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Insecticide Resistance Management Approaches and Naturally Derived Toxicants for the Control of Fall Armyworm, Spodoptera frugiperda

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INSECTICIDE RESISTANCE MANAGEMENT APPROACHES AND NATURALLY DERIVED TOXICANTS FOR THE CONTROL OF FALL ARMYWORM, *SPODOPTERA FRUGIPERDA*

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Table of Contents

Acknowledgements ii

Abstract iv

Chapter 1. Literature Review 1
1.1. Fall armyworm (*Spodoptera frugiperda*) Pest Status 1
1.2. Insecticides for FAW Control 2
1.3. Insecticide Resistance and Management of FAW 5
1.4. Natural Products as Insecticides 9
1.5. Chromene Inspired Chemistries 11
1.6. Overarching Hypothesis to be Tested 13
1.7. Objectives of the Study 13

Chapter 2. Reduced neuronal sensitivity and susceptibility of the fall armyworm, *Spodoptera frugiperda*, to pyrethroids in the absence of known knockdown mutations 15
2.1. Introduction 15
2.2. Methods 17
2.3. Results 24
2.4. Discussion 33
2.5. Supplemental Figures 39
2.6. Supplemental Tables 40
2.7. Acknowledgments 41
2.8. Notes 42

Chapter 3. Toxicological and neurophysiological characterization of natural product based chromene analogs to *Spopdoptera frugiperda* (Fall armyworm) 43
3.1. Introduction 43
3.2. Methods 46
3.3. Results 49
3.4. Discussion 55

References 58

Vita 67

Abstract

Spodoptera frugiperda, the fall armyworm (FAW), is a major agricultural pest causing billions of dollars in damage annually to staple crops. Agricultural losses stemming from this pest continues to increase as resistance to commercialized insecticide classes evolves and spreads. Considering this, novel chemistries with new modes of action as well as novel resistance management strategies need to be developed to achieve continued control of FAW populations. This project consisted of two main goals. First, we aimed to develop a neurophysiological assay to enable a more precise understanding of pyrethroid and organophosphate resistance at the level of the nerve. We collected FAW from whorls of late-season corn and compared neural sensitivity and susceptibility to lambda-cyhalothrin and chlorpyrifos. Field collected FAW were >700-fold resistant to lambda-cyhalothrin and had >1700-fold reduced neural sensitivity yet, no known kdr mutations were identified. These data suggest the absence of *kdr* mutations does not necessarily indicate pyrethroid susceptibility and vice versa and therefore, the inclusion of neurophysiological assays into existing resistance monitoring programs is likely to provide a more accurate assessment of resistance mechanisms. In the second goal, we aimed to test the neural potency and toxicity of natural product based chromene (Benzopyran) analogs to FAW to determine if chromene based scaffolds may represent a base for FAW directed insecticide development. Chromene testing included intrathoracic injections, feeding assays, and neurophysiological recordings. Select chromenes induced acute toxicity and induced abnormalities upon adult eclosion such as partial eclosion and twisted wings, indicating these molecules may interfere with development. Therefore, chromene based molecules represent a series of scaffolds that may be used to develop FAW directed insecticides and warrant further study.

Chapter 1. Literature Review

1.1. Fall armyworm (*Spodoptera frugiperda*) Pest Status

The fall armyworm (FAW), *Spodoptera frugiperda* (Lepidoptera: Noctuidae), is a polyphagous herbivore that is recognized as one of the most damaging agricultural pests of row crops with economic losses estimated to be at $6 billion dollars per year (Yu, Nguyen, and Abo-Elghar 2003). Global trade and travel has facilitated the expansion of the FAW and ranges from the Americas to nearly 100 countries, which has threatened the food security of millions of people due to the ability of FAW to cause 100% loss of maize and rice crops if left uncontrolled (Goergen et al. 2016; Sharanabasappa et al. 2018; Wu et al. 2019; Cairns et al. 2013). The ability of *S. frugiperda* to feed on a wide range of host plants, the occurrence of multiple generations in a single growing season, and their ability to migrate make *S. frugiperda* one of the most significant economic pests of the Western Hemisphere (Okuma et al. 2018b). *S. frugiperda* feeds on more than 80 species of agricultural row crops including maize, rice, cotton, sorghum, and other types of grasses (Pannuti et al. 2016). In addition to the economic damage, *S. frugiperda* is a serious threat to the food security of millions of people as FAW populations have recently become established across Africa, India, and China (Goergen et al. 2016; Sharanabasappa et al. 2018; Wu et al. 2019), which rely heavily on maize and rice as staple food crops. *S. frugiperda* has also established itself at a severe pest in Brazil due to the rapid destruction of corn crops resulting from consistent larval feeding (Pannuti et al. 2016). The lack of diapause in this pest results in the migration of FAW to warmer climates which, in turn, later leads to reinvasions of crops and greater resistance is then developed (Pannuti et al. 2016). Although FAW is cold intolerant, it surprisingly manages to survive and repopulate through winter months (Nagoshi et al. 2017; Pannuti et al. 2016). The success of this pest to repopulate and reinvade the Americas is due to migration to

subtropical climates such as Florida and southern Texas where it is warmer year-round (Nagoshi et al. 2017). *S. frugiperda* has risen greatly as a severe pest and has more recently established itself in countries around the world, making it one of the most important pests across the globe for management practices.

1.2. Insecticides for FAW control

The most common approach employed by FAW control programs is the use of a combination of synthetic insecticides and *Bacillus thuriengiensis* (Bt) expressing plants to maintain pest populations below the economic damage threshold (Blanco, Chiaravalle, Dalla-Rizza, Farias, García-Degano, et al. 2016; Nagoshi et al. 2017). Over 136 synthetic insecticides are used for control of FAW when Bt plants are not established as a control method (Carvalho et al. 2013; Gutiérrez-Moreno et al. 2019) with the most important and widely used synthetics include pyrethroids, organophosphates, diamides, spinosads, and carbamates (U.N.L. 2017).

Plants expressing *Bacillus thuringiensis* insecticidal genes (Bt crops) will express Bt proteins that are similar to the proteins that the bacterium produce (Blanco, Chiaravalle, Dalla-Rizza, Farias, García-Degano, et al. 2016). Bt protein expressing plants display tremendous species specificity (Blanco, Chiaravalle, Dalla-Rizza, Farias, García-Degano, et al. 2016). Additionally, this bacterium contains four highly toxic Cry proteins (Cry1Aa, Cry1Ab, Cry1Ac, and Cry2), as well as Bt spores, that erode the midgut epithelium to induce toxicity ("Bacillus Thuringienses Mode of Action"). FAW intoxicated by Cry toxins will stop eating in minutes due to hemolymph poisoning and extensive midgut damage ("Bacillus Thuringienses Mode of Action"). Additionally, midgut wall cells are damaged from Cry toxin crystals solubilizing and forming pores, which results in sepsis and FAW death in a matter of 1-3 days ("Bacillus Thuringienses Mode of Action").

The use of genetically modified Bt expressing crops has significantly reduced the widespread use of insecticides for farmers for over 20 years and continues to mitigate insecticide resistance development in *S. frugiperda* ("Bacillus Thuringienses Mode of Action" ; Blanco, Chiaravalle, Dalla-Rizza, Farias, García-Degano, et al. 2016). However, FAW management with Bt crops alone is becoming less sustainable due to resistance to Bt proteins and thus, inclusion and/or rotation of synthetic insecticides with Bt technology is imperative.

Pyrethroids are one of the most common synthetic insecticide classes used against FAW and are voltage-gated sodium channel agonists (VGSC) (Clark and Symington 2012; U.N.L. 2017) that alter neuron polarization and sensitivity. Pyrethroids disrupt nerve function by altering the rapid kinetic transitions between conducting (open) and nonconducting (closed or inactivated) states of voltage-gated sodium channels that underlie the generation of nerve action potentials, which ultimately prevents or slows inactivation (Soderlund 2010; Soderlund and Bloomquist 1989). The pyrethroid class is divided into two types depending on the alcohol substituent in the molecule. Type 1 pyrethroids are broadly defined and includes pyrethroids containing descyano-3-phenoxybenzyl or other alcohols. Type 2 pyrethroids are more narrowly defined in regards to their chemical structure as they contain an α-cyano-3-phenoxybenzyl moiety, which increases insecticidal activity by approximately 10-fold (Nasuti et al. 2003; Soderlund 2010; Soderlund and Bloomquist 1989). The signs of intoxication after exposure to pyrethroids usually present rapidly after poisoning and different syndromes exist for type 1 and type 2 pyrethroids (Ross 2011; Soderlund 2010; Soderlund and Bloomquist 1989). Type 1 pyrethroids induce hyperexcitability and convulsions within insects and a whole-body tremor (T syndrome) in mammals (Ross 2011; Soderlund 2010; Soderlund and Bloomquist 1989). Type 2 pyrethroids induce uncoordinated movements, lethargy and spurts of excitability in insects with mammals exhibiting CS syndrome

highlighted by salivation and gastrointestinal problems (Ross 2011; Soderlund 2010; Soderlund and Bloomquist 1989). These differing symptomologies are due to their mechanism of action at the sodium channel where the duration of modified sodium currents by Type 1 compounds lasts tens or hundreds of milliseconds, while those of Type 2 compounds last for minutes or longer (Soderlund 2010; Soderlund and Bloomquist 1989). Thus, Type 1 compounds induce multiple spike discharges in central and peripheral neurons whereas Type 2 pyrethroids depolarize the axon membrane potential, which reduces the amplitude of the action potential and eventually leads to a loss of electrical excitability (Soderlund 2010; Soderlund and Bloomquist 1989).

Organophosphorus insecticides, which are oftentimes referred to as organophosphates (OPs), are a structurally diverse class of chemicals that all inhibit acetylcholinesterase enzyme in the arthropod central nervous system (Siegfried and Scharf 2001). OPs are bioactivated by cytochrome P450 monooxygenases and the oxidized metabolite reacts with a serine hydroxyl group in the AChE active site that inactivates the catalytic activity of the enzyme (Ellison et al. 2012). The inhibition of AChE prevents hydrolysis of acetylcholine from the post synaptic receptor and leads to increased concentrations of acetylcholine within the synaptic cleft that leads to hyperexcitation and long trains of action potentials that arise from single stimuli (Siegfried and Scharf 2001). OPs are a commonly used insecticidal class to control populations of FAW with chlorpyrifos being the most common OP used against this pest in corn crops around the world (Corona 2019).

Diamides are the most recently commercialized class of insecticide control of lepidopteran pests and are inhibitors of ryanodine receptors (RyR) (Boaventura et al. 2020b). This class of insecticides are derived from the water soluble plant extract ryania that contains ryanodine and 9, 21-dehydroryanodine, which are both known to be highly toxic to insects (Wang et al. 2014). The

two primary diamide insecticides commercialized for *S. frugiperda* control are chlorantraniliprole and flubendiamide (Boaventura et al. 2020b), which are anthranilic and phthalic acid diamides, respectively and have a similar mechanism of action of ryanodine but are not structural analogs (John T. Andaloro 2010). Diamides activate calcium release in the sarcoplasmic reticulum and the prolonged open state of the calcium channels increases the intracellular concentration of calcium within the muscle fiber and induces a sustained contraction of insect body wall muscle, leading to paralysis and death (Cordova et al. 2006).

1.3. Insecticide Resistance and Management of FAW

FAW resistance to Bt planted crops is likely multifactorial and has been documented to be resultant of reduced activity or inactivation of the midgut Bt receptors (Ingber 2017). Since the introduction of Bt expressing crops, insecticide use decreased by 47.8% (Brookes and Barfoot 2017), yet FAW have gained resistance to varying Bt proteins such as Cry1F, Cry1Ac, Cry1Ab, Cry1A.105, Cry1F, and Cry1Ab in Puerto Rico, the U.S, Brazil, and even Argentina (Blanco, Chiaravalle, Dalla-Rizza, Farias, García-Degano, et al. 2016; Gutiérrez-Moreno et al. 2019). Consequently, development of Bt-resistant strains of FAW has risen, and the increased Cry1F resistance alleles within FAW populations (Santos-Amaya et al. 2017; Blanco, Chiaravalle, Dalla-Rizza, Farias, Garcia-Degano, et al. 2016) has forced farmers to significantly increase the frequency of insecticide applications to maintain low FAW levels. Unsurprisingly, the increased frequency of application has driven the evolution of resistance to multiple insecticidal classes, including pyrethroids, organophosphates, carbamates, benzoylureas, spionsyns, and diamides (Ríos-Díez and Saldamando-Benjumea 2011; Okuma et al. 2018a; Jia et al. 2009; Carvalho et al. 2013; Boaventura et al. 2020a). The first report of insecticide resistance in *S. frugiperda* was to

the carbamate insecticide carbaryl (Young and McMillian 1979). After this first report, high levels of resistance have been reported to all classes of insecticides licensed for FAW control (Carvalho et al. 2013).

Pyrethroid resistance in *S. frugiperda* is multifactorial and results from a combination of target-site insensitivity, enhanced detoxification mechanisms, and reduced cuticular penetration (Gutiérrez-Moreno et al. 2019; McComic et al. 2020; Ottea and Holloway 1998b; Ríos-Díez and Saldamando-Benjumea 2011; Vontas, Small, and Hemingway 2001). Metabolic detoxification usually occurs in two different phases: (I) hydrolysis and oxidation of insecticide and (II) products from phase I conjugate (merge) with endogenous compounds which result in excretion of the xenobiotics from the body (Indyra F. Carvalho `2018). Most importantly, FAW metabolic enzymes are efficient in metabolizing pyrethroids so most commercial products require the addition of a P450 inhibitor, such as piperonyl-butoxide (PBO), to increase the bioavailability of the insecticide (Gist and Pless 1985; Gleave et al. 2018). Studies have shown that elevated levels of Glutathione-S-Transferases (GSTs), microsomal oxidases, and carboxylesterases in FAW are associated with increased pyrethroid resistance through metabolic detoxification (Vontas, Small, and Hemingway 2001; Richardson et al. 2020).

In addition to metabolic detoxification, target site insensitivity of pyrethroids, known as knockdown resistance (kdr*)*, results from point mutations in the nucleotide sequence of the VGSC (Carvalho et al. 2013; McComic et al. 2020; Singh et al. 2011). The kdr mutations found within the VGSC for *S. frugiperda* are T929I, L932F, and L1014F (housefly numbering) amino acid substitutions (Carvalho et al. 2013). L1014F was first identified in the house fly (*Musca domestica* L.) where it was characterized as “knockdown”, and is the most noted VGSC mutation within arthropod species (Davies and Williamson 2009). All three *kdr* mutations intensify the closed-state

inactivation of the VGSCs, resulting in a decrease in the number of channels available and a reduction in the binding affinity of pyrethroids (Vais et al. 2000). Lambda (λ)-cyhalothrin resistance in FAW is driven by multiple recessive genes and additional studies have identified three different *kdr* and super *kdr*-type mutations (Carvalho et al. 2013; Ríos-Díez and Saldamando-Benjumea 2011) that are analogous to known house fly gene regions causing insensitivity to pyrethroid insecticides (Miyazaki et al. 1996; Soderlun and Lee 2001). Although metabolic and non-metabolic mechanisms are known to contribute to pyrethroid susceptibility, the majority of efforts to determine mechanisms of resistance have focused on genetic analyses that identify target site mutations analogous to *kdr* mutations established in model arthropod pests. Although this is a general predictor of insecticide sensitivity within that population, the presence or absence of target-site mutations does not necessarily predict the neuronal sensitivity (sensitivity and susceptibility of the central nervous system to neurotoxic insecticides) or resistance ratios (RR) to the insecticide within the population of interest (McComic et al. 2020). Considering this, the inclusion of phenotypic assays that quantify the neural sensitivity of neurotoxic insecticides combined with genotypic assays is critical for generating a more holistic understanding of resistance mechanisms and driving management approaches (McComic et al. 2020).

