

NATÁLIA DE SOUZA RIBAS

**EFFECTS OF Cry1Fa BT RESISTANCE ON THE  
REPRODUCTIVE OUTPUT OF *Spodoptera frugiperda***

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

VIÇOSA  
MINAS GERAIS - BRASIL  
2018

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

T

R482e  
2018  
Ribas, Natália de Souza, 1990-  
Effects of Cry1Fa Bt resistance on the reproductive output  
of *Spodoptera frugiperda* / Natália de Souza Ribas. – Viçosa,  
MG, 2018.  
x, 30f : il. (algumas color.) ; 29 cm.

Orientador: Eraldo Rodrigues de Lima.  
Dissertação (mestrado) - Universidade Federal de Viçosa.  
Referências bibliográficas: f. 25-30.

1. Lagarta-do-cartocho - Reprodução. 2. *Spodoptera  
frugiperda*. 3. Plantas transgênicas. 4. Milho - Resistência a  
doenças e pragas. I. Universidade Federal de Viçosa.  
Departamento de Entomologia. Programa de Pós-graduação em  
Entomologia. II. Título.

CDD 22 ed. 595.752

NATÁLIA DE SOUZA RIBAS

**EFFECTS OF Cry1Fa BT RESISTANCE ON THE  
REPRODUCTIVE OUTPUT OF *Spodoptera frugiperda***

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

APROVADA: 23 de fevereiro de 2018.

---

Marcelo Gustavo Lorenzo

---

Og Francisco Fonseca de Souza

---

Eraldo Rodrigues de Lima  
(Orientador)

A meus pais, que sempre se  
doaram para a realização dos  
meus sonhos, dedico essa  
conquista!

“Desconfie do destino e acredite em você.  
Gaste mais horas realizando que sonhando,  
fazendo que planejando, vivendo que esperando.  
Porque, embora quem quase morre esteja vivo,  
quem quase vive, já morreu.”  
Sarah Westphal

## Agradecimentos

A Deus pelo milagre de cada instante, pela saúde, força e possibilidades de atuação.

A meus pais, por serem o meu porto seguro, a minha inspiração, por estarem sempre ao meu lado e nunca me deixarem desistir.

A meus irmãos, Bruna e Milton, por todo amor, carinho e apoio. Amo muito vocês!

A Wanderson, por toda ajuda, carinho, amor e confiança.

A todos da minha família, que sempre me apoiaram e nunca me deixaram desistir. Em especial, a minha vó Liquinha, meu vô Antônio, a Paulinha e ao Vinicio. Família, muito obrigada!

A Universidade Federal de Viçosa e ao Programa de Pós-graduação em Entomologia pela oportunidade e estrutura concedida para realização desse trabalho.

A CAPES, pelo concessão de bolsa durante meu mestrado.

Ao Professor Eraldo Lima, que com muita boa vontade me ensinou muito no decorrer destes anos. Seu apoio, incentivo e confiança no meu trabalho foram fundamentais para minha formação pessoal e científica.

Ao Professor Jeremy McNeil, pelo apoio, sugestões e esclarecimentos.

Aos colegas de laboratório que fizeram parte dessa longa jornada. Em especial, gostaria de agradecer o Hernane, a Josie, o Manuel e a Morgana. Sem eles não seria possível ter enfrentado todos os desafios dessa dissertação. Sou muito grata a todos que de alguma forma contribuíram não só para a realização dessa dissertação, mas também pelo meu engrandecimento pessoal através da convivência com todos.

Aos estagiários que incansadamente me ajudaram: Janice, Gislaine, Lucas, Bruna, Vinicio e Mikaelison. Muito Obrigada!

Aos colegas do laboratório do Professor Elizeu, onde tive o prazer de dividir experiências e aprender muito. Em especial, gostaria de agradecer

o Clebson e o Thadeu, pois sem eles tudo seria mais difícil. Ganhei duas pessoas muito importantes na minha vida. Muito obrigada por tudo!

Ao Arne e ao Vinícius pelo suporte nas análises estatísticas.

Ao Professor Og de Souza, pelo uso do equipamento para tirar fotos dos espermatóforos.

Aos amigos dos Estados Unidos por sempre me incentivarem e serem verdadeiras fontes de inspiração.

Aos amigos de Viçosa por entenderem a minha ausência e por toda amizade.

Aos amigos e amigas que a Agronomia me deu e que estão sempre ao meu lado.

A todos os que colaboraram de alguma forma para o êxito desse trabalho, o meu eterno agradecimento e estima.

# Contents

	Page
<b>Abstract</b> . . . . .	<b>vii</b>
<b>Resumo</b> . . . . .	<b>ix</b>
<b>1 Introduction</b> . . . . .	<b>1</b>
<b>2 Materials and Methods</b> . . . . .	<b>7</b>
2.1 Insects . . . . .	7
2.2 Virgin males . . . . .	8
2.2.1 Fecundity and fertility . . . . .	8
2.2.2 Spermatophore . . . . .	8
2.3 Mated males . . . . .	9
2.3.1 Fecundity and fertility . . . . .	9
2.3.2 Spermatophore . . . . .	9
2.4 Statistical analysis . . . . .	10
<b>3 Results</b> . . . . .	<b>11</b>
3.1 Virgin males (fecundity and fertility) . . . . .	11
3.2 Virgin males (spermatophore weight) . . . . .	11
3.3 Mated males (fecundity and fertility) . . . . .	12
3.4 Mated males (Spermatophore weight) . . . . .	13
<b>4 Discussion</b> . . . . .	<b>22</b>
<b>5 References</b> . . . . .	<b>25</b>

## Abstract

RIBAS, Natália de Souza, M.Sc., Universidade Federal de Viçosa, February, 2018. **Effects of Cry1Fa Bt resistance on the reproductive output of *Spodoptera frugiperda***. Adviser: Eraldo Rodrigues de Lima.

The transgenic Bt corn producing the Cry1Fa toxin is the principal strategy for the control of The fall armyworm *Spodoptera frugiperda*, that is the most important lepidopteran pest of corn in South American countries and has evolved resistance to this toxin. It has been shown that insects resistant to Bt toxins can have costs of reproduction resulting in a reduction of offspring production. One way of assessing the possible effects of resistance on the reproduction of *S. frugiperda* is by evaluating the fecundity and fertility of females. So, we mated susceptible and resistant virgin and mated males of different ages with virgin females and measured the fecundity and fertility. The fecundity and fertility were lower for resistant females mated with resistant mated males than for susceptible females mated with susceptible experienced males. To figure out if the spermatophore transferred by males has some effect on the results above, we mated virgin and mated males of different ages with virgin females and measured the weight of the spermatophores. The spermatophore weight was lighter for resistant females mated with resistant mated males than for susceptible females mated with susceptible mated males. Since the spermatophore also contains nutrients that are used by the females to fecundate their eggs, it can explain the fecundity results. Our

data provided clear evidence that the development of Bt resistance affected the reproductive capacity of resistant mated males of *S. frugiperda* and that this effected the reproductive output of females mated with this males.

## Resumo

RIBAS, Natália de Souza, M.Sc., Universidade Federal de Viçosa, fevereiro de 2018. **Efeitos da resistência à toxina Bt Cry1Fa na reprodução de *Spodoptera frugiperda***. Orientador: Eraldo Rodrigues de Lima.

