LAURA MARCELA MACHUCA MESA

PHEROMONE BLEND AND SEXUAL ATTRACTION IN BT-SUSCEPTIBLE AND RESISTANT FALL ARMYWORM (*Spodoptera frugiperda*)

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para obtenção do título de *Doctor Scientiae*.

VIÇOSA
MINAS GERAIS - BRASIL
2019

Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Câmpus Viçosa

T

Machuca Mesa, Laura Marcela, 1987-

M151p Pheromone blend and sexual attraction in Bt-susceptible
2019 and resistant fall armyworm (*Spodoptera frugiperda*) / Laura
Marcela Machuca Mesa. – Viçosa, MG, 2019.
xiv, 77 f. : il. ; 29 cm.

Texto em inglês.
Orientador: Eraldo Rodrigues de Lima.
Tese (doutorado) - Universidade Federal de Viçosa.
Referências bibliográficas: f. 58-77.

1. *Bacillus thuringenisis*. 2. Feromônios sexuais. 3. Insetos - Reprodução. 4. Insetos - Atração sexual. 5. Comunicação.
I. Universidade Federal de Viçosa. Departamento de Entomologia. Programa de Pós-Graduação em Entomologia.
II. Título.

CDD 22. ed. 579.362

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APROVADA: 12 de abril de 2019.

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(Orientador)

Dedico a minha mãe por me acompanhar sempre e me ensinar a lutar pelos sonhos.

Epígrafe

"He who is not courageous enough to take risks will accomplish nothing in life.". *Muhammad Ali*.

Agradecimentos

A minha mãe por cada palavra de força todos os dias. A meu pai por me ensinar que o perdão faz parte de uma vida melhor, e por me apoiar nos caminhos que eu decido empreender. As minhas famílias por estarem sempre ao meu lado.

A María Agenis Bonilla e Carolina Becerra por me mostrarem o maravilhoso mundo da ecologia e por incentivarem o estudo com insetos.

A Organização dos Estados Americanos, a Universidade Federal de Viçosa, ao Departamento de Entomologia e CAPES, pela oportunidade de fazer meu doutorado e pelo apoio financeiro.

Ao professor Eraldo, por me aceitar como orientanda, por confiar em mim para o projeto com Spodoptera, por mostrar o mundo do comportamento de insetos, pelas palavras de força quando necessitei. Obrigada pela ajuda acadêmica e pessoal.

Ao Eliseu por sua ajuda no desenvolvimento do projeto, na análise dos resultados e por sempre estar disposto a me ajudar.

Ao professor Jeremy McNeil, por seus múltiplos ensinamentos, pela ajuda com minhas inquietudes e por ser essa pessoa simples e especial.

Ao professor Renato Sarmento, Raimundo Wagner, seus grupos de pesquisa e Marciane Dotto pela ajuda, disponibilidade de laboratório, ensinamentos e por me permitir conhecer um pouco mais do Tocantins.

Ao Evandro e Josie, pela ajuda com a criação e demais. Muito obrigada por sempre estarem dispostos a ajudar.

As minhas "chicoritas" Cata e Stephy, vocês mostraram que a amizade vence barreiras. Vocês sempre passaram para mim toda a boa energia. Amo vocês!

A Amalia por ser minha nova amiga, pela ajuda com as suas aulas personalizadas e pelo apoio incondicional. Foram muitos caminhos descobertos e com certeza muitos que virão.

Ao Lucas (mon amour), por ser meu amigo incondicional, pela companhia nos experimentos, por estar presente para as discussões, pelo apoio, pela ajuda com os gráficos e por me escutar nos momentos que precisei.

Ao Mika (Gatinho), pela amizade, pelo carinho por ser uma estrela no pensamento de experimentos, por me ajudar com o trabalho e por fazer parte dos momentos tristes e felizes.

A Gislaine (Gis) pela ajuda com os bichos, pela companhia em todo momento, por liderar e organizar o time.

Ao Diego pelo carinho, pela ajuda com os experimentos, por estar sempre comigo e por trazer um pouquinho do calor humano da bahia.

A Janice, por seu apoio no cuidado da criação, pelo apoio e companhia nos experimentos. A Afonso, Lucas Fárias e Mozart pela amizade e ajuda durante seu tempo na UFV. Ao Manuel, Hernane, Antônio e Carla e pelos ensinamentos.

A Eugênio, Claudia e Nerilda, pelo carinho e por compartilhar comigo momentos de "família".

A Angélica, Juliana, Luis Carlos pelo apoio e ajuda com os documentos.

A todos os que de uma forma ou outra contribuíram para que eu conseguisse caminhar em busca deste objetivo.

Contents

List of Figures

Abstract

MACHUCA-MESA, Laura Marcela, D.Sc., Universidade Federal de Viçosa, April, 2019. **Pheromone blend and sexual attraction in Bt-susceptible and resistant fall armyworm (*Spodoptera frugiperda*)**. Adviser: Eraldo Rodrigues de Lima. Co-adviser: Eliseu José Guedes Pereira.

Location and attraction of sexual partner is a necessary condition for sexual reproduction in insects. Among moths, chemical signals called sex pheromones are used to find a potential mate. Female moths produce pheromones active over long distances, which induce mate-location and mating. Biotic and abiotic factors influence production and emission of female sex pheromones, and male responsiveness. Age, pathogens, conspecific pheromones, mating status, temperature, day length, light intensity, relative humidity, wind speed, atmospheric conditions, geographic variations, host plants, and insecticide resistance affect sexual communication. In this thesis, we focused on the changes in sex communication of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) caused by the resistance to maize hybrids expressing Bt toxins (Cry1Fa). First, we evaluated the sex pheromone production of resistant and susceptible (homozygous and heterozygous) individuals. We observed differences among the genotypes in the amount of major component (Z9-14: Ac) and the relative proportion of the minor component during calling days and hours of scotophase. The main changes were observed in the titer of crucial minor component (Z7-12: Ac). The variations in the minor sex pheromone component can generate differences in sexual partner attraction. Second, we evaluated the male response to the calling female in the laboratory and the field. We showed that susceptible males respond less to resistant females both in the laboratory and field. Our results showed that Bt-resistance modify the sexual communication variables including pheromone production and male response. Both the pheromone variations and differences in male responsiveness can generate assortative mating among the insect genotypes. Assortative mating between resistant females and resistant males increase the resistance allele in the population favoring field resistance development to the Bt crops. Finally, the assortative mating negatively affects the high dose/refuge strategy proposed to delay the resistance in the field.

Resumo

MACHUCA-MESA, Laura Marcela, D.Sc., Universidade Federal de Viçosa, abril de 2019. **Feromônio e atração sexual em mariposas (*Spodoptera frugiperda*) suscetíveis e resistentes a Bt**. Orientador: Eraldo Rodrigues de Lima. Coorientador: Eliseu José Guedes Pereira.

Localizar e atrair o parceiro é uma condição necessária para a reprodução sexual em insetos. Em mariposas, sinais químicos denominados feromônios sexuais são usados para encontrar o potencial parceiro. Fêmeas de mariposas produzem feromônios que, em ação a longas distâncias, atraem machos para as proximidades e agem a longas distâncias localizando parceiros e desencadeam comportamentos de cópula. A produção e emissão de feromônio sexual nas fêmeas, e a resposta de machos são influenciadas por fatores bióticos e abióticos. A idade, patógenos, feromônios coespecíficos, status de acasalamento, temperatura, horas de luz, intensidade luminosa, umidade relativa do ar, velocidade do vento, condições atmosféricas, variações geográficas, plantas hospedeiras e resistência a inseticidas afetam a comunicação sexual. Neste trabalho, estudou-se as variações da comunicação sexual de *Spodoptera frugiperda* (Lepidoptera: Noctuidae) causadas pela resistência a culturas de milho que expressam toxinas Bt (Cry1Fa). Primeiro, avaliou-se a produção de feromônio sexual de indivíduos resistentes e suscetíveis (homozigotos e heterozigotos). Foram observadas diferenças na quantidade do componente principal (Z9-14: Ac) e na proporção relativa do componente minoritário nos dias de chamamento e nas horas da escotofase. As principais alterações foram observadas na proporção do componente minoritário (Z7-12: Ac), que é indispensável para a atração de machos. As variações encontradas no componente minoritário do feromônio sexual podem gerar diferenças na atração e resposta do parceiro. Em seguida, avaliou-se a resposta de machos a fêmeas chamando no laboratório e no campo. Mostrou-se que os machos suscetíveis respondem menos as fêmeas resistentes tanto no laboratório quanto no campo. Os resultados mostraram que a resistência à toxina Bt pode modificar as

variáveis da comunicação sexual incluindo a produção de feromônio e a resposta de machos. Tanto as variações de feromônio quanto as diferenças na resposta do macho podem contribuir para acasalamentos não aleatórios entre os genotipos. O acasalamento seletivo entre fêmeas resistentes e machos resistentes aumenta a presença dos alelos resistentes na população, favorecendo o desenvolvimento da resistência nas culturas Bt presentes no campo. Finalmente, neste caso o acasalamento seletivo afeta negativamente a estratégia de alta dose e refúgio proposta para retardar a resistência no campo.

General Introduction

Animals receive a great deal of information from the environment around them to find resources like food and refuge. The information can also be received from conspecifics or from other species. Intraspecific and interspecific communication is fundamental for the individual survival. Communication can be defined as the transfer of information between a sender and the receiver using a signal. In purposeful communication, the information transfer is not accidental because it benefits the sender, receiver or both. The signal in the communication can be defined as an act or structure that influence or modify the behavior of other individuals "receivers" (Wilson, 1970; Ryan and Cummings, 2005; Stevens, 2013b). Animals can use different signals to communicate such as electric, light, magnetic, mechanical, and chemical (Ali and Morgan, 1990; Stevens, 2013b).

The general term used for molecules involved in chemical communication is semiochemicals (Law and Regnier, 1971; Dicke and Sabelis, 1988). Semiochemicals that act in individuals from other species are called allelochemicals. The allelochemicals are divided according to the cost or benefits in the emitter and the receiver. Allomones are substances that benefit the emitter but not the receiver; when the odor benefits the receiver but not the emitter, it is named kairomone, and the synomones are semiochemicals benefiting both the sender and the receiver. Specifically, pheromones are a subclass of semiochemicals used in intraspecific communication. The pheromones are divided by function and effect they have. They are aggregation, alarm, and trail pheromones and those pheromones involved in mate-finding or attraction and partner acceptance are called sex pheromones (Ali and Morgan, 1990; Wyatt, 2010, 2014).

Sex pheromones are not only compounds that cause attraction and location of the individuals of the opposite sex resulting in mating, but also may elicit courtship (Baker, 1989). Sex pheromones can carry information about the degree of relatedness (Smith, 1983), quality and quantity of resources, nuptial gifts (Dussourd et al., 1991), bilateral symmetry (Thornhill, 1992), dominance status (Moore et al., 1997), and body size (Shine et al., 2003). In Lepidoptera, the studies have been focused in moth sex pheromones because chemical attraction is their critical signal to sexual recruitment (Cardé and Baker, 1984; Wicker-Thomas, 2011). Moths produce long and close-range sex pheromones (Costanzo and Monteiro, 2007), and both males, and females can produce sex pheromones. Female sex pheromones are from the abdominal glands (Hartlieb and Anderson, 1999; Wyatt, 2017) and act by attracting males from long distances (Greenfield, 1981; Cardé and Baker, 1984; Löfstedt, 1993; Smadja and Butlin, 2009). Sex pheromones from moth males commonly act in short distances to recognize the partner and begin acceptance (Fitzpatrick et al., 1988; Birch et al., 1990; Smadja and Butlin, 2009), and in a few cases, moth males use sex pheromones to attract females. The communication using sex pheromones is under constant selection process to maintain the connection between female signal and male response (Greenfield, 1981; Lassance and Löfstedt, 2009; Groot et al., 2016a).

Female moths commonly produce multicomponent sex pheromones (Wyatt, 2005) over specific hours during the night. Sex pheromones blends can have different ratios and components (Lima and McNeil, 2009), providing a unique communication signal to each species. Furthermore, male moths present high capacity to detect, discriminate and recognize the right sex pheromone blends (Svensson, 1996). However, signal production comes with energetic and physiological costs (Svensson, 1996; Stoddard and Salazar, 2011; Steiger and Stökl, 2014), and signals such as mating calls will expose female moths to eavesdropping (Stevens, 2013a). Harari et al. (2011) showed that the calling *Lobesia botrana* (Lepidoptera: Tortricidae) incurs in costs and requires female conditions. The cost of signaling depends on female conditions because extensive signaling affects the number of eggs laid by small females. However, it is unclear whether the cost is in the sex pheromone biosynthesis or in the energy spent during the calling (Harari et al., 2011). Additionally, the male response to female chemical signals is a high-cost venture (Acharya, 1995).

The production, emission, perception, and male responses to chemical signals are influenced by several factors (McNeil, 1991; Svensson, 1996). Some biotic and abiotic variables such as age, mating status, calling periodicity, host plants, temperature, light intensity, wind speed, and humidity affect sexual communication (McNeil, 1991). Both females and males of many moth species are sexually mature 1 to 2 days after emergence. Sex pheromone glands often contain a low amount of sex pheromone on the first night after emergence and increase on the second and third nights (Pope et al., 1982, 1984; Heath et al., 1991; Gemeno and Haynes, 2000; Xiang et al., 2010). Old females commonly exhibit low levels of sex pheromone (Groot, 2014), and in many species the onset calling is earlier in young females (Kanno, 1979; Turgeon and McNeil, 1982; Webster and Cardé, 1982; Howlader and Gerber, 1986; Kou and Chow, 1987; Delisle, 1992; Kamimura and Tatsuki, 1993; Spurgeon et al., 1995), to increase the mating likelihood (Kanno, 1979; Webster and Cardé, 1982). Nevertheless, calling time decreases with age (Castrovillo and Cardé, 1979; West et al., 1984; Delisle and McNeil, 1986; Schal and Cardé, 1986), this can occur because of the costs associated with this behavior. In male moths, the response to sex pheromones increases with age, and the receptivity window is wider than in the female calling (McNeil, 1991).

