marker can identify F1 interstrain hybrid males, the homogametic sex (ZZ), but it is not able to detect interstrain hybrid females, who are hemizygous (ZW). Although strain assignments using these two markers are generally consistent, discordance between them has been reported in up to 24% of moths (Nagoshi, 2012); 10% of which consisting of *Tpi* heterozygotes, and 14% in which had an opposite strain assignment between the *Tpi* and *COI* markers. This discordance may be the result of interstrain hybridization, followed by several generations of backcrossing. With such a high percentage of moths showing a mismatch between the two strain identification methods, more molecular

markers are needed to both confirm the strain assignments of field collect moths, and get a better idea of the interstrain hybridization rate in the field.

Recent advances in sequencing and real-time PCR allow for the development of improved molecular diagnostic assays based on single nucleotide polymorphisms (SNPs) with alleles unique to each fall armyworm strain. Fluorogenic Real-Time PCR-based TaqMan® assays, originally developed by Livak et al. (1995), are a rapid and effective method for identifying the specific allele at a known SNP locus within an organism's genome. In these assays, two highly specific fluorescent probes are introduced into a PCR reaction. Each probe is bound to a unique fluorophore at the 5' end, and a quencher at the 3'end (Figure 3.1a). Whilst the flourophore is in close proximity to the quencher, no fluorescence is emitted. During the PCR reaction, DNA is denatured and the probe binds to its target sequence. Forward and reverse primers bind to the DNA sequence and Taq DNA polymerase begins amplifying the sequence of interest (Figure 3.1b). When the Taq reaches the bound probe, the fluorophore is released, and emits its fluorescent signal. As the PCR repeats, the fluorescent signal increases, until it passes the threshold that can be detected on a real-time PCR machine. The number of cycles necessary to reach this threshold is known as the Cq value (Figure 3.1c).

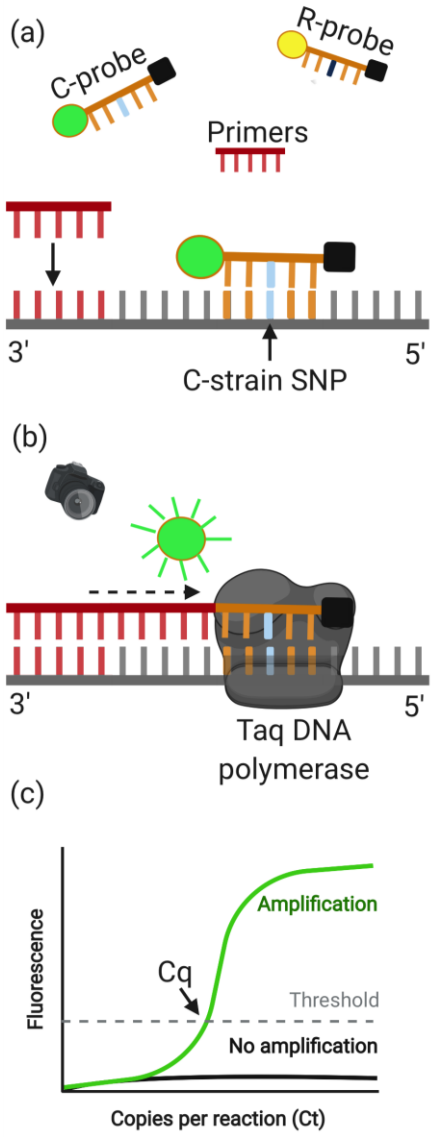

Figure 3.1 Illustration of Real-Time PCR based TaqMan assays to identify the strain-specific allele present at a SNP locus. (a) After DNA denaturation, the probe containing a strain specific SNP allele binds to the DNA. Following the probe binding, primers anneal to their complimentary sequences. (b) Taq DNA polymerase begins replicating the DNA. Upon reaching the probe, the flourophore is released from its association with a quencher, emitting a fluorescent signal that can be detected on a Real-Time PCR machine. (c) As the number of PCR cycles increases, the fluorescent signal increases. The number of cycles required for the fluorescent signal to reach intensity above background noise (e.g. threshold) is the fluorophore Cq value (Figure created using Biorender.com).

In the current study we use SNP data collected in Chapter 2 to develop four new Real-Time PCR based TaqMan® diagnostic assays to rapidly and reliably differentiate between strains. We validate these assays using fall armyworm samples collected from across both the eastern and central US flyways and then compare the accuracy of our diagnostic assays to the two most commonly used methods for strain differentiation, *Tpi* and *COI*. Finally, we employ these new diagnostic assays to test the hypothesis that allochonic differences in nightly activity occur between the two fall armyworm strains in the field.

3.2. Methods

3.2.1. TaqMan® assay development

3.2.1.1. Diagnostic SNP identification and TaqMan® Real-Time PCR assay development

All sequencing data used to develop real-time PCR assays were the same as those used in Chapter 2. Sequences were obtained from 426 moths collected from 5 locations across the central US. Genomic DNA from the thorax of all samples was extracted using the Gentra Puregene Tissue Kit (Qiagen), and sequenced using double digest restriction site associated DNA sequencing (ddRADseq) (Peterson et al., 2012). Libraries were prepared by digesting genomic DNA with *MseI* and *EcoRI* and then selecting fragments that ranged from 300-500bp. All fragments were sequenced on an Illumina NovaSeq 6000 to obtain 150bp paired end reads. Sequencing was conducted by Texas A&M AgriLife Genomics and Bioinformatics Services (TxGen), who provided demultiplexed raw reads and FastQC v.0.11.7 reports.

Raw reads were initially aligned to both the published C-strain and R-strain *S. frugierda* genomes (Gouin et al., 2017) using FastQ Screen v.0.14.0 with the BWA aligner order to remove any potential contaminant DNA. All sequences that did not match uniquely to either genome were removed from the analysis. The repair function in BBMap v3.8.08 (Chaisson & Tesler, 2012) was used to match together the resulting forward and reverse reads and the dDocent v.2.2.16 SNP-calling pipeline was used to identify genomic loci containing SNPs (Puritz et al., 2014). The dDocent pipeline removed low quality bases using trimmomatic, and then mapped all reads to a published *S. frugiperda* chromosome map (Liu et al., 2019) using a BWA aligner. Genomic loci containing SNPs and indels were identified using the program FreeBayes and then written in a VCF file.

All indels were removed and SNPs were filtered to a minimum PHRED score of 30 using VCFtools v.0.1.16 (Danecek et al., 2011). The dDocent_filters script (https://github.com/jpuritz/dDocent/blob/master/scripts/dDocent_filters) was run as the final SNP filtering step. All SNPs that did not match to a specific chromosome on the Liu et al. (2019) chromosome map were removed from the analysis. In total, 5,439 mapped SNPs were used for further analysis. Using a smartPCA and *f3* outgroup statistics, we identified 271 individuals as C-strain, 146 individuals as R-strain and 7 individuals as interstrain hybrids. All hybrids were removed when identifying potential loci that were diagnostic between strains.

To identify strain specific SNP loci, fixation indices (F_{st}) between the C- and R-strain individuals were calculated for each of the 5,439 mapped SNPs using R

v.3.6.2/genepop (Rousset, 2008). All SNPs with F_{st} values greater than 0.7 were identified for further analysis. Each of these SNPs were mapped to the Liu et al. (2019) chromosome map in Geneious v.11.0.2, and 250 bp upstream and downstream of each SNP was extracted (501bp region total). If any of the other 5,439 identified SNPs were present in this 501bp region, the reference nucleotide was denoted as 'N'. In several cases, divergent SNPs were in close proximity to one another in the genome. In these cases, the DNA segment 250bp upstream of the most 5' SNP and downstream of the most 3' SNP was extracted.

All extracted sequences containing divergent SNPs were manually examined in Geneious to assess the variability in each region and the possibility of designing high quality primer and probe sequences. Of the 41 initial SNPs with F_{st} values >0.7, seven were selected to test for TaqMan® Real-Time PCR assay development. In preliminary tests, three of these assays either failed to show any amplification, or showed equal binding of both probes across all individuals assessed. Thus, we focused exclusively on validating the remaining 4 assays (referred to here as SNP A, B, C, and D for convenience).

The 501bp sequence containing each divergent SNP was uploaded to the Custom TaqMan® Assay Design Tool offered through ThermoFisher Scientific (https://www.thermofisher.com/order/custom-genomic-products/tools/genotyping/). This program identifies the optimal primer and probe sequences for Real Time PCR based SNP genotyping. In order to increase the melting temperature (Tm) of each hydrolysis probe while maintaining the short length, each probe was designed with a minor groove

binder (MGB) moiety at the 3' end. Each probe contained either a FAM or VIC fluorescent reporter dye on the 5’ end and a non-fluorescent quencher (NFQ) on the 3’ end. All probes designed to bind to the R-strain and C-strain SNPs were bound to a FAM or VIC fluorophore, respectively. Primer and probe sequences, as well as the ThermoFisher assay IDs are listed in Table 3.1. The development of these real time PCR assays followed MIQE guidelines whenever possible (Bustin et al. 2009).

Table 3.1 Primer and probe sequences used in each TaqMan® Real Time PCR assay. All assays can be purchased from ThermoFisher Scientific using the listed Assay ID. Additionally, the chromosomal position of each SNP within the Liu et al. (2019) fall armyworm chromosome map is indicated.

SNP	Chrom	Position (bp)	Description	Sequence	ThermoFisher Assay ID
SNP A	1/Z	14,104,488	Forward Primer	5'-GCAAGTGCAATTTTCCCATCTGATG	ANEP2UM
			Reverse Primer	5'-CAAGCCGTTCGCGGTTAG	
			FAM Probe Sequence	5'-FAM-AGACCAAAAGGACTCAT-MGB-NFQ	
			VIC Probe Sequence	5'-VIC-CTAGACCAAAAAGACTCAT-MGB-NFQ	
SNP B	1/Z	4,933,322	Forward Primer	5'-GGGAACTCATATACTAAAATCGGAAAAACCT	ANFVWEJ
			Reverse Primer	5'-ACACTCGCATTATTTGTGTGCAATT	
			FAM Probe Sequence	5'-FAM-CCGCAGTAGCGTATGT-MGB-NFQ	
			VIC Probe Sequence	5'-VIC-TCCGCAGTACCGTATGT-MGB-NFQ	
SNP C	1/Z	4,683,787	Forward Primer	5'-TGACAGCATTGATGTGCTGGAT	ANAAHJC
			Reverse Primer	5'-CGCCGGAGCGTTACAGA	
			FAM Probe Sequence	5'-FAM-CGCTACCAAAGCCAG-MGB-NFQ	
			VIC Probe Sequence	5'-VIC-CGCTACCAGAGCCAG-MGB-NFQ	
SNP D	16	14,134,047	Forward Primer	5'-TGAGTGCCAACAGCTATCTTCTG	ANH6JJE
			Reverse Primer	5'-GCAGTCCATTACAGCTGGTGAA	
			FAM Probe Sequence	5'-FAM-AGCTCATGTCCTACTCC-MGB-NFQ	
			VIC Probe Sequence	5'-VIC-AGCTCATGTCGTACTCC-MGB-NFQ	

3.2.2. Diagnostic assay validation

3.2.2.1. Insects

We conducted three rounds of assay validation using 1) previously sequenced samples of known genotypes, 2) samples of unknown strain from the central US flyway, and 3) samples of unknown strain collected from the eastern US flyway. In the initial validation, 20 individuals that had previously been sequenced as part of the assay development were selected. These included, 8 samples with the C-strain genotype, 8 with the R-strain genotype and 4 samples that were F1 interstrain hybrids. In the second round of validation, DNA was extracted from 48 moths that were collected across the central flyway between 2017 and 2020. These moths had not previously been sequenced and the strain genotype was unknown. For the final validation, DNA from 44 moths was provided by Dr. Juan Luis Jurat-Fuentes at the University of Tennessee. These moths had been collected between 2012 and 2017 at several locations in the eastern US. The collection information of all moths that were used in assay validation can be found in Table 3.2.

Table 3.2 Collection information for all samples used to validate the four TaqMan® Real Time PCR assays.