O milho transgênico que expressa a toxina Cry1Fa é a principal estratégia para o controle da lagarta-do-cartucho *Spodoptera frugiperda*, que é a principal praga do milho nos países da América do Sul e evoluiu resistência a esta toxina. Tem sido demonstrado que essa resistência decorre em um custo reprodutivo para esses insetos, o que resultaria em uma redução de sua prole. Uma maneira de estimar os possíveis efeitos da resistência na reprodução de *S. frugiperda* é avaliando a fecundidade e fertilidade das fêmeas. Assim, acasalamos machos virgens e experientes, suscetíveis e resistentes, de diferentes idades com fêmeas virgens suscetíveis e resistentes, respectivamente, e medimos a fecundidade e a fertilidade das mesmas. A fecundidade e a fertilidade foram menores para fêmeas resistentes acasaladas com machos resistentes experientes do que para fêmeas suscetíveis acasaladas com machos experientes suscetíveis. Para descobrir se o espermatozóide transferido pelos machos tem algum efeito sobre os resultados acima, acasalamos machos virgens e experientes, suscetíveis e resistentes, de diferentes idades, com fêmeas virgens suscetíveis e resistentes, respectivamente, e pesamos os espermatozóides transferidos pelo macho. Machos experientes resistentes acasalados com fêmeas resistentes produziram espermatozóides mais leves do que machos experientes

susceptíveis acasalados com fêmeas suscetíveis. Uma vez que o espermatóforo também contém nutrientes que são utilizados pelas fêmeas para fecundar seus ovos, isso explica os resultados da fecundidade. Nossos dados forneceram evidências claras de que o desenvolvimento da resistência Bt afetou a capacidade reprodutiva de machos experientes resistentes de *S. frugiperda* e que isso afetou a sua geração de descendentes.

# 1 Introduction

The use of transgenic expressing insecticidal proteins from *Bacillus thuringiensis* (Bt) is the most successful biotechnological strategy for the control of important lepidopteran pest of corn, cotton and soy (Gould, 1998; James, 2007; Okumura *et al.*, 2013). The genetically modified Bt-cultures produce a toxic protein, called Cry that dissolves in the alkaline lumen of the midgut of the insect and participates in the formation of pores in the bilayer lipid membrane of midgut cells. The disruption of this membrane integrity happens because of these pores and later kills the cells. Therefore, insects die because they stop to feed or because of septicemia (Pardo-Lopez *et al.*, 2012).

The fall armyworm, *Spodoptera frugiperda* (J. E. Smith 1797) is the most important lepidopteran pest of corn in South American countries (Cruz & Valicente, 1990; Pogue, 2002; Blanco *et al.*, 2016). Due to the difficulty of controlling the caterpillar inside the whorl of the plant, hindering contact with insecticides, and due to its evolutionary history of adaptation to chemicals compounds, transgenic corn was rapidly adopted worldwide to control this pest (Yu, 1991; Diez-Rodríguez & Omoto, 2001; Siebert *et al.*, 2008). The Bt technology was first introduced in Brazil in 2009 for the control of *S. frugiperda* and during the 2016/2017 crop season, transgenic corn comprised 88.4% of the total area cultivated (Céleres, 2017).

However, it is known that *S. frugiperda* showed resistance to most of the Bt proteins from the Cry1 group (such as Cry1F protein) in Puerto Rico, the United States, and Brazil (Storer *et al.*, 2010; Farias *et al.*, 2014; Huang *et al.*, 2014). Resistant populations of this pest had been reported for Cry1F protein in seven different Brazilian states (Farias *et al.*, 2014).

Consequently, the success of the transgenic cultures that produce this protein will depend on the evolution of resistance in *S. frugiperda* populations, once it has compromised the effectiveness of this kind of control.

In order to mitigate the resistance in insect population, the resistance management plan, known as the high dose and refuge strategy has been used (Gould, 1998; Storer *et al.*, 2010). This strategy attempts to ensure that concentration of Cry protein enables the death of offspring of the crosses individuals (susceptible x resistant) and the refuge provides that there are susceptible insects to mate with resistant insects and generate heterozygous insects that are killed by the Bt-corn. However, may happens failures in the high dose/refuge strategy due to the genetic basis of resistance, that may not meet some of the assumptions required for proper function of this strategy.

Also, the resistance may carry a fitness cost for the insects, for example, when the resistant insects show a lower fecundity and fertility, compared with susceptible ones (Carrière *et al.*, 2006; Gassmann *et al.*, 2009). This cost was reported for resistant populations of *Helicoverpa armigera* (Lepidoptera: Noctuidae), *S. frugiperda* and *Diatraea saccharalis* (Lepidoptera: Crambidae) (Liang *et al.*, 2008; Jakka *et al.*, 2014; Zhang *et al.*, 2014). Although in these cases there was a fitness cost on the insect development, but changes have also been reported in reproductive behavior and reproductive physiology of resistant insects (Zhao *et al.*, 2009; Zhang *et al.*, 2014). Further, if there is a fitness costs, susceptible individuals are more likely to survive and reproduce in the non-Bt area than resistant individuals, therefore it may reduce the allelic frequency of resistant insects (Fatoretto *et al.*, 2017).

The changes in the reproductive behavior and physiology can affect both sexes. For example, Delisle & Bouchard (1995) showed that resistant females of *Choristoneura rosaceana* (Lepidoptera: Tortricidae) produced less pheromone, initiated calling much later in the night, and called for a shorter period than susceptible ones. In another example, Higginson *et al.* (2005) showed that susceptibles males of *Pectinophora gossypiella* (Lepidoptera:

Gelechiidae) leaned to mate more often than resistant ones. In both cases, there was a change in the reproductive behavior of the resistant moths.

Furthermore, the reproductive physiology of these insects may also present alterations. For example, Zhang *et al.* (2015) showed that Cry1Ac resistant *H. armigera* females had a significantly delayed ovary development. Carrière *et al.* (2006) showed that Cry1Ac resistant *P. gossypiella* males transferred less sperm than susceptible ones. Therefore, the reproductive changes caused by the resistance to the Bt protein showed a trade-off between the resistance and the reproductive capacity of the resistant insects.

During mating, male moths can transfer sperm indirectly to the females through a spermatophore, which is formed inside the females bursa copulatrix (Boggs & Gilbert, 1979; Proctor, 1998). In addition to transferring sperm to fertilize females eggs, the spermatophore contains nutrients that are used by them to produce eggs, such as proteins, amino acids, and hydrocarbons (Friedel & Gillott, 1977; Engebretson & Mason, 1980; Marshall & McNeil, 1989). Spermatophore size is related to the male reproductive capacity since the larger ones have more sperm and nutritious content than the smaller ones (He & Miyata, 1997; Svärd & Wiklund, 1986). Also, some important factors influence spermatophore size, such as number of matings, in some lepidopterans the spermatophore size decrease with the number of matings (Fig 1); age of male, in some lepidopteras how much older the male in its first mating bigger is his spermatophore tranfered (Fig 2); mating duration, when the male transfers a much bigger spermatophore size in his longer mating than in his shorter mating and weight of males, when the bigger male transfers a bigger spermatophore size (Royer & McNeil, 1993; Delisle & Bouchard, 1995; Butler IV *et al.*, 2015; Duploux & Hanski, 2015). The fecundity and fertility of females can also be affected by the spermatophore size (Royer & McNeil, 1993; Svärd & McNeil, 1994). Wedell & Karlsson (2003), showed that females of *Pieris napi* (Lepidoptera: Pieridae) that received a larger spermatophore produced more eggs. Cahenzli & Erhardt (2013), showed

that females of *Coenonympha pamphilus* (Lepidoptera: Nymphalidae) produced a larger mass of hatched larvae when the males transferred a larger spermatophore.

