

University of São Paulo
“Luiz de Queiroz” College of Agriculture

**Ecological Modelling of *Spodoptera frugiperda* Genotypes Larval Dispersal
as Tool to Understand and Management Resistance in Bt Cotton
Landscapes**

José Bruno Malaquias

Thesis presented to obtain the degree of Doctor in
Sciences. Area: Entomology

Piracicaba
2019

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RESUMO

Modelagem Ecológica da Dispersão Larval de Genótipos de *Spodoptera frugiperda* como Ferramenta para Compreender e Manejar a Resistência em Paisagens do Algodão Bt

A demanda por informações de forma sistematizada sobre ecologia de insetos-praga alvos de plantas transgênicas tem crescido nas últimas décadas devido ao proeminente interesse nesta tecnologia no manejo de pragas. A mobilidade larval em ecossistemas agrícolas quando na ocorrência de contaminação de plantas Bt, de forma intencional ou não intencional, tem sido assunto de estudo por diversos Pesquisadores, entretanto informações sobre a mobilidade larval em condições tropicais são ainda escassas. Este cenário criou oportunidades para testes de hipóteses em aspectos comportamentais de *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae) em paisagens com co-ocorrência espaço-temporal de plantas de algodão Bt e não Bt. O principal objetivo desta pesquisa foi estudar a dispersão larval da praga em condições de campo e de laboratório em ordem para inserir componentes de modelagem computacional para descrever a evolução da resistência ao algodão Bt. No primeiro capítulo desta tese, é apresentada uma introdução geral. No segundo capítulo, foi estudado o comportamento alimentar associado com a dispersão larval de genótipos de *S. frugiperda* e possíveis efeitos na distribuição espacial de indivíduos resistentes em campos de algodão Bt e não Bt. Foram analisadas no terceiro capítulo as consequências para o progresso da resistência do padrão de dispersão de genótipos suscetíveis, resistentes a Cry1F e heterozigotos de *S. frugiperda* em paisagens artificiais com pureza e com contaminação. No quarto capítulo, avaliou-se o impacto da dispersão por balonismo combinado dispersão por caminhamento na evolução da resistência em condições de mistura de plantas de algodão não Bt e algodão Bt em eventos com alta e baixa dose. No último capítulo, foi comparada a dinâmica de movimentação de genótipos de *S. frugiperda* entre as temperaturas de 28°C e 32°C, e com um modelo baseado no indivíduo analisou-se a possibilidade se um dos genótipos poderia persistir e levar o outro a exclusão em áreas de refúgio com plantas de algodão não Bt. Baseado na dispersão larval de genótipos de *S. frugiperda*, os resultados encontrados ressaltam a necessidade de implementação de estratégias para evitar contaminação por meio de práticas agronômicas inadequadas tais como destruição de plantas após a colheita, controle de plantas voluntárias, e o ato de se salvar as sementes após a colheita. Todas informações geradas nesta tese poderão contribuir para otimização de manejo da resistência regional dentro de um conceito de controle de insetos em grandes áreas.

Palavras-chave: Movimento larval; Lagarta militar; Dinâmica evolutiva; Planta Bt

ABSTRACT

Ecological Modelling of *Spodoptera frugiperda* Genotypes Larval Dispersal as Tool to Understand and Management Resistance in Bt Cotton Landscapes

The demand for information in a systematic way about ecology of target insect species of transgenic plants has increased in recent decades due to the growing interest in this new technology in pest management. Larval mobility in agricultural ecosystems when on occurrence of contamination of Bt crops, intentionally or unintentionally - has been subject of study by several researchers, however information about larval mobility in tropical conditions are still scarce. This scenario created opportunity for hypothesis testing on behavioural aspects of *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae) in landscapes with spatial-temporal co-occurrence of Bt and non-Bt cotton plants. The main objective of the current research was to study the larval dispersal of the pest in field and laboratory conditions in order to insert computational modelling components to describe the evolution of resistance to Bt cotton. In the first chapter of this thesis, it is presented a general introduction. In the second one, we studied the feeding behaviour associated with larval dispersal of *S. frugiperda* genotypes and possible effects on spatial distribution of the resistant individuals in Bt and non-Bt cotton fields. We analyzed in the third chapter the consequences to resistance progress of the dispersal pattern of susceptible, Cry1F-resistant and heterozygous genotypes of *S. frugiperda* in pure and contaminated artificial landscapes. In the fourth chapter we assessed the impact of the dispersal by ballooning combined with walking dispersal on resistance evolution in conditions of plant mixture with non-Bt and Bt cotton plants in events with high and non-high dose. In the last chapter, we compared the movement dynamics of *S. frugiperda* genotypes between 28°C and 32°C, and with an individual-based model we analyzed the possibility if one of the genotypes could persist and would lead the other to the exclusion on refuge areas with non-Bt cotton plants. In a general way, faced on the larval dispersal of *S. frugiperda* genotypes, the results found here highlight the importance of implementation of strategies to avoid contamination through inadequate agronomic practices such as destruction of cotton plants after harvest, volunteer plant control, and seed saving after harvest. All information generated in this thesis could contribute in the optimization of regional resistance management within a concept of insect population control in wide areas.

Keywords: Larval movement; Fall armyworm; Evolutionary dynamics; Bt plant

1 General Introduction

Cotton is host of a complex lepidopteran species. These species defoliate and destroy the plant reproductive structures. High populational densities result in large economic loss for the crop (Ramalho *et al.* 2011). Within the species causing damage to cotton, *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae) has featured due the destructive capacity in the main cotton producer regions in Brazil. The utilization of genetically modified cotton varieties resistant to cotton caterpillars has been purposed as alternative to chemical control. These cotton plants express genes of *Bacillus thuringiensis* (Berliner) (Bt), which are lethal when ingested by the caterpillar (Vachon *et al.* 2012). Since the release of commercial Bt plants in the United States, a lot of farmers have adopted this strategy aiming effective increase on agricultural production. However, the Bt toxin continuous expression on plants provides a high selection pressure on target insect population. As consequence, the host selection behaviour could be modified by the Bt crop, this may be an important factor to influence the resistance evolution specially under conditions where the initial allele frequency is considered high (Bernardi *et al.* 2015).

Some cases of resistance of pests to Bt toxins were already documented in some countries. Dhurua & Gujar (2011) found in India, resistance of *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) to cotton that express Cry1Ac in an isolated way or combined with Cry2Ab2. Gassmann *et al.* (2011), verified also in field the strain of *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) resistant to Bt corn that express Cry3Bb1 or Cry34/35Ab1. Storer *et al.* (2010) verified resistance of *S. frugiperda* to Cry1F toxin in corn fields in Puerto Rico. It was also observed in the United States of America the *S. frugiperda* resistance to corn that express Cry1F (Huang *et al.* 2014).

Spodoptera frugiperda resistance to Cry1F of insects collected in Puerto Rico is recessive, controlled by autosomal genes, and with moderate cross resistance to Cry1Ab and Cry1Ac (Storer *et al.* 2010), that are also used in Brazil. It's possible that, particularly, in Puerto Rico the reasons for resistance have been because the synergy between the local factors, such as, geographic isolation, tropical weather, intensive planting of corn and extensive use of Bt with spraying. After control failures detection in corn fields in Brazil, Farias *et al.* (2014a) observed that the resistance ratio was 5,000 times higher than a susceptible population. Besides, in Brazil the resistance inherence of *S. frugiperda* to Cry1F is

controlled by autosomal genes, and the resistance alleles are in the same locus. Faced on this contextualization, the implementation of resistance management strategies aiming the technology sustainability for *S. frugiperda* controlling is emergent. Not different of Puerto Rico, the cotton planting system and corn in “Brazilian cerrado” is greatly intensive, mainly under irrigation. The irrigation activity favors the insect generation overlapping compromising the pest management, specially by the significative occurrence of gene flow between *S. frugiperda* populations from cotton and corn when are cultivated in the same geographic region (Martinelli *et al.* 2006). In general, this scenario is characterized by the absence of genetic variability, thus this aspect suggests that the same population may be attacking the same crops in Brazil (Martinelli *et al.* 2007). Therefore, resulting in decreasing susceptibility of pest to Cry1 toxins from regions where this planting system occurs (Farias *et al.* 2014b).

In addition to high selection pressure of Bt crops to *S. frugiperda* populations, another great challenge to resistance management in Brazilian conditions is the seed contamination effect. The contamination can occur in many cases, mostly if the Bt seeds or non-Bt seeds or both are contaminated by seed mixture, being intentional or non-intentional, or still by gene flow (Ellstrand *et al.* 1999). This contamination can occur on refuge or on Bt fields. The refuge is the cultivated area that does not have Bt toxin, with the aim of provide the survival of susceptible insects for crossing with resistant insect that survive on Bt fields. This strategy aims to delay the resistance evolution of insects to Bt toxins. The refuge may be considered structured or non-structured. The structured refuge consists of non-Bt plants in previously defined areas. The non-structured refuge has been purposed as refuge in the bag (RIB), that is the procedure that consists in an intentional seed mixture in different proportions of Bt with non-Bt seeds (Zancanaro *et al.* 2012).

The business segment of international sphere has discussed the possibility of adoption of the RIB on resistance management based on seed mixture, however the information scarcity about larval mobility in tropical condition unfeasibles the adoption of this strategy. In refuge fields, the contamination by Bt plants increases the selection for resistant individuals, therefore, hasten the resistance evolution. On the other hand, is hoped that the contamination of Bt fields with non-Bt plants has opposite effect, that is of always reduce the resistance evolution rate. However, this not always happen, mainly if the larvae move between plants within of same Bt field. Then, the high movement rates can reverse the contamination effect

on Bt fields to delay the resistance evolution (Glaum *et al.* 2012). High contamination levels can occur fast when on the seed production process there is no adoption of rigidus criterion (Haygood *et al.* 2003). This can also occur during the seed utilization by the farmers together with the low infra-structure and educational preparation of the farmers that use this biotechnology.

The procedure of many farmers in developing countries harvest and use their Bt seed on the following years to eliminate the costs to buy the seeds, can result in contamination of Bt fields with non-transgenic materials (Glaum *et al.* 2012). Besides, other agronomic practices have been targeted of discussion in the implementation of resistance management strategies, because after the end of cotton harvest, still can be observed intensive re-growth of cotton plants (Christoffoleri *et al.* 2006). The lack of destruction of plants after harvest of cotton can compromise the cotton production on local and in regional scales. The efficacy of this procedure can be achieved with the integration between mechanic, cultural and chemical methods (Silva *et al.* 2011). However, it is possible to observe constantly failures on adoption of these procedures on producer regions that provide the occurrence of cotton volunteer plants on subsequent planting of the other crops such as corn and soybean. The Bt field contamination deserves attention, since the larvae that hatch in transgenic plants can do the feed test and after that may dispersal (Goldstein *et al.* 2010).

The larval movement of pest population of targeted pest between Bt and non-Bt plants is the main factor to be considered in the intentionally or non-intentionally seed mixture (Wangila *et al.* 2012). Wangila *et al.* (2012), found that the seed mixture favors the production of refuge comparable than structured refuge to *Diatraea saccharalis* (Fabr., 1794) (Lepidoptera: Pyralidae). On the other hand, in Brazil, recent studies showed that the dispersal and/or feeding behaviour of *Alabama argillacea* (Hübner) (Lepidoptera: Noctuidae) is different between Bt and non Bt plants (Ramalho *et al.* 2014), endorsing the special attention in scenarios where happen the seed mixture.

Environmental factors such as temperature should be also considered in studies about the potential risk of resistance evolution. High temperatures provide decreasing effects on Bt toxin expression, but in low temperatures there is a significantly increasing on the toxin expression (Chen *et al.* 2012). These factors affect the feeding behaviour of target pest and, consequently, the resistance evolution (Chen *et al.* 2012). Other studies about the impact on feeding behaviour and larval dispersal of *S. frugiperda* on Bt cotton plants combined with

different temperatures are necessary to determine the consequences of seed mixture on transgenic crops, when compared to other scenarios, such as structured refuge or absence of refuge. Hereby, is relevant the assessment of feeding behaviour and dispersal of *S. frugiperda* neonate on Bt and non-Bt cotton plants in different temperatures and under different landscapes scenarios.

The modelling of the dispersal capacity of insects such as the computer simulation that can predict the resistance evolution are valuable tools to understand the behaviour process. The modelling of dispersal and resistance evolution is a necessary procedure to guide professionals to design an effective program of resistance on different situations, constituting a tool that can reveal the technology efficacy accordance to landscape structure – in this case the refuge. On this way, the seed mixture can represent environmental risk because may provide high resistance evolution risk of *S. frugiperda* to toxins present in transgenic cultivars; however, information in this direction is still incipient in neotropical conditions, motivating and justifying the search for this knowledge by this research.

The first step to assess the gap of information was the conduction of bioassays on laboratory, with the aim to study the host acceptance of the pest on Bt cotton. The posterior step was to know if the dispersal was associated with a previous consumption of the plant by the larvae or if the dispersal is a natural event without association with the plant. Besides, it was of the great importance also to understand how happen the dispersal dynamic on field experiments. These variables were approached within a context with the interactions involving the host plant, temperature and the pest strain. According to this theoretical contextualization showed, this study had the following goals:

- (i) to analyze the feeding behaviour and larval dispersal of susceptible and Cry1F-resistant strains of *S. frugiperda* on Bt and non-Bt cotton varieties, and to understand the possible effects of Bt and non-Bt cotton fields contamination on the dispersal and infestation capacity of *S. frugiperda* larvae by using a computer model (**second chapter**).
- (ii) to assess the influence of Bt and non-Bt cotton plants on the larval dispersal and survivalship of susceptible, Cry1F-resistant and heterozygous genotypes of *S. frugiperda* in pure and contaminated artificial micro-landscape, and to

analyze the consequences of *S. frugiperda* mobility on resistance evolution (**third chapter**).

