

NATALY DE LA PAVA SUÁREZ

BEHAVIORAL RESPONSES OF BT -RESISTANT AND
-SUSCEPTIBLE *Spodoptera frugiperda* MALES TO FEMALE
SEX PHEROMONE

Dissertação apresentada à Universidade
Federal de Viçosa, como parte das exigên-
cias do Programa de Pós-Graduação em
Entomologia, para obtenção do título de
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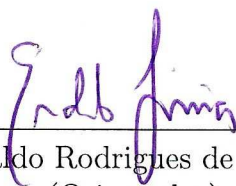
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Eliseu José Guedes Pereira



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Eraldo Rodrigues de Lima
(Orientador)

Ao meus pais, Maria del Carmen
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Biografia

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Resumo

DE LA PAVA, Nataly Suárez, M.S., Universidade Federal de Viçosa, janeiro de 2016. **Respostas comportamentais dos machos de *Spodoptera frugiperda* ao feromônio sexual da fêmea em populações resistentes e suscetíveis ao Bt.** Orientador: Eraldo Rodrigues de Lima. Coorientadores: Eliseu José Guedes Pereira e Og Francisco Fonseca de Souza.

Spodoptera frugiperda é a praga mais importante da cultura do milho em áreas tropicais do continente Americano. Plantas geneticamente modificadas que produzem toxinas de *Bacillus thuringiensis* (Bt) tem sido utilizadas como principal estratégia no controle desta praga. No entanto, apesar do uso da estratégia de manejo da resistência (refúgio/alta dose) nas culturas, insetos resistentes ao Bt ainda podem ser selecionados. Esta adaptação diferencial pode levar a diferenças comportamentais, por exemplo, na comunicação espécie-específica entre populações resistentes e suscetíveis ao Bt. Obstáculos para o encontro de uma fêmea de genótipo diferente pode levar a uma redução na eficácia da estratégia de refúgio. Neste trabalho nós mostramos que o comportamento reprodutivo de machos de *S. frugiperda* muda quando submetidos à pressão de seleção de uma cultura Bt. O comportamento de voo de machos resistentes e suscetíveis em resposta ao extrato de glândulas de feromônio sexual de fêmeas de ambos os genótipos foi avaliado em túnel de vento. Os machos alcançaram a fonte de odor em maior frequência quando testados com o feromônio de fêmeas de seu próprio genótipo. A atração

cruzada entre genótipos também ocorreu, mas em menor frequência. Machos suscetíveis foram mais atraídos pelo feromônio de fêmeas resistentes que o contrário. Nossos resultados sugerem que a pressão de seleção gerada por culturas Bt causam o isolamento reprodutivo em populações de *S. frugiperda* e redução na efetividade da estratégia de refúgio.

Abstract

DE LA PAVA, Nataly Suárez, M.S., Universidade Federal de Viçosa, February, 2016. **Behavioral responses of Bt -resistant and -susceptible *Spodoptera frugiperda* males to female sex pheromone.** Adviser: Eraldo Rodrigues de Lima. Co-advisers: Eliseu José Guedes Pereira and Og Francisco Fonseca de Souza.

Spodoptera frugiperda is the most important economic pest of corn in tropical areas of the American continent. Genetically modified crops that produces *Bacillus thuringiensis* (Bt) toxins are the main strategy for the pest control. But, despite the use of resistance management strategy (refuge/high dose) on these crops, Bt-resistant insects can still be selected. This differential adaptation can lead to behavioral changes, for example, in the species-specific communication between Bt-resistant and -susceptible populations. An obstacle in finding a different genotype female could lead to a reduction of the efficacy of refuge strategy. Here we show that the reproductive behavior of *S. frugiperda* males changes when subjected to a selection pressure from a Bt crop. The flight behavior of Bt -resistant and -susceptible males towards female sex pheromone gland extract from both genotypes were evaluated in a wind tunnel. The males reached the source of odor in the higher frequency when tested with their own genotype pheromone. Cross-attraction between genotypes also occurred, but at a lower frequency. Susceptible males had a higher attraction to the resistant female pheromone than resistant males

flying to susceptible female pheromone. Our results suggest that the selective pressure generated by Bt crops cause a reproductive isolation in *S. frugiperda* populations and reduction of effectiveness of refuge strategy.

1 Introduction

The genetically modified Bt-corn contains a gene from the soil bacterium *Bacillus thuringiensis*, which results in constitutive production of particular insecticidal Cry1 proteins in the plant. These proteins are most effective against lepidopteran larvae (Van den Berg *et al.*, 2013) and these was released for the first time in the United States to improve control of *Ostrinia nubilalis* (Lepidoptera: Crambidae) (Ostlie *et al.*, 1997) and *Diatraea grandiosella* (Dyar) (Lepidoptera: Crambidae) (Archer *et al.*, 2001). This technology was first introduced in Brazil in 2009 to control the fall armyworm, *Spodoptera frugiperda* (J.E. Smith) considered the major pest of corn crops in Brazil (Cruz & Valicente, 1998). The fall armyworm is native to the tropical regions of the western hemisphere from the United States to Argentina (Buntin, 1986; Mitchell *et al.*, 1991; Azevedo *et al.*, 2012; Nagoshi *et al.*, 2014). The control of this pest was mainly based in insecticides (Cruz *et al.*, 2002; Crubelati-Mulati *et al.*, 2014), however, this method has not been effective, primarily because of the insect behavior: the first instar larva enters into the plant and remains protected of the application of any contact insecticide (Harrison, 1984; Buntin, 2008).