Although there is some study on the subject, little is known about the effect of the resistance to Cry1Fa toxin in the reproductive capacity of males, once this parameter is rarely considered in studies of Bt resistance. *S. frugiperda* has some biological characteristics which support the invasion of this pest on corn throughout the year, such as high reproductive capacity and multiple generations (Farias *et al.*, 2014; Nagoshi *et al.*, 2014). However, if such effects occur, the change in the reproductive capacity of resistant males could reduce its generation of offspring and decrease the resistant allele frequency in the field. One way of assessing the possible effects of resistance on the reproduction of *S. frugiperda* is by evaluating the fecundity and fertility of females. So, we hypothesized that resistant females mated with resistant males would have lower fecundity and fertility than susceptible females mated with susceptible males and if this is proved to, we expected that resistant males transferred lighter spermatophores than susceptible ones. Since, the higher the spermatophore, the greater the amount of nutrients and sperm transferred by it, that are used by the females to fecundate and fertilize their eggs, this could explain the possible results. Therefore, we undertook a laboratory study to determine if there is a difference between resistant and susceptible female's fecundity and fertility and if this happened because of the spermatophore weight of resistant and susceptible males of *S. frugiperda*



Figure 1: Spermatophores of *S. frugiperda* on successive matings, showing the decrease in size.



Figure 2: Spermatophores of *S. frugiperda* virgin males in their first mating with 3, 5 and 7 days old, showing the increase in size.

## 2 Materials and Methods

### 2.1 Insects

Initially, a *S. frugiperda* population susceptible to Cry1Fa (SS), that exists for 15 years without exposure to pesticides, was obtained from the Laboratory of Insect Ecotoxicology and Management of Embrapa Maize Sorghum (Sete Lagoas, MG, Brazil). The resistant population (RR) was provided by the laboratory of DuPont Pioneer in Brasília, Federal District. The DuPont Pioneer technicians did a collection of approximately 200 larvae from four corn producing regions in Brazil (Cascavel, Sorriso, Brasília, and Morrinhos). These populations were maintained separately for two generations and then sent to the Federal University of Viçosa (UFV, Viçosa, state of Minas Gerais, Brazil). In the F3 generation under laboratory rearing, moths of the different populations were reciprocally crossed as described by (Santos-Amaya *et al.*, 2016) resulting in a resistant genotype (RR). To maintain the resistance in this population, larvae of RR were exposed to Cry1Fa corn leaves every five generations.

The insects were then reared following Kasten Jr *et al.* (1978), with adaptations. About 150 moths were maintained in PVC cages (30 cm height x 20 cm in diameter) with sulfite paper on the inner walls for egg laying. A piece of cotton soaked in a 10% sugar and 1% ascorbic acid solution was offered *ad libitum* as food, which was changed every two days. Eggs were collected every two days and stored in 500 ml plastic bags until hatching. 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 Ind. e Comércio de Plásticos

Ltda) until pupation. The insects were kept at a controlled temperature set at  $27 \pm 1^\circ\text{C}$ ,  $70 \pm 15\%$  relative humidity and 14L:10D photoperiod.

Newly emerged female moths were individually placed in a clear plastic jar and kept fed in a distinct room until the experiments. The males were weighed on an analytical balance (Shimadzu AUW220D) as soon as they emerged and before being fed then placed in a clear plastic jar and kept fed in a distinct room until the experiments.

## 2.2 Virgin males

### 2.2.1 Fecundity and fertility

We tested whether females fecundity and fertility are affected by the males age in its first mating, mating duration and male weight in two genotypes: resistant and susceptible to the Cry1Fa toxin. For the experiment, resistant and susceptible males were offered to mate with resistant and susceptible virgin females, respectively, at the fourth hour of the scotophase. 3, 5 and 7 days old virgin males were mated with 3 days old virgin females ( $n = 25$  per treatment). After the mating in each assay, the females were individualized in 75 ml PVC cages internally coated with sulfite paper for egg laying. Food was available *ad libitum*. To assess the fecundity, the number of eggs laid was counted two days after the cages were assembled. To assess the fertility, the number of caterpillars was counted as soon as they hatched from the egg every day until the fifth day.

### 2.2.2 Spermatophore

We tested whether the *S. frugiperda* spermatophore weight is affected by male age in its first mating, mating duration and male weight in two genotypes: resistant and susceptible to the Cry1Fa toxin. For the experiment, resistant and susceptible males were offered to mate with resistant and susceptible virgin females, respectively, at the fourth hour of the scotophase. 3,

5 and 7 days old virgin males were mated with 3 days old virgin females (n = 25 per treatment). After each mating in each assay, the females were dissected with micro scissors for the removal and weighing of the spermatophore placed by males. The mating duration of each mating was recorded to see if it affected spermatophore weight. The spermatophores were weighed on the same analytical balance (Shimadzu AUW220D), where the males were weighed before mating.

## 2.3 Mated males

### 2.3.1 Fecundity and fertility

We tested whether females fecundity and fertility are affected by the males number of mating, mating duration and male weight in two genotypes: resistant and susceptible to the Cry1Fa toxin. For the experiment, resistant and susceptible males were offered to mate with resistant and susceptible virgin females, respectively, at the fourth hour of the scotophase. 3 days old virgin male were mated with 3 days old virgin females, the same male were mated again in the fifth day in his second mating, with another 3 day old virgin female and for the third mating on the seventh day with another 3 day old virgin female (n = 8 per treatment). After the mating in each experiment, the females were individualized in 75 ml PVC cages internally coated with sulfite paper for egg laying. Food was available *ad libitum*. To assess the fecundity, the number of eggs laid was counted two days after the cages were assembled. To assess the fertility, the number of caterpillars was counted as soon as they hatched from the egg every day until the fifth day.

### 2.3.2 Spermatophore

We tested whether the *S. frugiperda* spermatophore weight is affected by males number of mating, mating duration and male weight in two genotypes: resistant and susceptible to the Cry1Fa toxin. For the experiment, resistant

and susceptible males were offered to mate with resistant and susceptible virgin females, respectively, at the fourth hour of the scotophase. 3 days old virgin males were mated with 3 days old virgin females, the same males were mated again in the fifth day in his second mating with another 3 day old virgin female and for the third mating on the seventh day with another 3 day old virgin female ( $n = 8$  per treatment). After each mating in each experiment, the females were dissected with micro scissors for the removal and weighing of the spermatophore placed by males. The mating duration of each mating was recorded to see if it affected spermatophore weight.

The spermatophores were weighed on the same analytical balance (Shimadzu AUW220D), where the males were weighed before mating.

## 2.4 Statistical analysis

In the experiments with virgin males, we needed to determine if the age, the mating duration, and male weight affect the number of eggs laid, the number of caterpillars hatched, and the weight of the spermatophores in two genotypes: resistant and susceptible to the Cry1Fa toxin. We built a generalized linear model (GLM) under Poisson, Binomial or Gaussian distribution of errors, respectively.

In the experiments with mated males, we needed to determine if the number of mating, the mating duration and male weight affects the number of eggs laid, the number of caterpillars hatched, and the weight of the spermatophores in two genotypes: resistant and susceptible to the Cry1Fa toxin. We also built a generalized linear model (GLM) under Poisson, Binomial or Gaussian distribution of errors, respectively.

Both analyses were performed and followed by residual analyses to confirm the error distribution and the suitability of the model. They were performed with the R statistical program (R Development Core Team, 2014).