- (iii) to study the impact of dispersal by ballooning associated with walking movement on resistance evolution in plant mixture with non-Bt and Bt cotton plants in events with high and non-high dose (**fourth chapter**).
- (iv) to characterize with video tracking system for automation in behavioural experiment the movement dynamics of *S. frugiperda* genotypes on Bt cotton and its non-Bt isoline in 2 temperatures (28°C and 32°C), and to test the hypothesis if one of the genotypes could persist and would lead the other to the exclusion on refuge areas using an individual-based model (**fifth chapter**).

The computational resources used in the current research were compatible to allow bold designs that promoted interface areas such as dispersal behaviour, evolutionary dynamics and resistance management. That point is important because given the extensive problems facing resistance to Bt crops, the demand for the solutions of the resistance problems has been complex and increasing. Thus, integrative and multidisciplinary approach is a powerful alternative to provide solutions in short-term. Each chapter of this thesis was written following the manuscript submission guidelines of different journals, as follows: Scientific Reports (Chapter 2), Journal of Pest Science (Chapter 3), Nature Biotechnology (Chapter 4) and Entomologia Experimentalis et Applicata (Chapter 5).

References

- Bernardi, O., Bernardi, D., Ribeiro, R.S., Okuma, D.M., Salmeron, J.F., Medeiros, F.C.L., Burd, T., Omoto, C. 2015. Frequency of resistance to Vip3Aa20 toxin from *Bacillus thuringiensis* in *Spodoptera frugiperda* (Lepidoptera: Noctuidae) populations in Brazil. *Crop Protection*, 76: 7–14.
- Chen, Y., Wen, Y., Zhang, X., Chen, D. 2012. The effects of the relative humidity on the insecticidal expression level of Bt cotton during bolling period under high temperature. *Field Crops Research*, 137: 141–147.
- Christoffoleri, P.J., Foloni, L.L., Carvalho, L.H., Carvalho, S.J.C. Manejo químico de soqueiras gera ganhos operacionais. *Visão Agrícola*. In: USP ESALQ, ano 3, Jul, 2006.
- Dhuraa, S., Gujar, G. 2011. Field-evolved resistance to Bt toxin Cry1Ac in the pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae), from India. *Pest Management Science*, 67: 898–903.
- Ellstrand, N.C., Prentice, H.C., Hancock, J.F. 1999. Gene flow and introgression from domesticated plants into their wild relatives. *Annual Review of Ecology and Systematics*, 30:539–563
- Farias, J.R., Andow, D.A., Horikoshi, R.J., Sorgatto, R.J., Santos, A.C., Omoto, C. 2014a. Field-evolved resistance to Cry1F maize by *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Brazil. *Crop Protection*, 64: 150-158.
- Farias, J.R., Horikoshi, R.J., Santos, A.C., Omoto, C. 2014b. Geographical and temporal variability in susceptibility to Cry1F toxin from *Bacillus thuringiensis* in *Spodoptera frugiperda* (Lepidoptera: Noctuidae) populations in Brazil. *Journal of Economic Entomology*, 107: 2182-2189.
- Gassmann, A., Petzold-Maxwell, J.L., Keweshan, R.S., Dunbar, M.W. 2011. Field-Evolved resistance to Bt maize by western corn rootworm. *PLoS ONE*, 6 (7). doi: 10.1371/journal.pone.0022629.

- Glaum, P.R., Ives, A.R., Andow, D.A. 2012. Contamination and management of resistance evolution to high-dose transgenic insecticidal crops. *Theoretical Ecology*, 5:195–209.
- Goldstein, J.A., Mason, C.E., Pesek, J. 2010. Dispersal and movement behavior of neonate European corn borer (Lepidoptera: Crambidae) on non-Bt and transgenic Bt corn. *Journal of Economic Entomology*, 103: 331-339.
- 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., Yang, F., Danga, V. 2014. Cry1F resistance in fall armyworm *Spodoptera frugiperda*: single gene versus Pyramided Bt Maize. *PLoS ONE*, 9 (11): e112958.
- Martinelli, S., Barata, R.M., Zucchi, M.C.S.F., Omoto, C. 2006. Molecular variability of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) populations associated to maize and cotton crops in Brazil. *Journal of Economic Entomology*, 99: 519-526.
- Martinelli, S., Clark, P.L., Zucchi, M.I., Silva-Filho, M.C., Foster, J.E., Omoto, C. 2007. Genetic structure and molecular variability of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) collected in maize and cotton fields in Brazil. *Bulletin of Entomological Research*, 97: 225–231.
- Ramalho, F.S., Azeredo, T.L., Nascimento, A.R.B., Fernandes, F.S., Nascimento Júnior, J.L., Malaquias, J.B., Silva, C.A.D., Zanuncio, J.C. 2011. Feeding of fall armyworm, *Spodoptera frugiperda*, on Bt transgenic cotton and its isoline. *Entomologia Experimentalis et Applicata*, 139: 207–214.
- Ramalho, F.S., Pachu, J.K.S., Lira, A.C.S., Malaquias, J.B., Zanuncio, J.C. 2014. Feeding and dispersal behavior of the cotton leafworm, *Alabama argillacea* (Hübner) (Lepidoptera: Noctuidae), on Bt and non-Bt cotton: Implications for evolution and resistance management. *PLoS ONE* 9(11): e111588. doi:10.1371/ journal.pone.0111588
- Silva, O.R.R.F., Lamas, F.L., Ferreira, A.C.B., Sofiatti, V., Medeiros, J.C. 2011. Manejo dos restos culturais do algodoeiro. In: Freire, E.C. Algodão no cerrado do Brasil. 2ed. ABRAPA. Cap. 10, p. 331-344.
- 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.

Vachon, V., Laprade, R., Schwatz, J.L 2012. Current models of the mode of action of *Bacillus thuringiensis* insecticidal crystal proteins: A critical review. *Journal of Invertebrate Pathology*, 111:1–12.

Wangila, D.S., Leonard, B.R., Bai, Y., Head, G.H., Huang, F., 2012. Larval survival and plant injury of Cry1Ab-susceptible, -resistant, and -heterozygous genotypes of the sugarcane borer on transgenic corn containing single or pyramided Bt genes. *Crop Protection*, 42: 108–115.

Zancanaro, P.O; Buchweitz, E.D; Boica Júnior, A.L; Moro, J.R 2012. Avaliação de tecnologias de refúgio no cultivo de milho Bt. *Pesquisa Agropecuária Brasileira*, 47: 886-891.

2 Larval Dispersal of *Spodoptera frugiperda* Strains on Bt Cotton: A Model for Understanding Resistance Evolution and Consequences for its Management

Abstract

High dispersal of Lepidoptera larvae between non-Bt and Bt cotton plants can favour the evolution of insect resistance; however, information on host acceptance of neonates in tropical transgenic crops is scarce. Therefore, the purposes of this study were as follows: (i) to investigate the feeding behaviour of susceptible and Cry1F-resistant strains of *Spodoptera frugiperda* (J.E. Smith) on Bt and non-Bt cotton (*Gossypium hirsutum* L.) varieties and (ii) to understand the possible effects of cotton field contamination on the dispersal and infestation capacity of *S. frugiperda* larvae by using an individual-based model. The main results of this paper are as follows: (1) the highest post-feeding larval dispersal of the Cry1F-resistant strain occurred at an exposure time of 18–24 h; (2) via video tracking assays, we found that the least distance moved was by larvae resistant to Cry1F on non-Bt cotton; and (3) the model indicated differences in mobility capacity between Bt and non-Bt cotton. We conclude that resistant neonates exhibit sedentary behaviour. Our report represents the first findings concerning the fitness cost of larval behaviour traits of *S. frugiperda* associated with Cry1F resistance in Brazilian populations. The current chapter was written following the manuscript submission guidelines of the journal Scientific Reports.

Keywords: Movement; Fall armyworm; Contamination; Cotton fields

2.1 Introduction

The cropping system in Brazil is intensive, which favours the overlapping of generations of insect pests such as *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae). This overlap increases the selection for resistance to insecticides and transgenic crops expressing *Bacillus thuringiensis* Berliner (Bt) toxins due to gene flow between *S. frugiperda* populations from maize and cotton fields in the same geographical region¹. Another challenge to resistance management is seed contamination. Contamination may occur for many reasons, especially if Bt seeds, non-Bt seeds or both are contaminated by mixing seeds or by gene flow between cotton varieties via pollen flow². In developing countries such as Brazil, the presence of volunteer plants, cross contamination between different plant species, or gene flow between Bt and non-Bt varieties, coupled with the practice of harvesting and saving seeds for use in the following year, are common causes for concern. Depending on the contamination degree and whether larvae move among plants within a field, resistance evolution may be accelerated³. In addition, insect-resistant genetically modified crops can affect some behavioural traits, including locomotion and feeding behaviour, as well as larval dispersal⁴.

The resistance of *S. frugiperda* to insecticides and Bt toxins has been characterized in Brazil under laboratory and field conditions⁵⁻⁸. Therefore, there is an urgent need to implement resistance management strategies. In recent decades, the demand for systematic information about the behavioural ecology of *S. frugiperda* on transgenic crops to explain the reasons for control failure has increased. With potential seed contamination, the movement from non-Bt plants to Bt plants within a field may promote insect survival in the last instars and consequently favour resistance evolution⁹; in this condition, the main reason for larval survival on Bt plants in the last instars is the brief exposure time during which the insect does not ingest a sufficient amount of insecticidal proteins to cause mortality before metamorphosis to the pupal stage¹⁰. This behaviour was observed in *S. frugiperda* larvae that were fed non-Bt cotton for 18 days and were subsequently transferred to Bt cotton expressing Cry1Ac and Cry1F toxins, where they reached pupal and adult stages⁹. Similarly, *S. frugiperda* strains in the last instars survived on Bt maize expressing Vip3Aa20 and Cry1Ab¹⁰.

The incorporation of knowledge about the movement of insect pests within large areas is essential for pest management, especially due to the lack of information on where, when, and

why these pests move¹¹. Larval dispersal has been investigated considering risk formulation of seed mixing because when larvae are stimulated by Bt plants, they may move a longer distance than expected towards non-Bt cotton plants, and resistance could evolve within a few years¹². Knowledge about the movement of Lepidoptera larvae between Bt and non-Bt cotton plants is based on the configuration of the refuge^{13,14}. Pre-feeding dispersal and post-feeding dispersal (*PF**D*) of neonates is common when the host contains toxins, as observed from studies with Bt¹⁵. For pests with low mobility capacity, the seed mixture may provide a refuge comparable to a structured refuge, leading to longer delays to resistance¹³. Nevertheless, for pests with a high dispersal capacity, further studies to determine the consequences of the feeding behaviour of pests on Bt crops are needed, especially under conditions of non-structured or different degrees of seed contamination.

Theoretical models that consider the movement of Lepidoptera larvae are relevant for predicting the population dynamics of pests in agricultural systems, allowing one to study the effects of different refuge proportions and the movements of larvae on cotton crops with various resistance allele frequencies¹⁶. Modelling is a necessary procedure to guide professionals in designing effective programmes for resistance management in different scenarios and a helpful tool to explain the efficacy of a technology according to the landscape structure, i.e., the refuge, and to evaluate the potential consequences of contamination of Bt and non-Bt cotton fields.

Computational models that integrate information about the ecological traits of pest strains are necessary, especially in tropical conditions. The inclusion of information about ecological traits in modelling is essential to explain the fast resistance evolution of *S. frugiperda* to Bt cotton in Brazilian agroecosystems or similar systems. Although the fitness costs were not evaluated for the biological parameters of Cry1F-resistant strains of *S. frugiperda*¹⁷ collected from Brazilian agroecosystems, the formulation of a hypothesis based on the possible existence of differences in feeding behaviour between larval phenotypes motivates the comparison of these ecological traits between susceptible and Cry1F-resistant strains of *S. frugiperda* on Bt cotton. Therefore, this study had two main questions of interest. Does the feeding behaviour differ between susceptible and Cry1F-resistant strains of *S. frugiperda*? Does the fitness cost associated with Cry1F resistance in *S. frugiperda* affect the spatial pattern in accordance with contamination of Bt and non-Bt cotton? In the context of the importance of assessing the feeding behaviour and neonate dispersal of *S. frugiperda* on Bt and non-Bt cotton, this study aimed to quantify some behavioural ecological traits of *S.*

frugiperda larvae on Bt cotton and to investigate the effects of larval movement on population distribution. The overall goal was to gain an understanding of the rapid evolution of resistance and the possible implications for resistance management based on these behavioural traits. We studied the following variables: dispersal rate, *PFD*, individual proportions that were found on varieties and feeding on plant tissues (*IFP*), survival (via a host acceptance bioassay), distance moved, mean velocity, and continuous mobility period (via a video tracking assay). Larval dispersal and larval density on Bt and non-Bt cotton were also spatially simulated using an individual-based model.