Previous studies have used lepidopteran *ace*-1 encoding primers to determine if the target-site mutations within the AChE conferring resistance to organophosphates were present in *S. frugiperda* (Carvalho et al. 2013). Consequently, three amino acid substitutions were found that are known to confer resistance to organophosphates: A201S, G227A, and F290V (Carvalho et al. 2013). The A201S mutation is known to confer insensitivity to organophosphates (and carbamates), rendering AChE less sensitive to the organophosphate paraoxon (Carvalho et al. 2013). Although this mutation renders the AChE less sensitive, it only results in low levels of

organophosphate resistance within *S. frugiperda* when compared to the G227A and F290V mutations (Carvalho et al. 2013). The G227A and F290V mutations in *S. frugiperda* populations occur in higher frequencies than A201S, resulting in more modest levels of resistance (Carvalho et al. 2013). Due to *ace-1* target site mutations and metabolic detoxification, organophosphate resistance continues to threaten effective control programs and limits the availability of insecticidal classes to control FAW.

Diamides were commercialized around 2007 and have enhanced management of FAW due to its novel target site action that circumvents existing resistance mechanisms (Richardson et al. 2020; Ebbinghaus-Kintscher et al. 2007). However, the increased use of diamides has led to increased selection pressure that has driven the evolution of resistance in several lepidopteran species, including FAW (Richardson et al. 2020). A recent study has provided evidence that a field population of FAW in Correntina, Bahia has developed tolerance to chlorantraniliprole which suggests the formation of resistance alleles (Richardson et al. 2020). Increased levels of P450s, carboxylesterases, and GSTs found in FAW and other insects exposed to diamides suggest that metabolic detoxification could play a part in this resistance (Richardson et al. 2020). In addition to metabolic detoxification mechanisms of diamides, *S. frugiperda* target site resistance involving amino acid polymorphisms within the RyR channel are found to be associated with decreased diamide efficacy (Richardson et al. 2020). The elevated resistance seen in these FAW colonies to synthetic insecticides, including diamides, is due to heavy application of commercial products (Richardson et al. 2020).

In addition to Correntina, Bahia, farms in Puerto Rico showed reduced product efficacy with chlorantraniliprole and flubendiamide, two commercialized diamide insecticides (Gutiérrez-Moreno et al. 2019; Richardson et al. 2020), which mirrors a recent report that has shown high

levels of resistance due to target site mutations (Boaventura et al. 2020b). Despite reduced product efficacy, control failures in the field have remained low, with the LD80 of chlorantraniliprole still residing below the recommended dose value (Richardson et al. 2020). The increased levels of resistance to Bt crops in areas around Brazil have led to the high application of insecticides, which is more than likely the result of increased resistance to these synthetic products, including diamides (Richardson et al. 2020). These recent diamide resistance discoveries further highlight the need for new chemistries targeting novel sites to slow down the regulatory pressure and globalization of this pest (Richardson et al. 2020).

Considering the dwindling efficacy of commercialized chemical classes to FAW populations, there is need to ensure that insecticide resistance detection and management adequately addresses the evolving landscape of resistance mechanisms. Thus, we aim to address existing deficiencies in the field of FAW resistance and resistance management in Chapter 2 by developing an electrophysiological assay to measure the spontaneous activity of the FAW central nervous system and provide proof-of-concept method for the rapid quantification of reduced potency within a field-collected population of FAW at the level of the nerve.

1.4. Natural Products as Insecticides

The identification of new chemistries that augment the suite of products commercialized for FAW management is of significant interest considering the expansion of FAW across the world, the few available classes of chemistry, and the rapid development of resistance to these chemistries (Carvalho et al. 2013; Committee 2016; Goergen et al. 2016; Sharanabasappa et al. 2018). Natural products (NPs), derived from the primary or secondary metabolites of living cells in plants, fungi, and bacteria, have been used to develop new insecticides because of the

evolutionary arms race between plants and insects (Sparks et al. 2020; Sparks, Crouse, and Durst 2001; Sparks, Hahn, and Garizi 2017). Not surprisingly, NPs have grown to serve as models for a number of pest control agents currently on the market such as synthetic pyrethroids and the strobilurin fungicides (Sparks, Hahn, and Garizi 2017; Sparks, Crouse, and Durst 2001; 'Natural Products as Drugs and Leads to Drugs: An Introduction and Perspective as of the End of 2012' 2014). Approximately 65 percent of major insecticide classes today have origins to a NP model or derivative (Sparks, Crouse, and Durst 2001; Sparks, Hahn, and Garizi 2017). Although NPs are a great source for inspiration of synthetic insecticides and an abundance of new NPs are discovered each year, the commercialization of natural chemistry remains low due to concerns regarding chemical complexity, expensive synthesis costs, and poor pharmacokinetic properties (Sparks, Hahn, and Garizi 2017). Commercialization of natural products for agrochemical use is rare due to poor chemical properties that limit field use, such as low photostability, poor cuticular penetration, or low biological activity (Sparks, Crouse, and Durst 2001; Sparks, Hahn, and Garizi 2017). However, the fact that numerous insecticides are based on NPs highlights their relevance and importance to the agrochemical industry because they can serve as models and inspiration for chemical development (Sparks, Hahn, and Garizi 2017).

Pyrethrum is the only plant-based NP insecticide that has inspired the development of synthetic derivative products: pyrethroids (Sparks, Hahn, and Garizi 2017; Ross 2011). Pyrethroids have similar modes of action to pyrethrum but are much more stable in sunlight and the environment, rendering them more suitable for commercial application when compared to natural pyrethrins (Ross 2011). Considering this, plant-based NPs serve as an important source of new chemistry development because of the impacts already displayed in the insecticide world.

Furthermore, it is important that we continue to use NPs as a means of developing new chemistries, with new modes of action, which can potentially be used to control insect pest populations.

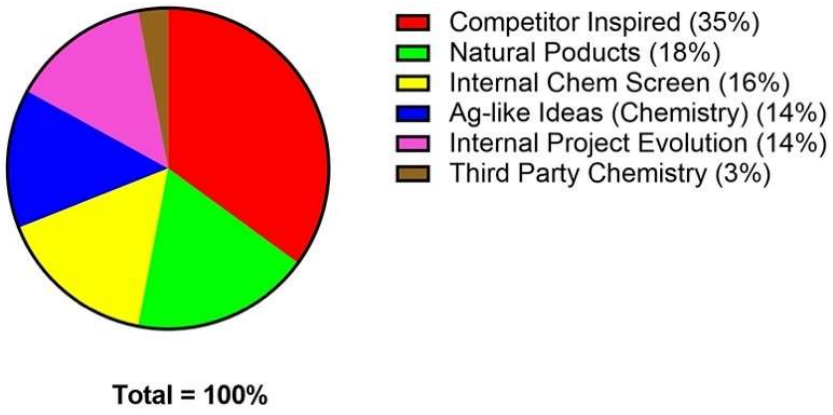


Figure 1.1. Varying approaches to agrochemical discovery and their origins since 1990 (Sparks and Lorsbach 2017).

1.5. Chromene Inspired Chemistries

Chromenes are heterocyclic compounds that consist of a benzene ring fused to a pyran nucleus (Majumdar et al. 2015). The use of chromenes has grown in medical and agrochemical fields due to their potential for mitigating illnesses, as well as providing toxic attributes for potential insecticides. The most commonly used chromenes as natural products, 2H-Chromenes (2H-1-Benzopyran derivatives), provide several biological activities that make them essential for use in a variety of products (Majumdar et al. 2015). Its unique structural motif (Costa et al. 2016) is displayed in many of the current natural products (polyphenols and tannins found in teas, vegetables, and fruits) and medicines. Due to its important biological activities, the 2H-Chromene skeleton has arisen in numerous products showing anticancer, anti-inflammatory, antitumor, anti-microbial, antioxidant, diuretic, and anti-HIV activity properties, while including itself in several others as well (Majumdar et al. 2015; Patil et al. 2013). Many of the anticancer and antitumor related 2H-Chromenes were extracted from plants and provided extremely effective toxicity to

cancer cell lines throughout the body (Costa et al. 2016). Additionally, antioxidant chromene activity was specific in its inhibition of generation of superoxide anions (Costa et al. 2016). Furthermore, these have appeared to act on physiological aspects (potassium channels) and biological aspects (enzyme inhibition) in humans, while also developing as agents in the technical field (lasers, optical brighteners, fluorescence probes, etc.) (Majumdar et al. 2015). Thus, the potential presented in the benzopyran moiety of chromenes has generated interest regarding its utility as a structural backbone of new insecticides (Costa et al. 2016; Majumdar et al. 2015).

As mentioned above, 2H-chromenes serve in a multitude of products and medicines and could even have the potential to be incorporated as active agents in insecticides. Consequently, natural products from various plants containing these chromenes have been researched and tested as possible insecticides (Meepagala, Estep, and Becnel 2016; Meepagala et al. 2011). In particular, *Amyris texana* (Rutaceae) is a flowering plant that contains biologically active secondary metabolites that have antimicrobial and insecticidal components (Meepagala et al. 2011). Considering this, a chromene amide, N-[2-(2,2-Dimethyl-2H-chromen-6-yl)-ethyl]-3,N-dimethylbutyramide, (Fig. 1.2) was extracted, purified, and was found to be moderately toxic to termites (Meepagala et al. 2011). Additionally, a second study showed that specific chromene analogs possess repellent properties to *Aedes aegypti* at a similar potency rate to N,N-Diethyl-meta-toluamide (DEET), but with a longer duration (Meepagala et al. 2013). Further, these analogs were also found to be toxic to *Ae. aegypti* larvae and adults.

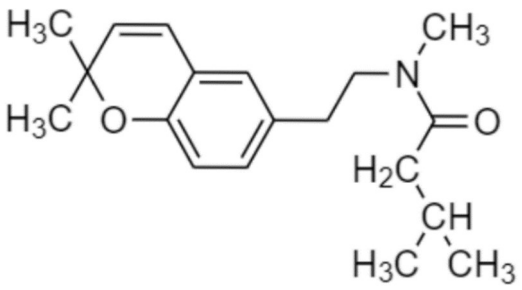


Figure 1.2. Structure of chromene amide used for chromene analog derivatives (Meepagala et al. 2011).

1.6. Overarching Hypothesis to be Tested

Considering 1) resistance to multiple insecticide modes of action within multiple FAW populations is established across multiple continents, 2) insecticide resistance management decisions are heavily influenced by genotypic screens and not phenotypic analyses, and 3) chromene structures are implemented into many natural products, medicines, long-term illness treatments, and potential insecticides (Costa et al. 2016; Majumdar et al. 2015; Meepagala et al. 2013; Meepagala, Estep, and Becnel 2016; Meepagala et al. 2011), we hypothesized that neurophysiological assays would provide a more holistic perspective in regards to the mechanism of resistance and a more direct comparison to changes in acute toxicity and ultimately enhance IPM strategies. Secondly, we hypothesized that natural product-based chromene analogs isolated from Texas Torchwood (*Amyris texana*) would have insecticidal and feed deterrent activity against *Spodoptera frugiperda*.

1.7. Objectives of Study

The overarching goals of this study were to determine the predictability of high pyrethroid resistance by the *kdr* genotype and to characterize the toxicological profile of naturally derived chromene analogs to FAW.

1. Define and address deficiencies in insecticide resistance monitoring approaches for fall armyworm
2. Determine the toxicant effects of natural product-derived chromene analogs through injection, ingestion, and neurophysiological application

Chapter 2. Reduced neuronal sensitivity and susceptibility of the fall armyworm, *Spodoptera frugiperda,* to pyrethroids in the absence of known knockdown mutations

2.1. Introduction

The fall armyworm (FAW), *Spodoptera frugiperda* (Lepidoptera: Noctuidae), is a polyphagous herbivore that is known to be a major insect pest of multiple economically important row crops, such as corn, cotton, sorghum, and rice (Koffi et al. 2020; Montezano et al. 2018). The ability of FAW to feed on a wide range of host plants, the occurrence of multiple generations in a single growing season, and their ability to migrate make FAW one of the most significant economic pests of the Western Hemisphere. If left uncontrolled, FAW has been documented to cause up to 100% crop yield loss and global economic losses have been estimated to be upwards of $6 billion USD annually (Blanco, Chiaravalle, Dalla-Rizza, Farias, Garcia-Degano, et al. 2016; International 2017). In addition to the economic damage, FAW is a serious threat to the food security of millions of people as FAW populations have recently become established across Africa, India, and China (Goergen et al. 2016; Sharanabasappa et al. 2018; Wu et al. 2019; Koffi et al. 2020), which rely heavily on maize and rice as staple food crops. To mitigate these global economic and food security concerns, synthetic insecticides remain a significant component of FAW control programs (Brookes and Barfoot 2016; Blanco, Chiaravalle, Dalla-Rizza, Farias, Garcia-Degano, et al. 2016; Gutiérrez-Moreno et al. 2019) despite the use of *Bacillus thuringiensis* technologies. Unfortunately, insecticide use rates have been documented to be extreme as is evidenced by Mexican maize farmers using an estimated 3,000 tons of synthetic insecticides per year to control FAW (Blanco et al. 2014) and African countries using copious amounts of synthetic insecticides as an emergency response to slow immigration into new regions of the continent (Fotso Kuate et al. 2019).

As with other insect pests, the evolution of insecticide resistance is likely to be amplified in FAW by the high use rates and limited availability of registered mechanisms of action. Thus, current resistance management practices suggest alternating between five foliar insecticide mechanism of actions based on pre-planting, planting, vegetative, and reproductive stages of the plant as well as incorporating seed treatments and transgenic technologies (Committee 2016). However, despite the implementation of resistance management practices, field-evolved resistance to multiple classes of synthetic insecticides has occurred in many populations across the world (Gutiérrez-Moreno et al. 2019), which threatens the efficacy of current FAW control paradigms. Furthermore, although multiple insecticidal classes are available and suggested for use in FAW control programs, pyrethroids and organophosphates remain the most commonly used chemical insecticide classes ("Agrofit: Sistema de Agrotóxicos Fitossanitários" 2013).

Pyrethroid resistance in lepidopteran pests is multifactorial and considered to be due to reduced penetration, increased metabolism, and altered target-site sensitivity (Carvalho et al. 2013; Ottea, Ibrahim, Younis, Leonard, et al. 1995; Nicholson and Miller 1985a; Yu, Nguyen, and Abo-Elghar 2003). Furthermore, lambda (λ)-cyhalothrin resistance in FAW was shown to be driven by multiple recessive genes and additional studies have identified three different *kdr*- and super *kdr*-type mutations (Carvalho et al. 2013; Ríos-Díez and Saldamando-Benjumea 2011) that are analogous to known house fly gene regions causing insensitivity to pyrethroid insecticides (Miyazaki et al. 1996; Soderlun and Lee 2001). Although metabolic and non-metabolic mechanisms are known to contribute to reduced pyrethroid susceptibility, the majority of efforts to determine mechanisms of resistance have focused on genetic analyses that identify target-site mutations analogous to *kdr* mutations established in model arthropod pests. Although this is a general predictor of insecticide sensitivity within that arthropod population, the presence or

absence of target-site mutations does not necessarily predict the neuronal sensitivity or resistance ratios (RR) to the insecticide within the population of interest. Therefore, this study aimed to develop an electrophysiological assay to measure the spontaneous activity of the FAW central nervous system and provide a proof-of-concept method for the rapid quantification of reduced potency within a field-collected population of FAW at the level of the nerve.

2.2. Methods

2.2.1. Compounds and Compound Synthesis

Permethrin, λ-cyhalothrin, and dichlorvos were all purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The organophosphates chlorpyrifos and chlorpyrifos-oxon were purchased through ChemService Inc. (West Chester, PA, USA). All compounds were > 95% purity. Karate Z® was generously donated by Dr. Sebe Brown (Assistant Professor, Louisiana State University). The solvents dimethyl sulfoxide (DMSO) and absolute ethanol were purchased from Sigma-Aldrich Chemical Co. A molecular sieve OP type 3Å was purchased from Sigma-Aldrich and used to prevent water absorption within the DMSO stock.

2.2.2. Spodoptera frugiperda Stock and Rearing Conditions

The laboratory colony of FAW used in this study was initially established in 2005 from cotton fields at the Macon Ridge Research Station in Winnsboro, Louisiana. The colony has been maintained in the Department of Entomology at Louisiana State University and will be referred to as LSU-Lab-1 hereafter. LSU-Lab-1 was genetically confirmed as being the corn-strain (Gordy et al. 2015) and was maintained as previously described (Gordy et al. 2015). Briefly, larvae were reared on artificial diet (Stonefly Heliothis Diet, Ward's Natural Science, Rochester, NY, USA) in

30-mL plastic cups. Eggs were deposited on the cheesecloth and 20-30 neonates were placed in 8-cell trays (Bio-Serv, Frenchtown, NJ, USA) as they emerged. Larvae for experiments were kept on diet for approximately 7-8 d until they exhibit signs of molting. Cohorts of molting larvae were synchronized at head capsule slippage and starved overnight before use in insecticide toxicity bioassays.