The length of the day or scotophase can also affect sexual communication in moths. Generally, when nights are longer, the female callings start later (Delisle and McNeil, 1986; Gerber and Howlader, 1987). However, Haynes and Birch (1986) showed that changes in the photoperiod do not affect calling time in *Platyptilia carduidactyla* (Lepidoptera: Pterophoridae) and *P. williamsii* females. In male moths, the length of the day affects sexual maturation. In shorter days, *Pseudaletia unipuncta* (Lepidoptera: Noctuidae) males matured 24h later, when compared with long days conditions (McNeil, 1991), and Kanno (1979) showed that *Chilo suppressalis* (Lepidoptera: Pyralidae) males responded later in the scotophase during short days than males kept on longer days.

Change in the temperature can generate asynchronous sexual communication because males and females respond differently to changes in temperature (Baker and Cardé, 1979; Cardé and Baker, 1984; Giebultowicz et al., 1992; Linn, 1997). Low temperature can generate inhibition in the callings and high temperatures, a delay in the callings at night (Baker and Cardé, 1979). In males, the sexual maturation is also affected with the temperature as males of *Trichoplusia ni* (Lepidoptera: Noctuidae) and *Pseudaletia unipuncta* maintained at 25°C, matured faster than males maintained at 15°C (Bollinger et al., 1977; Turgeon et al., 1983). Moreover, other environmental factors such as light intensity (McNeil, 1991), wind speed (Conner et al., 1985; Elkinton et al., 1987; Conner and Best, 1988), and humidity, also affect communication between male and female moths (Baker and Cardé, 1979; Kanno, 1979; Royer and McNeil, 1993).

Host plants stimulate sex pheromone production (Raina et al., 1992) and its release (Riddiford, 1967), and pheromone titer in some species, even though *Pseudaletia unipuncta* does not present differences in the pheromone amount in females fed on distinct host plants (McNeil, 1991). Male moths are commonly attracted to host plants because there they can find females to mate with (Kaae et al., 1977; Underhill et al., 1982). McNeil (1991) reported that sometimes host plants can also modify the male response because *Sitotroga cerealella* (Lepidoptera: Gelechiidae) males responded more to traps with corn extracts than traps with only sex pheromone. Additionally, the presence of pathogens can vary in the steps of male courtship behavior (Sweeney and McLean, 1987), but the effect of the pathogens in sex pheromone synthesis, emission and calling have not yet been studied. The chemical environment and pheromones from conspecifics influence sexual communication. *Heliothis subflexa* (Lepidoptera: Noctuidae) females presented more inhibitory compounds when emerged in odors of sister species (*H. virescens*) (Groot et al., 2010). Furthermore, females can delay the calling behavior in the presence of sex pheromones in the atmosphere (Noguchi and Tamaki, 1985). However, *Pseudaletia adultera* (Lepidoptera: Noctuidae) isolated females presented a short calling window and started calling at an older age than female groups not isolated (Rehermann et al., 2016). In the males, the presence of conspecifics can inhibit sexual behavior in other males and reduce the male competition (Hirai et al., 1978).

Insecticide resistance is a factor that has generated changes in sexual communication, specifically in sex pheromones (Campanhola et al., 1991; Delisle and Vincent, 2002; Sousa, 2016). Insecticide resistance is known as a significant decrease in a population's susceptibility to an insecticide (Gassmann et al., 2009). Campanhola et al. (1991) reported that *Heliothis virescens* (Lepidoptera: Noctuidae) females resistant to pyrethroids presented lower of sex pheromones than susceptible females. Additionally, both *Choristoneura rosaceana* (Lepidoptera: Tortricidae) and *Plutella xylostella* (Lepidoptera: Plutellidae) females resistant to organophosphorus, azinphosmethyl, tebufenozide and, abamectin, also presented lower sex pheromone titer (El-Sayed et al., 2001; Trimble et al., 2004; Xu et al., 2010). The response in resistant males of *Heliothis virescens* and *Plutella xylostella* to female sex pheromones were less than susceptible males (Campanhola et al., 1991; Xu et al., 2010). The male ability to locate females or sources of sex pheromones decrease when they were resistant (El-Sayed et al., 2001; Trimble et al., 2004; Xu et al., 2010).

Since 1996, *Bacillus thuringiensis* (Bt) genes expressed in transgenic crops is commercialized for pest control (Siebert et al., 2008; Storer et al., 2012). Transgenic crop plants expressing Bt are an attractive tool for insect management (Carrière et al., 2010; James et al., 2015; Santos-Amaya et al., 2015). Bt plants were genetically modified to produce high doses of Bt toxins in green tissues (Jouanin et al., 1998; Carriére et al., 2001). These Bt crops offer benefits such as reduced use of conventional insecticides against the target pest, increased yield (Cattaneo et al., 2006; Marvier et al., 2007; Hutchison et al., 2010), and are harmless to most non-target organisms (Lu et al., 2012; Pardo-López et al., 2013). Nevertheless, Bt crops must be implemented together with the refuge strategy to prevent the rapid resistance evolution. The refuge strategy consists of planting crops without toxins, where the susceptible insects can feed. Thereby maintain susceptible alleles in the population (Gould, 2000). The susceptible insects from the refuge would mate with resistant insects from Bt plants, where the resistant alleles will be diluted (Gould, 2000; Vélez et al., 2016).

Nevertheless, the high adoption of Bt crops permanent presence of Bt plants, and low refuge adoption, impose high selection pressure for rapid development of resistance (Santos-Amaya et al., 2015). Insect resistance Bt toxins may be associated with fitness costs and these can aid to delay the development of resistance if these costs select the resistant individuals in refuge areas (Gassmann et al., 2009). The fitness costs of Bt resistance may affect developmental and reproductive variables in the insects. The presence of fitness costs depends on the pest population and the geographical region. For example, Vélez et al. (2016) reported that a population of fall armyworm (Lepidoptera: Noctuidae) resistant to Cry1Fa from Puerto Rico did not show costs in developmental and reproductive variables such as pupal weight, development time, larval growth rate, number of spermatophore per male and the number of eggs and larvae per female. However, Dangal and Huang (2015) reported that resistant populations of fall armyworm from Florida and Puerto Rico had costs in survival and development time. Groeters et al. (1993) reported costs of the Bt resistance in sexual behavior of resistant males of *Plutella xylostella*, which mated fewer times than susceptible males.

Our group has studied the possible influence of Cry1Fa Bt resistance on variables associated with the sexual communication of fall armyworm in Viçosa (De la Pava Suárez, 2016; Sousa, 2016). *Spodoptera frugiperda* resistant populations from Brazil shows reproductive advantages including time spent calling, calling bouts and number of mature eggs, when compared with susceptible females (Sousa, 2016). Additionally, De la Pava Suárez (2016) reported differences in male response to the sex pheromone gland extract in wind tunnel. Resistant males responded to susceptible sex pheromone in lower proportions than susceptible males and vice versa. Furthermore, the refuge strategy assumes random mating between resistant and susceptible insects, but the differences in female calling and male response may compromise this assumption. According to the previous statement, our objectives were to assess the amount of sex pheromone components among resistant (R), susceptible (S) and hybrid (♀S X ♂R and ♀R ♂S) females and evaluated the attraction of resistant and susceptible males to female calling in the laboratory and field. The identification of variations in the female sex pheromone and male response to female will facilitate the understanding of the resistance, development of resistance and possible mechanisms for field management.

Study model

Spodoptera frugiperda (J.E. Smith) (Lepidoptera: Noctuidae), the fall armyworm, is distributed in the American, Asian and African continents (Sparks, 1979; Busato et al., 2005; Virla et al., 2008; Goergen et al., 2016; Cock et al., 2017; Sharanabasappa et al., 2018). Fall armyworm is an important pest of the Poaceae family, with more of 80 hosts. Injury by fall armyworm causes economic losses in corn, rice, soybean cotton, wheat, and some Solanaceae. Corn crops are good host plants for *S. frugiperda* larvae, which feed primarily on whorl leaves. Cotton plants are usually attacked on floral leaves and flower "buds," and on soybean plants the larvae attack leaves and pods in the initial phase (Barros et al., 2010). In intensive agricultural systems, different crops are sown nearby which may have different phenology and may facilitate the movement of *S. frugiperda* among crops (Nagoshi, 2009). The previous fact contributes to the increased frequency of occurrence of the pest.

In general, the reproductive behavior of fall armyworm consists in females emitting sex pheromone and attracting the males over long distances. The males emit short-distances sex pheromones from the abdominal hair pencil for courtship (Schöfl et al., 2009). The sex pheromone of *S. frugiperda* consists in a major component (Z)-9- Tetradecenyl acetate (Z9-14:Ac) (Groot et al., 2016b) and the critical minor component (Z)-7-Dodecenyl acetate (Z7-12Ac) (Tumlinson et al., 1986; Batista-Pereira et al., 2006; Groot et al., 2008; Lima and McNeil, 2009). Another component is (Z)-11-Hexadecenyl acetate (Z11-16:Ac), but the behavioral effect of this component is unclear and can vary with the geographical region (Tumlinson et al., 1986; Pashley and Martin, 1987; Andrade et al., 2000; Malo et al., 2001; Batista-Pereira et al., 2006).

Spodoptera frugiperda exhibits two host strains, corn and rice. These strains were identified by Pashley et al. (1985); Pashley (1986) from larvae collections from Puerto Rico. These strains were determined by a polymorphism with an esterase allozyme marker (Pashley, 1986). Actually, these two strains show differences in gene fragments, including cytochrome oxidase I (COI) and NADH dehydrogenase 1 (ND1) (Pashley and Ke, 1992;

Lu and Adang, 1996; Levy et al., 2002), and in amplified fragment length polymorphisms (AFLP) (McMichael and Prowell, 1999; Busato et al., 2004; Prowell et al., 2004), restriction length fragment polymorphisms (RFLP) (Lu et al., 1992) and polymorphisms in the triose phosphate isomerase gene (Nagoshi, 2010). Additionally, females of corn and rice strains also exhibit differences in the amount of the sex pheromone components and sexual behavior (Groot et al., 2008; Lima and McNeil, 2009; Unbehend et al., 2013).

In Brazil, *S. frugiperda* is a principal insect pest of corn. Since 2009, Brazil started to commercialize Bt seeds to control *S. frugiperda* in corn crops (Storer et al., 2012). Nevertheless, Farias et al. (2014) reported individuals of *S. frugiperda* resistant to Bt-corn in the field and Horikoshi et al. (2016) reported resistance for Bt-corn and Bt-cotton varieties.

Chapters

General Introduction

This section presents the central topic of the thesis and the specific questions developed in the following chapters. The next two chapters are manuscripts, where we tested reproductive variables of resistant, susceptible and hybrids insects.

Chapter 1. Sex pheromone blend of Bt-susceptible and resistant fall armyworm (*Spodoptera frugiperda* - Lepidoptera: Noctuidae)

In chapter 1, the relative proportion of three main components of the sex pheromone of resistant to Bt (Cry1Fa), susceptible and hybrids of *S. frugiperda* was evaluated. Bt resistance was associated with differences in the pheromone blend among the genotypes. The relative proportion of the minor component (Z)-7-Dodecenyl acetate (Z7-12:Ac) changed between resistant and susceptible females. The changes in sex pheromone blend can affect the male response and assortative mating, low efficiency of Bt, refuge strategy and rapid evolution of the resistance in the field.

Chapter 2. Sexual attraction between Bt-susceptible and resistant fall armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae)

In this chapter, we evaluated the male attraction to susceptible and resistant (Cry1Fa) females in the laboratory and the field. Bt resistance was associated with differences in the male responsiveness, which preferred females with similar genotype. The differences in sexual communication between resistant and susceptible insects may generate assortative mating compromising refuge strategy.

Chapter 1

Sex pheromone blend of Bt-susceptible and resistant fall armyworm (*Spodoptera frugiperda* - Lepidoptera: Noctuidae)

Abstract

Female moth sex pheromones are potentially important for reproductive isolation among species and for speciation. Sex pheromone synthesis, composition, and emission are modified with environmental and biotic factors such as temperature, daylength, light intensity, wind speed, relative humidity, atmospheric conditions, host plants, pathogens, conspecific pheromones, and insecticide resistance. Bt (*Bacillus thuringesis*) crops have been widely adopted for insect control. Brazil introduced Bt crops as an alternative tool for *Spodoptera frugiperda* control. Nevertheless, the insects were able to develop resistance in approximately three years. The fitness costs of Bt-resistance in *S. frugiperda* sexual communication are currently being investigated. We evaluated the changes in sex pheromone components and our results showed differences in the amount of the major component among genotypes, but those differences were not reflected in the blend proportion. Furthermore, minor component differed in the genotypes studied, both in the calling days and hours of scotophase. Minor sex pheromone component has an important role and his proportion in the sex blend modify the number of males attracted. We show here that resistant alleles should modify sexual communication as sex pheromone blend and consequently modify the male attraction. Differences in sex pheromone can be linked with assortative mating between resistant and susceptible individuals and increase the resistant alleles in the field. High resistant allele frequency and assortative mating impair the high dose/refuge strategy used to decrease field resistance.

Key words: Bt resistance, fitness cost, sex pheromone blend, sexual communication .

