FERNANDA FREITAS SOUSA

CALLING BEHAVIOR AND OVARIAN DEVELOPMENT OF *Spodoptera frugiperda* POPULATIONS RESISTANT TO BT TOXINS

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*.

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APROVADA: 17 de fevereiro de 2016

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Eraldo Rodrigues de Lima
(co-orientador)

Eugênio Eduardo de Oliveira
(orientador)

À memória de meus avós Aparecida, Waldemar e José Maurício,
com amor, todo amor,
dedico.

Aos meus pais, por todo apoio desde a minha mais tenra infância,
ofereço

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(Madre Teresa de Calcuta)

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BIOGRAFIA

FERNANDA FREITAS SOUSA, filha de Fernando Luis de Sousa e Maria Helena Freitas de Sousa nasceu em Cataguases, Minas Gerais, em 06 de novembro de 1981.

Foi criada em Além Paraíba, onde estudou no colégio dos Santos Anjos durante quase todo seu ensino fundamental.

Cursou o Ensino Médio no COLUNI, o Colégio de Aplicação da UFV na turma de 1997-1999.

Em 2001 iniciou o curso Ciências Biológicas na Universidade Federal de Juiz de Fora, sendo que transferiu-se em 2003 para a Universidade Federal de Viçosa.

Durante a graduação, estagiou no laboratório de Feromônios e Comportamento de Insetos sob orientação do professor Eraldo Rodrigues de Lima. Graduou-se Bacharel em março de 2007 e Licenciou-se em agosto do mesmo ano.

Em agosto de 2007 começou a ministrar aulas de Biologia no cursinho pré-vestibular Vivenciar, em Coimbra-MG, função esta que manteve até dezembro de 2010.

Em 2008 iniciou estágio no Laboratório de Manejo integrado de Pragas, sob supervisão do professor Marcelo Coutinho Picanço, onde desenvolveu trabalhos com manejo integrado de pragas de hortaliças, grandes culturas, fruteiras e ornamentais. Possuiu bolsa de Apoio Técnico de agosto de 2008 a julho de 2009.

Em agosto de 2009 ingressou no curso de Mestrado em Entomologia na UFV, curso que concluiu em julho de 2011 sob orientação do professor Eliseu José Guedes Pereira.

Possuiu bolsa de DTI com a Professora Madelaine Venzon de setembro de 2011 a março de 2012 na EPAMIG.

Em 2012 iniciou o Doutorado em Entomologia, curso o qual está concluindo em fevereiro de 2016.

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RESUMO

SOUSA, Fernanda Freitas, D.Sc., Universidade Federal de Viçosa, fevereiro de 2016. **Comportamento de chamamento e desenvolvimento ovariano de populações de *Spodoptera frugiperda* resistentes a toxinas de Bt.** Orientador: Eugênio Eduardo de Oliveira. Coorientador: Eraldo Rodrigues de Lima.

Culturas transgênicas têm sido adotadas para controle de pragas desde 1996 e entre os benefícios do uso desta tecnologia estão o controle efetivo das pragas alvo e o baixo risco para organismos não-alvo. Entretanto, a evolução da resistência é uma grande ameaça para a continuidade a longo prazo de tal tecnologia. Insetos resistentes a tecnologia Bt podem manifestar custo fisiológico o que ajuda a reverter a ocorrência da resistência de culturas Bt quando refúgio está presente. *Spodoptera frugiperda* é a praga mais destrutiva de milho em regiões tropicais das Américas e já foram documentados casos de resistência a eventos de milho Bt que produzem toxina Cry1F. Já foi observado custo adaptativo associado à resistência Cry1Fa em características do ciclo de vida, porém não foi observado custo em parâmetros reprodutivos. Por outro lado, observou-se custo associado à resistência ao evento piramidado de milho Bt em características de ciclo de vida e parâmetros de fertilidade. No entanto, características comportamentais de reprodução (e.g, chamamento) têm sido pouco estudadas como possíveis características de custo. Assim, este trabalho foi realizado com o objetivo de estudar o comportamento de chamamento e comparar o desenvolvimento dos ovários de fêmeas de *Spodoptera frugiperda* suscetíveis e resistentes ao milho transgênico expressando Cry1Fa e a outro evento que expressa toxinas Cry2Ab2 e Cry1A.105. As fêmeas resistentes à toxina Cry1Fa iniciaram o chamamento mais cedo, além de passarem mais tempo chamando e geralmente apresentarem maior número de acessos de chamamento e acessos mais duradouros que as fêmeas suscetíveis durante a escotofase. Não houve diferença entre o diâmetro dos oócitos basais entre as fêmeas resistentes e suscetíveis, mas as fêmeas resistentes apresentaram mais ovos maduros do que as suscetíveis na mesma idade após a emergência, além de apresentarem ovos maduros um dia antes das fêmeas suscetíveis. As fêmeas resistentes às toxinas Cry2Ab2 e Cry1A.105 não apresentaram diferença no tempo médio do início do chamamento. Apesar disso, as fêmeas resistentes

geralmente passam mais tempo chamando além de apresentar um maior número de acessos de chamamento durante a escotofase. Às 12h após a emergência, as fêmeas resistentes apresentam maior diâmetro dos oócitos basais quando comparado aos óocitos das suscetíveis, valor que se torna igual às 24h. No início da vida adulta, as fêmeas resistentes e suscetíveis não diferiram em termos da presença de número de ovos maduros, no entanto após 72 horas este número é maior nas fêmeas suscetíveis. Portanto, apesar de haver vantagens no chamamento, existe custo da resistência ao milho Bt piramidado expressando as toxinas Cry2Ab2 e Cry1A.105 na população de *S. frugiperda* testada em relação ao número de ovos maduros. No entanto, não houve custo da resistência à Cry1Fa na população de *S. frugiperda* testada, e as fêmeas resistentes apresentaram um maior desempenho que as suscetíveis.

ABSTRACT

SOUSA, Fernanda Freitas, D.Sc., Universidade Federal de Viçosa, February, 2016. **Calling behavior and ovarian development of *Spodoptera frugiperda* populations resistant to Bt toxins** Adviser: Eugênio Eduardo de Oliveira. Co-Adviser: Eraldo Rodrigues de Lima.

Transgenic crops have been adopted for pest control since 1996 and among the benefits of using this technology are the effective control of target pests and low risk to non-target organisms. However, the evolution of resistance is a major threat to the long-term efficacy of such technology. Insect resistant can express physiological cost, which helps reverse the occurrence of resistance to Bt crops when refuge is present. *Spodoptera frugiperda* is the most destructive pest of maize in tropical regions of the Americas and have been documented cases of resistance to Bt corn events that produce Cry1F toxin. It has already been observed fitness cost associated with resistance Cry1Fa in life cycle characteristics, but was not observed cost in reproductive parameters. Moreover, it was observed cost associated with resistance to Bt maize stacked event in the life cycle characteristics and fertility parameters. However, behavioral features (e.g., calling) have been little studied as possible characteristics of cost. This work was carried out to study the calling behavior and compare the ovarian development of *Spodoptera frugiperda* female susceptible and resistant to GM maize expressing Cry1Fa and other event that expresses toxins Cry2Ab2 and Cry1A.105. Resistant to first-generation event expressing Cry1Fa toxin females started calling earlier, spend more time calling and generally have higher number and most enduring calling bouts than susceptible female during scotophase. There was no difference between the of the basal oocytes width between resistant and susceptible females, but resistant females showed more mature eggs than susceptible at the same age after emergence, besides presenting mature eggs one day before. Resistant females to second-generation Bt event expressing Cry2Ab2 and Cry1A.105 toxins showed no difference in the mean onset time of calling. Nevertheless, the resistant females generally spend more time calling in addition to present a larger number of call bouts during the scotophase. At 12 hours after emergence, resistant females have larger basal oocytes width compared to susceptible, and this value becomes equal at 24 hours after emergence. In early adulthood, the resistant and susceptible females did not differ in number of mature eggs, but after 72 hours this number is higher in susceptible females. Therefore, although there are advantages in the calling behavior, there is a cost in the number of mature eggs associated with Bt stacked expressing Cry2Ab2 and Cry1A.105 toxins resistance in the population of *S. frugiperda* tested. However, no cost Cry1Fa resistance, and resistant females showed a higher performance than susceptible.

GENERAL INTRODUCTION

Spodoptera frugiperda (Lepidoptera: Noctuidae) (J. E. Smith, 1797) is a polyphagous species which attacks many economically important crops in several countries in the Neotropics (Luginbill, 1928; Sparks, 1979; Casmuz *et al.*, 2010). In Brazil, it can attack rice, cotton, soybeans, wheat, beans, peanuts, among others, and is considered one of the main pests of maize and it is reported that *S. frugiperda* reduction in the production of this crop may reach 34% and the losses could reach 500 million dollars annually (Waquil *et al.*, 2002; Cruz *et al.*, 2010).

Populations of the fall armyworm feeding on different plant species can be classified as two distinct host-associated strains according to their host preference: the corn strain feeds primarily on corn and a rice strain that feeds primarily on forage grasses and rice (Pashley, 1986; 1988). There are differences in susceptibility to insecticide (Pashley e Martin, 1987) and Bt toxin (Adamczyk Jr *et al.*, 1997) between the two fall armyworm host associated strains. So, maybe to an efficient resistance management it may be necessary to consider the fall armyworm strain. The control of *S frugiperda* is difficult because of resistance development to most of conventional chemical control tactics (Diez-Rodriguez e Omoto, 2001; Siebert *et al.*, 2008).

So, in 1996 it was introduced commercially in the US genetically modified maize expressing Cry1Ab toxin of *Bacillus thuringiensis* to control the main lepidopteran maize pests populations (Koziel *et al.*, 1993; Siegfried *et al.*, 2007). The development and use of transgenic crops expressing toxins from *Bacillus thuringiensis* represent one of the most significant changes in pest management practices since synthetic pesticides and have provided positive impacts on global agricultural production (Shelton *et al.*, 2002; Romeis *et al.*, 2008).

Among the advantages of using these transgenic Bt plants regarding the conventional insecticides is the high specificity of the insecticidal protein, causing minimal effect on non-target organisms, lower input in pesticide application costs, reduced environmental impact besides the easier use of this technology by farmers (Macintosh *et al.*, 1990; Noteborn *et al.*, 1996; Naranjo *et al.*, 2005; O'callaghan *et al.*, 2005; Romeis *et al.*, 2006).

