MOLECULAR BASIS OF INSECTICIDE RESISTANCE IN THE DIAMONDBACK MOTH, *PLUTELLA XYLOSTELLA*, (LEPIDOPTERA: PLUTELLIDAE) IN GEORGIA AND FLORIDA WITH EMPHASIS ON DIAMIDE INSECTICIDES

by

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(Under the Direction of DONALD CHAMPAGNE)

ABSTRACT

The diamondback moth (DBM), *Plutella xylostella*, is a key pest of Cole crops in the Southeastern USA. Insecticide resistance, in particular diamide resistance, is a major concern for DBM populations in Georgia and Florida based on recent maximum dose bioassays conducted during the course of this research. Colonies were established from resistant field populations from Tift County (LTF), Colquitt County (NP), and Crisp County (CSP) in Georgia, as well as Manatee County (MAN) in Florida. The LTF, NP, and MAN colonies were highly resistant to chlorantraniliprole (2,813 to 4,298-fold), while the CSP colony only showed intermediate resistance (109-fold). Intermediate resistance to both cyantraniliprole (50 to 108-fold) and spinetoram (29 to 217-fold) was also determined for the colonies. The G4946E, a target site mutation of the DBM ryanodine receptor, was confirmed for these colonies. Allele frequency estimates of the G4946E were determined to be 90%, 61%, 53%, and 32%.

INDEX WORDS: diamondback moth, *Plutella xylostella,* insecticide resistance, molecular, bioassay methods, insecticide resistance management (IRM), Lepidoptera, Plutellidae

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DEDICATION

For my family and friends who have encouraged me to further my education. Without their support, I would not have been able to complete the rigorous coursework, writing, and research required for this program.

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I would like to thank my advising professor and committee members who assisted me with this project. Without their guidance and encouragement, I would not have been able to complete this project and all the accompanying work. I would also like to thank my coworkers for all their efforts in keeping this lab running.

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CHAPTER 1. INTRODUCTION

The Diamondback moth (DBM), *Plutella xylostella* (Lepidoptera: Plutellidae), is an insect pest that specializes in feeding on plants in the family Brassicaceae. These include Cole crops such as cabbage, broccoli, collards, and kale (Talekar and Shelton 1993, Furlong et al. 2013). DBM are well known for their ability to rapidly develop resistance to insecticides. Currently, over 95 insecticide products have experienced insecticide resistance when implemented against DBM populations (APRD 2020). Resistance to specific insecticides may vary from population to population, which in turn complicates insecticide resistance management (IRM) strategies. Documented mechanisms of DBM insecticide resistance include avoidance behaviors, such as leg autonomy (Moore, Tabashnik, and Stark 1989), as well as genetic factors in the form of target site mutations and enzyme upregulation (Guo et al. 2014, Troczka et al. 2012, Gao et al. 2018). There is currently limited research of DBM insecticide resistance mechanisms in Georgia and Florida, but resistance to insecticides is a recurring problem in this area. Agricultural practices are dependent upon the use of insecticides to control DBM; therefore, this information could be essential in developing integrated pest management (IPM) programs to control DBM.

DBM can be found throughout most of the world, although temperature has been shown to influence fecundity. The optimal temperature for DBM development is around 19.4 °C (Shi, Li, and Ge 2012), but Marchioro and Foerster (2011) demonstrated that DBM are tolerant to temperatures ranging from 6.1 °C to 32.5 °C. At a constant

temperature of 20 °C, adult DBM (Fig. 1.3) were shown to survive for 17.7±2.34 days (Salinas 1986). Female DBM oviposit 150 eggs on average (Capinera 2000) and will typically oviposit their eggs on the underside of brassica crop foliage, most often targeting crevices (Justus and Mitchell 1996). Although wild brassicas are adequate hosts for DBM, cultivated brassica crops tend to be favored over wild plants (Marchioro and Foerster 2014). Rather than damaging crop foliage when feeding like DBM larvae, DBM adults feed on nectar found in nearby blossoms. The average development times of DBM eggs (Fig. 1.1), 1st, 2nd, 3rd, 4th instar larvae (Fig. 1.2) and pupae are 5.8±0.9, 4.5±1.0, 2.8±0.7, 3.4±0.5, 4.5±0.7, and 9.1±0.7 days, respectively. Average sex ratios of DBM have also been determined at males: females = 1:1.5. All of the developmental and sex ratio averages previously mentioned were determined with development occurring at 20 °C constant temperature (Salinas 1986).

Figure 1.1. Diamondback moth eggs on a brassica crop leaf (Photo Credit: John E. Bennett and Thomas P. Dunn).

Figure 1.2. A 3rd instar diamondback moth larvae feeding on a brassica leaf (Photo Credit: John E. Bennett and Thomas P. Dunn).

Figure 1.3. A diamondback moth adult on a brassica crop stem (Photo Credit: David G. Riley).

The economic importance of DBM is established when observing crop damage and how it affects the global economy. Furlong (2013) reports that brassica crops contribute more than US$26 billion to the global economy, and global DBM crop damage costs anywhere from US$4-5 billion every year (Zalucki et al. 2012). Recently, China experienced a 20-fold increase (0.16 million ha to 3.35 million ha) in the area of land used to grow brassica crops. Consequentially, the area of crops damaged by DBM also increased from 0.15 million ha to 2.23 million ha (Li et al. 2016).

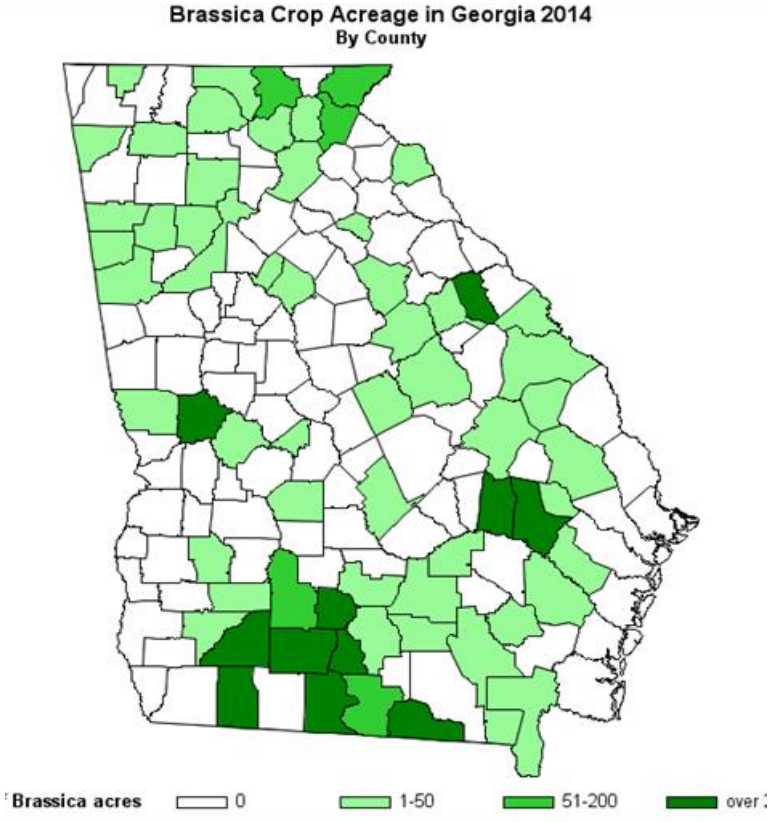

Figure 1.4. Distribution of Cole crop acreage in Georgia in 2014 (Photo Credit: David G. Riley).

The use of transplants to establish vegetable crops in the state of Florida has recently increased (McAvoy and Ozores-Hampton 2019), as has use in the state of Georgia. Some benefits of transplant production include increased speed of stand establishment, as well as avoiding unfavorable conditions (McAvoy and Ozores-Hampton 2019). While transplant production is continuing to increase, the possibility of propagating resistance factors through transport may be an issue. The transport of contaminated transplants with DBM larva has been reported (Shelton et al. 1996). This may contribute to the spread of resistance factors if resistant larva are transported to new areas. DBM migration could also play a role in the spread of insecticide resistance. DBM in China and Europe have been observed migrating long distances (Wei et al. 2013, Chapman et al. 2002); however, there is currently no data documenting the long migration of DBM in the United States, though it is likely to occur.

Figure 1.5. Damaged brassica leaf caused by diamondback moth larvae (Photo Credit: David G. Riley and Alton "Stormy" Sparks).

As previously mentioned, brassica crop damage occurs when DBM larva feed on foliage. Figure 1.5 depicts extensive damage to a collard leaf due to DBM larval feeding. The U.S. No. 1 and U.S. Commercial systems are the grading systems provided for brassica crops (Coolong and Kelley 2000). If the criteria of the chosen system are not met during grading, the product may be rejected to prevent its use for consumption. Under both systems, extensive damage to the product leaves is justifiable for rejection. Produce rejection may also occur in the event that live larva are found within the florets, regardless of the amount of damage done to the leaves (Capinera 2000). Since leaf damage and larval presence could potentially lead to rejection of brassica products, control of DBM in the field is necessary to allow maximum yield.

Pesticides are heavily integrated into the field of agriculture as a means of protection against pests. However, over reliance on the use of pesticides often leads to the development of pesticide resistance. Reports of DBM insecticide resistance can be found as far back as 1953, when DBM began showing signs of resistance to DDT (Johnson 1953). In 1986, DBM populations from Taiwan began showing signs of resistance to pyrethroid, organophosphate, and carbamate insecticides (Sun et al. 1986). These reports were soon followed by extreme levels of resistance to pyrethroids, as well as resistance to organophosphates, carbamates, and cyclodiene insecticides in a DBM population from Hastings, Florida during 1991 (Yu and Nguyen 1992). DBM resistance to indoxacarb, avermectin, and spinosyn insecticides have been documented (Sayyed and Wright 2006, Pu et al. 2010, Zhao et al. 2002, Zhao et al. 2006), as well as resistance to *Bacillus thurengiensis* (*Bt*) insecticides (Tang et al. 2001). The more recently developed diamide insecticides (IRAC 28) have been of great concern when discussing resistance

development in DBM. The first diamide insecticide, flubendiamide, was developed by Bayer CropScience AG and Nihon Nohyaku Co., Ltd (Yan et al. 2014), and the first registration of the product occurred in the Philippines in 2006 (Troczka et al. 2017). Cases of DBM control failure with diamide insecticides first appeared in the Bang Bua Thong district of Thailand in 2009. These reports of DBM cross-resistance to flubendiamide and chlorantraniliprole occurred just 18 months after the release of flubendiamide in Thailand in 2007 (Troczka et al. 2017). Wang and Wu (2012) also reported the development of chlorantraniliprole resistance in Chinese DBM populations collected from 2010 to 2011.

Reports of diamide resistance in Asian DBM populations were followed by studies of possible resistance mechanisms. These studies provided knowledge of target site mutations in the DBM ryanodine receptor (RyR) which are associated with diamide insecticide resistance. Three mutations, the E1338D, Q4594L, and I4790M, were discovered in Asian DBM populations and have yet to be found elsewhere (Guo et al. 2014). However, the fourth mutation, referred to as the G4946E, has been reported on multiple continents since its discovery in 2012 (Troczka et al. 2012, Steinbach et al. 2015). Metabolic detoxification has also been studied in DBM populations as a mechanism of insecticide resistance. The upregulation of two cytochrome P450 enzymes (P450) has been shown to occur after exposure to five different insecticides, including chlorantraniliprole (Gao et al. 2018). There have been similar reports of DBM insecticide resistance in the Southeastern United States, especially in regards to diamide resistance (Riley et al. 2020, Bhandari et al. 2020). Despite these reports, studies of possible genetic

contributors to insecticide resistance have not occurred for DBM populations in Georgia and Florida.

The goal of this study is to identify genetic mechanisms associated with DBM diamide insecticide resistance in Georgia and Florida. Resistance to diamide insecticides has appeared in Georgia and Florida DBM populations, and knowledge of which resistance mechanisms are present may assist in forming better management practices.

The hypotheses tested in this thesis associated with each objective are as follows:

Objective 1. Control failures with diamide insecticides are common around the world, but there have been recent reports of resistance specifically in Georgia & Florida. In China and Southeast Asia, control failures have been associated with specific target site mutations in the ryanodine receptor (RyR). These mutations are referred to as the E1338D, Q4594L, I4790M/K, and G4946E mutations of the RyR. The presence of these or other novel mutations in Georgia & Florida has not been determined. We plan to screen colonies derived from insecticide resistant field populations for these mutations.

Hypothesis 1:

The diamondback moth colonies derived from the resistant field populations in Georgia and Florida contain target site mutations associated with diamide insecticide resistance.

Approach: LC_{50} values for chlorantraniliprole and cyantraniliprole will be determined for the colonies to examine resistance levels compared to a control

population commercially available from Frontier Inc. Polymerase chain reaction will be used to observe segments of the DBM RyR from these populations to potentially identify target site mutations associated with diamide resistance. This work will be covered in Chapter 3.

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CHAPTER 2. LITERATURE REVIEW

2.1 The importance and biology of the diamondback moth

The diamondback moth (DBM), *Plutella xylostella,* is a major pest of cruciferous crops. It is estimated that $4-5 billion dollars of worldwide crop damage occurs from DBM outbreaks annually (Zalucki et al. 2012). Damages to brassica crop products occur when DBM larva feed on leaf tissue (Capinera 2000). These damages, as well as produce contamination by pupating DBM, can result in the rejection of potential food products (Troczka et al. 2017). Thus, failure to control DBM can result in economic setbacks in the world of agriculture.

At a constant temperature of 20 °C, adult DBM (Fig. 3) were shown to survive for 17.7±2.34 days (Salinas 1986). The average development times of DBM eggs, 1st, 2nd, 3rd, 4th instar larvae, and pupae are 5.8±0.9, 4.5±1.0, 2.8±0.7, 3.4±0.5, 4.5±0.7, and 9.1±0.7 days, respectively. Average sex ratios of developing DBM have also been determined at males: females = 1:1.5. All of the developmental and sex ratio averages previously mentioned were determined with development occurring at 20 °C constant temperature (Salinas 1986).

DBM can be found throughout most of the world, although temperature has been shown to influence fecundity. The optimal temperature of DBM development is around 19.4 °C (Shi, Li, and Ge 2012), however Marchioro and Foerster (2011) demonstrated that DBM are tolerant to temperatures ranging from 6.1 °C to 32.5 °C.