To compare insecticide susceptibility between laboratory and field strains, late corn was planted at the Macon Ridge Research Station in Winnsboro, Louisiana and FAW larvae were collected in whirls of corn plants. The field strain of FAW is referred to as LSU-Field-1 hereafter. Larvae were raised to adulthood, mated, and reared as described above. The F1 generation was used for insecticide toxicity bioassays or neurophysiological recordings.

2.2.3. Insecticide Toxicity Bioassays

The laboratory susceptibility of third-instar larvae (approximately 10 mg/individual) to λ-cyhalothrin and chlorpyrifos was determined with a topical bioassay where larvae were treated with 1 μL of 95% ethanol (control) or ethanol containing the insecticide applied to the thoracic dorsum using a hand-held pipette (Gilson, Middleton, WI, USA). Treated larvae were held at 25 °C with a 14:10 h (light:dark) photoperiod and mortality was assessed at 24-h post treatment. Mortality was defined as the inability for coordinated movement within 10 sec after prodding with a needle. The dose required to kill 50% of the population (lethal dose, LD_{50}) was calculated using GraphPad Prism (GraphPad Software, San Diego, CA) and was constructed using 6-9 concentrations that ranged from 0% to 100% mortality. Each concentration consisted of 3 replicates of 10 larvae/replicate. A total of three LD_{50} values were obtained from separate cohorts, and the mean LD_{50} value was used for the generation of the resistance ratio (field colony

LD_{50}/laboratory colony LD_{50}). For all toxicity bioassays, control mortality never exceeded 10% and Abbots formula (Abbot 1925) was used to correct for control mortality.

Karate Z® (Syngenta Crop Protection, Greensboro, NC, USA), a λ-cyhalothrin formulated product, toxicity was evaluated following previously established methods (Leonard et al. 1990). Susceptibility to Karate Z® was compared between laboratory- and field-collected strains at the highest recommended label rate (1.92 oz/acre) by dissolving 1.92 oz of product into 20 gal of water. Third-instar FAW were treated with 1 µL aliquot on the thoracic dorsum and toxicity was assessed at 24 h as described above. Mean percent mortality was determined based on three individual replicates that were conducted on separate days where each replicate consisted of 3 replicates of 30 individuals. The mortality of field and laboratory strains were statistically compared using an unpaired *t*-test with significance at $p < 0.05$. The toxicity of chlorpyrifos formulated product was not able to be determined due to a limited number of F1 progeny from field-collected individuals.

2.2.4. Neurophysiological Assays

Extracellular recordings of spontaneous neuronal activity from the FAW central nervous system (CNS) were performed based on methods described for *Drosophila melanogaster* (Swale et al. 2018; Bloomquist, Roush, and ffrench-Constant 1992) and *Heliothis virescens* (Ottea, Ibrahim, Younis, Leonard, et al. 1995; Nicholson and Miller 1985a; Ottea and Holloway 1998a). The central nervous system was dissected from third-instar FAW as shown in Figure 2.1.A and immediately bathed in 200 µL physiological saline for lepidopteran neural systems (Salgado et al. 1998) containing: 140 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 5 mM HEPES, 28 mM D-glucose, pH: 7.4. The CNS was manually transected between the second and third ganglia to disrupt the blood

brain barrier that enhanced chemical penetration (Swale et al. 2018; Chen and Swale 2018). Glass pipette electrodes were pulled from borosilicate glass capillaries on a P-1000 Flaming/Brown micropipette puller (Sutter Instrument, Novato CA, USA) and peripheral nerve trunks from the thoracic or abdominal ganglia were drawn into the suction electrode. Electrical activity was monitored from descending nerves and was amplified by an AC/DC amplifier (Model 1700, Systems, Inc., Carlsborg, WA, USA). Descending electrical activity was subjected to window amplitude discrimination and converted on-line into a rate plot, expressed in Hertz (Hz), using LabChart7 Pro (ADInstruments, Colorado Springs, CO, USA). Noise (60 Hz) was eliminated using Hum Bug (A-M Systems, Sequim, WA, USA). Activity was monitored for 10 min to establish a constant baseline firing rate and, after a baseline was established, the CNS preparation was directly exposed to test compounds by adding 200 µL of solution to the bath containing 200 µL of saline. The mean baseline firing rate for recordings used to study sensitivity to insecticides was 25-40 Hz and any recordings outside of this range were not included in the data analysis. The final concentration of solvent in the bath was 0.1% DMSO or less. Frequencies were measured for 5-8 min for each concentration prior to the addition of the next drug concentration. Individual concentrations were performed on individual preparations to ensure the effect to CNS firing was not due to sequential application of increasing drug concentrations. Mean spike discharge frequencies for each concentration were used to construct concentration-response curves to determine EC_{50} values that were calculated by non-linear regression (variable slope) using GraphPad Prism™ (GraphPad Software, San Diego, CA, USA). Each drug concentration was replicated 5-10 times.

2.2.5. RNA extraction and cDNA synthesis

The RNA extraction area and materials were treated with RNase-zap® (Ambion Inc., Foster City, CA). Twenty individuals of each strain were used for the following steps. The samples were preserved in 95% ethanol at -80 °C and needed to be rehydrated before extraction. Each larva was immersed in decreasing concentrations of ethanol prepared with nuclease-free water (70%, 50%, 30%, 10%) for 2 min before being transferred to nuclease-free water for 2 min and proceeding to the extraction. RNA extraction was conducted using Tri Reagent RT (Molecular Research Center, Inc., Cincinnati, OH) and 1 µg of RNA per 20 µL reaction volume was reverse transcribed using the iScript cDNA reverse transcription kit following manufacturer recommendations (Biorad, Hercules, CA).

2.2.6. Semi-nested PCR and product sequencing

The PCR amplification of *ace,* the gene encoding acetylcholinesterase (AChE), and *para*, the gene encoding voltage-gated sodium channel (VGSC), regions containing the main mutations suspected to confer resistance to organophosphate or pyrethroid insecticides in FAW, were conducted following previous methods (Carvalho et al. 2013). Two microliters of the cDNA produced for each individual larva were used in a first amplification step using the AceF2Lep and AceSfR1 primers following a second PCR using a different reverse primer, AceSfR2, with the PCR product from the first reaction serving as template for the second PCR. The product of the second PCR amplification was *ca*. 1 kb. Temperature cycling conditions were 95 °C for 1 min followed by 35 cycles of 95 °C for 30 sec, 55 °C for 30 sec and 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. The amplification of the *vgsc* (or sodium channel) also followed the same protocol using cDNA and NaChF1Sf and NAChR1Sf in the primary PCR and NaChF2Sf and NaChR1Sf in the second PCR as well as the product of the first amplification as a

template. The final product of this amplification was 350 bp. The temperature cycle was similar to that for *ace* amplification, except the annealing temperature was 58 °C. All PCRs were conducted with a GoTaq® Flexi DNA polymerase kit (Promega, Madison, WI) on a S1000 Biorad thermal cycler. All primers were synthesized by Sigma-Aldrich Chemical Co.. The 1 kb *ace* and 350 bp *vgsc* PCR products were visualized for 20 individuals on a 1.2% agarose gel run for 70 min at 76 V and 400 mAmps followed by purification using a QIAquick® gel extraction kit (Qiagen, Germantown, MD) using manufacturer's recommendations. Sequencing was conducted by Genewiz (location) using the primers of the second PCR for each target.

2.2.7. Sequence alignments and identification of mutations in the laboratory and field population

All sequences were aligned with the Clustal Omega multiple alignment tool (Sievers et al. 2011) and compared to the published sequences for organophosphate- and pyrethroid-susceptible and -resistant strains of FAW (Carvalho et al. 2013), NCBI accession numbers for the published sequences are: KC435023.1 (FAW *ace* susceptible), KC435024.1 (FAW *ace* resistant), KC435025.1 (FAW *vgsc* susceptible), and KC435026.1 (FAW *vgsc* resistant). Additional visualizations were conducted using Geneious Prime® 2020.0.5 and simplified alignment files corresponding to the positions of interest were generated for each mutation.

2.2.8. Quantitative PCR of detoxification transcripts of the field and laboratory populations

The cDNA produced for the sequencing experiment was also used for the quantitative PCR (qPCR) and originated from nine individuals, including three laboratory-strain individuals and six field-collected individuals. The field-collected individuals were selected as follows: five presented point mutations in the *ace* sequence with two heterozygotes with A201S, one homozygote with

G227A, and two heterozygotes with G227A. The last individual had no mutation. Primers are described in Suppl. Table 1 and were selected for genes that were found to significantly differ between insecticide-susceptible and -resistant laboratory strains (Carvalho et al. 2013). Six detoxification genes (EST-9555, EST-0974, GST-1950, GST-0801, GST-0968, CYP-9131) were selected due to their expected expression patterns between insecticide-susceptible and -resistant FAW as previously described (Carvalho et al. 2013). The two housekeeping genes 28S ribosomal RNA (28S) and elongation factor 1 (EF1) were used as reference genes. All primers were synthesized by Sigma-Aldrich Chemical Co. Primer efficiency and qPCR were conducted on a Biorad CFX Connect Real-Time System with iTaq Universal SYBR® Green Supermix. The PCR cycle protocol replicated the program from Carvalho et al (Carvalho et al. 2013). Each reaction consisted of 2 µL of cDNA, 0.5 µL of each primer at 10 µM, 5 µL of SYBR® green and 2 µL of water, for a total volume of 10 µL. The cDNA used was diluted to obtain 10 ng per reaction and each condition was repeated in technical triplicates. Outlier CT values were removed before data analysis.

Two analyses were conducted, including a MCMC.qPCR package (Matz, Wright, and Scott 2013) in R and $2^{-\Delta\Delta CT}$ to measure statistical significance based on non-overlapping 95% confidence intervals (*13*). The MCMC.qPCR package gathers information from primer efficiency and CT values to estimate copy numbers using a generalized linear mixed model (GLMM). The housekeeping genes 28S and EF1 were used as reference genes. The population status was the factor evaluated with two levels, including the field population and the susceptible population, which served as the reference. *P*-values were corrected for multiple tests by applying the method of Benjamini and Hochberg (1995) with a false discovery rate of 5%.

2.3. Results

2.3.1. Spontaneous firing rate of thoracic and abdominal ganglia of 3rd-instar FAW

To determine the ideal ganglia to use for determinations of potency for insecticides, we compared the spontaneous discharge frequency of abdominal and thoracic ganglia over a 60-min period. For thoracic ganglia, the mean firing rate was found to be 25 ± 6 Hz at 0-10 min and increased to an average firing rate of 31 ± 5 Hz, 31 ± 4 Hz, 29 ± 3 Hz for 10-20 min, 20-30 min, and 30-40 min, respectively, which were not significantly different from each other (Fig. 2.1.C). A significant ($p < 0.05$) reduction in firing rate was observed with a mean discharge rate of 16 ± 5 Hz at 40-50 min and 11 ± 2 Hz at 50-60 min (Fig. 2.1.C). This suggests recordings from the thoracic ganglia should be terminated 40 min after the initiation of recording.

The mean firing rate of the thoracic ganglia was significantly ($p < 0.05$) greater than the firing rate from abdominal ganglia for time points 0-40 min (Fig. 2.1.C). The mean firing rate at 0-10 min was found to be 8 ± 2 Hz, which was not significantly different from 10-20 min or 20-30 min time points. A statistically significant ($p < 0.05$) increase in firing rate was observed at 30-40 min with a mean rate of 14 ± 7 Hz, but this rate is still significantly lower than that observed from the thoracic ganglia. No statistically significant difference between thoracic and abdominal ganglia was observed at 40-50 min or 50-60 min (Fig. 2.1.C). Representative recordings for thoracic and abdominal ganglia are shown in Fig. 2.1.D and 2.1.E, respectively.

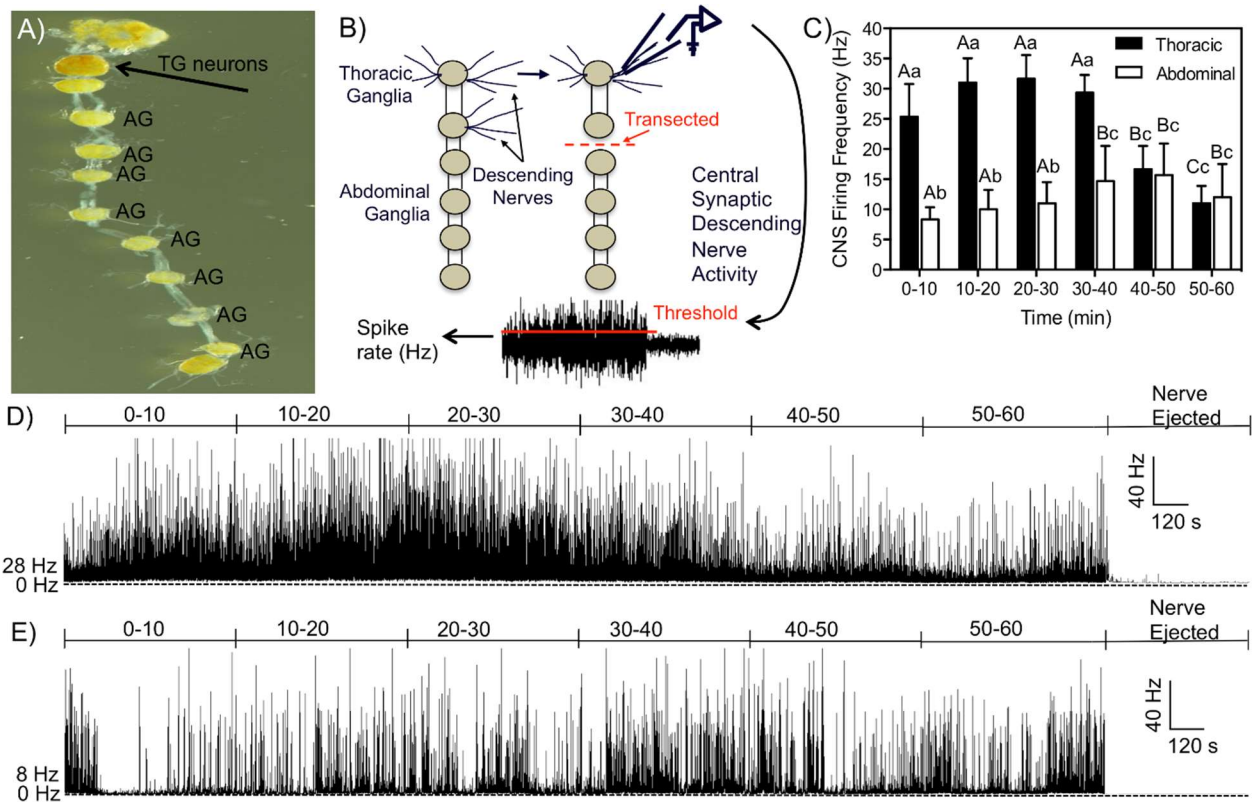


Figure 2.1. Neurophysiological recording methodology. **A)** Dissection of FAW CNS showing thoracic ganglia (TG) and abdominal ganglia (AG) along with descending neurons originating from the TG. **B)** Schematic diagram showing the recording arrangement, tissue preparation, and configuration of the electrodes. **C)** Mean (n > 10 individual recordings) spike discharge frequencies of the FAW CNS from the thoracic (black bars) and abdominal (white bars) ganglia in 10-min increments for a total recording period of 60 min. Statistical significance is denoted by letters where bars labeled with different uppercase letters denotes significance ($p < 0.05$) between ganglionic types across time points and bars labeled by different lowercase letters denotes significance ($p < 0.05$) between ganglionic type within the same time point. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by a multiple comparison post-test. **D-E)** Representative recordings for thoracic and abdominal ganglia, respectively.