Currently, the transgenic crops (Bt crops) are the most successful biotechnological insect management application (Gould, 1998). This technology allowed an improvement in pest management, reducing pesticide use and increasing cultivated areas (Télliez-Rodríguez *et al.*, 2014). In general, the action of Cry protein occurs in the larval midgut, when Bt spores are consumed. The Bt spores germinate and the crystals associated with them dissolve in the alkaline lumen of the midgut. The soluble toxin molecules undergo various interactions with the molecules in the epithelial membranes

in the midgut of the insect, then the toxin participates in the formation of pores in the bilayer lipid membrane. The disruption of membrane integrity eventually kills the cells. The eventual death of the larva is generally attributed to starvation which is likely exacerbated by proliferation of Bt and other microorganisms in the damaged midgut (Heckel *et al.*, 2007).

However, it has been recognized that the insect may develop resistance to this Bt protein, affecting any of the processes linked to the time of ingestion of the protein, causing reduction in usefulness of the long term culture (Gould, 1998; Storer *et al.*, 2010). It was reported for *S. frugiperda* populations from the United States, Puerto Rico and Brazil (Storer *et al.*, 2010; Farias *et al.*, 2014; Huang *et al.*, 2014).

Some authors argue that the resistance acquisition carries a fitness cost, when resistant species showing lower development in relation to the susceptible species (Carrière *et al.*, 2006; Gassmann *et al.*, 2008; Konopka *et al.*, 2012). Nevertheless, Vélez *et al.* (2014) reported that *S. frugiperda* did not express significant difference in fitness associated with resistance to protein Cry1F either in heterozygous or homozygous resistant groups. This might result into new mutations conferring higher resistance levels but lower fitness cost (Cao *et al.*, 2014). This low fitness cost has been reported for resistant populations of species like *S. frugiperda* and *Diatraea saccharalis* in Bt corn (Jakka *et al.*, 2014; Zhang *et al.*, 2014).

To manage this adaptation, the use of plants with high doses of Bt and the implementation of a refuge area with non-Bt plants was established as a strategy (Gould, 1998; Storer *et al.*, 2010). Assuming the hypothesis that this strategy would delay increasing resistance in these cultures, keeping insect population not exposed to the Bt protein could result in emerging insects from the refuge area that could mate with few resistant insects emerging from Bt crop (Mallet & Porter, 1992; Monsanto, 2012). Consequently, the heterozygous progeny can still be susceptible to Bt toxin dose (Télliez-Rodríguez *et al.*, 2014).

Also, the refuge strategy assumes that Bt-susceptible insects migrate to the Bt area and mate with the reduced population of resistant insects. However, it is unknown whether this really happens. Dalecky *et al.* (2006) hypothesized that the emerging insect is in a dilemma: should it mate after or before dispersal?, however, dispersal before mating can carry the risk of not finding a sexual partner. Dalecky *et al.* (2006) evaluated the dispersion precopulatory and mating rate between populations of *O. nubilalis* in Bt-corn. It was found that resident females mated regardless if males dispersed previously or not. Consequently, almost 67% of the resident females mated before dispersion and mostly with males of the same area. This is proportional to the amount of resident males. Nonetheless, resident males rarely mated with immigrant females, who were mostly already mated.

Seemingly, the proximity of both areas of cultivation in the refuge strategy does not assure that the insects mate and decrease the selection pressure generated by Bt culture. Furthermore, it is known that certain environmental and agricultural pressures can modify reproductive behavior of insects (Haynes, 1988; Gould, 1998; Guedes *et al.*, 2009). However, not much is known about the effect of the Bt resistance on this behavior. It has only been reported for *Helicoverpa armigera* (Lepidoptera: Noctuidae) that the selection pressure due to cotton CryAc crops changed the female calling behavior (Zhao *et al.*, 2009). It suggests that the selection pressure produced by Bt cultures can modify the communication channels of a target species and potentially lead to isolation within a population.

The sexual communication in moths is mediated by sex pheromones. Females usually produce species-specific sex pheromones that exclusively attract conspecific males over long distances (Cardé & Baker, 1984; Cardé & Haynes, 2004; Unbehend *et al.*, 2014). That pheromone signals can convey information about species identity, social status, and mate choice (Wyatt, 2003; Lassance, 2010). The pheromone is released as a part of a specialized “calling behavior”, which occurs during a well-defined period of the day shown to follow the circadian rhythm (Cardé & Haynes, 2004). The detection of

the pheromone triggers a stereotypic search behavior in the male who will fly upwind to locate the odor source (Linn Jr & Roelofs, 1995).

In the majority of the species investigated, the female signals appear to be highly species-specific (Löfstedt, 1993; Cardé & Haynes, 2004). Also, signal-specificity is viewed as a mechanism to prevent mating mistakes and enhance the ability of individuals to maximize their reproductive investment and locate potential mates before competitors (Lassance, 2010). To find a suitable mating partner, males need to respond to a specific chemical signal that is emitted by a conspecific female (Löfstedt, 1993; Cardé & Haynes, 2004).