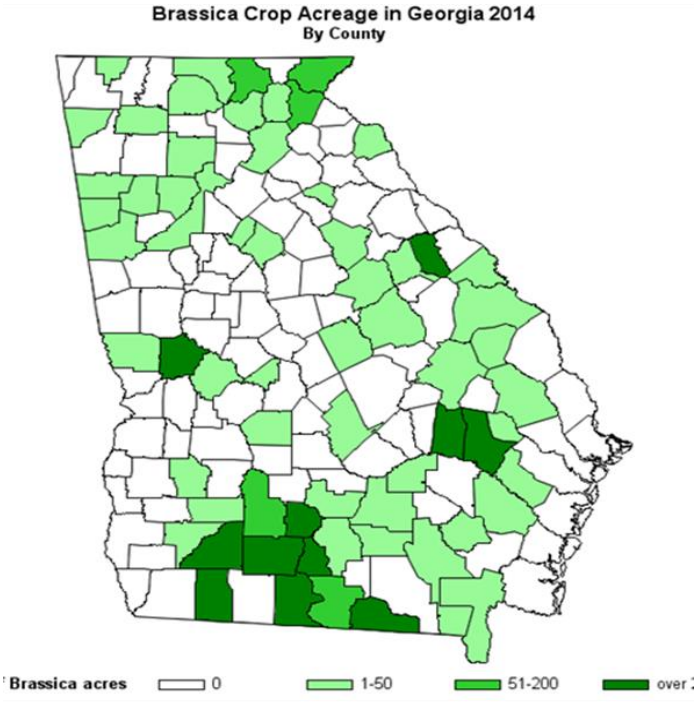

Figure 2.1. Cole crop distribution in Georgia in 2014 (Photo Credit: David G. Riley).

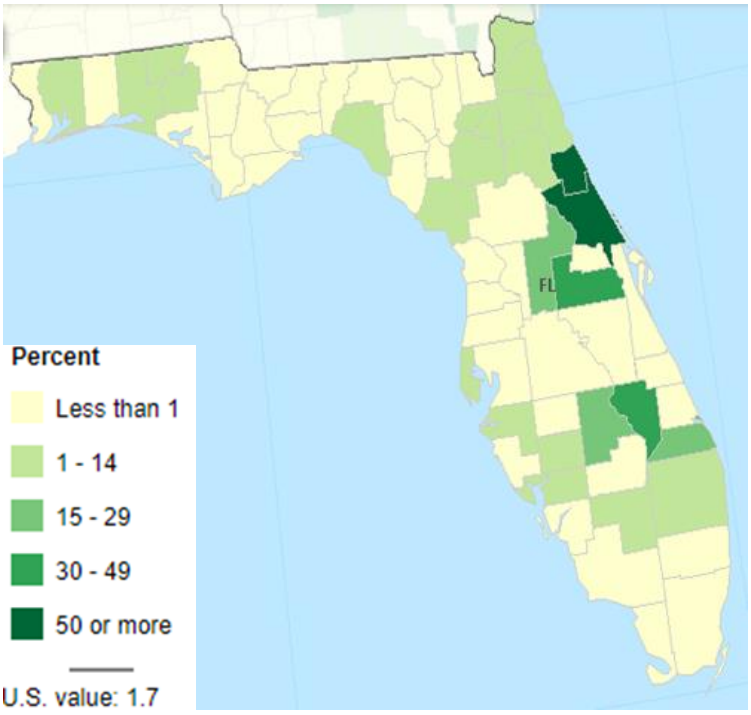

Figure 2.2. Acres of cabbage harvested for sale in Florida in 2017 (Photo Credit: https://www.nass.usda.gov/Publications/AgCensus/2017/Online_Resources/Ag_Census_Web_Maps/index.php Accessed 10/19/2020).

DBM mating generally occurs between dusk and midnight, and female DBM adults often oviposit 150 eggs over an average of 10 days. Rather than ovipositting eggs in batches, DBM oviposit their eggs individually and tend to target crevices on the underside of foliage (Justus and Mitchell 1996). As mentioned earlier, DBM target brassicaceous crops such as broccoli, cabbage, collards, and kale (Talekar and Shelton 1993, Furlong et al. 2013). These are vital cool season commodity crops that are grown all over Georgia (Fig. 6) and Florida (Fig. 7), giving the DBM populations plenty of host plants during growing seasons. Between growing seasons, wild brassicas are adequate hosts for DBM, but cultivated brassica crops seem to be favored over wild plants (Marchioro and Foerster 2014). When studied by Marchioro and Foerster (2014), analysis shows that wild radish and turnipweed are suitable hosts for DBM, however cultivated crops had more success in regards of larva reaching adulthood. Justus and Mitchell (1996) reports that host selection by DBM is heavily influenced by contact stimuli. This stimulus can occur via chemosensilla on tarsi, antennae, and even ovipositors in the case of female DBM. There is also a set line of behaviors performed by female DBM before oviposition. These behaviors include antennal rotation, antennation, ovipostional sweeping, and finally ovipostion (Justus and Mitchell 1996). It is believed these behaviors utilize the chemosensilla to detect chemicals such as host plant volatiles (HPV), which have been shown to increase egg deposition.

2.2 Insecticidal control and insecticide resistance development of the diamondback moth

The Insecticide Resistance Action Committee (IRAC) is an organization that works to prevent the formation of insecticide and acaricide resistance (https://irac-

online.org/about/irac/). IRAC has two main goals; to communicate with and educate the public on insecticide resistance, and to support the development of insecticide resistance management (IRM) strategies that assist in sustaining agriculture (Sparks and Nauen 2015). The MoA classification scheme, which is maintained by IRAC, provides a system of grouping insecticides based off of similarities and differences of MoA and target sites (https://irac-online.org/about/irac/). When utilizing tactics that mitigate insecticide resistance development, the classifications and the ease of access make it an important tool of IRM.

Insecticides encompassing a wide range of IRAC classes have been used to control DBM around the world. Organophosphates (IRAC 1B), pyrethroids (IRAC 3A) indoxacarb (IRAC 22A), benzoylureas (IRAC 15), and *Bacillus thuringiensis* (*Bt*) (IRAC 11A) insecticides are all commonly used for DBM control (Atumurirava, Nand, and Furlong, 2016). Spinosyn insecticides (IRAC 5) have also been used for DBM control in the United States (Zhao et al. 2006), and the more recently developed diamide insecticides (IRAC 28) were highly effective against lepidopteran pests upon their release (Roditakis et al. 2017, Itagaki and Sonoda 2017). Reports of DBM insecticide resistance can be found as far back as 1986, when Taiwanese populations began showing signs of resistance to pyrethroid, organophosphate, and carbamate insecticides (Sun et al. 1986). These reports were soon followed by extreme levels of resistance to pyrethroids, as well as resistance to organophosphates, carbamates, and cyclodiene insecticides in a DBM population from Hastings, Florida during 1991 (Yu and Nguyen 1992). DBM resistance to indoxacarb (Sayyed and Wright 2006) and avermectin insecticides have been documented (Pu et al. 2010, Zhao et al. 2006), as well as spinosad resistant DBM

populations in Hawaii, Georgia, and California during the early 2000's (Zhao et al. 2006). In total, there has been documentation of resistance to at least 95 different insecticide formulations in DBM (APRD 2020).

Diamide insecticides are a relatively new class of insecticides on the market. In 2006, flubendiamide was introduced for use as an insecticide (Roditakis et al., 2017; Troczka et al. 2017) followed by chlorantraniliprole and cyantraniliprole. The first reported cases of failed DBM control with diamides were from the Bang Bua Thong district of Thailand in 2009. These reports occurred just 18 months after the release of flubendiamide in Thailand. Wang and Wu (2012) also reported the development of chlorantraniliprole resistance in Chinese DBM populations collected from 2010 to 2011. Chlorantraniliprole was labeled for use in Georgia (U.S.) in 2008 (Bhandari et al. 2020), and was highly efficacious against DBM. Riley (2014) highlighted the importance of insecticide rotations as a means of preventing DBM resistance development for diamide insecticides in the U.S. Regardless, the high levels of efficacy of diamides against DBM in the U.S. were short lived, as reports of diamide resistant DBM in Mississippi began to appear in 2013 (IRAC Newsletter 33). These reports of diamide resistance in Mississippi were soon followed by reports of diamide resistance in other nearby states. Riley et al. (2020) conducted a study of multiple DBM populations in multiple counties in Georgia and Florida. This study occurred from 2016-2019, and resistant DBM populations that caused widespread damage to crops were collected for study. Maximum dose bioassay data indicated low levels of DBM larval control from chlorantraniliprole application, while cyclaniliprole and cyantraniliprole still provided good DBM control. These low levels of chlorantraniliprole control compounded with the increasing chlorantraniliprole

LC_{50} values in Georgia determined by Bhandari et al. (2020) suggests the development of diamide insecticide resistance in these DBM populations.

2.3 Factors influencing the development of insecticide resistance of the diamondback moth

The rapid development of insecticide resistance among DBM populations has been a serious issue in IPM. Development of resistance in insect populations is generally linked to over use in the field. Ffrench-Constant et al. (2004) describes insecticide selection as an example of an artificial selection pressure. Intense insecticide selection of a population may reduce the frequency of susceptible traits within that population, resulting in higher frequencies of resistant traits. The use of insecticide rotations can potentially mitigate selection pressures that may result in insecticide resistance development (Zhao et al. 2010, Riley 2014). Insecticide rotations refer to applying separate insecticide MoA's to an insect population during a generation in order to prevent exposure of that population to a single MoA multiple times within a single generation. This method of IRM is dependent on utilizing 'window' periods in which a separate MoA is used to control the newest generation of pests. Applying during this window prevents exposure to multiple exposures to a single MoA during a single generation and is thought to reduce the development of insecticide resistance (Zhao et al. 2010, Riley 2014). Zhao et al. (2010) also suggests that mosaic applications, which are applications of different MoA to different areas of a field during a single generation of a pest, should be avoided to prevent exposure of one pest generation to multiple MoA. In Georgia, there are generally three DBM generations during the spring and three in the fall (Riley and Sparks 2011). However, Riley (2014) reported an increase from four DBM generations in the

spring of 2010 to six DBM generations in the spring of 2012, so changing weather patterns must also be taken into account.

Generally, alleles associated with resistance are also associated with fitness costs (Steinbach, Moritz, and Nauen 2017). Steinbach, Moritz, and Nauen (2017) demonstrated that a DBM population (Sudlon-Tfm strain) with resistance to diamide and benzoylurea (BPU) insecticides had longer generation times after exposure to BPUs when compared to another diamide resistant population (Sudlon strain). Since the Sudlon-Tfm strain was derived from the Sudlon strain and exposed to BPUs for 10 generations, it was suggested that the developed BPU resistance may be associated with the fitness cost of significantly longer generation time. Ribeiro et al. (2014) proposed that chlorantraniliprole resistance in a DBM population from Brazil may be associated with fitness costs. This was concluded after observing little stability in the resistance of the populations, as well as the elongation of the larval stage and reduced weight in resistant larva when compared to susceptible larva. Interestingly, DBM populations from Japan that exhibited extreme levels of resistance (> 10,000-fold) to flubendiamide, chlorantraniliprole, and cyantraniliprole showed no significant differences in terms of egg hatchability, larval development, or fecundity when compared to susceptible lab strains (Fukada et al. 2020).

Reversion, otherwise referred to as the loss of resistance, has been found to occur in insect populations and is often associated with fitness costs (Jan et al. 2015). As demonstrated in Yang et al. (2014), insecticide resistance of the brown planthopper, *Nilaparvata lugens*, decreased over generations with lessened exposure to insecticides. Lepidopteran relatives of DBM have also reverted back to susceptibility after reduced insecticide exposure. Jan et al. (2015) reported that resistant populations of the spotted

bollworm, *Earias vittella fabricius*, experienced reversion to spinosad, cypermethrin, and deltamethrin after just eight generations without selection pressure from these insecticides. Similar results of reversion occurred with the tobacco cutworm, *Spodoptera litura*. A field collected population of *S. litura* developed up to 2,358.6-fold resistance to methoxyfenozide after thirteen generations of methoxyfenozide exposure. Cross resistance was also recorded for abamectin and deltamethrin, at 12.87 and 28.82-fold respectively. However, after five generations with no selection pressure, resistance to methoxyfenozide decreased from 2,358.6 to 163.9-fold (Rehan and Freed 2014). Reversion of resistance traits associated with chlorantraniliprole resistance have been observed in DBM. Itagaki and Sonoda (2017) reported increasing frequencies of a target site mutation of the DBM RyR, referred to as the G4946E, from spring into summer. The G4946E frequencies would then decrease into the fall, where they would remain consistent until the next spring. The increase of G4946E frequencies during growing seasons seems to suggest insecticide selection may be a direct influence, however the cause of the decreased G4946E frequencies still remains unclear. As mentioned earlier, some evidence of fitness costs associated with diamide resistant DBM populations suggests reversion of resistance in these populations (Ribeiro et al. 2014, Steinbach, Moritz, and Nauen 2017). However, conflicting results have been reported in more recent studies of DBM populations confirmed to possess the G4946E mutation (Fukada et al. 2020). Although reversion has been observed in many different insect populations, May and Dobson (1986) suggests that reversion of insect populations will occur more slowly than the development of insecticide resistance and that reverted insect populations will regain resistance traits faster than the initial resistance developed.

Environmental factors have the potential to influence resistance within populations. Refuges are a percentage of a field where non-treated crops are grown to prevent the loss of susceptibility within a population (USDA 2017). Refuges are commonly utilized when growing *Bt* crops and allow susceptible individuals to reproduce (Tang et al. 2001). In the case of *Bt* corn, the Environmental Protection Agency (EPA) requires a certain percentage of the crop be planted as a refuge in order to slow resistance development in certain pests (USDA 2017). There are different strategies of refuge implementation, which include structured, seed blend, and natural refuges (EPA 2021). Natural refuges include weeds, wild host plants, or other cultivated crops that the insect pest may target instead. Wild hosts, such as wild radishes and turnipweeds, are suitable for DBM growth and development (Marchioro and Foerster 2014). Seed blend refuges involve a mixture of *Bt* and non-*Bt* seeds, meaning the refuge will be spread throughout the field. Finally, structured refuges involve the coordination and planting of non-*Bt* crops in a certain area of the field, thereby providing an area which allows the survival of susceptible individuals (EPA 2021).