In 2007, it was regulated in Brazil the procedures for commercial planting of cotton and maize expressing insecticidal proteins from *Bacillus thuringiensis* for caterpillar control in these cultures (Ctnbio, 2007). The first generation of Bt consists of transgenic plants expressing only one protein with insecticidal action. The first events released in Brazil were MON810 and BT11, both expressing Bt Cry1Ab toxin, and in 2009 was released the event expressing Cry1F, which was more effective in controlling *S. frugiperda* (Ctnbio, 2007; 2008a; 2008b; 2009). The positive impacts of using Bt crops have led to a high adoption of this technology, so the planted area has increased worldwide since its first use by US farmers in 1996 and this large-scale cultivation can be configured in an environment with high pressure selection for resistance (James, 2014).

One strategy used to prevent resistance development is the "high dose refuge". This strategy assumes that homozygous resistant individuals surviving in Bt crops will mate randomly with homozygous susceptible individuals originating from areas containing plant non-Bt (refuge). However, the success of this strategy depends on key factors as: the plant should produce toxin at doses sufficient to kill most heterozygous resistant insects; besides that, the initial frequency of resistance alleles should be low and resistance must be recessive and monogenic. In addition, there must be an abundant refuge area near to Bt crops and resistant individuals must present reduced fitness on Bt and non-Bt plants (Tabashnik *et al.*, 1994; Gould, 1998; Carriére e Tabashnik, 2001; Bourguet *et al.*, 2003).

The first Bt generation was a great success, although there are documented cases of resistance in the field, resulting in loss of susceptibility and pest control failures (Storer *et al.*, 2010; Dhurua e Gujar, 2011; Tabashnik *et al.*, 2013; Farias *et al.*, 2014; Huang *et al.*, 2014).

Then began using a second generation of Bt events, called pyramided events, intending to maximize pest control and delay resistance (Tabashnik *et al.*, 2013). In these new events approved in Brazil in 2009 are expressed two or more Bt toxins, with different modes of action, aimed control of a same group of target pests and must be no cross-resistance between the toxins expressed (Roush, 1997; 1998; Zhao *et al.*, 2003; Ctnbio, 2009; Tabashnik *et al.*, 2013). This strategy has provided a new approach to delay pest resistance, for the development of resistance to a single toxin can be faster than to the multiple toxins (Zhao *et al.*, 2003).

Efficience of pyramided events can decrease if this strategy is adopted after the selection of resistance, but in some cases it can be used to remedy resistance problems already documented for any of the toxins from the event (Tabashnik *et al.*, 2013). For greater effectiveness the pyramided events should not be planted next to transgenic crops that express one of its toxins (Zhao *et al.*, 2005).

Resistance studies are important not only for the integrated management programs of pests, but also as models of evolution newly adapted phenotypes and their physiological and genetic changes associated, among them the existence of cost associated with resistance (Coustau *et al.*, 2000; Raymond *et al.*, 2001). Resistance to *B. thuringiensis* toxin is a particular case of resistance, because besides the direct losses associated with resistance, can also prevent the use of transgenic plants expressing such toxins. Insect resistant Bt toxins can show physiological costs and individuals who have resistance alleles have lower performance in the absence of Bt toxin than the susceptible (Coustau *et al.*, 2000).

Presence of cost associated with resistance may help reverse or prevent the spread of resistance alleles when refuges are present because it can reduce the coefficient of selection favoring susceptible individuals instead the resistant ones (Gould, 1998; Carriére e Tabashnik, 2001; Carrière *et al.*, 2010). The existence of costs is commonly assessed by comparing life cycle characteristics, such as survival, development and fertility parameters (Gassmann *et al.*, 2009; Crespo *et al.*, 2010; Pereira *et al.*, 2011; Jakka *et al.*, 2014; Vélez *et al.*, 2014), although cost on aspects of development and reproductive behavior have been little studied.

In Lepidoptera, the partners recognition for mating occurs through the perception of chemical signals, and it is the most ancient form of communication (Bradbury e Vehrencamp, 1998; Wyatt, 2003). Among the advantages of this kind of communication are: chemical signals are transmitted over long distances, crossing obstacles and are an efficient form of communication in the darkness (Svensson, 1996). Chemical communication by pheromones are important in the species-specific mate recognition and in the majority of moth species, females emit the signals and the males are attracted by these signals (Tamaki, 1985; Svensson, 1996).

As defined by Wyatt (2003), "pheromones are a subclass of semiochemicals, which are used for communication within the same species (intraspecific communication) and are widely used by a variety of organisms". The period in which matings occur follow a daily rhythm in many animals, so females release the pheromone at a certain time of day and males respond to this stimulus at same time (Silvegren *et al.*, 2005). Therefore, the sexual behaviors occur during a narrow window of time, the so-called “circadian gate” (Pittendrigh e Skopik, 1970). The mating rhythmicity probably portrays selective pressures on the behaviors to occur at a propitious time

So, females attract the male by emitting pheromones and this is called calling behavior. It has been observed that calling behaviour can be affected by endogenous and exogenous factors such as photoperiod and temperature (Delisle e Mcneil, 1987a; 1987b; Gómez e Rojas, 2006; Mozūraitis e Būda, 2006; Soufbaf *et al.*, 2013) body weight and feeding (Sadek, 2012) detection of conspecific pheromone (Lim *et al.*, 2007) host plant (Mcneil e Delisle, 1989; Sadek e Anderson, 2007) and also by resistance to insecticides (Zhao *et al.*, 2009).

There is a relationship between calling and ovarian development of Lepidoptera (Cusson e Mcneil, 1989a) due to the fact that the juvenile hormone (JH) to be associated with ovarian development as well as play an important role in the production of the sex pheromone and calling behavior expression in virgin females(Cusson e Mcneil, 1989b; Mcneil *et al.*, 1996). It has been reported that a minimum ovarian development is required to initiate synthesis and emission of pheromone (Cusson e Mcneil, 1989a; Cusson *et al.*, 1994).

The presence of cost associated on these parameters can influence the reproductive success and alter the reproductive window. The changes in reproductive window can influence randomness in the choice and therefore, favor assortative mating, spoiling one of the basic assumptions of resistance management, the randomness of mating between resistant and susceptible individuals from the refuge and it could affect the selection of resistance.

Therefore, the objectives of this study are to verify the presence of possible costs in ovarian development and behavioral aspects of *Spodoptera frugiperda* female call associated with resistance to the corn first generation Bt (expressing Bt Cry1Fa toxin) and second Bt generation (expressing Cry2Ab2 and Cry1A.105 toxins).

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CHAPTER 1

Possible costs in the calling behaviour and ovarian development associated with Cry1Fa resistance in *Spodoptera frugiperda*

INTRODUCTION

Spodoptera frugiperda (Lepidoptera: Noctuidae) (J. E. Smith, 1797), known as fall armyworm, is the most destructive pest of maize in Brazil and another tropical regions of the Americas (Buntin, 1986; Cruz et al., 1999). The control of this pest using most of conventional chemical control tactics is not good enough, because of resistance development (Diez-Rodriguez e Omoto, 2001; Siebert *et al.*, 2008). So, as an alternative to control this pest, transgenic maize (Zea mays L.) expressing the Cry1F protein from Bt (TC1507 event) (and another toxins) have been used since 2009 in Brazil and was quickly adopted in large scale (Ctnbio, 2009).

However, the large-scale cultivation of Bt plants, in addition to agricultural practices adopted, sets a strong selection pressure for resistance in pest populations and resistance is a major threat to long-term efficacy of Bt toxins (Tabashnik *et al.*, 1994; Gould, 1998; Ferré *et al.*, 2008; James, 2014).

It has already been reported cases of resistance and control failure of *S. frugiperda* in Brazil, USA and Puerto Rico and this evolution of resistance may be favored by characteristics aspects of this specie as multivoltinism and high reproductive rate (Storer *et al.*, 2010; Storer *et al.*, 2012; Farias *et al.*, 2014; Huang *et al.*, 2014).

Resistant insects may present physiological costs and thus, it may help reverting or preventing the spread of resistance alleles when refuge areas are present because it can reduce the coefficient of selection, favoring susceptible individuals rather than resistant ones (Gould, 1998; Carriére e Tabashnik, 2001; Carrière *et al.*, 2010). Some studies haven't shown fitness costs(Jakka *et al.*, 2014; Vélez *et al.*, 2014; Santos-Amaya, Tavares, Rodrigues*, et al.*, 2016) however Dangal e Huang (2015) reported fitness cost on developmental aspects associated with the Cry1F resistance in *S. frugiperda* populations.

Fitness costs is commonly evaluated by comparing life-cycle traits parameters (Gassmann *et al.*, 2009; Crespo *et al.*, 2010; Pereira *et al.*, 2011; Jakka *et al.*, 2014; Vélez *et al.*, 2014), however the costs in behavioral characteristics of reproduction have been little studied. Therefore, the aim of this work was to study the calling behaviour and compare the ovarian development of *Spodoptera frugiperda* females susceptible and resistant to first generation transgenic maize producing Cry1Fa toxin to assess possible time delay in the ovarian development and behavioral changes in response to resistance.

METHODS

Insects

Two population of *Spodoptera frugiperda*, one resistant to an event expressing Cry1Fa Bt toxin (MTH) and its susceptible isoline (MT) were used. Resistant individuals were obtained from laboratory selected colonies (Santos-Amaya, Tavares, Monteiro*, et al.*, 2016) and all individuals were reared on an artificial diet modified from Greene *et al.* (1976), at controlled conditions (27 ± 2 °C, 70 ± 15 % RU and 14L:10D photoperiod) up to pupation.

Pupae were sexed (Capinera, 2000) and the female ones were collected and daily observed to verify emergence. When the females emerge, they were placed at individual cages made by an acrylic recipient covered at one side with voil and fed by 10% sugar 1% ascorbic acid solution. The feed was exchanged every two days

The experiments were carried out under local conditions.

Calling behavior

To record the calling behaviour, newly emerged female were observed for six calling nights. The first night of calling behaviour was the one that the female called for the first time. Calling behaviour was recognized by the lifted wings, curved abdomen and exposed pheromone gland.