## 3 Results

### 3.1 Virgin males (fecundity and fertility)

The genotype ( $F = 2.14$ ;  $df = 1, 123$ ;  $p = 0.1463$ ) and the age of the male in their first mating ( $F = 0.38$ ;  $df = 1, 124$ ;  $p = 0.5360$ ) did not affect the fecundity. However, there was an interaction between genotype in relation to the fecundity and the age of the male in its first mating ( $F = 13.43$ ;  $df = 1, 122$ ;  $p = 0.0003$ ) (Fig 3). This interaction showed that the fecundity of resistant females mated with resistant males decreased more than the fecundity of susceptible females mated with susceptible males. The genotype ( $F = 0.71$ ;  $df = 1, 124$ ;  $p = 0.3994$ ) and the age of the male in its first mating ( $F = 0.28$ ;  $df = 1, 123$ ;  $p = 0.5995$ ) did not affect the fertility of females.

### 3.2 Virgin males (spermatophore weight)

The mating duration and the genotypes did not affect the weight of the spermatophore transferred by males in the mating duration assay.

In the male weight assay, there was a significant interaction between the genotypes in relation to the spermatophore weight and the male weight. This interaction showed that the weight of spermatophores of resistant males decreased more, how heavy the males, than the weight of spermatophores of susceptible males (Fig 4).

In the assay to verify the effect of age, there was also an interaction between the genotype in relation to spermatophore weight and the male age. This interaction showed that for both genotypes, the older the male in their first mating the heavier the spermatophore transferred by him and that the

weight of spermatophores of resistant males increased more, with an increased in the males age in their first mating than the weight of spermatophores of susceptible males (Fig 5). The analysis of each variable that affected and not affected the spermatophore weight of *S. frugiperda* males are summarized in Table 1.

Table 1: Effect of age, mating duration and male weight on spermatophore of resistant and susceptible males of *S. frugiperda* to the Cry1Fa toxin (GLM, F-test). \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$

		<i>df</i>	F-value	p-value	
	Age	1,147	128.21	0.0001	***
Age	Genotype	1,148	0.68	0.4123	
	Interaction	1,146	16.76	0.0001	***
Mating duration	Mating duration	1,147	0.09	0.7642	
	Genotype	1,148	0.34	0.5585	
	Interaction	-	-	-	
Male weight	Male weight	1,147	0.009	0.92501	
	Genotype	1,148	0.35	0.55494	
	Interaction	1,146	4.70	0.03168	*

### 3.3 Mated males (fecundity and fertility)

The SS females laid more eggs than the RR females ( $F = 5.8797$ ;  $df = 1, 39$ ;  $p = 0.02005$ ). For both genotypes, the number of eggs laid decreased after the first mating ( $F = 6.7581$ ;  $df = 1, 40$ ;  $p = 0.01311$ ) (Fig 6).

The SS genotype presented a higher proportion of emerged caterpillars per number of eggs laid than the RR genotype ( $F = 7.1357$ ;  $df = 1, 40$ ;  $p = 0.01097$ ). The number of mating did not affect the fertility ( $F = 0.0003$ ;  $df = 1, 39$ ;  $p = 0.98744$ ) (Fig 7).

### 3.4 Mated males (Spermatophore weight)

The mating duration did not affect the weight of the spermatophore transferred by males in the mating duration assay. However, RR males took longer to transfer the same spermatophore weight for females than SS ones (Fig 8).

In the male weight assay, the weight of the spermatophore transferred by Susceptible males was higher than in Resistant males. For both genotypes the lighter male weight transferred the heavier spermatophore weight and the the heavier male weight transferred the lighter spermatophore weight (Fig 9).

In the number of previous matings assay, in each treatment SS males transferred heavier spermatophores than RR males. For both genotypes, the higher the number of matings, the smaller the weight of the spermatophore transferred for the female (Fig 10).

The analysis of each variable that affected and not affected the spermatophore weight of *S. frugiperda* males are summarized in Table 2.

Table 2: Effect of number of previous mating, mating duration and male weight on spermatophore of resistant and susceptible males of *S. frugiperda* to the Cry1Fa toxin (GLM, F-test). \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$

		<i>df</i>	F-value	p-value	
Age	Number of previous mating	1,45	52.49	< 0.001	***
	Genotype	1,46	10.87	0.0019	***
	Interaction	-	-	-	
Mating duration	Mating duration	1,45	1.54	0.22	
	Genotype	1,46	59.56	<0.001	***
	Interaction	-	-	-	
Male weight	Male weight	1,45	6.00	0.02	*
	Genotype	1,46	5.68	0.02	*
	Interaction	-	-	-	

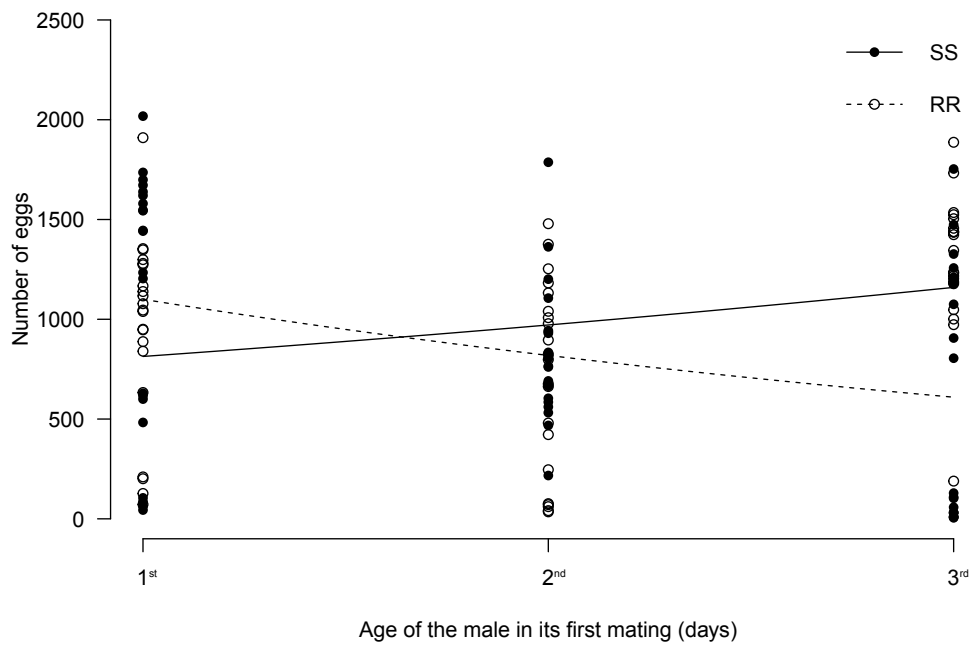


Figure 3: Fecundity (number of eggs laid) of resistant and susceptible *S. frugiperda* females on mating with resistant (RR) and susceptible (SS) males, respectively, with 3, 5, and 7 days old ( $n = 21$  per treatment). Filled circles represent (SS) males, and open circles represent (RR) males, regression lines for SS and RR in plain and dotted black, respectively.