	Location	Date	# Samples	Strain if known
Validation 1: Known samples, sequences used in assay creation	Weslaco, TX	December 11, 2018	3	R
	Corpus Christi, TX	May 13, 2018	1	Hybrid
	Corpus Christi, TX	July 10, 2018	2	C
	Corpus Christi, TX	October 19, 2018	2	R
	College Station, TX	October 24, 2017	1	Hybrid
	College Station, TX	May 18, 2018	3	R
	College Station, TX	June 28, 2018	2	C
	Lubbock, TX	May 2, 2018	2	C
	Lubbock, TX	June 12, 2018	1	Hybrid
	Rosemount, MN	September 12, 2017	1	Hybrid
	Rosemount, MN	August 21, 2018	2	C
Validation 2: Central Population Unknowns	College Station, TX	October 20, 2018	12	?
	College Station, TX	June 13, 2020	24	?
	Lubbock, TX	May 2, 2018	6	?
	Rosemount, MN	August 21, 2018	6	?
Validation 3: Eastern Population Unknowns	Collier, FL	February 15-24, 2012	9	?
	Tifton, GA	August 7, 2014	10	?
	Jarretsville, MD	August 3-30, 2017	9	?
	Charleston, SC	June 30, 2017	10	?
	Roper, NC	November 16, 2017	6	?

3.2.2.2. Strain determination using COI and Tpi

There are two markers that are commonly used to determine fall armyworm strains; *COI* and *Tpi*. Prior to determining the genotype of each individual at our four SNP loci, we assessed the strain of all individuals at these previously described markers using RFLP analysis.

To assess the *COI* strain marker in each individual, a 568bp fragment of the *COI* gene was amplified using the *JM-76/JM-77* primer pair described by Levy et al. (2002). In this reaction, initial denaturation occurred for 2min at 94°C, followed by 28 cycles of the following protocol: 94°C for 30sec, 59°C for 30 sec, 72°C for 1 min. The reaction was then held at 72°C for three minutes for the final extension. Two ul of PCR product were then added to both 5units of *MspI* (New England BioLabs) diluted in 1x CutSmart® buffer (New England BioLabs) and 5 units of *SacI* (New England BioLabs) diluted in 1x NEBuffer 1.1™ (New England BioLabs) in accordance with the manufacturers protocol. These reactions were incubated at 37°C for 1hr and then run on a 1.8% agarose gel. This amplified product from the C-strain has one *MspI* cut site, but is not cut by *SacI*, whereas the R-strain shows the reciprocal pattern (Nagoshi et al., 2006). Because *COI* is maternally inherited, hybrids cannot be detected (Figure 3.2).

To determine an individual's strain using the *Tpi* marker, an approximately 600bp fragment of the fall armyworm *Tpi* gene was amplified using the primer pair *Tpi-632 F/Tpi-1195R* described by Nagoshi (2012). In this reaction, initial denaturation occurred for 1min at 94°C, followed by 32 cycles of the following protocol: 92°C for 30sec, 57°C for 30 sec, 72°C for 1 min. The reaction was then held at 72°C for three minutes. Two ul of PCR product were then added to 5units of *MspI* (New England BioLabs) diluted in 1x CutSmart® buffer (New England BioLabs) in accordance with the manufacturers protocol. This reaction was held at 37°C for 1hr and then the resulting product was run on a 1.8% agarose gel. The resulting banding patterns for C-strain, R-strain and hybrid individuals can be seen in Figure 3.2.

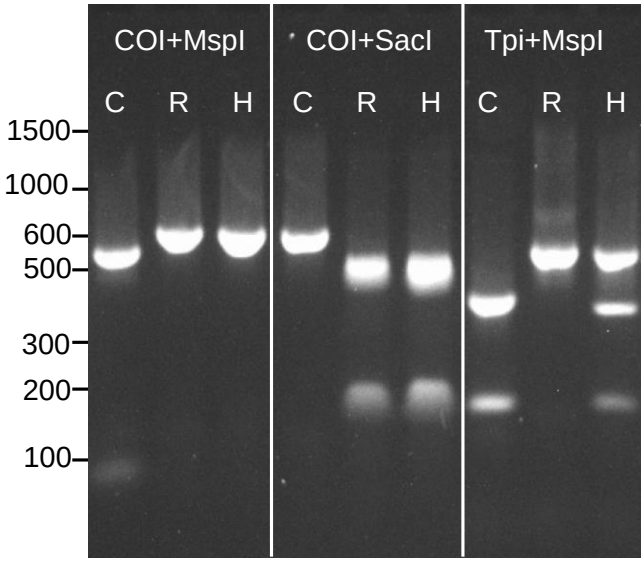

Figure 3.2 Example RFLP analysis for *COI* digested with MspI, *COI* digested with SacI, and *Tpi* digested with MspI of three fall armyworms; one of the C- strain, one of the R strain, and one known interstrain hybrid (H). Since mtDNA is maternally inherited, the hybrid could not be distinguished from the R-strain using COI alone.

3.2.2.3. Strain determination using TaqMan® Real-Time PCR assays

Real-Time PCR assays were conducted as 10ul reactions in 384 well plates. Each reaction contained 1ul of template DNA diluted to 20ng/ul, 5.00ul of TaqMan® Genotyping Master Mix (Applied Biosystems), 0.5ul of 40x Custom ThermoFisher TaqMan® assay containing the primers and hydrolysis probes (assay IDs are listed in Table 1 along with the primer and probe sequences), 0.05ul of Precision Blue Real Time PCR Dye (BioRad), and 3.45ul of TE buffer. One ul of TE was added instead of DNA to create non-template controls, and all reactions were conducted in duplicate. The Real-Time PCR program began by holding samples at 95°C for 10 min. This was followed by

40 cycles oscillating between 95°C for 15sec to 60°C for 1 min. After being held at 60°C for 1 min, fluorescence was recorded across all four channels of a CFX384 Touch Real-Time PCR Detection System (BioRad). All samples were assessed individually at each of the 4 diagnostic SNP loci (SNP A, B, C, and D).

All real-time PCR data were input into the CFX Masetro software to determine the Cq values for both FAM and VIC in each reaction. CFX Maestro assigned each individual as either homozygous for allele 1 (R-strain), homozygous for allele 2 (C-strain), or heterozygous at the locus of interest (hybrid) based on the relative fluorescence of each fluorophore detected in a reaction. If both fluorophores were detected in a reaction, the difference between the Cq values of each flourophore was calculated for each individual (ΔCq). The ΔCq across all homozygous individuals was then averaged for each allele and is denoted as $\Delta Cq_{(R)}$ and $\Delta Cq_{(C)}$ (Table 3.4). For most reactions the $\Delta Cq_{(R)}$ differed slightly from the $\Delta Cq_{(C)}$, indicating that one of the probes had slightly higher specificity that the other. The ΔCq for all heterozygote individuals across all SNP based TaqMan® assays ranged from 0-2, indicating relatively equal expression of both fluorophores.

A consensus strain determination was made for all unknown individuals. This consensus was reached if a minimum of 5 out of the 6 diagnostic assays (4 real time PCR + 2 RFLP analyses) agreed on the strain call. The number of individuals that matched the consensus sequence was calculated for each assay. A chi-square test in JMP 14 was used to determine if there was a statistically significant difference in accuracy

between the six diagnostic assays. In this case, accuracy was defined as the proportion individuals matching the consensus strain assignment.

3.2.2.4. Real-Time PCR assay sensitivity analysis

The sensitivity of all Real-Time PCR assays was assessed using serial dilutions of DNA extracted from 3 R-strain and 3 C-strain fall armyworm moths. The samples selected for sensitivity analysis were all previously sequenced, and thus the allele present at each of the SNP sites was known. The DNA concentration was measured using a Fluorometer Spectrophotometer (DeNovix) and each sample was then diluted to 100ng/ul. Six serial dilutions were prepared by adding 1ul of DNA to 9ul of nuclease free water resulting in DNA concentrations of: 100, 10, 1, 0.1, 0.01, 0.001, 0.0001ng/ul. The resulting Cq values of two duplicate runs were averaged for each SNP. No assay was able to detect fluorescence at 0.0001ng/ul, so this concentration was removed from further analysis. The average Cq values across biological replicate were plotted against the log transformed DNA concentration. A linear regression was then fit in JMP ® Pro 14.0.1 (SAS Institute Inc., Cary, NC) to determine the slope, y-intercept, and correlation (R^2) between DNA concentration and assay sensitivity.

3.2.3. Testing strain activity at two times of the night

Having developed and validated diagnostic SNP assays, we demonstrated their utility by conducting an experiment to test for allochronic separation between the C- and R- strains using the SNP assays to genotype field collected individuals active at different times during the night.

3.2.3.1. Insect collections

S. frugiperda moths were collected over the course of four consecutive nights from 3-7 September 2020 using universal green moth bucket traps, baited with a Scentry PSU 2-component lure (Scentry Biologicals, Billings, MT). All traps were placed a minimum of 300m apart along the edges of agricultural fields at the Texas A&M AgriLife Research and Extension Center in Lubbock, Texas, USA. Nearby host plants are reported in Table 3.3. Moths were collected from each of the traps at three different times spanning a 11 hour overnight activity period. Traps were initially set out just before sunset, which occurred at approximately 2010 hours. Traps were first checked and emptied five hours later (0110-0130 hours), again two hours later (0310-0330 hours), and a third time four hours later, just after sunrise. Collected moths were preserved in 95% ETOH separately for each trap and collection time. The collection periods before 0130 hours and after 0330 hours will henceforth be referred to as the early and late collections, respectively. The intermediate period was considered a buffer between the early and late activity periods, and the 15 individuals trapped during this period were not used in the analyses.

Table 3.3 List of all major crops within approximately 50 meters of each trap. The total number of moths collected in each trap across all sampling days is also included.

	Trap #					
	1	**2**	**3**	**4**	**5**	**6**
Sunflower	X					
Sorghum (Booting)						X
Sorghum (Mature)	X		X	X		
Cotton		X	X	X	X	X
Peanut		X	X		X	
Soybean		X				
# Moths collected	1	37	9	17	4	16

3.2.3.2. Strain determination using Real-Time PCR assays

A total of 84 moth samples were genotyped; 67 from early in the night, and 17 from late in the night. The thorax or abdomen of each moth was placed in a 1.5ml Eppendorf tube, frozen in liquid nitrogen and then crushed with a sterilized plastic pestle. DNA was extracted from the crushed thorax or abdomen using a Qiagen DNeasy Blood & Tissue Kit in accordance with the manufacturers protocol with the inclusion of the optional RNase A treatment. The DNA concentration was measured using a NanoVue Plus Spectrophotometer (General Electric). Since all sample concentrations were between 3ng/ul and 30ng/ul, no further dilutions were performed.

For each sample, TaqMan assays for the four SNPs (A, B, C, and D) were conducted individually as 10ul reactions as described above. The only modification to this protocol was for SNP D, in which off target binding of the FAM-labeled probe was detected after 35 cycles a few samples with DNA concentration >25ng/ul. For this assay,

allelic discrimination was evaluated after 33 temperature oscillation cycles. Each reaction was performed in duplicate, and all reactions were manually checked for consistency across technical replicates.

All Real-Time PCR data was input into CFX Maestro (BioRad) to determine which allele was detected for each of the SNPs. CFX Maestro compares the fluorescents of each fluorophore to that of a non-template control. Based on the relative fluorescent units detected in each reaction, every sample was labeled as either allele 1 (primarily expressing Fam), or allele 2 (primarily expressing Vic). In all four assays, allele 1 corresponded with the R-strain specific variant, and allele 2 corresponded with a C-strain specific variant.

3.2.3.3. Data analysis

There were three factors that could have influenced the proportion of each strain collected throughout the night; time (early and late), specific trap location, and night that the collection occurred. We used a generalized linear model with a logit link and binary distribution to assess the effects of trapping time, trap location, and night collected on the probability of collecting each strain. Because the factors trap location and night collected, were not significant, we ran a Fisher's exact test to determine if there were differences in the proportion of each strain trapped during the two collection periods.

3.3. Results

3.3.1. TaqMan assay validation for four SNPs

In the first round of validation using samples of known genotypes, all four SNP based TaqMan assays were able to accurately differentiate between C-strain and R-strain individuals. Additionally, the SNP A and SNP D assays accurately identified all five hybrid individuals, whilst our SNP B assay identified two and the SNP C assay identified one known hybrid as homozygous for the C strain allele. Although these individuals were hybrid across most of their genome, upon further examination of the SNP data for these particular individuals at the two loci of interest (SNP B and C), we found that these individuals were indeed homozygous for the C-strain allele, indicating our assays correctly identified the individual genotype at the locus of interest, but hybrid individuals may not always be heterozygous at these loci.