Introduction

Pheromones are chemical compounds used for communication among individuals of the same species (Wyatt, 2014). Sex pheromones help in the communication between males and females because the sexual reproduction requires location and recruitment of mate. Many groups of insects commonly use sex pheromones to attract a potential mate. The moths are a group where the chemical attraction is the main means of sexual recruitment (Cardé and Baker, 1984; Wicker-Thomas, 2011). Frequently, the female moths release volatiles to attract the males, and in a few cases, the male moths attract the female or emit aggregation pheromones. The female sex pheromone act to attract males over long distances (Greenfield, 1981; Cardé and Baker, 1984; Löfstedt, 1993; Smadja and Butlin, 2009). The sex pheromone is produced in a sex pheromone gland located at dorsal and ventral of the intersegmental membrane between 8^{th} and $9t^{th}$abdominal segments (Hollander et al., 1982). The gland exposition and sex pheromone release behavior are know as "calling" (Cardé and Willis, 2008). The sex pheromone in moth species commonly presents multicomponents (Wyatt, 2005) with different ratios or/and constituents (Lima and McNeil, 2009). The female sex pheromones are important in the reproductive isolation among species and speciation (Roelofs and Rooney, 2003; Cardé and Kenneth F, 2004; Symonds and Elgar, 2008).

Different abiotic and biotic factors (McNeil, 1991) may influence the production and emission of sex pheromones. Age is an important factor that affects female pheromone biology. In some species, the females call with 1 day (24h) of emergence (Webster and Cardé, 1982; West et al., 1984) but in other moth species vary considerably (Howlader and Gerber, 1986; Turgeon and McNeil, 1982; Delisle, 1992). Younger females of leaf roller moth (*Platynota stultana*) presented more pheromone in the gland (Webster and Cardé, 1982) and attracted more males in the field than older females (Aliniazee and Stafford, 1971). Younger females of cabbage looper, *Trichoplusia ni* also presented a higher rate of pheromone release and a longer time of calling than older females (Bjostad et al., 1980). Similarly, the hour of scotophase can also change the pheromone titer in the gland (Underhill et al., 1982; Raina et al., 1986; Snir et al., 1986; Delisle and McNeil, 1987; Schal et al., 1987). However, the changes in sex pheromone emission during the calling are not known (McNeil, 1991).

In some species, the mating status also affects the female calling and pheromone titer. Raina et al. (1986) showed that the mating suppressed the sex pheromone in females of *Heliothis zea* (Lepidoptera: Noctuidae) and 24h after mating the calling and pheromone titer increase anew. The temperature during pupal development (Turgeon and McNeil, 1982) and adult life (Turgeon and McNeil, 1982; Delisle and McNeil, 1987; Gerber and Howlader, 1987) affects both the age of the first calling and the duration of the calling period (Webster and Cardé, 1982; Webster, 1988). Other environment variables such as day length, photoperiod (Delisle and McNeil, 1987; Gemeno and Haynes, 2001), wind speed, relative humidity (Royer and McNeil, 1991), light intensity, and atmospheric conditions can also modify the sexual communication in moths (McNeil, 1991). Furthermore, the sexual communication varies with geographical variations (McElfresh and Millar, 1999; Wu et al., 1999; Gemeno et al., 2000) and changes in the sex pheromone blend could generate reproductive isolation and posteriorly speciation (Phelan, 1992; Baker, 2002; Smadja and Butlin, 2009; Allison and Cardé, 2016).

Biotic variables such as host plants, pathogens, and conspecific pheromones also affect the female calling and pheromone blend in moths (McNeil, 1991). Host plants stimulate the sex pheromone production (Raina et al., 1992) and released (Riddiford, 1967) in moths as *Helicoverpa zea* and *An-*

theraea polyphemus. The pheromone titer is also affected by modifications in the diet in species such as *Chilo suppressalis* and *Adoxophyes fasciata* (Lepidoptera: Tortricidae) but *Pseudaletia unipuncta* did not show differences in the pheromone titer in the glands of females fed in various host plants (McNeil and Delisle, 1989). The calling behavior can be modified by the presence of sex pheromone in the atmosphere or autodetection (Ochieng et al., 1995). *Choristoneura fumiferana*, (Lepidoptera, Tortricidae) virgin females exposed to sex pheromone call earlier than exposed to clean air (Palaniswamy and Seabrook, 1985). However, Noguchi and Tamaki (1985) reported that *Adoxophyes* sp. and *Homona magnanima* (Lepidoptera: Tortricidae) delay the calling behavior in the presence of sex pheromone in the atmosphere and Rehermann et al. (2016) showed that *Pseudaletia adultera* (Lepidoptera: Noctuidae) females extended their calling "window" and started calling at a lower age than isolate females.

Insecticide resistance is another variable that can modify both the sexual communication and development (Campanhola et al., 1991; Delisle and Vincent, 2002; Wei and Jia-wei, 2004; Ferrari and Georghiou, 1981; Unbehend et al., 2013; Santos-Amaya et al., 2017a). Campanhola et al. (1991) showed that *Heliothis virescens* (Lepidoptera: Noctuidae) susceptible females to pyrethroids produced more amount of sex pheromone than resistant females. The sex pheromone components were also lower in the obliquebanded leafroller (Lepidoptera: Tortricidae) females resistant to insecticides, including organophosphates (El-Sayed et al., 2001) and azinphosmethyl (Trimble et al., 2004). Xu et al. (2010) also reported similar results in diamondback moth (*Plutella xylostella*) females resistant to tebufenozide and abamectin.

Nowadays, new biotechnologies are used to insect control in the field. Some crop plants are genetically modified to express genes from *Bacillus thuringiensis* (Bt) and to produce high doses of Bt toxins (Jouanin et al., 1998; Carriére et al., 2001). The Bt crops offer some benefits including low or negligible impacts to most non-target organisms (Lu et al., 2012; Pardo-López et al., 2013), increased yield and reduced use of conventional insecticides (Cattaneo et al., 2006; Marvier et al., 2007; Hutchison et al., 2010). Plants such as corn, cotton, and soybean are the Bt crops most commercialized, and countries such as the United States, Brazil, Argentina, and Canada present a high adoption of Bt cultivars (ISAAA, 2016).

The Bt crops to insect control are recommended to be implemented with the refuge strategy to prevent Bt resistance evolution. The refuge strategy consists of planting non-Bt cultivars to provide plants without toxin where bt susceptible insects can survive, thereby maintaining susceptible alleles in the insect population (Gould, 2000). In this case, resistant insects emerging from Bt crop would mate with susceptible insects from the refuge, thereby the resistant alleles should be diluted, by generating heterozygous insect progeny that would die in the Bt crop in the same or the next season (Gould, 2000; Vélez et al., 2016). However, the resistance of *S. frugiperda* to Cry1Fa corn in the field was already reported in the United States (Huang et al., 2014), Brazil (Farias et al., 2014) and Puerto Rico (Storer et al., 2010). The possible causes of the rapid resistance evolution are believed to be the low adoption of the refuge and an intense selection pressure by the large-scale adoption of Bt crops (Santos-Amaya et al., 2015; Tabashnik and Carrière, 2017).

In terms of evolutionary ecology, Bt resistance sometimes generates fitness cost in the development and reproductive characteristics (Kliot and Ghanim, 2012; Vélez et al., 2016), and if strong, these costs can help in the management of the resistance evolution (Gassmann et al., 2009). Pupal weight, developmental time, growth rate, sex proportion are the variables of development usually compared between resistant and susceptible individuals. The number of eggs/larvae per female, number of spermatophores per male are some reproductive characteristics that have received attention in published studies. For instance, Vélez et al. (2016) showed that *Spodoptera frugiperda* resistant populations to Cry1Fa from Puerto Rico did not present costs in variables such as pupal weight, development time, larval growth rate, number of spermatophore per male and the number of eggs and larvae per female. However, Dangal and Huang (2015) reported that *S. frugiperda* resistant populations from Florida and Puerto Rico had costs in survival and development time. Experiments done by Groeters et al. (1993) did not show differences in the mating success between susceptible and resistant females of diamondback moth (Lepidoptera: Plutellidae), but they did not consider specifically female attraction or mating choice.

Bt corn producing the Cry1Fa Bt toxin is commercialized in Brazil since 2009 (Storer et al., 2012) to control *S. frugiperda* because it is one of the most important pests of corn crops in Latin America. However, *S. frugiperda* developed resistance to Bt corn rapidly (3 years). The warm climate conditions may have caused the fast evolution of Cry1Fa Bt resistance in Brazil, multiple crop cycle per year and low adoption of refuge to reduce the selection pressure (Farias et al., 2014; Santos-Amaya et al., 2016). The Cry1Fa resistant populations from Brazil have been studied to determine causes, fitness cost, and resistance management (Horikoshi et al., 2015, 2016; Santos-Amaya et al., 2016, 2017a,b). The resistant population studied by Santos-Amaya et al. (2017b) showed an absence of cost in developmental variables, fecundity, and progeny production.

Additionally, Sousa (2016), showed that the Cry1Fa resistance in fall armyworm could come with advantages in reproductive variables such as time spent calling, calling bouts, and the number of mature eggs. Here, we further evaluated the amount of the three main sex pheromone components in females of the Cry1Fa resistant (R), susceptible (S) and hybrids (♀R X ♂S and ♀S X ♂R) genotypes from same fall armyworm strain (Sousa, 2016; Santos-Amaya et al., 2017b). The identification of variations in the sex pheromone blend allows us to interpret the potential phenotypic changes linked to the resistance and their fitness, cost to the sexual communication in fall armyworm moths.

Fall armyworm sex pheromone consists of a mixture of three main components. The major component is (Z)-9-tetradecenyl acetate (Z9-14: Ac), the minor component is (Z)-7-dodecenyl acetate (Z7-12: Ac) and the third more abundant component is (Z)-11-hexadecenyl acetate (Z11-16: Ac). Z7-12: Ac and Z9-14: Ac is important in male attraction, but the effect of the third component (Z11-16: Ac) in the male behavior remains unclear (Groot et al., 2016b) and vary in the geography regions (Andrade et al., 2000; Batista-Pereira et al., 2006). The presence of the minor component in *S. frugiperda* sex pheromone increased male response in populations from Central America and Z7-12: Ac as is named as the crucial sex component. In other species, the minor component also as small changes induces greater changes in male response and has an essential role in the interspecific reproductive isolation (Mozuraitis, 2000; Yang et al., 2009; Uehara et al., 2014) and the male attractiveness of the pheromone mixture (Downham et al., 2003; Chen et al., 2018). Nevertheless, the specificity of sex pheromone is determined by the whole mixture of components emitted by conspecific individuals, including abundant and minor components (Chen et al., 2018).

Material & Methods

Insects

Colonies of fall armyworm susceptible and resistant to Cry1Fa were provided by the Laboratory of insect-plant interaction from Federal University of Viçosa (Viçosa, MG, Brazil). The susceptible colony was derived from collections in non-*Bt* corn crops in the field in 2010 and share the same genetic architecture of the resistant colony. The later was derived from the susceptible strain exposing the individuals throughout larval development to Cry1Fa toxin (TC1507 event) until reaching high levels of resistance to Bt toxins as described Santos-Amaya et al. (2016, 2017b).

Resistant and susceptible colonies were reared as described in Kasten et al. (1978) with adaptations. The adult moths were placed in PVC cages (20 cm in height x 15 cm in diameter) having white paper on the inner walls for egg-laying. A cotton soaked in a solution of 10% sugar and 1% ascorbic acid was introduced. Eggs were collected every 2 days and stoked in plastic cups until hatching. Neonates were fed in artificial diet (Kasten et al., 1978) and transferred to 500mL plastic cups until the 2^{nd} or 3^{rd} instar larvae and placed in pairs in 16 PVC cells until pupation. The colonies were kept at 26 ± 2 °C, 14L:10D photoperiod and a relative humidity (RH) of 70 ± 10 %.

Hybrid females were obtained by reciprocal crosses of susceptible females × resistant males (♀S × ♂R) and resistant females × susceptible males (♀R × ♂S). The management of hybrid populations were similar with susceptible and resistant genotypes.

Pheromone extraction

The quantification and periodicity of pheromone production were obtained through the analysis of sex pheromone gland extractions from resistant, susceptible and hybrid virgin females with 1, 3, 5 and 7 days of calling. The glands were extrated at 3^{rd}, 6^{th} and 9^{th} hours of scotophase, totaling 12 treatment combinations (4 ages of calling x 3 periods of the scotophage) per genotype and 15 glands per treatment. Sex pheromone glands, localized in the last abdominal segments (Sekul and Sparks, 1967), were excised with micro scissors. Each gland was transferred to a glass vial with 5 μL hexane with 40 ng of n-heptyl acetate (IS) for 30 minutes.

Gas Chromatography and Quantification

Extracts were analyzed by gas chromatography with flame ionization detector (GC-FID) using a Shimadzu GC-17A equipped with a Rtx-5 crossbond 5% diphenyl-95% dimethyl polysiloxane capillary column (30 m, 0.25 mm i.d. and 0.25 μm film thickness; Thames Restek UK Ltd). One microliter of each sample was injected in splitless mode with the injector at 250°C. The column oven was maintained at 80°C for 2 min, and then the temperature was increased to 180°C at 30°C/min, and finally the temperature was increased to 250°C at 5°C/min, and held for 2 min. Helium was used as the carrier gas at a constant flow rate of 8 ml/min. The amount of the three pheromone compounds, (Z7-12:Ac, Z9-14:Ac and Z11-16:Ac) was determined based on an analytical curve at 0.1, 0.5, 0.7, 1.0, 10, 30, 50, 70 and 100 ng/μl with an authentic standard of Z7-12:Ac, Z9-14:Ac and Z11-16:Ac.