2.2 Results

2.2.1 Feeding behaviour of *S. frugiperda* strains on WideStrike and non-Bt cotton varieties. We selected two components of the eigenvalues of the correlation matrix according to the criterion of Kaiser¹⁸. The first principal component (PC1) is represented by the contrast between the variables dispersal rate (eigenvector = 0.7026 x_1) and *IFP* (eigenvector = 0.5577 x_2). PC2 includes the contrast among *PFD* (0.4469 z_1), the weighted average of *IFP* (eigenvector = -0.5958 z_2) and survival (eigenvector = -0.6429 z_3). PC1 and PC2 explain 39.42 and 33.79% of the total variation in the data, respectively.

The results of Pearson's correlation analysis and the arrangement of the vectors in the biplot showed a negative correlation (Pearson's correlation coefficient = -0.5253) between dispersal and *IFP*. Characterizing treatments via biplot enables us to determine the highest survival rate and greatest *PFD* for susceptible insects that were kept on non-Bt cotton plants for 6 and 12 h and for Cry1F-resistant insects that were kept on non-Bt cotton plants for 6 h. A tendency towards a low dispersal rate and a high proportion of plant tissue feeding was revealed on the biplot for the Cry1F-resistant insects at 12, 18, and 24 h of exposure to Bt cotton and at 12 and 18 h of exposure to non-Bt cotton (Fig. 2.1).

Larval survival differed significantly between cotton varieties ($F_{1,46} = 11.90$; $P = 0.0410$) (Table 2.1 and Fig. 2.2a). The boxplot in Figure 2.2a shows the effect of cotton varieties on the survival of *S. frugiperda*. Larval survival was greater than 70% on non-Bt cotton and lower than 60% on Bt cotton. The survival pattern of *S. frugiperda* larvae as a function of time was strain dependent ($F_{3,46} = 3.81$; $P = 0.0199$) (Table 2.1). In fact, the regression curves reveal that the survival response of *S. frugiperda* larvae was higher for susceptible larvae than for Cry1F-resistant larvae; however, there was an overlapping of curves at 18 h. At 24 h, the survival rates were 34 and 52% for susceptible and Cry1F-resistant strains, respectively (Fig. 2.2b). According to the analysis of variance of the proportion of *S. frugiperda* larvae found on cotton plants (host acceptance), no significant differences were found between Bt and non-Bt varieties ($F_{1,46} = 2.29$; $P = 0.1369$) (Table 2.1). There were no significant interactions among variety, strain and length of time ($F_{3,46} = 0.42$; $P = 0.7374$) (Table 2.1). On the other hand, the effect of exposure time on the percentage of larvae found on plants depended on the strain ($F_{3,46} = 6.04$; $P = 0.0024$). In fact, from 12–18 h, the percentage of larvae of the susceptible strain recovered from the plants significantly decreased compared to that from 0–6 h. In the comparison of percentages of Cry1F-resistant

S. frugiperda neonates recovered from plants over time, there was no difference between exposure times; however, from 12–18 h and 18–24 h, there was a greater percentage of host acceptance than in the susceptible strain (Table 2.2).

The percentages of *IFP* were significantly affected by strain versus time ($F_{3,46} = 5.08$; $P = 0.0053$) (Table 2.1); therefore, this variable was summed across Bt and non-Bt varieties. The average percentage of *IFP* for the Cry1F-resistant strain was lower at an exposure time of 6 h than at other exposure times (Fig. 2.3a). We summed the percentages of *IFP* across all time intervals because of the significant strain-versus-variety interaction ($F_{3,46} = 7.25$; $P = 0.0111$) (Table 2.1). The percentage of *IFP* was higher for the Cry1F-resistant strain than for the susceptible strain on Bt cotton (Fig. 2.3b).

We analysed the effect of the strain-versus-time interaction on the percentage of *PFD* ($F_{3,46} = 3.11$; $P = 0.0422$) (Table 2.1), and we observed that exposure time did not affect this percentage for the susceptible strain. However, there was a difference between the Cry1F-resistant and susceptible strains at an exposure time of 18–24 h (Fig. 2.3c). At exposure times of 12–18 and 18–24 h, we found the lowest and highest percentages of *PFD* for the Cry1F-resistant larvae, respectively (Fig. 2.3c). The percentages of *PFD* were affected by variety ($F_{1,46} = 6.64$; $P = 0.0155$) (Table 2.1); we observed a higher percentage of *PFD* on non-Bt cotton than on Bt cotton (Fig. 2.3d).

2.2.2 Feeding behaviour of Cry1F-resistant *S. frugiperda* on WideStrike, TwinLink, and non-Bt cotton. The distance matrix based on proportion data allowed the observation of treatment clusters by average linkage method. Dissimilarity was studied based on proportion data for survival, dispersal, *PFD*, and *IFP*. This method allowed a comparison between the pattern of feeding behaviour and the susceptibility of *S. frugiperda* Cry1F-resistant strains among treatments. By observing the dendrogram from left to right and inserting a cut near 1.15, we observed the existence of two groups inside the cluster that were well-defined based on average distances. This finding indicates that the feeding behaviour and survival of *S. frugiperda* are similar when they are maintained on TwinLink (for 6 and 12 h) and WideStrike (for 6 h) (Fig. 2.4).

By analysing each group from bottom to top, we verified that the feeding behaviour of *S. frugiperda* was similar within non-Bt and WideStrike varieties for 12 and 18 h and between these varieties for 24 h in the second subgroup. Following the same procedure led to the

observation that the highest dissimilarity occurred with TwinLink for 18 h and with the non-Bt variety for 6 h because there was a longer distance within this group (Fig. 2.4).

2.2.3 Movement analysis of the *S. frugiperda* strains with video tracking. The mean velocity and continuous mobility period of larvae were not affected by cotton variety (mean velocity: $F_{1,1} = 2.61$; $P = 0.2044$; continuous mobility period: $F_{1,1} = 3.22$; $P = 0.1157$) or strain (mean velocity: $F_{1,1} = 0.22$; $P = 0.6850$; continuous mobility: $F_{1,1} = 1.56$; $P = 0.2523$). However, the distance moved was affected by cotton variety ($F_{1,1} = 8.21$; $P = 0.0242$). In fact, neonates of the Cry1F-resistant strain kept on non-Bt cotton moved shorter distances than did larvae of the same strain exposed to Bt cotton ($P = 0.0129$) or in relation to susceptible larvae from Bt cotton ($P = 0.0452$) (Fig. 2.5). Therefore, the effect of cotton variety on distance moved depends on the strain ($F_{1,1} = 4.61$; $P = 0.0500$).

2.2.4 Spatial model. Based on data indicating that the movement distance of Cry1F-resistant larvae was approximately three times higher on Bt cotton than that on non-Bt cotton (Fig. 2.5), we assumed that each Cry1F-resistant larva could move within a region of 7×7 cells (radius 3) in Bt cotton varieties and within a region of 3×3 cells (radius 1) in non-Bt cotton varieties and represented this movement in the spatial model (Fig. 2.6). The mean distance of larvae from the centre of the population distribution was significantly different when Bt and non-Bt cotton varieties with the same levels of contamination were compared, but it was similar within the same cotton variety under different levels of contamination (Fig. 2.7a). Larval density was significantly different only when Bt and non-Bt cotton varieties with contamination levels of 10 and 20% were compared (Fig. 2.7b).

2.3 Discussion

In the current study, we verified that the dispersal behaviour of the susceptible and Cry1F-resistant strains varied according to the exposure time. The host acceptance rate was higher from 0–6 h after infestation and lower from 12–18 h for the susceptible strain, resulting in high abandonment rates. The Cry1F-resistant strain of *S. frugiperda* showed a similar pattern of host acceptance among exposure times. By comparing this trait between strains from 12–18 h and 18–24 h, the highest percentage of host acceptance was found for the Cry1F-resistant strain. Although there is evidence that the dispersal behaviour of Lepidoptera larvae is the result of genetic programming^{19,20}, the effect of toxins on survival, combined with larval behaviour, at different exposure times intervals also strongly affects the dispersal rate of neonates^{12,19}.

Regarding the preferential response of neonates to feeding on plant tissues, a higher *FPD* was generally found for non-Bt cotton than for Bt cotton. The lowest percentage of *IFP* was recorded on Bt cotton for the susceptible strain. It was common for host plants that contain toxins to stimulate neonate dispersal well before feeding (pre-feeding dispersal) and/or after feeding (*FPD*)¹⁵, but this was species specific. At an exposure time of 0–6 h, we observed the lowest percentage of *IFP* for Cry1F-resistant larvae. From 0–6 h, there was no difference in host acceptance of *Alabama argillacea* Hübner (Lepidoptera: Noctuidae) larvae between Bt and non-Bt cotton plants¹². In our study, the highest dispersal rate for the Cry1F-resistant strain associated with plant tissue ingestion was found 18–24 h after plant infestation.

The adaptation of Cry1F-resistant insects to WideStrike and the possible fitness cost found for resistant larvae on non-Bt cotton are likely explanations for the shorter distance moved by *S. frugiperda* larvae in the absence of selection pressure (non-Bt cotton) observed via video tracking with EthoVision. According to Vélez *et al.*¹⁴, there is no strong evidence of differences between susceptible and Cry1F-resistant *S. frugiperda* and *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) strains; however, a low percentage of susceptible strains of both species abandoned the plant tissue expressing Cry1F. Horikoshi *et al.*¹⁷ showed no associated fitness costs of development time, survival and reproduction variables of the Cry1F-resistant population of *S. frugiperda*.

We observed similarity in the feeding behaviour of *S. frugiperda* on WideStrike and TwinLink cotton at the beginning of the exposure period (0–6 h) after hatching/infestation. During this exposure time, there was a relatively low percentage of insects that dispersed post-feeding (in comparison to 24 h) and a low number of insects feeding on plant tissues. The high dispersal at the beginning of the exposure time increases the probability of resistance evolution and loss of the TwinLink technology, mainly in agroecosystems where intentional or non-intentional seed mixing occurs (contamination through inadequate agronomic practices such as destruction of cotton plants after harvest, volunteer plant control, and seed saving after harvest). The implementation of strategies based on resistance management aiming to preserve both Bt technologies for WideStrike and TwinLink should use the information generated in the present study concerning the behaviour of the Cry1F-resistant strain of *S. frugiperda*. Pyramided cotton containing Cry1Ac and Cry1F was effective against susceptible and heterozygous insects but not against Cry1F-resistant insects²¹. In some cases, chemical control of *S. frugiperda* has been necessary in Brazilian agroecosystems using WideStrike because Cry1F-resistant *S. frugiperda* survives on WideStrike²². TwinLink was effective in promoting mortality of individuals resistant to the proteins Cry1F (in Herculex maize – HX), Cry1A.105 and Cry2Ab (in YieldGard VT PRO - VT), and Vip3 (in Agrisure Viptera - VIP) and of heterozygous individuals from crosses between the susceptible population with strains resistant to HX, VT, and VIP²². Technologies combining new insecticide proteins without cross resistance to and high activity against key pests applied in Brazilian agroecosystems or similar systems have been proposed^{22,23}. The use of Vip3Aa20 protein has also been recommended for maximizing the durability of Cry proteins in *S. frugiperda* control, but tactics based on the high-dose/refuge strategy are necessary to avoid the loss of Vip3Aa20 protein with resistance under field conditions²⁴. We would like to emphasize that knowledge of the behavioural ecological traits of *S. frugiperda* is also relevant for the establishment of effective resistance management programmes, but it is important to collect data relative to the behaviour of multiple pests¹⁴, especially in neotropical agroecosystems.

Here, we presented computational support for conclusions concerning the effects of larval movement in different scenarios in Bt and non-Bt cotton fields. Simulations confirmed that the mean distance of larvae to the centre of the population was smaller on non-Bt cotton plants than on Bt cotton plants, irrespective of the contamination degree. Therefore, because of the different rates of larval movement, there was lower expansion of the larval population

on non-Bt cotton fields than on Bt cotton fields. However, the levels of contamination did not interfere significantly with the results. These results can be explained as follows: first, in this modelled system, the rate of larval movement was the only variable in the simulations because the model allowed us to isolate the variable of interest. Thus, it should be noted that the model indicated that considering only the differences between the rates of larval movement of *S. frugiperda* in Bt and non-Bt cotton would not be sufficient to identify a difference in the dispersion of the whole population at the studied contamination levels (10–20%). Additionally, larval dispersal is a short-scale movement, and therefore, it is expected that its effect on the dispersion of the whole population is only observed when extreme situations are compared (e.g., most of the area contains either Bt or non-Bt crops) and not under slightly different conditions (e.g., different levels of contamination of non-Bt cotton when Bt cotton is the dominant crop).

In conclusion, the present study demonstrated that the dispersal of the Cry1F-resistant strain of *S. frugiperda* was affected by cotton varieties over time. The highest rate of *PFD* of Cry1F-resistant larvae occurred after 18 h of exposure; therefore, our results support the idea that the *PFD* at different time intervals is strain specific. In addition, at the beginning of exposure (up to 6 h) of larvae to plants with toxins, there was high degree of similarity in the feeding behaviour between populations of *S. frugiperda* kept on WideStrike and those kept on TwinLink. With respect to the sedentary behaviour of the Cry1F-resistant neonates, there is evidence that Cry1F-resistant neonates moved shorter distances when reared on non-Bt cotton than on Bt cotton; in addition, independent of contamination degree, the dispersal capacity of Cry1F-resistant larvae was smaller on non-Bt cotton than on Bt cotton. According to our findings regarding the fitness costs of *S. frugiperda* on non-Bt cotton linked to behavioural ecological traits, the present study motivates further research on the expression of genes responsible for the ethological activities of *S. frugiperda* strains on different hosts.