2.3.2. Potency of select pyrethroid and organophosphate insecticides

Pyrethroids and organophosphates continue to be the most commonly used insecticide classes for FAW population control and, thus, the aim was to establish baseline potency values for standard pyrethroid and organophosphate insecticides to CNS firing rates of LSU-Lab-1 strain of FAW. Permethrin was found to be moderately potent with 1 μM resulting in peak firing rate that was a 3.3-fold increase over baseline firing. Concentration dependency of permethrin is shown in

Fig. 2.2.A and the concentration required to induce 50% increased firing rate (EC_{50}) was found to be 418 nM (95% CI: 306-571 nM, Hillslope: 3.9, r^2: 0.84). An inhibition of nerve firing was observed at a concentration of 3 μM (Fig. 2.2.A). The type 2 pyrethroid, λ-cyhalothrin, was shown to be 199-fold more potent to the FAW CNS when compared to permethrin (Fig. 2.2.B), but the peak firing rate was greater for permethrin than λ-cyhalothrin. Peak firing rate after exposure to λ-cyhalothrin occurred at 10 nM with no significant increase in firing at concentrations up to 300 nM. Concentration dependency of λ-cyhalothrin is shown in Fig. 2.2.B and the EC_{50} was found to be 2.1 nM (95% CI: 0.7-7 nM, Hillslope: 1.3, r^2: 0.8). In contrast to permethrin, inhibition of nerve firing was not observed at any concentration studied (Fig. 2.2.B). Representative recordings for permethrin and λ-cyhalothrin are shown in Fig. 2.2.A and 2.2.B, respectively.

Dichlorvos was found to be highly potent to FAW (LSU-Lab-1) CNS with 100 nM resulting in peak firing rate that was 2-fold over baseline firing rates with no significant changes in firing rates up to 1 μM. Concentration dependency of dichlorvos is shown in Figure 2.2.C and the EC_{50} was found to be 24 nM (95% CI: 13-45 nM, Hillslope: 1.1, r^2: 0.87). Chlorpyrifos-oxon was found to have similar potency when compared to dichlorvos with an EC_{50} of 15 nM (95% CI: 8-27 nM, Hillslope: 1.2, r^2: 0.86), which is shown in Fig. 2.2.D. However, 9 of the 11 preparations exposed to 300 nM chlorpyrifos-oxon had a significant increase in firing rate followed by a rapid cessation of firing and subsequent nerve death (representative trace, Fig. 2.2.D), which was not observed with dichlorvos (representative trace, Fig. 2.2.C).

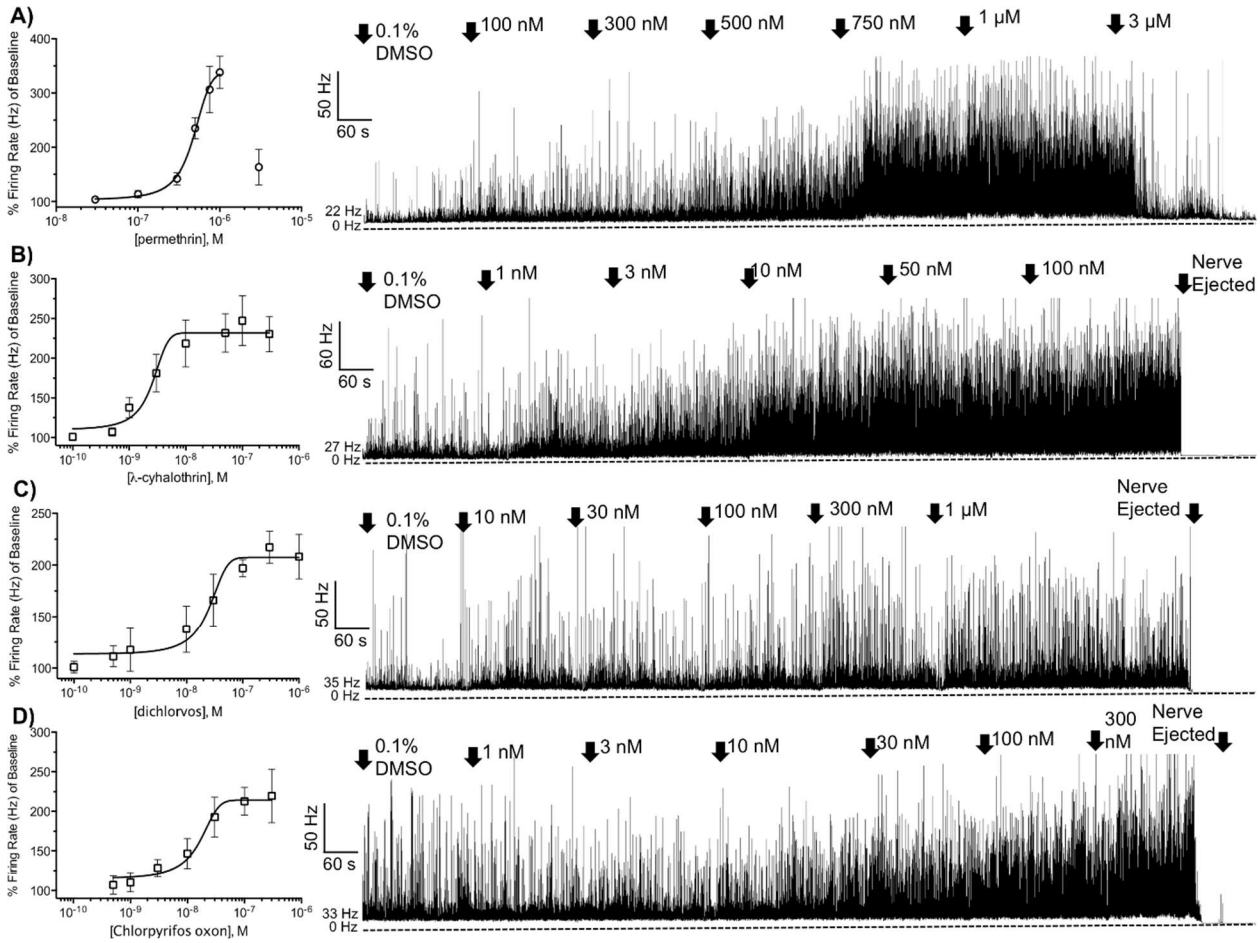


Figure 2.2. Potency determinations for pyrethroid and organophosphate insecticides to *S. frugiperda* CNS firing rates. Concentration-response curves and representative recordings for permethrin **(A)**, λ-cyhalothrin **(B)**, dichlorvos **(C)**, and chlorpyrifos-oxon **(D)**. Baseline firing rates are denoted to the left of each representative recording and 0 Hz is represented by the dashed line. Data points represent means from replicated recordings (n = 5-12 preparations per curve, with each concentration replicated a minimum of 4 times). Data points represent mean percentage increase of baseline firing rate and error bars represent SEM. For permethrin, the inhibition of CNS firing at 3 µM was excluded from the non-linear regression analysis to ensure accurate generation of an EC_{50}.

2.3.3. Differential target-site sensitivity between laboratory and field strains of S. frugiperda

A premise of this work was to validate the extracellular CNS recording as an appropriate method for rapid quantification of insecticide resistance levels within a field-collected population of FAW at the level of the nerve. Thus, the survival rates and CNS sensitivity were compared between the LSU-Lab-1 strain to F1 progeny of field-collected FAW exposed to λ-cyhalothrin and

chlorpyrifos. λ-Cyhalothrin was observed to be highly toxic to LSU-Lab-1 (Fig. 2.3.A) with a dose required to kill 50% of the population (LD_{50}) of 51 pg/mg of insect (95% CI: 37-69 pg/mg, Hillslope: 1.3, r^2: 0.94). A 767-fold reduction of mortality was observed with LSU-Field-1 (LD_{50}: 38 ng/mg of insect, 95% CI: 8-91 ng/mg, Hillslope: 0.62, r^2: 0.89) after topical exposure to λ-cyhalothrin when compared to LSU-Lab-1, which is shown in Fig. 2.3.A. The high RR after topical exposure to λ-cyhalothrin provided a comparison of λ-cyhalothrin potency to CNS activity. Similarly, LSU-Field-1 strain was found to be 1,749-fold less sensitive to λ-cyhalothrin (EC_{50}: 3.6 μM, 95% CI: 1.5-6.8 μM, Hillslope: 1.1, r^2: 0.74) when compared to LSU-Lab-1 strain (Fig. 2.3.B), which indicates resistance to this pyrethroid insecticide at the target site. To determine if a topical toxicity RR of 767 (Fig. 2.3.A) and a 1700-fold neuronal RR (Fig. 2.3.B) results in product failure, the percent mortality was measured with Karate Z®, a formulated product of λ-cyhalothrin. As expected, LSU-Lab-1 resulted in 100 ± 0% mortality whereas 16 ± 9% mortality was observed with LSU-Field-1 L3 FAW (Fig. 2.3.C) and, in turn, the likelihood for product failure in the field.

In addition to λ-cyhalothrin, the ability of the neurophysiological assay to detect differential potency to the organophosphate insecticide chlorpyrifos was tested between susceptible and resistant strains of FAW. The LSU-Lab-1 strain was highly sensitive to chlorpyrifos with an LD_{50} value of 9.9 ng/mg of insect (95% CI: 7-13 ng/mg of insect, Hillslope: 2.1, r^2: 0.98). Differential toxicity was observed in the LSU-Field-1 strain when compared to LSU-Lab-1 as LSU-Field-1 was 12.5-fold less sensitive to chlorpyrifos with an LD_{50} value of 124 ng/mg of insect (95% CI: 98-155 ng/mg of insect, Hillslope: 1.8, r^2: 0.97). The dose-response curves of chlorpyrifos for the two strains are shown in Fig. 2.3.D. To determine if the differential toxicity was due to reduced nerve sensitivity to chlorpyrifos, the potency of chlorpyrifos was

compared to CNS firing rates through the extracellular recording. Although the shift in potency between laboratory and field strains was less than observed for λ-cyhalothrin, a significant shift in potency was observed for chlorpyrifos as LSU-Field-1 was 7.7-fold less sensitive (EC_{50}: 218 nM, 95% CI: 147-321 nM, Hillslope: 2.3, r^2: 0.67) (Fig. 2.3.E). The toxicity of chlorpyrifos formulated product was not able to be determined due to a limited number of F1 progeny from field-collected individuals. The susceptibility differences of chlorpyrifos formulated product was not able to be determined due to a limited number of individuals of field-collected individuals.

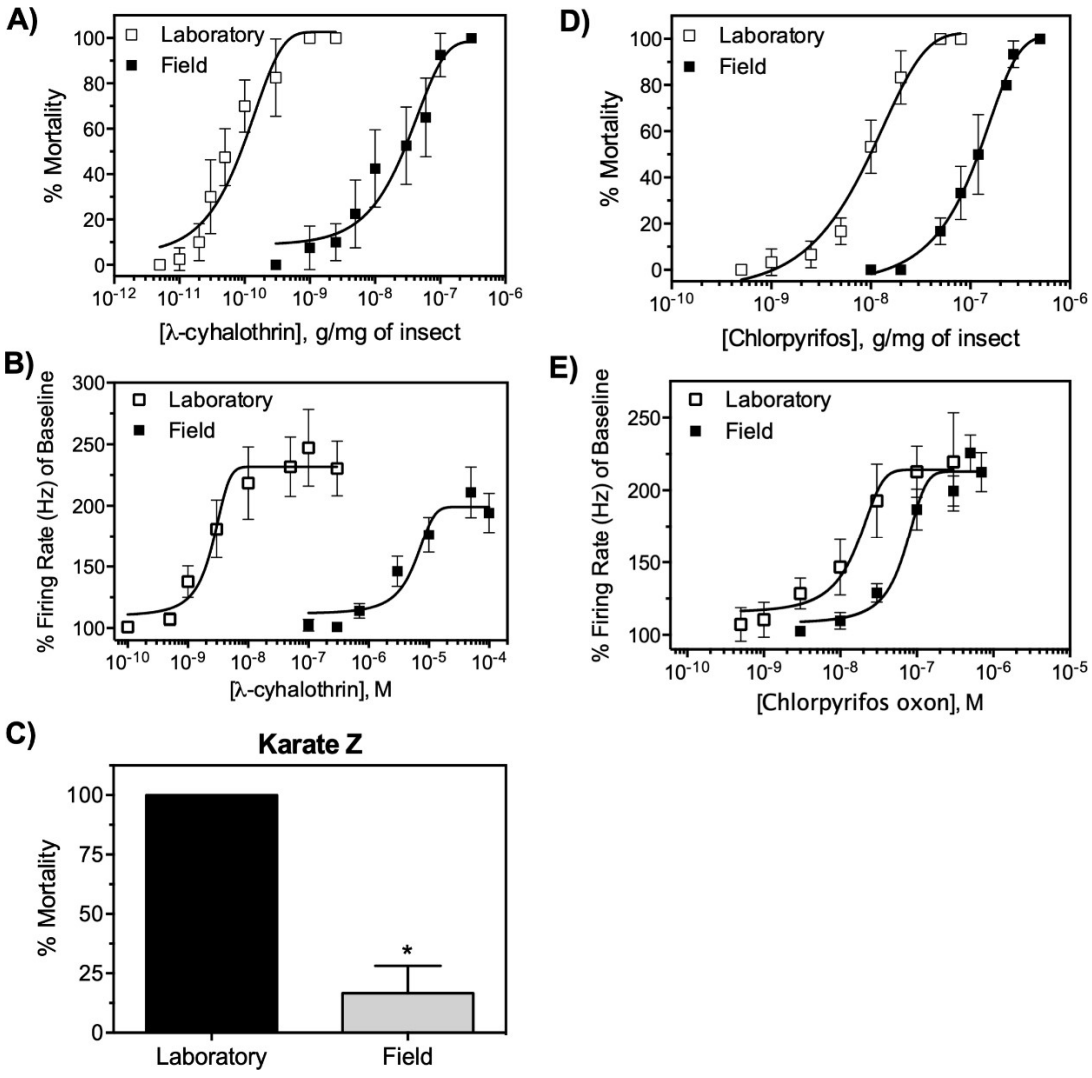


Figure 2.3. Comparison of topical toxicity and neuronal resistance ratios in two distinct populations of FAW. **A)** Topical toxicity of λ-cyhalothrin to 3rd-instar LSU-Lab-1 (open square)

and LSU-Field-1 (closed square) FAW. **B)** Neurophysiological recordings to determine differences of λ-cyhalothrin potency between LSU-Lab-1 (open square) and LSU-Field-1 (closed square) populations. **C)** Percent toxicity of Karate Z at the highest label rate for FAW where bars represent mean toxicity from 90 individuals. **D)** Topical toxicity of chlorpyrifos to 3rd-instar LSU-Lab-1 (open square) and LSU-Field-1 (closed square) FAW. **E)** Neurophysiological recordings to determine differences of chlorpyrifos oxon potency between LSU-Lab-1 (open square) and LSU-Field-1 (closed square) populations. For toxicity curves in panels A and D, data points represent mean percent mortality for each drug concentration and was determined by three independent replicates where each replicate was comprised of 3 treatment groups of 30 individuals each, thus each data point is comprised of 270 individuals. For neural potency curves in panels B and E, data points represent mean firing rate of baseline from replicated recordings (n = 5-12 preparations per curve, with each concentration replicated a minimum of 4 times).

2.3.4. Molecular genetic analyses of genes encoding VGSC and AChE

Complete nucleotide alignments for genes encoding FAW VGSC and AChE are shown in Suppl. Files 1 and 2, respectively. Twenty individuals were sequenced for each field and laboratory strain. Suppl. Fig. 2.5.1.-2.5.3. representing the sequencing data of *ace* for each individual and is simplified to highlight the region of alignment where mutations are expected based on published literature (A201S, G227A, and F290V) (Carvalho et al. 2013). Suppl. Fig. 2.5.1.-2.5.3. represents a consensus sequence generated for LSU-Lab-1 and LSU-Field-1 and is compared to KC435024.1 and KC435023.1, which represent published sequences for OP-resistant and OP-susceptible FAW, respectively. Suppl. Fig. 2.5.1. highlights the A201S mutation described for OP-resistant FAW and LSU-Lab-1 was found to be consistent with the OP-susceptible sequence (KC435023.1 (Carvalho et al. 2013)). However, the *ace* sequences of the 20 individuals from LSU-Field-1 show 18 of 22 individuals having a GCG or GCA at codon 201, which both encode alanine as observed with OP-susceptible individuals (KC435023.1). However, two individuals possessed multiple nucleotides at codon 201 that resulted in an alanine (GCG or GCA) or in a serine with the mutation TCA. These individuals are likely heterozygotes for this allele, which is consistent with the mutation found in the resistant population represented by KC435024.1 (TCG: serine). Fig. 2.5.2.

highlights the region containing the mutation G227A in KC435024.1, encoded by GCA, showing that LSU-Lab-1 is consistent with the published susceptible sequence with expression of GGA (glycine) at the codon 227. However, LSU-Field-1 was shown to possess either a GCA (encodes alanine) or GGA (encodes glycine) as observed for the A201S mutation. For the LSU-Field-1 population, 60% of the tested individuals carried a G to C point mutation in second position of the codon that results in a glycine to alanine substitution, which has been described in OP-resistant individuals (Carvalho et al. 2013). Interestingly, 67% of the individuals possessing the G227A mutation also possessed a G in the second position of the codon suggesting heterozygosity at this codon. The remaining 33% of the individuals possessing G227A mutation carried a C in the second position, which indicates they are homozygous for the allele GCA that encodes alanine. The *ace* mutation F290V to confer OP-resistance was F290V was examined, but no individuals of LSU-Lab-1 or LSU-Field-1 were detected to have this mutation (Fig. 2.5.3.).