Tang et al. (2001) demonstrated that differences in strategies of refuge use may also affect crop yield. The study found that using a 20% refuge of non-*Bt* broccoli crops resulted in less defoliation of *Bt* broccoli than using percentages of 0, 3.3, 10, and 100% when allowing DBM larva to feed. It was also observed that keeping the refuge separated from the *Bt* crops assisted in the effectiveness of the strategy. Natural enemies are also an important environmental factor with potential to affect resistance development. The natural enemies that target DBM most often are either Braconid or Ichneumonid parasitoid wasps (Capinera 2000), but other insects such as beetles may also feed on

DBM. Liu et al. (2014) demonstrated the importance of refuges and natural predators in preventing DBM *Bt* insecticide resistance. The study utilized *Bt* broccoli that expressed Cry1Ac proteins, as well as the ladybird beetle, *Coleomegilla maculata*, which acted as a natural predator. Observations of DBM fecundity showed that *Bt* broccoli without a non-*Bt* refuge or predators began experiencing *Bt* resistant DBM by the third generation and averaged 51 DBM per broccoli plant. This same group began experiencing over 100 DBM per plant after just six generations. In comparison, *Bt* plants that included a non-*Bt* refuge with no natural predators exceeded 50 DBM per plant on average after six generations. However, the use of a non-*Bt* refuge alongside *C. maculata* resulted in < 2 DBM per plant on average after six generations, suggesting that synergistic use of both non-*Bt* refuges as well as natural predators may assist in control of DBM.

Secondary plant compounds are defense mechanisms utilized to prevent damages and often target insect pests (Lucas-Barbosa et al. 2011). Glucosinolates (GS) are a secondary plant compound that are associated with brassicaceous plants (Fahey et al. 2001). Although GS may be common among brassica plants, GS levels can vary among species as well as cultivars. As previously mentioned, DBM are specialist pests of cruciferous crops. Therefore plants compounds closely associated with crucifers, such as GS, would be expected to influence DBM during host plant selection. The suggested influences of GS compounds have shown potential roles in both attracting and repelling DBM from host plants. Gols et al. (2008) demonstrated elevated GS levels in wild *Brassica oleracea* plants when compared to *B. oleracea* cultivars. The study also demonstrated that while the effects were not extreme, DBM larvae that fed on wild *B. oleracea* experienced longer development and reduced adult body mass. This may

provide insight as to why DBM tend to favor cultivated brassica crops over wild brassicas (Marchioro and Foerster 2014). Robin et al. (2017) demonstrated that brassica plant genotypes possessing the GS compounds glucobrassicin, glucoiberin and glucoiberverin repelled DBM larva, while the opposite was found for genotypes possessing 4-hydroxyglucobrassicin, glucoerucin, glucoraphanin, progoitrin and gluconapin. The results of this study suggested that certain GS compounds seem to be more associated with resistance or susceptibility to DBM, rather than total GS content or classification (Indolic or aliphatic GS) as the amount of GS did not seem to affect DBM larva. Ratzka et al. (2002) determined that DBM encode glucosinolate sulfatase enzymes which affect GS compounds. These enzymes were demonstrated to convert sinigrin, glucotropaeolin, and glucobrassicin into desulfo-glucosinolates.

Other studies have demonstrated the importance of GS compounds in DBM oviposition (Renwick and Radke 1990), and chemosensilla of the antennae, tarsi, and ovipositor have also shown importance via DBM ovipositional behaviors (Justus and Mitchell 1996). Yellow Rocket, *Barbarea vulgaris,* is a particularly interesting plant when researching DBM and their relationship with plant compounds. It is believed that GS produced by yellow rocket causes female DBM to oviposit on leaf surfaces, as GS have been shown to occur on the leaf surface of *Barbarea spp.* However saponins, another plant compound known to deter the feeding of DBM larva, were found in leaf tissues of *Barbarea spp.*, but not on the leaf surface. This suggests that GS content on the surface of yellow rocket leaves causes oviposition by females DBM, but the saponin content within the leaves causes larval death upon feeding (Badenes-Pérez et al. 2011).

Physical structures may also deter feeding from insect pests. Trichomes, also known as leaf hairs, originate from epidermal cells which act as physical deterrents to pests (Lazniewska, Macioszeck, and Kononowicz 2012). Numerous studies reviewed in Levin (1973) have shown how effective trichomes are in deterring insect pests. These hair-like structures may also act as a barrier for catching infectious microorganisms, such as fungi (Lazniewska, Macioszeck, and Kononowicz 2012). Transgenic lines of rapeseed, *Brassica napus,* engineered to express higher trichome densities have shown resistance to DBM larva (Alakahoon et al. 2016). The hairy (AtGL3+) and ultra-hairy (K-5-8) varieties were compared to their parent cultivar, referred to as Westar. Female DBM ovipostional preferences as well as larval feeding preferences were tested in this study. The results indicated that female DBM adults showed no preferences between Westar and AtGL3+ cultivars at either age of the plant (cotyledons or true leaves). However, the adult females seemed to prefer the K-5-8 second and third leaves over Westar leaves of similar age. This seemed to be the only preference shown by adult females among cultivars, as well as cultivar ages. Variations due to ovipostional preferences of female DBM on plants with high trichome densities have been noted before. Talekar et al. (1994) reported a positive relationship between oviposition and trichome density, while Handley et al. (2005) has reported a negative relationship. In regards to larval feeding, results suggested that antibiosis factors may have influenced DBM larval avoidance of the AtGL3+ line of *B. napus.* Unfortunately, no results were produced for the K-5-8 line regarding larval feeding (Alakahoon et al. 2016).

2.4 Mechanisms of insecticide resistance to different insecticides of the diamondback moth

The global outbreaks of resistant DBM have led to multiple studies of possible mechanisms of resistance, providing information on factors that could be contributors. These factors include target site mutations, enzymatic detoxification, and avoidance behavior among others (Roditakis et al. 2017, Troczka et al. 2017, Gao et al. 2018, Li et al. 2017).

Table 2.1: Selected specific changes to alleles associated with insecticide resistance in the diamondback moth, Plutella xylostella, 2016-2021

Insecticide {IRAC Group#}	Resistance trait	Reference
Chlorantraniliprole and flubendiamide {28}	Rf34 and Rh36, PxRyR associated with resistance	Qin et al. 2018
Chlorantraniliprole {28}, cypermethrin {3}, dinotefuran {4}, indoxacarb {22} and spinosad {5}	Commonly responding over-transcribed genes were two cytochrome P450 genes (Cyp301a1 and Cyp9e2) and nine cuticular protein genes.	Gao et al. 2018
Chlorantraniliprole and flubendiamide {28}	Resistance associated with a point mutation (G4946E) in the RyR gene	Kang et al. 2017 & Yan et al. 2014
Mevinphos {1}	Mutation of the *Pxace1* gene	Lin et al. 2017
Organophosphate and carbamate {1}	Resistance associated with G227A point mutation	Guo et al. 2017
Chlorantraniliprole {28}	Over expression of UGT2B17 enzymes	Li et al. 2017
Chlorpypifos {1}	2 CarE cDNAs (Pxae18 and Pxae28) associated with resistance	Xie et al. 2017
Spinosad {5}	A three amino acid deletion of the nicotinic acetylcholine receptor	Wang et al. 2016
Benzoylureas/novaluron {15}	A point mutation (I1042M) in the chitin synthase 1 (CHS1) gene associated with resistance	Douris et al. 2016
Across multiple resistant genotypes	ATP-binding cassette transporter genes	Qi et al. 2016
Abamectin {6}	Over expressed cytochrome P450 gene CYP340W1 associated with resistance	Gao et al. 2016
Indoxacarb and metaflumizone {22}	Point mutations (F1845Y and V1848I) in the sixth segment of domain IV of the PxNav protein associated with resistance	Wang et al. 2016c

Table 1 provides some of the more recent studies involving genetic insecticide resistance in DBM. The recently developed diamide insecticides were effective against lepidopteran pests including DBM, however recent control failures in the field, as well as increased LC_{50} values have raised much concern. The ryanodine receptor (RyR) is the target site of diamide insecticides. The RyR is a calcium channel located within the sarcoplasmic reticulum (SR) membrane that regulates the flow of calcium ions (Ca^{2+}) from internal stores within the SR (Lahm et al. 2007, Ebbinghaus-Kintscher et al. 2006). Diamides target the RyR via modulation of its structure, causing it to remain in the open conformation (Teixeira and Andaloro 2013). This leads to an influx of Ca^{2+} which results in feeding cessation, paralysis, and eventually leads to death (Lahm et al. 2007).

Cross-resistance of diamides is also a concern for resistant DBM populations. A DBM population from Thailand reportedly exhibited cross-resistance to both chlorantraniliprole and flubendiamide shortly after their release in the country (Troczka et al. 2017). Qi and Casida (2013) demonstrated species differences in specific binding sites of diamides in *Musca domestica*, *Apis mellifera*, *Heliothis virescens,* and *Agrotis ipsilon* via radioligand binding studies. Interestingly, differences were noticed when comparing *M. domestica* and *A. mellifera* to *H. virescens* and *A. ipsilon.* Binding of [^{3}H] chlorantraniliprole was shown to be stimulated by ryanodine and flubendiamide in *M. domestica* and *A. mellifera.* The opposite was seen in *H. virescens* and *A. ipsilon*, with [^{3}H] chlorantraniliprole inhibition occurring in the presence of flubendiamide, cyantraniliprole, and chlorantraniliprole. [^{3}H] Flubendiamide binding in *H. virescens* and *A. ipsilon* was also found to be inhibited by ryanodine, chlorantraniliprole, cyantraniliprole, and flubendiamide. Lobster and rabbit RyR were also shown to bind

ryanodine, but bound neither chlorantraniliprole nor flubendiamide. The lack of insecticide binding to the rabbit RyR may be explained by the little homology between the three mammal RyR isoforms and the one insect RyR isoform (Puente et al. 2000). These results may suggest that lobster and rabbit RyR lack chlorantraniliprole and flubendiamide binding sites, *M. domestica* and *A. mellifera* RyR have two distinct binding sites for chlorantraniliprole and flubendiamide, and *H. virescens* and *A. ipsilon* RyR have two distinct sites for ryanodine and chlorantraniliprole/flubendiamide (Qi and Casida 2013).

2.5 Target site mutations of the diamondback moth associated with insecticide resistance

Four target site mutations of the DBM RyR have been discovered in Asian DBM populations and are associated with diamide resistance (Troczka et al. 2012, Guo et al. 2014). All four target site mutations are point mutations, which result when a single base pair within a codon is exchanged (Troczka et al. 2017, Guo et al. 2014). The G4946E mutation of the DBM RyR, discovered by Troczka et al. (2012), is perhaps the most widespread of the known target site mutations. This occurs when the GGG codon that results in a glycine (G) at the 4,946th position of the amino acid chain is exchanged for a GAG codon that leads to a glutamate (E) in that position instead. Troczka et al. (2015) cloned DBM RyR and expressed them in fall armyworm, *Spodoptera frugiperda,* cell lines. The cloned receptors contained either the GGG codon (wt) or the GAG codon (mt). Exposure to an EC_{50} of 17nM± 2 nM of chlorantraniliprole resulted in increased Ca^{2+} concentrations for the wt RyR, while exposure to an EC_{50} of 3,715 nM ± 776 nM was

needed to affect the mt RyR. This suggests that the G4946E mutation contributes to some degree of chlorantraniliprole resistance in the DBM RyR.

The possibility of the independent evolution of the G4946E in separate DBM populations was suggested by Troczka et al. (2012) after observing the G4946E in two populations from Thailand and the Philippines. Since this discovery, Steinbach et al. (2015) revealed the presence of the G4946E in 11 different countries, including the U.S. The percentages of heterozygous and homozygous alleles of these mutations from these populations were also determined. 100% homozygous resistant alleles were discovered in populations from Mississippi (U.S.) and Vietnam, while 90%, 88%, 85%, and 70% homozygous resistant alleles were discovered in DBM populations from Thailand, Japan, the Philippines, and Korea, respectively (Steinbach et al. 2015). Itagaki and Sonoda (2017) observed changes in the proportion of the G4946E mutation in Japanese populations during different seasons. The results indicate that the proportion of DBM with the G4946E mutation increases during the spring and summer and decreases during the fall. The study suggests these fluctuations may occur due to selection with diamides during growing seasons, however migration may also play a role in these fluctuations. There has been documentation of long distance migration of DBM from mainland Europe to the United Kingdom (Chapman et al. 2002) and China (Wei et al. 2013), however long distance migrations of DBM in the U.S.A. has not yet been documented.

Three additional DBM RyR point mutations associated with diamide resistance have only been identified in Asian DBM populations as of now. These include the E1338D, Q4594L, and I4790M mutations, all three of which were discovered in a diamide resistant DBM population of the Yunnan province of China (Guo et al. 2014).

The E1338D mutation occurs when the GAA codon that results in a glutamate (E) at the 1,338th position of the amino acid chain is exchanged for a GAT codon that leads to an aspartic acid (D) in that position. The E1338D mutation is located near the N-terminus of the RyR which is not thought to be a functional region of the receptor, however Guo et al. (2014) claims this area requires a certain structural integrity for the success of diamide-induced RyR activation. The Q4594L mutation occurs when the CAG codon that results in a glutamine (Q) at the 4,594th position of the amino acid chain is exchanged for a CTG codon that leads to a leucine (L). The Q4594L mutation is located near a diamide sensitive area in the loop between transmembrane domains 1 and 2, which suggests its potential role in diamide resistance (Guo et al. 2014). Finally, the I4790M mutation occurs when the ATA codon that results in an isoleucine (I) at the 4,790th position of the amino acid chain is exchanged for an ATG codon that leads to a methionine (M). Lin et al. (2020) revealed that the I4790M mutation occurs in transmembrane helix S2, which is in the same area of the receptor as the G4946E mutation. Since the distance between the G4946E and I4790M is only 15 Å, Lin et al. (2020) suggests that the I4790M mutation may also play a crucial role in the disruption of diamide insecticide binding. The results of Guo et al. (2014) suggest that the combination of these three mutations, as well as the G4946E mutation, is a contributing factor to chlorantraniliprole resistance in DBM populations from China. However, after mapping all four mutations on structural models, Lin et al. (2020) suggests that the E1338D and Q4594L mutations may be less likely to cause structural changes of the RyR.