In our final two validations using individuals of unknown genotypes from both the eastern and central US flyways, our assays detected a relatively even mix of C- and R-strain individuals with 42 individuals being consistently identified as the R-strain, and 50 individuals being consistently identified as the C-strain in at least 3 out of our 4 SNP assays.

The average Cq value for both fluorophores across all four SNP assays ranged from 22.91 to 23.93 in homozygous individuals. In most reactions, some level of fluorescence was detected for both FAM and VIC fluorophores by the end of cycle 40. We calculated the difference in the Cq value for each flourophore given each allele assignment (C and R) and denoted these as $\Delta Cq_{(R)}$ and $\Delta Cq_{(C)}$ (Table 3.4). For SNP A,

the FAM reporter dye was never detected in individuals with allele 1, and for SNP C the VIC reporter dye was never detected in individuals with allele 2. For SNP B, some fluorescence was detected by both FAM and VIC regardless of the individual's allele, however the fluorescence of the off target probe was always detected more than 15 cycles after the fluorescence of the target probe, which is sufficient to accurately identify the strain. For SNP D, FAM and VIC fluorescence was detected in all individuals regardless of the allele present, and the fluorescence of the off target probe was often detected only 3 cycles after fluorescence of the target probe. This off target probe binding could result in an overlap of fluorescence between the two reporter dyes, especially in assays with high DNA concentration. As a result, this assay may be less effective than the other three at differentiating between C- and R-strain individuals.

Table 3.4 Mean $\Delta Cq_{(R)}$ and $\Delta Cq_{(C)}$ from reactions in which both fluorophores were detected. In the SNP A assay, only VIC was detected for all individuals with the R-strain allele and in the SNP C assay only FAM was detected in individuals with the C-strain allele.

	Mean $\Delta Cq_{(R)}$ ± SD	Mean $\Delta Cq_{(C)}$ ± SD
SNP A	N/A	7.05 ± 0.89
SNP B	16.26 ± 1.26	17.12 ± 1.38
SNP C	10.32 ± 0.97	N/A
SNP D	3.67 ± 0.29	5.77 ± 0.45

3.3.2. Comparison between TaqMan® assays, COI, and Tpi markers

In addition to the four SNP based Real-Time PCR diagnostic assays, we also determined the strain of all fall armyworm samples collected in both the central and eastern US

flyways, using two previously described diagnostic RFLP analyses (*COI* and *Tpi*). In 97.3% of individuals assessed, at least 5 of the 6 combined SNP and RFLP diagnostic assays were in agreement with the strain call. In these cases, the consensus strain was determined for each individual. Three individuals did not show a consensus between assays. Two of these were previously sequenced individuals that were known to be hybrids and one was an unsequenced individual from Collier, FL that we suspect may also have been of hybrid descent. Since the genotype of the individual from Collier, FL was not known, this sample was removed from assessments of assay accuracy.

For each assay, we determined the number of individuals that disagreed with the consensus strain call. The *Tpi* marker had the lowest accuracy with 7 individuals showing disagreement from the consensus strain, and SNP A had the highest consistency with 2 individuals showing disagreement with the consensus strain. We used a Pearson's chi square test to determine if proportion of accurate calls significantly differed amongst our six assays, and no differences were detected (Figure 3.3, $X^2= 17.14$, $p=0.07$).

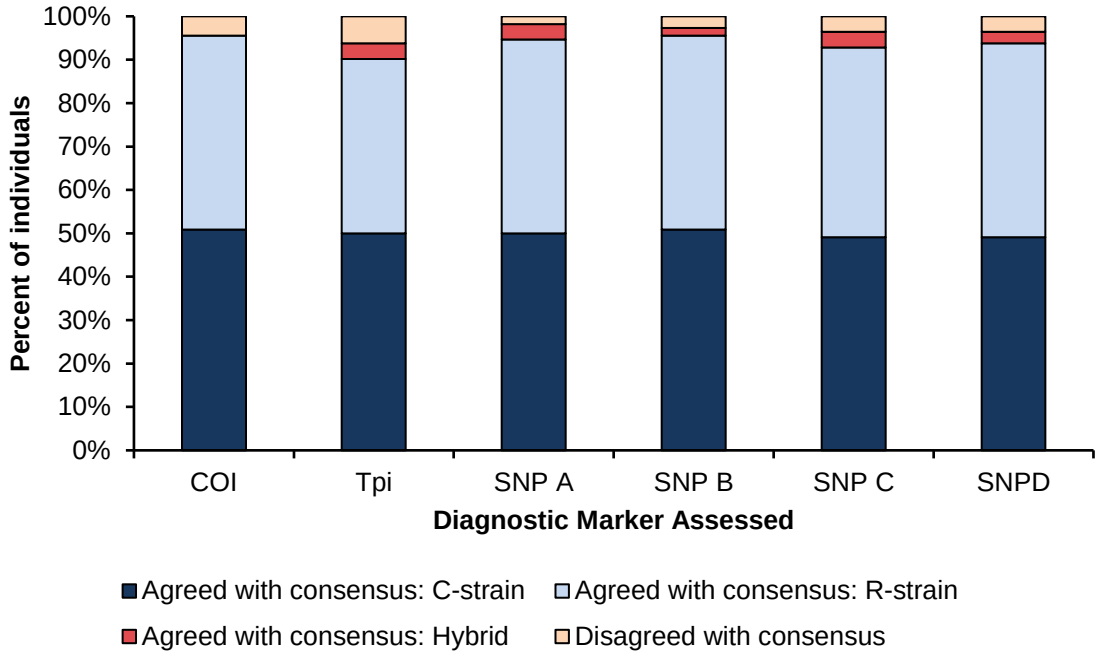

Figure 3.3 Percent of individuals for each diagnostic assay that were in agreement with the consensus strain. Each bar is split into those that were determined to be R-strain, C-strain, or hybrid. No significant difference was detected in accuracy between the six assays (p=0.07).

3.3.3. Sensitivity analysis results

Serial dilutions of genomic DNA from known R- and C-strain individuals were used to assess the sensitivity of all four TaqMan® real-time PCR assays to initial DNA concentration. Across all assays, no fluorescence was detected when <0.001ng of DNA was added to the reaction. When more than 0.001ng of DNA was added, there was a linear increase in the Cq of each reaction as DNA concentration decreased, with R^2 values ranging from 0.982-0.999 across all assays and fluorophores (Figure 3.4). These

results indicate that all four of our TaqMan® Real-Time PCR assays are capable of detecting the allele present at each loci when provided between 0.001-100ng of gDNA.

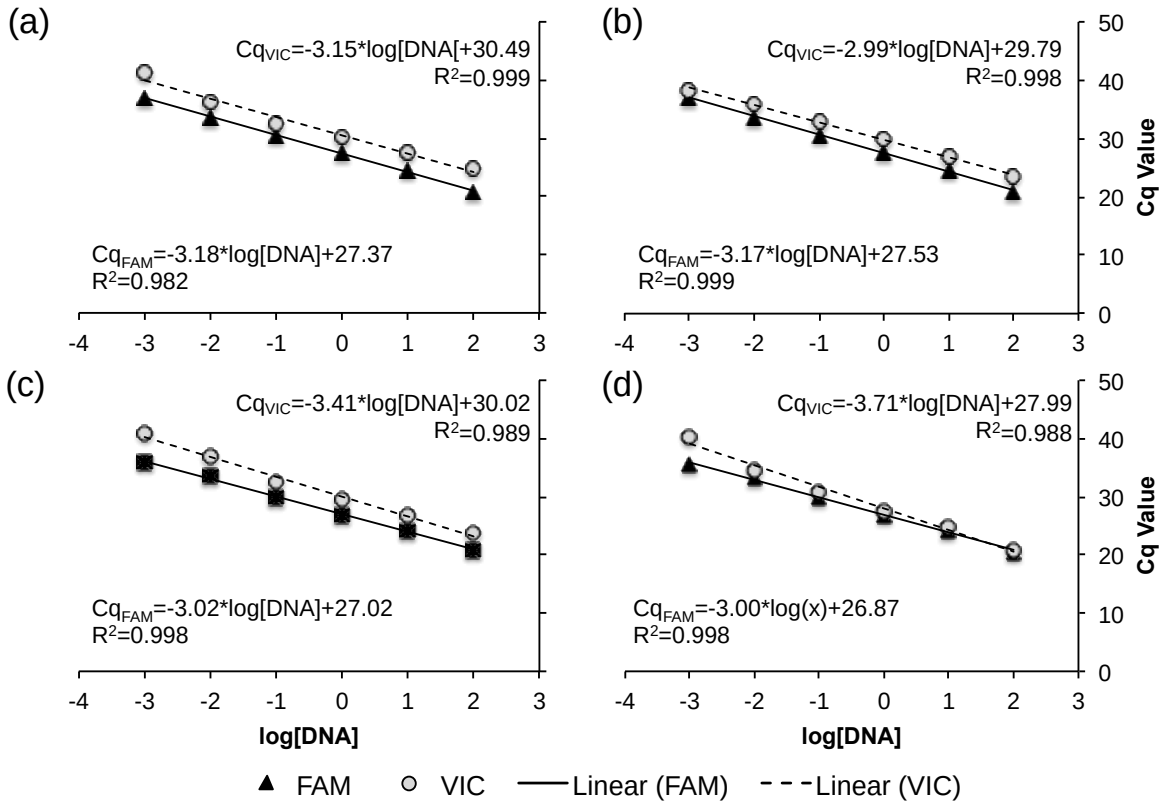

Figure 3.4 Standard curves relating Cq value to the DNA concentration for both the C-strain (solid line) and R-strain (dashed line). The standard curve for each TaqMan® Real-Time PCR assays is demonstrated as a separate panel: (a) SNP A assay, (b) SNP B assay, (c) SNP C assay, and (d) SNP D assay. Corresponding slopes and R^2 values are reported in each panel.

3.3.4. Testing strain activity at two times of the night

Over the course of four nights, we collected 84 fall armyworm moths; 67 during the early collection and 17 during the late collection. All four newly developed TaqMan®

Real-Time PCR assays were used to determine the strain of each moth collected. For all individuals, a minimum of three out of the four assays resulted in the same strain call, allowing us to determine a consensus strain for each individual. For all samples, the SNP A and SNP C assays agreed with the consensus strain call. The SNP B assay called one individual as R-strain when all other assays determined this individual to be the C-strain, and there were four instances where the SNP D assay disagreed with the consensus strain. In three of these cases the SNP D assay called the individual as R-strain when the consensus was that that individual was C-strain. In one case SNP D called an individual as C-strain when all other assays had marked the individual as a hybrid heterozygote.

Of the 84 individuals collected, 78 were C-strain, 5 were R-strain, and 1 was heterozygous at 3 of the 4 diagnostic loci (putative hybrid). All 5 R-strain individuals and the putative hybrid were collected during the late collection time (Figure 3.5). Because we only collected a single putative hybrid, this individual was removed from statistical analysis. The generalized linear model with a binary distribution and logit link indicated that there was no effect of trap, or night collected on the probability of collecting a C- or R-strain individual, however, there was a significant effect of time (Table 3.5). Using a Fisher's exact test to test the hypothesis that the proportion of C-strain and R-strain varied across our two collection times, we confirmed that there was a strong significant effect of collection time on strain abundance between early and late periods of the night ($p=0.0002$).