For the gene encoding the FAW VGSC, no point mutations were identified in the laboratory or field strains that were previously described (Carvalho et al. 2013) to confer target site resistance (Fig. 2.5.4. and 2.5.5.). Specifically, the coding sequence of ACC to encode a threonine (T) at codon 929 and TTG to encode a leucine (L) was observed in LSU-Lab-1 and LSU-Field-1 as well as the OP-susceptible sequence KC435025.1 (Carvalho et al. 2013) (Fig. 2.5.4.). Similarly, the laboratory and field strains of FAW used in this study did not have the L1014F mutation that was previously described in the resistant FAW population (KC435026.1) (Carvalho et al. 2013) (Fig. 2.5.5.).

2.3.5 qPCR of detoxification genes shows differences between field and laboratory populations

GLMM-based method indicated three genes were differentially expressed between strains,

with over 5-fold differences (Fig. 2.4.A, Suppl. Table 2.6.1.). The expression of Est-0974 and GST-0968 was significantly higher ($P < 0.05$) in the susceptible individuals whereas GST-1950 was more abundant in field-collected individuals. The $2^{-\Delta\Delta CT}$ method was used, in addition to the GLMM-based method, to compared 95% confidence intervals. The expression of GST-0801 and GST-0968 was different between the two populations. Although the expression of GST-0801 was significantly higher ($P < 0.05$) in the field-collected individuals compared to the susceptible larvae, the expression of GST-0968 was significantly higher ($P < 0.05$) in the laboratory population (Fig. 2.4.B, Suppl. Table 2.6.3.).

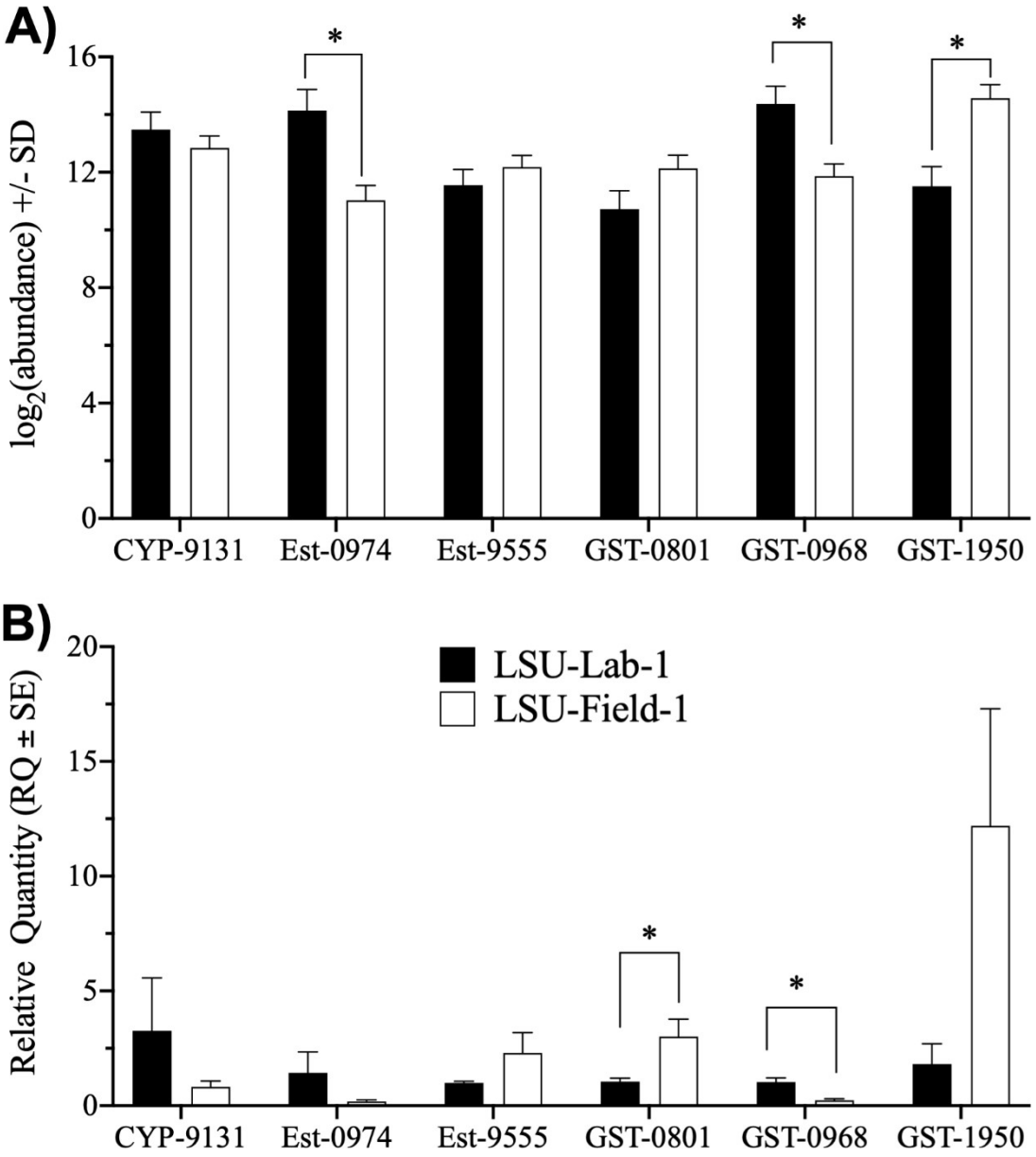


Figure 2.4. Gene expression patterns cytochrome P450s (CYP), esterases (EST), and glutathione *S*-transferases (GST) from laboratory-reared (LSU-Lab-1) and field-collected (LSU-Field-1) FAW**.** Vertical bars represent the **A)** $\log_2$(abundance) standard deviation and **B)** mean relative quantity standard error for each gene. Asterisks above the bars represent a significant difference between LSU-Lab-1 and LSU-Field-1**.**

2.4. Discussion

Previous studies have investigated the biochemical, molecular, and genomic characteristics of pyrethroid and organophosphate resistance in FAW with data indicating multiple mechanisms

for insecticide resistance (Carvalho et al. 2013; Yu, Nguyen, and Abo-Elghar 2003). Multiple mechanisms of pyrethroid resistance of other lepidopteran pests, such as *H. virescens*, has also been documented and it was suggested that expression of enhanced metabolism in the absence of reduced target-site sensitivity is inadequate to protect the pest from insecticide intoxication (Ottea, Ibrahim, Younis, Leonard, et al. 1995; Wilkinson and McCaffery 1991). The high correlation of target-site insensitivity and product failures in the field has driven genetic and genomic studies to identify point mutations that confer insensitivity to insecticides. For instance, previous studies have revealed the presence of point mutations in the alpha subunit of the voltage-gated sodium channel (VGSC) and acetylcholinesterase (AChE) enzyme that are known to confer pyrethroid and organophosphate resistance in dipteran insects, respectively (Carvalho et al. 2013; Negre et al. 2006). Yet, these studies simply conclude these mutations may modify target-site pharmacokinetics for the described model insects, but descriptions of these mutations do not specifically define changes in target-site sensitivity or toxicity of FAW to the neurotoxic insecticides. This is due to a myriad of reasons that include "silent" single nucleotide polymorphisms (SNPs) that do not confer reduced potency of ligands (ffrench-Constant et al. 1998) and that the resistance allele frequency required to yield insensitivity to the insecticide is dependent on the species. For instance, it has been shown for horn flies and mosquitoes that the heterozygous *kdr* genotype does not result in pyrethroid resistance and product failure (Foil et al. 2005; Brito et al. 2018; Martins et al. 2012), yet aphids heterozygous for the L1014F mutation in the sodium channel are resistant to pyrethroids (Foster et al. 2014). Furthermore, the lack of point mutations in a target site known to cause insecticide resistance, such as L1014F in the VGSC, has been shown to not correlate to nerve sensitivity or susceptibility (Abdalla et al. 2008; Matambo et al. 2007). Considering this, the aim of this study was to build on previous molecular work

conducted with FAW by employing neurophysiological recordings that quantify neuronal sensitivity of neurotoxic insecticides to facilitate direct comparisons between insecticide potency and differential toxicity across lab and field strains of FAW.

Previous neurophysiological assays have been developed to measure neuronal sensitivity of *H. virescens* to allethrin (Ottea and Holloway 1998a; Nicholson and Miller 1985a), but the expanding range and increased pest status of FAW has underscored the need to develop an easy, reproducible assay to measure neuronal sensitivity to neurotoxic insecticides for this lepidopteran pest. Previous work did not describe differences of baseline firing rates between thoracic and abdominal ganglia in lepidopterans, but our data indicate the thoracic ganglia is more amendable to neurophysiological recordings due to their high firing rates and prolonged stability when compared to abdominal ganglia (Figure 2.1.). These data may be slightly different than the stability of *Manduca sexta* as desheathed abdominal ganglia were used to determine the mode of action for spinosad (Salgado et al. 1998).

The observed levels of mortality suggest larvae from the LSU-Field-1 population were highly resistant (767-fold) to λ-cyhalothrin when compared to LSU-Lab-1. This level of resistance is more than 7-fold higher than those previously documented (Ríos-Díez and Saldamando-Benjumea 2011), which is potentially due to the differential sensitivities of laboratory colonies or resistance estimates for different FAW generations. The resistance to λ-cyhalothrin in FAW is autosomal recessive (Jia et al. 2009; Ríos-Díez and Saldamando-Benjumea 2011) and indicates resistance levels will increase in a non-additive manner across generations. Unfortunately, the generational stage is unknown for LSU-Field-1 due to collection of larvae from late-whorl stage corn that prevents direct comparisons of insecticide susceptibility. It is also important to note that the Hillslope for the dose-response curve of LSU-Field-1 after topical exposure to λ-cyhalothrin

was 0.6, which is half the Hillslope for LSU-Lab-1 and, in turn, may indicate heterogeneity in the population (Fig. 2.3.A). Although, concentration-response curves with λ-cyhalothrin against the spontaneous firing of the CNS produced a Hillslope of near unity (Fig. 2.3.B), which indicates heterogeneity of the population is not likely to be based at the target site. On the contrary, LSU-Field-1 was significantly less resistant to the organophosphate chlorpyrifos when compared to λ-cyhalothrin with a topical toxicity RR of *ca*. 12-fold. This level of resistance is similar to others described for methomyl (Diez-Rodriguez and Omoto 2001; Ríos-Díez and Saldamando-Benjumea 2011), a carbamate insecticide that inhibits the same target as organophosphate insecticides. The differing toxicity RR for λ-cyhalothrin and chlorpyrifos enabled a comparison of differential neuronal sensitivity between LSU-Lab-1 and LSU-Field-1.

Neurophysiological recordings with chlorpyrifos-oxon show the LSU-Field-1 to be *ca.* 7-fold less potent at the neuron, which likely accounts for the majority of the 12-fold acute toxicity RR between the two populations (Fig. 2.3.D-E). The remaining 5-fold difference in toxicity could be due to increased vigor of the field strain compared to a laboratory strain, metabolic detoxification enzyme levels, or other mechanisms. Surprisingly, neural sensitivity to λ-cyhalothrin in the LSU-Field-1 population was 1700-fold less when compared to LSU-Lab-1 (Fig. 3B), yet we only observed a 700-fold reduction in toxicity (Fig. 2.3.A). This finding was unanticipated as a greater shift in acute toxicity was expected between the two strains and not neural sensitivity, which was observed for chlorpyrifos. A greater neuronal RR when compared to acute toxicity RR could potentially be explained by LSU-Lab-1 having increased esterase hydrolysis activity than LSU-Field-1, an artifact of *in vivo* versus *in vitro* methodologies, or the LSU-Lab-1 and LSU-Field-1 populations being different strains (i.e., corn versus rice) that are known to have differential sensitivities to insecticides (Pashley 1986; Ríos-Díez and Saldamando-

Benjumea 2011).

To test if the poor relationship between neuronal RR and acute toxicity RR is due to differential metabolic enzyme expression patterns, the expression of known CYP, esterase, and GST genes was compared between LSU-Lab-1 and LSU-Field-1. The qPCR results indicate differential esterase activity between the two populations may account for the non-linear relationship between neuronal and toxicity RR because one of the two esterase-encoding genes tested showed significantly increased expression in LSU-Lab-1 when compared to LSU-Field-1. However, other genes were also significantly different between the two populations of interest. Two glutathione *S*-transferase (GST)-encoding transcripts were differentially expressed when using the GLMM-based method, one confirmed with the $2^{-\Delta\Delta CT}$ method, but their expression patterns were opposite. Moreover, the expression of GST-0968 was discordant with the literature (Carvalho et al. 2013; "Agrofit: Sistema de Agrotóxicos Fitossanitários" 2013). This may indicate that changes in expression of GST-encoding genes, previously thought to be part of insecticide resistance in FAW (Carvalho et al. 2013; Nicholson and Miller 1985b; "Agrofit: Sistema de Agrotóxicos Fitossanitários" 2013), may not play a critical role in the susceptibility of LSU-Field-1 to these insecticides. The discrepancies in gene expression between previous work (Carvalho et al. 2013; "Agrofit: Sistema de Agrotóxicos Fitossanitários" 2013) and this study demonstrate a variability between populations, particularly when comparing laboratory-reared resistant populations (Carvalho et al. 2013; "Agrofit: Sistema de Agrotóxicos Fitossanitários" 2013) and field-collected populations that may not present the same resistance mechanisms. Additionally, the outcomes of the two analyses conducted were different and illustrate the need for reliable analyses with gene expression comparisons.