More recently, another mutation at the same point of the I4790M mutation was discovered in Japanese DBM populations. The KA17 strain which possessed the I4790K

mutation exhibited extremely high levels of resistance to chlorantraniliprole (> 20,833-fold), flubendiamide (> 38,461-fold), and cyantraniliprole (66,200-fold). In comparison, the KU13 strain which possessed the G4946E mutation exhibited extremely high levels of resistance to chlorantraniliprole (20,833-fold) and flubendiamide (>38,461-fold), while showing intermediate resistance to cyantraniliprole (678-fold) (Jouraku et al. 2020). Wang et al. (2020) utilized CRISPR/Cas9 system to generate a new resistant DBM strain (I4790M-KI) via knock-in of the I4790M mutation from a susceptible lab strain (IPP-S). However, dose response assays comparing the two strains determined resistance ratios much lower than that of Jouraku et al. (2020). The resistance ratios were 40.5, 6.0, 7.7, 0.97, and 1.33 in response to flubendiamide, chlorantraniliprole, cyantraniliprole, indoxacarb, and beta-cypermethrin, respectively. Fukada et al. (2020) confirmed the presence of the I4790K mutation in DBM from two separate populations in Japan during 2017 and 2018, however no DBM from either site were shown to possess the I4790K mutation in 2019.

Other insects, such as the tomato leaf miner (TLM), *Tuta absoluta*, have been studied via genotyping and point mutations comparable to that of DBM have been discovered. The TLM RyR mutations were referred to as the G4903E and I4746M mutations, which correspond to the DBM RyR mutations G4946E and I4790M respectively (Roditakis et al. 2017). These two RyR mutations are thought to contribute to TLM diamide resistance. The beet armyworm, *Spodoptera exugia*, is another lepidopteran pest with similar RyR mutations. The I4743M and G4900E were discovered in the Shandong province of China and were demonstrated to confer comparable levels of

diamide resistance to the mutations of both *T. absoluta* and *P. xylostella* (Zuo et al. 2020).

A mutation of the DBM nicotinic acetylcholine receptor (nAChR) which may influence insecticide resistance has recently been discovered. This three amino acid deletion of the nAChR does not affect diamide efficacy, but is associated with spinosyn resistance in DBM (Wang et al. 2016). Target site mutations conferring resistance to organophosphate and carbamate insecticides have also been documented for DBM (Guo et al. 2017). These insecticides target acetylcholinesterase (AChE) enzymes that catalyze the degradation of acetylcholine. Guo et al. (2017) discovered a G227A within the AChE gene thought to confer resistance to both organophosphates and carbamates. Analysis of RNA-Seq data suggested that the G227A mutation was positively associated with resistance to both insecticides. Benzoylureas (BPU), which inhibit chitin biosynthesis, have also experienced some trouble with DBM resistance (Douris et al. 2016). An I1042M mutation discovered in the *CHS1* gene of BPU resistant DBM is thought to be associated with resistance to these insecticides. This mutation was found at relatively high frequencies in field resistant populations located in cabbage fields from China and India (Douris et al. 2016).

2.6 Metabolic detoxification associated with insecticide resistance

Another mechanism of insecticide resistance is the upregulation of detoxification enzymes, such as cytochrome P450 monooxygenase (P450), Uridine diphosphate-glucuronosyltransferases (UGT), and glutathione-S-transferases (GSTs) (Liu et al. 2018, Li et al. 2017, Gao et al. 2018). Detoxification of foreign compounds via enzyme

upregulation relies on systems of enzymes working together for detoxification. These systems can be split into Phase I, Phase II, and Phase III for better understanding.

P450's are a large superfamily of enzymes that have been found across multiple insect species (Feyereisen 1999). P450's are well known for their general roles in insecticide detoxification (Feyereisen 2015), however the functions of these enzymes include more than protection from toxic substances. The involvement of P450's in the biosynthetic pathways of both juvenile hormone and ecdysteroid suggests their importance in the growth and development of insects (Feyereisen 1999). Regardless, their function in insecticide resistance across multiple insect species makes them all the more important to IRM.

P450 enzymes are associated with Phase I detoxification (Danielson 2002, Hodges and Minich 2015). Phase I detoxification refers to the addition of reactive groups, such as hydroxyls or carboxyls, to the molecule in need of excretion. These groups are added via reduction, oxidation, or hydrolysis reactions (Danielson 2002). More recently, a study of P450's in *Anopheles gambiae* revealed their role in cuticular hydrocarbon (CHC) production. The results of this study showed the *CYP4G16* P450 found in oenocytes of pyrethroid resistant *A. gambiae* produced CHC. Oenocytes are a cell thought to produce and release hydrocarbons, and therefore are important in regards to insect cuticles. Interestingly, the pyrethroid resistant *A. gambiae* had a thicker epicuticular layer, as well as a higher CHC content in comparison with a susceptible strain. This suggests that *CYP4G16* production of hydrocarbons may play a role in resistance to pyrethroids in *A. gambiae* via cuticular thickening (Balabanidou et al. 2016).

The role of P450's in hydrocarbon production as a mechanism of insecticide resistance further demonstrates the importance of this enzyme family in IRM.

Multiple studies have demonstrated the upregulation of P450's in DBM after insecticidal exposure. Significant levels of upregulation occurred for eight P450 families in response to sub-lethal doses of cypermethrin within a resistant DBM population. This was compared to the upregulation of one P450 family in an insecticide-susceptible DBM population (Baek, Clark, and Lee 2010). Gao et al. (2018) observed the upregulation of two P450 enzymes in DBM in response to exposure to five different insecticides. The two genes, known as *Cyp301a1* and *Cyp9e2*, were observed to be upregulated in response to chlorantraniliprole, cypermethrin, dinotefuran, indoxacarb, and spinosad. Gao et al. (2018) speculated that these two P450's may be generalist enzymes that are expressed in response to a broad spectrum of xenobiotics. Other insects have also shown differences in P450 expression in comparisons of susceptible and resistant strains. A deltamethrin-resistant strain of *Tribolium castaneum* was shown to upregulate the *CYP6BQ9* gene more than 200-fold than a susceptible lab strain (Zhu et al. 2010). Further study in which transgenic *D. melanogaster* were transformed to express the *CYP6BQ9* gene showed the transformed *D. melanogaster* exhibited higher survivorship upon exposure to deltamethrin in comparison to non-transformed *D. melanogaster* (Zhu et al. 2010). This suggests the role of the *CYP6BQ9* gene in resistance to deltamethrin.

While no current insecticide MoA targets P450's, metabolic detoxification via P450's can potentially threaten the efficacy of any insecticide class (Feyereisen 2014). The implementation of synergists, such as piperonyl butoxide (PBO), has assisted insecticidal efficacy by inhibiting P450 detoxification. Although PBO is perhaps the most

common P450 synergist, other synergists may need to be implemented for certain P450's since PBO does not inhibit all P450's equally (Feyereisen 2014). The differences in PBO interactions between different P450 enzymes demonstrates the need for identification of P450's heavily involved in insecticidal detoxification.

Phase II enzymes, also known as conjugation enzymes, function by adding hydrophilic compounds to foreign molecules. The addition of groups like glucuronic acid and glutathione make the molecule more water-soluble and can lead to faster excretion via urine and bile in humans. These hydrophilic groups are often added via specific enzymes with glucuronic acid being added via UGT enzymes and glutathione being added via GST enzymes (Xu, Yong-Tao, and Kong 2005). Phase II enzymes have also been studied for their upregulation in DBM following insecticidal exposure. Li et al. (2017) observed UGT expression in DBM after exposure to multiple insecticides. The expression of these UGT genes, referred to as *UGT40V1, UGA33AA4,* and *UGT45B1* were all induced in a resistant DBM population, suggesting a possible role in insecticide resistance. A recent study also demonstrated that *UGT33AA4* may be closely linked chlorantraniliprole resistance in some DBM populations (Li et al. 2017, Li et al. 2018). GST enzymes are also known for increasing water solubility of certain compounds to assist with excretion (Habig, Pabst, and Jakoby 1974). Differences in midgut expression levels of GST enzymes between insecticide-resistant and susceptible DBM strains have been observed (Huang et al. 1998), and resistance to several insecticides associated with GST's has been recorded in different insects (Enayati et al. 2005).

Carboxylesterase (CarEs) enzymes are often involved in the detoxification of environmental xenobiotics in insects (Xie et al. 2017). Xie et al (2017) revealed two

CarE genes in DBM, Pxae18 and Pxae28, which were highly expressed upon low dose chlorpyrifos exposure. Both *Pxae18* and *Pxae28* belonged to the α-esterase class of CarEs, which are associated with the detoxification of xenobiotics and insecticides. Xie et al. (2017) also demonstrated RNAi mediated knockdown of both *Pxae18* and *Pxae28* resulted in increased mortality of DBM larva upon exposure to chlorpyrifos. Some Phase I and Phase II enzyme inducers have been shown to share mechanisms of transcriptional activation, which seems to suggest regulatory coordination (Xu, Yong-Tao, and Kong 2005).

Phase III is associated with transporters of Phase II metabolites for excretion. Transporters such as ATP binding cassette (ABC) transporters can act as importers or exporters of substrates, however eukaryotic organisms lack importers (Xu, Yong-Tao, and Kong 2005). This means the function of ABC transporters is restricted to exporting substrates in DBM. ATP-binding cassette (ABC) transporter genes encode important transmembrane proteins that transport compounds across intra and extra cellular membranes (Qi et al. 2016). *Bt* toxin resistance is associated with the ABC transporter *ABCC2* in some lepidopteran pests (Baxter et al. 2011), and may also be found in combination with two other ABC genes, *ABCC3* or *ABCG1* (Guo et al. 2015a, Guo et al. 2015b). In addition, RNAi-mediated knockdown of the DBM gene, *ABCH1*, resulted in increased larval and pupal mortality after exposure to the Cry1Ac toxin (Guo et al. 2015c).

2.7 Other mechanisms of insecticide resistance of the diamondback moth

MicroRNAs (miRNA) are short, non-coding RNAs that modulate bodily processes via post transcriptional regulation (Ambros 2001). This can be done either by

inhibiting messenger RNA (mRNA) or through the degradation of mRNA (Carthew and Sontheimer 2009). Li et al. (2015) not only determined that the upregulation of RyR mRNA was associated with DBM chlorantraniliprole resistance, but that two miRNAs, miR-7a and miR8519, regulate this expression. RNAi mediated knockdown of the RyR gene was also shown to decrease tolerances to chlorantraniliprole in DBM larva. However, a study of RNAi repression of the RyR gene in the whitebacked plant hopper, *Sogatella frucifera*, resulted in decreased mortality after chlorantraniliprole exposure (Yang et al. 2014).

The diversity and success of insects can be partially explained by their interactions with beneficial microorganisms (Engel and Moran 2013b). These microorganisms can assist insects via digestion of food and protection from pathogens and parasites just to name a few functions. Microorganisms of the insect gut have been shown to mediate different lifestyles, depending on the type of host in which these microorganisms live (Engel and Moran 2013b). Termites of the genus *Naustitermes* rely on gut symbionts for the degradation of cellulose (Warnecke et al. 2007). In the honey bee, *Apis mellifera*, pectin degradation as well as B12 biosynthesis occurs due to gut bacteria (Engel et al. 2012, Engel and Moran 2013a). Most interestingly in the case of DBM, a gut symbiont of the bean bug, *Riptortus pedestris*, known as *Burkholderia* has been shown to degrade the insecticide fenitrothion (Kikuchi et al. 2012).

The insect gut is an important location of interaction between the target insect and implemented insecticide. Tang et al. (1997) studied Florida DBM populations which developed resistance to *Bt* toxins. The colony known as Loxa A, established from a field-resistant population in Loxahatchee, Florida, exhibited high levels of feeding tolerance to

the insecticidal crystal proteins Cry1A(a), Cry1A(b), and Cry1A(c). This resistance is thought to be associated with decreased binding of the toxic proteins to the receptor located in the midgut. Sayyed et al. (2004) had similar findings when exposing DBM to *Bt* toxins and performing binding assays. These assays revealed that Cry1A(b) and Cry1A(c) toxins experienced decreased binding to midgut membrane receptors in comparison to other Cry toxins.

Previous studies have explored the contents of the DBM gut microbiome in order to find symbionts that may play a role in insecticide detoxification. Through the use of metagenomics, Xia et al. (2017) was able to more completely study the DBM gut microbiome. It was discovered that DBM gut symbionts produce the amino acids threonine and histidine, which DBM cannot synthesize on their own. It was also discovered that DBM gut symbionts assist in the detoxification of host plant volatiles (HPV) found within brassica crops. Xia et al. (2017) reports an aerobic pathway leading to the degradation of catechol, another brassica crop HPV. The metagenomic analysis of DBM gut symbionts lead to the discovery of multiple genes, including catechol 1,2-dioxygenase, muconate cycloisomerase, muconolactone D-isomerase, and 3-oxoadipate enol-lactonase. These genes are expressed by *Enterobacter asburiae* and *Enterobacter cloacae*, which are thought to provide protection against plant defense compounds. It was also observed that gut symbionts assisted in the detoxification of phenolic compounds and reactive oxidative species (ROS).

Avoidance behavior is another interesting aspect of DBM insecticide resistance. Nansen et al. (2016) demonstrated behavioral avoidance of spinetoram and gamma-cyhalothrin in both larval and adult DBM. The two resistant strains used in this

experiment were referred to as the single resistant strain (SRS) and the double resistant strain (DRS). These strains exhibited resistance to gamma-cyhalothrin or spinetoram and gamma-cyhalothrin, respectively. Female adults of both resistant strains exhibited selective oviposition, opting to ovisposit on untreated leaves rather than leaves treated with gamma-cyhalothrin and spinetoram. Larva of the SRS exhibited significantly faster movement when placed on leaves treated with either insecticide as compared to the DRS. It was also noted that in treatments where only 50% of the leaves were treated with insecticide, that SRS larva would avoid treated leaves. This suggests that lower resistance levels in the SRS may lead to stronger selection pressures, leading to the development of behavioral avoidance of insecticidal compounds. DBM have also exhibited a behavior known as leg autotomy (Moore, Tabashnik, and Stark 1989). This behavior refers to metathoracic leg dropping in response to tarsal contact with insecticides. Moore, Tabashnik, and Stark (1989) studied adult DBM leg autotomy in response to tarsal exposure to fenvalerate. By implementing ^{14}C-labeled fenvalerate, it was observed that leg dropping resulted in significantly lower presences of insecticide in DBM that dropped legs compared to those that did not. It was also observed that autotomized legs contained more than 10 times the concentration of insecticide than the body of the adult, demonstrating the effectiveness of this resistance behavior.