Statistical analysis

The amount of Z9-14:Ac and relative proportion of Z7-12:Ac, Z9-14:Ac and Z11-16:Ac in each treatment were analyzed with a Generalized Linear Model (GLM) with a normal distribution of errors, followed by analysis of residues to check for the suitability of error distribution and the final model. The adequate model was obtained by extracting non-significant terms from the final models composed by the significant explicative variables and their interactions ($p < 0.05$). Finally, we performed a contrast analysis to determine which means differed in the amount of Z9-14:Ac and the relative proportions of the three components among the four genotypes (Crawley, 2012). All analyses were performed with the R statistical program (R Development Core Team, 2008).

Results

The amount of the major component (Z9-12:Ac) changed as a function of the genotype, calling days, genotype with calling days interaction and calling days with hour interaction. The amount of (Z9-12:Ac) decreased with the calling days in the four genotypes (Fig.1.1 and 1.2). The amount of Z9-12: Ac was significantly higher in 1-day resistant females than in 1-day susceptible females. Additionally, 3 and 5-days of calling resistant females in the middle and end of the scotophase also had a higher amount of (Z9-12:Ac)(Fig.1.1). The amount of (Z9-12:Ac) in the hybrid females (♀S x ♂R and ♀R x ♂S) was similar in the calling days and the hour of scotophase, except in the 9th hour of scotophase from the 5th day of calling, where hybrid females ♀S x ♂R presented the highest amount(Fig.1.2).

Blend components (mixture) were different in the four genotypes. The minor component (Z7-12: Ac) proportion was higher in susceptible females than in resistant and hybrid females (Fig.1.3). The Z9-12: Ac proportion was lower in susceptible, resistant and hybrid females ♀R x ♂S than in hybrid ♀S x ♂R and vice-versa with Z11-16: Ac where susceptible, resistant and hybrid females ♀R x ♂S presented the highest amount.

The proportion of Z7-12:Ac changed in the genotypes with days of calling, and the hour of scotophase. The relative proportion of Z7-12:Ac was less on the first day of calling for the four genotypes. The relative proportion of Z7-12:Ac increased with 3 and 5 days of calling but decreased when the females were 7 days of calling (Fig. 1.4). Only, at 3th hour of scotophase susceptible females presented higher relative proportion of Z7-12:Ac than resistant females (Fig. 1.5). The hybrid females (♀S x ♂R and ♀R x ♂S) always presented less relative proportion of Z7-12:Ac than resistant and susceptible females (Fig. 1.5).

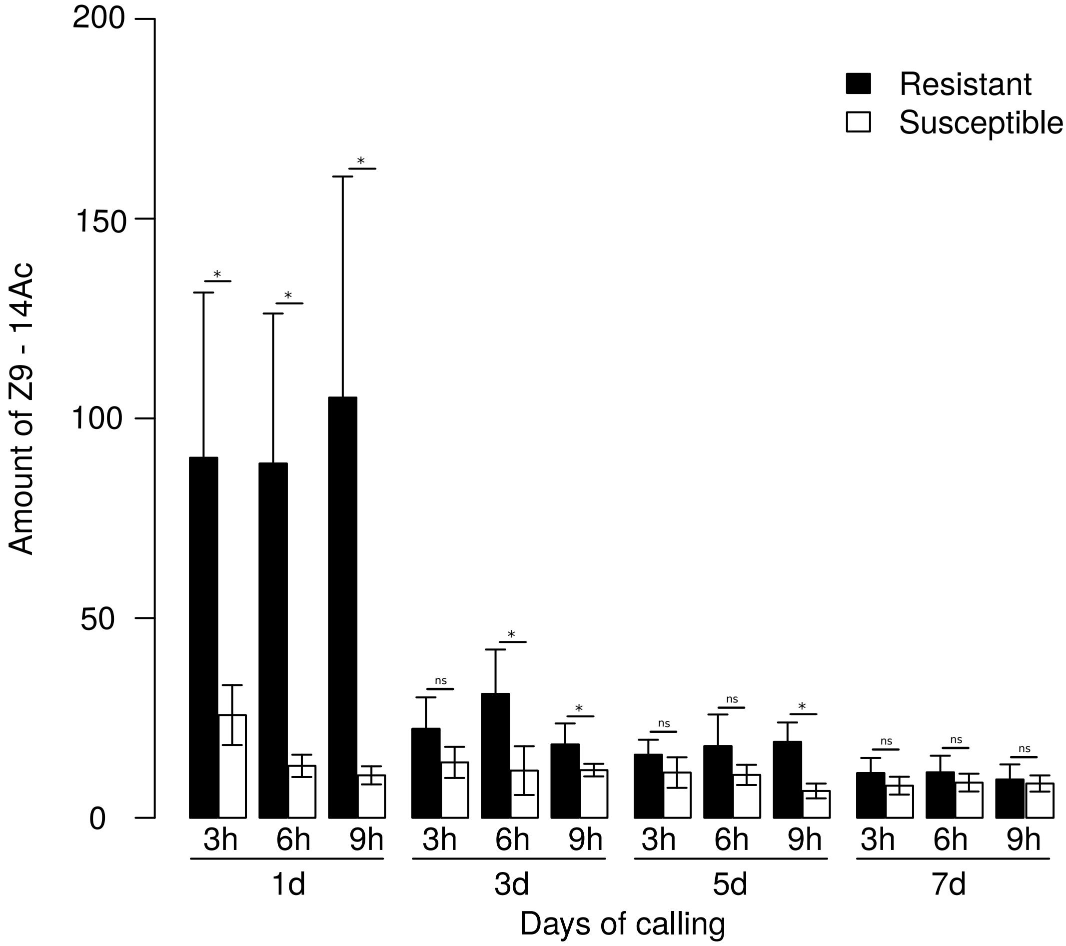

Figure 1.1: **Amount of Z9-14Ac (Mean±SEM) in pheromone gland of resistant and susceptible females**. Pheromone glands from virgin, calling females were extracted at 3^{rd}, 6^{th} and 9^{th} hours of scotophase and were analyzed and quantified by gas chromatography. * *significant differences between genotypes and* ns *not significant differences (*$p > 0.05$ *GLM with a normal distribution and contrast tests)*

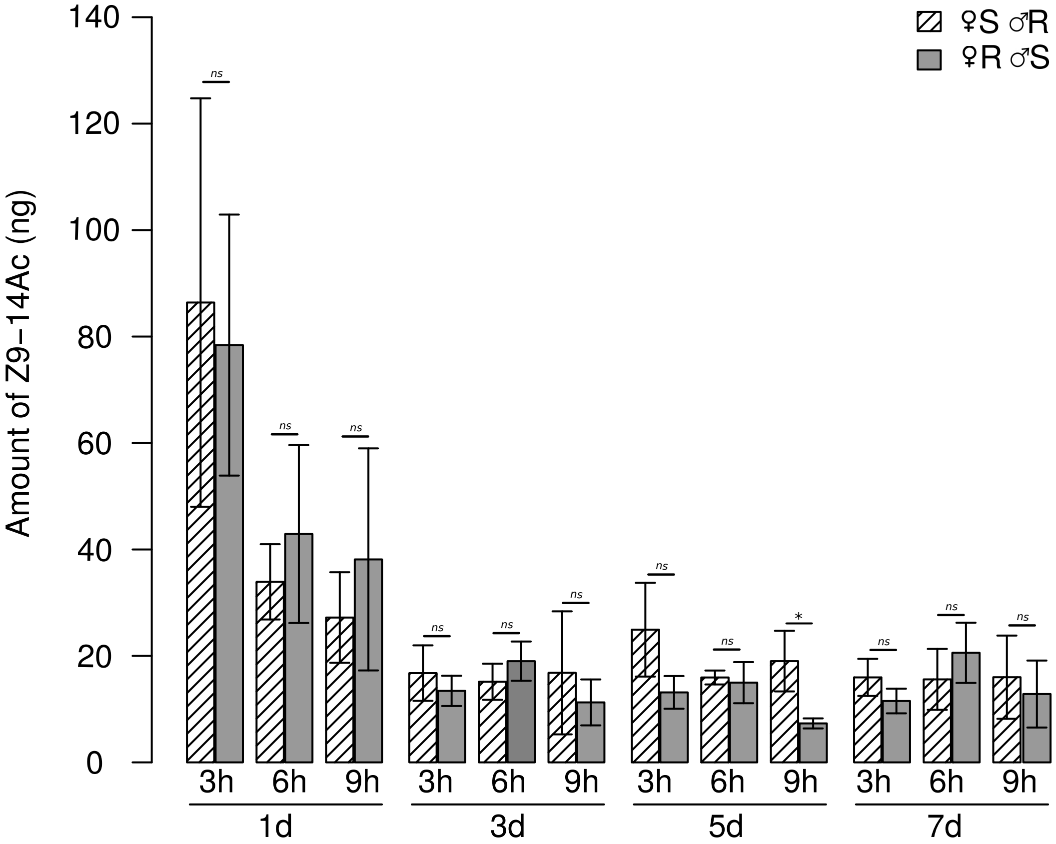

Figure 1.2: **Amount of Z9-14Ac (Mean±SEM) in pheromone gland of hybrid females**. Pheromone glands from virgin, calling (♀S x ♂R and ♀R x ♂S) females were extracted at 3^{rd}, 6^{th} and 9^{th} hours of scotophase and were analyzed and quantified by gas chromatography. ** significant differences between genotypes and ns not significant differences (*$p > 0.05$ *GLM with a normal distribution and contrast tests)*

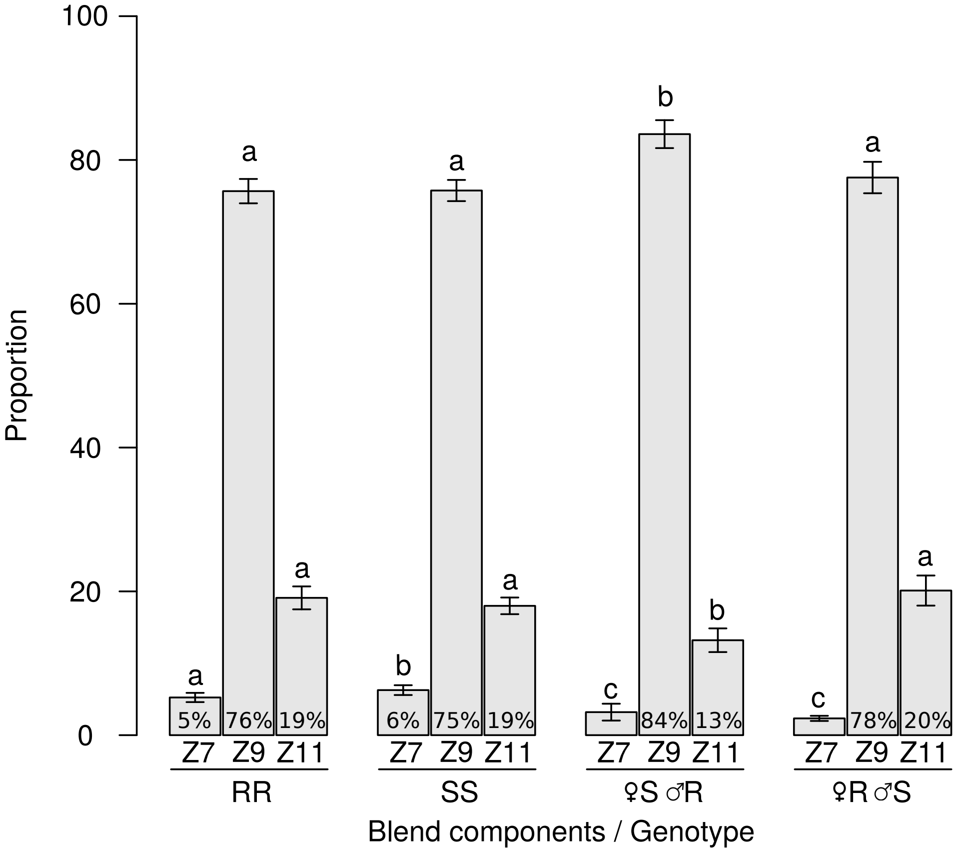

Figure 1.3: **Blend proportion (Mean±SEM) in pheromone gland of resistant, susceptible and hybrid females**. Pheromone glands from virgin females with 1, 3, 5 and 7 calling days were extracted at 3^{rd}, 6^{th} and 9^{th} hours of scotophase and were analyzed and quantified by gas chromatography. *Letters are comparisons among genotypes. Different letters show significant differences (p* $>$ 0.05 *GLM with a normal distribution and contrast tests)*