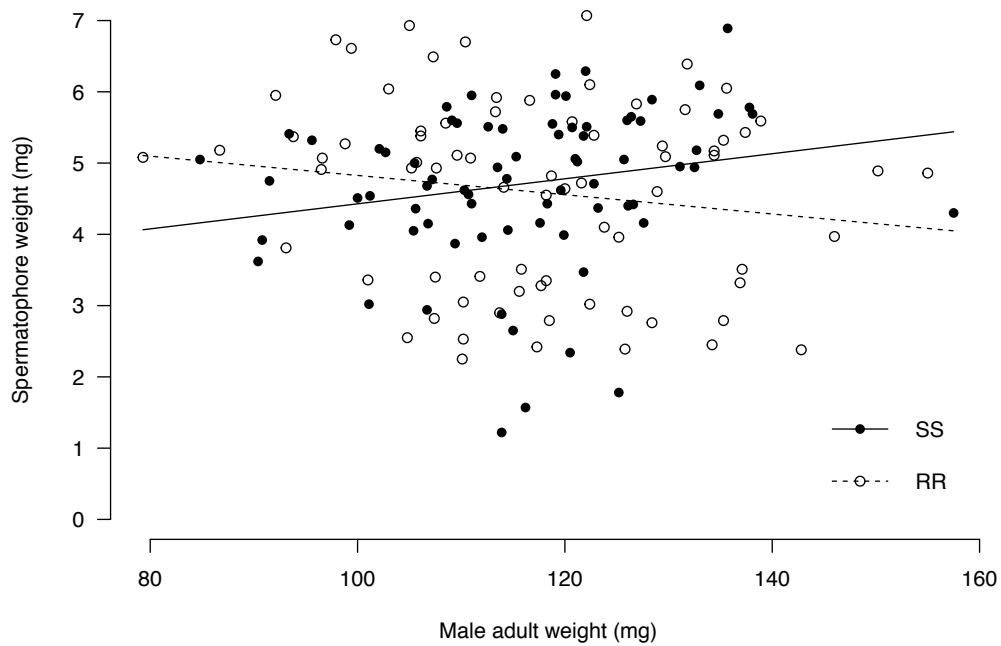


Figure 4: The relationship between the weight of the spermatophore transferred by *S. frugiperda* (resistant and susceptible) virgin males and the adult male weight regardless of the age of the male in their first mating ( $n = 75$  per treatment). Filled circles represent susceptible males (SS), and open circles represent resistant males (RR), regression lines for SS and RR in plain and dotted black, respectively.

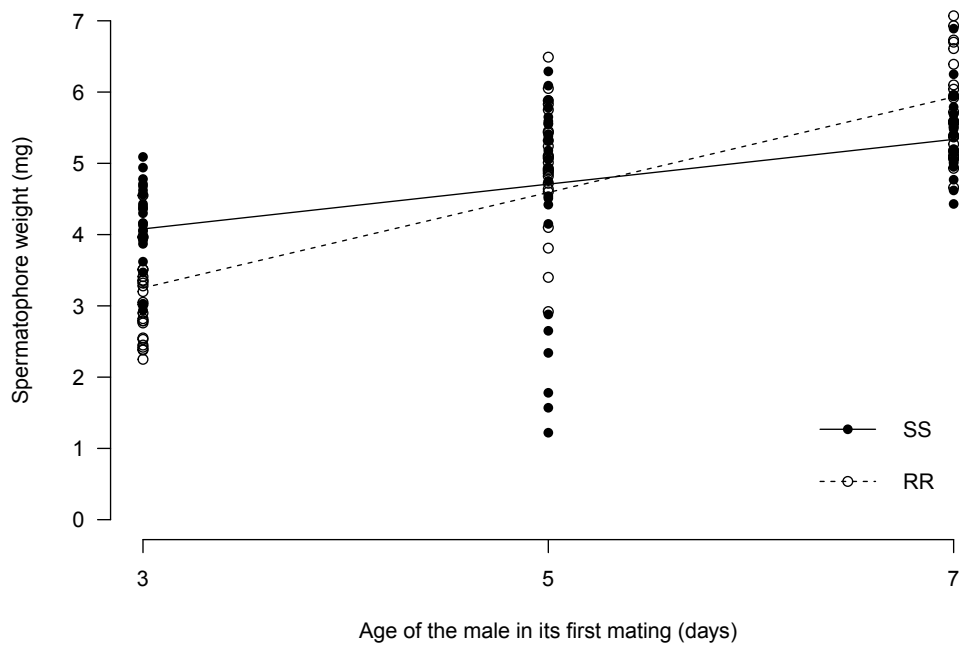


Figure 5: The weight of the spermatophore transferred by resistant and susceptible *S. frugiperda* males in their first mating with 3, 5 and 7 days old ( $n = 25$  per treatment). Filled circles represent susceptible males (SS), and open circles represent resistant males (RR), regression lines for SS and RR in plain and dotted black, respectively.

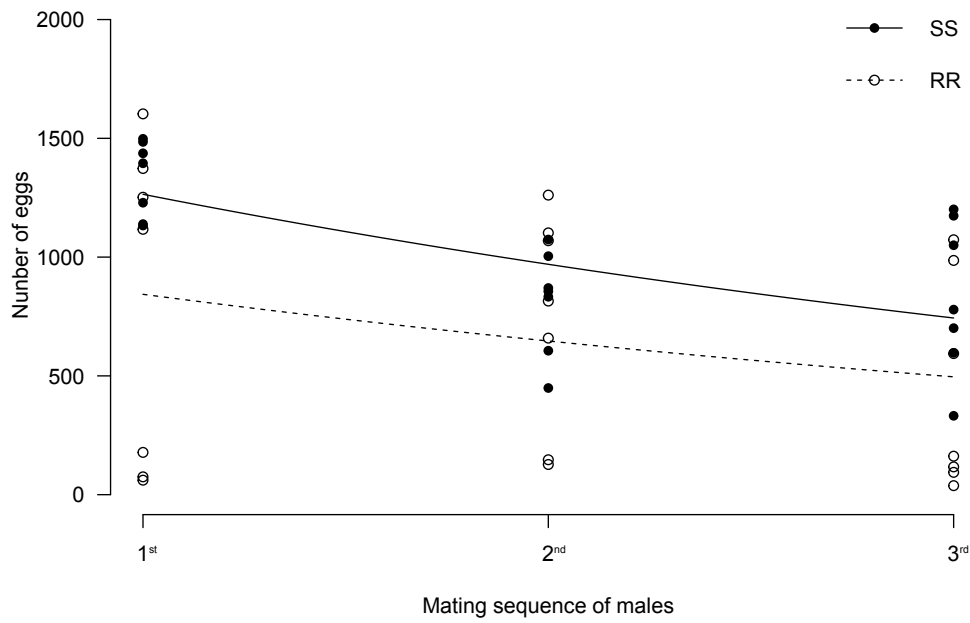


Figure 6: Fecundity (number of eggs) of resistant and susceptible *S. frugiperda* females on successive mating with resistant and susceptible males, respectively ( $n = 7$  per treatment). Filled circles represent susceptible males (SS), and open circles represent resistant males (RR), regression lines for SS and RR in plain and dotted black, respectively.

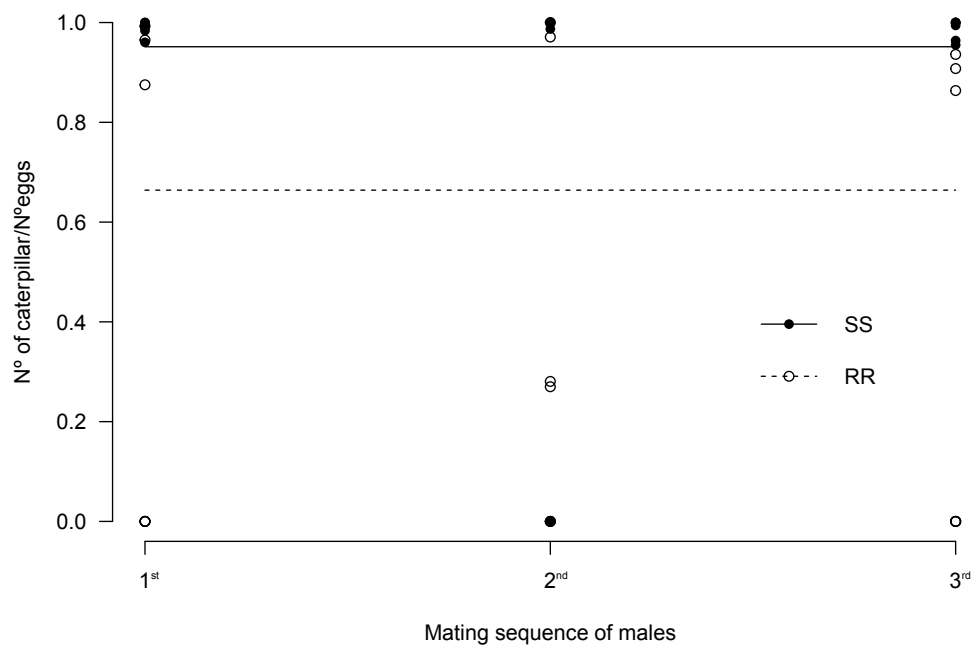


Figure 7: Fertility (number of caterpillars emerged/number of eggs laid) of resistant and susceptible *S. frugiperda* females on successive mating with resistant and susceptible males, respectively ( $n = 7$  per treatment). Filled circles represent susceptible males (SS), and open circles represent resistant males (RR), regression lines for SS and RR in plain and dotted black, respectively.