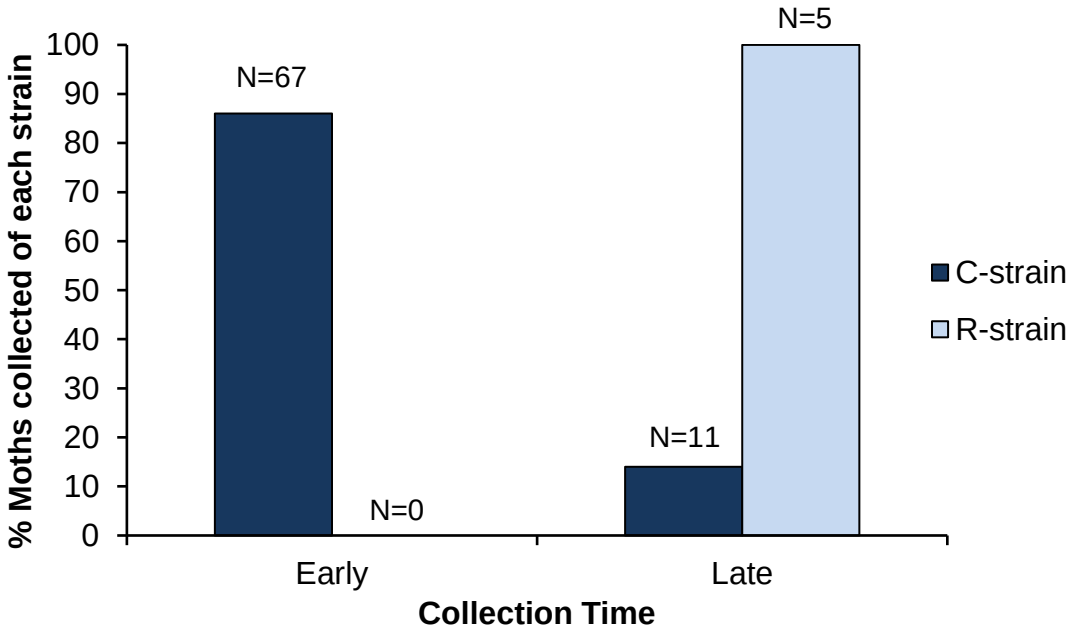

Figure 3.5 Percent of C-strain and R-strain moths collected during the two collection time points: early (before 0130hours) and late (after 0330hours). The number of individuals collected from each strain during each collection time is listed.

Table 3.5 Statistical significance of the factors trap location, night collected, or time of night on the probability of collecting R-strain moths as determined by a generalized linear model with a logit link and binary distribution.

	DF	X^2	p-value
Time	1	19.18	<0.0001
Trap	5	3.70	0.59
Night	2	0.21	0.21

3.4. Discussion

The fall armyworm is comprised of two genetically distinct strains, widely referred to as the Corn or C-strain and the Rice or R-strain (Pashley, 1986). Because there are no strain specific morphological differences, molecular markers are necessary to differentiate these strains. The first aim of this study was to identify strain specific SNP markers that could differentiate between strains, and then develop real-time PCR based TaqMan® assays that could rapidly and effectively provide a strain assignment for fall armyworm samples. Accurate strain identification is imperative for conducting field-based studies on the ecology and behavior of these strains and their hybrids. Additionally, accurate and efficient strain diagnostics could facilitate the implementation of strain specific management programs to better control this species in agricultural fields.

Here, we present four new diagnostic assays to differentiate between the two fall armyworm strains. All four assays could effectively assign a fall armyworm strain when provided as little as 0.001ng of DNA. In one assay, SNP D, the ΔCq value was between 3-6 cycles indicating that both the target and non-target probes were binding to the DNA sequence containing the SNP. Although the target probe bound preferentially and was evident at least 3 cycles before the off target probe, very high DNA concentrations may result in homozygous individuals being identified as hybrid heterozygotes due to a lack of probe specificity. When only 20ng of DNA were added to the reaction, this assay preformed as well as the rest at assigning strain. The probes for all other assays were more specific resulting in lower off target probe fluorescence regardless of DNA concentration.

Prior to this study, the two most commonly used molecular markers to identify fall armyworm strains relied on polymorphisms in the mitochondrial gene *COI* or the Z-linked gene *Tpi* (Nagoshi, 2010; Nagoshi et al., 2006). However, discordance between these markers often caused confusion in the strain assignment. All four of the new SNP-based diagnostic assays were equally if not more effective at assigning strain when compared to the original *COI* and *Tpi* assays. Therefore, the addition of four new diagnostic assays will allow for increased confidence in the strain assignment. Furthermore, *COI* is maternally inherited and thus cannot identify hybrid heterozygotes, and *Tpi* is Z-linked and thus can only detect hybrids in males who have two copies of the Z chromosomes (ZZ). Three of our four diagnostic SNPs (SNPs A-C) were also located on the Z-chromosome and thus have the same limitations as *Tpi*, however SNP D is located on chromosome 16, and thus could be used to identify hybrid heterozygotes of both males and females. Across all individuals screened, at least five of the six diagnostic assays were in agreement (4 SNP assays + *COI* + *Tpi*). Therefore, to streamline the diagnostic process and reduce the amount of reagents needed, only three diagnostic assays are required to confidently identify strain. Since all TaqMan® assays require essentially the same reagents and have the same reaction conditions, they can be assessed simultaneously on the same plate in a single real-time PCR run. Therefore, it is most efficient to assess the strain of field collected fall armyworm samples using three of our SNP based diagnostic assays.

Although only individuals that would have originated at the Texas overwintering site (the central population) were used for SNP identification and diagnostic assay

development, all four diagnostic markers were capable of identifying strain of insects across both the central and eastern populations. This indicates that our assays are highly diagnostic, with strain specific markers being conserved across fall armyworm populations despite originating from different overwintering locations. These overwintering populations were once thought to be genetically distinct based on consistent differences in the ratios of mitochondrial haplotype that occur in each population (Nagoshi et al., 2017b, 2012, 2008b). However, more recent genomic studies using SNP data have shown very little genomic differentiation between the two geographic overwintering populations. This likely indicates there is some level of population panmixia, despite geographic differences in overwintering location (Schlum et al., 2020). The efficacy of our diagnostic markers across both overwintering populations may further support the idea that interbreeding occurs between these two geographic populations.

The second objective of this study was to assess the utility of our SNP based diagnostic assays as a research tool by testing a hypothesis about strain behavior in the field. Specifically, we were interested in whether C-strain and R-strain moths in the field exhibited allochronic differences in their nightly activity. This idea was originally proposed by Pashley (1992) based upon laboratory observations of the two strains. Although the behavioral differences were assumed to apply to field populations as well, this hypothesis has never been empirically tested in the field. In this study, we find that the majority of C-strain moths (86%) were collected before 0130hours, whilst all R-strain individuals were collected after 0330hours. Despite only recovering 5 R-strain

moths total, these findings indicate that there are significant differences in the proportion of each strain flying in the early and late time periods. Given that both strains were recovered in the same fields, but exhibited differences in activity patterns throughout the night, our data indicate that allochronic differences are likely a more important driving factor of reproductive isolation between strains than habitat isolation due to differences in strain host use.

The combined evidence presented here and in Chapter 2, suggests the term 'host-associated' strains is likely an ecologically inaccurate designation for these genetically distinct groups. As such, we propose that these allochronic strains be referred to as the crepuscular (C-strain) and resurgent (R-strain) strain, reflecting their early and later night activity periods, respectively.

4. PROTEIN-CARBOHYDRATE REGULATION AND NUTRITIONALLY – MEDIATED RESPONSES TO BT ARE AFFECTED BY CATERPILLAR POPULATION HISTORY*

4.1. Introduction

Bt crops that express insecticidal Cry and Vip proteins derived from the bacterium *Bacillius thuringensis* are widely used for the management of certain insect agricultural pests. The widespread adoption of Bt crops, notably corn and cotton, has resulted in increased selection on the target pest populations to overcome these insecticidal proteins, potentially allowing for the rapid evolution of resistance (Gould, 1991; Tabashnik et al., 2003). When monitoring for insect resistance in the field, both discriminating dose assays and dose response artificial diet assays are used. In these assays, neonate larvae are fed on diet containing either a single toxin concentration high enough to kill all susceptible and heterozygous insects (discriminating dose), or a gradient of toxin concentrations (dose response) to determine the concentration of toxin required to kill 50% of the population (i.e. LC_{50})(Huang, 2006). The response of field collected insect populations to these toxins are then compared to susceptible lab colonies to calculate resistance ratios and determine if the field insects are resistant to the Bt toxins on which they were screened (Tabashnik et al., 2009). This method assumes the underlying

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physiology of a species is inherently the same across populations and differences in susceptibility among populations is due to allelic variation. In this case, a population is defined as a group of potentially interbreeding individuals of the same species sampled at a given place and time. Therefore, a cohort of individuals maintained as a lab colony would be considered a unique population.

Insect colonies have been established and maintained in laboratories around the world for a number of research applications, including both physiological assays and insecticide and Bt resistance studies. However, several researchers have shown that insect colonies maintained in the lab can exhibit significant physiological differences from their wild counterparts (Mason et al., 1987). For example, because of differences in the sensitivity of chemoreceptors, lab colonies of *Manduca sexta* will accept host plants that are rejected by field insect populations (Schoonhoven, 1967). Also, *Heliothis virescens* moths reared in the lab exhibit significant differences in their time to oviposition and mating frequency compared with the field populations (Raulston, 1975). As a final example, genetic differentiation between locust rearing colonies from different laboratories around the world has been implicated as a confounding factor underlying variable physiological and phenotypic experimental results, leading researchers to question their relevance to natural field populations (Berthier et al., 2010). These population level effects, or effects of laboratory domestication, are often overlooked, both when conducting studies on the nutritional physiology of an insect species and when comparing insect responses to Bt in resistance monitoring experiments.

Nutrition is fundamental to all aspects of an insect herbivore's survival and fitness, and a balanced macronutrient intake of proteins (p) and carbohydrates (c) can optimize development, fecundity and immune responses (Lee et al., 2006; Roeder and Behmer, 2014). Although many insect pests, notably caterpillars, are confined to a single plant during development, plant tissues show significant variation in their p and c content, making a single plant a heterogeneous nutritional landscape (Deans et al., 2016; Deans et al., 2018; Machado et al., 2015). For instance, within a cotton plant, the ratio of p:c in the leaves can change overtime, resulting in young foliage having a different nutritional profile than the mature foliage (Deans et al., 2016). Developing flowers (squares) and fruit (bolls) offer even more nutritional variation. Insects can then forage between different plant tissues to actively regulate their intake of p and c and achieve a specific balance of these macronutrients. The self-selected optimal balance of multiple nutrients is known as an intake target (IT), and it can be quantified using approaches developed within the Geometric Framework for Nutrition (GFN) (Simpson and Raubenheimer, 2012, 1993). Many studies that employ the GFN use lab colonies to determine a species-specific IT, or the ratio of nutrients that enhances fitness or performance (e.g. Deans et al., 2015; Merkx-Jacques et al., 2008; Tessnow et al., 2018; Thompson and Redak, 2005). This method assumes that the underlying physiology of a species is inherently the same across populations and that they all share a similar IT, regardless of if the population is from the field or a lab colony.

Both an insect pest's nutritional environment and physiology have been shown to affect its susceptibility to Bt toxins. In recent studies, two caterpillar pests, *Helicoverpa*

zea and *H. armigera*, exhibited reduced susceptibility to the Bt toxin Cry1Ac when consuming diets that matched their species-specific nutritional intake target (Deans et al., 2017; Tessnow et al., 2018). This indicates that the macronutrient intake an insect selects may be important in determining the insect's susceptibility to Bt crops. Although these studies served as a foundation for exploring nutritionally mediated susceptibility, it is not clear if these findings can be extrapolated to other lepidopteran insect pests such as the fall armyworm, *Spodoptera frugiperda* (J.E. Smith). The fall armyworm is a highly polyphagous pest with a propensity for long distance migration (Sparks, 1979). Although native to North and South America, this species was introduced into Africa in 2016 and has since become established across most of Africa and Asia, making it a current global concern (Ganiger et al., 2018; Goergen et al., 2016; Jing et al., 2019; Otim et al., 2018). This pest also exhibits field-evolved practical resistance to the Bt toxin Cry1F across multiple regions and countries in the Western Hemisphere (Chandrasena et al., 2018; Farias et al., 2014; Huang et al., 2014; Storer et al., 2010). Because fall armyworm populations are commonly kept as laboratory cultures and used in Bt resistance studies, we assessed if populations of this species exhibited differences in their macronutrient foraging behavior and nutritionally mediated response to Cry1F. Specifically, we focused on three *S. frugiperda* populations that differed in the number of generations they had been in the lab and their susceptibility to Cry1F.

We focused on two overarching questions. First, do different populations of *S. frugiperda* forage for different protein-carbohydrate ratios, indicating differences in their nutritional physiology? Second, does protein-carbohydrate intake impact the insect's

susceptibility to Cry1F? To address these questions, we first conducted choice assays to determine the self-selected p:c intake target for the three populations of *S. frugiperda*. We then used no choice assays in the absence of Cry1F to verify the importance of nutritional intake on survival and development of each population. Lastly, we preformed diet incorporation dose response assays to determine the susceptibility of each population to Cry1F when fed two diets differing in their composition of protein and carbohydrates.