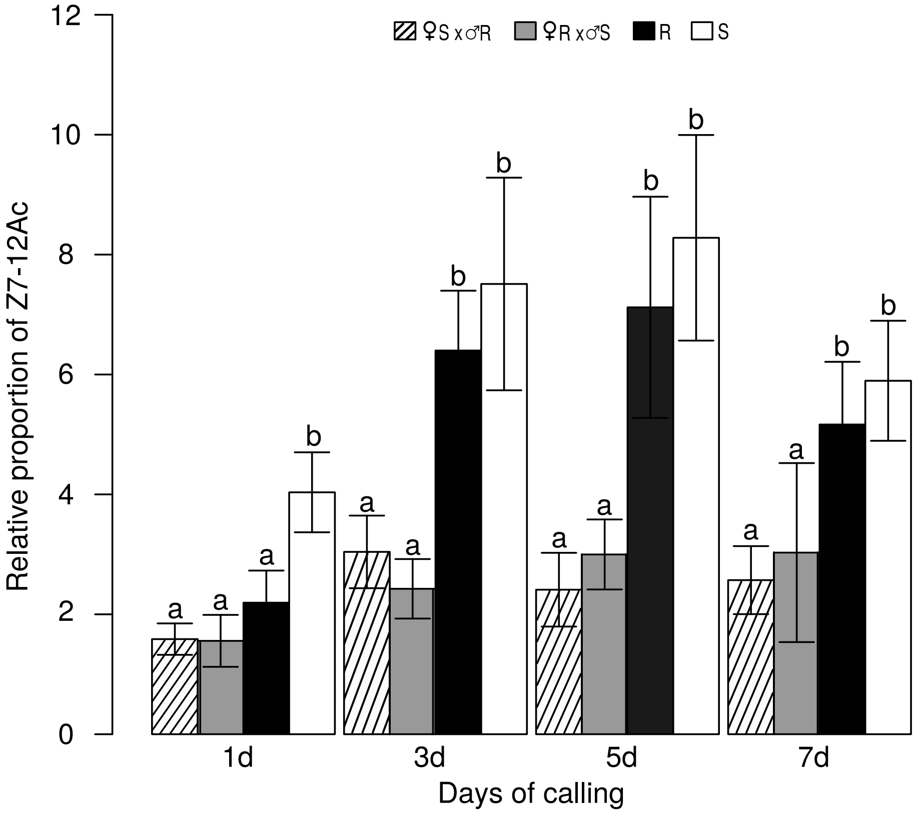

Figure 1.4: **Relative proportion (Mean±SEM) of Z9-12:Ac as a function of the calling days for resistant (R) and susceptible (S) and hybrid females (♀S X ♂R and ♀R X ♂S)**. Pheromone glands from virgin females with 1, 3, 5 and 7 calling days were extracted at 3^{rd}, 6^{th} and 9^{th} hours of scotophase and were analyzed and quantified by gas chromatography. *Letters are comparisons among genotypes on calling days. Different letters show significant differences (*$p > 0.05$ *GLM with a normal distribution and contrast tests)*

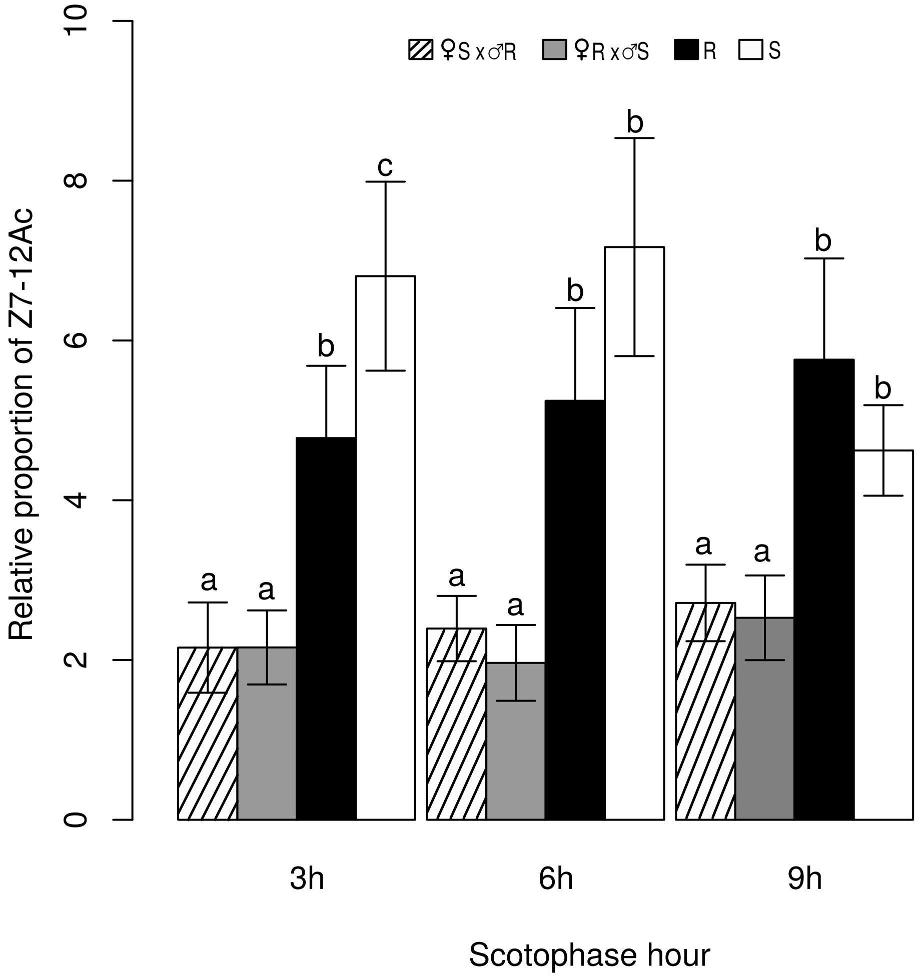

Figure 1.5: **Relative proportion (Mean±SEM) of Z9-12:Ac as a function of the hour of scotophase for resistant (R) and susceptible (S) and hybrid females (♀S X ♂R and ♀R X ♂S)**. Pheromone glands from virgin females with 1, 3, 5 and 7 calling days were extracted at 3^{rd}, 6^{th} and 9^{th} hours of scotophase and were analyzed and quantified by gas chromatography. *Letters are comparisons among genotypes on hours of scotophase. Different letters show significant differences (p* $>$ 0.05 *GLM with a normal distribution and contrast tests)*

Discussion

We found that there are significant changes in the sex pheromone blend of *S. frugiperda* resistant to Cry1Fa toxin. The amount of Z9-14:Ac was higher in the first three calling days than in the last two calling days. This was an unexpected result because Lima and McNeil (2009) showed that the amount of Z9-14:Ac increased between 3^{rd} and 5^{rd} calling days, then later decreased. However, the amount of the sex pheromone components vary according to geographic region and type of populations (Groot et al., 2008; Lima and McNeil, 2009; Unbehend et al., 2013). Additionally, the amount of Z9-14:Ac in 1 calling day resistant females and hybrid females (♀S x ♂R, ♀R x ♂S) was higher than susceptible females. Z9-14:Ac is the major component in the sex pheromone blend found in fall armyworm from several regions in North and South America and in individuals with different host plant (Groot et al., 2008). Malo et al. (2004); Batista-Pereira et al. (2006) showed that increasing doses of Z9-14:Ac also increased the male antenna response. However, pheromone traps with Z9-14:Ac as single component captured only 5% of males. Female sex pheromone components can act together (mixture) and present a synergistic effect (Groot et al., 2016b) because traps with two and/or three components captured more males (Andrade et al., 2000; Batista-Pereira et al., 2006).

The blend (components mixture) in the four genotypes were different in at least one component. The Z9-14:Ac titer was not different among resistant, susceptible and hybrid ♀R x ♂S females. This result verified that the amount Z9-14:Ac was different among resistant and susceptible females but the ratio did not change. Additionally, the Z7-12:Ac titer in the blend was different among susceptible, resistant and hybrids females (♀S x ♂R, ♀R x ♂S). The Z7-12:Ac is a critical secondary component (Tumlinson et al., 1986; Groot et al., 2008; Lima and McNeil, 2009; Batista-Pereira et al., 2006) and its presence in the mixture can increase up to 10x the male capture (Andrade et al., 2000). Little changes in the Z7-12:Ac ratios had a significant effect on the male attraction (Andrade et al., 2000; Batista-Pereira et al., 2006).

Susceptible moths with one calling day showed a higher relative proportion of Z7-12:Ac than other genotypes. The changes found in Z7-12:Ac component between resistant and susceptible females may contribute to differences in the attraction of resistant and susceptible males. Additionally, Sousa (2016) also found that resistant and susceptible females differ in the number of mature eggs as a function of the time after emergence. In moths, the number of mature eggs and basal oocyte width are often associated with the sex pheromone production and pheromonal communication (Cusson and McNeil, 1989). If young resistant and susceptible females present differences in the minor pheromone component and the males perceive these differences, it can generate assortative mating. A greater resistant offspring in the field does not favor Bt resistance management as expected when deploying refuges to reduce the selection pressure. There no differences in the Z7-12:Ac component in resistant and susceptible females of three to seven calling days, indicating that the changes in the sexual communication presented in the first calling days. The hybrid females (♀S X ♂R and ♀R X ♂S) always presented a lower amount of Z7-12:Ac than resistant and susceptible females. This indicates the absence of hybrid vigor in the Z7-12:Ac production in contrasts to that was observed for Santos-Amaya et al. (2017b) in the developmental characteristics.

Our results also showed changes in the relative proportion of Z7-12:Ac during the night. The difference in the proportion of Z7-12:Ac between resistant and susceptible females were only observed at 3rd hour of scotophase. This indicates that on the first hours of scotophase exist differences in the calling and mating between resistant and susceptible individuals. The differences of Z7-12:Ac between resistant and susceptible females can cause that resistant females are more attracted to resistant males early in the night. Mating between individuals of the same genotype increases the frequency of the resistance alleles in the field, accelerating the rate of the resistance evolution. Additionally, in all genotypes, we found that sex pheromone titer changes with the age and hour of scotophase. Changes in the pheromone titer with the age and hour of scotophase occur in several moth species (Bjostad et al., 1980; Webster and Cardé, 1982; Raina et al., 1986; Snir et al., 1986; Delisle and McNeil, 1987; Schal et al., 1987)

The proportion mean of Z11-16:Ac in our work did not present differences among resistant, susceptible and hybrid ♀S x ♂R females. Furthermore, Batista-Pereira et al. (2006) reported that the addition of Z11-16:Ac to the blend, did not increase male attraction. The relative proportions of Z7-12:Ac and the amount of Z9-14:Ac that we reported in this work, presented inter-individual differences. These inter-individual differences are frequent in some Lepidoptera species (Löfstedt et al., 1985; Barrer et al., 1987; Ono et al., 1990), but in our work were lower than in other species.

In conclusion, we found changes in the sex pheromone blend in resistant population to Cry1Fa toxin. These differences were mainly in the minor component Z7-12Ac in young females and in the first hours of scotophase. These results are important to understand the changes in sex pheromones and in sexual communication. Finally, the differences in the pheromone blend could potentially modify the communication channels, the male attraction, and mating between resistant and susceptible individuals in the field and contribute to fast resistance evolution.

Chapter 2

Sexual attraction between Bt-susceptible and resistant fall armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae)

Abstract

In insects, usually, the signals used in sexual partner attraction and orientation are sex pheromones. In many moth groups, females release sex pheromone and males respond over long distances. Male perception and responsiveness may be influenced by variables such as age, temperature, day length, light intensity, wind speed, relative humidity, atmospheric conditions, host plants, pathogens, and conspecific pheromones. However, insecticide exposure and insecticide resistance are other factors that can affect sexual communication in moths. In Brazil, crops expressing Bt (*Bacillus thuringesis*) insecticidal toxins has increased as an alternative tool for *Spodoptera frugiperda* (Lepidoptera: Noctuidae) the most important pests of corn crops in Latin America but, Bt-resistance also has increased. The costs of Bt-resistance in sexual communication variables of *S. frugiperda* are now being investigated. Here, we studied the pattern of the attraction of resistant and susceptible males of *S. frugiperda* to the female calling. Resistant males respond less to susceptible females in the laboratory and this response is consistent with those observed in the field experiments of partner attraction. Our result showed that Bt-resistance can modify sexual communication variables such as the male response. Our results also showed that resistant females attracted a high number of resistant males and this increased the resistant alleles in the population, thus favoring field resistance development to the Bt crop. These results give us an example of speciation mechanisms caused by sexual communication modifications and help us to understand how phenotypic changes associated with resistance itself may increase the rate of resistance evolution, and to propose new strategies for insecticide resistance. These results are an example of speciation mechanisms caused by sexual communication modifications and help us to understand how phenotypic changes associated with resistance itself may increase the rate of resistance evolution, and to propose new strategies for insecticide resistance.

Key words: Fitness cost, sex attraction, Sexual communication, male response.

Introduction

Location and recruitment of the mate is necessary in sexual reproduction. Many insect groups use pheromones such as signals to attract and orient their partner's movements. In moths, the females predominantly emit sex pheromones and the males receive and respond over long distances (Greenfield, 1981; Cardé and Baker, 1984; Löfstedt, 1993; Smadja and Butlin, 2009; Wicker-Thomas, 2011). Male moths can also produce sex pheromones, which operate in short distances and aid in the recognition and acceptance by females (Fitzpatrick et al., 1988; Birch et al., 1990; Smadja and Butlin, 2009). In moths, sex pheromone communication undergoes a constant selection process to maintain the relationship between the female pheromones and the male's ability to identify the conspecific blend (Greenfield, 1981; Lassance and Löfstedt, 2009; Groot et al., 2016a).

The male perception and response may be influenced by different environmental and biotic factors. One important factor is its age because the male receptivity changes on age. Generally, the male responsiveness increases with age and the receptivity window is wider than the female calling (McNeil, 1991). The mating status also affects the male response, modifying the neuromodulators and/or antennal sensitivity (Duportets et al., 1998; Gadenne et al., 2001; Reyes et al., 2015). In many moth species, the temperature affects the male sexual maturation and the response to female sex pheromones. Males of *Trichoplusia ni* and *Pseudaletia unipuncta* maintained at 15°C required more days to reach sexual maturation than males maintained at 25°C (Bollinger et al., 1977; Turgeon et al., 1983). Other abiotic factors such as day length, light intensity, wind speed, relative humidity, and atmospheric conditions can also affect the sexual communication in moths (McNeil, 1991).