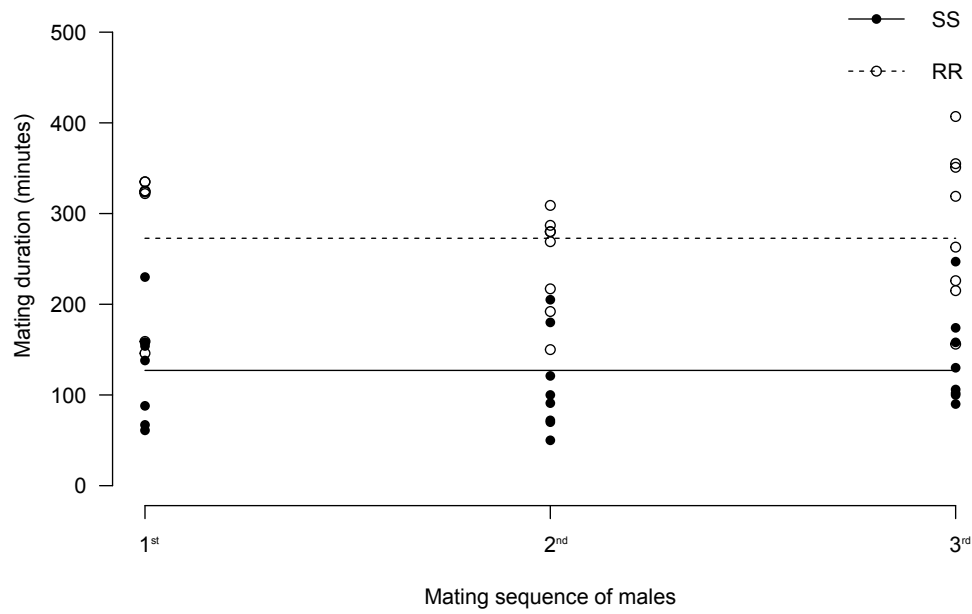


Figure 8: The relationship between mating duration by *S. frugiperda* (resistant and susceptible) males and the mating sequence of matings ( $n = 8$  per treatment). Filled circles represent susceptible males (SS), and open circles represent resistant males (RR), regression lines for SS and RR in plain and dotted black, respectively.

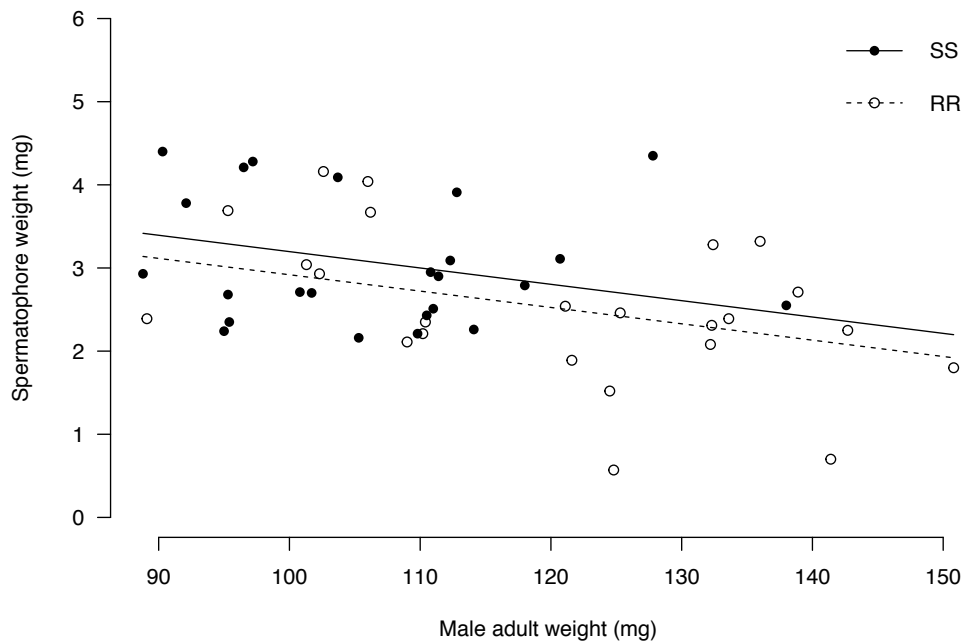


Figure 9: The relationship between the weight of the spermatophore transferred by *S. frugiperda* (resistant and susceptible) males and the adult male weight on successive mating regardless of the number of previous mating ( $n = 24$  per treatment). Filled circles represent susceptible males (SS), and open circles represent resistant males (RR), regression lines for SS and RR in plain and dotted black, respectively.

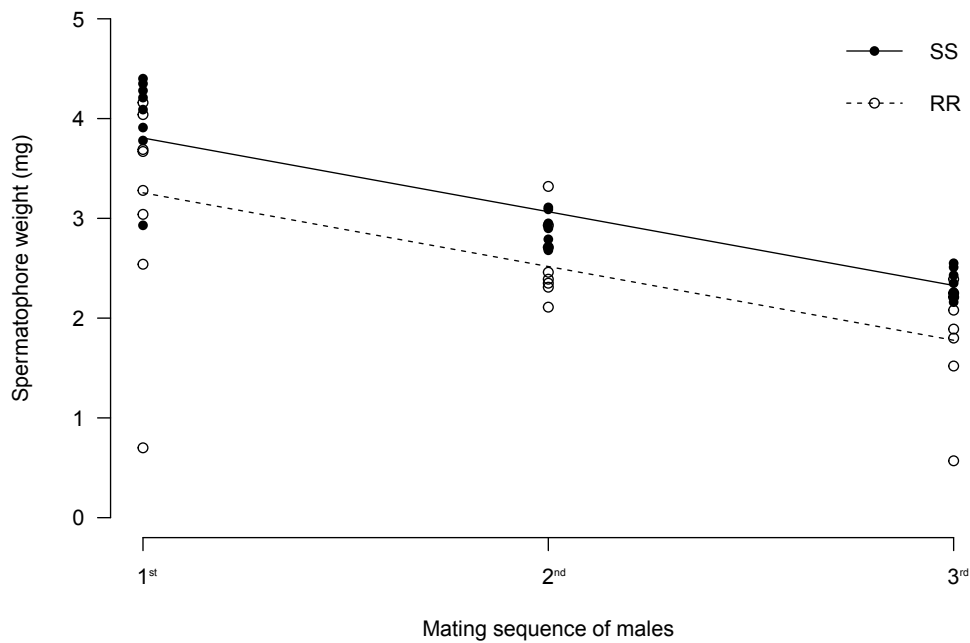


Figure 10: The weight of spermatophore (mg) transferred by resistant and susceptible *S. frugiperda* males on successive mating ( $n = 8$  per treatment). Filled circles represent susceptible males (SS), and open circles represent resistant males (RR), regression lines for SS and RR in plain and dotted black, respectively.