4.2. Methods

4.2.1. Insect populations and colony maintenance

Three *S. frugiperda* populations were used in this study: (1) BENZ-90, (2) NCBT-13, and (3) CSTX-16. Although *S. frugiperda* has two host-associated strains (Pashley, 1986), the populations used in this study were solely comprised of corn-strain fall armyworms. The BENZ-90 population was purchased from Benzon Research Inc. (Carlisle, PA). This population was originally collected from Stoneville, MS in 1990 prior to the commercial release of Bt transgenic crops, and has been in culture for over 200 generations (Storer et al., 2010). The NCBT-13 population was originally collected as larvae feeding on Bt corn in 2013 in Hyde, NC and had been in continuous culture in the Reisig lab at North Carolina State University for approximately 25 generations prior to this study (Huang et al., 2014). The CSTX-16 population was established from egg masses collected off sorghum in 2016 in College Station, TX. Neonates were reared individually on artificial diet through pupation, and then mated with individuals from

different egg masses in order to reduce inbreeding. All experiments involving this population were conducted in the first 8-generations after establishment in the lab.

All *S. frugiperda* colonies were housed in the Texas A&M Entomology Research Laboratory in College Station, TX. Colonies were maintained at 25±2°C with a 12:12 (L:D). Approximately 40 adults were housed together in 19cm x 13cm x 10cm plastic mating cages lined with paper and secured with mesh. Moths were provided constant access to cotton soaked in a 10% honey solution. Egg masses were collected daily from the paper lining and placed in bags to hatch. Upon hatching, approximately 50 neonates were moved into a 500ml plastic deli cup filled with 100ml of Southland artificial diet (Southland Products, Lake Village, AR). Five days after hatching, neonates were transferred from the rearing cohort into individual wells of a 32-well rearing tray with 1.5g of the same artificial diet. Insects were checked daily and additional diet was added as needed. Larvae pupated in the rearing wells.

4.2.2. Diets

The commercial Southland diet manufactured for *S. frugiperda* (Southland Products, Lake Village, AR) was used for all general rearing. This diet contained a total protein and carbohydrate composition (p+c) of 62.8% at a ratio of 1 part protein to 2.3 parts carbohydrates (p1:c2.3)(Deans et al., 2016).

Choice and no-choice assays were conducted using an artificial diet developed by (Ritter and Nes, 1981) and modified by (Jing et al., 2013; Ritter and Nes, 1981)). Each experimental diet only varied in its ratio of soluble proteins (p) and digestible carbohydrates (c). These diets were modified by altering the amounts of casein and

sucrose, while maintaining the same concentrations of all other ingredients. All experimental diets contained a total p+c of 42%. The recipes for all choice and no choice diets are provided (Table A-4).

The diet used for all dose response assays was developed by modifying the commercial Southland *S. frugiperda* diet. These modifications were made by adding casein and cellulose to the commercial diet mix in order to obtain the desired p+c, while keeping the amount of all other ingredients constant (Table A-5)(Deans et al., 2017). After modification, all diets contained 42% p+c, and differed only in their ratio of p:c. It should be noted that since different diets were used between the choice and no choice assays and the dose response assays, the protein source between these experiments slightly differed. Since there was sufficient protein in the diets of each of these experiments that no single amino acid would have been limiting (i.e., >8% protein)(Schoonhoven et al., 2005), this difference should not confound our results.

4.2.3. Choice assays

Simultaneous choice tests were conducted to empirically determine the nutritional intake target (IT) for each *S. frugiperda* population. The IT is the p:c ratio that the insect self-selects when given access to multiple nutritionally complimentary food sources. Newly molted final instar larvae were removed from rearing trays, weighed, and transferred to a 10cm petri dish containing two diet blocks that differed in their composition of p and c. These diet blocks were weighed and placed on opposite sides of the dish. Preliminary assays indicated that *S. frugiperda* larvae preferred a slightly protein biased p:c intake ratio similar to those determined for other Lepidoptera (Tessnow et al., 2018) and thus

two unique diet pairings were selected accordingly: 1) p35:c7 w/ p7:c35 and 2) p35:c7 w/ p14:c28. Two different diet pairings were used to assess if the larvae's nutritional intake was similar even when the insects were provided different choices, indicating active regulation of p and c. Twenty larvae from each of the three *S. frugiperda* populations (BENZ-90, NCBT-13, and CSTX-16) were assessed simultaneously on each diet pairing and allowed to feed freely until pupation. Diets were changed every 1-2 days throughout the experiment to ensure the insects had constant access to both diet choices. Assays were preformed in an incubator (Model I-41NL, Perceval Scientific, Perry, IA) at 25°C and 12:12 (L:D). Three insects from three different treatments died during the course of the experiment and were removed from the analysis. Thus, the final sample sizes were 19-20 larvae per treatment.

One day after pupation, pupae were weighed and sexed, and all leftover diet was dried and weighed. A linear regression was used to convert the initial wet mass of the diet to dry mass. The total amount of p and c consumed was calculated by subtracting the final dry diet weight from the initial dry diet weight, and multiplying by the proportion of p and c in each diet cube (Deans et al., 2015; Lee et al., 2006).

4.2.4. No choice assays

No-choice assays in which larvae were restricted to feeding on one of five diets differing in their ratio of p:c were conducted to test for effects of nutritional variation on larval survival and development in the absence of Cry1F. Within 12 hours of hatching, neonates were transferred to individual 60ml cups. Each cup contained a single block of diet made up of one of five p:c ratios that ranged from carbohydrate biased to protein

biased (p12:c30, p18:c24, p24:c18, p28:c14, or p30:c12). All three *S. frugiperda* populations (BENZ-90, NCBT-13, and CSTX-16) were assessed simultaneously, and 36-37 neonates of each population were placed on each diet treatment. All diet cups were kept in a 25°C growth chamber (Model PR505755L, ThermoScientific, Waltham, MA) with 12:12 (L:D), and larvae were checked daily. Time until pupation, mortality, pupal mass, and sex were recorded. Diets were changed every three days and the assays ran for 50 days. Individuals that failed to molt past the 4th instar at 50 days or escaped from their containers were removed from the analysis. So, of the 544 insects initially setup in the no choice assays, 527 were used in the final data analysis (Table A-6).

4.2.5. Dose response assays

To determine if a diet's protein-carbohydrate content affected the susceptibility of *S. frugiperda* to Cry1F, a diet incorporation dose response assay was conducted with each population using two diets that differed in their p:c ratio. The first diet approximately matched the populations' p:c IT as determined by the choice assay (p1.3:c1 for BENZ-90; p1.7:c1 for both NCBT-13 and CSTX-16; see Results). These diets will henceforth be referred to as the IT diet for each population. The second diet matched the c-biased p:c ratio found in the Southland commercial rearing diets (0.43p:1c). This carbohydrate-biased rearing diet will henceforth be referred to as the CB diet. Both the IT and CB diets contained 42% p+c.

Trypsin-activated HPLC purified Cry1F was provided by Dr. Marianne Pusztai-Carey at Case Western Reserve University (Cleveland, OH) and stored at -80°C. Prior to each dose response assay, 2 mg of Cry1F were added to 1ml RO water.

From this initial solution, five 1:10 serial dilutions were performed with 100ul of the initial solution being added to 900ul of RO water. 300 ul of each concentration was mixed with 6g of refrigerated diet as described previously (Deans et al., 2017). This resulted in diets containing one of seven concentrations of Cry1F (0, 0.01, 0.1, 1, 10, 100ppm). A piping bag was used to load the diet into each well of a 96-well plate, starting with the lowest Cry1F concentration and ending with the highest concentration to avoid contamination.

During the first assay all populations were exposed to these initial six concentrations of Cry1F. Since, both the BENZ-90 and CSTX-16 populations were highly susceptible to Cry1F, the toxin was further diluted for all additional trials to the following lower concentrations of 0, 0.001, 0.01, 0.1, 1.0 and 10ppm. A total of five trials were conducted for the BENZ-90 and CSTX-16 populations, and three trials were conducted for the NCBT-13 population. Mortality data did not differ between trials, so data for each population were pooled across trials for analysis.

After the diet was added, a paintbrush was used to move a single neonate into each well of the 96-well plate. The plates were sealed with heat activated adhesive lid, and placed at 25°C with 12:12 (L:D). Mortality was recorded after 7 days and death was defined as no response when the insect was stroked with a camelhair brush. In each trial, 16 insects were setup on each Cry1F concentration on each diet (IT and CB).

4.2.6. Statistical analysis

4.2.6.1. Choice assay

To establish that insects were feeding non-randomly between the two diets, a t-test was performed comparing the amount of each diet block consumed within the choice assay. If there were significant differences in at least one trial, it was concluded that the insects were feeding non-randomly and attempting to regulate their p:c intake.

A MANCOVA model was fit to determine the effects of population, diet pairing, and sex on the total p and total c consumed by each insect. For this model, total p and total c were the response variables, population, diet pairing, and sex were fixed factors, and larval mass was a covariate. Pairwise linear contrasts were used to test population specific differences in total p and total c consumed.

An ANCOVA model was fit to determine the effects of population, diet pairing and sex on the log normalized intake ratio of p:c . This model included ln(p:c) as the response variable, population, diet pairing, and sex as the fixed factors, with the initial larval mass as a covariate. Tukey HSD was used to determine specific differences between populations.

All choice data was analyzed in JMP ® Pro 12.0.1 (SAS Institute Inc., Cary, NC).

4.2.6.2. No choice assay

Survival curves were used to visualize the proportion of individuals that pupated over time on different diets across the three populations. Only individuals that survived until pupation were considered and no data points were censored. Individual nonparametric

log rank tests were used to determine if diet had a significant effect on the time it took the larvae to pupate within each population.

A full factorial ANOVA model was used to determine if diet, sex or population impacted the insects' pupal weight. Insignificant interaction terms were removed from highest to lowest order, until no interactions remained in the model. In the final model, pupal weight was the response variable and sex, diet, and population were fixed effects. Diet was a significant predictor of pupal weight, so Tukey's HSD was used as a posthoc test. All data met the assumption of homogenous variances.

A generalized linear model with a binary distribution and logit link was used to analyze the effects of population, diet, and the population by diet interaction on larval mortality. Because the interaction between population and diet was significant, independent linear contrasts were used to assess the specific differences between diets within each population. All ANOVA's and generalized linear models were preformed in JMP Pro 12.0.1.

4.2.6.3. Dose response assay

Mortality data were corrected using Abbott's formula (Abbott, 1925). The concentration required to kill 50% of larvae (LC_{50}) and 95% fiducial limits were calculated by Probit Analysis (Finney, 1947) using the Proc Probit procedure in SAS 9.4 (SAS Institute Inc., Cary, NC). LC_{50} values were considered significantly different when the 95% fiducial limits did not overlap.

4.3. Results

4.3.1. Choice assay

To establish that insects were feeding non-randomly with respect to p and c in their diets, a t-test was preformed comparing the amount of each diet block consumed within the choice assay. If there was a significant difference in consumption between the two diet blocks in at least one pairing, it was concluded that the insects were attempting to regulate their intake of p and c. All populations of *S. frugiperda* fed non-randomly on at least one diet pairing, indicating that the insects were actively selecting their food resource based on the p and c content (Table 4.1). Across all populations, insects showed a significant preference for p35:c7 over p7:c35 resulting in a slightly p-biased feeding pattern similar to other Lepidoptera (Tessnow et al., 2018). On the p35:c7 and p14:c28 diet pairing, both BENZ-90 and CSTX-16 showed random feeding while NCBT-13 preferred the p-biased diet. Both an ANCOVA and MANCOVA were used to test the effects of population, diet pairing, and sex on the insects' selected intake ratio of p:c and their total consumption of p and c while accounting for the initial larval mass as a covariate. In both cases, population was a significant predictor of p and c intake, while diet pairing, sex and the initial mass of the larva did not significantly impact the diet choice (Tables A-7 & A-8). This indicates that regardless of diet pairing, all individuals within a population converged to a single point in nutritional space, which was the p:c intake target. However, there was variation among populations ($p=0.0388$). The selected intake target ratios of p:c were p1.3:c1 for the BENZ-90 population, p1.85:c1 for the CSTX-16, and p1.68:c1 for the NCBT-13 (Figure 4.1).

Table 4.1. Assessment of non-random feeding between two diets in the choice assay. Reprinted from Tessnow et al. (2020).