Females were observed every 10 minutes during the scotophase using a flashlight covered with two red cellophane layers. If female called just one of two observations, they were considered calling for ten minutes and if they were calling during two or more sequential observations, we considered they have been calling for 20 minutes or more.

The observed parameters were: the mean time onset calling (MTOC), number of calling bouts (NCB), the mean time of calling bouts (MTCB) and absolute time that female spent calling (TSC).

To be sure about results we made two trials. At the first trial, for each population, 80 new-emerged females were observed until the 6th calling day, and at second trial 70 females were observed.

All calling statistical analysis was done using R (**R version 3.2.1, R Development Core Team**). To account for pseudorepetition (repeated females in days), we are using ’mixed model’. Since calling days are not independent from each other, residuals are autocorrelated. To account for this non-independence, data were subjected to linear mixed-effects modelling, using the *lmer* function from the lme4 package. Parameters were estimated using the maximum likelihood (ML) method. To assess the validity of the mixed effects analyses, likelihood ratio tests compared the models with fixed effects to the null models with only the random effects.

Initially, the full model was constructed and then simplified with a model simplification in which the explanatory variables are sequentially removed. A variable was retained in the minimal adequate model only if it caused a significant increase in deviance when it was removed from the current model. Further model simplification was done through contrast analyses, lumping treatment levels together, and making single degree of freedom comparisons (Crawley, 2002). Models were compared with analyses of variance between models, using an χ^2 test of the maximum likelihood ratio.

Ovarian development

To evaluate ovarian development, ten (10) virgin females from both populations were frozen at 12h, 24h, 48h, 72h and 96 hours after emergence. The females were posteriorly dissected and one ovary was removed and stained in Grenacher Borax Carmine (Humason, 1972) for 30 minutes. After that, the ovary was washed two times in 70% ethanol.

Chorionated eggs present in the ovaries and lateral oviducts were counted and duplicate and added added to those present in the common oviducts to represent the real egg amount per female. To estimate oocyte diameter, it was took a picture and the penultimate basal oocyte of two ovarioles had its width measured using the software Leica Application Suite 4.5.0.

The statistical analysis were done using R (**R version 3.2.1, R Development Core Team**) following the method of Crawley (2005a). The effect of either population (MTH vs MT) or hours after emergency, on the diameter of basal oocyte and the number of eggs by female was tested with a Generalized Linear Modelling (GLM) under quasipoisson distribution. In all analyses, a full model was fitted, from which terms were deleted in a stepwise fashion to obtain the minimal adequate model (MAM). Signifcance ($P<0.05$) was assessed by testing the change in deviance after the removal of a term from the model. Differences among treatment levels were examined by contrast analysis.. Models were checked by residual analyses, and eventually corrected for overdispersion as well as for the correctness of the assumed distribution (Crawley, 2005a).

RESULTS

Calling behavior

Resistant females initiate calling earlier at scotophase than susceptible ones in trial 1 ($\chi^2= 90.308$; df = 1; $P < 0.001$) (Fig 1 A) and 2 ($\chi^2 = 34.382$; df = 1; $P < 0.001$) (Fig 1 B) on successive calling days. Furthermore, in both MTH and Lab females the mean time onset calling advanced on successive calling days ($\chi^2 = 173.08$; df = 5; $P < 0.001$; $\chi^2 = 236.037$; df = 5; $P < 0.001$) regardless of trials (Fig 1 A, B), so older females begin to call early than the younger. But the interaction between treatment and calling days was not significant ($P > 0.05$) (Fig 1A and 1B).

In addition, resistant females spent more time calling than susceptible in both trials ($\chi^2 = 101.89$; df = 1; $P < 0.001$; $\chi^2 = 51.893$; df = 1; $P < 0.001$) (Fig. 2A, B). Another factor that affects the time spent calling is the calling day, and almost every day there was an increase in the time that female spent calling during scotophase ($\chi^2 = 325.96$; df = 5; $P < 0.001$; $\chi^2 = 333.26$; df = 5; $P < 0.001$) (Fig.

2A and B). As observed before in MTOC, the interaction between treatment and calling days was not significant ($P > 0.05$) in this parameter.

The number of calling bouts increased on successive calling days in Trial 1 ($\chi^2 = 356.31$; df = 5; $P < 0.001$) and Trial 2 ($\chi^2 = 222.79$; df = 5; $P < 0.001$) (Fig 3A and B). Moreover, resistant females present more bouts during scotophase than susceptible in both Trial 1 ($\chi^2 = 35.484$; df = 1; $P < 0.001$) and Trial 2 ($\chi^2 = 15.174$; df = 1; $P < 0.001$) (Fig 3A e 3B). Following the same trend of the previous parameters, the interaction between treatment and calling days was not significant ($p > 0.05$) in the number of calling bouts.

In addition to a greater number of bouts, resistant females have longer lasting bouts than susceptible ones in both Trial 1 ($\chi^2 = 84.484$; df = 1; $P < 0.001$) and Trial 2 ($\chi^2 = 42.888$; df = 1; $P < 0.001$) (Fig 4A and B). Beyond that, the duration of bouts tend to increase with the age of the females ($\chi^2 = 91.275$; df = 5; $P < 0.001$; $\chi^2 = 132.12$; df = 5; $P < 0.001$), so, older females present more longstanding bouts than the younger ones. Contrary to what occurred in all previous parameters, the interaction between resistance and calling days was significant for the mean time of calling bouts in both trials ($\chi^2 = 11.679$; df = 5; $P = 0.03946$; $\chi^2 = 11.226$; df = 5; $P = 0.04708$) (Fig 4A and B).

Ovarian development

There was no difference of the basal oocyte width between MTH and MT ($F_{1;40} = 0.83$ $P > 0.05$) but the width increases with hour after emergency ($F_{2;38} = 4.16$ $P = 0.0237$) in both resistant and susceptible females(Fig 5 B). The interaction between these two factors was also non-significant ($F_{2;36} = 0.35$; $P = 0.71$).

Resistant females present more mature eggs than susceptible ones at the same time after emergence ($F_{1;98}$ = 13.909 $P < 0.001$), besides having mature eggs one day before the susceptible females (Fig 5 D). Time after emergence was significant too ($F_{4;94}$ = 54.376 $P < 0.001$), and the contrast analysis shows that all levels (12h, 24h, 48h, 72h and 96h) are different, so the number of mature eggs increased with the time after emergence. There is no significant effect of the interaction of these two factors too ($F_{4;90}$ = 2.10 $P > 0.05$).

DISCUSSION

Here, it was assessed the possible fitness costs in ovarian development and calling behaviour of *S. frugiperda* females with the same genetic background but different susceptibility to Cry1Fa Bt toxin. It is important study fitness cost of resistance because it is a substantial information to effective resistance management (Carrière *et al.*, 2010). Some fitness cost of Bt resistance studies evaluating parameters of development and fertility table have already been made with *S. frugiperda* and another Lepidoptera (Crespo *et al.*, 2010; Pereira *et al.*, 2011; Jakka *et al.*, 2014; Vélez *et al.*, 2014), but there few works about behavioral aspects of reproduction.

In previous studies it was observed presence (Dangal e Huang, 2015) or absence of fitness cost associated with Cry1Fa resistance in *S. frugiperda* populations (Jakka *et al.*, 2014; Vélez *et al.*, 2014; Horikoshi *et al.*, 2015). In a recent study Santos-Amaya, Tavares, Rodrigues*, et al.* (2016) observed no fitness cost of Cry1Fa resistance in development and population growth in the same *S. frugiperda* strains used at this work.

Resistant females called earlier in the scotophase, besides spent more time calling and generally have higher numbers and most enduring calling bouts. These results are the opposite of that found for *Helicoverpa armigera* resistant to the toxin Cry1Ac, when it was verified fitness cost in calling behavior and ovarian development (Zhao *et al.*, 2009; Zhang *et al.*, 2014). In both studies mentioned above there was a delay in observed behaviors of resistant females when compared with the susceptible.

Females of *S. frugiperda* emerge before the males (Cruz, 1995) and when male are able to mate the females are already receptive to mating. Thus, initiate calling early as well as call for longer time can be favorable because increase the reproductive window, and it may influence the randomness of mating.

Sexual communication is effective if there is synchrony between female calling and male response. However, a large temporal response window of male could be advantageous in locating both early and late calling females (Linn Jr, 1997).

On the other hand, older females also called earlier than younger, as well as present longer time spent calling and greater number of calling bouts. It can probably increase the chance of mating, because older females can release suboptimal amounts of pheromone (Kanno, 1979; Webster e Cardé, 1982) and so, calling earlyer they avoid competing with younger females.

Recognizing potential partners is essential for mating success, so the earlier onset time of calling as well as longer time spent calling can increase the reproductive window in the resistant female, which could make these females more attractive so it can enhance the mating frequency of the resistant female. Moreover, the higher mating frequency may increase the frequency of resistant alleles (Sisterson *et al.*, 2004; Téllez-Rodríguez *et al.*, 2014).

The presence of mature eggs was not determinant to calling occur because female start calling before reaching sexual maturity. In a previous study, it was observed that a minimum ovarian development is required to call (Cusson e Mcneil, 1989a) and these females begin to call 24 hour after emergence, when the basal oocyte reaches a minimum value (about 0.33mm).

In addition to differences in calling behaviour, resistant females present more mature eggs than susceptible ones at the same time after emergence besides having mature eggs one day before the susceptible females. However, no significant differences were observed in fecundity and progeny production between resistant and susceptible strains and crosses (Santos-Amaya, Tavares, Rodrigues*, et al.*, 2016).

High dose/refuge is used in resistance management of pests to Bt toxins and to the efficacy of this tactic is necessary random mating between individuals surviving in culture and individuals susceptible presents at the refuge (Roush, 1997; Gould, 1998).

Furthermore, it is important to assess whether the males exhibit preference among females susceptible and resistant. If there is difference in attractiveness/preference, it can favor assortative mating.

So, for a better understanding of changes in reproductive behaviour caused by resistance and how this can influence the resistance management further studies need to be performed, among them studies focused on the blend of pheromones and beyond the acceptance of females to males.