2.4 Methods

Spodoptera frugiperda and cotton plants were grown at the Insect Ecology and Forestry Entomology Laboratory (ESALQ/USP), Piracicaba, São Paulo, Brazil. We used the susceptible (SS) and Cry1F-resistant (RR) strains of *S. frugiperda* characterized by Farias *et al.*²⁵ Larvae-rearing stock and plants were kept in a climate-controlled chamber at 25°C with a relative humidity of 65 ± 10% and a 12-h photophase. Cotton plants expressing the genes for the Bt proteins Cry1Ab/Cry2Ae [variety FM 940 GLT (TwinLink®)] and Cry1Ac/Cry1F [variety FM 975 (WideStrike)][®] and its non-Bt isoline [variety FM 993] were used in this study. All cotton varieties were planted in plastic pots 25 cm in diameter and 40 cm in height.

2.4.1 Feeding behaviour of *S. frugiperda* strains on WideStrike and non-Bt cotton varieties. This bioassay was conducted to quantify the proportion of *S. frugiperda* neonates that dispersed and fed on Bt and non-Bt cotton plants. We used two varieties: the Bt cotton WideStrike and non-Bt cotton (untransformed isoline FM 993). The experiment was conducted in 2 × 2 × 4 factorial randomized blocks with total of 4 blocks, where each block was divided into one combination of strains (SS or RR) and variety (Bt or non-Bt) and four time intervals (6, 12, 18, and 24 h after artificial infestation). We assessed each time interval in different plants independently to avoid repeated sampling in the same experimental unit. The experimental unit consisted of a Bt or non-Bt cotton plant that reached the six-leaf stage and received 20 neonates of *S. frugiperda* (0–24 h old) released on a leaf in the apical region of the plant. Each plant was covered with an organza bag. According to each time interval, the bags were inspected, and the larvae were removed with a brush. Each larva was categorized into insects found on the plant or outside the plant. Insects that fed on plant tissues and were found outside the plants were classified as *PFD*, while neonates that fed on plant tissues and were found on plants were classified as *IFP*. To determine whether the larvae had fed, we used microscope slides according to the method adopted by Razze *et al.*¹⁹ and Ramalho *et al.*¹²

2.4.2 Feeding behaviour of Cry1F-resistant *S. frugiperda* on WideStrike, TwinLink, and non-Bt cotton. An experimental design similar to that mentioned for the first bioassay was used here. We used one population resistant to Cry1F and three varieties of cotton: Bt cotton varieties (WideStrike and TwinLink) and non-Bt cotton (untransformed

cotton variety FM 993). We quantified the following variables: survival, dispersal, *PFD*, and *IFF*; in addition, we calculated the distance matrix based on proportion data.

2.4.3 Movement analysis of the *S. frugiperda* strains with video tracking. We conducted a bioassay to examine the differences between the behavioural traits of *S. frugiperda* neonates exposed to Bt and non-Bt cotton plants. We used an experimental design structured as randomized blocks, with two varieties (Bt and non-Bt cotton plants) and two strains (RR and SS) of *S. frugiperda*. The movement behaviour of larvae was tracked with automated video-tracking software (EthoVision[®])²⁶ for 12 h.

An infrared camera allowed EthoVision to locate the bodies of *S. frugiperda* larvae. Recordings of the paths of individual larvae, so-called “tracks”, were made for each individual larva. We used four replicates (= larvae) per treatment. The experimental unit consisted of 1 larva; each insect was placed in a Petri dish with one leaf of Bt or non-Bt cotton collected at the six-leaf stage. The insects were kept in a climate chamber regulated as mentioned before. EthoVision was used to calculate the variables related to the distance moved by the larvae and to the velocity of neonates. The program generated the following variables: distance moved (cm), mean velocity (cm/s), and continuous mobility period (s)²⁷.

2.4.4 Spatial model. To verify the influence of different movement behaviours studied in first and second bioassays on the distribution of the larval population of *S. frugiperda*, we used a spatial model programmed in C and developed by Garcia *et al.*¹⁶. All probability functions defined by Garcia *et al.*¹⁶ are presented in Supplementary Equations S1. In this model, a lattice of cells was created to represent the dynamics of immature (larvae and pupae) and adult (only females) insects. Each cell represented either a Bt or a non-Bt cotton plant, and immature and adult insects could occupy the same cell without interfering with each other. In relation to the dynamics of the immature stage, each cell could be occupied by an immature insect (receiving a value equal to 1) or remain empty (receiving a value equal to 0). In relation to the dynamics of the adult stage, each cell could be empty (0) or occupied by a maximum of 10 female adult insects.

A cell occupied by an immature insect in the lattice could become empty due to larval mortality or metamorphosis. Likewise, a cell not occupied by an immature insect could become occupied due to the oviposition of a female adult. A cell not occupied by a female adult could become occupied due to the metamorphosis of an immature insect in the cell. A

cell occupied by an adult could become empty due to mortality. We assumed that each adult could randomly move to any cell within a radius of 35 cells from its own cell. In each step, a cell within this radius was sorted out, and in cases where the carrying capacity was not reached, the adult moved towards it.

In this work, six different conditions were simulated by using this model. Within group 1, we had the following conditions: **a1.** resistant individuals in Bt areas without contamination of non-Bt cotton, **a2.** resistant individuals in Bt areas contaminated with 10% non-Bt cotton, and **a3.** resistant individuals in Bt areas contaminated with 20% non-Bt cotton. Within group 2, we had the following conditions: **b1.** resistant individuals in non-Bt areas without contamination of Bt cotton, **b2.** resistant individuals in non-Bt areas contaminated with 10% Bt cotton, and **b3.** resistant individuals in non-Bt areas contaminated with 20% Bt cotton.

The functions were the same in non-Bt cotton and Bt cotton areas. However, larval dispersal was different in each area, considering the results obtained in the bioassay of movement analysis with video tracking. Each simulation was repeated 50 times, and for each time, we calculated the mean distance of larvae represented in the lattice from the centre of the distribution of the larval population after 300 time steps. Larval density was also calculated for each condition.

2.4.5 Statistical analysis. The data proportions of *PFD*, *IFP*, survival, and dispersal (from the first bioassay: feeding behaviour of *S. frugiperda* strains on WideStrike® and non-Bt cotton varieties) were submitted to principal component analysis (PCA). PCA and Pearson's correlation analysis were performed by following the SAS Procedures²⁸ PRINCOMP and CORR, respectively. Regarding the PCA, the data were standardized by dividing the difference of each data point and the arithmetic mean of each variable by the standard deviation of the variable.

Survival data of *S. frugiperda* were subjected to analysis of deviance to assess the significance of the interactions among the factors strain, variety and time interval ($P = 0.05$) with a quasi-binomial generalized linear model. The goodness of fit was evaluated using half-normal plots with a simulated envelope²⁹ using R³⁰. Survival data were also submitted to logistic regression with PROC GENMOD²⁸. Additionally, data of *PFD*, *IFP*, and dispersal from the same bioassay were subjected to analysis of variance with PROC GLM²⁸ to determine whether there were interactions between factors, and these data were processed

through the Box-Cox method³¹. The hypothesis of equality was tested by Tukey's test ($P = 0.05$).

According to data from the bioassay of the feeding behaviour of *S. frugiperda* resistant to Cry1F on WideStrike, TwinLink, and non-Bt cotton varieties, the similarity and clustering treatments were analysed by cluster analysis using the average linkage method and TREE procedures²⁸.

The variables calculated by the movement analysis of the *S. frugiperda* strains with video tracking were subjected to analysis of variance with PROC GLM²⁸ to determine whether there were interactions between strain and variety. The hypothesis of equality was tested by Tukey's test ($P = 0.05$). The equality between the results of each simulation from the spatial model was tested by using the Tukey-Kramer test ($P = 0.05$).

References

1. Martinelli, S., Barata, R. M., Zucchi, M. I., Silva-Filho, M. C. & Omoto, C. Molecular variability of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) populations associated to maize and cotton crops in Brazil. *J. Econ. Entomol.* **99**, 519–526 (2006).
2. Ellstrand, N. C., Prentice, H. C. & Hancock, J. F. Gene flow and introgression from domesticated plants into their wild relatives. *Annu. Rev. Ecol. Syst.* **30**, 539–563 (1999).
3. Glaum, P. R., Ives, A. R. & Andow, D. A. Contamination and management of resistance evolution to high-dose transgenic insecticidal crops. *Theor. Ecol.* **5**, 195–209 (2012).
4. Han, P., Velasco-Hernández, C. M., Ramirez-Romero, R. & Desneux, N. Behavioral effects of insect-resistant genetically modified crops on phytophagous and beneficial arthropods: a review. *J. Pest Sci.* **89**, 859–883 (2016).
5. Nascimento, A. R. B., Farias, J. R., Bernardi, D., Horikkoshi, R. J. & Omoto, C. Genetic basis of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) resistance to the chitin synthesis inhibitor lufenuron. *Pest Manag. Sci.* **72**, 810–815 (2016).
6. Omoto, C., Bernardi, O., Salmeron, E., Sorgatto, R. J., Dourado, P. M., Crivellari, A., Carvalho, R. A., Willse, A., Martinelli, S. & Head, G. Field-evolved resistance to Cry1Ab maize by *Spodoptera frugiperda* in Brazil. *Pest Manag. Sci.* **72**, 1727–1736 (2016).
7. Bernardi, O., Bernardi, D., Horikoshi, R. J., Okuma, D. M., Miraldo, L. L., Faretto, J., Medeiros, F. C. L., Burdc, T. & Omoto, C. Selection and characterization of resistance to the Vip3Aa20 protein from *Bacillus thuringiensis* in *Spodoptera frugiperda*. *Pest Manag. Sci.* **72**, 1794–1802 (2016).
8. Farias, J. R., Andow, D. A., Horikoshi, R. J., Bernardi, D., Ribeiro, R. D., Nascimento, A. R., Santos, A. C. & Omoto, C. Frequency of Cry1F resistance alleles in *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Brazil. *Pest Manag. Sci.* **72**, 2295–2302 (2016).
9. Sorgatto, R. J., Bernardi, O. & Omoto, C. Survival and development of *Spodoptera frugiperda* and *Chrysodeixis includens* (Lepidoptera: Noctuidae) on Bt cotton and implications for resistance management strategies in Brazil. *Environ. Entomol.* **44**, 186–192 (2015).

10. Miraldo, L. L., Bernardi, O., Horikoshi, R. J., Amaral, F. S. A., Bernardi, D. & Omoto, C. Functional dominance of different aged larvae of Bt-resistant *Spodoptera frugiperda* (Lepidoptera: Noctuidae) on transgenic maize expressing Vip3Aa20 protein. *Crop Prot.* **88**, 65–71 (2016).
11. Allen, A. M. & Singh, N. J. Linking movement ecology with wildlife management and conservation. *Front. Ecol. Evol.* **3**, 155; 10.3389/fevo.2015.00155 (2016).
12. Ramalho, F. S., Pachú, J. K. S., Lira, A. C. S., Malaquias, J. B. & Zanuncio, J. C. Feeding and dispersal behavior of the cotton leafworm, *Alabama argillacea* (Hübner) (Lepidoptera: Noctuidae), on Bt and non-Bt cotton: implications for evolution and resistance management. *PLoS ONE.* **9**, e111588; 10.1371/journal.pone.0111588 (2014).
13. Wangila, D. S., Leonard, B. R., Bai, Y., Head, G. H. & Huang, F. Larval survival and plant injury of Cry1Ab-susceptible, -resistant, and -heterozygous genotypes of the sugarcane borer on transgenic corn containing single or pyramided Bt genes. *Crop Prot.* **42**, 108–115 (2012).
14. Vélez, A. M., Alves, A. P., Blankenship, E. E. & Siegfried, B. D. Effect of Cry1F maize on the behavior of susceptible and resistant *Spodoptera frugiperda* and *Ostrinia nubilalis*. *Entomol. Exp. Appl.* **159**, 37–45 (2016).
15. Zalucki, M. P., Clarke, A. R. & Malcolm, S. B. Ecology and behavior of first instar larval Lepidoptera. *Annu. Rev. Entomol.* **47**, 361–393 (2002).
16. Garcia, A. G., Cõnsoli, F. L., Ferreira, C. P. & Godoy, W. A. C. Predicting evolution of insect resistance to transgenic crops in within-field refuge configurations, based on larval movement. *Ecol. Complex.* **28**, 94–103 (2016).
17. Horikoshi, R. J., Bernardi, O., Bernardi, D., Okuma, D. M., Farias, J. R., Miraldo, L. L., Amaral, F. S. & Omoto, C. Near-isogenic Cry1F-resistant strain of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) to investigate fitness cost associated with resistance in Brazil. *J. Econ. Entomol.* **109**, 1–6 (2015).
18. Kaiser, H. F. An index of factorial simplicity. *Psychometrika.* **39**, 31–36 (1974).