Additionally, biotic factors such as host plants, pathogens, and conspecific pheromones also affect the male response. Many times the males are attracted to host plants because they may find females to copulate with on these plants (Kaae et al., 1977; Underhill et al., 1982). The host plants can also modify the male response, e.g. traps with sex pheromones and corn extracts, captured more *Sitotroga cerealella* (Lepidoptera: Gelechiidae) males than traps with only sex pheromones (reviewed by McNeil (1991)). Some pathogens can also modify the steps of male reproductive behavior (wing-fanning, take off, lock on and land and, fligh), expressed by healthy males (Sweeney and McLean, 1987). Furthermore, the presence of conspecific male sex pheromones can also be related to the male-to-male inhibition in *Pseudaletia unipuncta* (Lepidoptera: Noctuidae), to reduce mate competition (Hirai et al., 1978).

Insecticide exposure is another factor that can affect communication between moths (Delisle and Vincent, 2002; Wei and Jia-wei, 2004). Resistance and sub-lethal insecticide affect both the development and reproduction of the insects (Ferrari and Georghiou, 1981; Campanhola et al., 1991; Unbehend et al., 2013; Santos-Amaya et al., 2017b). Campanhola et al. (1991) showed that less number of resistant *Heliothis virescens* males responded to female sex pheromones. The ability of resistant *Choristoneura rosaceana* males to locate females and sources of pheromones were affected negatively (Trimble et al., 2004). However, Xu et al. (2010) found that resistant diamondback male moths presented higher responsiveness to female sex pheromones than susceptible males.

Both synthetic insecticides and "natural" insecticides are used for pest control. In the last decade, transgenic plants or crops expressing Bt (*Bacillus thuringesis*) insecticidal toxins has increased as an alternative tool (Carrière et al., 2010; James et al., 2015; Santos-Amaya et al., 2015). The Bt crop genes encoding Bt toxins, have been incorporated in plant genomes such as corn, cotton, soybean, sugarcane, rice (Tabashnik et al., 2009; James, 2013) and other crop plants (ISAAA, 2019). The Bt plants express the Bt proteins, and when pest insects consume the plant, they die. Bt crops are often advantageous, because they conserve the natural enemies of arthropod pests (Lu et al., 2012), are harmless to most non-target organisms (Pardo-López et al., 2013), and can increase yield and reduce use of conventional insecticides (Cattaneo et al., 2006; Marvier et al., 2007; Hutchison et al., 2010).

However, the large-scale adoption of Bt crops and the permanent presence of Bt plants in the field, have increased the risk of resistance imposing a strong selection pressure (Santos-Amaya et al., 2017b). The evolution of resistance to Bt proteins is caused by exposure of the insect populations to the toxins in the field (Tabashnik et al., 2013). It can be rapid if this is a high selection pressure in multiple generations of insect pest per year, the presence of similar Bt technologies in the field and no adoption of refuge (Santos-Amaya et al., 2017b). The refuge strategy to delay Bt resistance, consists in planting non-Bt crop near Bt crop to provide susceptible homozygous insects. These susceptible insects would mate with eventual resistant insects emerging from Bt crops, producing susceptible heterozygotes that would be eliminated by the Bt crop in the same or next growing season (Vélez et al., 2016). The refuge strategy is based on three requirements: resistance recessively inherited, low initial frequency of resistant alleles, and random mating between resistant and susceptible individuals (Gould, 1998; Carriére and Tabashnik, 2001; Onstad et al., 2002; Carrière et al., 2004). In past years, several cases of insect resistance to Bt crops have been reported, especially in the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae), which have evolved resistance to the Cry1Fa Bt toxin in different countries like the United States (Huang et al., 2014), Puerto Rico (Storer et al., 2010) and Brazil (Farias et al., 2014; Vélez et al., 2016).

Bt resistance can be associated with fitness cost in resistant insects in relation to Bt susceptible ones, and these costs can help to delay the resistance because they select against the resistant individuals in refuge areas (Gassmann et al., 2009). These fitness costs can be associated with developmental or reproductive traits. Life-history traits like pupal weight, developmental time, growth rate, lifetime fertility, number of spermatophores per male, sex proportion, and population parameters are compared between resistant and susceptible insects (Vélez et al., 2016). Studies in Florida, Puerto Rico and Brazil showed that Bt resistance can or cannot present fitness cost (Vélez et al., 2013; Jakka et al., 2014; Dangal and Huang, 2015; Santos-Amaya et al., 2017b). Vélez et al. (2013) showed that *Spodoptera frugiperda* (Lepidoptera, Noctuidae) resistant populations to Cry1Fa from Puerto Rico did not present costs in pupal weight, development time, larval grow rate, number of spermatophores per male and the number of eggs and larvae per female. However, Dangal and Huang (2015) reported that the fall armyworm resistant populations from Florida and Puerto Rico have costs in survival and development time. The discrepancy among these results can be due to differences of the alleles that confer the resistance (Vélez et al., 2016). In contrast, fitness costs of Bt resistance affecting behavioral variables have been little investigated although long ago. Groeters et al. (1993) showed cost of the Bt resistance in the sexual behaviour *Plutella xylostella* (Lepidoptera: Plutellidae), having the resistant males mated fewer times than the susceptible males.

Cry1Fa Bt corn cultivars have been commercialized in the United States since 2003 (Siebert et al., 2008; Storer et al., 2012), but Brazil in 2009 (Storer et al., 2012). The main target insect of Cry1Fa corn was the fall armyworm, which is one of the most important pest of corn crops in Latin America (Farias et al., 2014; Santos-Amaya et al., 2016). Nevertheless, fall armyworm populations developed resistance in three years. This fast evolution of resistance is believe to be associated with the climate conditions and multiple crop cycle per year (Farias et al., 2014; Santos-Amaya et al., 2016).

Santos-Amaya et al. (2017b) studied the possible fitness costs of Cry1Fa resistance in developmental variables, fecundity and progeny production and found no costs in *S. frugiperda* populations from Brazil.

Fall armyworm moth males resistant to Cry1Fa showed differences in the response to sex pheromone gland extract in the wind tunnel. The resistant males reached to susceptible sex pheromone in lower proportions than susceptible males and viceversa (De la Pava Suárez, 2016), suggesting possible non-random or assortative mating. However, the refuge strategy assumes random mating (mating between individuals where the choice of partner is not influenced by the genotypes) between resistant and susceptible insects such that the difference in the male response could lead to a biased mating system, and thus increasing the rate of resistance evolution. This hypothesis of non-random mating in Cry1Fa Bt resistant fall armyworm moths can be tested by identifying variations in the male responses to female pheromone, which was the goal in this study.

Specifically, we tested the following hypotheses: resistant males respond less to susceptible females in the laboratory and this response is consistent with those observed in the field experiments of partner attraction. The pattern of attraction of resistant and susceptible males of *S. frugiperda* to female calling in the laboratory and the field were consistent with the non-random mating between fall armyworm moths carrying Cry1Fa Bt resistant alleles. These results have important implications for resistance evolution and development of the main resistance management strategy, which is discussed in this chapter.

Material & Methods

Insects

Colonies of fall armyworm susceptible and resistant to Cry1Fa were provided by the Laboratory of insect-plant interaction from Federal University of Viçosa (Viçosa, MG, Brazil). The susceptible colony was derived from collections in non-*Bt* corn crops in the field in 2010 and share the same genetic architecture of the resistant colony. The later was derived from the susceptible strain exposing the individuals throughout larval development to Cry1Fa toxin (TC1507 event) until reaching high levels of resistance to Bt toxins as described Santos-Amaya et al. (2016, 2017b).

Resistant and susceptible colonies were reared as described in Kasten et al. (1978) with adaptations. The adult moths were placed in PVC cages (20 cm in height x 15 cm in diameter) having white paper on the inner walls for egg-laying. A cotton soaked in a solution of 10% sugar and 1% ascorbic acid was introduced. Eggs were collected every 2 days and stoked in plastic cups until hatching. Neonates were fed in artificial diet (Kasten et al., 1978) and transferred to 500mL plastic cups until the 2^{nd} or 3^{rd} instar larvae and placed in pairs in 16 PVC cells until pupation. The colonies were kept at 26 ± 2 °C, 14L:10D photoperiod and a relative humidity (RH) of 70 ± 10 %.

Wind tunnel experiment

To evaluate the virgin males response to virgin female in the laboratory, we tested resistant and susceptible males in the wind tunnel. At 3^{rd}, 6^{th} and 9^{th} hours of scotophase, one virgin female that was 2 or 3 days of calling was transferred into the wind tunnel. When the female was calling, one virgin male that was 1, 3, 5 or 7 days old was transferred into the wind tunnel. We observed male behaviour for two minutes and the males that reached the female were registered. Fifteen males were tested in each age and hour of scotophase. The wind tunnel was a Plexiglas construction (3 m in length x 1 m in width x 1 m in height), through which cleaned air was pushed by a fan producing a 0.3 m/s laminar airflow. The experiments were performed at 24 ± 2 °C, 60 ± 10 % RH and at 1 Lux light intensity.

Field experiments

Corn crops

The field experiment was conducted in the São Manuel farm (11º55’3”S, 48º49’1”W), located in Peixe (Tocantins State, Brazil). There was two nearby corn crops, one with 3 ha of non-*Bt* corn hybrid LG6310 (LIMAGRAIN BRASIL S.A, Curitiba, PR, Brazil) and the other with 20 ha of *Bt* corn AG7088 VT PRO. The corn crops were not irrigated and normal procedures were used to grow the plants (Borém et al., 2017). No pesticides applied.

Determining whether there were Cry1F Bt-resistant individuals in the local field population

Larvae were collected from non-*Bt* corn and were fed in corn leaves of the non-Bt corn hybrid until pupation. Using one laboratory-selected, Cry1F-resistant fall armyworm colony and the field-collected armyworms reciprocal crosses were done as follows. For the crosses, the adult females were mated with resistant males and adult males were mated with resistant females. One couple was individualized in PVC cages (7 cm in height × 10 cm in diameter). The cages were covered with paper bond for egg-laying and cotton soaked in a solution of 10% sugar and 1% ascorbic acid for food. Eggs were collected for 5 days and maintained in plastic containers until hatching.

The Cry1F-suscetibility of the F1 neonates was determined using diet-overlay bioassays, similar to Santos-Amaya et al. (2017a) and Marçon et al. (1999). Bioassays were performed using 128-well trays (each well measuring 16 mm in diameter, 16 mm in height, CD International, Pitman, NJ). One milliliter of the artificial diet was introduced to each well and left to solidify. Diagnostic concentration (2000ng/cm^2) of the purified Cry1Fa plus toxin-free control were used (Santos-Amaya et al., 2017a). The Cry1Fa protein used in the diet bioassays was obtained from Dr. Marianne P. Carey (Case Western Reserve University, OH). Dilution was prepared in 0.1% Triton X-100 (nonionic detergent) to obtain uniform spreading on the surface of the diet. Each well was treated superficially with 30mL of the diagnostic concentration and the control was treated with 30mL of 0.1% Triton X-100 only.

One neonate (<24 hours after hatching) was introduced into each well. The wells were covered with self-adhesive plastic vented lids (CD International Inc., Pitman, NJ). A total of 48 neonates (F1) per couple were tested individually with the Cry1Fa diagnostic and 16 neonates for experiment control. Couples that produced <48 neonates were considered nonviable. The bioassay was maintained in a controlled climate chamber at 26 ± 2 °C, 60 ± 10 % RH, and a 24 hours of scotophase. Mortality and weight were observed 7 days after exposure. Larvae with weight <0.1 mg or larvae that had not grown beyond the first instar were considered dead. The criterion for mortality used was severe growth inhibition and larvae mortality (Marçon et al., 1999).

The mortality expected at the diagnostic concentration depended on the individual genotype collected in the corn field. (*i*) If the individual were resistant (RR) to Cry1Fa, all the progeny would be resistant (RR) and 100% survival would be expected at the diagnostic concentration. (*ii*) If the individual were susceptible homozygotic (SS) for Cry1Fa, all the progeny would be susceptible heterozygotic (SR) and lead to 100% mortality at the diagnostic concentration. Finally, (*iii*) if the individual was susceptible heterozygotic (SR) for Cry1Fa, the progeny expected was 50% susceptible heterozygote (SR) and 50% resistant (RR), which would result in 50% mortality at the diagnostic concentration (Gould et al., 1997; Santos-Amaya et al., 2017a; Vélez et al., 2013).

In addition, the neonate susceptibility of the F1 also was determined using leaf tissue bioassays with Cry1Fa corn (30F35H, event TC1507) and non-*Bt* corn (LG6310-LIMAGRAIN BRASIL S.A, Curitiba, PR, Brazil)) in V3–V6 stage. A total of 32 individuals neonates of each couple were assayed using Cry1Fa Bt and non-Bt leaf tissue. Briefly, corn leaf sections of 340 mg were placed in each well (5.6 X 3.6 X 3 cm) of 16-well PVC trays (Advento do Brasil, Diadema, SP). One neonate (<24 hours after hatching) were transferred to each well using a fine hair brush. The corn foliage provided to the larvae was replaced daily. The bioassay trays were maintained at 26 ± 2 °C, 60 ± 10 % RH, and a 24 hours of scotophase. Mortality and larval weight were assessed 7 days after exposure. The outcomes of this assay were similar to that of the diagnostic concentration previously explained.

We found susceptible and resistant insects in the field. A total of 69 % (11 individuals) were susceptible and 31 % (5 individuals) were resistant insects and the frequency of resistance alleles was 0,55 %.