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FIGURES

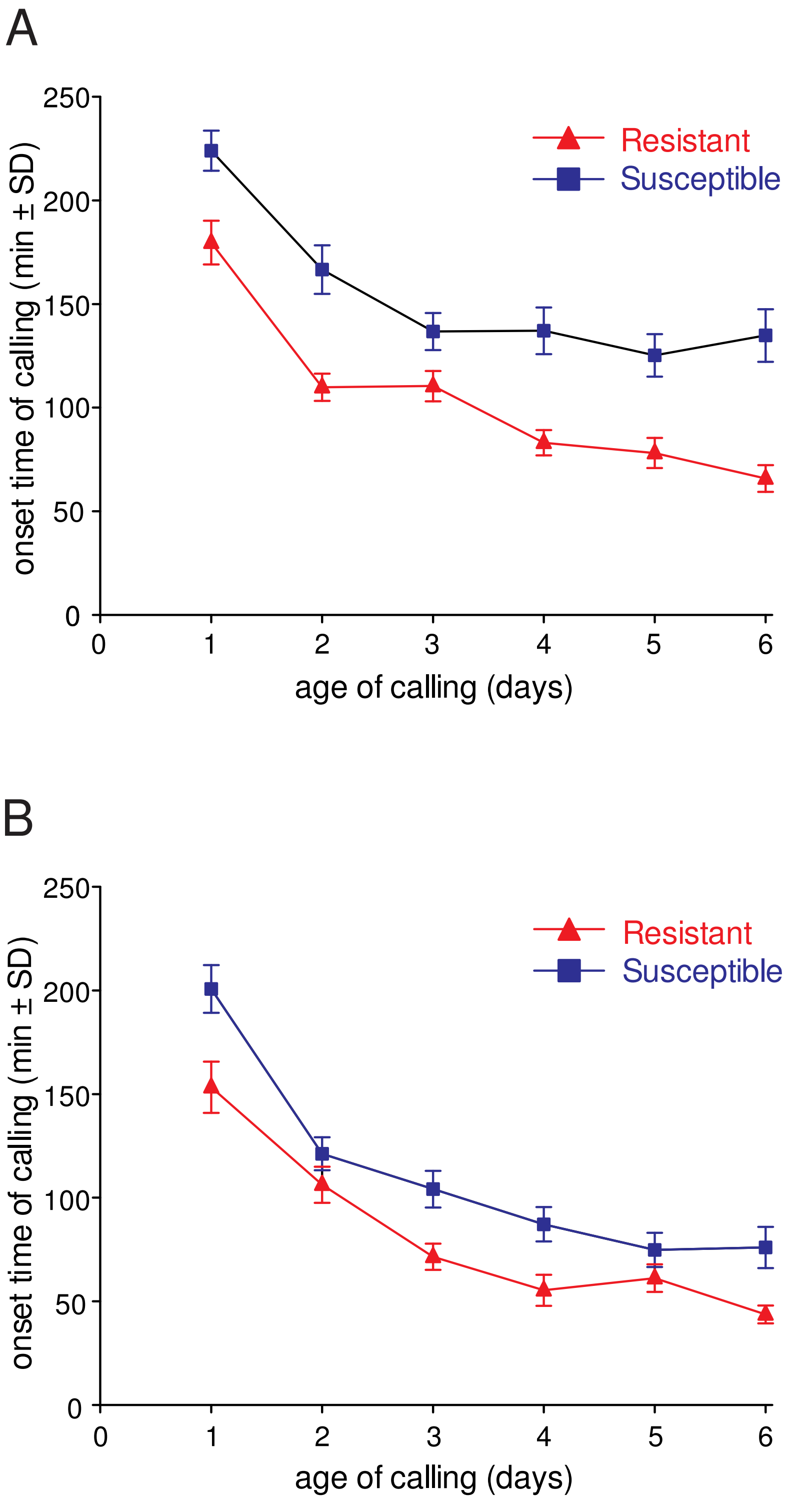

Figure 1. Mean time of onset calling (X ± SD) (in minutes after scotophase) on subsequent nights of calling of susceptible and resistant to Cry1Fa Bt toxin populations of *Spodoptera frugiperda*. A) Trial1; B) Trial2.

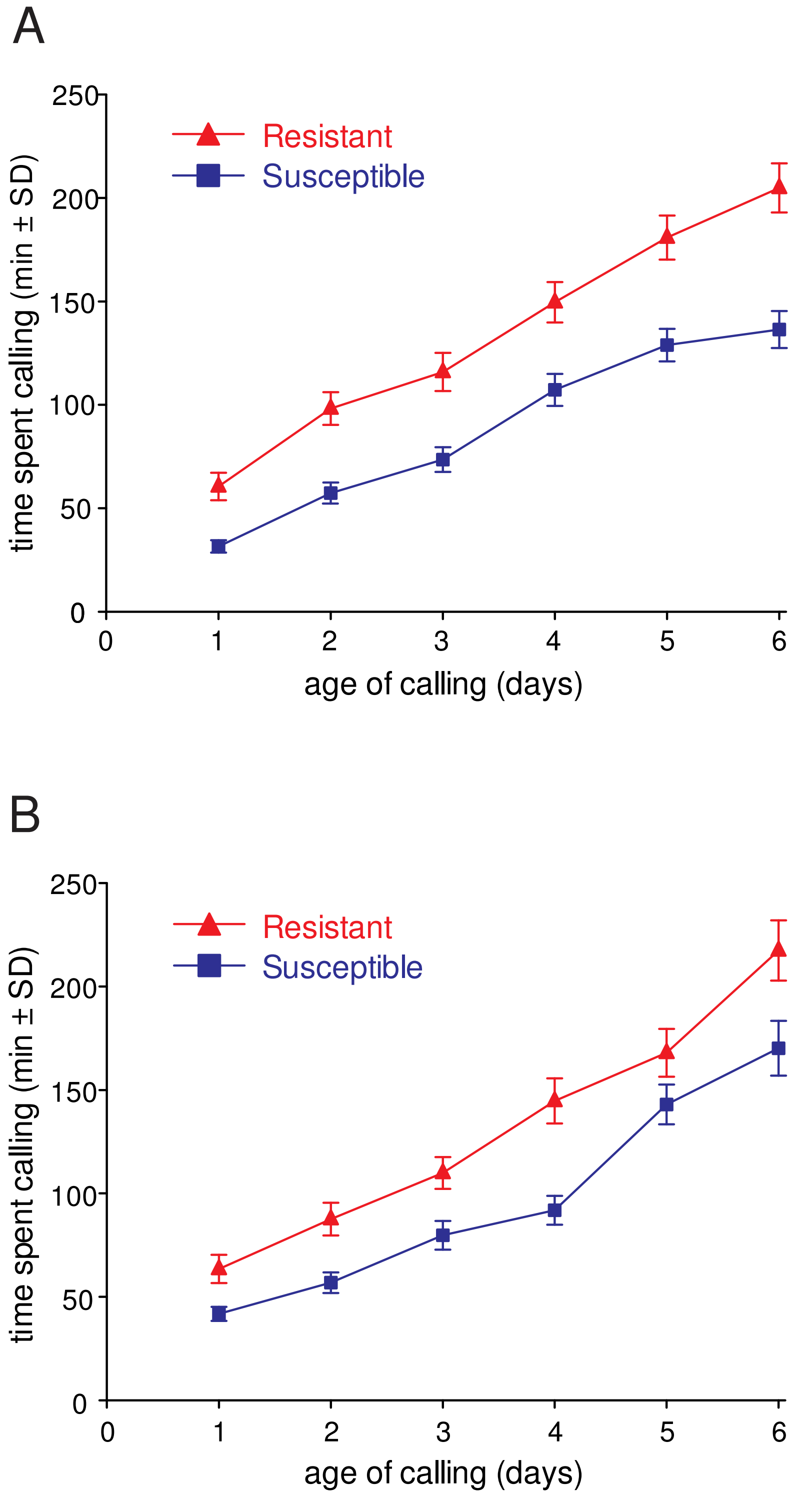

Figure 2. Time spent calling (X ± SD) (in minutes after scotophase) on subsequent nights of calling of susceptible and resistant to Cry1Fa Bt toxin populations of *Spodoptera frugiperda*. A) Trial1; B) Trial2.