19. Razze, J. M., Mason, C. E. & Pizzolato, T. D. Feeding behavior of neonate *Ostrinia nubilalis* (Lepidoptera: Crambidae) on Cry1Ab Bt corn: implications for resistance management. *J. Econ. Entomol.* **104**, 806–813 (2011).
20. Goldstein, J. A., Mason, C. E. & Pesek, J. Dispersal and movement behavior of neonate European corn borer (Lepidoptera: Crambidae) on non-Bt and transgenic Bt corn. *J. Econ. Entomol.* **103**, 331–339 (2010).
21. Yang, F., Kerns, D. L., Brown, S., Kurtz, R., Dennehy, T., Braxton, B., Head, G. & Huang, F. Performance and cross-crop resistance of Cry1F-maize selected *Spodoptera frugiperda* on transgenic Bt cotton: implications for resistance management. *Sci. Rep.* **6**, 28059; 10.1038/srep28059 (2016).
22. Horikoshi, R. J., Bernardi, D., Bernardi, O., Malaquias, J. B., Okuma, D. M., Miraldo, L. L., Amaral, F. S. A. & Omoto, C. Effective dominance of resistance of *Spodoptera frugiperda* to Bt maize and cotton varieties: implications for resistance management. *Sci. Rep.* **6**, 34864; 10.1038/srep34864 (2016).
23. Farias, J. R., Horikoshi, R. J., Santos, A. C. & Omoto, C. Geographical and temporal variability in susceptibility to Cry1F toxin from *Bacillus thuringiensis* in *Spodoptera frugiperda* (Lepidoptera: Noctuidae) populations in Brazil. *J. Econ. Entomol.* **107**, 2182–2189 (2014).
24. Bernardi, O., Bernardi, D., Horikoshi, R. J., Okuma, D. M., Miraldo, L. L., Faretto, J., Medeiros, F. C. L., Burdc, T. & Omoto, C. Selection and characterization of resistance to the Vip3Aa20 protein from *Bacillus thuringiensis* in *Spodoptera frugiperda*. *Pest Manag. Sci.* **72**:1794–1802 (2016).
25. Farias, J. R., Andow, D. A., Horikoshi, R. J., Sorgatto, R. J., Fresia, P., Santos, A. C. & Omoto, C. Field-evolved resistance to Cry1F maize by *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Brazil. *Crop Prot.* **64**, 150–15 (2014).
26. Noldus, L. P., Spink, A. J. & Tegelenbosch, R. A. EthoVision: a versatile video tracking system for automation of behavioral experiments. *Behav. Res. Meth. Ins. C.* **33**, 398–414 (2001).

27. Noldus, L. P., Spink, A. J. & Tegelenbosch, R. A. Computerised video tracking, movement analysis and behaviour recognition in insects. *Comput. Electron. Agr.* **35**, 201–227 (2002).
28. SAS Institute Inc., *SAS/STAT: 9.3 User's Third Edition*, SAS Institute Inc, Cary, NC (2010).
29. Demétrio, C. G., Hinde, J. & Moral, R. A. Models for overdispersed data in entomology in *Ecological Modelling Applied to Entomology*, (ed. Ferreira C. P. & Godoy W. A.) pp. 219–259 (Springer, 2014).
30. R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. <http://www.R-project.org/>.
31. Box, G. E. P. & Cox, D. R. An analysis of transformations. *J. Roy. Stat. Soc. B. Met.* **26**, 211–252 (1964).

Figure Legends

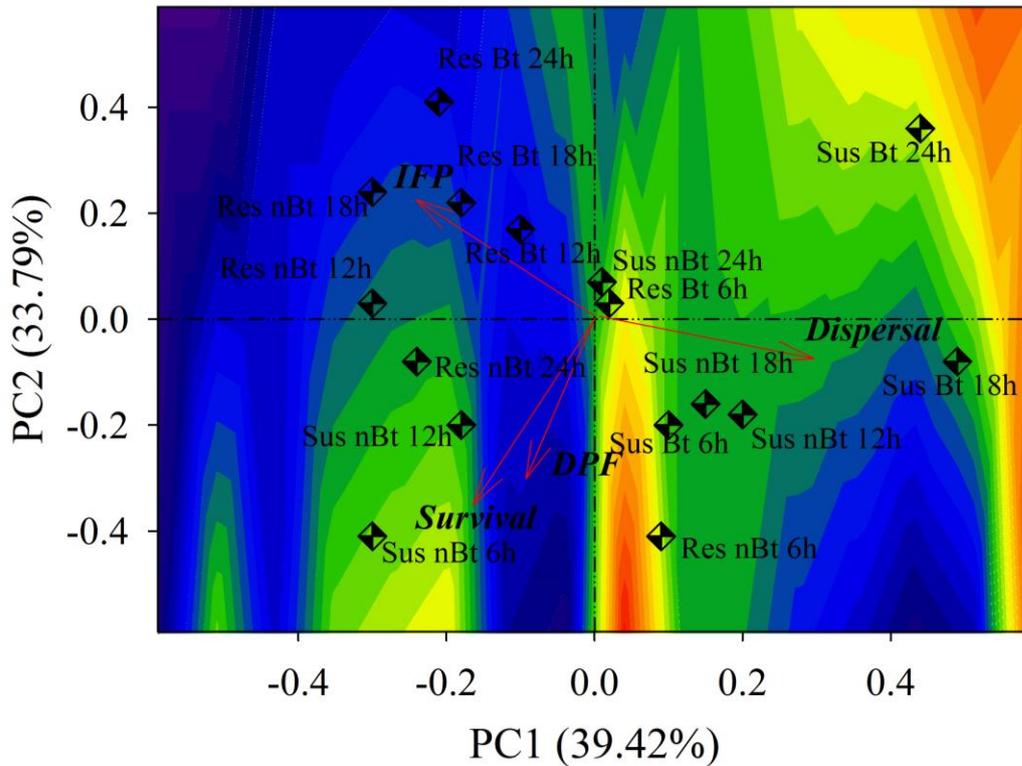


Figure 2.1. Biplot of variables: survival, dispersal rate, post-feeding dispersal (PFD) and individual proportions of *Spodoptera frugiperda* neonate that were found on varieties and feeding on plant tissues (IFP). The dots represent the following treatments: larvae of susceptible strain on Bt cotton (Sus Bt) and non-Bt cotton (Sus nBt) and Cry1F-resistant larvae on Bt cotton (Res Bt) and non-Bt cotton (Res nBt) at the time intervals 0–6 h, 6–12 h, 12–18 h, and 18–24 h. Contour area was defined based on the eigenvectors of each component.

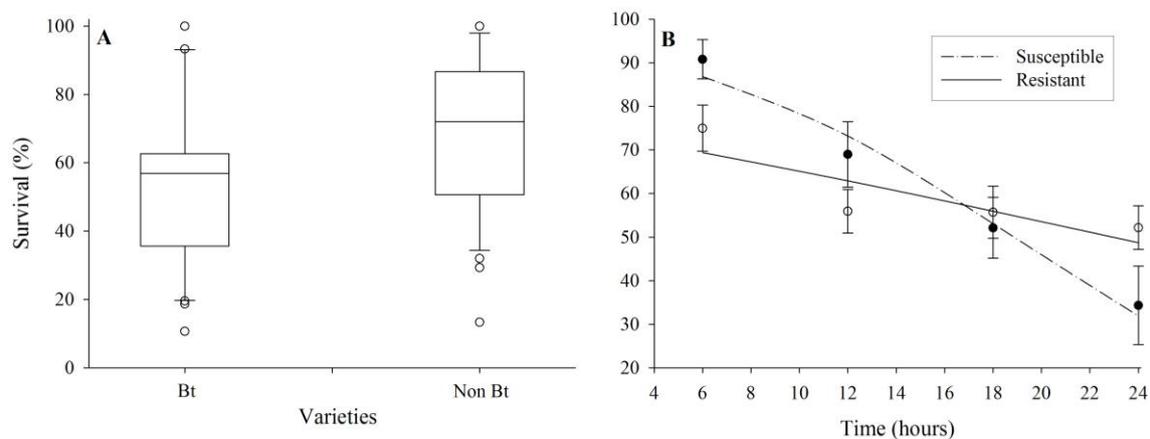


Figure 2.2 (a) Boxplot of *Spodoptera frugiperda* survival (%) on Bt (WideStrike) and non-Bt cotton. (b) Survival (%) (mean \pm SE) of susceptible (exp-0.1470time+2.7690/1+exp-0.1470time+2.7690) and Cry1F-resistant (exp-0.0483time+1.1089/1+exp-0.0483time+1.1089) strains as a function of time. Original data.

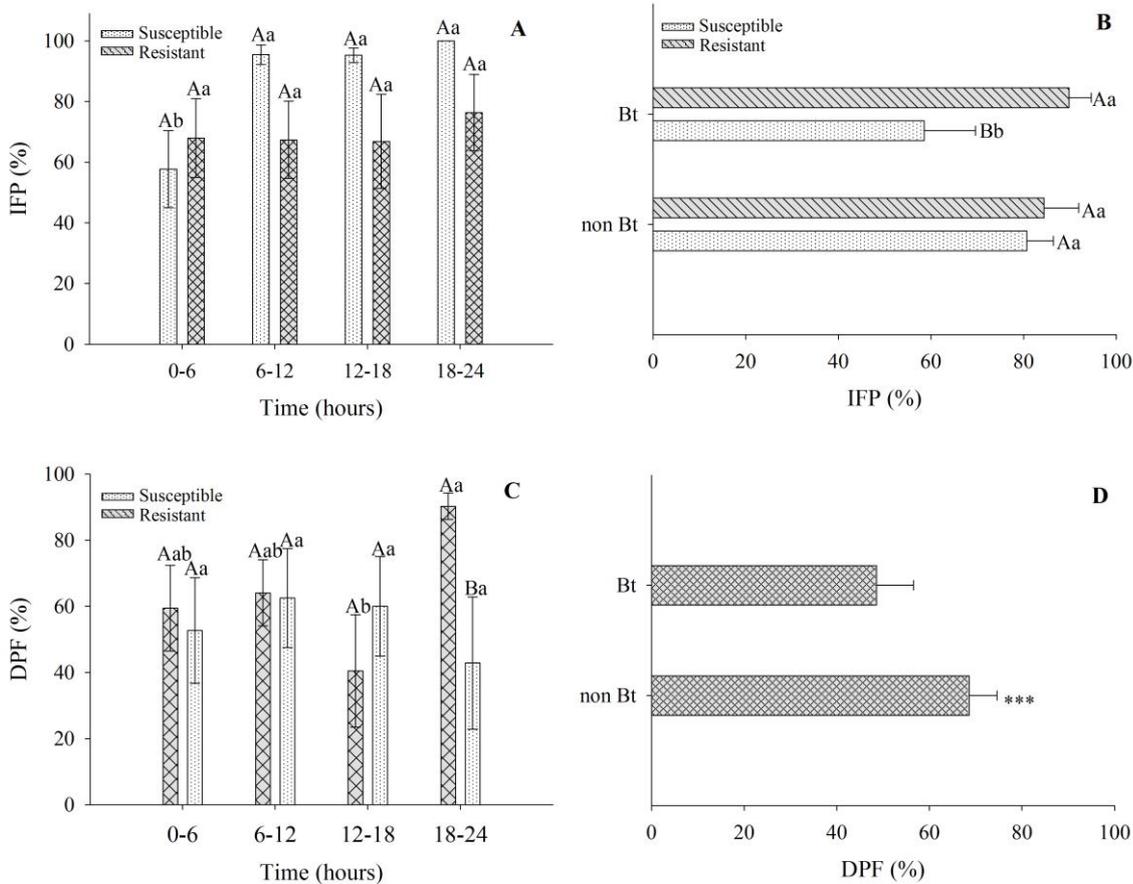


Figure 2.3. (a) Percentage (mean \pm SE) of neonates of susceptible and Cry1F-resistant *Spodoptera frugiperda* strains that were found on plants and that fed on plant tissues (IFP) at the time intervals 0–6 h, 6–12 h, 12–18 h and 18–24 h or (b) that fed on plant tissues from Bt or non-Bt cotton near isoline plants. (c) Percentage (mean \pm SE) of post-feeding dispersal (DPF) of *S. frugiperda* strains at different time intervals (h) or (d) that fed on Bt and non-Bt cotton. Means followed by the same capital letter (comparing bars of the different strains on same axis) and lowercase letter (comparing bars of time (Fig. 3a and Fig. 3c) or variety (Fig. 3c) in the same strain on different axis) are not significantly different as determined by Tukey's test ($P = 0.05$). *Asterisks on Figure 3d represent differences between means. Original data.**

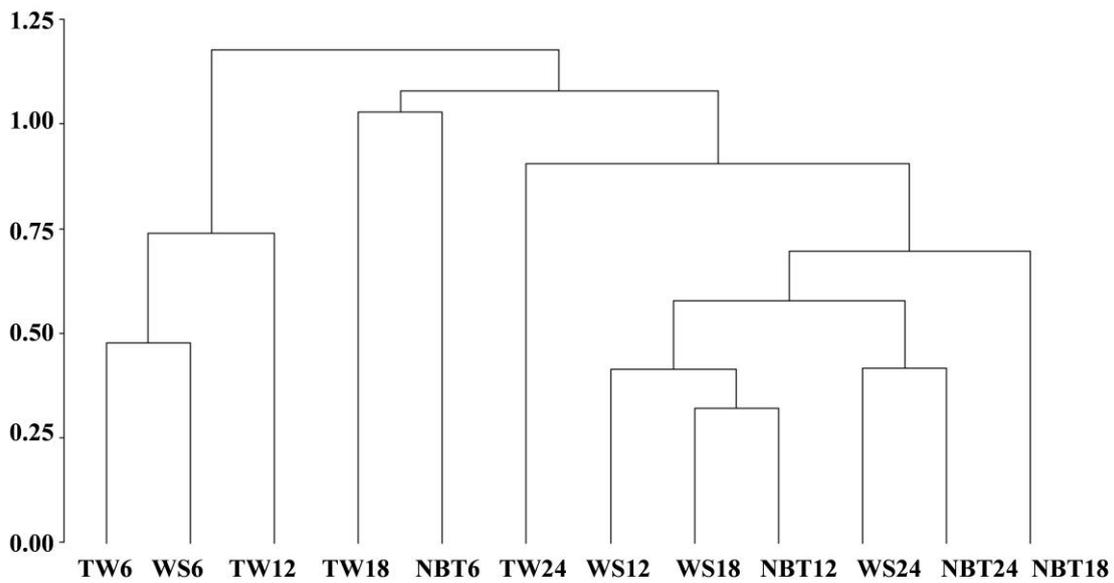


Figure 2.4. Mean distance between clusters represented by the treatments TwinLink (TW), WideStrike (WS) and non-Bt (NBT) cotton at four time intervals after artificial infestation: 0–6 h (6), 6–12 h (12), 12–18 h (18) and 18–24 h (24). Cluster average linkage method. Distance matrix based on proportion data for survival, dispersal, post-feeding larval dispersal and neonates of *Spodoptera frugiperda* found on plants and that fed on plant tissues.