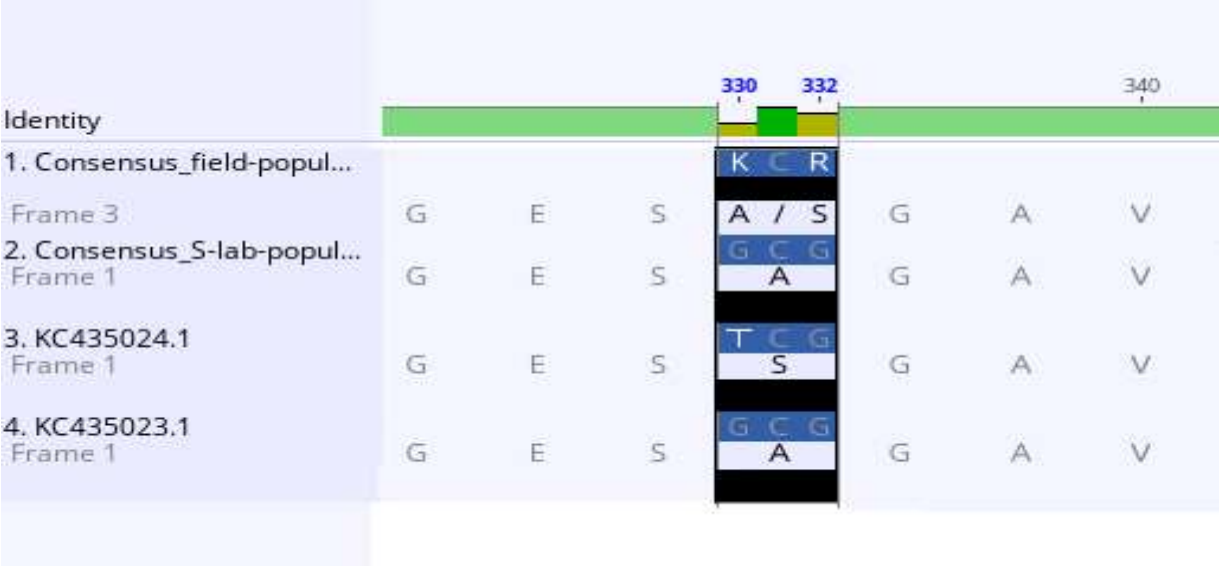
The lack of evidence for LSU-Field-1 resistant to λ-cyhalothrin based on enhanced

detoxification suggest product failure of Karate Z® (Fig. 2.3.C) may be due to target-site insensitivity for FAW populations in Northern Louisiana. This observation supports previous findings for other lepidopteran pests of Louisiana (Ottea and Holloway 1998a; Ottea, Ibrahim, Younis, Leonard, et al. 1995). The mechanism of reduced neural sensitivity to insecticides is often assumed to be due to point mutations located in the target site(s) that confer insecticide insensitivity (Soderlun and Lee 2001). For example, L1014F (*kdr*) and M918T (super-*kdr*) are two point mutations in homology domain II of the house fly Vssc1 VGSC alpha subunit that confer pyrethroid resistance via reduced potency at the target site (Soderlun and Lee 2001; Kasai, Sun, and Scott 2017; Dong 2007; Williamson et al. 1993). These analogous mutations have been shown to result in high levels of pyrethroid resistance in FAW populations (Carvalho et al. 2013). It was hypothesized that the 767-fold resistance (Fig. 2.3.A) and 1700-fold reduction in neural sensitivity (Fig. 2.3.B) to λ-cyhalothrin observed in the LSU-Field-1 strain was due to point mutations in the VGSC. Neither M918T nor L1014F mutations were detected in the LSU-Field-1 individuals suggesting M918T and L1014F are not the only predictors for neural insensitivity to pyrethroids and pyrethroid resistance. These data suggest that the absence of *kdr* in FAW populations does not necessarily indicate neural sensitivity and, in turn, susceptibility to pyrethroid insecticides.

Overall, this study provides data suggesting the reliance on genotypic bioassays may not be sufficient to drive insecticide resistance management decisions for FAW. While there is a strong correlation between the presence of *kdr* and super-*kdr* and a reduced pyrethroid sensitivity of VGSC, the data presented here indicate that significant reduction of neural sensitivity and high resistance ratios can still be observed in field-collected FAW populations despite the absence of key point-mutations in the VGSC. Thus, neurophysiological assays combined with molecular genetic analyses of FAW target sites will provide a better understanding of mechanisms for

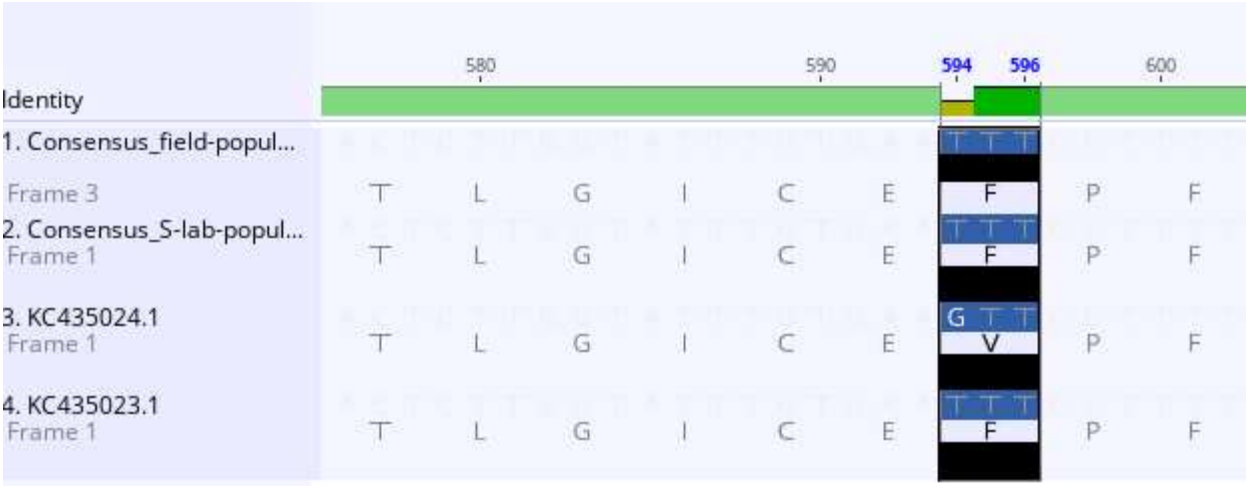
insecticide resistance in FAW. While there are some limitations for the use of neurophysiological recordings to predict insecticide resistance, a combination of these methods will provide important contributions insecticide resistance management strategies for FAW, a critical need when considering the extreme reliance on a few insecticide modes of action to control this global pest.

2.5. Supplemental Figures

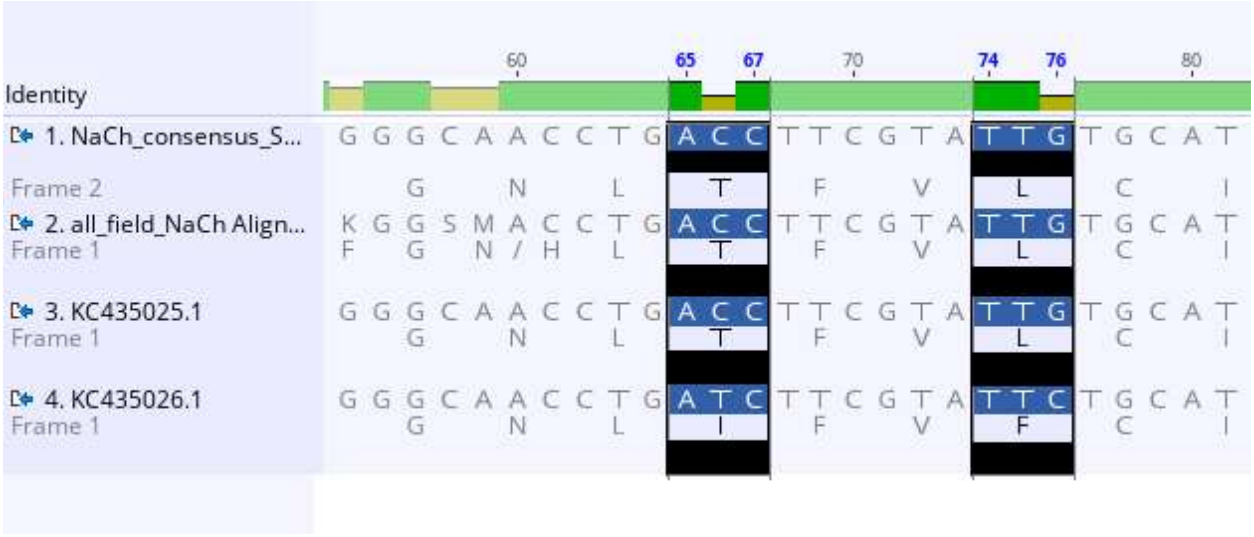


Supplemental Figure 2.5.1.: Location of the putative mutation A201S of the AChE sequence in the two sequences from Carvalho et al. 2013, and the laboratory and field populations described in this study. The sequence 1 represents the consensus for the field population and carries both alleles. K (in KCR) represents either G or T, and R represents either A or G.

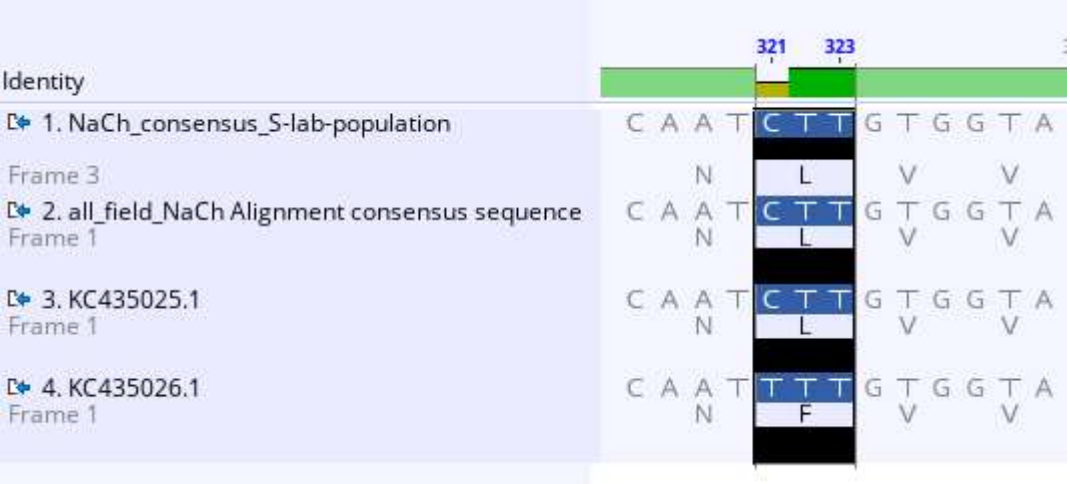
Supplemental Figure 2.5.2.: Location of the putative mutation G227A of the AChE sequence in the two sequences from Carvalho et al. 2013, and the laboratory and field populations described in this study. The sequence 1 represents the consensus for the field population and carries both alleles. S (in GSA)



Supplemental Figure 2.5.3.: Location of the putative mutation F290V of the AChE sequence in the two sequences from Carvalho et al. 2013, and the laboratory and field populations described in this study. The mutation replacing F by V was not found in our samples, and only identified in the resistant population (Carvalho et al 2013).



Supplemental Figure 2.5.4.: Location of the putative mutations T929I and L932F of the NaCh sequence in the two sequences from Carvalho et al. 2013, and the laboratory and field populations described in this study. None of the sequenced individuals carried the previously identified mutations.



Supplemental Figure 2.5.5.: Location of the putative mutation L1014F of the NaCh sequence in the two sequences from Carvalho et al. 2013, and the laboratory and field populations described in this study. None of the sequenced individuals carried the previously identified mutation.

2.6. Supplemental Tables

Table 2.6.1.: Primer sequences, calculated efficiency, and estimated product size**.** GST-1950 was annotated as pyruvate dehydrogenase in the publication but confirmation by a BLAST search revealed a match with a GST instead. Unknown* refers to the product size of Est-0974, not indicated in the publication and not identified from a BLAST search.

Gene names	Forward sequence	Reverse sequence	Calculated efficiency in %	Product size in base pair (bp)
28S (HKG)	TGATGGCTGCATGTTACCTC	ATAAGACCGGCTTTCTGTGC	102.7	99
EF1 (HKG)	TCGCTGTGGGTGTAATCAAG	GCTACTTCTTGCCCTTGGTG	99.6	96
Est-9555	TCAGCTACCCTGGAATGGTC	AGTTCCGGTCGTTGAGTAGC	100.8	92
GST-1950	CGATTTGGGCACCTTGTATC	TGTGGCCCTCTAGGAAAGTG	101.4	137
GST-0801	AGCCGGTGAACATCTCACTC	TCCTTTCCGTAGGTGTCAGC	98.2	135
CYP-9131	TACGGACACAGTGGCAAGAC	CTGGGTTCATCGCTAGTTGG	99.2	136
Est-0974	TAGGGCTTCTGAGCTGATGG	AGGCTGTTCTCTGGCACTAAAC	101.3	Unknown*
GST-0968	CGTCCTATGCGGTTTAGTGG	ACTCCTTGACCCCGGTAGAC	96.4	118

Table 2.6.2.: Main GLMM-estimated parameters for each gene of interest and significance levels between the susceptible strain used as a baseline and the field individuals. Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ns’ 1. Genes in bold are significantly different between susceptible and field populations.

Genes	Mean fold change (susc. to field)	Mean fold change (field to susc.)	*P*-value	Adjusted *P*-value	Significance
Est-0974		8.752	0.0001	0.0003	***
GST-0968		5.747	0.0001	0.0003	***
GST-1950	7.929		0.0001	0.0003	***
GST-0801	2.672		0.034	0.067	.
CYP-9131		1.599	0.279	0.389	
Est-9555	1.517		0.292	0.389	

Table 2.6.3.: Main parameters calculated for the 6 genes of interest based on the $2^{-\Delta\Delta CT}$ method. Yes/No indicate the overlap between confidence intervals, "No" corresponds to genes differentially expressed between susceptible and field population. RQ: relative quantity, St err: standard error, St dev: standard deviation, CI: confidence intervals, upper and lower correspond to the highest and lowest values based on the 95% confidence intervals.

Gene	Status	average RQ	St err	St dev	CI 95 %	upper	lower	overlap
EST9555	Susceptible	1.01	0.07	0.12	0.14	1.14	0.87	Yes
	Field	2.31	0.89	2.17	1.74	4.05	0.57	
GST1950	Susceptible	1.82	0.89	1.55	1.75	3.57	0.06	Yes
	Field	12.22	5.12	12.54	10.04	22.26	2.19	
GST0801	Susceptible	1.05	0.16	0.27	0.31	1.35	0.74	**No**
	Field	3.10	0.76	1.87	1.50	4.60	1.60	
CYP9131	Susceptible	3.27	2.30	3.98	4.50	7.77	-1.23	Yes
	Field	0.82	0.26	0.63	0.50	1.33	0.32	
EST0974	Susceptible	1.43	0.91	1.57	1.78	3.21	-0.34	Yes
	Field	0.19	0.07	0.18	0.14	0.33	0.04	
GST0968	Susceptible	1.03	0.18	0.32	0.36	1.39	0.68	**No**
	Field	0.24	0.07	0.16	0.13	0.37	0.11	

2.7. Acknowledgements

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2.8. Notes

1. This chapter was published under the same title in *Pesticide Biochemistry and Physiology* (PMID: 32828370) and the full citation is included below.

2. McComic, S. E., Rault, L. C., Anderson, T. D. & Swale, D. R. Reduced neuronal sensitivity and susceptibility of the fall armyworm, *Spodoptera frugiperda*, to pyrethroids in the absence of known knockdown mutations. *Pesticide biochemistry and physiology* **169**, 104652, doi:10.1016/j.pestbp.2020.104652 (2020).

Chapter 3. Toxicological and neurophysiological characterization of natural product based chromene analogs to *Spodoptera frugiperda* (Fall armyworm)

3.1. Introduction

The fall armyworm (FAW), *Spodoptera frugiperda* (Lepidoptera: Noctuidae), is recognized as one of the most damaging agricultural pests of row crops with economic losses estimated to be $6 billion dollars per year, which primarily stems from FAW infestation of maize crop (Yu, Nguyen, and Abo-Elghar 2003). Global trade and travel has facilitated the expansion range of FAW from the Americas to nearly 100 countries, which has threatened the food security of millions of people due to the ability of FAW to cause 100% loss of maize and rice crop if left uncontrolled (Goergen et al. 2016; Sharanabasappa et al. 2018; Wu et al. 2019; Cairns et al. 2013). FAW control programs rely on a combination of synthetic insecticides and *Bacillus thuringiensis* (*Bt*) expressing plants to maintain pest populations below the economic damage threshold (Brookes and Barfoot 2016; Blanco, Chiaravalle, Dalla-Rizza, Farias, Garcia-Degano, et al. 2016; Gutiérrez-Moreno et al. 2019). An increase in Cry1F resistance alleles within FAW populations throughout the Americas (Santos-Amaya et al. 2017; Blanco, Chiaravalle, Dalla-Rizza, Farias, Garcia-Degano, et al. 2016) has prompted farmers to significantly increase the frequency of pyrethroid and organophosphate applications to maintain low FAW levels. Unsurprisingly, the increased frequency of application drove the evolution of resistance to multiple insecticidal classes, including pyrethroids, organophosphates, carbamates, benzoylureas, spionsyns, and diamides (Ríos-Díez and Saldamando-Benjumea 2011; Okuma et al. 2018a; Jia et al. 2009; Carvalho et al. 2013; Boaventura et al. 2020a). The widespread resistance to multiple classes of insecticides emphasizes the need to identify novel chemical scaffolds that can augment control of FAW populations.