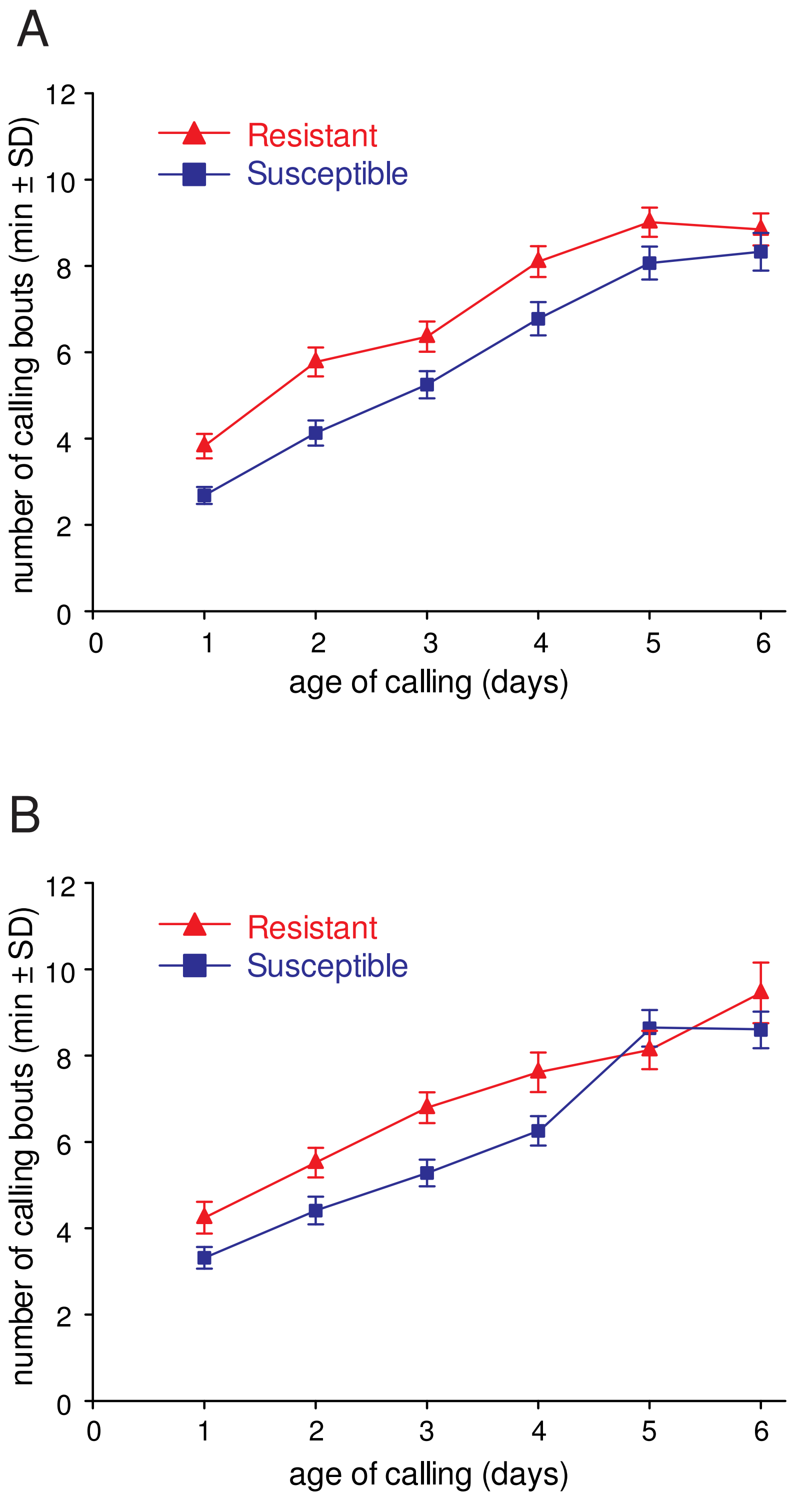

Figure 3. Number of calling bouts (X ± SD) on subsequent nights of calling of susceptible and resistant to Cry1Fa Bt toxin populations of *Spodoptera frugiperda*. A) Trial1; B) Trial2.

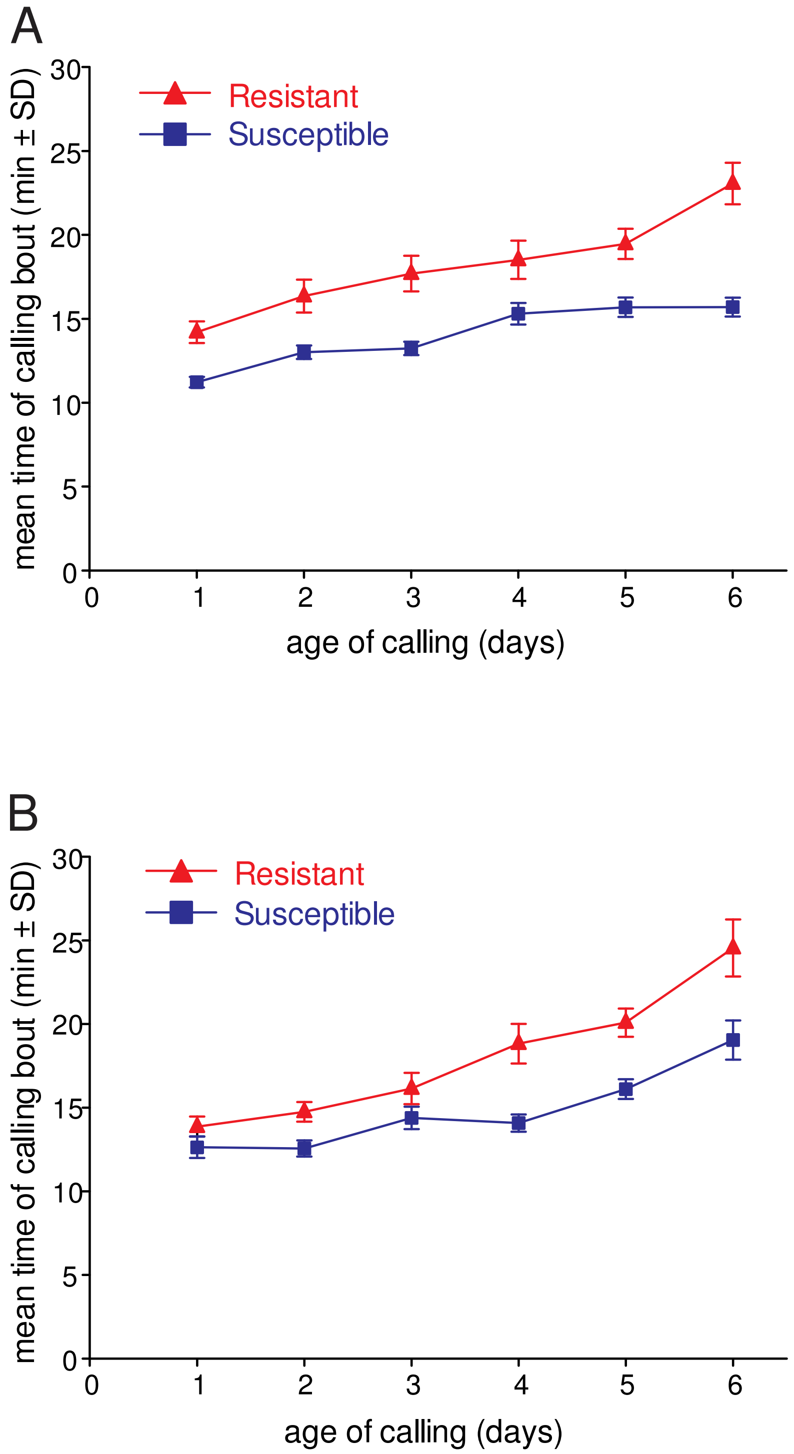

Figure 4. Mean time of calling bouts (X ± SD) on subsequent nights of calling of susceptible and resistant to Cry1Fa Bt toxin populations of *Spodoptera frugiperda*. A) Trial1; B) Trial2.

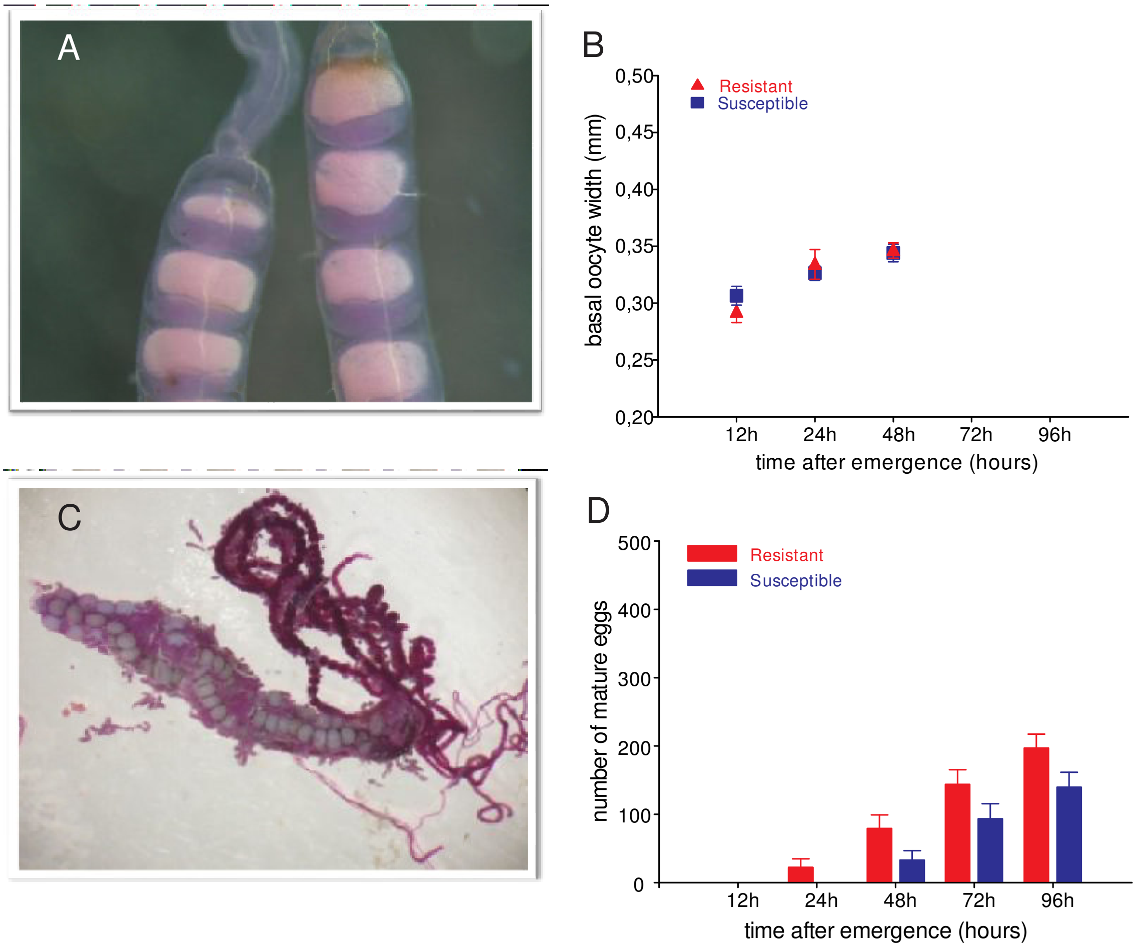

Figure 5. Ovarian development of susceptible and resistant *Spodoptera frugiperda* female. A) oocyte B) basal oocyte width (X ± SD) of resistant to Cry1Fa (red triangles) and susceptible (blue squares) female C) ovary with mature eggs D) number of mature eggs (X ± SD) of resistant to Cry1Fa (red bars) and susceptible (blue bars) female.

CHAPTER 2

Is there a cost of resistance in the calling behavior and ovarian development of *Spodoptera frugiperda* resistant to pyramided Bt maize expressing Cry2Ab2 and Cry1A.105 toxin?