Population	Diet Pairing	N	df	t-stat†	p-value‡
BENZ-90	p35:c7 w/ p7:c35	20	19	3.802	0.0012**
	p35:c7 w/ p14:c28	19	18	-1.143	NS
NCBT-13	p35:c7 w/ p7:c35	19	18	3.487	0.0026**
	p35:c7 w/ p14:c28	20	19	3.081	0.0062**
CSTX-16	p35:c7 w/ p7:c35	19	18	4.923	0.0001***
	p35:c7 w/ p14:c28	20	19	1.523	NS

† Positive t-stat values indicate a preference for the protein-biased diet.
‡ p-values <o.1 are indicated by **, p-values <0.001 are indicated by ***, NS indicates not significant

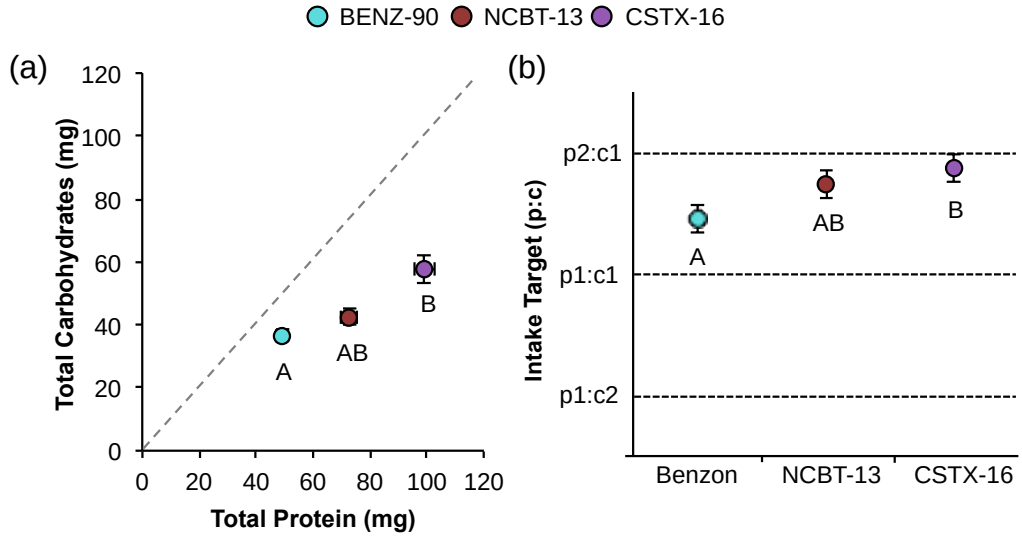

Figure 4.1 (a) Average amount of protein and carbohydrates consumed by each *S. frugiperda* population during the choice assays. The dotted line indicates a balanced p1:c1 nutritional rail. (b) Self-selected intake target (IT) illustrated as a ratio of p:c for each population of *S. frugiperda*. Error bars represent SEM and letters that do not connect indicate significant differences between populations. Reprinted from Tessnow et al. (2020).

4.3.2. No choice assay

A no-choice assay was used to test for effects of diet on the development and survival of the three populations of *S. frugiperda* larvae. A generalized linear model was used test for the effects of diet, population and their interaction on larval mortality (Table A-9). Mortality rates significantly differed between populations (p=0.0172). NCBT-13 had the lowest mortality rate with 20% of larvae dying prior to pupation. The other two populations exhibited higher mortality with 33% of CSTX-16 and 39% of BENZ-90 larvae dying prior to pupation. Additionally, there was a significant interaction between population and diet (p=0.0016). Due to the significant interaction, linear contrasts were used to independently assess the effects of diet on the mortality within each population. Mortality significantly varied across diets in all populations (Figure 4.2). In all three populations, the high p diets tended to have the lowest mortality with the most insects dying on the p12:c30 and p18:c24 diets (Figure 4.2).

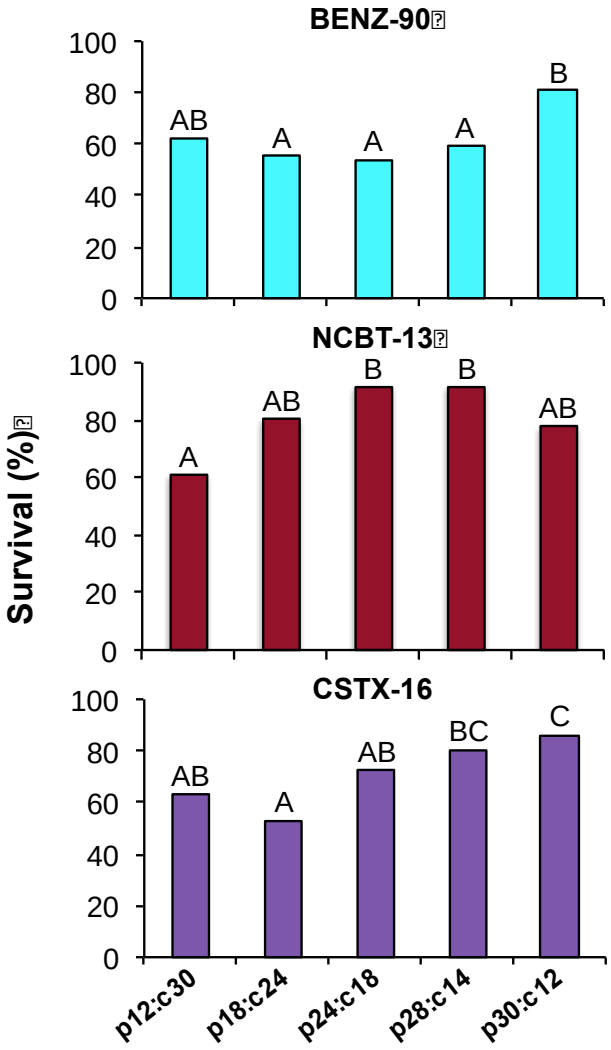

Figure 4.2 Percent of larvae from each population surviving on each diet treatment over the course of the no-choice experiment. Diet had a significant effect on mortality in all populations determined by generalize linear model. Letters that do not overlap indicate significant differences in survival between diet treatments within a population. Reprinted from Tessnow et al. (2020).

Survival plots were fit to visualize the effects of diet on the proportion of individuals that pupated over time (Figure 4.3). In all populations diet significantly impacted the amount of time it took individuals to pupate (Table A-10). Developmental time was directly proportional to the p:c ratio of the diet, with individuals on the high p diets pupating first, and individuals on the low p diets pupating last.

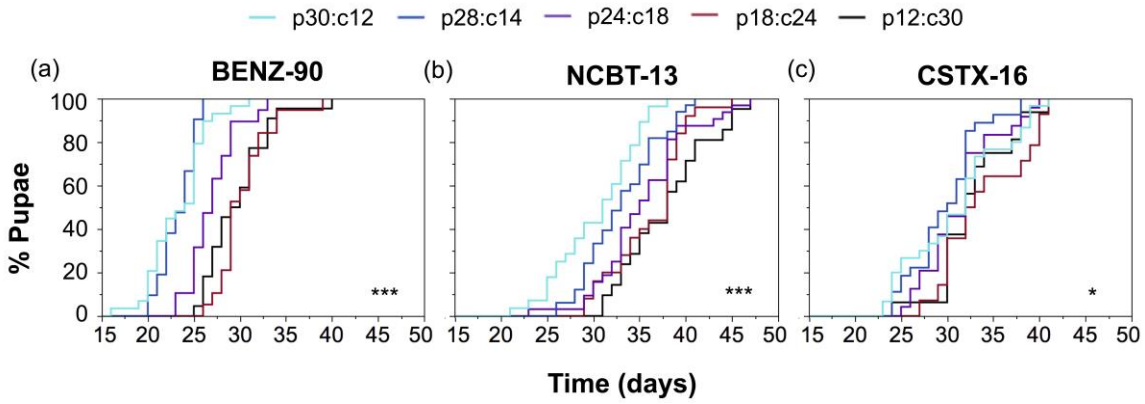

Figure 4.3 Proportion of insects that pupated over the course of the experiment when fed each diet treatment. Each population is visualized in a separate panel; a) BENZ-90, b) NCBT-13, c) CSTX-16. Significant differences in time until pupation between diet treatments are indicated by asterisks; * for p-value <0.001, and * for p-value <0.05. Reprinted from Tessnow et al. (2020).**

In addition to developmental time, we explored the effects of diet, population and sex on the insects' pupal weight using an ANOVA. There were no significant interactions between factors and all factors were significant (Table A-11) so a Tukey's HSD was fit to identify differences within each factor. In the case of population, CSTX-16 pupae averaged 184.1mg and were significantly smaller than either the BENZ-90 or NCBT-16, which averaged 214.1mg and 212.3mg respectively. Additionally, males

were general larger than females averaging 210.5 mg compared to 196.6 mg. In terms of diet, the most extreme p:c ratios (p12:c30 and p30:c12) had the smallest pupal weights while diets that were more balanced in their makeup of protein and carbohydrate seemed to facilitate higher pupal weights (Figure 4.4). Within the more balanced p:c (p18:c24, p24:c18, p28:c14), diets that were higher in c tended to have higher pupal masses (Figure 4.4).

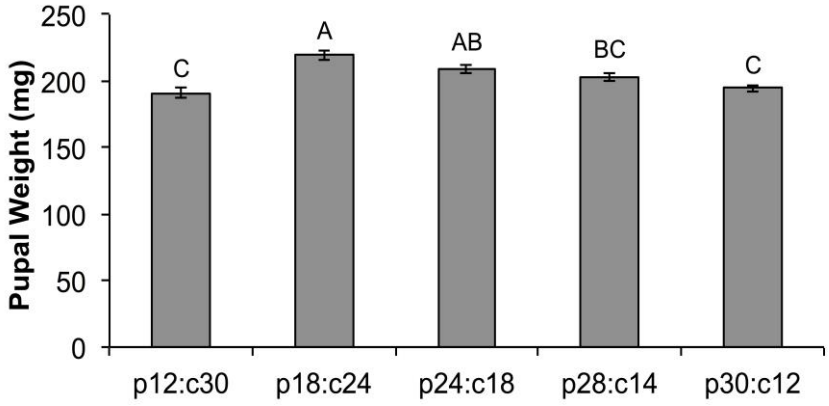

Figure 4.4 Average pupal weight (mg) for insects fed each diet in the no-choice assay. There was no significant interaction between population and diet so all populations were pooled for analysis. Different letters indicate significant differences between treatments (α=0.05). Reprinted from Tessnow et al. (2020).

4.3.3. Dose response assay

The concentration required to kill 50% of larvae (LC_{50}) was calculated for all three populations of *S. frugipdera* on two different macronutrient backgrounds. The first was a c-biased commercial rearing diet (0.43p:1c). The second was a p-biased diet that more closely matched the insects self-selected IT calculated in the choice assays above. For

BENZ-90, there was a significant effect of diet on the $LC_{50,}$ with insects feeding on the CB diet exhibiting a higher $LC_{50,}$ than those feeding on the IT diet (Table 4.2). There was no significant effect of diet in either the CSTX-16 or NCBT-13 populations, although the CSTX-16 population showed a similar trend to BENZ-90. There was no significant difference in Cry1F susceptibility between the field-collected CSTX-16 population and the laboratory reared BENZ-90 population. As expected the NCBT-13 population was significantly less susceptible to Cry1F compared to either BENZ-90 or NCBT-13 (Table 4.2, Figure 4.5).

Table 4.2 Dose dependent mortality response for three populations of *S. frugiperda* when screened against Cry1F on two different nutritional backgrounds: a carbohydrate biased diet (CB) and an intake target diet (IT). Reprinted from Tessnow et al. (2020).

Population	Diet	Diet (p:c)	n	Slope +SE	LC_{50} (95% FL)†
BENZ-90*	CB	p18:c24	437	0.65± 0.06	0.70 (0.41-1.26)
	IT	p24:c18	429	0.63±0.06	0.19 (0.11-0.33)
NCBT-13	CB	p18:c24	256	1.15 ±0.14	6.47 (3.92-10.74)
	IT	p26.5:c15.5	253	0.95 ±0.12	9.76 (5.59-17.90)
CSTX-16	CB	p18:c24	470	1.23±0.12	1.49 (1.03-2.21)
	IT	p26.5:c15.5	442	0.85±0.08	0.67 (0.42-1.11)

*significant difference in LC_{50} between diets, indicated by non-overlapping fiducial limits (α=0.05).
† LC_{50} units are recorded in ppm with 95% fiducial limits are in parentheses.