The discovery and development of new active ingredients to control arthropod populations and decelerate the inevitable evolution of insecticide resistance has been of consistent interest to the field of insecticide science. Development of molecules with similar structures to existing, known as competitor inspired or "Me-Too Chemistry", has been especially useful as a source for developing new agrochemicals (Sparks, Hahn, and Garizi 2017). One caveat to this method is reduced innovation and, with the exception of isoxazolines and meta-diamides, has not resulted in the identification of insecticides with novel modes of action (Swale and Bloomquist 2019). Thus, avenues should be explored to identify novel chemical scaffolds that act on unexploited targets.

Natural products (NPs) have served as models or inspiration for the discovery of many of the weed, plant pathogen, and insect pest control agents in use today, including the prime example of synthetic pyrethroid insecticides being directly derived from pyrethrum scaffold (Sparks, Hahn, and Garizi 2017). Other examples of NPs that were known to have insecticidal activity, but were not used directly as insecticides, include physostigmine, nereistoxin, insect juvenile hormone, deoxypyrrolmycin, stemofoline, and N-isobutylamides (Sparks, Hahn, and Garizi 2017). Among these NPs, all except the N-isobutylamides have given rise to fully synthetic mimics that include the N-methylcarbamates, nereistoxin analogs, juvenile hormone mimics, chlorfenapyr, and flupyradifurone, respectively (Sparks, Hahn, and Garizi 2017). Additionally, there are NP scaffolds existing (NPSEs) for several insecticide classes that were not originally based around those models (Sparks, Hahn, and Garizi 2017). Taken together, 64% of all insecticides are plant-based or have a plant NPSE suggesting that studies testing the secondary metabolites of plants holds significant potential for the development of novel insecticidal scaffolds and/or novel mechanisms of toxicity (Sparks, Hahn, and Garizi 2017).

Amyris texana P. Wilson (Rutaceae), also known as "Texas Torchwood", is a flowering plant distributed mainly in the arid regions in the southwestern United States and Mexico and has been shown to have insect repellent and toxicant properties to multiple insect species (Meepagala et al. 2013; Meepagala, Estep, and Becnel 2016). Considering this, previous work has aimed to isolate and purify the molecular constituents of the plant extracts (Meepagala et al. 2013; Meepagala, Estep, and Becnel 2016). Chromene amides (chromene benzopyran with a carbonyl group attached to a nitrogen; derived from carboxylic acid and an amine) were identified in the extracts that have been shown to have various biological activities in vertebrate and invertebrate systems (Meepagala, Estep, and Becnel 2016). The chromene moiety is an important structural component within various natural chemistry and the derivatives of benzopyran moiety has been shown to interact with multiple cellular targets (Majumdar et al. 2015). This relatively non-specific binding, or ability to bind to several cellular target sites in the body, likely contributes to their range of biological activities such as antitumor, antihepatotoxic, antioxidant, anti-inflammatory, diuretic, anticoagulant, antispasmolytic, estrogenic, antiviral, antifungal, antimicrobial, anti-helminthic, hypothermal, vasodilatory, anti-HIV, antitubercular, herbicidal, anticonvulsant and analgesic activity (Costa et al. 2016; Majumdar et al. 2015). The USDA-ARS Natural Products Utilization Research Unit (Oxford, MS) has generated a series of synthetic-derivatives of the identified chromene amides, known as chromene analogs, that were previously shown to have biological activity to mosquitoes (Meepagala et al. 2013) and termites (Meepagala, Estep, and Becnel 2016). Thus, the objective of this study was to test the insecticidal activity of chromene analogs to FAW in an effort to identify novel chemical scaffolds that can augment the current control programs.

3.2. Methods

3.2.1. Compounds and Compound Synthesis

Permethrin was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Chromene analogs **1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12,** and **13** were synthesized by Kumudini Meepagala (Research Chemist, USDA-Stoneville, MS) as previously described (Meepagala et al. 2013; Meepagala, Estep, and Becnel 2016) and were donated for toxicity assessments against FAW. The solvent dimethyl-sulfide (DMSO) was purchased through Sigma-Aldrich Chemical Co. Phosphate Buffered Saline 1X (PBS), pH 7.4 was purchased through GIBCO, Thermo Fisher Scientific (Waltham, MA, USA). The chemicals used to produce the neurophysiological saline were all purchased through Sigma-Aldrich Chemical Co. (sodium chloride, potassium chloride, calcium chloride, HEPES, and D-(+)-Glucose).

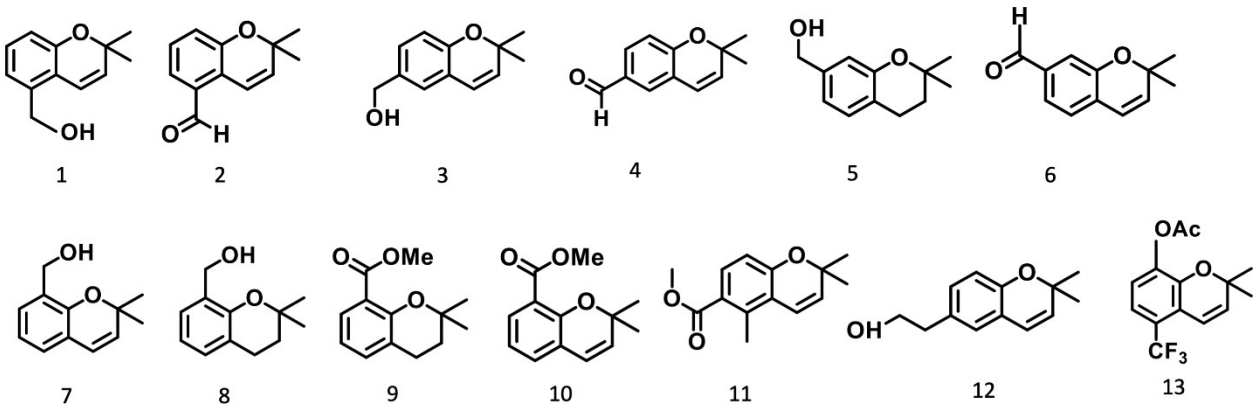

Figure 3.1. Chromene analog structures used in this study.

3.2.2. Spodoptera frugiperda and Rearing Conditions

The fall armyworm larvae used for these experiments were purchased from Benzon Research Inc. (Carlisle, PA, USA) and were reared from eggs to third instars. Cheesecloths with

approximately 100 eggs were each placed into 8 cell trays (Bio-Serv, Frenchtown, NJ, USA) for 48 hours until they exhibited signs of emergence. One to two neonates each were placed into 30-mL plastic cups that included 15-mL of artificial diet (Stonefly Heliothis Diet, Ward's Natural Science, Rochester, NY, USA). Rearing conditions involved a 14:10 h (light:dark) photoperiod and a 25 °C temperature. Once reaching the third instar (6 to 7 days on diet) the larvae were extracted and starved overnight before being used for these experiments.

3.2.3. Microinjection Toxicity Bioassays

We assessed toxicity of chromene analogs through two methods, microinjection and ingestion. Microinjection of chromene analogs were performed by dissolving chromenes in DMSO and solubilized in phosphate-buffered saline (PBS) that includes $CaCl^{2+}$ with pH 7.4. Control treatments were microinjection of saline and DMSO only. Glass pipettes for injection were generated from 1.14-MM glass capillary tubes (World Precision Instruments, Sarasota, FL, USA) and were pulled with a P-1000 Flaming/Brown micropipette puller (Sutter Instrument, Novato CA, USA). 1 µL of chemical solution was injected into the thorax using a SMARTTouch MICRO2T microinjector (World Precision Instruments, Sarasota, FL, USA) at a rate of 120 nL/sec. A total of 6 concentrations were used to construct ID_{50} (injected dose killing 50%) values where each concentration consisted of 10 individuals and 3 replicates. Total percentage of mortality was assessed at 48 hours post injection.

3.2.4. Chromene Incorporated Ingestion Assays

A diet incorporation assay was performed to assess larval mortality via ingestion. Each chromene was mixed into 15-mL of artificial diet at a concentration of 10 µg/mL and one neonate

was placed into each cup and reared on the chromene-infused diet until pupation. Larvae mortality and fold increase in weight was assessed at each instar stage. The pupae were then allowed to progress into adulthood to enable assessment of percent emergence and abnormalities. A total of 3 replicates with 10 larvae per replication were used to enable statistical comparisons. Percent mortality (percentage of larvae dying before pupation), weight (mg), percentage of adults able to fly (defined as adult with short wings rendering flight inability or wings that do not move correctly and cause adult to spin in circles), percentage of eclosed pupae, and overall adult abnormalities (pupal casing still attached to body, short wings, legs not fully extended) were all statistically compared within each other using one-way ANOVA with $p < 0.05$.

3.2.5. Neurophysiological Assays

Extracellular neurophysiological recordings were performed in a similar manner to Diptera (Bloomquist, Roush, and ffrench-Constant 1992; Swale et al. 2018) and *Heliothis virescens* (Ottea and Holloway 1998b; Ottea, Ibrahim, Younis, Young, et al. 1995) studies. Third instar larvae were bathed in 200 µL of neurophysiological saline and their central nervous system (CNS) was dissected. This saline included 140 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 5 mM HEPES, 28 mM D-glucose, and pH: 7.4. Glass capillary tubes were pulled into electrodes using the P-1000 Flaming/Brown micropipette puller (Sutter Instrument, Novato CA, USA). Thoracic ganglia nerves extending from the CNS were pulled into the electrode and monitored for electrical activity. Electrical activity was amplified by an AC/DC amplifier (Model 1700, Systems, Inc., Carlsborg, WA, USA). LabChart 7 Pro (ADInstruments, Colorado Springs, CO, USA) was used to reconstruct the electrical activity into a readable signal, Hertz (Hz) and a baseline firing rate was determined. Noise (60 Hz) was lessened or eliminated by Hum Bug (A-M Systems, Sequim, WA, USA). Once

the baseline firing rate was constant (25-40 Hz) for about ten minutes (in the 0.1% DMSO 200 µL control solution), the various chromene analog concentrations and standard chemicals were added in addition to the 200 µL saline bath, making a total of 400 uL. The total concentration of DMSO added to the saline bath never exceeded 0.1%. Mean spike discharge frequencies were recorded for individual concentrations and were used to develop a concentration-response curve to enable the construction of EC_{50} values. EC50 values were determined by non-linear regression analysis in GraphPad Prism™ (GraphPad Software, San Diego, CA, USA). Final EC_{50} values were determined by averaging 5-7 replicates.

3.3. Results

3.3.1. Toxicity of chromenes after microinjection and ingestion

Microinjection of permethrin resulted in an ID_{50} of 50 ng/mg insect (Table 3.1.; 95% CI: 22-72 ng/mg of insect, Hillslope: -1.2, r^2: 0.85). **1** and **2** were the most toxic chromenes tested with ID_{50} values of 101 ng/mg (Table 3.1.; 95% CI: 20-211 ng/mg of insect, Hillslope: -1.4, r^2: 0.82) and 138 ng/mg insect (Table 3.1.; 95% CI: 52-155 ng/mg of insect, Hillslope: -1.1, r^2: 0.87), respectively. **6** was found to be two-fold less toxic than **1** and **2** with an ID_{50} of 205 ng/mg insect (Table 3.1.; 95% CI: 151-274 ng/mg of insect, Hillslope: -0.99, r^2: 0.81). Alternatively substituted alcohols or aldehydes did not result in appreciable mortality at doses up to 300 ng/mg insect (Table 3.1.).

Ingestion after incorporation of chromenes into the larval diet showed **1** and **2** were the two most toxic chromenes with both having an LC_{50} value of 15 µg/mL (95% CI: 6-21 µg/mL, Hillslope: -1.5, r^2: 0.88) for both (Table 3.1.). Although not toxic after microinjection, **11** and **12** were found to be toxic after ingestion with LC_{50} values of 14 µg/mL (95% CI: 5-22 µg/mL,

Hillslope: -1.2, r^2: 0.8) and 35 µg/mL (95% CI: 12-61 µg/mL, Hillslope: -1.3, r^2: 0.85) (Table 3.1.). No statistical significance was found between LC_{50} values of **1**, **2**, **11,** and **12**. All other chromenes studied were found to elicit less than 50% mortality at solubility limits.

Table 3.1. Toxicity of chromenes to L3 fall armyworm after ingestion and microinjection. Lowercase letters represent statistical significance at P<0.05 across compounds within treatment group whereas uppercase letters represent statistical significance of synergized toxicity compared to non-synergized toxicity. Significance was determined by one-way ANOVA with Tukeys multiple comparison test.

Compound	**Ingested Toxicity**	**Injected Toxicity**	**Injected Tox + PBO**
	LC_{50}; µg/mL (95% CI)	**ID_{50}; ng/mg of insect (95% CI)**	
Permethrin	0.8 (0.4-1.3)	50 (22-72) a	18 (2-40) A
1	15 (6-21) b	138 (50-211) b	105 (45-172) B
2	15 (6-21) b	101 (52-155) b	112 (66-175) B
3	>100 c	>300 c	>300 C
4	>100 c	>300 c	>300 C
5	90 (72-135) d	>300 c	> 300 C
6	>100 c	205 (141-274) b	241 (145-312) B
7	>100 c	>300 c	>300 C
8	>100 c	>300 c	>300 C
9	>50 d	>300 c	>300 C
10	>100 c	>300 c	>300 C
11	14 (5-22) b	>300 c	>300 C
12	35 (12-61) b	>300 c	>300 C
13	>70 c	>300 c	>300 C

3.3.2. Signs of intoxication

The alcohol and aldehyde substitutions on the benzene ring of the benzopyran moiety resulted in contrasting behavior after injection. The Methanol substitution at the C1 position (**1**) resulted in lethargy and flaccid paralysis, yet the aldehyde substitution at the C1 position (**2**) resulted in hyperactivity and tonic contraction of the larval body wall (Figure 3.2.A-B). The pattern of Methanol substitution yielding flaccid paralysis and aldehyde substitution yielding tonic contraction was also observed with substitutions at C2 position albeit with reduced strength (Figure 3.2.C-D). Interestingly, substitutions of Methanol or aldehyde at the C3 position were found to induce no observable change in behavior or phenotype (Figure 3.2.E-F).

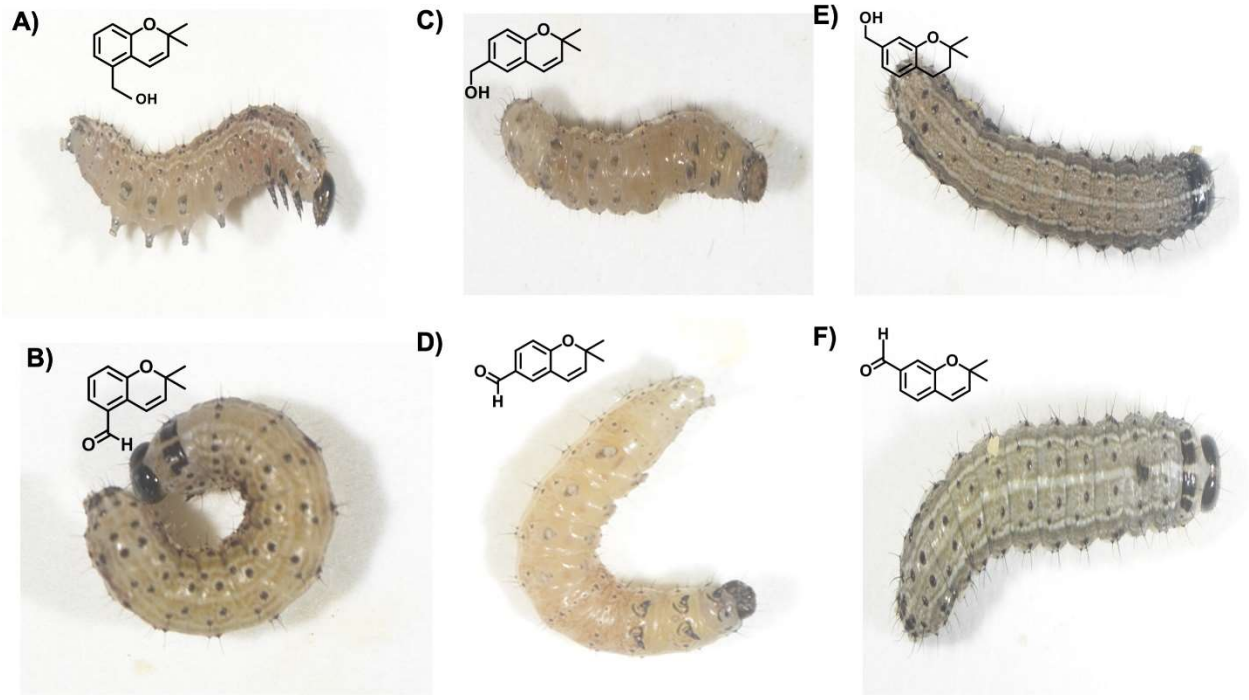

Figure 3.2. Phenotypic responses of larvae after injection of **1** (A), **2** (B), **3** (C), **4** (D), **5** (E), **6** (F). Images were taken 1 hour post injection.