Although it is known that there are multiple mechanisms of insecticide resistance, it is not known which resistant populations possess these mechanisms in the southeastern U.S.A. Since these resistance factors vary from population to population, it is important to understand what factors occur in specific locations. Identification of resistance mechanisms in resistant field populations could assist IRM programs by providing

specific actionable information for mitigating the development of insecticide resistance in DBM populations. Currently, neither target site mutations nor the upregulation of enzymes in DBM have been studied for populations found in the states of Georgia or Florida, hence the need for this research.

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CHAPTER 3: A TARGET SITE MUTATION ASSOCIATED WITH DIAMIDE INSECTICIDE RESISTANCE IN THE DIAMONDBACK MOTH (LEPIDOPTERA: PLUTELLIDAE) IS WIDESPREAD IN SOUTH GEORGIA AND FLORIDA POPULATIONS

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Abstract: Diamondback moth (DBM) larvae were collected from four sites in Georgia and Florida where diamide, specifically chlorantraniliprole, insecticide resistant populations were recently documented, and used to establish laboratory colonies. Dose/response experiments established these colonies exhibited 109- to 4,298-fold resistance to chlorantraniliprole, compared to a commercially available susceptible control colony. Similar dose/response experiments established these colonies exhibited 50- to 107-fold resistance to cyantraniliprole compared to a commercially available susceptible control. All colonies were screened for the presence of four known mutations in the ryanodine receptor (RyR), the target of diamide insecticides, previously associated with resistance in DBM populations from Asia. One mutation, G4946E, which is believed to confer resistance to chlorantraniliprole, was identified in colonies from all four sites. Three additional RyR target site mutations, E1338D, Q4594L, and I4790M, were not identified in any of the screened samples. The allele frequency of the G4946E mutation in these samples ranged from 32% to 90%. These data are consistent with recently reported chlorantraniliprole control failures in Georgia and Florida. It is likely that the G4946E mutation is currently a very important contributing factor to chlorantraniliprole resistance in Georgia and Florida DBM populations.

Key Words: *Plutella xylostella*, insecticide resistance management (IRM), chlorantraniliprole, cyantraniliprole, target site, mutation, G4946E

3.1 Introduction

The Diamondback Moth (DBM), *Plutella xylostella*, is a major pest of cruciferous crops worldwide (Furlong et al. 2013, Talekar and Shelton 1993). Annual worldwide damage estimates due to DBM outbreaks range from US $4-5 billion (Zalucki et al. 2012); thus effective insecticide resistance management (IRM) strategies are critical to mitigate these damages. Documentation of insecticide resistance has shown that at least 95 different insecticides have experienced control failure when used against DBM (APRD 2020). Multiple mechanisms of insecticide resistance have been discovered in DBM, ranging from avoidance behaviors to metabolic detoxification of insecticidal compounds (Moore et al. 1989, Nansen 2016, Gao et al. 2018). Target site mutations, which reduce binding by the insecticide molecule to its molecular target, have recently been shown to adversely affect insecticide control in DBM as well (Richardson et al. 2020).

Diamides (IRAC Group 28) are class of insecticides which target the ryanodine receptor (RyR). The RyR is a calcium channel located within the sarcoplasmic reticulum (SR) membrane that regulates the flow of calcium ions (Ca^{2+}) from internal stores within the SR resulting in muscle contraction (Lahm et al. 2007, Ebbinghaus-Kintscher et al. 2006). Diamide insecticides target the RyR via modulation of its structure, causing it to remain in the open conformation (Teixeira and Andaloro 2013). This leads to an influx of Ca^{2+} which results in feeding cessation, paralysis, and eventually death (Lahm et al. 2007). The phthalic acid diamide flubendiamide, released in 2006, was the first diamide insecticide to be developed (Lahm et al. 2009). By 2008, both flubendiamide and the newly developed anthranilic diamide chlorantraniliprole (trade name Coragen®) were

registered for use in multiple countries (Lahm et al. 2009). Another anthranilic diamide, cyantraniliprole (trade name Exirel®), shares chemical similarities to chlorantraniliprole, while providing control to a wider range of insect pests (Troczka et al. 2017). However, products utilizing cyantraniliprole were not registered for use until 2012 (Troczka et al. 2017).

In 2009, DBM populations in the Bang Bua Thong district of Thailand began showing signs of flubendiamide resistance, as well as chlorantraniliprole resistance. This occurred just 18 months after the release of flubendiamide in Thailand in 2007 (Troczka et al. 2017). Wang and Wu (2012) reported the development of chlorantraniliprole resistance in DBM populations from China that were collected from 2010-2011. Reports of diamide resistant DBM populations in Taiwan, Brazil, and the United States also occurred from 2011 to 2013 (IRAC Newsletter 33). Richardson et al. (2020) provided the most comprehensive review of diamide resistance in multiple insects to date. While diamide insecticides were initially effective against lepidopteran pests (Lahm et al. 2007), constant use without IRM has likely led to a decline in their efficacy (Teixeira and Andaloro 2013).

Studies of the DBM RyR have revealed four target site mutations associated with diamide insecticide resistance. The first and most prevalent mutation is G4946E, which was discovered by Troczka et al. (2012). Association of the G4946E mutation with insecticide resistance was confirmed by Troczka et al. (2015) when the DBM RyR with the G4946E mutation was cloned and expressed in *Spodoptera frugiperda* (Sf9) cell lines. Exposure to an EC_{50} of 17nM ± 2 nM of chlorantraniliprole resulted in a 50% increase in Ca^{2+} mediated intracellular fluorescence for the wild type (wt) RyR, while

exposure to an EC_{50} of 3,715 nM ± 776 nM was needed to similarly affect the mutant (mt) RyR. Steinbach et al. (2015) documented the presence of the G4946E mutation in 10 different countries, including Japan, India, and one population in the US.

Additional target site mutations, discovered by Guo et al. (2014) in a highly (2,128-fold) chlorantraniliprole-resistant DBM population from Yunnan, include the E1338D, Q4594L, and I4790M mutations. All three mutations were present at a frequency of 100%, compared to only 20% for the G4946E mutation in the same population. Further, ligand binding assays showed markedly reduced chlorantraniliprole binding to the RyR containing multiple mutations from this resistant population. Lin et al. (2020) modeled the DBM RyR and found I4790M was located in transmembrane helix S2, only 15 Å distant across a cavity from where G4946E faces from near the end of transmembrane helix S4 and the beginning of the physiologically important S4-S5 linker. This proximity suggests that the cavity includes the diamide binding pocket. The I4790M mutation makes the cavity shallower, while the G4946E mutation narrows the opening and makes the surface charge of the pocket more negative. Altogether this suggests that I4790M and G4946E may interact to reduce diamide binding to the RyR. Wang et al. (2020) used CRISPR/Cas9 to produce a knock-in DBM strain with only the I4790M mutation and measured 6.0-fold resistance to chlorantraniliprole, 7.7-fold resistance to cyantraniliprole, and 40.5-fold resistance to flubendiamide, confirming the participation of this mutation in diamide resistance and providing evidence for a differential effect on the efficacy of different diamide insecticides. In contrast, E1338D and Q4594L are more distant and located in flexible regions of the receptor, and are less likely to result in functional changes to the RyR (Lin et al. 2020).

The documentation of specific resistance mechanisms in insect populations is an important component for developing IRM strategies. Knowledge of specific resistance mechanisms in DBM populations as well as relative susceptible wild type and resistant mutant allele frequencies could better inform insecticide rotation strategies for managing DBM (Riley 2014). The G4946E mutation was first found in the USA in a single DBM population from Mississippi (Steinbach et al. 2015), but has not been formally reported in the major Cole crop production areas of Georgia and Florida. Recently, diamide insecticide resistance has become a serious obstacle to managing DBM in Cole crops in both of these states (Riley et al. 2020). The goal of this study was to quantify the level of resistance for the anthranilic diamides chlorantraniliprole and cyantraniliprole in colonies founded from resistant DBM populations in Georgia and Florida, and assess the prevalence of the known target site mutations specifically conferring diamide insecticide resistance. Our hypothesis was that mutations associated with chlorantraniliprole resistance elsewhere would also occur in chlorantraniliprole and cyantraniliprole-resistant populations of DBM in the sample areas in Georgia and Florida.

3.2 Materials and Methods

Plutella xylostella were collected from resistant field populations throughout South Georgia and Florida in 2018 as part of a previous study (Riley et al. 2020), and used to establish laboratory colonies. Larva and pupa were collected from Tift County (designated the LTF colony), Colquitt County (NP colony), and Crisp County (CSP colony) in Georgia, as well as a population from Manatee County, Florida (MAN colony). A susceptible strain, commercially available from Frontier Genomics (FT colony), was used as a control. Lab colonies were established at the Coastal Plains

Experiment Station located in Tifton, Georgia. These colonies were reared on mustard (*Brassica juncea*) and collard (*Brassica oleracea var. acephala*) plants, grown in Percival growth chambers with a temperature of 26.0 ± 1 °C and a day and night cycle of 12:12. Adult DBM were given 10% honey solution via soaked cotton balls placed in 37.0 mL SOLO cups. DBM colony cages were placed under lights on a 10:14 day and night cycle, and the room temperature was held constant at 68 °F.

Toxicological Bioassays. In order to assess the level of resistance to chlorantraniliprole and cyantraniliprole, LC_{50} values were determined for each colony (LTF, MAN, NP, and CSP, with FT acting as the susceptible control population). Leaf-dip bioassays were used to expose DBM larva to varying concentrations of chlorantraniliprole (Table 3.1) or cyantraniliprole (Table 3.2). Bioassays were set up in petri dishes with 38 mm (1-1/2 inch) diameter holes cut into the lids for ventilation. The hole was covered with hot-glued nylon chiffon to ensure the larva could not escape. Circular Whatman filter papers were placed into the bottom of the petri dish and sprayed with water until damp to prevent leaf discs from drying out. A 70 mm (2-3/4 inch) diameter circular leaf disc was cut out from a fully-grown collard (*Brassica oleracea var. acephala*) leaf and dipped into a 0.25 L aqueous solution consisting of either chlorantraniliprole or cyantraniliprole at the concentration of interest (Tables 3.1 and 3.2), with 0.25 mL Kinetic adjuvant to ensure even spreading and adherence. The treated leaf disc was placed into the petri dish on top of the damp filter paper and allowed to air dry for 30 minutes. Once dried, 10 3rd instar larvae were placed on the leaf and the top of the petri dish was secured with rubber bands to prevent escape. Each replicate was checked every 24 hours for live, moribund, pupated, and dead larvae until 72 hours had

passed. Data was analyzed in SAS Enterprise Guide 7.1 (SAS Institute, Raleigh, NC) via Proc Probit analysis to determine LC_{50} values. SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA) was also used to generate dose response curves for comparisons of different colony resistance levels for both chlorantraniliprole and cyantraniliprole.

Genetic analysis. Each colony was analyzed for the presence of the four known diamide-related target site mutations. Three to four independent cDNA replicates were synthesized and analyzed for each colony. For each replicate, five late 3rd instar DBM larvae were stored in 1.0 mL of RNAlater™ (Thermo Fisher Scientific, Waltham, MA) for transportation to Athens, Georgia and subsequently stored at -80 °C until they were processed for mRNA extraction. Initially, DBM mRNA was extracted using TRIzol™ (Ambion, Thermo Fisher Scientific, Waltham, MA) following the manufacturer's protocol. Subsequently, the use of a Dynabeads™ mRNA DIRECT™ Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA) proved to be more successful for mRNA extraction in this study. cDNA was synthesized with SuperScript IV VILO MasterMix™ (Invitrogen) following the manufacturer's protocol and stored in a -20 °C freezer until needed for polymerase chain reactions (PCR).

A BIO-RAD C1000 Touch™ (Life Science, Hercules, California) thermal cycler was used to amplify cDNA samples. PCR samples were amplified using high-fidelity Phusion Taq polymerase and 5X HF Reaction Buffer while following the manufacturer's protocol. Primers to generate amplicons spanning the four mutation sites of the DBM RyR were designed using Primer3Plus (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (Table 3.3), and indicated primer combinations, extension times, and annealing temperatures were used for each mutation (Table 3.4).

PCR reactions were analyzed in a 1.5% agarose gel to ensure a single product of the expected size had amplified. Purification of PCR products was accomplished via Monarch® PCR and DNA Cleanup kit (Promega, Madison, WI). The purified PCR products were sent to Eurofins Genomics in Louisville, Kentucky for Sanger sequencing.

Sequencing chromatograms and ab1 files (read using FinchTV 1.4.0 (Geospiza, Inc., Seattle, WA)) were inspected for evidence of each of the RyR mutations. Mixtures of wild-type and mutant haplotypes in a sample were indicated by the presence of superimposed peaks at the position of the mutation, with the relative height of the peaks indicating the approximate proportions of each allele in the sample (Figure 3.3A). In that event, the relative frequency of the two alleles was ascertained by cloning and sequencing a minimum of ten clones from each of three independent replicates of cDNA samples from each colony, as described above. To generate clones for sequencing, purified PCR products produced as described above were cloned using a CloneJET PCR Cloning Kit (Thermo Fisher Scientific, Waltham, MA) according to the product protocol. Following transformation in NEB turbo competent *E. coli,* and overnight growth on LB/ampicillin plates, colonies were selected and grown overnight at 37 ^{o}C in 5 ml of LB/ampicillin. Plasmids were extracted via GeneJet Plasmid Miniprep Kit (ThermoScientific) following the manufacturer's protocol, quantified by Nanodrop, and Sanger sequenced (Eurofins Genomics, Louisville, Kentucky. The sequences inspected and identified as encoding either the susceptible wild type codon (GGG) or the resistant mutant codon (GAG). Sigma Plot 11.0 was used to run a One-Way ANOVA for determination of significant differences among group means. The Holm-Sidak method was used for Pairwise Multiple

Comparisons of each group. The One-Way ANOVA also provided means, standard deviations, and critical values (CV) for data analysis.