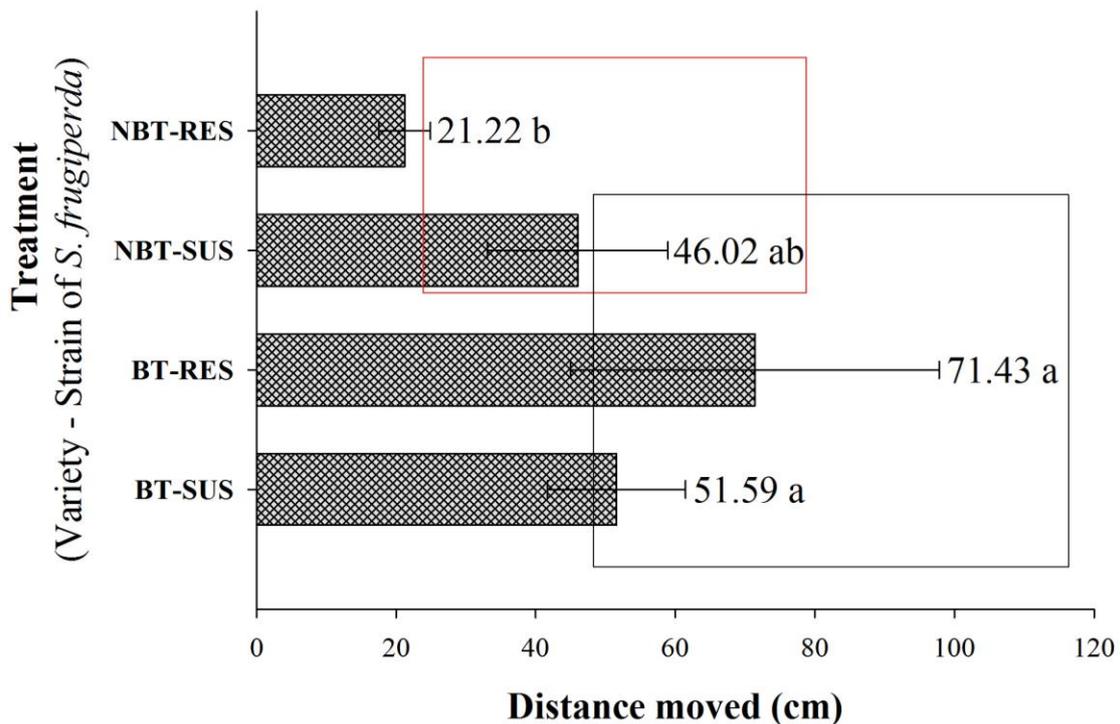


Figure 2.5. Distance moved (mean \pm SE) by susceptible and Cry1F-resistant *Spodoptera frugiperda* strains on Bt and non-Bt cotton. Means followed by the same letters or by rectangles of the same colour were not significantly different as determined by Tukey's test ($P = 0.05$). Original data.

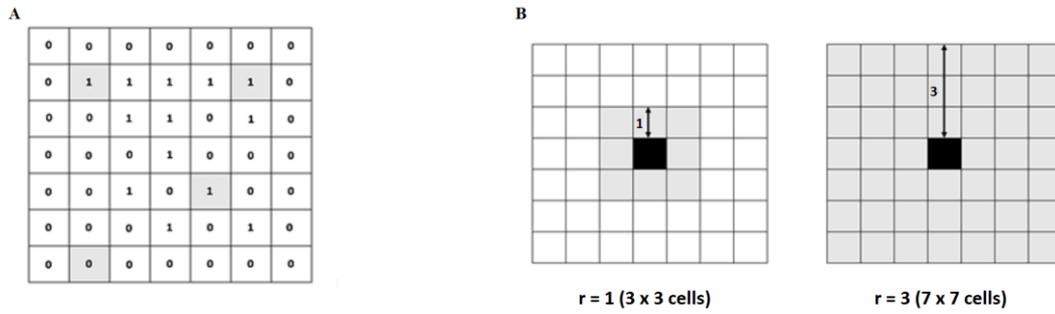


Figure 2.6. Spatial model representation. Grey cells represent the contamination in a homogeneous landscape. (a) Cells receiving a value equal to 0 are empty, and cells receiving a value equal to 1 are occupied by an insect. Cells dynamics are determined by the probability functions. (b) Representation of the neighbourhood in which larva dispersal occurred on non-Bt cotton and Bt cotton crops.

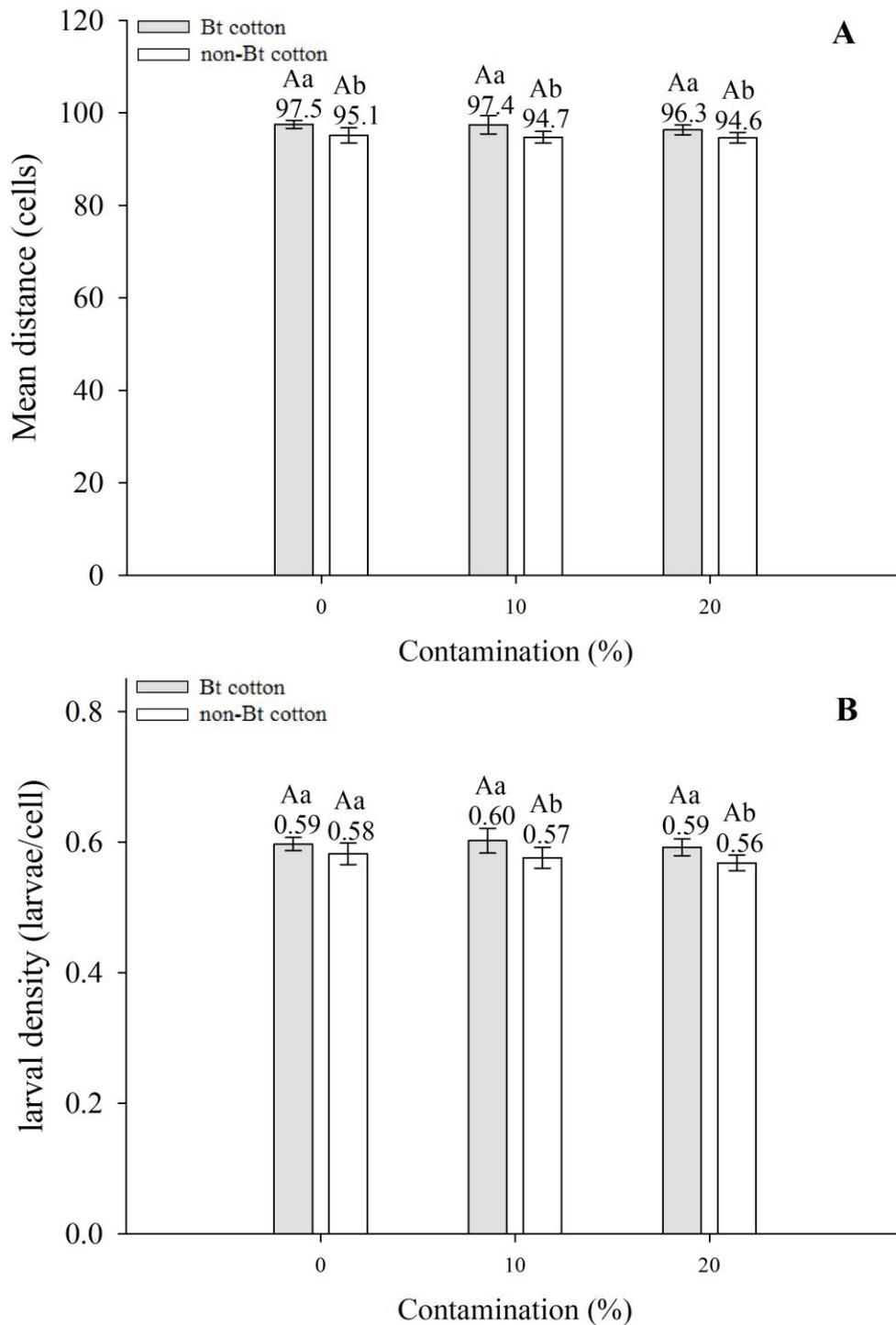


Figure 2.7. (a) Distance (mean \pm SE) of *Spodoptera frugiperda* larvae from the centre of the population distribution. (b) Larval density (mean \pm SE) of *S. frugiperda* in the lattice of cells. Means followed by different uppercase letters above contamination levels from the same type of crop and different lowercase letters above different types of crops with the same contamination level were significantly different as determined by Tukey's test ($P = 0.05$).

Effect	Df	Survival		Host acceptance		IFP		PFD	
		<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Block	3	0.74	0.5357	0.34	0.7954	12.47	< 0.0001	7.72	0.0003
Variety	1	11.90	0.0410	2.29	0.1369	1.40	0.2433	6.64	0.0155
Strain	1	0.26	0.6119	15.15	0.0003	9.10	0.0042	0.49	0.4888
Time	3	1.11	0.2981	0.74	0.5343	3.96	0.0137	0.55	0.6527
Variety <i>x</i> Strain	1	1.52	0.2009	1.41	0.2412	7.25	0.0111	1.04	0.3150
Variety <i>x</i> Time	3	0.44	0.7254	1.57	0.2100	0.41	0.7467	0.89	0.4533
Strain <i>x</i> Time	3	3.81	0.0199	6.04	0.0024	5.08	0.0053	3.11	0.0422
Variety <i>x</i> Strain <i>x</i> Time	3	0.38	0.7670	0.42	0.7374	0.33	0.8062	0.09	0.9642

Table 2.1. Summarized model of analysis of deviance for effects of the variety of cotton¹, strain of insect², and exposure time interval³ of neonate to plants on survival of *Spodoptera frugiperda* and analysis of variance (ANOVA) on host acceptance, percentage of neonates that were found on plants and that fed on plant tissues (IFP) and percentage of post-feeding dispersal (PFD).

¹Varieties: Bt (WideStrike) and non-Bt near isoline cotton.

²Strains: Susceptible and resistant to Cry1F.

³Time intervals: 0–6 h, 6–12 h, 12–18 h, and 18–24 h.

Strain	Exposure time interval of neonate larvae to cotton plants (h)			
	0–6	6–12	12–18	18–24
Susceptible	64.83 ±4.50Aa	59.00 ±7.50Aba	33.50 ±7.70Bb	47.98 ±9.40ABb
Cry1F- Resistant	62.68 ±5.30Aa	65.80 ±5.60Aa	75.80 ±6.50Aa	73.38 ±5.50Aa

Table 2.2. Mean percentage (\pm SE) of neonates of *S. frugiperda* that were found on the plant on Bt cotton and non-Bt cotton at the different tested time intervals. Means within the same time interval column with the same lowercase letter or means between strains within the same row with the same capital letters are not significantly different ($P = 0.05$, Tukey's test). Original data.

3 Experimental and Theoretical Landscape Influences on *Spodoptera frugiperda* Movement and Resistance Evolution in Contaminated Refuge Areas of Bt Cotton

Abstract

Transgenic cotton plants producing *Bacillus thuringiensis* (Bt) (Berliner) insecticidal proteins have contributed to the management of the main targeted lepidopteran pests. High dispersal rates between non-Bt and Bt plants in landscapes with seed contamination can speed the evolution of insect resistance. We evaluated the effect of Bt and non-Bt cotton plants on the larval dispersal pattern and survival of susceptible, Cry1F-resistant, and heterozygous genotype of *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) in pure and contaminated artificial micro-landscape. A computer model was used to analyze the consequences of *S. frugiperda* dispersal behaviour on resistance evolution in refuge areas with different contamination levels and migration of different adult genotype combinations. The biological data from artificial micro-landscape experiments were used in simulations of macro-landscape scales. The Cry1F-resistant genotype avoided non-Bt cotton. The heterozygote had a similar dispersal behaviour as the susceptible genotype when non-Bt cotton was the natal plant. Our simulations provide evidence that in refuge areas contaminated with Bt cotton plants, the evolution of resistance may be > 75-fold faster in relation to a contamination-free refuge. In conclusion, *S. frugiperda* resistance management practices in regional scales with contamination-free refuges are important to prevent losses in different crops. The current chapter was written following the manuscript submission guidelines of the journal Journal of Pest Science guidelines.