3.3.3. Neurophysiological spontaneous nerve firing reactions to chromenes

The most toxic chromenes noted post-injection were applied to the central nervous system (CNS) of *Spodoptera frugiperda* to assist in a better understanding of how these affect this pest.

Additionally, these chromenes were tested to determine if any of the toxic chromenes were neuroactive. Compounds **1** and **2** resulted in neuroexcitation and neural inhibition (Figure 3.3), respectively, which mirrored the opposite signs of intoxication observed after larval exposure to these molecules. **1** was found to inhibit *S. frugiperda* CNS spike discharge frequency with an IC_{50} value of 340 μM (95% CI: 172-510 μM; Hillslope: -1.6; R^2: 0.98) whereas **2** was found to increase firing rates with an EC_{50} value of 87 μM (95% CI: 44-131 μM; Hillslope: -0.9; R^2: 0.97) (Figure 3.3.). Additional chromenes were studied in the neurophysiological assay but did not elicit significant changes to neural activities.

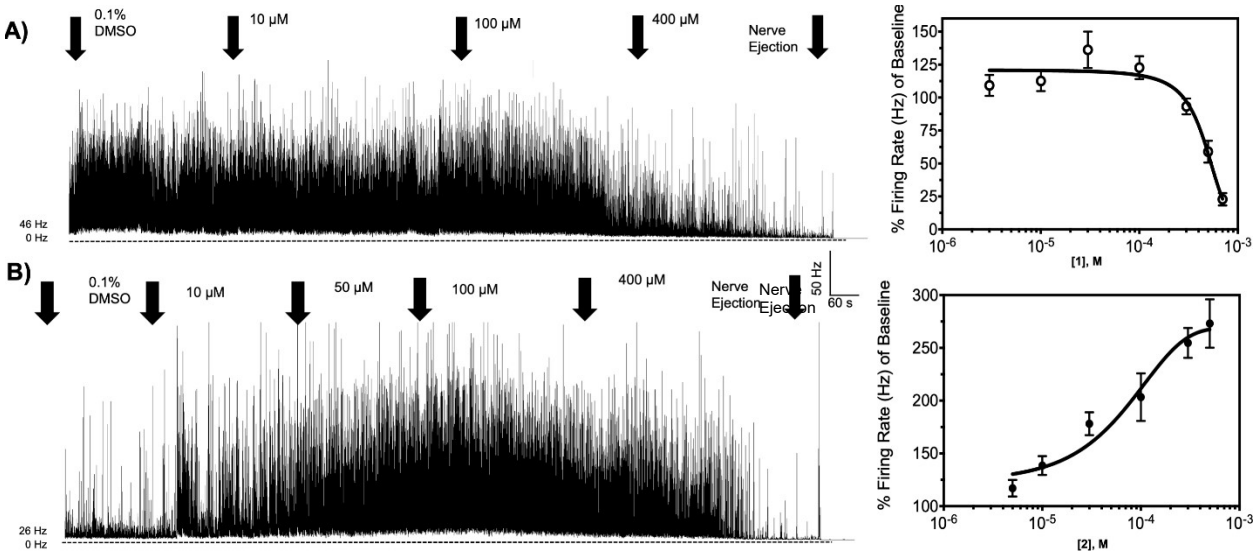

Figure 3.3. Potency determinations for 1 and 2 to FAW CNS firing rates. Representative traces and concentration-response curves and representative recordings for **1 (A)** and **2 (B)**. Data points represent means from replicated recordings (*n* = 5-12 preparations per curve, with each concentration replicated a minimum of 4 times). Data points represent mean percentage increase of baseline firing rate and error bars represent SEM.

3.3.4. Effect of chromenes to larval development

To measure the influence of ingestion of chromenes on larval weight gain during each instar, we incorporated chromenes into the larval diet and measured weight gain over each instar. Chromenes were not found to influence larval weight gain from the L1 to L2 life stage when compared to solvent control treated larvae (Figure 3.4.A). From the L2 to L3 life stage, only **4** was found to significantly ($P<0.05$) reduce larval increase in mass with a 1.3-fold reduction when compared to control groups (Figure 3.4.B). Interestingly, **3, 4, 10,** and **12**, were all found to significantly ($P<0.05$) reduce weight gain during the L3-L4 life stage by up to 1.8-fold when compared to control groups (Figure 3.4.C). These same compounds as well as **9** were shown to significantly ($P<0.05$) reduce weight gain across the L4-L5 life stage by up to 2.2-fold when compared to control groups (Figure 3.4.D).

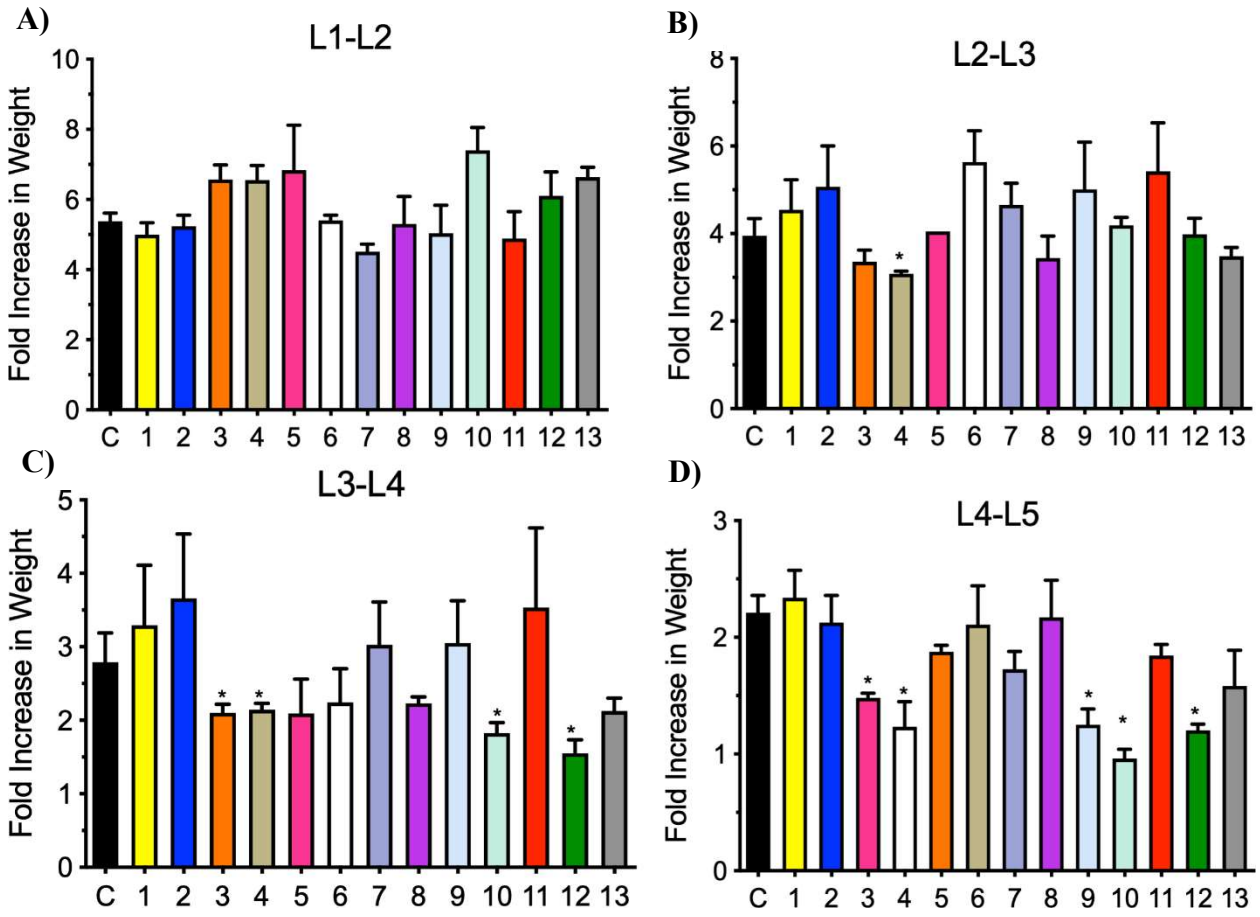

Figure 3.4. Effect of chromenes to larval development**.** Bars represent mean (n=3) weight gain for each larval instar and error bars represent SEM. Asterisks represent statistical significance at P<0.05 as determined by unpaired t-test compared to control.

3.3.5. Effect of chromenes on adult eclosion and development

To measure the sub-lethal effect of chromenes on FAW, we tested aspects of development such as the emergence percentage and the functional capacity of emerged adults. We observed no statistical difference between the percent pupae that attempted to emerge to an adult for any chromene studied (Figure 3.5.A); yet **4, 9,** and **13** were found to significantly increase the percent of the emerged adults that had exhibited developmental abnormalities (Figure 3.5.B). Control moths displayed abnormalities upon emergence at a rate of 8 ± 4%, with **4**, **9,** and **13** displaying abnormalities at 29 ± 6%, 53 ± 14%, and 35 ± 6%, respectively, all significantly greater (P<0.001) than control (Figure 3.5.B). Representative images for abnormalities of **4**, **9,** and **13** are shown in Figures 3.5. C, D, and E, respectively.

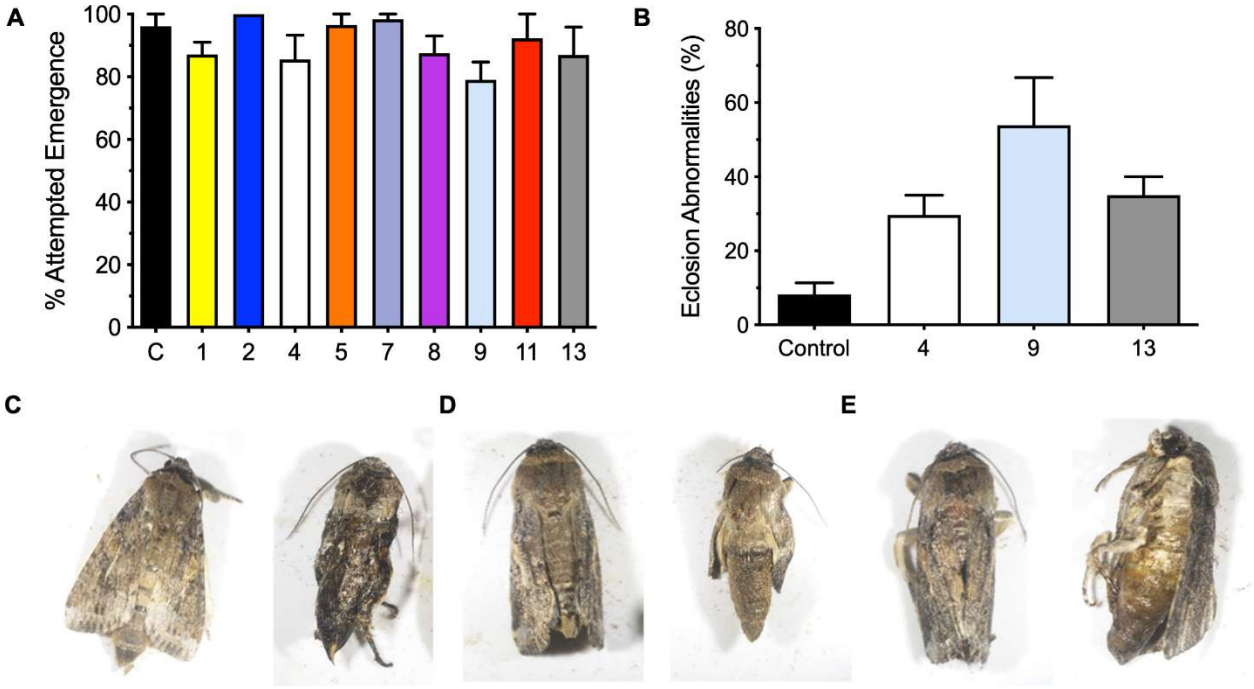

Figure 3.5. Developmental abnormalities after eclosion. **A)** Bars represent mean (n=30-50 individuals) adults that attempted adult eclosion. **B)** Percentage of eclosion abnormalities observed from **4, 9,** and, **13**. **C-E)** Representative images of adults affected by larval exposure to **4, 9,** and, **13**.

3.4. Discussion

Natural products or naturally derived chemistry has been used for decades in the agrochemical industry for control of various pests (Sparks, Hahn, and Garizi 2017). The use of chromene (Benzopyran) is an interesting aspect when developing new structures that are potentially toxic. The chromene is present in several of natural products in various markets market (polyphenols found in tannins and teas, fruits, and numerous vegetables) (Costa et al. 2016) and further, 2H-Chromenes have been extracted from their host plants and have been shown to possess biological activity in anti-cancer studies, insect repellent and toxicant assays, and others (Costa et al. 2016; Majumdar et al. 2015). 2H-Chromene skeletal structures are important in medical research and this activity has suggested it may hold relevance in the agrochemical industry for insect control.

Amyris texana (Family: Rutaceae) is a dense shrub whose leaves possess the aroma of citrus ("Texas Torchwood, Torchwood, Rue, Lantrisco, Chapotillo, Texas Torchwood Amyris" 2020) and has been shown to have various insecticidal properties against dipteran pests, yet the toxicological relevance to lepidopteran pests remains unknown. Previous studies have shown that various analogs from this plant produce toxicological effects and even high mortality against mosquitoes (Meepagala et al. 2013) and termites (Meepagala, Estep, and Becnel 2016), in both larval and adult stages. A number of chromene analogs were synthesized from the chromene amide structure, and 13 of them were tested for toxicity or effects to development against FAW (Meepagala et al. 2013; Meepagala, Estep, and Becnel 2016).

Select chromenes resulted in high toxicity when injected thoracically or incorporated into the larval diet. Chromene **2** presented the highest toxicity after microinjection and most larvae affected were hyperactive, curling into a "C" shape, and/or quickly trying to crawl out of their holding chamber. Furthermore, contrasting phenotypes were observed between **1** and **2** post-injection that suggests the substitution of a Methanol to an aldehyde on this benzene structure, can result in different mechanisms of action. Additionally, low mortality occurred with the other 11 chromenes injected, but signs of intoxication were seen from chromenes **3** and **4**. Chromenes **3** and **4** had similar phenotypic responses as larvae would remain on their dorsal or ventral side with little to no movement, and this effect was shown to be dose dependent. The time spent in a flaccid state would persist up to 6 hours at high doses.

Larvae exposed to the chromenes through the diet incorporation were reared until pupation to enable analysis of changes to developmental trajectories. Pupae then emerged and any abnormalities were assessed regarding the adult moths. A small percentage of moths within select groups were observed to have twisted wings, partial eclosion, pupal casing still attached, or other defects.

The contrasting signs of intoxication observed by **1** and **2** were mirrored by opposite influence to central neuron firing rates that further indicates these two molecules possess two distinct modes of action, despite small changes in side chain substitutions. Interestingly, although **3** and **4** showed similar signs of intoxication post-injection, **4** did not present any inhibitory nerve activity when **3** did, that suggests the structure activity of this substitution is quite precise. Chromenes **5** and **6** were also applied to the FAW CNS and were found to not influence the firing rates of central neurons, suggesting that the methanol and the aldehyde on the C3 position of the benzene ring are not potent ex vivo and this lack of potency results in poor toxicity.

Overall, chromene (Benzopyran) natural products have proven to be efficient in their use as potential insecticides. The 13 chromene analogs tested for toxicity against *Spodoptera frugiperda* do possess some potential qualities that could be further analyzed into possible insecticides, yet the chemicals studied resulted in relatively low toxicity and had poor in vitro potencies against the CNS nerve firing. However, these compounds did alter the developmental trajectory of FAW by significantly reducing the number of non-deformed adults upon eclosion, indicating these molecules may also alter the endocrine pathways during metamorphosis and may represent a potential growth regulator for FAW.

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Vita

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