3.3 Results

Toxicological Bioassays. Comparisons of LC_{50}-values for each DBM colony with the susceptible FT colony revealed particularly high levels of chlorantraniliprole resistance in the NP, MAN, and LTF colonies. These resistance ratios were determined to be 4,298-fold, 2,845-fold, and 2,813-fold, respectively (Table 3.5). On the other hand, the CSP colony had an intermediate resistance ratio of 109-fold when compared to the other colonies. Cyantraniliprole LC_{50}-values were also determined for the NP, MAN, and CSP colonies, and the susceptible FT control colony. The cyantraniliprole resistance ratios were determined to be 67-fold, 108-fold, and 50-fold, respectively (Table 3.5). Figures 3.1 and 3.2 illustrate dose response curves generated for chlorantraniliprole and cyantraniliprole respectively against DBM colonies. The CSP slope of the response curve for chlorantraniliprole appeared flatter suggesting a more heterogenous population (Fig. 3.1).

Genetic analysis. The presence of mixtures of alleles could be seen as superimposed peaks in sequencing chromatograms (Ab1 files). This was illustrated with four different proportions of the E codon (GAG) and the G codon (GGG), produced by sequencing known mixtures of clones of susceptible and resistant alleles at the site of the G4946E mutation in the RyR (Figure 3.3A). When the four colonies were screened by sequencing PCR-generated amplicons covering the sites of the four known resistance-associated mutations in the RyR gene, only the G4946E mutation was identified (Fig. 3.3B). The experiment was replicated with three or four independently generated cDNA

samples (5 larvae/replicate) for each colony and comparable results were observed; two representative replicates are shown in Figure 3.3. Relative heights of the G or A peak indicate that the mutant (resistant) GAG codon was dominant in the MAN and NP colony samples, with little representation of the susceptible wild-type GGG codon. For the LTF colony three of four replicates showed the resistant allele to be most abundant, and one had the wild type allele predominating with the resistant allele present at a lower frequency. All replicates of the CSP colony showed a mixture of codons, with the wild-type GGG somewhat more abundant. As expected, the susceptible control FT colony only yielded the wild-type GGG codon. In contrast, the three additional RyR target site mutations, E1338D, Q4594L, and I4790M (Guo et al. 2014), were not identified in any of the screened samples, which showed only the susceptible wild-type sequence (Fig. 3.3C-E).

Since sequencing results of PCR products suggested the G4946E mutation was present in each colony population, relative frequencies of resistance alleles were estimated by cloning, and sequencing 10 clones from each of three cDNA samples from each colony. The mean allele frequencies (with standard deviation and standard error in parenthesis) of GAG alleles in the colonies were as follows: LTF, NP, MAN, and CSP samples were LTF 0.587 (0.168, 0.097); NP 0.533 (0.057, 0.033); MAN 0.90 (0.173, 0.10), and CSP 0.319 (0.105, 0.61) (Fig. 3.4). The results of the One-Way ANOVA show a significant difference among group means ($p = 0.005$). The Holm-Sidak method Pairwise Multiple Comparison results showed MAN vs. CSP (CV = 0.009, $p = <0.001$) as the only significantly different allele frequencies among the colony comparisons. The comparisons of MAN vs. LTF (CV = 0.013, $p = 0.022$), MAN vs. NP (CV = 0.010, $p =$

0.010), LTF vs. CSP (CV = 0.017, $p = 0.041$), NP vs. CSP (CV = 0.025, $p = 0.088$), and LTF vs. NP (CV = 0.05, $p = 0.641$) were all determined to have no significant differences.

3.4 Discussion

Widespread reports indicate that DBM resistance to diamide insecticides, in particular chlorantraniliprole resistance, have been a recurrent and growing problem. Initially, multiple reports of chlorantraniliprole resistance in DBM populations from Asia occurred not long after its release (Trockzka et al. 2017, Wang and Wu 2012). These reports were soon followed by reports of the G4946E mutation (Troczka et al. 2012), as well as several other DBM RyR target site mutations (Guo et al. 2014) associated with resistant populations. Troczka et al. (2015) demonstrated that the G4946E mutation reduced chlorantraniliprole binding, and reduced the effect of the insecticide on Ca^{2+} flux in *Spodoptera* cells expressing the mutant RyR relative to the wild-type RyR. This strongly implicates the G4946E mutation is a contributing factor to diamide resistance in DBM populations. Although the mutations identified by Guo et al. (2014) have not been reported outside of limited Asian DBM populations, reports of the G4946E mutation have occurred in multiple countries, including one site in Mississippi in the U.S. (Steinbach et al. 2015).

Recently, chlorantraniliprole resistance has become an obstacle in control of Georgia and Florida DBM populations (Riley et al. 2020). Field assays in which DBM larvae were exposed to the maximum label rate of chlorantraniliprole, cyantraniliprole, and other insecticides (referred to as “maximum dose bioassays”) revealed that resistance to chlorantraniliprole, and to a lesser extent cyantraniliprole, is widespread in the area.

To facilitate further study, selected populations that tolerated exposure to the maximum label rate for chlorantraniliprole were sampled and laboratory colonies were established in 2018.

Toxicological bioassays were used to assess the levels of resistance in each colony to chlorantraniliprole and cyantraniliprole. The results of the dose response assays showed very high levels of chlorantraniliprole resistance in the MAN, NP, and LTF colonies, all of which exceeded 2,800-fold relative to the susceptible FT lab colony. The CSP colony had the lowest chlorantraniliprole resistance ratio at 109-fold. It is worth noting that these LC_{50} values are much higher than what was found in 2018 collections and assays from similar areas of South Georgia (Bhandari et al. 2020). However, levels of chlorantraniliprole resistance similar to that of the NP, MAN, and LTF colonies were reported in the diamide-resistant Sudlon strain from Cebu Island, in the Philippines, as well as the diamide-resistant ThaiR strain from the Bang Bua Thong district of Thailand (Troczka et al. 2012). Interestingly, both the Sudlon and ThaiR strains were among the first DBM populations reported to possess the G4946E mutation (Troczka et al. 2012). These resistance ratios are consistent with the maximum dose bioassay results of the field populations in which these colonies were derived (Riley et al. 2020). As the colonies are removed from environmental influences that might have been present in the field, and the resistance persisted over several generations (from establishment in 2018 until experimentation in 2020) without exposure to insecticide, the observed resistance is certainly due to heritable (i.e. genetic) factors.

Much more modest levels of resistance to cyantraniliprole, between 50- and 108-fold, was detected in the MAN, NP, and CSP colonies. There appeared to be little or no

cross-resistance between chlorantraniliprole and cyantraniliprole for these colonies, i.e., high level of resistance to one did not correlate with high levels of resistance to the other, despite both being anthranilic diamide insecticides that target the same RyR.

Reports of specific target-site mutations associated with chlorantraniliprole resistance (Troczka et. al., 2012, Guo et al. 2014) led us to hypothesize that these mutations may also be associated with resistance in Georgia and Florida DBM populations. In this study we confirmed the presence of the G4946E mutation in all four studied colonies. The proportion of the allele resulting in the resistant G4946E version of the RyR varied considerably between colonies, in a manner that correlates with the level of resistance. In particular, the highly resistant (2,880- to 4,300-fold) colonies (NP, MAN, and LTF) had the highest proportion of the mutant (GAG) allele (53, 90, and 61% respectively). The CSP colony with a lower level of resistance (109-fold) had a lower representation (32%) of the mutant allele. In contrast, the E1338D, Q4594L, and I4790M mutations were not identified in any of our colony samples. It is worth mentioning that a mix of CAG and CAA haplotypes was seen at the position of the Q4594L mutation. However these are synonymous codons, both resulting in a glutamine (Q) and so the susceptible phenotype. Over all, these results from DBM colonies representing populations distributed over the Cole crop producing areas of Georgia and Florida (Figure 4) indicate that chlorantraniliprole resistance is widespread and is likely due to the widespread presence of the G4946E mutation.

Although the correlation between chlorantraniliprole resistance and the proportion of the G4946E mutation across colonies is high, it is interesting that the NP colony, which has the highest level of resistance (4,298-fold), was found to have a lower

proportion of the G4946E allele (53%) compared to the MAN colony (90% G4946E, 2,845-fold resistant). This may simply reflect the fact that our protocol provided only an estimate of the actual G4946E representation in the colony. Alternatively, it may indicate the presence of additional resistance mechanisms in the NP colony that add to the resistance afforded by the G4946E mutation. Such potential mechanisms, particularly metabolic resistance due to detoxification enzymes (Gao et al. 2018), will be the topic of a forthcoming paper.

The low levels of cyantraniliprole resistance in the colonies, and apparent absence of cross-correlation with chlorantraniliprole resistance, suggest that resistance mechanisms which affect chlorantraniliprole efficacy have less of an impact on cyantraniliprole efficacy. In particular, it is of interest that high frequencies of the G4946E mutation seems to confer little resistance to cyantraniliprole toxicity in our colonies. Our results resemble those of Douris et al. (2017), who studied diamide resistant *Tuta absoluta* from Greece and found high levels of resistance to chlorantraniliprole (9,329-fold) and flubendiamide (4,969-fold) but only moderate levels of resistance to cyantraniliprole (191-fold). The G4946V mutation (equivalent to G4946E in DBM) was present in this population with a frequency of ~79%, and the frequency of I4790M was ~21%. Each of these mutations was introduced into *Drosophila melanogaster* using CRISPR/Cas9. The G4946V mutation conferred 194.7-fold resistance to chlorantraniliprole compared to only 5.4-fold resistance to cyantraniliprole. The I4790M mutation conferred 7.5-fold resistance to chlorantraniliprole and 2.3-fold resistance to cyantraniliprole. Similarly, Jouraku et al (2020) selected two *P. xylostella* strains, one (KA17) with over 66,000-fold resistance to

cyantranilirole compared to 678-fold for the second (KU13) strain. Both strains had very similar (~20,000-fold) resistance to chlorantraniliprole. The KA17 strain was homozygous for a novel mutation, I4790K, and the KA13 strain was homozygous for the G4946E mutation, demonstrating the importance of these two mutations in the differential resistance to diamides in DBM. It is likely that the G4946E and the I4790M (or I4970K) mutations alter the diamide binding pocket described by Lin et al (2020) differently, resulting in differential inhibition of binding by cyantraniliprole or chlorantraniliprole. Taken together these results suggest that it is likely the absence of mutations affecting I4790 in our colonies that accounts for the lower level of resistance to cyantraniliprole compared to the high level of resistance to chlorantraniliprole conferred by the G4946E mutation.

Our results suggest that chlorantraniliprole resistance, often at a high level, is widespread across southern Georgia and Florida, as is the G4946E mutation previously associated with resistance in DBM populations from Asia. However, these results are based on colonies established from resistant field populations in 2018, and so our estimates of the frequency of susceptible and resistant alleles may not accurately reflect the current field situation. We plan to extend this study by directly sampling field collections from across the study area to ascertain the prevalence of the G4946E mutation and possible mutations affecting I4790, and the corresponding level of resistance to diamide insecticides, particularly chlorantraniliprole and cyantraniliprole, as determined by maximum dose bioassays (Riley et al. 2020). We expect that such field studies will further reveal the correlation between G4946E prevalence and chlorantraniliprole resistance, and open the possibility of using a molecular diagnostic approach to predict

control success/failure. A similar study in Japan indicated that G4946E frequencies above 40% were associated with resistance to flubendiamide (Sonoda et al. 2017) and that frequencies of this mutation in the field fluctuate according to season (Itagaki and Sonoda 2017) and decline in the absence of diamide use (Fukada et al. 2020). This genetic monitoring approach could contribute significantly to IPM by avoiding use of insecticides likely to fail to control the pest. It could also contribute to insecticide resistance management by limiting chlorantraniliprole use in specific populations where the proportion of G4946E genotypes is high enough to suggest resistance is likely to become problematic. Further, monitoring for the appearance of mutations affecting I4790 could help to forestall the development of resistance to cyantraniliprole and prolong the usefulness of this important diamide insecticide.

Acknowledgements

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Table 3.1: Concentrations used in determining chlorantraniliprole LC_{50} values.

Concentrations (mg_ai/L)	**Frontier**	**LTF**	**MAN**	**NP**	**CSP**
Very High	78	3200	3200	3200	640
High	7.8	640	640	640	320
Medium High	.78	160	320	320	160
Medium	.078	78	160	160	78
Medium Low	.0078	7.8	78	78	7.8
Low	.00078	N/A	7.8	7.8	0.78
Very Low	.000078	N/A	0.78	0.78	0.078
Check	0	0	0	0	0

Range in values used in dose responses bioassays were determined from results of maximum dose bioassays. Concentrations were in mg ai/L.

Table 3.2: Concentrations used in determining cyantraniliprole LC50 values.

Concentrations (mg_ai/L)	**Frontier**	**LTF**	**MAN**	**NP**	**CSP**
Very High	105	N/A	400	400	400
High	10.5	N/A	105	105	105
Medium High	1.05	N/A	10.5	10.5	10.5
Medium	.105	N/A	1.05	1.05	1.05
Medium Low	.0105	N/A	.105	.105	.105
Low	.00105	N/A	.0105	.0105	.0105
Very Low	.000105	N/A	.00105	.00105	.00105
Check	0	N/A	0	0	0

Range in values used in dose responses bioassays were determined from results of maximum dose bioassays. Concentrations were in mg ai/L.

Table 3.3: The sequences of the primers and their association with each mutation.

Mutation	Primer Name	Primer Sequence
E1338D	E1338D-F	5’-ACGAAGGACCAGCCCATTTT-3’
E1338D	E1338D-R	5’-GCTTTGAGCGCCAGATTCAT- 3’
Q4594L	Q4594L-F	5’-AGAACCCACGGAACAGGAGA-3’
Q4594L	RyR3-R	5’-AGAACAGCAGCACAAAGT-3’
I4790M	I4790M-F	5’-CTGGTGAAGGGTCCGGTATC-3’
I4790M	G4946E-R	5’-GAAGTTGAACGCGATGACCG-3’
I4790M	RyR4-F	5’-CTCGCCAGGAAGTTCTACA-3’
I4790M	G4946E-R	5’-GAAGTTGAACGCGATGACCG-3’
G4946E	G4946E-F2	5’-AAGAAGGTCCGCGTCAAGTA-3’
G4946E	G4946E-R	5’-GAAGTTGAACGCGATGACCG-3’

Primers spanning each DBM RyR mutation site were designed using Primer3.