Keywords: Larval dispersal; Fall armyworm; non-Bt fields; Local adaptation

3.1 Introduction

Cotton cultivars containing lepidopteran-active insecticidal proteins derived from the bacterium *Bacillus thuringiensis* (Berliner) (Bt) have been effective against the main targeted insect pests. Current Brazilian populations of *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) are no longer controlled by some proteins present in specific transgenic events (Horikoshi et al. 2016). The intensive planting system in Brazil, mainly under irrigation, creates high selection intensity, probably resulting in rapid resistance evolution (Farias et al. 2014a; Martinelli et al. 2017). The high selection pressure associated with a small refuge area has contributed to *S. frugiperda* resistance to Cry1-proteins, as documented in the recent years to Cry1Ab (Omoto et al. 2016), Cry1F (Farias et al. 2014b), and cross resistance to Cry1Ab and Cry1A.105 (Bernardi et al. 2015). Refuge areas with non-Bt cotton are vital components of the Insect Resistance Management (IRM) programs and are a source of large numbers of susceptible target insects of Bt cotton (Head and Dennehy 2010). They are placed within a certain distance of Bt cotton fields so that any resistant insects emerging from the Bt cotton areas will likely mate with susceptible insects from the non-Bt refuge areas (Head and Dennehy 2010).

In developing countries like Brazil another significant concern about *S. frugiperda* resistance management for Bt crops is the common practice of saving seeds and using them in the following years, creating contamination scenarios in Bt cotton fields and refuge areas (Malaquias et al. 2017). The movement of target pests between non-Bt and Bt cotton in a seed mixture may affect the effective dominance of Bt cotton (Ramalho et al. 2014, 2017) and hasten resistance evolution (Mallet and Porter 1992). The impact of Bt crops on arthropod behavioural traits such as immature insect feeding, and locomotion have recently been studied (Han et al. 2016). This information may improve IRM programs (Ramalho et al. 2017); particularly, when in some conditions, such as intentional or non-intentional use of seed mixtures combined with the high dispersal rates of many Lepidoptera between non-Bt and Bt plants, which can favor the evolution of insect resistance (Caprio et al. 2016). The intentional seed mixture, also called refuge in the bag, is the mixing of a percentage of non-transgenic seeds directly into bags with transgenic plants (Yang et al. 2014). Additionally, the seed mixture may occur in a non-intentional way through cross-pollination (Caprio et al. 2012; Yang et al. 2014), presence of volunteer plants in Bt fields and the practice of harvesting and saving seed for use in the following year (Malaquias et al. 2017).

Compliance with the requirement for structured refuges areas with non-Bt crops could contribute to delay the evolution of resistance of *S. frugiperda* to Cry1 insecticidal proteins in South America (Martinelli et al. 2017). In addition, *S. frugiperda* is a strong migrant, and the spread of Cry1 resistant individuals between different geographic regions is possible (Miller and Sappington 2017). The impact of seed mixtures on insect movement and survival has also been a subject of scientific discussions, considering both cases of intentional or unintentional seed mixtures (Malaquias et al. 2017). However, there is a gap of information about the impact of the neighborhood structure shaped by the seed mixture on the mobility, survival and consequences of these ecological behavioural traits on resistance evolution in neo-tropical lepidopteran species such as *S. frugiperda*. Both larval interplant movement and adult migration from other areas to Bt fields and refuge areas have strong effects on resistance evolution of lepidopteran pests to Bt cotton (Head and Dennehy 2010). Nevertheless, no study has yet investigated the combination of information about larval dispersal in refuge areas within micro-landscapes with simulated scenarios of contamination and differential genotypic migration on evolution of resistance of *S. frugiperda* at a macro-landscape level.

It is possible to hypothesize that the contamination of non-Bt cotton refuges with Bt cotton plants along with the high dispersal rates of Cry1F-resistant *S. frugiperda* immature and adults may cause loss of the essential function of the refuge, which is the production of susceptible insects (Head and Greenplate 2012). A second hypothesis in our current research is that Cry1F-resistant larvae may have a lower exploitation of sites with non-Bt cotton than susceptible and heterozygous genotypes. Malaquias et al. (2017) showed that Cry1F-resistant *S. frugiperda* larvae exhibit low movement on non-Bt cotton leaves, but their study did not examine the effects of multiple-choice conditions on dispersal and survival of the genotypes, and the heterozygous genotype was not considered in this study. In addition, some genetic models suggest that the dominance of pest resistance to Bt crops may be increased for a seed mixture (Mallet and Porter 1992), therefore, a third hypothesis is that the contamination of refuge areas with Bt cotton increases the dominance and consequently accelerates the resistance evolution of *S. frugiperda*.

Simple behavioural responses in local interactions may be incorporated in computational analysis to produce large-scale patterns such as neighborhood effects on larval dispersal to understand the mechanisms involved in the dispersal process (Lima et al. 2009). Thus, the combination of biological information related to larval dispersal patterns in micro-landscapes with computer simulations applied at the macro-landscape level may be an

important guide for regulators in decision making regarding resistance management of *S. frugiperda* in South America (Garcia et al. 2016). In this context, the aim of this research was to study the impact of the artificial micro-landscapes with Bt and non-Bt cotton plants in choice and no-choice treatments on larval dispersal pattern and survival of *S. frugiperda* genotypes. In addition, we implemented a computer model of selection with functions of probability and associated with a non-parametric bootstrap to estimate the effects of larval dispersal in *S. frugiperda* on the resistance frequency in refuge areas simulating different contamination levels with hypothetical combinations of adult genotypic migration.

3.2 Material and Methods

Spodoptera frugiperda and cotton plants were grown at Insect Ecology and Forestry Entomology Laboratory (ESALQ/USP), Piracicaba, São Paulo, Brazil. We used susceptible, Cry1F-resistant, and heterozygous genotypes characterized in *S. frugiperda* Brazilian populations (Farias et al. 2014b). The procedures for constructing the near-isogenic Cry1F-resistant *S. frugiperda* and for producing of the heterozygote were the same used by Horikoshi et al. (2015).

Larvae-rearing stock and plants were kept in a climate-controlled Phytotron at 25°C with a relative humidity of 70±10% and a 12-h photophase. Cotton plants expressing Cry1Ac/Cry1F [cultivar FM 975 (WideStrike®)] and its non-Bt isoline [cultivar FM 993] were used in this study. All cotton cultivars were planted separately in plastic pots (25 cm in diameter and 40 cm in height) with organic substrate. The plants were used in the experiment when they reached the four-leaf stage.

3.2.1 Effects of the artificial micro-landscapes on dispersal and survival of *S. frugiperda* genotypes

The effect of Bt or non-Bt plants on neonate dispersal and larval survival was evaluated with choice and no-choice treatments. In this study recently hatched neonate ≤ 24 h old were used. The experimental design was a randomized complete block with following treatments:

- **T₁**: non-Bt cotton plant in the centre (**C**) of the arena, and non-Bt cotton plants in the adjacent sites to centre of the arena (*NW*, *W*, *SW*, *N*, *S*, *NE*, *E* and *SE*) (Figure 3.1).

<i>NW</i>	<i>N</i>	<i>NE</i>
<i>W</i>	C	<i>E</i>
<i>SW</i>	<i>S</i>	<i>SE</i>

Fig. 3.1 Grid representation of the sites that received Bt or non-Bt cotton plants. **C** cell represents the centre of the arena. The neighbourhood cells adjacent to the central plant are represented by the following cells (coordinates of the wind rose): *NW*(NorthWest), *W*(West), *SW*(SouthWest), *N*(North), *S*(South), *NE*(NorthEast), *E*(East) and *SE*(SouthEast)

- **T₂**: non-Bt cotton plant in the centre and Bt cotton plants in the adjacent cells.

- **T₃**: Bt cotton plant in the centre and non-Bt cotton plants in the adjacent cells.
- **T₄**: Bt cotton plant in the centre and Bt cotton plants in the adjacent cells.

The treatments 1 (with only non-Bt cotton) and 4 (with only Bt cotton) were considered no-choice treatments, while 2 and 3 were called choice treatments. We used choice treatments with alternation of Bt and non-Bt cotton to simulate scenarios of contamination between these cultivars. We considered each treatment as an artificial micro-landscape, we used 4 replications for each treatment. An arena received 80 neonates of *S. frugiperda* released on C cell of the arena (**Fig. 3.1**). Each arena was covered with a PVC bag (40 cm in diameter and 60 cm in height). The bags were inspected 24 h after releasing the neonates in the arenas to quantify the proportion of the larvae in the different plants in each micro-landscape (treatments). The larvae were removed with a brush, each larva was categorized into insects found in three sites: insects found in the centre, in the adjacent plants or any other sites in the bags, such as floor, wall or ceiling.

The frequency distribution of *S. frugiperda* genotypes on adjacent plants in each directional coordinate was used to test the hypothesis of a uniform circular distribution of larvae per treatment. This experiment was carried out to investigate how heterogeneous micro-landscape, in terms of natal (centre) and adjacent plants, could affect the dispersal pattern of *S. frugiperda* genotypes. In other words, if after the release of larvae on the centre plant with Bt or non-Bt cotton plants, there was a bias in the *S. frugiperda* genotypic distribution around the adjacent plants while searching for a good food source (perhaps due to intraspecific interactions such as competition) or whether the population follows a uniform or random spread to the adjacent plants.

Insects were fed during the entire larval stage on the cultivar that they were found. Larvae found on the bag were fed with the same cultivar of the adjacent plants for that treatment. The insects were kept in 50-mL transparent plastic cups. A seedling of cotton was inserted inside of the cup and covered with an acrylic cover. Larval mortality was recorded daily until pupation of the insects.

3.2.2 Statistical analysis

We carried out all statistical analyzes using R (version 3.4.3; R Foundation for Statistical Computing, Vienna, AT, 2017). A binomial generalized linear model was used to assess the effects of significance ($P = 0.05$) of the interactions among the following factors:

treatments and genotype of insect for proportion of survival of the larval stage; And a quasi-binomial generalized linear model was used to assess the effects of significance ($P = 0.05$) of the interactions among treatments and genotype of insect on dispersal rate. The goodness of fit was evaluated using half-normal plots with a simulated envelope (Demétrio et al. 2014) employing the *hnp* package (Moral et al. 2017).

Frequency distributions of insects on the directional coordinates were used to test the hypothesis of uniform circular and approximately uniformly distributed (von Mises distribution) with the Watson's test using the *circular* (Agostinelli and Lund 2017) and *CircStats* packages (Agostinelli 2012). The mean distribution in each treatment was expressed as rose diagrams. In addition, we showed the Bayesian Confidence Region (*BCR*) in **Fig. 3.2, 3.3** and **3.4**.

The Bayesian Confidence Region (*BCR*) were calculated using the observations of the treatment, given $Y = y$, the interval $[a, b]$, being $(1-\alpha)100\%$ Bayesian Credible Interval (*BCI*) for X , then if the posterior probability of X being in $[a, b]$ is equal to $(1-\alpha)$, therefore, $(1-\alpha) = P(a \leq X \leq b | Y = y)$. Therefore, the *BCR* consists of the range between the 2.5% and 97.5% quantiles. For the analysis of the *BCR*, we used 30,000 iterations using a Monte Carlo and Markov chains (MCMC) process with three strings for each parameter and a burn-in of 5,000 samples. The convergence of the chains was checked by means of graphical analysis (data not included). The parameters were estimated using the *R2Openbugs* package (Sturtz et al. 2005).

3.2.3 Population genetic model

3.2.3.1 The impact of *S. frugiperda* adult genotype migration and contamination in refuge areas on resistance evolution

A computer model was implemented in R (version 3.4.3; R Foundation for Statistical Computing, Vienna, AT, 2017). The model is capable of examining the impact of contamination in refuge areas with Bt cotton and the effect of migration of the three genotypes. We considered two Bt toxins, with resistance to each of the two Bt-toxins being governed by diallelic, independently segregating loci. The model focused on the change in frequency of a single resistance locus for each toxin. We are assuming that genes conferring resistance are already present in the population and that resistance to each toxin is coded by a single gene. Therefore, there is one single locus associated with resistance for each of the two

toxins (toxin 1 and toxin 2). Each locus is characterized by two alleles. We assumed in both cases (1 and 2) that there was no linkage, and denoted R_1 and R_2 corresponding to alleles conferring resistance to Bt-toxin 1 and Bt-toxin 2, respectively. The same denotation was applied to alleles that promote susceptibility (S_1 and S_2). The total different genotypes of offspring are: $S_1S_1S_2S_2$ (1); $S_1S_1S_2R_2$ (2), $S_1R_1S_2S_2$ (3), $S_1R_1S_2R_2$ (4), $S_1S_1R_2R_2$ (5), $S_1R_1R_2R_2$ (6), $R_1R_1S_2S_2$ (7), $R_1R_1S_2R_2$ (8) and $R_1R_1R_2R_2$ (9).