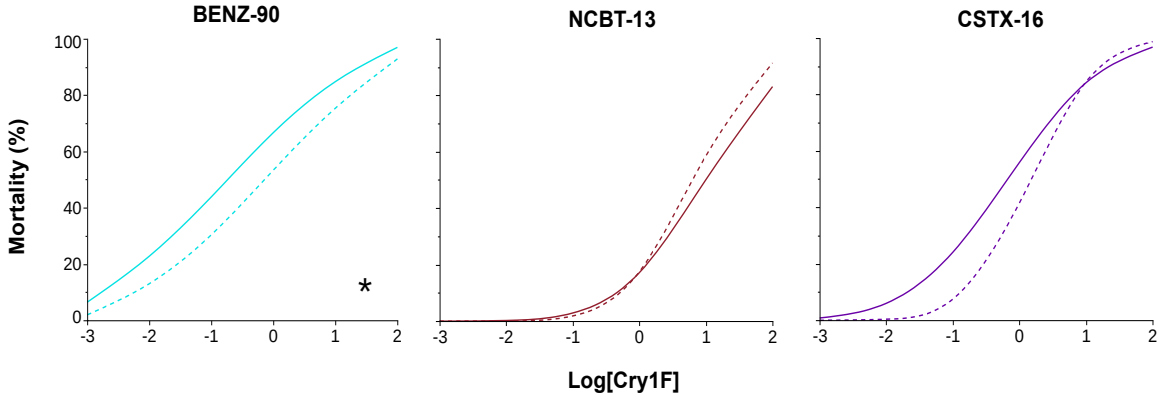

Figure 4.5 Cry1F dose response curves for three *S. frugiperda* populations (a) BENZ-90, (b) NCBT-13, (c) CSTX-16 on two different macronutrient backgrounds. Solid lines indicate response on the IT diet and dashed lines indicate response on the CB diet. Significant differences in LC_{50} between diet treatments are indicated by asterisks (α=0.05). Reprinted from Tessnow et al. (2020).

4.4. Discussion

This study constitutes the first assessment of the protein-carbohydrate intake and regulation in *S. frugiperda*. We demonstrated that similar to other caterpillar species (Tessnow et al., 2018), *S. frugiperda* selected for a protein-biased intake target. With the exception of extreme grass specialists such as *S. exempta* (Lee et al., 2004), caterpillars tend to self-select protein-biased diets when provided a choice. Eating a protein-rich diet during the larval stage is correlated with higher survival and greater reproduction (Behmer, 2009; Deans et al., 2015; Lee et al., 2006, 2002; Merkx-Jacques et al., 2008; Tessnow et al., 2018; Thompson and Redak, 2005). An additional benefit linked to eating a protein-biased diet is reduced developmental time, which shortens the amount of time an individual spends as a caterpillar; feeding during the larval stage is especially risky for lepidopterans (Bernays, 1997). Generally, caterpillars are at high risk of being

predated or parasitized, exposed to pesticides and plant chemicals, and under some circumstances over exploiting resources and starving. In contrast, once an individual becomes an adult, they are more mobile and have a higher probability of surviving to reproduction. Thus, reducing the amount of time that an individual is a caterpillar has been associated with increased fitness (Bernays, 1997; Bernays and Woods, 2000; D'Amico et al., 2001; Simpson and Raubenheimer, 2012).

We found significant variation in the protein-carbohydrate intake among the three *S. frugiperda* populations we sampled. Interestingly, as the number of generations in culture increased, the protein-carbohydrate intake of our different populations became less protein-biased. The BENZ-90 population that had been in culture for over 200 generations preferred a significantly higher ratio of carbohydrates than the CSTX-16 population that was recently collected from the field. Intriguingly, we found that the NCBT-13 population that had been in culture for approximately 25 generations exhibited an intermediate nutritional foraging preference. We propose two non-mutually exclusive hypotheses about why this could be. First, since lab colonies are housed in an environment free from predators and parasitoids, rapid development may no longer enhance the insect's fitness within the population. Rather in the absence of risk, factors such as pupal weight, which was higher on the slightly carbohydrate-biased diets and has been correlated with fecundity in *S. exigua* (Greenberg et al., 2001), play a greater role in the insect's fitness and thus have been selected for over time. Second, both the BENZ-90 and the NCBT-13 populations had been continuously maintained on carbohydrate-biased commercial rearing diets (Deans et al., 2017). After many generations of

selection, these insects may have shifted their nutrient requirements to more closely align with their cultural lab rearing diet. This is similar to findings by Warbrick-Smith et al. (2009) who showed that diamondback moth caterpillars, *Plutella xylostella* (Linnaeus), kept in culture for more than 350 generations selected for a protein and carbohydrate intake that matched their cultural rearing diet (p1:c1)(Warbrick-Smith et al., 2009). Our data suggest fundamental differences may exist in the nutritional physiology of field and lab populations perhaps due to differential selection pressures. This information should be taken into account when designing experiments using lab colonies, or when extrapolating data collected using lab colonies to field pest populations. That said, underlying genetic variation between the three populations used in this study cannot be ruled out as causing the differences in nutrient intake. Ultimately, more data on the nutritional intake from *S. frugiperda* populations that have been in culture for varying numbers of generations will be needed to support this claim.

Several recent studies have established connections between the nutritional intake of *Helicoverpa* caterpillars and their susceptibility to the Bt toxin Cry1Ac (Deans et al., 2017; Tessnow et al., 2018). These findings suggest that changes in the environment, including a caterpillar's nutritional state, can cause a single genotype to produce a range of phenotypes known as phenotypic plasticity (West-Eberhard, 1989; Whitman and Agrawal, 2009). In this study, we explored the possibility that the Cry1F susceptibility of *S. frugiperda* might exhibit nutrition-mediated plasticity, and we found mixed results. Contrary to our expectations, the BENZ-90 population showed a slight increase in susceptibility to Cry1F when allowed to feed on the higher protein diet that

matched its self-selected intake target. The Cry1F response of the other two populations was not significantly affected by the change in protein-carbohydrate intake. These findings may suggest that nutrition-mediated changes in susceptibility are less pronounced in *S. frugiperda* than in the *Helicoverpa* species assessed in previous studies. Given the mode of action of Cry1Ac and Cry1F is similar with some cross-resistance reported between toxins (Hua et al., 2001; Vélez et al., 2013, 2016), and evidence of a shared binding site (Jakka et al., 2016), these differing patterns of nutritionally mediated plastic responses to two Cry toxins were unexpected. One possible explanation for this difference between species is that there are underlying differences in the ecology, physiology and feeding behavior of *Helicoverpa spp.* and *S. frugiperda.* Comparative studies on the nutrient balancing strategies and efficiency of post-ingestive nutrient utilization are needed to further clarify the differences in the nutritionally mediated response of these caterpillar species to Bt.

Our study highlights population-level variation in the nutritional physiology of *S. frugiperda* caterpillars and its affect on Cry1F susceptibility. Additional studies on the mechanisms underlying nutritionally mediated variation in Bt susceptibility in *Spodoptera* and *Helicoverpa* species will help us to better understand the impact of individual nutrition on the evolution of resistance and its monitoring in the field. Additionally, future work should integrate transcriptomics with studies on the nutritional mediated response of caterpillars to toxins. This would facilitate exploration of how an insect's nutrient intake alters gene expression patterns that in turn lead to a shift in Bt susceptibility.

5. CONCLUSIONS

The overarching goal of this dissertation research was to enhance our understanding of the genetic structure, behavior, and nutritional physiology of the fall armyworm in the central US. The insights gained from this research will serve both to inform fall armyworm management programs and facilitate future studies on the ecology and behavior of the two fall armyworm host strains.

5.1. Population genetics of fall armyworms in the central US

In Chapter 2 of this dissertation, I used population genomics to characterize the population structure of fall armyworms across multiple locations in the central US. Due to the high mobility of this pest species, no population structure could be attributed to either geographic location or collection time. The only two genetically distinct populations that I recovered in my data were identified as two previously described fall armyworm strains; the C-strain and the R-strain. This provides strong evidence that fall armyworms in the US are made up of two sympatric strains, or potentially incipient species. Each strain is a panmictic population with individuals dispersed from South Texas to Minnesota.

5.2. Current status of fall armyworm strains

Using population genomics, I found a strong signal of genetic differentiation between the two fall armyworm strains. There are two hypotheses that have been proposed to explain the primary pre-reproductive factor maintaining genetic isolation between

strains: 1) habitat isolation due to differences in host range, and 2) allochonic separation due to differences in nightly activity between stains.

Originally, these two strains were described as host associated, indicating the primary factor maintaining strain divergence is habitat isolation due to differences in host range. However, our trapping data from Chapter 2 and 3 recovered moths of both strains inhabiting the same fields, an observation that is not uncommon in the literature (Nagoshi and Meagher 2004, Meagher and Nagoshi 2004). Due to this overlap in locality, it seems unlikely that habitat isolation is the primary factor preserving genetic differentiation between strains and the term 'host strain' may be ecologically inaccurate.

Previous studies from Dorthy Pashley and Astrid Groot's labs have indicated that the timing of moth activity differs between the two fall armyworm strains (Pashley 1992, Hänniger et al. 2017). When I assessed the strain specific activity of field-collected moths in Chapter 3, I noted similar differences. In my study, the majority of C-strain individuals (86%) were captured in the first 5 hours after the onset of the scotophase, whereas all R-strain moths were captured more than 7 hours after the onset of the scotophase. This suggests that allochronic differences in strain activity are likely a driving force maintaining genetic isolation between strains. If so, the fall armyworm could be a new model system for studying allochronic speciation.

Although I found a strong signal of genetic divergence between the two fall armyworm strains, there was evidence of hybridization. Five of the collected individuals exhibited significant interstrain admixture when tested using *f3* outgroup statistics. Another 32 individuals showed differences in strain assignment when I used the

mitochondrial haplotype as opposed to the nuclear genome to assign strain. Interestingly, all 32 of these individuals had the R-strain mtDNA, but were assigned to the C-strain based on their nuclear genomes. This indicates that hybrid females with R-strain maternal origin successfully mate with C-strain males in the field, however hybrids with C-strain maternal origin are not backcrossing to the R-strain. This is evidence for unidirectional hybrid inviability, a post-zygotic barrier to gene flow that may solidify the genetic divergence between strains.

Using the SNP data collected from my population genomic study, I was also able to determine that patterns of strain divergence were not evenly distributed across the insect's genome. Rather, the majority of SNPs that exhibited high levels of divergence between the two strains mapped to the Z-chromosome. This is indicative of the large Z-effect, which is commonly observed in species undergoing the process of speciation. This observation may explain why virtually all loci that have been identified to differentiate between strains have been sex linked (Prowell 1998), and these strains may be more accurately described as incipient species.

5.3. Utility of lab populations for studying insect pests

The majority of fall armyworm behavioral and physiological studies that are used to inform pest management strategies are conducted in the lab using colonies of insects that have been in culture for tens to hundreds of generations. Even though there is a lack of genetic structure within each fall armyworm strain in the field, differential selection pressures exist on lab and field populations of this species, potentially compromising the utility of these lab colonies as models for the wild population. Fall armyworm lab

colonies have even been shown to exhibit moderate levels of genetic differentiation (Fst=0.06) when compared to field populations (Schlum 2020). Due to this difference, it is critical that we compare the behavior and physiology of these lab reared insect colonies to their wild counterparts, prior to using them to draw general conclusions about the species' behavior and physiology.

In Chapter 4, I assessed the nutritional foraging behavior of three populations of fall armyworms that differed in the number of generations since they had been established in the lab. I then used dose response assays to test if protein-carbohydrate intake altered the susceptibility of each of these populations to the Bt protein, Cry1F. I found that each of these populations foraged for slightly different protein-carbohydrate ratios when provided a choice, with colonies that spent more time in culture selecting a significantly higher proportion of carbohydrates. Additionally, the field colonies responded similarly to the Cry1F toxin across different diets, whilst the lab population showed a decrease in Cry1F susceptibility when the diet contained a higher proportion of carbohydrates. These differences in nutritional physiology between lab and field caterpillar populations highlight the potential consequences of drawing general conclusions about a species' behavior using lab-reared colonies. Although lab colonies are convenient and can be an important tool to understand insect pests, they have limitations when drawing broader conclusions about species ecology. Regularly supplementing these colonies with field caught individuals may be necessary if we hope to use these colonies to enhance our understanding of these insects as pests in the wild.