Nevertheless, the environment and physiological factors also play a role and can change the specificity of the communication channel and are therefore essential to the mating system (Delisle & McNeil, 1987; Wyatt, 2003; Lassance, 2010). Thus, changes in the communication channel can affect the pheromone signal of the female and may result in a reproductive isolation and can lead to speciation (Cardé & Roelofs, 1973; Smadja & Butlin, 2009). In the same way, the males response depends on many factor, including pheromone blend, temperature, relative humidity and male age. This will involve changes in the sensitivity of the nervous system implicated in the pheromone communication (Traynier, 1970; Cardé & Roelofs, 1973; Matsumoto & Hildebrand, 1981; Witzgall *et al.*, 2008).

Some authors reported changes in the pheromone communication system in *O. nubilalis* caused by geographical isolation. In this species, there are two strains, *Z* and *E*, which are morphologically undifferentiated but have a variation in the pheromone compounds ratio. In addition, the behavioral response of males corresponds with that difference, isolating both strains (Klun *et al.*, 1973; Kochansky *et al.*, 1975; Roelofs *et al.*, 1987; Smadja & Butlin, 2009; Lassance, 2010). In the particular case of *S. frugiperda*, Pashley (1986) reported the occurrence of two strains, *Corn* and *Rice*. Just like *O. nubilalis*, some authors reported differences in the proportion of sex pheromone compounds produced and female calling behavior. Nevertheless,

despite this differentiation, males do not show a preferential attraction to females of their own strain (Pashley, 1988; Lima & McNeil, 2009; Unbehend *et al.*, 2014).

However, if the variation in the insect reproductive behavior occurs in the same geographical area, it could be expected a selection pressure generated by Bt crops. It can cause changes in the pheromone communication channels of this species because the high sensitivity of the system to changes in the environment.

In that sense, the effect of the fitness cost associated with the Bt resistance on lepidopterous reproductive behavior has not yet been investigated. If such effects occur, the change in the behavioral response of resistant male could reduce its mating frequency. Also, it could lead to a difference in the pheromone communication between Bt-susceptible and -resistant genotypes. This differences may reduce the level of hybridization, which is the basis for refuge strategy and control of resistances.

Accordingly, the purpose of this study was to know what cost is associated with the acquisition of Bt resistance in the behavioral response of *S. frugiperda* males towards the female sex pheromone to each resistant and susceptible genotype. The behavior test analyzed the male behavioral response in the wind tunnel, which included the following behavioral responses: time to take-off, oriented flight toward the source at 200 cm and landing. The experimental results indicated that, compared with the Cry1F -susceptible genotype, the male attraction changes when exposed to female pheromone gland extract of different genotypes. It suggests that there is a cost associated with the male response of Cry1F resistant genotype.

2 Materials and Methods

2.1 Insects

The Cry1F resistant and susceptible *S. frugiperda* populations were obtained as described by Santos-Amaya *et al.* (2016). The resistant population used in this experiment came from the selected line MT (progeny resulting from the population crosses Cascavel x Sorriso and Brasília x Morrinhos - The resulting baseline strain was designated as MT). To maintain the resistance, larvae of the resistant population were exposed to Cry1F corn leaves every 5 generations.

The insect rearing was adapted from Kasten Jr *et al.* (1978) and Santos-Amaya *et al.* (2016). About 150 moths were maintained in PVC cages (30 x 20 cm) with sulfite paper on the inner walls for egg laying. The adults were fed with a 10% sugar and 1% ascorbic acid solution soaked in cotton. It was offered *ad libitum* and changed every two days. Eggs were removed from the cage every two days and stored in 500 ml plastic cups. Groups of neonates were transferred to an artificial diet (Kasten Jr *et al.*, 1978) in plastic cups of the same capacity until the 2nd instar and then individually placed in 16-cell PVC trays (Advento do Brasil, Diadema, SP) until pupation. The insects were kept in climate-controlled chambers at $26 \pm 2^\circ\text{C}$, $70 \pm 15\%$ relative humidity (RH) and 14L:10D photoperiod with a light intensity of 208 lux during the photophase. Newly emerged male moths were individually placed in a clear plastic jar and kept fed in a isolated room for female moth until the experiments in the wind tunnel. The insects used in the experiments were not separated according to the strain reported for *S. frugiperda*, i.e., they could be corn or rice strain.

2.2 Pheromone Extraction

The sex pheromone extract of susceptible (n=80) and resistant (n=33) *S. frugiperda* females was obtained by direct sex pheromone gland extraction. The gland is located between the VII and IX abdominal segment. The dissection was made with 5 days old virgin females at the third and fourth hour of the scotophase, when the calling behavior is at its peak (Lima & McNeil, 2009). Groups of 20 glands were excised and transferred to a conical vial with 40 μl of HPLC grade hexane and held for 10 minutes. The samples were maintained at -20°C until analyses.