Table 3.4: Primer combinations, annealing temperatures, and extension times used to screen cDNA samples for each of the four RyR mutations via PCR.

Mutation	**Primer Combination**	**Annealing Temperature (°C)**	**Extension Times (s)**	**Product Size (bp)**
E1338D	E1338D-F x E1338D-R	59	40	570
Q4594L	Q4594L-F x RyR3-R	54	40	542
I4790M	I4790M-F x G4946E-R	61	60	779
I4790M	RyR4-F x G4946E-R	59	60	889
G4946E	G4946E-F2 x G4946E-R	59	30	393

Annealing temperatures were measured in degrees Celsius (°C**),** extension times were measured in seconds (s), and PCR product sizes were measured in the number of nucleotide base pairs (bp).

Table 3.5: LC_{50} values determined for each population when exposed to chlorantraniliprole and cyantraniliprole.

Insecticide	**Population**	**LC_{50} (mg_ai/L)**	**N larva tested**	**95% Fiducial Limits**	**Resistance Ratio**
Chlorantraniliprole	FT	0.14	240	0.06 – 0.28	N/A
Chlorantraniliprole	NP	601.74	280	316.2 – 1,232	4,298
Chlorantraniliprole	MAN	398.25	240	252.6 – 602.7	2,845
Chlorantraniliprole	LTF	393.79	150	195.27-704.90	2,813
Chlorantraniliprole	CSP	15.24	290	4.90 - 36.8	109
Cyantraniliprole	FT	0.17	420	0.08-0.25	N/A
Cyantraniliprole	MAN	18.29	470	10.76-28.86	108
Cyantraniliprole	NP	11.41	310	1.52-35.48	67
Cyantraniliprole	CSP	8.46	280	3.87-15.17	50

Data was analyzed via Probit analysis in SAS Enterprise Guide (64-bit). LC_{50} concentrations are measured in milligrams of active ingredient per Liter (mg_ai/L). The susceptible FT population was used for comparison and determination of resistance ratios (LC_{50} of FT/ LC_{50} of colony

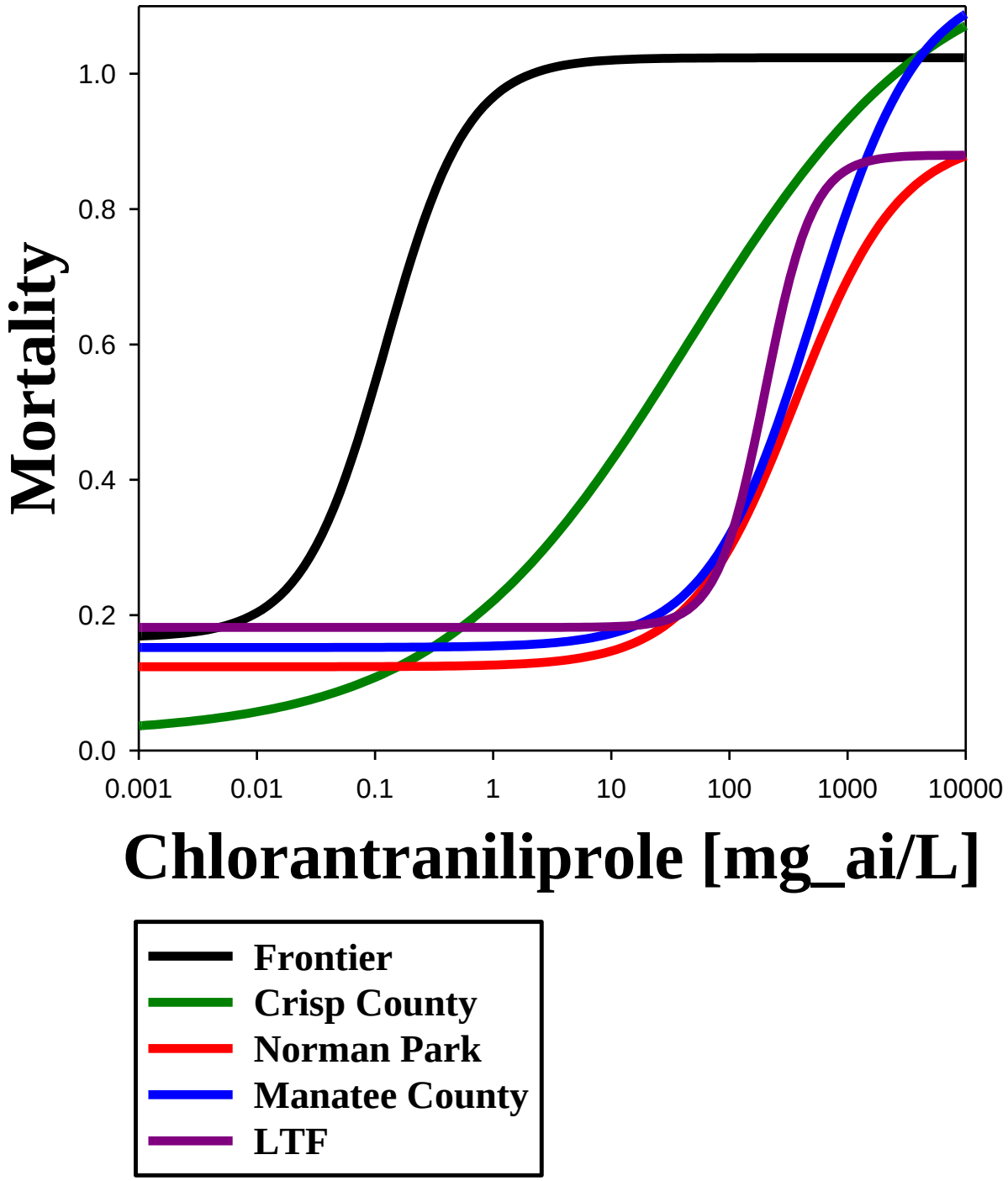


Figure 3.1. Chlorantraniliprole dose response curves (SigmaPlot 11.0) that show differences in resistance levels among the resistant colonies (LTF, MAN, NP, CSP), as well as the susceptible control (FT) (Fiducial limits in Table 3.5).

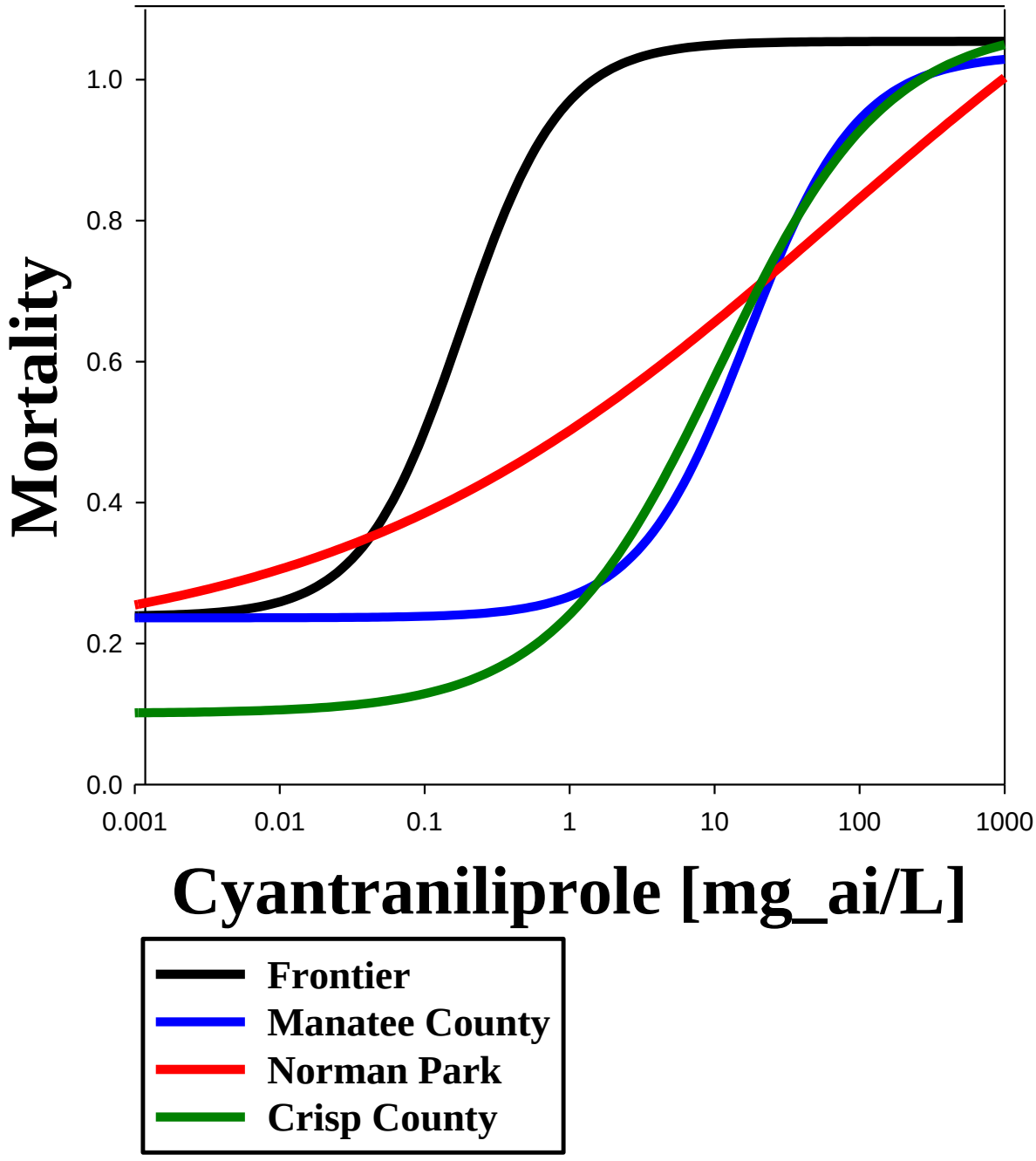
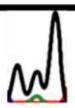
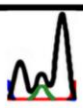
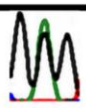
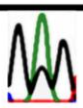
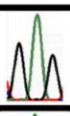
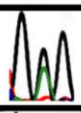
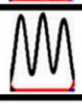
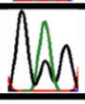
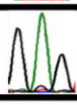
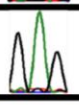
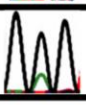


Figure 3.2. Cyantraniliprole dose response curves (SigmaPlot 11.0) that show differences in resistance levels among the resistant colonies (MAN, NP, CSP), as well as the susceptible control (FT) (Fiducial limits in Table 3.5).

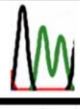
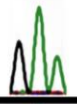
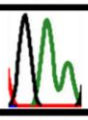
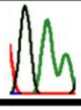
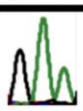
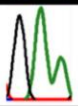
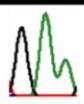
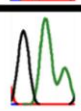
A

GE Proportions	20% E / 80% G	40% E / 60% G	60% E / 40% G	80% E / 20% G
GE Chromatogram				

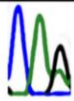
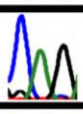
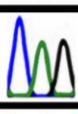
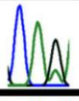
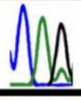
B

G4946E	FT	LTF	MAN	NP	CSP
Sample 1					
Sample 2					
Codon	G G G	G A G	G A G	G A G	G G G

C

E1338D	FT	LTF	MAN	NP	CSP
Sample 1					
Sample 2					
Codon	G A A	G A A	G A A	G A A	G A A

D

Q4594L	FT	LTF	MAN	NP	CSP
Sample 1					
Sample 2					
Codon	C A G	C A G	C A G	C A G	C A G

E

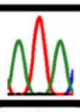
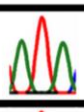
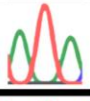
I4790M	FT	LTF	MAN	NP	CSP
Sample 1					
Sample 2	N/A				
Codon	A T A	A T A	A T A	A T A	A T A

Figure 3.3 A-E. Ab1 files received from Eurofins Genomics depicting proportions of the G4946E mutation in four different samples. B. Ab1 files that depict the G4946E mutation site from each population. Wild type (susceptible) alleles are represented by the three dark blue peaks (GGG), while mutant (resistant) alleles are represented by two outer dark blue peaks and a center green peak (GAG). C. Ab1 files that depict the E1338D mutation site from each population. Wild type (susceptible) alleles are represented by a dark blue peak followed by two green peaks (GAA), while mutant (resistant) alleles are represented by a dark blue peak, a center green peak, followed by a red peak (GAT). D. Ab1 files that depict the Q4594L mutation site from each population. Wild type (susceptible) alleles are represented by a light blue peak, a center green peak, and a dark blue peak (CAG), while mutant (resistant) alleles are represented by a light blue peak, a center red peak, and a dark blue peak (CTG). E. Ab1 files that depict the I4790M mutation site from each population. Wild type (susceptible) alleles are represented by a green peak, a center red peak, and another green peak (ATA), while mutant (resistant) alleles are represented by a green peak, a center red peak, and a dark blue peak (ATG).