Capture of males in the corn crops

In order to test male response to female sex pheromones in corn crops, we placed susceptible and resistant female traps in *Bt*, non-*Bt* and transition areas. We captured the males that arrived to the female trap and took them to the laboratory. The female trap consisted of one female with two or three days of calling. One female was individualized inside tubes with cloth in the extremes and located at the crop plants' top. Each female trap was distanced 40 m. The experiment was realized for four nights (May 18^{th}, 19^{th}, 23^{rd} and 24^{th} and the atmosphere conditions were 27 ± 0.5 °C (start experiment), 24 ± 0.3 °C (end experiment), 60 ± 3 % RH, and 12 hours of scotophase. The experiment was conducted in the new moon phase to avoid light effect in the moth behavior. The female traps were installed at 6:30 pm and the calling female was inspected. The males were captured realized at 3^{rd} and 6^{th} hours of scotophase.

Identification of the male genotype captured in the traps

The males captured in the corn crops were transported to Federal University of Tocantins for their identification. Each male was mated with one laboratory-resistant female in PVC cages (7 cm in height X 10 cm in diameter). The cages were internally covered with paper bond for egg-laying. Eggs were collected for 5 days and stored in plastic bags until hatching. The male genotype was indentified as previously described using diagnostic concentration bioassays and using leaf tissue bioassays.

To eliminate false positives, larvae that survive the diagnostic concentration were maintained in artificial diet until pupation. Afterwards, the adults from were mated and their offspring were tested using the diagnostic concentration to confirm resistance (Gould et al., 1997; Santos-Amaya et al., 2017a).

Statistical analysis

The proportion of resistant and susceptible males that flew to the female in the wind tunnel as function of female genotype, hour os scotophase and male age were analyzed using a generalized linear modelling (GLM) with a binomial distribution of errors (Crawley, 2012). Model simplification was done removing variables or interactions that did not have statistical significance ($p < 0.05$). The total number of resistant and susceptible males captured in the traps placed in the field as function of female genotype, hour of scotophase and culture were analysed with a GLM with Poisson distribution of errors (Crawley, 2012). All analyses were performed with the R statistical program (R Development Core Team, 2008).

Results

Wind tunnel experiments

The male response to virgin female in the wind tunnel was affected by the male genotype ($\chi^2 = 138.18; p < 0.001$), hour of scotophase and male genotype interaction ($\chi^2 = 124.87; p = 0.001$) and and the interaction male age x female genotype x male genotype ($\chi^2 = 107.07; p < 0.001$).

The percentage of 1-day-old-susceptible males reaching susceptible females and resistant females was not among the hour of scotophase ($p >$ 0.05). However, a greater number of resistant males responded to resistant and susceptible females (66% and 53%, respectively) at the 6th hour of scotophase (Fig. 2.1). At the 3rd hour of scotophase, a similar percentage of 1-day-old males were captured in the 4-couple combinations. At the 9th hour of scotophase a greater number of resistant males responded to resistant females (70%; Fig. 2.1).

The percentage of three-day-old susceptible males reaching susceptible females were greater at the 3rd hour of scotophase (80%) than at the 6th and the 9th hours ($p < 0.001$; Fig. 2.2). Resistant males reaching resistant and susceptible females did not present differences among the hours of scotophase ($p = 0.53, p = 0.54$ respectively). The response from susceptible males to resistant females were greater (70%) at the 6th hour than at the 3rd and the 9th hours of scotophase ($p = 0.02$). Male captured were similar in each hour of the scotophase for the four couple combinations.

Percentage of 5-day-old-susceptible males reaching susceptible females were similar in the hours of scotophase ($p = 0.31$), and the number of resistant males reaching susceptible females also did not differ among the hour of scotophase ($p = 0.13$). However, the proportion resistant males reaching resistant females were greater at the 6th hour than at the 3rd and the 9th hours of scotophase ($p = 0.0254$); Fig. 2.3). At the 3rd and the 9th hours of scotophase, a similar percentage of males were captured for the 4-couple combinations. At the 6th hour of scotophase a greater number of resistant and susceptible males responded to resistant females (67% and 70% respectively; Fig. 2.3).

Percentage of 7 day old susceptible and resistant males reaching susceptible and resistant females were similar in all the hours of scotophase ($p > 0.05$; Fig. 2.4). At the 6th and 9th hours of scotophase a greater number of resistant males were captured responding to resistant and susceptible females (40%, 46% and 33% respectively).

Field experiments

Capture of males in corn crops

The resistant females attracted more resistant than susceptible males and susceptible females attracted more susceptible males (Fig. 2.5). The number of males captured in field for four nights was affected by the interaction between female genotype and male genotype (χ^2 = 5.32, df = 1, p= 0.04). The hour of scotophase and the crop did not any effect on the atractance of fall armyworm males ($p > 0.05$).

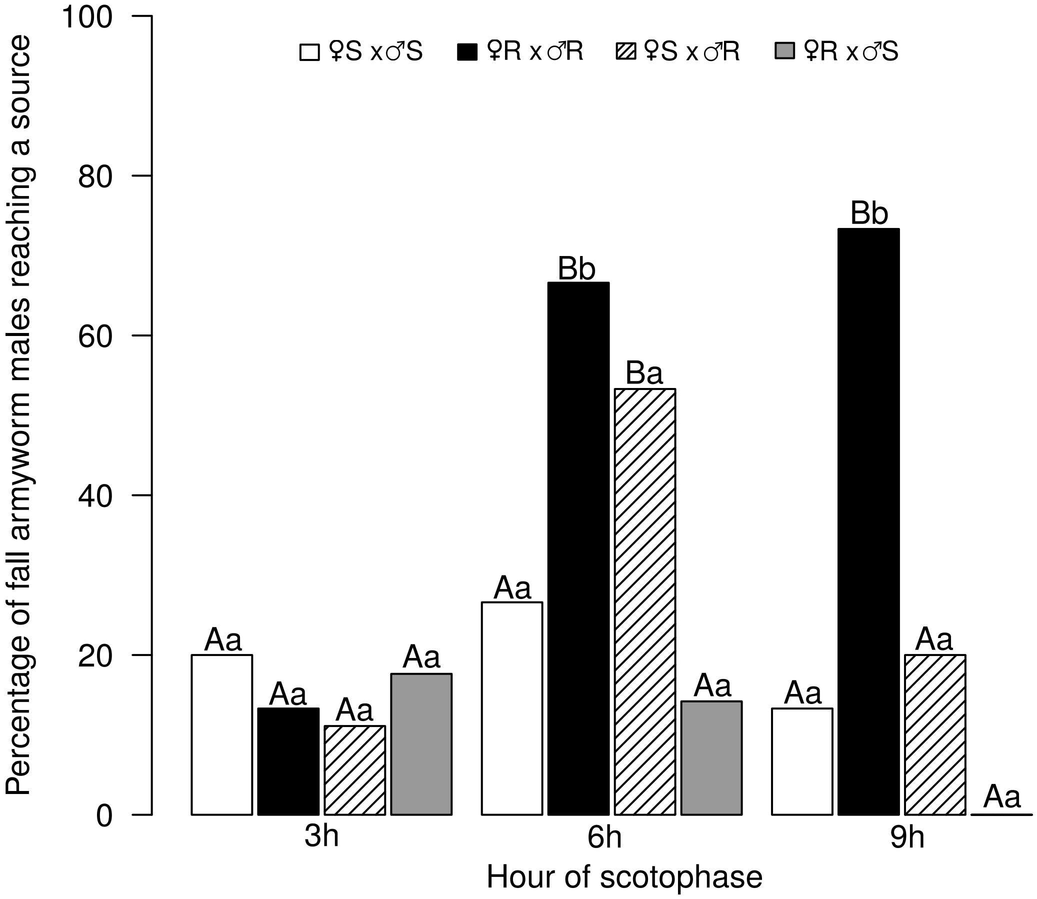

Figure 2.1: **Response of 1-day-old male moths to calling females moths of fall armyworm of specific genotypes in wind-tunnel assays.** Virgin, calling females of 2 or 3 days of calling were placed in the wind tunnel and 1-day-old males were placed in the oposite side of the wind tunnel to observe their response. **Capital letter are comparisons of the same genotype among hour of scotophase. *Lowercase are comparisons of the four genotypes on each hour of scotophase. Same letters = not significant differences (p* $>$ 0.05 *GLM with a binomial distribution and contrast tests).*

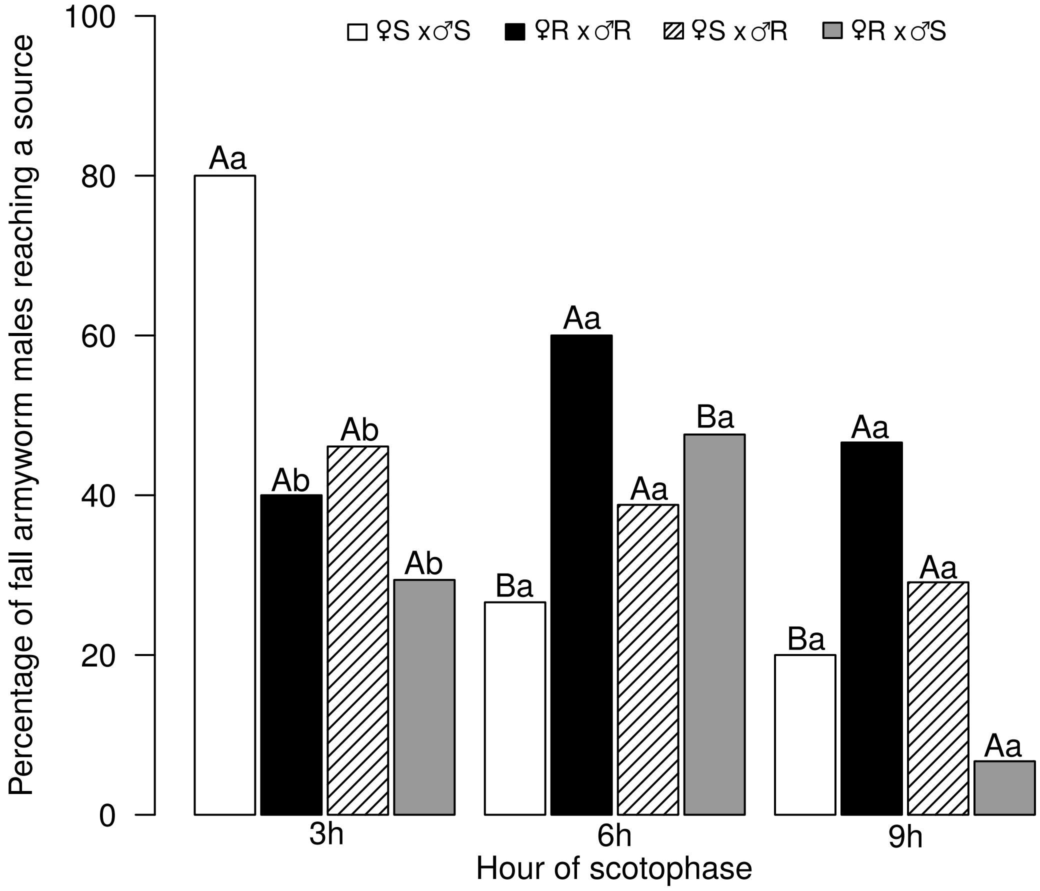

Figure 2.2: **Response of 3-day-old male moths to calling females moths of fall armyworm of specific genotypes in wind-tunnel assays.** Virgin, calling females of 2 or 3 days of calling were placed in the wind tunnel and 1-day-old males were placed in the oposite side of the wind tunnel to observe their response. **Capital letter are comparisons of the same genotype among hour of scotophase. *Lowercase are comparisons of the four genotypes on each hour of scotophase. Same letters = not significant differences (p* $>$ 0.05 *GLM with a binomial distribution and contrast tests).*

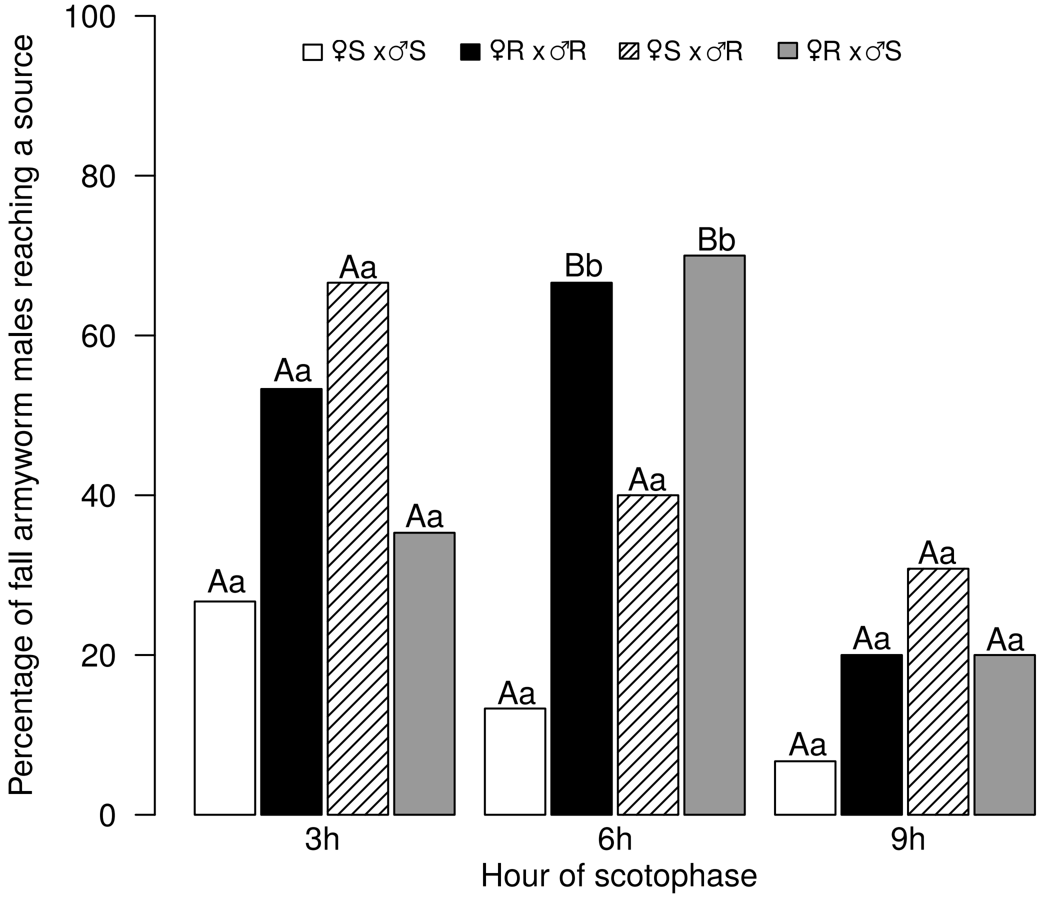

Figure 2.3: **Response of 5-day-old male moths to calling females moths of fall armyworm of specific genotypes in wind-tunnel assays.** Virgin, calling females of 2 or 3 days of calling were placed in the wind tunnel and 1-day-old males were placed in the oposite side of the wind tunnel to observe their response. **Capital letter are comparisons of the same genotype among hour of scotophase. *Lowercase are comparisons of the four genotypes on each hour of scotophase. Same letters = not significant differences (p >* 0.05 *GLM with a binomial distribution and contrast tests).*