2.3 Dose determination of female pheromone gland extract to use in the wind tunnel

Several female equivalent (**fe**) doses were tested to determine which of them was more attractive to males. The following corresponding doses were evaluated: 2, 1, 0.6, 0.5, 0.3, 0.2, 0.15, 0.1 and 0.05 $\text{fe}/\mu\text{l}$. The flight behavior of virgin males towards each dose (30 males/dose) was tested in a wind tunnel at the fifth hour of the scotophase. The 0.15 $\text{fe}/\mu\text{l}$ dose attracted the higher percentage of males (65%). To observe how the dynamics of the main component of the sex pheromone, (Z)-9-tetradecenyl acetate (Z9-14Ac), varies in females, each sample was 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). 1 μl of each sample was injected in splitless mode with the injector at 250°C . The column oven was kept at 60°C for 1 min, increased to 200°C at a rate of $7^{\circ}\text{C}/\text{min}$ and then to a final temperature of 280°C at a rate of $15^{\circ}\text{C}/\text{min}$. Synthetic standards of (Z)-7-dodecenyl acetate (Z7-12:Ac), (Z)-9-tetradecenyl acetate (Z9-14:Ac), (Z)-11-tetradecenyl acetate (Z11-16:Ac) compounds were injected to get relative retention times for comparison with

female pheromone. A 1 μl aliquot of Z9-14:Ac (100 ng/ μl) synthetic was used as an external standard to quantify the Z9-14Ac pheromone compound. Absolute amounts were calculated from the integrated peak area of each component relative to that of the external standard. Regardless of the proportion of Z9-14Ac found in the gland of each genotype, the same **fe** dose was used for each combination male/pheromone extract genotype tested in the wind tunnel.

2.4 Wind Tunnel Bioassays

In order to evaluate the flight behavior of virgin males in response to the female sex pheromone source, Cry1F protein resistant and susceptible males were tested through the sex pheromone gland extract of the females of both genotype in a wind tunnel. One hour before the onset of the scotophase, the virgin males, individually placed in glass tubes (15 x 3 cm) covered with gauze, were transferred to a climate-controlled chamber with a wind tunnel (300 cm long x 100 cm wide x 100 cm high) at $23 \pm 2^\circ\text{C}$ and $60 \pm 25\%$ RH. On one side of the tunnel was placed the virgin male, 2 m apart from the odor source. The odors tested were delivered through a microinjection pump (CMA/100) linked to an ultrasonic sprayer (El-Sayed *et al.*, 1999) at a 10 $\mu\text{l}/\text{min}$ rate. The wind speed inside the tunnel was 35 cm/s. The glass tube and the ultrasonic sprayer were placed on a stand 30 cm high.

It was observed the flight behavior in response to 0.15 fe/ μl sex pheromone gland extract from susceptible and resistant females. Virgin males of 1, 3, 5 and 7-days-old from both genotypes were tested, with a total of 1990 insects. The experiment was conducted during three periods of scotophase (2-4, 5-7 and 8-10 h). At least 40 individuals of each age were tested at each period. Each individual was given 3 min to respond and each insect was tested only once. The following behavioral responses of male fall armyworm were recorded: time to take-off, oriented flight towards the source at 200 cm and landing. Also, the time it took to develop each behavior was timed.

2.5 Statistical analysis

The variables observed during the flight behavior were subjected to a generalized linear modelling (GLM) under binomial and gaussian distribution of errors. The analysis was performed and followed by residual analyses to confirm the error distribution and the suitability of the model.

To determine if the age, female and male genotype affect the percentages of male reached the source, in each scotophase period, we built a linear model (GLM) with a binomial distribution, followed by a contrast analysis to determine which means differed (Crawley, 2012).

To determine if the time to male take off and the distance reach by males that not landing at sprayer differ influenced by variables, we built a linear model (GLM) with a gaussian distribution.

All analyses were performed with the R statistical program (R Development Core Team, 2013).

3 Results

3.1 Pheromone quantification

The mean amount of Z9-14Ac produced in the fourth hour of the scotophase (peak productivity) was 0.516 ± 0.073 ng for susceptible females and 1.048 ± 0.207 ng for resistant females.

3.2 Frequency of behaviors

The percentage of *S. frugiperda* males that showed attraction response to the female sex pheromone extract of each genotype changed according to age and period of scotophase. Resistant males had a higher percentage of individuals responding to each behavior (Figure 3.5).

3.3 Take off response to the sex pheromone gland extract

There were significant differences on the take off response between the periods of the scotophase. A significant genotype *vs* period interaction (Table 1) indicated that the response in these periods differed between genotypes. The resistant males took less time to respond in the first period of scotophase (2^{nd} - 4^{th} h) than the susceptible males (32.52 ± 1.85 and 37.95 ± 1.96 , respectively). In the following two periods (5^{th} - 7^{th} h and 8^{th} - 10^{th} h), both genotypes showed the same response time ($P > 0.05$).

Table 1: Generalized linear model (GLM) with identity link function and gaussian distribution showing the factors that influenced the taking off response by two male *Spodoptera frugiperda* genotypes (resistant and susceptible to Bt) under a 14L:10D photoperiod.