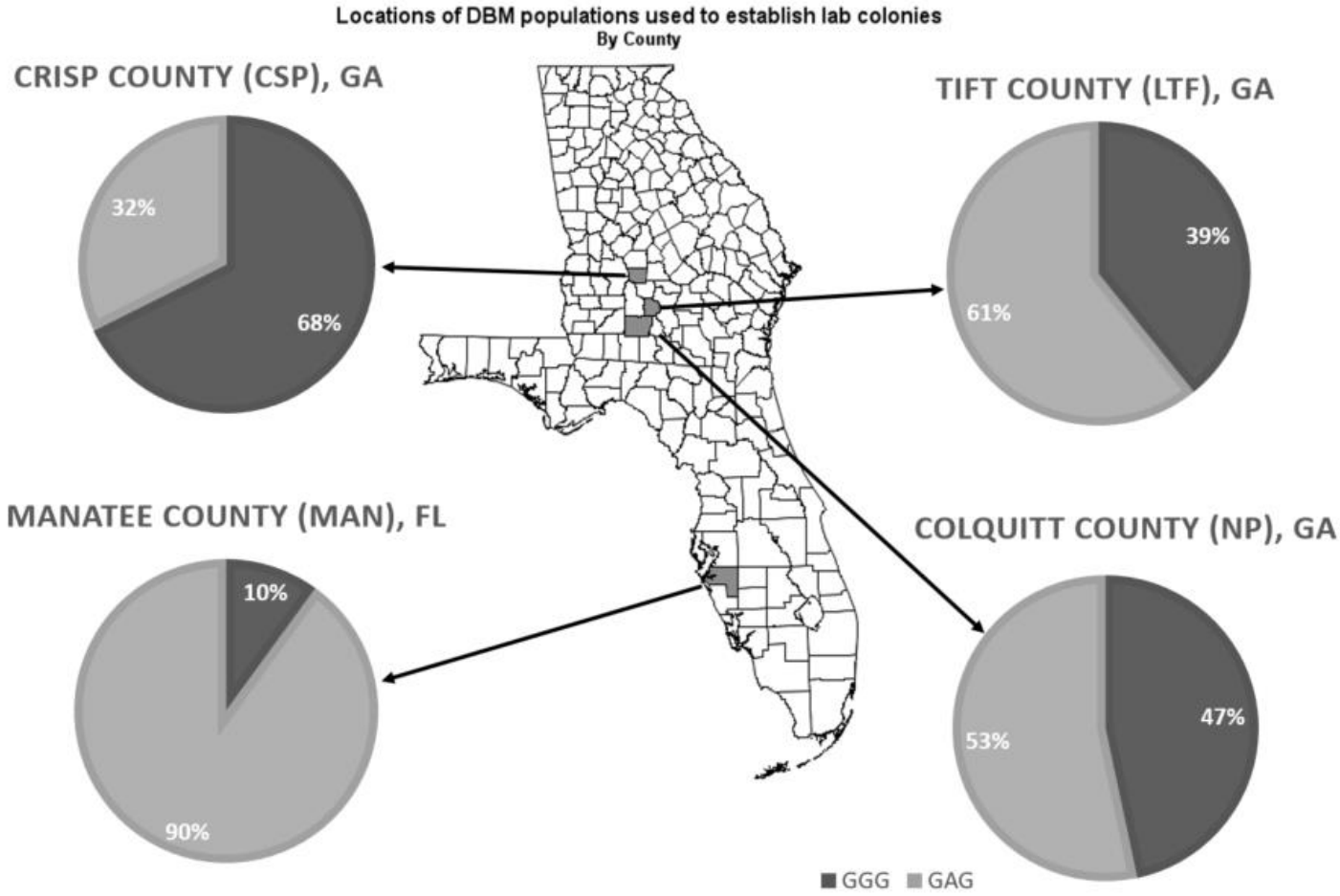


Figure 3.4. Locations and frequencies of G4946E alleles from resistant field populations in Georgia and Florida used to establish lab colonies for toxicological and genetic analysis.

SUMMARY

The diamondback moth (DBM), *Plutella xylostella,* is a major pest of cruciferous crops worldwide. The development of resistance to multiple insecticide modes of action has been a recurrent problem with DBM, and more recently developed insecticide products, such as chlorantraniliprole (Coragen), have failed to control this pest in certain DBM populations. The goal of this research was to determine if DBM colonies derived from diamide resistant field populations across South Georgia and Florida possessed genetic resistance factors known to contribute to diamide resistance in the field. The discovery of target site mutations of the DBM ryanodine receptor (RyR) from resistant DBM populations from Asia provided evidence of genetic mutations which contribute to diamide insecticide resistance. While the G4946E mutation, discovered by Troczka et al. (2012), has been identified in DBM populations from multiple countries (including a population in Mississippi) (Steinbach et al. 2015), three other mutations referred to as the E1338D, Q4594L, and I4790M have only been identified in Asian populations (Guo et al. 2014). This study involved both dose response assay techniques, as well as the genetic screening of DBM cDNA samples via polymerase chain reaction (PCR). Once identified, the development of protocols to identify resistance factors, such as target site mutations, would provide information that could be used to develop a long-term insecticide resistance management (IRM) program.

Four separate resistant field populations were selected for collection in Tift, Colquitt, and Crisp counties in Georgia, as well as Manatee County in Florida in 2018. The collected larva were used to establish lab colonies in Tifton, Georgia at the Coastal

Plains Experiment Station, and were referred to as LTF, NP, CSP, and MAN, respectively. Dose response assays were used to determine the levels of resistance in each colony to chlorantraniliprole and cyantraniliprole. Interestingly, the results of the dose response assays indicated little cross resistance between both diamide insecticides in the colonies. While resistance to chlorantraniliprole for LTF, MAN, and NP ranged from 2,813 to 4,298-fold in comparison to the susceptible Frontier Genomics (FT) strain, the CSP colony only showed 109-fold resistance in comparison to FT. By comparison, the resistance ratios of MAN, NP, and CSP for cyantraniliprole ranged from 50 to 108-fold. These findings are consistent with recent studies that display intermediate levels of cyantraniliprole resistance alongside high levels of chlorantraniliprole resistance in DBM (Riley et al. 2020).

Once LC_{50} values were determined, the each colony was screened for each of the four mutations. Third instar DBM larvae were collected at random from each colony and pooled in groups of five. These pooled larvae were then used for mRNA extraction and cDNA synthesis after transport to Athens, Georgia. Primers spanning the four regions containing the mutation sites of the DBM RyR were used to produce amplicons via PCR. The purified PCR products were sent to Louisville, Kentucky for genetic sequencing. The results of sequencing three to four PCR products (each from individual cDNA samples) from each colony for each mutation site revealed only the G4946E mutation in our samples. Each sequenced PCR sample from each of the colonies contained the G4946E mutation at varying proportions. All PCR products sequenced from MAN and NP showed the resistance mutation (GAG) as dominant, while the LTF colony showed the GAG as dominant in three samples and the susceptible genotype (GGG) as dominant in

one sample. The CSP colony was the only colony to show the GGG as dominant across all sampled PCR products. The E1338D, Q4594L, and I4790M mutations were not detected in any of our sequenced samples.

Once the G4946E mutation was confirmed through PCR, the estimates of allele frequencies were determined for each population. Three cDNA samples from each colony were used to transform NEB turbo competent *E. coli* cells via CloneJET procedure following the manufacturer's protocol. Plasmids were extracted via GeneJet Plasmid Miniprep Kit following the manufacturer's protocol. Extracted plasmid samples were sent to Eurofins genomics in Louisville, Kentucky for Sanger sequencing. The sequences were then used for the quantification of GAG alleles for each DBM colony. The results estimated GAG allele frequencies of 90, 61, 53, and 32% for MAN, LTF, NP, and CSP, respectively. The results of this study suggest the G4946E is widespread throughout Georgia and Florida DBM populations. Interestingly, the frequencies of the G4946E mutation seem to share a positive relationship with the levels of chlorantraniliprole resistance in each of the colonies. This relationship does not seem to be consistent with cyantraniliprole resistance in these colonies.

In the Appendix, we provided additional LC_{50} values for the colonies in response to spinetoram (Radiant). These LC_{50} values were produced for use in future studies for exposure of the colonies to sub-lethal doses of spinetoram. Resistance ratios for NP, MAN, and CSP were 59, 217, and 29-fold, respectively. These resistance ratios were similar to cyantraniliprole resistance ratios gathered for the colonies. While this data is of interest for future work, the levels of resistance to spinetoram should not be affected by mutations of the RyR. Spinetoram belongs to IRAC Class 5, which target the nicotinic

acetylcholine receptor (nAChR). Since this IRAC Class targets a completely different receptor, a relationship between spinetoram resistance and the frequency of GAG alleles in the colonies would not be expected.

Thomas P. “Sam” Dunn

Short Biography

Sam has been a student at UGA since January of 2021 where he began his master’s in Entomology. He completed his undergraduate degree at Abraham Baldwin Agricultural College and graduated with a BS in Biology in December of 2018. While studying at ABAC he worked multiple jobs, including working as a farm hand and temporarily working as a pharmacy technician. Eventually, he began working in David Riley’s lab as a student worker in February of 2018. After working in an Entomology lab for 10 months, he began his master’s degree under the direction of Donald Champagne in Athens, Georgia. He is set to graduate in July 2021.

Sam is known to love the outdoors. He is an avid hunter, and would constantly make trips home during deer, dove, and turkey hunting seasons. He also enjoys a variety of music. His favorite genre of music is the blues, and some of his favorite bands/artists are Led Zeppelin, ZZ Top, Stevie Ray Vaughn, and Jimi Hendrix.

APPENDIX: ADDITIONAL SPINETORAM DOSE RESPONSE DATA FOR SOUTH GEORGIA AND FLORIDA DIAMONDBACK MOTH COLONIES

Introduction

The Diamondback Moth (DBM), *Plutella xylostella,* is a major pest of cruciferous crops worldwide (Furlong et al. 2013, Talekar and Shelton 1993). Annual damage estimates due to DBM outbreaks range from US $4-5 billion around the world (Zalucki et al. 2012), thus effective insecticide resistance management (IRM) strategies are critical to mitigate this damage. Documentation of DBM insecticide resistance has shown that at least 95 different insecticides have experienced control failure when used against DBM (APRD 2020).

Resistance to spinosyns (IRAC Class 5) has been documented in multiple DBM populations. Spinosad resistance in the U.S. was documented in DBM populations from Hawaii, California, and Georgia in the early 2000's (Zhao et al. 2002, Zhao et al. 2006), and Bhandari et al. 2020 demonstrated a clear increase in spinetoram LC_{50} values of South Georgia DBM populations from 2012 to 2018. Wang et al. (2016) discovered a three amino acid deletion of the DBM nicotinic acetylcholine receptor (nAChR). This mutation was discovered after selecting against a susceptible field strain (SZ) from the Guangdong Province of China with spinosad for eighty generations, resulting in a new resistant strain (SZ-SpinR). Dose response assays revealed the SZ-SpinR strain was 940 and 1,060-fold resistant to spinosad and spinetoram, respectively. A recent study of

diamide resistant DBM populations from Georgia and Florida revealed high levels of resistance to chlorantraniliprole (109 to 4,298-fold) and intermediate levels of resistance to cyantraniliprole (50 to 108-fold) (Dunn et al. in prep.). These same colonies were used to determine LC_{50} values for spinetoram.

Materials and Methods

Diamondback Moth were collected from field resistant populations throughout South Georgia and Florida in 2018 as part of a previous study (Riley et al. 2020), and used to establish laboratory colonies. Larva and pupa were collected from Tift County (designated the LTF colony), Colquitt County (NP colony), and Crisp County (CSP colony) in Georgia, as well as a population from Manatee County, Florida (MAN colony). A susceptible strain, commercially available from Frontier Genomics (FT colony), was used as a control. Lab colonies were established at the Coastal Plains Experiment Station located in Tifton, Georgia. These colonies were reared on mustard (*Brassica juncea*) and collard (*Brassica oleracea var. acephala*) plants, grown in Percival growth chambers with a temperature of 26.0 ± 1 °C and a day and night cycle of 12:12. Adult DBM were given 10% honey solution via soaked cotton balls placed in 37.0 mL SOLO cups. DBM colony cages were placed under lights on a 10:14 day and night cycle, and the room temperature was held constant at 68 °F.

Toxicological Bioassays. Leaf-dip bioassays were used to expose DBM larva to varying rates of spinetoram. Chlorantraniliprole and cyantraniliprole LC_{50} values for these colonies were determined in a previous study (Dunn et al. in prep). Bioassays were

set up in petri dishes with 38 mm (1-1/2 inch) diameter holes cut into the lids for ventilation. The hole was then covered with hot-glued nylon chiffon to ensure the larva could not escape. Circular Whatman filter papers were placed into the bottom of the petri dish and sprayed with water until damp to prevent leaf discs from drying out. A 70 mm (2-3/4 inch) diameter circular leaf disc was cut out from a fully grown collard (*Brassica oleracea var. acephala*) leaf and dipped into a 0.25 L aqueous solution consisting of spinetoram at the concentration of interest. 0.25 mL of Kinetic adjuvant was also added to the solution to ensure even spreading and adherence of the insecticide. The treated leaf disc was placed into the petri dish on top of the damp filter paper and allowed to air dry for 30 minutes. Once dried, 10 3rd instar larvae were placed on the leaf and the top of the petri dish was secured with rubber bands to prevent escape. Each replicate was checked every 24 hours for larval mortality until 72 hours had passed. Data was analyzed via SAS Enterprise Guide 7.1 (64-bit) via Probit analysis to determine LC_{50} values (Table A.1). SigmaPlot (Version 11.0) was also used to generate dose response curves for comparisons of different colony resistance levels for spinetoram.

Results and Discussion

Lethal concentration 50 (LC_{50}) values were determined for each colony in response to spinetoram. Resistance ratios for CSP, NP, and MAN were determined to be 28.9, 59.1, and 216.9-fold resistant to spinetoram, respectively (Table A.1). The resistance levels determined in these dose response assays suggest more intermediate levels of resistance to spinetoram, similar to what was determined for cyantraniliprole in the previous chapter. Although extreme levels of spinetoram resistance may be possible in some DBM populations, the estimated allele frequencies of the G4946E mutation

would not be expected to play a role in this resistance as both modes of action target two different receptors. The dose response curves (Fig. A.1) demonstrate intermediate levels of resistance to spinetoram in all three tested colonies. Interestingly, the flatter curves of the NP and CSP colonies suggests more heterogenous populations when compared to the sigmoidal shapes in the MAN colony and FT strain.

Table A.1: Spinetoram LC_{50} values, 95% Fiducial Limits, and resistance ratios for each colony.

Insecticide	Population	LC50 (mg_ai/L)	N larva tested	Fiducial Limits	RS Ratio
Radiant	FT	0.03465	240	0.02 – 0.06	-
Radiant	NP	2.044	420	0.76 – 4.44	59.1
Radiant	MAN	7.505	320	3.93 – 12.99	216.9
Radiant	LTF	N/A	N/A	N/A	N/A
Radiant	CSP	1.00031	240	0.372-2.133	28.87

Data was analyzed via Probit analysis in SAS Enterprise Guide (64-bit). LC_{50} concentrations are measured in milligrams of active ingredient per Liter (mg_ai/L). The susceptible FT population was used for comparison and determination of resistance ratios (LC_{50} of FT/ LC_{50} of colony).

Figure Captions

Figure A.1. Dose response curves for spinetoram against the FT strain and CSP, NP, and MAN colonies. This graph was produced in SigmaPlot 11.0 using the Logistic 4 Parameter model.

Fig. A.1. Dose response curves for spinetoram against the FT strain and CSP, NP, and MAN colonies (Fiducial Limits in Table A.1).