## 4 Discussion

Our results provided for the first time clear evidence that the development of resistance to the Cry1Fa toxin affected the reproductive output of females mated with mated males. We also showed that the selection pressure produced by Bt cultures lead to a decrease in the reproductive capacity of *S. frugiperda* males, measured by their spermatophore weight. Based on both results presented above we concluded that the reduction in the reproductive output of resistant female mated with resistant mated males occurs due to a reduction in nutritional quality and viability of sperm transferred by the male to female during mating. This because the contribution in the fertility of females is entirely from the male who transfers the sperm to fertilize female's eggs and because there was no difference between the number of ovocytes and oocytes produced by susceptible and resistant females (Fernanda Freitas de Sousa et al., unpublished data).

In the assays with virgin males we saw that the fecundity of resistant females mated with resistant males decreased more than the fecundity of susceptible females mated with susceptible males and that the weight of spermatophores of resistant males increased more, with an increased in the males age in their first mating than the weight of spermatophores of susceptible males. It shows that even when the resistant males got old for his first mating, producing heavier spermatophores, the female mated with these males had a lower fecundity than susceptible females mated with susceptible males. It also may mean a reduction in nutritional quality of sperm transferred by resistant males to females during mating.

The results also showed that the number of times the male had previously mated correlated negatively with the number of eggs laid by the female but

did not affect the proportion of emerged caterpillars per number of eggs laid. This indicates that the weight of the spermatophore in consecutive mating is limited by the nutritional quality, not sperm quality, once the females showed a decrease in their number of eggs laid with the number of time the male mate but not in the fertility. It could be explained as a consequence of the decrease in the amount of substances needed, such as proteins to be produced in consecutive matings (Oberhauser, 1988).

The effect of the number of mating in the spermatophore weight is well known, because of its weight decreases with successive mating in some lepidopteran species (Svärd & Wiklund, 1986; Knight, 2007). For example, a study with *Ostrinia nubilalis* (Lepidoptera: Crambidae) has demonstrated that there was a decrease between the first and either the second or third mating in the spermatophore volume (Royer & McNeil, 1993). In agreement with these observations the spermatophore weight of mated *S. frugiperda* males correlated negatively with the number of the previous mating. This could be explained by the fact that *S. frugiperda* is a polyandric species, and it can lead to competition between males (Arnqvist & Nilsson, 2000). Thereby, to reduce the chance of subsequent mating, males tend to produce large spermatophore in their first mating (Wiklund *et al.*, 1993; Svärd & Wiklund, 1986; Morais *et al.*, 2012).

It was also found that, for mated males in their first mating, and regardless the genotype, occurred a decrease in the spermatophore weight with an increase in the male weight, what indicates a trade-off between male weight and reproduction. In polygynous species the spermatophore production is physiologically costly, we assumed that if the male invests in development, it will have less energy to reproduction. For virgin males the weight of spermatophores of resistant males decreased more, how heavy the males, than the weight of spermatophores of susceptible males. It shows that the cost of grown more is greater for resistant males than for susceptible males.

Also, the resistant males took longer to transfer the same spermatophore weight for females than susceptible ones. This is an important issue to be

studied in futures works, because how long they stay in the field mating, biggest the chance of being preyed.

In conclusion, our data reveal a cost in the reproductive output of *S. frugiperda* to the Cry1Fa toxin. Specifically, this study shows how the pressure generated by resistance in insects causes changes in the reproductive potential of males, based on their spermatophores weight and how this affected the female fecundity and fertility.

## 5 References

- Arnqvist, G. & Nilsson, T. (2000). The evolution of polyandry: multiple mating and female fitness in insects. *Animal behaviour*, 60, 145–164.
- Blanco, C.; Chiaravalle, W.; Dalla-Rizza, M.; Farias, J.; García-Degano, M.; Gastaminza, G.; Mota-Sánchez, D.; Murúa, M.; Omoto, C.; Pieralisi, B. *et al.* (2016). Current situation of pests targeted by bt crops in latin america. *Current opinion in insect science*, 15, 131–138.
- Boggs, C. L. & Gilbert, L. E. (1979). Male contribution to egg production in butterflies: evidence for transfer of nutrients at mating. *Science*, 206, 83–84.
- Butler IV, M. J.; Macdiarmid, A. & Gnanalingam, G. (2015). The effect of parental size on spermatophore production, egg quality, fertilization success, and larval characteristics in the Caribbean Spiny lobster, *Panulirus argus*. *ICES Journal of Marine Science*, 72, i115–i123.
- Cahenzli, F. & Erhardt, A. (2013). Nectar amino acids enhance reproduction in male butterflies. *Oecologia*, 171, 197–205.
- Carrière, Y.; Ellers-Kirk, C.; Biggs, R. W.; Nyboer, M. E.; Unnithan, G. C.; Dennehy, T. J. & Tabashnik, B. E. (2006). Cadherin-based resistance to *Bacillus thuringiensis* cotton in hybrid strains of pink bollworm: fitness

costs and incomplete resistance. *Journal of Economic Entomology*, 99, 1925–1935.

Céleres (2017). Follow-up on agricultural biotechnology adoption for the 2016/17 Crop (2015). Available: <http://www.celeres.com.br/>. Accessed: June 2017.

Cruz, I. & Valicente, F. (1990). Manejo da lagarta-do-cartucho, *Spodoptera frugiperda* em milho. In: *Embrapa Milho e Sorgo-Resumo em anais de congresso (ALICE)*. In: CONGRESSO NACIONAL DE MILHO E SORGO, 18., 1990, Vitória, ES. Resumos. Vitória: EMCAPA, 1990. p. 66.

Delisle, J. & Bouchard, A. (1995). Male larval nutrition in *Choristoneura rosaceana* (Lepidoptera: Tortricidae): an important factor in reproductive success. *Oecologia*, 104, 508–517.

Diez-Rodríguez, G. I. & Omoto, C. (2001). Herança da resistência de *Spodoptera frugiperda* (JE Smith)(Lepidoptera: Noctuidae) a lambda-cialotrina. *Neotropical Entomology*, 30, 311–316.

Duploux, A. & Hanski, I. (2015). Small spermatophore size and reduced female fitness in an isolated butterfly population. *Ecological Entomology*, 40, 167–174.

Engebretson, J. A. & Mason, W. H. (1980). Transfer of 65 zn at mating in *Heliothis virescens*. *Environmental entomology*, 9, 119–121.

Farias, J. R.; Andow, D. A.; Horikoshi, R. J.; Sorgatto, R. J.; Fresia, P.; dos Santos, A. C. & Omoto, C. (2014). Field-evolved resistance to Cry1F maize by *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Brazil. *Crop Protection*, 64, 150–158.

Fatoretto, J. C.; Michel, A. P.; Silva Filho, M. C. & Silva, N. (2017). Adaptive potential of fall armyworm (Lepidoptera: Noctuidae) limits bt trait

durability in brazil. *Journal of Integrated Pest Management*, 8, 17.

URL + <http://dx.doi.org/10.1093/jipm/pmx011>

Friedel, T. & Gillott, C. (1977). Contribution of male-produced proteins to vitellogenesis in *Melanoplus sanguinipes*. *Journal of Insect Physiology*, 23, 145–151.