	Df	Deviance	Resid	Df	Resid.Dev	F	Pr(>F)
NULL			1324		1041660		
Male - Genotype	1	9.0	1323		1041651	0.0116	0.91438
Period	2	5957.9	1321		1035693	3.8148	0.02229 *
Male-genotype x Period	2	5685.0	1319		1030008	3.6400	0.02652 *

Signif. Codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

3.4 Attraction to the odor source

There were significant differences in the relative proportion of males that reached the source according to the age of males (Table 2). The presence of a significant interaction between male *vs* female genotypes (Table 2) indicated that the percentage of male that reached the source differed between female's pheromone genotypes (Figure 3.5). Also, both male genotypes were more attracted (long flight and landing) to both female genotypes in the two final periods of the scotophase (5th - 7th h and 8th - 10th h), showing its influence on the insect behavior (Table 2, Figure 3.5). The resistant and susceptible males were most responsive when exposed to the gland extract of the same female genotype. Cross-attraction between genotypes also occurred, but at a lower frequency. Susceptible males had a higher attraction to the resistant female pheromone than resistant males flying to susceptible female pheromone (Figure 3.5).

There were not significant differences in the flight time between male genotypes that reached the odor source. However, there were significant differences in the flight time as a function of the female genotype. The males spent the same time to reach the source regardless its genotype and they had more flight time when the stimulus came from the sex pheromone gland extract of a susceptible female than from the resistant female (50.579 ± 4.156 and 40.672 ± 2.818 minutes, respectively).

Table 2: Generalized linear model (GLM) with logit link function and binomial distribution showing the factors that influenced two *S. frugiperda* male genotypes (resistant and susceptible to Bt) to reach the odor source under a 14L:10D photoperiod.

	Df	Deviance	Resid	Df	Resid.Dev	Pr(>Chi)
NULL			387		441.52	
Female - Genotype	1	1.356	386		440.16	0.24417
Male - Genotype	1	2.150	385		438.01	0.14256
Age	1	40.797	384		397.21	1.689e-10 ***
Period	2	6.736	382		390.48	0.03445 *
Male-genotype x Female-genotype	1	17.310	381		373.17	3.176e-05 ***

Signif. Codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

3.5 Variation in the upwind flight distance in the wind tunnel according to the scotophase period

The rest of males that did not reach the odor source showed not significant changes in the upwind flight distance in both male genotypes ($P > 0.05$). Also, in both male genotypes there were not significant changes in the male response regardless the female genotype exposed (Table 3). However, the male age and the scotophase period influenced in the upwind flight of that males (Figure 3.5). In males that not reached the source the flight distance was greater in the first and second period of the scotophase and when the males age exceeded the five days old ($P < 0.05$, $df = 2$) (Figure 3.5).

Table 3: Generalized linear model (GLM) with identity link function and gaussian distribution showing the factors that influenced the upwind flight distance in wind tunnel of two male *Spodoptera frugiperda* genotypes (resistant and susceptible to Bt) under a 14L:10D photoperiod.

	Df	Deviance	Resid	Df	Resid.Dev	F	Pr(>F)
NULL			1864		5780305		
Age	1	11872	1863		5768433	3.8478	0.04996*
Period	2	26095.4	1860		5733949	4.2376	0.01458 *
Signif. Codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1							