Introduction

Transgenic crops have been used to pest control since 1996 (James, 2014). It represents one of the most significant changes in pest management practices since synthetic pesticides and is the main tactic of pest control worldwide, presenting many advantages besides providing positive impacts on agricultural production (Shelton *et al.*, 2002; Huang *et al.*, 2005; Cattaneo *et al.*, 2006; Marvier *et al.*, 2007; Romeis *et al.*, 2008; Carrière *et al.*, 2010; Hutchison *et al.*, 2010). However, the long-term efficacy of Bt toxins is threated by selection of pest resistance (Tabashnik *et al.*, 1994; Gould, 1998).

Although the first generation of Bt cultivars expressing only Bt toxin has been fairly successful, it is already been observed that some pests populations already developed resistance to Bt crops, resulting in loss of susceptibility and pest control failures (Janmaat e Myers, 2003; Van Rensburg, 2007; Storer *et al.*, 2010; Dhurua e Gujar, 2011; Tabashnik *et al.*, 2013; Farias *et al.*, 2014).

Six years after commercialization of first generation Bt crops a second generation of transgenic plants, became available in the United States (Tabashnik *et al.*, 2009). This plants, so called pyramided, express more than one Bt protein that are active against the same pest have been used as a strategy to retard the development of resistance and obtain a more effective pest control (Sanahuja *et al.*, 2011; Tabashnik *et al.*, 2013). Pyramided crops may present a wide range of pests control, increase the effectiveness of control and decrease damages to crops (Adamczyk Jr e Gore, 2004) and its adoption crops increased rapidly and tend to increase more in the future (Carrière *et al.*, 2015).

It is expected that it can help to delay pest resistance most effectively than first generation Bt plants if the toxins does not cause cross-resistance, frequency of the alleles that confer resistance is low, resistance is recessive and incomplete (Zhao *et al.*, 2005; Gassmann *et al.*, 2009; Carrière *et al.*, 2010; Tabashnik *et al.*, 2013; Carrière *et al.*, 2015). Despite the inheritance of resistance is more recessive and the increase of abundance of non-Bt host plant refuges in pyramided Bt events (Tabashnik *et al.*, 2009) resistance can be selected and reduce the effectiveness of these events in pest control.

However, fitness costs associated with resistance may be important to delay or prevent the spread of alleles conferring resistance to Bt crops when refuges of non-Bt host plants are present because it can reduce the coefficient of selection, favoring susceptible individuals regarding resistant ones (Gould, 1998; Carriére e Tabashnik, 2001; Gassmann *et al.*, 2009; Carrière *et al.*, 2010). The existence of fitness costs is commonly evaluated by comparing life-cycle traits and fertility parameters (Gassmann *et al.*, 2009; Crespo *et al.*, 2010; Pereira *et al.*, 2011; Jakka *et al.*, 2014; Vélez *et al.*, 2014; Santos-Amaya, Tavares, Rodrigues*, et al.*, 2016) , but other important aspects as behavioral characteristics of reproduction have been neglected.

It is already been reported that calling behavior is affected by several exogenous and endogenous factors such as temperature and photoperiod (Delisle e Mcneil, 1987a; 1987b; Gómez e Rojas, 2006; Mozūraitis e Būda, 2006; Soufbaf *et al.*, 2013), feeding and body weight (Sadek, 2012), detection of conspecific pheromone (Lim *et al.*, 2007), host plant (Mcneil e Delisle, 1989; Sadek e Anderson, 2007) and also by resistance (Zhao *et al.*, 2009).

Spodoptera frugiperda (Lepidoptera: Noctuidae) (J. E. Smith, 1797) is the main pest of maize in tropical regions of the Americas and can cause significant losses in corn yield (Buntin, 1986; Cruz, 1995). A laboratory selected population of *S. frugiperda* resistant to a pyramided event (express Cry1A.105 and Cry2Ab2 Bt toxin) presents fitness costs on survival, developmental time, larvae and pupae biomass and reproductive aspects (Santos-Amaya *et al.*, In preparation).

There is a relation between calling behavior and ovarian development in Lepidoptera (Cusson e Mcneil, 1989a). It is important to check the presence of cost associates to resistance to Bt in these parameters because it may influence the randomness of choice and mating success, and compromise the management of resistance.

So, the aim of this work was to study the calling behaviour and compare the ovarian development of *Spodoptera frugiperda* females susceptible and resistant to a transgenic pyramided maize (expressing Cry2Ab2 and Cry1A.105 toxin) to assess possible fitness costs associated with resistance.

METHODS

Insects

Two population of *Spodoptera frugiperda*, one resistant to an event expressing Cry1A.105 + Cry2Ab Bt toxins (Bahia-Bt) and its susceptible isoline (Bahia-Cv) were used. All individuals were obtained from laboratory selected colonies (Santos-Amaya *et al.*, 2015) and reared on an artificial diet modified from Greene *et al.* (1976), at controlled conditions (27 ± 2 °C, 70 ± 15 % RU and 14L:10D photoperiod) up to pupation.

Pupae were sexed (Capinera, 2000) and the female ones were collected and daily observed to verify emergence. When the females emerge, they were placed at individual cages made by an acrylic recipient covered at one side with voil and fed by 10% sugar 1% ascorbic acid solution. The feed was exchanged every two days

The experiments were carried out under local conditions.

Calling behavior

To record the calling behaviour, 80 newly emerged female were observed for six calling nights. The first night of calling behaviour was the one that the female called for the first time. Calling behaviour was recognized by the lifted wings, curved abdomen and exposed pheromone gland.

Females were observed every 10 minutes during the scotophase using a flashlight covered with a two red cellophane layers. If female called just one of two observations, they were considered calling for ten minutes and if they were calling during two or more sequential observations, we considered they have been calling for 20 minutes or more.

The observed parameters were: the mean time onset calling (MTOC), number of calling bouts (NCB), the mean time of calling bouts (MTCB) and absolute time that female spent calling (TSC).

All calling statistical analysis was done using R (**R version 3.2.1, R Development Core Team**). To account for pseudorepetition (repeated females in days), we are using ’mixed model’. Since calling days are not independent from each other, residuals are autocorrelated. Parameters were estimated using the maximum likelihood (ML) method. To assess the validity of the mixed effects analyses, likelihood ratio tests compared the models with fixed effects to the null models with only the random effects.

Initially, the full model was constructed and then simplified with a model simplification in which the explanatory variables are sequentially removed. A variable was retained in the minimal adequate model only if it caused a significant increase in deviance when it was removed from the current model. Further model simplification was done through contrast analyses, lumping treatment levels together, and making single degree of freedom comparisons (Crawley, 2002). Models were compared with analyses of variance between models, using an χ^2 test of the maximum likelihood ratio.

Ovarian development

To evaluate ovarian development, ten (10) virgin females from both populations were frozen at 12h, 24h, 48h, 72h and 96 hours after emergence. The females were posteriorly dissected and one ovary was removed and stained in Grenacher Borax Carmine (Humason, 1972) for 30 minutes. After that, the ovary was washed two times in 70% ethanol.

Chorionated eggs present in the ovaries and lateral oviducts were counted and duplicate and added to those present in the common oviducts to represent the real egg amount per female. To estimate oocyte diameter, it was took a picture and the penultimate basal oocyte of two ovarioles had its width measured using the software Leica Application Suite 4.5.0.

The statistical analysis were done using R (**R version 3.2.1, R Development Core Team**) following the method of Models were checked by residual analyses, and eventually corrected for overdispersion as well as for the correctness of the assumed distribution Crawley (2005b). The effect of either population (Bahia-Bt vs Bahia-Cv) or hours after emergency, on the diameter of basal oocyte and the number of eggs by female was tested with a Generalized Linear Modelling (GLM) under quasipoisson distribution. In all analyses, a full model was fitted, from which terms were deleted in a stepwise fashion to obtain the minimal adequate model (MAM). Signifcance (*P*<0.05) was assessed by testing the change in deviance after the removal of a term from the model. Differences among treatment levels were examined by contrast analysis .(Crawley, 2005b).

RESULTS

Calling behavior

There were no significant differences in the mean onset time of calling between resistant and susceptible females (χ^2 = 1.2835; df = 1; *P* = 0.2572). Furthermore, the mean time onset calling was affected by calling days (χ^2 = 266.32; df = 5; *P* < 0.001) (Fig 1), and at the first calling day females took considerably longer time after the beginning of scotophase to call than in the days that followed. Regarding the interaction between resistance and the calling days was not found significance (χ^2 = 11.776; df = 5; *P* < 0,05).

Resistant female spent more time calling than susceptible ones (χ^2 = 28.328; df = 1; *P* < 0.001) (Fig. 2). Another factor that affects the time spent calling is the calling day (χ^2 = 205.49; df = 5; *P* < 0.001), so almost every day there was an increase in the time female spent calling during scotophase (Fig. 2). The interaction between treatment and calling days was not significant (*P* > 0.05) in this parameter.

Resistant females present more bouts during scotophase than susceptible ones (χ^2 = 17.662; df = 1; *P* < 0.001) (Fig 3). The number of calling bouts was also affected by calling day (χ^2 = 167.23; df = 5; *P* < 0.001) and so, older females presents more bouts than younger ones (Fig 3), however the interaction between treatment and calling days was not significant ($P > 0.05$) in the number of calling bouts.

In addition to a greater number of bouts, resistant females have longer lasting bouts than susceptible ones ($\chi^2 = 6.4918$; df = 1; $P = 0.01084$) (Fig 4). Beyond that, the duration of bouts tend to increase with the age of the females ($\chi^2 = 63.173$; df = 5; $P < 0.001$), so, older females present more longstanding bouts than the younger ones. The interaction between resistance and calling days was non significant for the mean time of calling bouts ($\chi^2 = 8.2237$; df = 5; $P = 0.1443$) (Fig 4).

Ovarian development

There was no difference of the basal oocyte width between RR-BA and SS-BA ($F_{1;32} = 2.9881$ $P > 0.05$) but there are a significant effect of hour after emergency ($F_{1;31} = 25.682$; $P < 0.0001$). Susceptible females showed low basal diameter of the oocyte 12 hours after emergence, but this value equals to the resistant females when observed at 24 hours after emergence. The interaction between these two factors was non-significant ($F_{1;30} = 2.9276$ $P = 0.097$). At 48 hours after emergence all the resistant and susceptible females showed mature eggs.