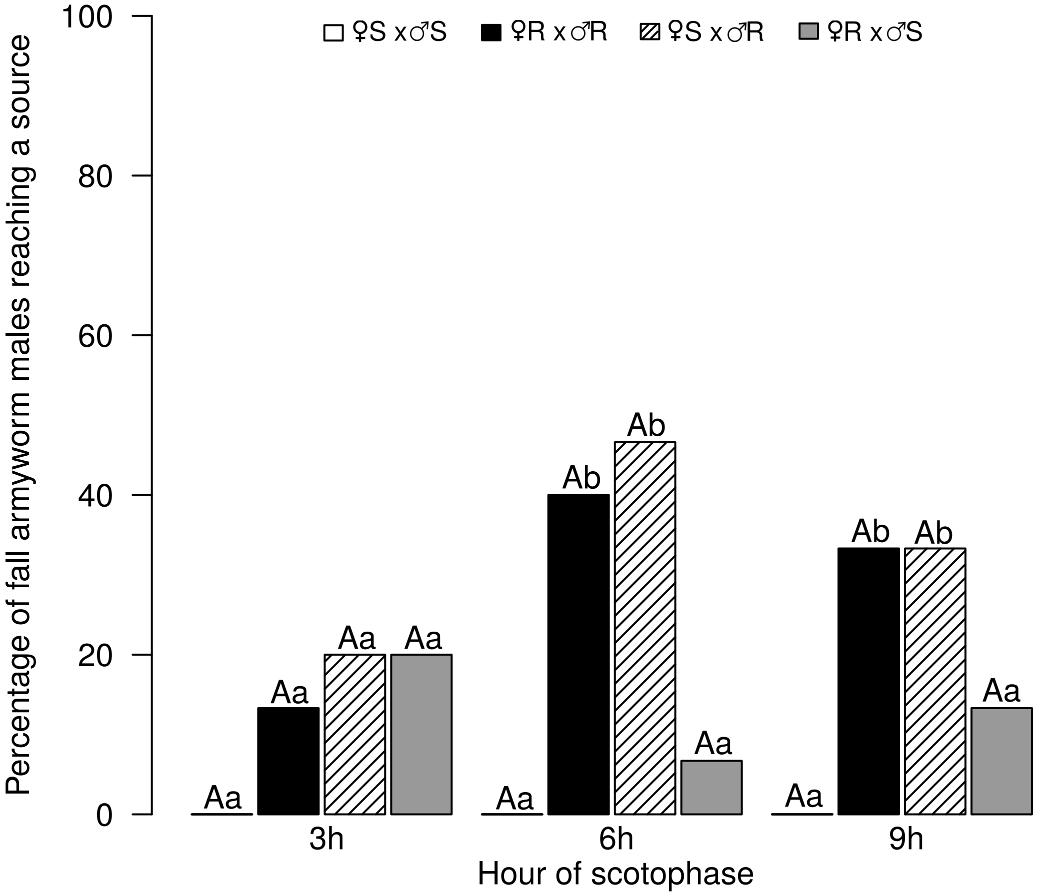

Figure 2.4: **Response of 7-day-old male moths to calling females moths of fall armyworm of specific genotypes in wind-tunnel assays.** Virgin, calling females of 2 or 3 days of calling were placed in the wind tunnel and 1-day-old males were placed in the oposite side of the wind tunnel to observe their response. **Capital letter are comparisons of the same genotype among hour of scotophase. *Lowercase are comparisons of the four genotypes on each hour of scotophase. Same letters = not significant differences ($p > 0.05$ GLM with a binomial distribution and contrast tests).*

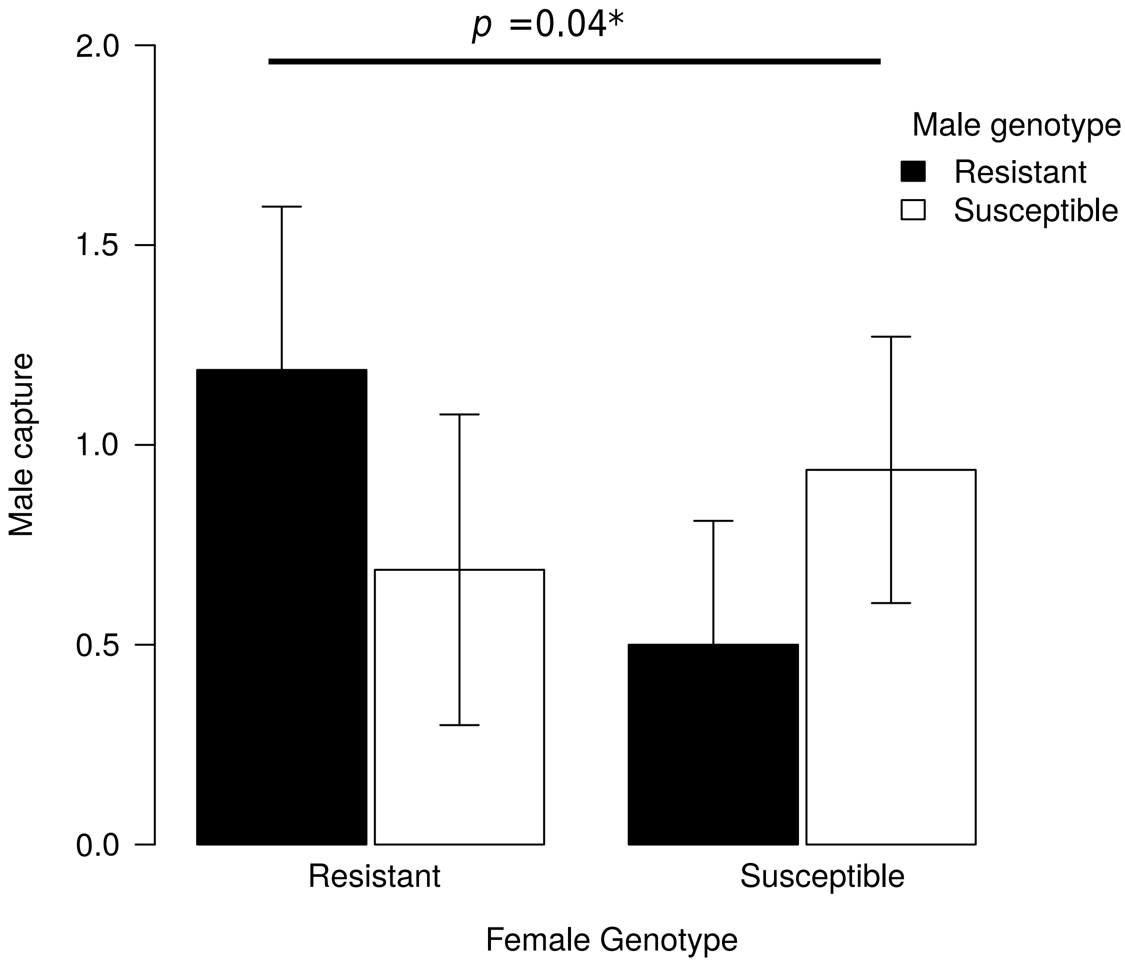

Figure 2.5: **Number of fall armyworm males caught (Mean±SEM) in the field as a function of female genotype and male genotype.** Traps of virgin, calling females of 2 or 3 days of calling were placed in the corn crops and the males attracted were caught.* *significant differences between genotypes*

Discussion

We observed that fall armyworm males that were susceptible and resistant to Bt (Cry1Fa) responded unlike to resistant and susceptible females in the wind tunnel and in the field (corn crops). We found that younger (1 day old) resistant males responded to resistant females in the middle and end of scotophase. One-day-old resistant males responded more to susceptible females in middle of the scotophase. However, susceptible males responded in a lower proportion to resistant and susceptible females. These results suggest that resistant males may mature quicker than susceptible males, that is, the Cry1F-resistant males are likely to respond to calling females before the susceptible males. Sousa (2016) found that Cry1Fa Bt resistant females from the same population matured and produced more eggs than susceptible females. These findings indicate that the sexual changes occur in male and female fall armyworms, and that the maturation of resistant males are better synchronized with the sexual maturation of resistant females. This synchronization may permit an effective sexual communication, metabolic costs and the predation risk (Groot, 2014).

The responsiveness of three-day-old susceptible males was higher than of one, five and seven-day-old ones. This shows that the age had influence in the male sexual maturation and perception. McNeil (1991) mentioned that the age is an important variable in the male receptivity to pheromones sources. The susceptible males responded more to susceptible females at the third hour of scotophase, and susceptible males responded more to resistant females at the sixth hour of scotophase. These differences may be related to the production and emission of sex pheromone from resistant and susceptible females (This topic was studied in the Chapter 1). Additionally, the males may track female blend pheromone changes , which may generate a distinct communication channels (Baker, 2002).

The response of five-day-old susceptible males to susceptible females decreased but susceptible males continued responding to resistant females. This result indicates that the temporal window of susceptible male response is wider than the female calling window. Commonly, a wider "window" in the male response permits to locate both early and late calling females (Linn, 1997). The wider response "window" of susceptible males allow that they mate with susceptible females early and with resistant females late. This seems to favor the refuge strategy for fall armyworm resistance management, maintaining the susceptible individuals in the field or generating hererozygous insects that would be died the Bt corn (Gould, 1998; Vélez et al., 2013).

However, there was an increase (55 to 70%) in the responsiveness of five-days-old resistant males at the third and the sixth hours of scotophase to resistant females increased. Furthermore, at the sixth and the ninth hours of scotophase, the seven-day-old susceptible males did not respond or responded less to susceptible and resistant females than the resistant males. These results show that resistant males have a response window wider than susceptible males. The wider response window of resistant males in all ages studied may lead them to spread the resistance allele because of the high probability of mating between resistant insects.

In the field experiment, the resistant females attracted a greater number of resistant males and vice-versa with susceptible insects. The field results were consistent with the laboratory and experiments in the wind tunnel and with a previous study by De la Pava Suárez (2016), where the males responded to extracts of pheromone gland of females with the same genotype. Futhermore, we also observed that resistant females attracted 37% males with a different genotype and susceptible females attracted 34% resistant males. This confirms again that the male response window is wider than female response window and that the males may also respond to wide range mixtures of female sex pheromones (Linn et al., 2003; Krokos et al., 2002). Mating between resistant and susceptible individuals critical for propose function of the current resistance management strategies in Bt crops. Other moths species such as *Heliothis virescens* resistant to pyrethroids (Campanhola et al., 1991) and of *Plutella xylostella* resistant to Bt (Groeters et al., 1993) did not show assortative mating. Other studies have reported that the resistance to insecticides did not present impact in the male's ability to acquire mates and in the random mate (Groeters et al., 1993; Delisle and Vincent, 2002).

Our field results showed that approximately 35% females attracted males with different genotype and this can be contributing to delay resistance evolution in the field assuming that the heterozygous insect progeny will die with the high dose of Bt toxin in transgenic plants. However, our results show that resistant females attracted a high number of resistant males and this would account to rapid increase the resistance allele in the population, thus favoring field resistance development to the Bt crop. We did not include in our statistical analysis the heterozygous males attracted by resistant and susceptible females but the heterozygotic males presence would also contribute to rapid resistance evolution. If the evolution of Cry1F Bt-resistance increases male response "window" and favors the assortative mating in the field, these features should have contribute to the rapid evolution of the resistance that Farias et al. (2014) reported in Brazil.

In conclusion, we found differences in the male response in fall armyworm moths resistant and susceptible to Bt. These results give us an example of speciation mechanisms caused by sexual communication modifications, and help us to understand how phenotypic changes associated with resistance may increase the rate of resistance evolution.

Chapter 3

General Conclusions

The results generated by the present work showed that Bt-resistant and susceptible insects present differences in the sex pheromone blend. The differences were in the minor component (Z7-12Ac) in young females and in the first hours of scotophase (Chapter 1). The differences both in the minor component and pheromone blend could potentially modify the communication channels and affect the male responsiveness in the laboratory and in the field. Male attraction in the field effectively was different between resistant and susceptible females, the males responded to female with the same genotype and this can be influencing the resistance control in the field (Chapter 2). Furthermore, the findings obtained by this thesis are an example when modifications in sexual communication can help in the speciation mechanisms.

Chapter 4

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