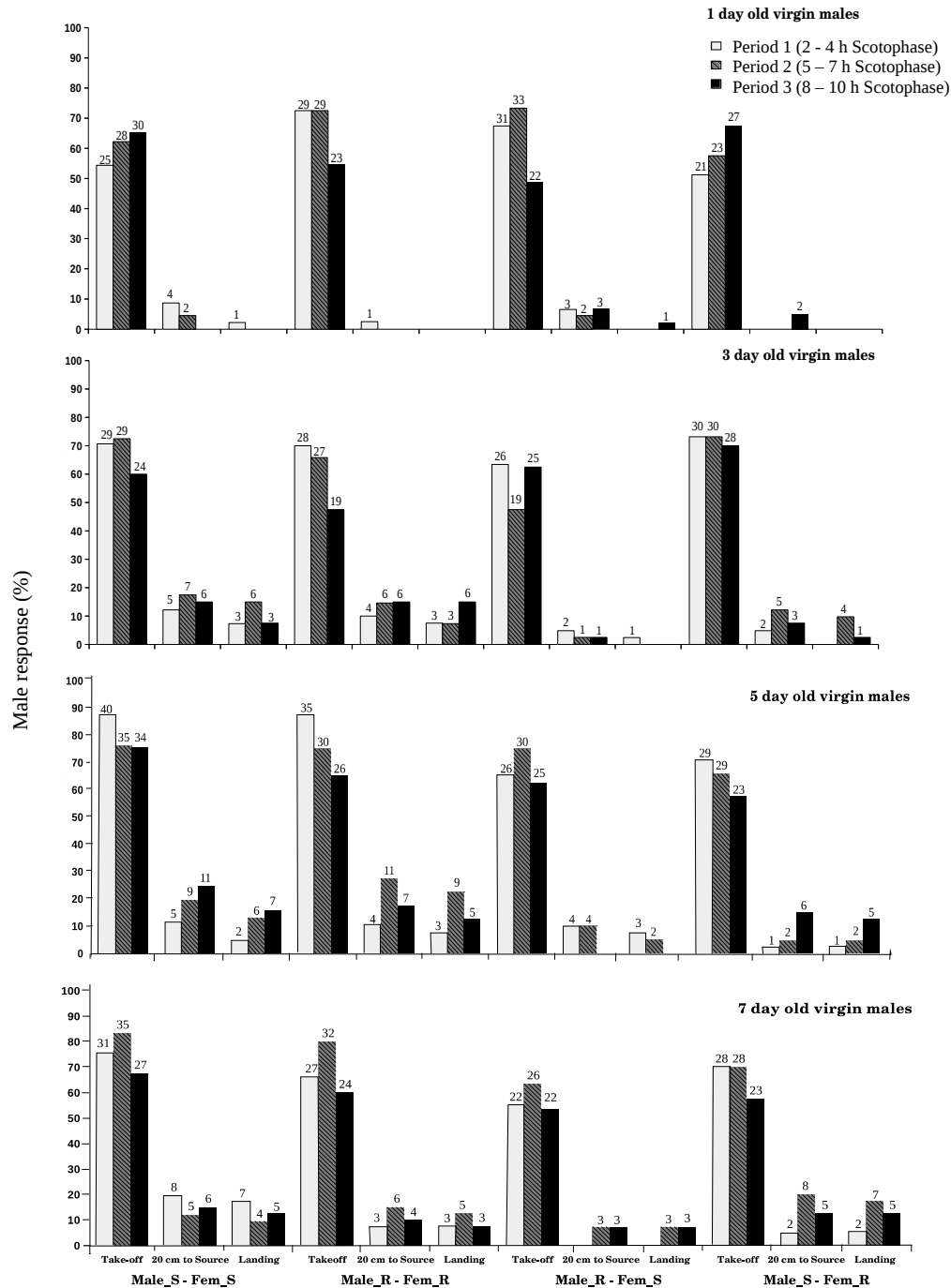


Figure 1: Percentage of *S. frugiperda* males that showed attraction response according to the age and period of scotophase to female gland extract of each genotype (n= 1990). All shown results are related to the resistant and susceptible genotype of males and the genotype of sex pheromone gland extract used in the sprayer for male attraction. The percentage of attracted males is also related to the male age and the period of the scotophase.