There is a significant effect of resistance ($F_{1,98} = 4.665$; $P < 0.05$) and time after emergence ($F_{4,94} = 107.56$; $P < 0.0001$) in the number of mature eggs. One of the resistant females evaluated already had mature eggs just 12 hours after the emergency. Evaluating females after 24h and 48h of emergency there was no statistical difference in this parameter despite resistant females present higher number of eggs, but after 72 hours of the emergency susceptible females had significantly higher numbers of mature eggs that resistant, showing the presence of physiological cost.

The contrast analysis shows that levels of time after emergence (12h-24h, 48h, 72h and 96h) are different. In spite of that, the interaction of these two factors was not significant ($F_{4,90} = 1.177$; $P > 0.05$).

DISCUSSION

The possible fitness costs in ovarian development and calling behaviour of *S. frugiperda* females with same genetic basis and differences in susceptibility to Cry1A.105+Cry2Ab2 Bt toxins were studied. Knowing about fitness cost is important for efficient management of resistance

Some fitness cost of Bt resistance studies have already been made with *S. frugiperda* and another Lepidoptera to Bt events that express one toxin (Crespo *et al.*, 2010; Pereira *et al.*, 2011; Jakka *et al.*, 2014; Vélez *et al.*, 2014; Santos-Amaya, Tavares, Rodrigues*, et al.*, 2016) however, there are few studies of fitness cost studies with pyramided events because this is a new technology and it was not been detected field resistance to this yet. Besides that, behavioral aspects of reproduction are being neglected to both first and second-generation Bt plants.

(Santos-Amaya *et al.*, In preparation) observed fitness cost associated with the resistance to event expressing Cy1A.105 + Cry2Ab2 in development and population growth in the same strain of *S. frugiperda* used in this work. So, this population may also present costs in reproductive development as well as behavioral aspects of reproduction

In our results resistant females presented an increase regarding some aspects of calling behavior when compared to susceptible females. There was no observed difference in the mean onset time of calling, however resistant females spent more time calling and generally have higher number of calling bouts than susceptible ones. These results are the opposite of what was found for *Helicoverpa armigera* resistant to the toxin Cry1Ac, which was verified fitness cost in calling behavior and ovarian development, and resistant females showed a delay in these behaviors when compared to susceptible ones (Zhao *et al.*, 2009; Zhang *et al.*, 2014).

Females of both population called earlier as well as present longer time spent calling from the second night of calling than at first night. These results are similar as in the works already cited (Zhao *et al.*, 2009; Zhang *et al.*, 2014). This can be important because the rate of pheromone released as well as pheromone titer and composition may change over calling days and older females may throw suboptimal amounts of pheromone (Kanno, 1979; Bjostad *et al.*, 1980; Charlton e Cardé, 1982; Webster e Cardé, 1982; Hunt e Haynes, 1990; Lima e Mcneil, 2009), then calling earlier, for longer time and having more calling bouts they would be more competitive with younger females.

However, with respect to the ovarian development resistant female presents larger oocyte diameter than the susceptible and this diameter already reaches the reported threshold to start calling (Cusson e Mcneil, 1989a) 12 hours after emergency. Besides that, one resistant female already presented mature eggs at this time. Despite this, there was no difference in the oocyte diameter and number of mature eggs between resistant and susceptible females at 24 hours after emergency and susceptible females had significantly higher numbers of mature eggs than resistant, after 72 hours of the emergency showing the presence of cost.

So, resistant females start development earlier, however, over time, susceptible females have higher number of mature eggs that resistant, showing a fitness cost associated with resistance. The delay in development, along with the increase in time spent calling and the number of calling bouts, added to early ovarian development of these females may favor the preferential mating between resistant individuals, which can speed up the rate of development of resistance. However, that is already observed cost on population growth in this population (Santos-Amaya *et al.*, In preparation) and this is a factor that helps in reversing the resistance.

To efficacy of high dose/refuge strategy in resistance management of pests to Bt toxins, is necessary random mating between individuals surviving in culture and individuals susceptible presents at the refuge (Roush, 1997; Gould, 1998). Thus, spent more time calling, present more and longer calling bouts can be favorable because increase the reproductive window which may favor assortative mating because of the greater availability of resistant female. Moreover, this higher mating frequency may increase the frequency of resistant alleles (Sisterson *et al.*, 2004; Téllez-Rodríguez *et al.*, 2014).

Females emerge before the males (Cruz, 1995) and when male are able to mate the females are already receptive to mating. There are differences in susceptibility to insecticide (Pashley e Martin, 1987) and Bt toxin (Adamczyk Jr *et al.*, 1997) so, when the resistance is selected may be selecting just individuals of corn strain. It is already reported that there are significant preference of males for females of the same strain (Pashley *et al.*, 1992).

However no studies regarding the attractiveness to males by resistant and susceptible females to the pyramided event that express Cry2Ab2+Cry1A.105 toxins.

So, for a better understanding of changes in reproductive behaviour and how this can influence the resistance management further studies on the blend of pheromones, consider the fall armyworm strain and the differences between susceptibility to Bt event, female choice made by male beyond the acceptance of females to males are necessary.

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FIGURES

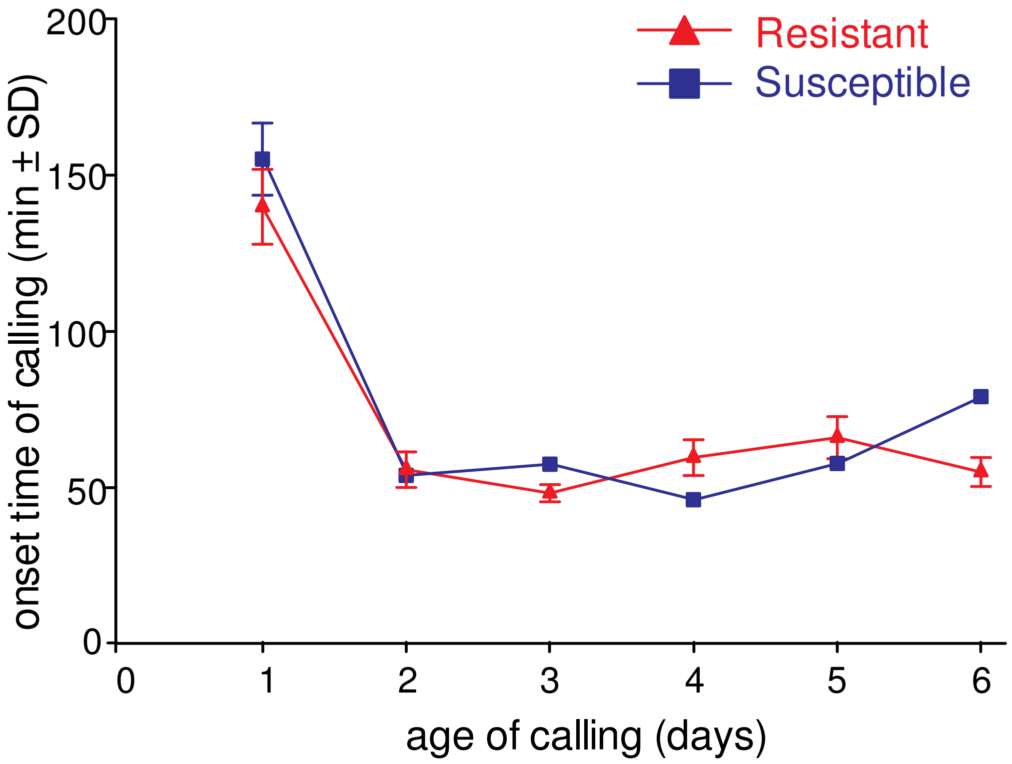

Figure 1. Mean time of onset calling (X ± SD) (in minutes after scotophase) on subsequent nights of calling of susceptible and resistant to Cry2Ab2+Cry1A.105 Bt toxins populations of *Spodoptera frugiperda*.

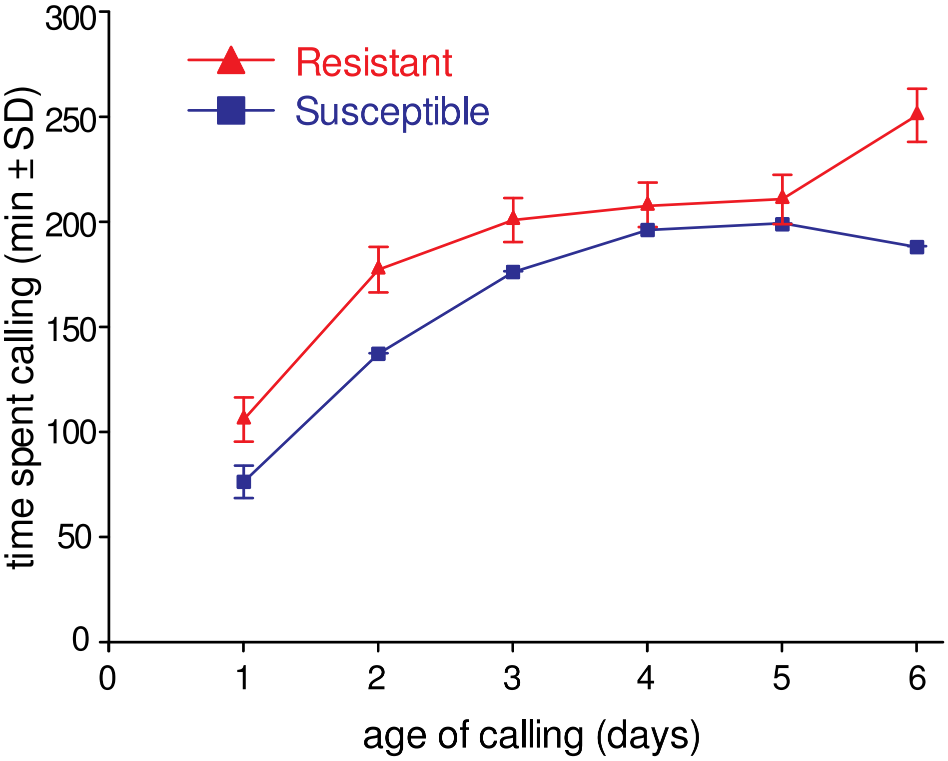

Figure 2. Time spent calling (X ± SD) (in minutes after scotophase) on subsequent nights of calling of susceptible and resistant to Cry2Ab2+Cry1A.105 Bt toxins populations of *Spodoptera frugiperda*.