Gassmann, A. J.; Carrière, Y. & Tabashnik, B. E. (2009). Fitness costs of insect resistance to *Bacillus thuringiensis*. *Annual Review of Entomology*, 54.

Gould, F. (1998). Sustainability of transgenic insecticidal cultivars: integrating pest genetics and ecology. *Annual Review of Entomology*, 43, 701–726.

He, Y. & Miyata, T. (1997). Variations in sperm number in relation to larval crowding and spermatophore size in the armyworm, *Pseudaletia separata*. *Ecological Entomology*, 22, 41–46.

Higginson, D. M.; Morin, S.; Nyboer, M. E.; Biggs, R. W.; Tabashnik, B. E. & Carrière, Y. (2005). Evolutionary trade-offs of insect resistance to *Bacillus thuringiensis* crops: fitness cost affecting paternity. *Evolution*, 59, 915–920.

Huang, F.; Qureshi, J. A.; Meagher Jr, R. L.; Reisig, D. D.; Head, G. P.; Andow, D. A.; Ni, X.; Kerns, D.; Buntin, G. D.; Niu, Y. *et al.* (2014). Cry1f resistance in fall armyworm *Spodoptera frugiperda*: single gene versus pyramided bt maize. *PLoS One*, 9, e112958.

Jakka, S.; Knight, V. & Jurat-Fuentes, J. (2014). Fitness costs associated with field-evolved resistance to bt maize in *Spodoptera frugiperda* (lepidoptera: Noctuidae). *Journal of Economic Entomology*, 107, 342–351.

James, C. (2007). *Global status of commercialized biotech/GM crops, 2007*, vol. 37. ISAAA Ithaca, NY.

- Kasten Jr, P.; Precetti, A. & Parra, J. (1978). Dados biológicos comparativos de *Spodoptera frugiperda* (je smith, 1797) em duas dietas artificiais e substrato natural. *Revista de Agricultura*.
- Knight, A. (2007). Multiple mating of male and female codling moth (lepidoptera: Tortricidae) in apple orchards treated with sex pheromone. *Environmental entomology*, 36, 157–164.
- Liang, G.-M.; Wu, K.-M.; Yu, H.-K.; Li, K.-K.; Feng, X. & Guo, Y.-Y. (2008). Changes of inheritance mode and fitness in *Helicoverpa armigera* (hübner)(lepidoptera: Noctuidae) along with its resistance evolution to cry1ac toxin. *Journal of invertebrate pathology*, 97, 142–149.
- Marshall, L. & McNeil, J. (1989). Spermatophore mass as an estimate of male nutrient investment: a closer look in *Pseudaletia unipuncta* (harrowth)(lepidoptera: Noctuidae). *Functional Ecology*, 605–612.
- Morais, R. M. d.; Redaelli, L. R. & Sant’Ana, J. (2012). Age and multiple mating effects on reproductive success of *Grapholita molesta* (Busck)(Lepidoptera, Tortricidae). *Revista Brasileira de Entomologia*, 56, 319–324.
- Nagoshi, R. N.; Meagher, R. L. & Hay-Roe, M. (2014). Assessing the resolution of haplotype distributions to delineate fall armyworm (Lepidoptera: Noctuidae) migratory behaviors. *Journal of Economic Entomology*, 107, 1462–1470.
- Oberhauser, K. S. (1988). Male monarch butterfly spermatophore mass and mating strategies. *Animal behaviour*, 36, 1384–1388.
- Okumura, R. S.; de Cinque Mariano, D.; Dallacort, R.; Zorzenoni, T. O.; Zaccheo, P. V. C.; Eugencirc, H.; da Silva Lobato, A. K. *et al.* (2013). Agonomic efficiency of textitBacillus thuringiensis (bt) maize hybrids in pests

control on lucas do rio verde city, state of mato grosso, brazil. *African Journal of Agricultural Research*, 8, 2232–2239.

Pardo-Lopez, L.; Soberon, M. & Bravo, A. (2012). *Bacillus thuringiensis* insecticidal three-domain cry toxins: mode of action, insect resistance and consequences for crop protection. *FEMS microbiology reviews*, 37, 3–22.

Pogue, M. G. (2002). *A world Revision of the Genus Spodoptera Guenée:(Lepidoptera: Noctuidae)*. American Entomological Society Philadelphia.

Proctor, H. (1998). Indirect sperm transfer in arthropods: behavioral and evolutionary trends. *Annual review of entomology*, 43, 153–174.

Royer, L. & McNeil, J. (1993). Male investment in the european corn borer, *Ostrinia nubilalis* (lepidoptera: Pyralidae): impact on female longevity and reproductive performance. *Functional Ecology*, 209–215.

Santos-Amaya, O. F.; Tavares, C. S.; Monteiro, H. M.; Teixeira, T. P.; Guedes, R. N.; Alves, A. P. & Pereira, E. J. (2016). Genetic basis of cry1f resistance in two brazilian populations of fall armyworm, *Spodoptera frugiperda*. *Crop protection*, 81, 154–162.

Siebert, M. W.; Babcock, J.; Nolting, S.; Santos, A.; Adamczyk Jr, J.; Neese, P.; King, J.; Jenkins, J.; McCarty, J.; Lorenz, G. *et al.* (2008). Efficacy of cry1f insecticidal protein in maize and cotton for control of fall armyworm (lepidoptera: Noctuidae). *Florida Entomologist*, 91, 555–565.

Storer, N. P.; Babcock, J. M.; Schlenz, M.; Meade, T.; Thompson, G. D.; Bing, J. W. & Huckaba, R. M. (2010). Discovery and characterization of field resistance to Bt maize: *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Puerto Rico. *Journal of Economic Entomology*, 103, 1031–1038.

- Svärd, L. & McNeil, J. N. (1994). Female benefit, male risk: polyandry in the true armyworm *Pseudaletia unipuncta*. *Behavioral Ecology and Sociobiology*, 35, 319–326.
- Svärd, L. & Wiklund, C. (1986). Different ejaculate delivery strategies in first versus subsequent matings in the swallowtail butterfly *Papilio machaon* l. *Behavioral Ecology and Sociobiology*, 18, 325–330.
- Wedell, N. & Karlsson, B. (2003). Paternal investment directly affects female reproductive effort in an insect. *Proceedings of the Royal Society of London B: Biological Sciences*, 270, 2065–2071.
- Wiklund, C.; Kaitala, A.; Lindfors, V. & Abenius, J. (1993). Polyandry and its effect on female reproduction in the green-veined white butterfly (*Pieris napi* l.). *Behavioral Ecology and Sociobiology*, 33, 25–33.
- Yu, S. (1991). Insecticide resistance in the fall armyworm, *Spodoptera frugiperda* (je smith). *Pesticide Biochemistry and Physiology*, 39, 84–91.
- Zhang, L.; Leonard, B. R.; Chen, M.; Clark, T.; Anilkumar, K. & Huang, F. (2014). Fitness costs and stability of Cry1Ab resistance in sugarcane borer, *Diatraea saccharalis* (F.). *Journal of Invertebrate Pathology*, 117, 26–32.
- Zhang, W.; Ma, L.; Zhong, F.; Wang, Y.; Guo, Y.; Lu, Y. & Liang, G. (2015). Fitness costs of reproductive capacity and ovarian development in a bt-resistant strain of the cotton bollworm *Helicoverpa armigera* (hübner)(lepidoptera: Noctuidae). *Pest Management Science*, 71, 870–877.
- Zhao, X. C.; Wu, K. M.; Guo, Y. Y. *et al.* (2009). Modified female calling behaviour in cry1ac-resistant *Helicoverpa armigera* (lepidoptera: Noctuidae). *Pest management science*, 65, 353–357.