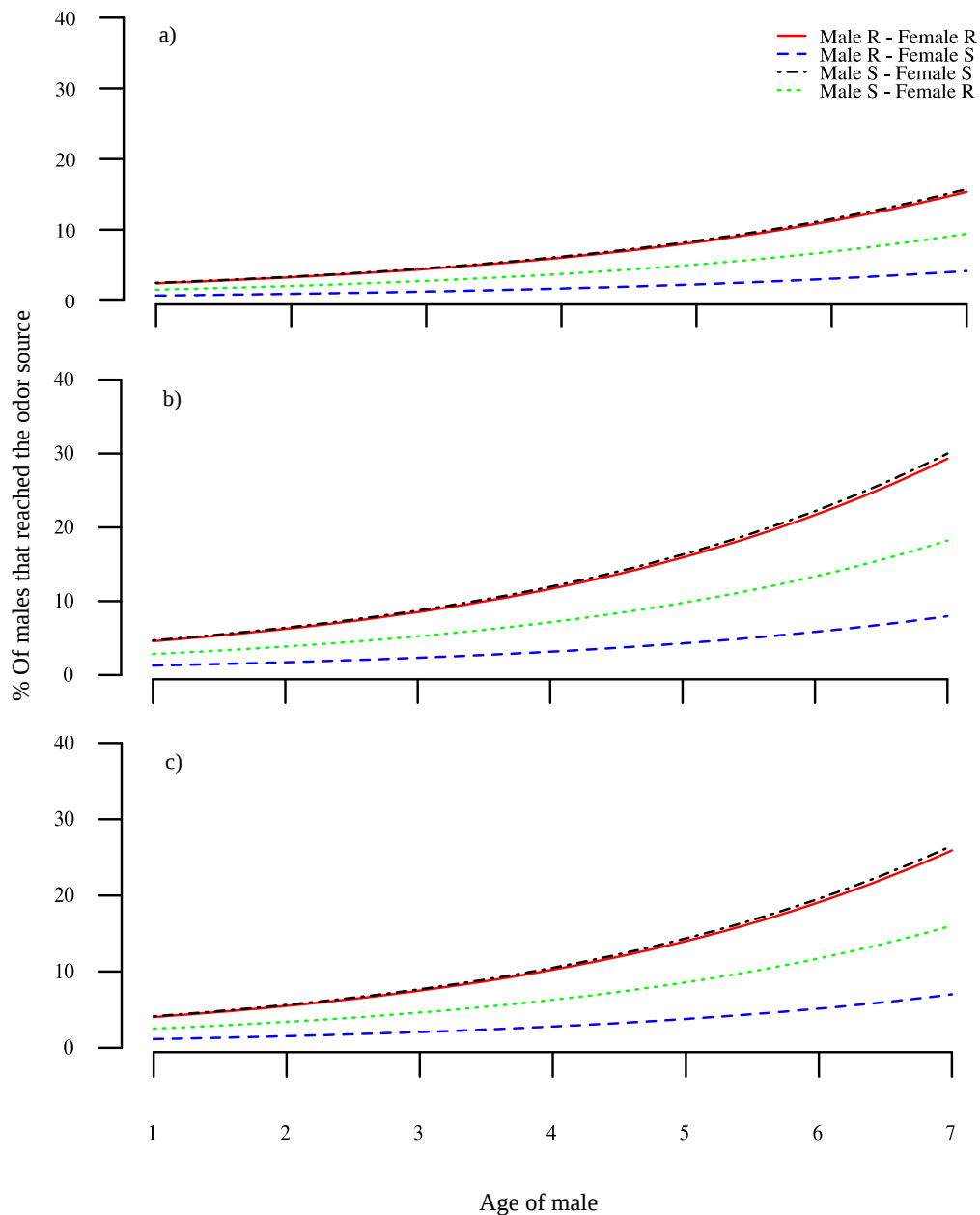


Figure 2: **Percentage of *S. frugiperda* males that reached the odor source depending on the period of the scotophase.** a) Percentage of males that reached the source on the first period of the scotophase (2^{nd} - 4^{th} h) (n=664); b) Percentage of males that reached the source in the second period of the scotophase (5^{th} - 7^{th} h) (n=660); c) Percentage of males that reached the source in the third period of the scotophase (8^{th} - 10^{th} h) (n=644). In all cases the response changed according to the age; older males showed better performance.

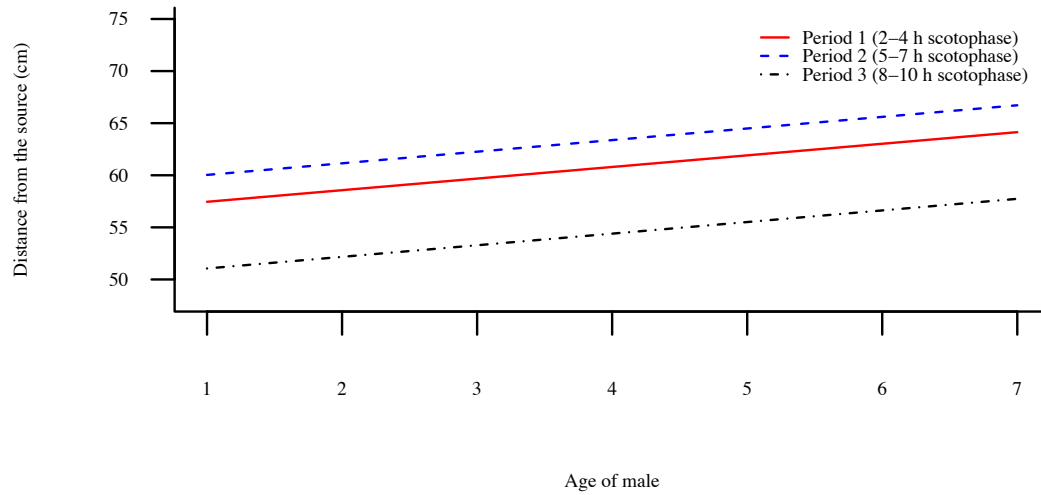


Figure 3: **Distance covered by *S. frugiperda* males that did not reach the source (n=1886).** The response of *S. frugiperda* males changed throughout the scotophase period and age. The males flew longer distances into the tunnel in the first and second period of the night ($p < 0.05$).

4 Discussion

Here, we demonstrated that the evolution of the Bt resistance causes changes in the reproductive behavior of *S. frugiperda*, measured by the male response to the sex pheromone gland extract in the wind tunnel.

The selective pressure by Cry1F corn under chronic exposure increasing survival and larval biomass and decreasing developmental time in *S. frugiperda* has been reported (Storer *et al.*, 2010; Santos-Amaya *et al.*, 2016). We showed that exists an effect and this can be observed in the reproductive behavior of the male too (Figure 3.5), represented by the males that reached the odor source. Modifications in the calling behavior of *H. armigera* females resistant to CryAc cotton have been reported (Zhao *et al.*, 2009). This suggests that the selection pressure produced by Bt cultures can modify the communication channels of target species and potentially lead to isolation within a population of a species. These channels depend generally on the variation in the pheromone production and male response (Cardé & Baker, 1984; Löfstedt *et al.*, 1991; Roelofs & Bjostad, 1984).

In the wind tunnel bioassays, the male response changed according to its age and scotophase period, which is common for Lepidoptera (Klun *et al.*, 1973; Guerin *et al.*, 1984; Droney *et al.*, 2012; Bengtsson *et al.*, 2014). The best response for each behavior was recorded in the seventh day of life, but this was expected, since when virgin insects become older the mating response increases (Simmons & Marti, 1992). However, under field conditions is expected that the insect mate in the first three days after emergence (Simmons & Marti, 1992; Hu, 2008). Therefore, the moth should be mated before getting that older. Anyway, we were able to see that no matter how

old the insect was, exist a greater attraction response to the sex pheromone gland extract of the same female genotype.

According to the observations, resistant males showed greater longevity and had a higher performance until the 7 days of life. Unlike, the susceptible genotype demanded a greater volume of insects in reserve to ensure the same number of insects of this age for wind tunnel experiment due to a higher mortality (De la pava *et al.*, unpublished data). So, it is probable that exists a variation in the genetic information transferred by males influenced by the resistant or susceptible genotype.