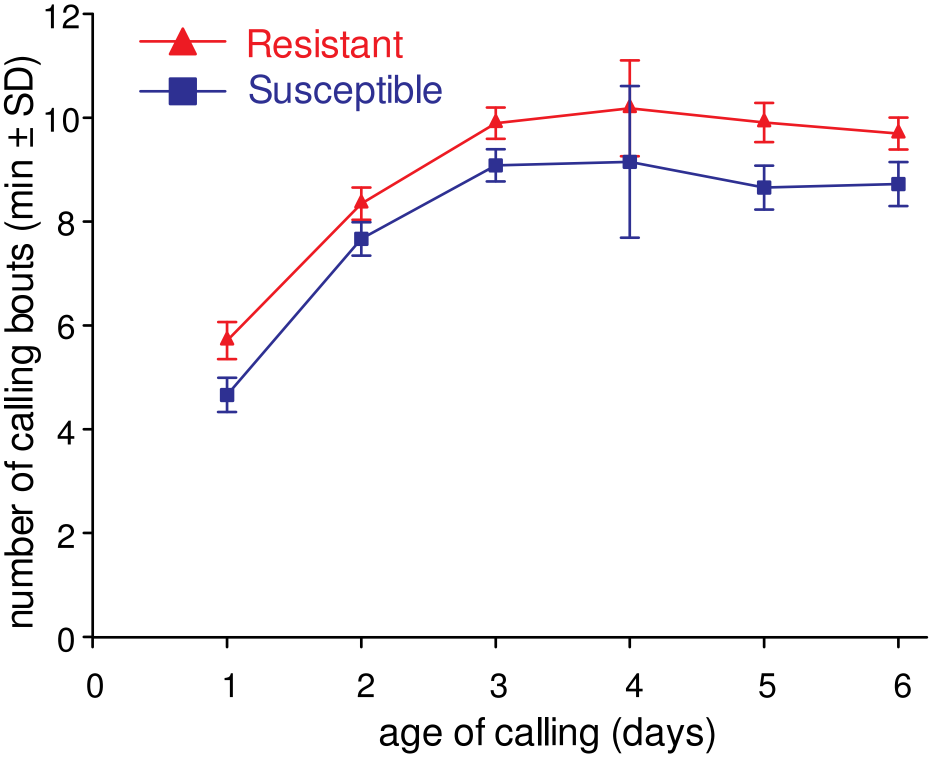

Figure 3. Number of calling bouts (X ± SD) on subsequent nights of calling of susceptible and resistant to Cry2Ab2+Cry1A.105 Bt toxins populations of *Spodoptera frugiperda*.

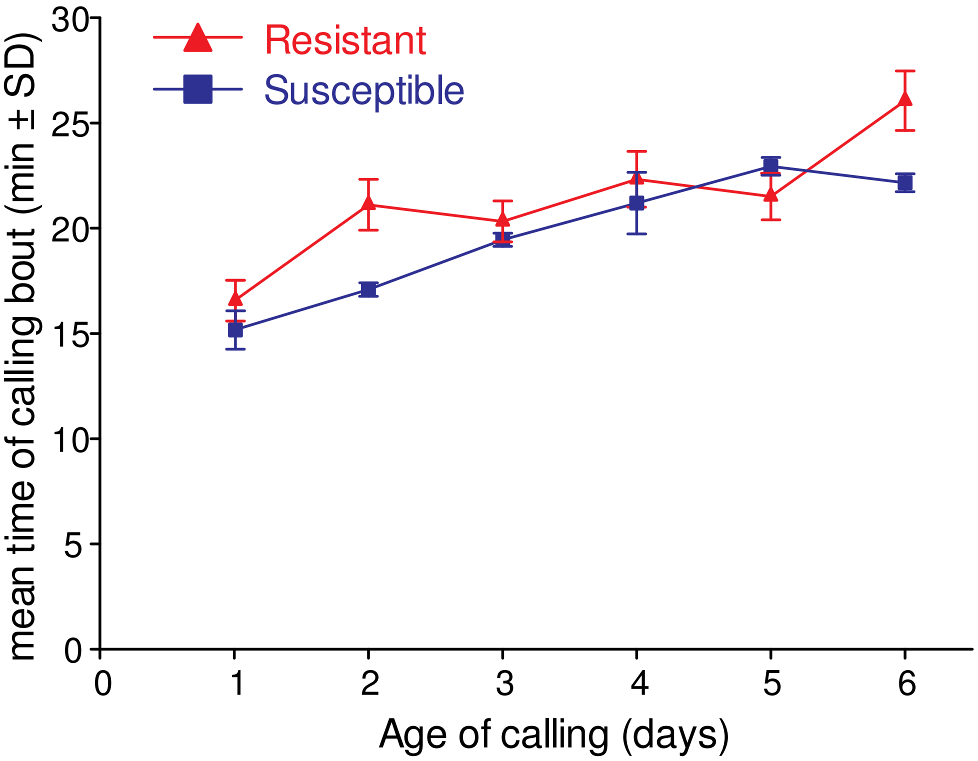

Figure 4. Mean time of calling bouts (X ± SD) on subsequent nights of calling of susceptible and resistant to Cry2Ab2+Cry1A.105 Bt toxins populations of *Spodoptera frugiperda*.

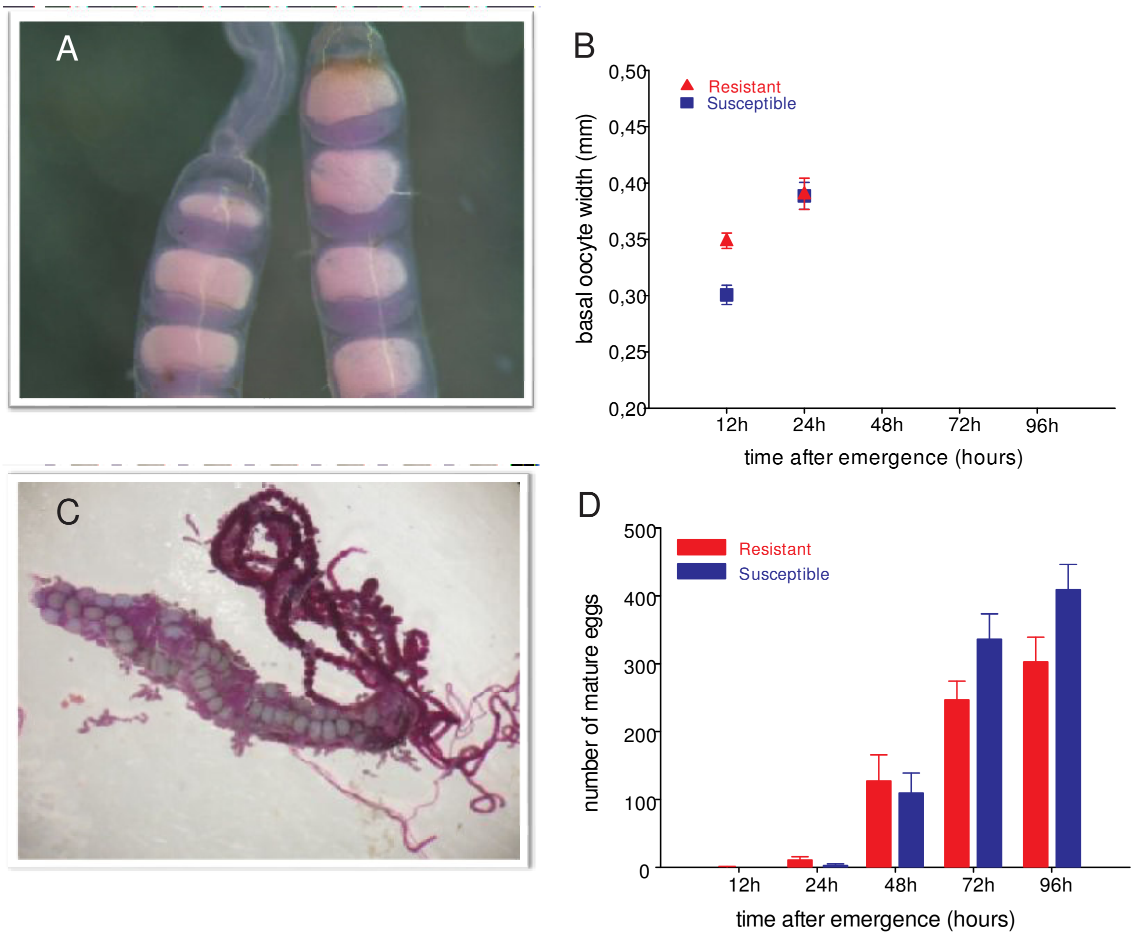

Figure 5. Ovarian development of susceptible and resistant *Spodoptera frugiperda* female. A) oocyte B) basal oocyte width (X ± SD) of resistant to Cry2Ab2+Cry1A.105 (red triangles) and susceptible (blue squares) female C) ovary with mature eggs D) number of mature eggs (X ± SD) of resistant to Cry2Ab2+Cry1A.105 (red bars) and susceptible (blue bars) female.

GENERAL CONCLUSIONS

- Resistance to different Bt events influence differently the calling behavior and ovarian development of *Spodoptera frugiperda* females.
- There is no cost associated to Cry1Fa resistance in the evaluated parameter on *S. frugiperda* population, in contrast, resistant females had more success than the susceptible when not exposed to selection pressure.
- Although an advantage regarding the time spent calling, the higher number and longer duration of the calling bouts per night, besides starting their ovarian development 1 day earlier, the females resistant to stacked event expressing Cry2Ab2 and Cry1A.105 present less mature eggs than susceptible in the final stage of ovarian development, setting up cost.
- Presence or absence of costs associated to resistance may influence the effective resistance management.
- Absence of cost to Cry1Fa can contribute to the rapid selection of resistance to this Bt toxin.
- There is a difference in susceptibility between different host strains of *S. frugiperda*, so there may be differential selection between strains
- For a better understanding of changes in reproductive behaviour and how this can influence the resistance management efforts are required studies on the blend of pheromones, consider the fall armyworm strain and the differences between susceptibility to Bt event, female choice made by male beyond the acceptance of females to males.