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APPENDIX

SUPPLEMENTARY TABLES FOR ALL RESEARCH CHAPTERS

Table A-1 Tracy Widom statistics and p-value for the first 10 principal components in the smart PCA.

PC	EigenValue	TW Stat	p-value
1	11.002	405.962	0.000
2	1.719	-0.038	0.175
3	1.708	-0.575	0.295
4	1.692	-1.681	0.631
5	1.665	-4.135	0.995
6	1.660	-4.008	0.993
7	1.652	-4.321	0.997
8	1.647	-4.248	0.996
9	1.628	-5.830	1.000
10	1.626	-5.476	1.000

Table A-2 Results for each individual of the outgroup *f3* test for the model *f3*(C-, R-; putative hybrid). Asterisks indicate individuals significant admixture.

#	Source 1	Source 2	Putative Hybrid Individual	*f3*	stderr	Zscore
1	C	R	2CCMAY_42*	-0.087	0.050	-1.762
2	C	R	2CSJUN_21*	-0.083	0.056	-1.475
3	C	R	2LBBJUN_23*	-0.072	0.055	-1.3
4	C	R	CSTXOCT_R02*	-0.072	0.052	-1.38
5	C	R	MNSEPT_R29*	-0.080	0.045	-1.782
6	C	R	2CCMAY_02	-0.008	0.018	-0.407
7	C	R	2CCMAY_08	0.026	0.022	1.174
8	C	R	2CCMAY_19	0.047	0.028	1.698
9	C	R	2CCMAY_46	-0.005	0.015	-0.345
10	C	R	2CSJUN_16	0.024	0.026	0.92
11	C	R	2LBBJUN_14	0.078	0.035	2.235
12	C	R	2LBBSEPT_03	0.013	0.022	0.591
13	C	R	2WESJUL_02	0.025	0.021	1.176
14	C	R	2WESMAY_05	0.096	0.039	2.448
15	C	R	2WESMAY_09	0.048	0.032	1.53
16	C	R	2WESMAY_14	0.019	0.034	0.553
17	C	R	2WESMAY_17	0.039	0.031	1.279
18	C	R	2WESMAY_18	0.019	0.025	0.779
19	C	R	2WESMAY_19	0.026	0.025	1.055
20	C	R	2WESMAY_23	0.041	0.028	1.434
21	C	R	CCSEPT_C02	-0.027	0.024	-1.115
22	C	R	CSTXMAY_R06	0.023	0.022	1.042
23	C	R	CSTXMAY_R11	0.022	0.023	0.951
24	C	R	CSTXMAY_R21	-0.014	0.018	-0.802
25	C	R	CSTXOCT_R10	0.000	0.019	-0.012
26	C	R	CSTXOCT_R11	0.053	0.029	1.837
27	C	R	CSTXOCT_R22	0.048	0.038	1.251
28	C	R	LBBJUN_R04	0.028	0.029	0.96
29	C	R	LBBJUN_R11	0.059	0.037	1.601
30	C	R	LBBJUN_R27	0.026	0.020	1.259
31	C	R	LBBJUN_R30	0.019	0.024	0.796
32	C	R	LBBJUN_R33	0.056	0.027	2.056
33	C	R	LBBSEPT_R01	0.063	0.032	1.934
34	C	R	LBBSEPT_R08	0.006	0.023	0.254
35	C	R	LBBSEPT_R10	0.024	0.020	1.189
36	C	R	MNSEPT_R12	-0.020	0.012	-1.691
37	C	R	MNSEPT_R28	-0.006	0.028	-0.23
38	C	R	MNSEPT_R31	0.037	0.026	1.433
39	C	R	LBBSEPT_R17	0.022	0.018	1.264

Table A-3 Chromosome position and NCBI BLAST matches for sequences containing SNPs that appeared as outliers (high Fst values) on the Manhattan plot (Figure 3a). Many of the SNPs that showed high levels of divergence were clustered into six groups. The remaining are listed as singletons in the bottom of the table.

Group	SNP	NCBI BLAST Match	Chrom.	Position (bp)	Fst	Mutation notes †	NCBI Reference
1	195	No significant match found	1/Z	10,927,561	0.78	N/A	N/A
	197			10,927,579	0.78		
2	201	Homologous to a protein coding region for a suppressor of cytokine signaling (SOCS) protein in *S. litura*. GO molecular function: 1-phosphatidylinositol-3-kinase regulator activity	1/Z	11,030,881	0.77	Synonomous	XM_022977237.1, XM_022977236.1, XM_021332332.1, XM_021332331.1
	203			11,030,973	0.77	Non-synonmous TYR to HIS	
	205			11,030,998	0.77	Synonomous	
	207			11,031,097	0.77	**CYS to stop codon**	
	208			11,031,103	0.77	Synonomous	
	209			11,031,124	0.77	Synonomous	
	210			11,031,163	0.78	Synonomous	
	211			11,031,172	0.78	Synonomous	
3	222	No significant match found	1/Z	12,671,645	0.72	N/A	N/A
	223			12,671,651	0.73		
	227			12,671,837	0.73		
4	250	Protein coding region of predicted Spodoptera litura 1-phosphatidylinositol 4,5-biphosphate phosphodiesterase gamma-1	1/Z	13,292,971	0.77	Synonomous	XM_022976465.1, XM_022976466.1
	253			13,293,046	0.76	Synonomous	
	254			13,293,061	0.77	Synonomous	
	258			13,295,934	0.74	Synonomous	
	264			13,296,117	0.77	Synonomous	

Table A-3 Continued

Group	SNP	NCBI BLAST Match	Chrom.	Position (bp)	Fst	Mutation notes †	NCBI Reference
5	418	No significant match found	1/Z	22,985,972	0.76	N/A	N/A
	422			22,986,192	0.76		
	423			22,986,226	0.76		
	424			22,986,239	0.76		
	425			22,986,294	0.76		
6	5103	Partial match to Spodoptera litura disco-interacting protein 2 (LOC111361098)	24	4725576	0.25	N/A	XM_022977441.1, XM_022977440.1, XM_022977439.1, XM_022977437.1, XM_022977436.1, XM_022977435.1, XM_022977434.1, XM_022977433.1, XM_022977432.1, XM_022977431.1
	5104			4725589	0.26		
	5105			4725594	0.26		
	5106			4725626	0.26		
	5107			4725676	0.26		
	5109			4725754	0.46		
	5111			4725770	0.25		
	5113			4725772	0.43		
	5114			4725800	0.25		
	5116			4725835	0.54		
	5121			4729356	0.35		
	5122			4729386	0.28		
Singletons	16	Predicted coding region for	1/Z	1,121,015	0.73	Synonymous	XM_022976921.1
	106	Matched to *S. litura* uncharacherized mRNA	1/Z	4,933,322	0.78	N/A	XM_022960480.1
	301	No significant match found	1/Z	14,104,488	0.79	N/A	N/A
	307	Upstream of coding region for LIM/homeobox protein Lhx2-like	1/Z	14,412,021	0.72	N/A	XM_026891428.1
	2757	No significant match found	12	12,456,628	0.72	N/A	N/A
	3686	Protein coding region of Solute carrier family 25 member 35-like isoform in *S. litura*	16	14,134,047	0.74	Synonomous	XM_022963959.1

Table A-4. Amount of each diet ingredient added to choice and no-choice experimental diets. All amounts are listed in grams unless otherwise specified. Reprinted from Tessnow et al. (2020).

COMPONENT	p35:c7	p30:c12	p28:c14	p24:c18	p18:c24	p14:c28	p12:c30	p7:c35
Vitamin-free casein	32.5	26.8	24.6	20.4	14	9.8	7.8	2.7
Wesson's salt	5	5	5	5	5	5	5	5
Sucrose	5.2	10	11.9	15.8	21.4	25	27.2	32
Cellulose	32	30	30	30	30	30	30	31
Cysteine HCl	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Choline chloride	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Myo-inositol	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Torula yeast	5	5	5	5	5	5	5	5
Dry milk (whole)	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
Vitamin mix	0.8125	0.8125	0.8125	0.8125	0.8125	0.8125	0.8125	0.8125
100% ethanol (mL)	5	5	5	5	5	5	5	5
dl-alpha-tocopherol acetate	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Cholecalciferol	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025
Menadione	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025
Linoleic acid (uL)	275	275	275	275	275	275	275	275
Alpha-linolenic acid (uL)	135	135	135	135	135	135	135	135
Sorbic acid	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
l-ascorbic acid	1	1	1	1	1	1	1	1
Methyl paraben	0.4875	0.4875	0.4875	0.4875	0.4875	0.4875	0.4875	0.4875
Chlorotetracycline HCl	0.0087	0.0087	0.0087	0.0087	0.0087	0.0087	0.0087	0.0087
Streptomycin sulphate	0.0087	0.0087	0.0087	0.0087	0.0087	0.0087	0.0087	0.0087
37% Formaldehyde (uL)	365	365	365	365	365	365	365	365
Cholorform (mL)	35	35	35	35	35	35	35	35
Cholesterol	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Distilled water (mL)	375	375	375	375	375	375	375	375
Vitamin Solution (mL)	30	30	30	30	30	30	30	30
Agar	10	10	10	10	10	10	10	10

Table A-5 Amount (g) of each ingredient added to experimental diets used in the dose response assays. The commercial diet was purchased from Southland Products (Lake Village, AR) and 250ml of boiling water was added to each diet. Reprinted from Tessnow et al. (2020).

Ingredient	CB *all pops	IT CSTX-16 &NCBT-13	IT BENZ-90
Commercial Diet	27.07	14.37	16.85
Cellulose	11.17	13.98	13.69
Casein	0.00	9.03	7.26
Agar	1.83	3.54	2.80
Methyl paraben	0.08	0.16	0.15
Sorbic acid	0.08	0.16	0.15
Chlorotetracycline	0.03	0.07	0.06

Table A-6 Total numbers of insects assessed on each diet in the no-choice assays. Reprinted from Tessnow et al. (2020).

	Diet (p:c)				
	p12:c30	p18:c24	p24:c18	p28:c14	p30:c12
BENZ-90	37	35	36	37	36
NCBT-13	36	34	35	36	36
CSTX-16	30	32	35	36	36

Table A-7 ANCOVA results testing the effects of sex, diet pairing, and population on the ln(p:c) intake target ratio for *S. frugiperda* considering the initial mass of the insect as a covariate. Reprinted from Tessnow et al. (2020).

Effect	DF	F-Ratio	p-value
Initial Mass	1	0.0006	NS
Population	2	3.3494	0.0388
Diet Pairing	1	0.0034	NS
Sex	1	0.2357	NS

Table A-8 MANCOVA results testing for the effects of sex, diet pairing, and population on the response variables total protein and total carbohydrates with the initial mass of the insect as a covariate. Reprinted from Tessnow et al. (2020).

Factor	DF	F-Ratio	p-value
Intercept	1	5.014	0.0271
Initial Mass	1	0.096	NS
Population	2	10.07	<0.0001
Diet Pairing	1	0.16	NS
Sex	1	0.023	NS

Table A-9 Significant predictors of larval mortality during the no-choice assay determined by a generalized linear model with a binary distribution and logit link. Reprinted from Tessnow et al. (2020).

Effect	DF	X^2	p-value
Strain	8	18.595	0.0172
Diet	2	16.324	0.0003
Strain*Diet	4	17.387	0.0016

Table A-10 Log rank chi square and p-value showing the effects of diet on the proportion of larvae that pupated over the course of the experiment. Diet significantly impacted the time it took each population to pupate as indicated by p-values <0.05. Only individuals that successfully pupated were considered in this analysis. Reprinted from Tessnow et al. (2020).

Population	DF	X^2	p-value
BENZ-90	4	81.808	<0.0001
NCBT-13	4	36.140	<0.0001
CSTX-16	4	10.043	0.0397

Table A-11 Significant predictors of pupal weight according to an ANOVA model. No interactions between effects were significant and thus were not included in the model. Reprinted from Tessnow et al. (2020).

Effect	DF	F-Ratio	p-value
Population	2	39.128	<0.0001
Diet	4	10.295	<0.0001
Sex	1	20.37	<0.0001