In Lepidoptera, is known that not just the age affects the male response towards the female pheromone (Traynier, 1970; Turgeon *et al.*, 1983), but also the scotophase (Dumont & McNeil, 1992; Unbehend *et al.*, 2014). We observed in both genotypes an increase in the frequency of each behavior in males during the second period of the scotophase (5th - 7th h) and this directly corresponds to the period of calling behavior and major pheromone production of *S. frugiperda* females (Lima & McNeil, 2009), as well as to the mating period (Simmons & Marti, 1992; Unbehend *et al.*, 2014).

The results suggest a change not just in the male response, but also in the sex pheromone production. Females are responsive to environmental changes and that could have affected the calling behavior and the proportion of the produced pheromone compounds, as reported to *O. nubilalis*, *S. frugiperda* and *Pseudaletia unipuncta* (Lepidoptera: Noctuidae) (Kochansky *et al.*, 1975; Turgeon & McNeil, 1982; Delisle & McNeil, 1987; Lima & McNeil, 2009).

If the female genotype did not affect the male response, we would have gotten the same answer for both genotypes. On the contrary, we found differences in the interaction between the male and female genotype. It is not unusual to expect a variation in the proportion of compounds from both genotypes, as the occurrence of pheromone races have been reported for several moths like *O. nubilalis*, *Zeiraphera diniana*, *Cydia splendana* and

Lobesia botrana (Lepidoptera: Tortricidae) (Klun *et al.*, 1973; Guerin *et al.*, 1984; Witzgall *et al.*, 2005; Bengtsson *et al.*, 2014).

The variation in the Z9-14Ac proportion produced by each female genotype and the differential male response can also be influenced by the *S. frugiperda* strains (rice and corn) (Pashley, 1986, 1988; Pashley *et al.*, 1992; Lima & McNeil, 2009; Unbehend *et al.*, 2014). The segregation of hosts (spatial isolation) can cause changes in the calling behavior and in the proportion of pheromone compounds produced by the female (Lima & McNeil, 2009; Unbehend *et al.*, 2014). This variable was not contemplated in the assay. Nevertheless, the male strain does not evidence a preference by its own female strain (Unbehend *et al.*, 2014). Then, we considered that the possible presence of one or both strains did not change our results.

The resistant males reached the susceptible odor source genotype in lower proportions. This indicates differences in male's perception, despite that their response window is wider than the variability observed in the female pheromone production (Haynes, 1988; Baker, 1989; Löfstedt *et al.*, 1991; Krokos *et al.*, 2002). A mutation in resistant males could have changed the odorant receptors and the pheromone recognition patterns. This phenomenon was reported in the reproductive isolation of the genus *Ostrinia* and *Heliothis* (Gould *et al.*, 2010; Leary *et al.*, 2012).

The moth pheromone communication system usually depends on the variation of the pheromone production and the male response (Cardé & Baker, 1984; Roelofs & Bjostad, 1984; Löfstedt *et al.*, 1991). We have shown that there were significant differences in the response rate of *S. Frugiperda* genotypes in homozygous individuals (rr - ss), when exposed to stimulus of different pheromone genotypes. The inheritance of the resistance to Bt in transgenic crops is typically recessive (Liang *et al.*, 1999) and is controlled by a single autosomal incomplete recessive allele (Sims & Stone, 1991). Likewise, the resistance in *S. frugiperda* is reported like autosomal and incomplete recessive (Leite *et al.*, 2012; Niu *et al.*, 2014; Santos-Amaya

et al., 2016). Therefore, this suggests that resistant hybrids of the population (as established by the refuge strategy) will express the same male response obtained in susceptible males. Nevertheless, the recessive allele expression depends on the environmental conditions (Sims & Stone, 1991). So, it is necessary studies of the progeny behavior.

The insect behavior modifications generated by the selection pressure of Bt crops, as shown for *H. armigera* females (Zhao *et al.*, 2009) and *S. frugiperda* males, implies a decrease in Bt crops productivity, since there is a male preference for its own genotype. Also, there may be a problem of dispersion that had not been contemplated in the refuge strategy (Dalecky *et al.*, 2006). This could explain the consequent increase of resistance on Bt corn crops of Brazil. Introduced into the country in 2009 (Storer *et al.*, 2010), the first resistant insects were reported in 2014, with nearly 50% of survival in populations from Bahia and Paraná States (Farias *et al.*, 2014). The differential response of the resistant male genotype can be associated to a selective pressure generated by the resistance together with the reports that refuge areas were being implemented below the recommended level or were not being used at all. This clearly increases the risk of reproductive isolation between the genotypes and reduces the productive life of Bt crops.

In conclusion, there are significant differences in attractiveness between genotypes of *S. frugiperda* male sex pheromones joined to another environmental and agronomic factors, and it seems to play a major role in the reproductive isolation between resistant and susceptible insects. Specifically, this study shows how the pressure generated by resistance in insects causes changes in the reproductive behavior of males, based in the attraction response to the sex pheromone gland extract in a wind tunnel. However, more research is required to determine what is the variation in the female behavior and in the pheromone production and how the environment can modulate the interaction between these genotypes in field. Future works

should also consider if the changes in the reproductive behavior is repeated in the progeny.

5 References

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