Model scenarios examined resistance evolution with three contamination levels (%) in refuge (non-Bt cotton) areas with eight possible combinations of adult genotypic migration. We only examined the outputs from non-Bt cotton areas, because the impact of contamination or seed mixtures on resistance evolution on Bt fields is already well known in Brazilian populations of *S. frugiperda* (Garcia et al. 2016).

The criterion for a high level of the resistant genotype in refuge areas was measured as the time until the resistance allele frequency became 30% of the entire refuge population in non-Bt cotton fields. This was an arbitrary criterion.

The aim of this model was to evaluate the impact of larval movement and the adult immigration of different genotypes on resistance evolution in *S. frugiperda*. A loop was inserted to simulate eight different migration combinations (Table 3.1) at a rate corresponding to 0.90 of adults from other areas to refuge areas only during the start of the cotton season. We assumed that *S. frugiperda* has four generations during each cotton cycle.

Table 3.1 Migration combinations simulated of *S. frugiperda* adult genotypes^a from other areas to non-Bt refuges at beginning of the cotton season

Combination ^b	Resistance Allele Frequency	Migration Scenario
$A_S A_{S[1]} / A_R A_{S[1]} / A_R A_{R[1]}$	0.50	Migration of $A_S A_S$, $A_R A_S$ and $A_R A_R$
$A_S A_{S[1]} / A_R A_{S[1]} / A_R A_{R[0]}$	0.25	Migration of $A_S A_S$ and $A_R A_S$
$A_S A_{S[1]} / A_R A_{S[0]} / A_R A_{R[0]}$	0.00	Migration of only $A_S A_S$
$A_S A_{S[0]} / A_R A_{S[0]} / A_R A_{R[0]}$	0.00	No migration
$A_S A_{S[0]} / A_R A_{S[0]} / A_R A_{R[1]}$	1.00	Migration of only $A_R A_R$
$A_S A_{S[0]} / A_R A_{S[1]} / A_R A_{R[1]}$	0.75	Migration of $A_R A_S$ and $A_R A_R$
$A_S A_{S[1]} / A_R A_{S[0]} / A_R A_{R[1]}$	0.50	Migration of $A_S A_S$ and $A_R A_R$
$A_S A_{S[0]} / A_R A_{S[1]} / A_R A_{R[0]}$	0.50	Migration of only $A_R A_S$

^a $A_S A_S$ = susceptible, $A_R A_S$ = heterozygote and $A_R A_R$ = resistant. ^b Combinations refer to: 1 = migration. 0 = no migration.

In order to simulate the impact of neonate movement in heterogeneous landscapes of non-Bt with Bt cotton, we assumed that each insect has a two-stage life cycle. The probability functions used in the current report were defined by Mallet and Porter (1992) (Table 3.2).

Table 3.2 Summary of the probabilities functions used in the simulations

Function	Event
$\alpha: F^2M_\alpha$	Probability of ovipositing on a non-Bt plant and moving to another toxin-free plant
$\sigma: F(1-M_\alpha)$	Probability of ovipositing on a non-Bt plant and not moving
$\gamma: F(1-F)M_\gamma$	Probability of ovipositing on a non-Bt plant and moving to a Bt plant
$\Psi: F(1-F)M_\Psi$	Probability of ovipositing on a Bt plant and moving to a non-Bt plant
$\rho: (1-F)^2M_\rho$	Probability of ovipositing on a Bt plant and moving to a Bt plant
$\varepsilon: (1-F)(1-M_\rho)$	Probability of ovipositing on a Bt plant and not moving

Here we are assuming that M may vary according to micro-landscape and genotype. Then, different values were given to M , as detailed on Table 3.3.

Table 3.3 Data used in the model on parametrization of the M^l given the probability function

Parameter associated to probability	Data used to parametrization
M_α	Insect proportion found on adjacent plants of treatment 1
M_γ	Insect proportion found on adjacent plants of treatment 2
M_Ψ	Insect proportion found on adjacent plants of treatment 3
M_ρ	Insect proportion found on adjacent plants of treatment 4

¹ M is the parameter of movement associated with these functions: the probability of oviposition on non-Bt plant and moving to other non-Bt plant (α), probability of oviposition on a non-Bt plant and moving to a Bt plant (γ), probability of oviposition on a Bt plant and moving to a non-Bt plant (Ψ) and the probability of oviposition on a Bt plant and moving to a Bt plant (ρ).

The first stage is laid on a free-toxin plant (non-Bt) with probability F (that corresponds to the proportion of non-Bt plants), thereafter it may move with probability M_α , in which case it ends up on a non-Bt plant again in the second stage with probability F . For instance, the probability of oviposition on a non-Bt plant and moving to other non-Bt plant is:

$$\alpha: F(FM_\alpha) \quad (\text{eqn 1})$$

Similarly, the insect may not change plants, with probability $1-M_\alpha$, then the probability of the insect being laid on a non-Bt plant and completing its life cycle on this same plant is:

$$\sigma: F(1-M_\alpha) \quad (\text{eqn 2})$$

The probability of oviposition on a Bt cotton plant is $(1-F)$. Therefore, considering all Bt plants adjacent to the natal plant, the probability of oviposition on a non-Bt plant and moving to a Bt plant is:

$$\gamma: F(1-F)M_{\gamma} \quad (\text{eqn 3})$$

Analogously, considering non-Bt plants on adjacent sites in relation to Bt as natal plant, the probability of oviposition on a Bt plant and movement to other non-Bt plant is:

$$\Psi: F(1-F)M_{\Psi} \quad (\text{eqn 4})$$

Likewise, the probability of oviposition on a Bt plant and moving to other Bt plant (ρ) or staying on the same Bt plant (ε) are given by:

$$\rho = (1-F)^2 M_{\rho} \quad (\text{eqn 5})$$

$$\varepsilon: (1-F)(1-M_{\rho}) \quad (\text{eqn 6})$$

The sum of all probabilities, each multiplied by its respective fitness in each condition, gives the survival of each genotype in the agroecosystem, as shown below for the susceptible genotype $A_S A_S$:

$$W_{ss} = \alpha_{ss} (s_{[a]}) + \sigma_{ss} (s_{[b]}) + \gamma_{ss} (s_{[c]}) + \Psi_{ss} (s_{[d]}) + \rho_{ss} (s_{[e]}) + \varepsilon_{ss} (s_{[f]}) \quad (\text{eqn 7})$$

We used the same procedure to calculate the fitness of the $A_R A_S$ (W_{RS}) and $A_R A_R$ (W_{RR}) genotypes. Therefore, we estimated that W_{RR} was not completely 1 because in the absence of selection pressure there was a fitness cost on dispersal capacity of Cry1F-resistant individuals (Malaquias et al. 2017 and data available in Table 6), as well as differentiated survivorship (Table 3.6 – results section).

Table 5 contains the dispersal values from laboratory experiments used in the simulations. In parametrization of the model, we considered survival would be given for each possible oviposition site (Bt or non-Bt plant) and based on larval movement observed in each treatment of the laboratory bioassay with artificial micro-landscapes. In other words, each probability function (α , σ , γ , Ψ , ρ and ε) was multiplied by survival given each condition [i]. We modelled the following conditions: $s_{[a]}$ larval survival of insects found on adjacent plants when released on a central non-Bt cotton plant and moved to another non-Bt cotton plant (adjacent plants of the treatment 1 – section bioassay); $s_{[b]}$ larval survival of insects found on the centre when released on non-Bt plants placed in the centre of the arena (treatment 1); $s_{[c]}$ larval survival of insects found on Bt plants when this cultivar was on adjacent plants with a non-Bt plant in the centre (treatment 2); $s_{[d]}$ larval survival of insects found on adjacent non-Bt plants when the central plant expressed Bt (treatment 3); $s_{[e]}$ larval survival of insects

found on adjacent Bt plants of treatment 4, and, finally, $s_{[f]}$ larval survival of insects found on centre Bt plant of the treatment 4.

Survival data for all genotypes (Table 3.6, results section) were used. For example, the $s_{[a]}$ survival rate equals 0.5671, because the survival percentage was 56.71% when susceptible larvae that were released on non-Bt cotton and moved to an adjacent plant, in this case other non-Bt cotton plants.

The fitness of the heterozygous genotype was measured by the dominance of resistance (h) relative to homozygous susceptible and resistant individuals and was calculated in accordance to method used by Brévault et al. (2015).

A function was programmed to assess the non-parametric bootstrap mean and confidence intervals of the fitness and dominance of the resistance trait. For bootstrapping, mean confidence limits were calculated based on 2,000 resamples of the simulation data from the model. The overall loss rates (LR) of each genotype ($_{gen}$) is:

$$LR_{gen} = 1 - W_{gen} \quad (\text{eqn 8})$$

We chose an initial frequency of the resistant allele of 10^{-6} . Biological parameters for oviposition rate, larva-adult development period and adult longevity were estimated based on data collected by Horikoshi et al. (2016). In the model assumptions we assumed random oviposition and mating within the refuge areas of Bt cotton. The fecundity rates varied by genotype (Horikoshi et al. 2016), therefore, in our algorithm we assumed that genotypes of the mating pairs determined the genotypes of the progeny, given the conditional probabilities of genotype fecundity rates, as shown below and in Table 3.4.

$$A_S A_S = x^2 F_{SS} + \frac{1}{2} xy F_{SS} + \frac{1}{2} xy F_{RS} + \frac{1}{4} y^2 F_{RS} \quad (\text{eqn 9})$$

$$A_R A_S = \frac{1}{2} xy F_{SS} + xz F_{SS} + \frac{1}{2} xy F_{RS} + \frac{1}{2} y^2 F_{RS} + \frac{1}{2} yz F_{RS} + xz F_{RR} + \frac{1}{2} yz F_{RS} \quad (\text{eqn 10})$$

$$A_R A_R = \frac{1}{4} y^2 F_{RS} + \frac{1}{2} yz F_{RS} + \frac{1}{2} yz F_{RS} + z^2 F_{RR} \quad (\text{eqn 11})$$

where x , y and z correspond, respectively to resistant, susceptible and heterozygous individuals, and F_{SS} , F_{RS} , and F_{RR} , are the reproduction rate capacity of $A_S A_S$, $A_R A_S$ and $A_R A_R$ genotypes, respectively.

Table 3.4 Probabilities of the mating and offspring genotype produced in each section of the random mating

Mating frequency		Offspring		
♀	♂	$A_S A_S$	$A_R A_S$	$A_R A_R$
x	x	$x^2 F_{SS}$	0	0
x	y	$\frac{1}{2} xy F_{SS}$	$\frac{1}{2} xy F_{SS}$	0
x	z	0	$xz F_{SS}$	0
y	x	$\frac{1}{2} xy F_{RS}$	$\frac{1}{2} xy F_{RS}$	0
y	y	$\frac{1}{4} y^2 F_{RS}$	$\frac{1}{2} y^2 F_{RS}$	$\frac{1}{4} y^2 F_{RS}$
y	z	0	$\frac{1}{2} yz F_{RS}$	$\frac{1}{2} yz F_{RS}$
z	x	0	$xz F_{RR}$	0
z	y	0	$\frac{1}{2} yz F_{RS}$	$\frac{1}{2} yz F_{RS}$
z	z	0	0	$z^2 F_{RR}$

Based on a paper published recently by Martinez et al. (2017), we incorporated the density dependence effects on resistance evolution. The maximum population growth rate, R_{max} , represented the maximum insect growth rate. We studied the effects of density dependence using the Hassell equation (Hassell 1975):

$$N_{t+1} = N_t R_{max} (1 + a N_t)^{-b} \quad (\text{eqn 12})$$

N is the population density larvae at time step t and $t+1$, the constant a is a scaling parameter (units = density⁻¹), and b is a unitless exponent that determines the strength with which population growth rate responds to departures from equilibrium population density. We assumed that $b = 1$, that means survival changes directly with population density, in other words we assumed that there is contest competition between *S. frugiperda* larvae (Andow et al. 2015). The b parameter could be less or greater 1. When b is less or greater than 1 there is undercompensation and scramble competition, respectively. The constant scaling a , was calculated by following formula:

$$a = \frac{R_{max}^{\frac{1}{b}} - 1}{K}, \quad (\text{eqn 13})$$

Where K is the carrying capacity. That procedure ensures that population densities are attracted to the K .

3.3 Results

3.3.1 Effects of the artificial micro-landscapes on dispersal and survival of *S. frugiperda* genotypes

A significant interaction occurred between treatments and insect genotypes on dispersal rate of *S. frugiperda* ($F_{6,33} = 221.06$, $P = 0.0179$). Considering the comparisons among the treatments, the lowest dispersal (56.17%) of the Cry1F-resistant insects was when the insects were kept with Bt cotton plants in the center and adjacent plants were non-Bt cotton plants (T3) (Table 3.5).

The highest dispersal percentage (69.05%) of heterozygous larvae was found on the treatment with only Bt cotton (T4) (Table 3.5). Comparing larval dispersal of the Cry1F-resistant and heterozygous genotypes, the unique difference between them was on pure non-Bt cotton plants (T1) (Table 3.5).

The dispersal rate of susceptible larvae was near to 80% when Bt cotton plants were the natal plants (T3 and T4). The dispersal percentage of susceptible larvae in these both treatments were higher than the dispersal of the same genotype larvae found on the other treatments (T1 and T2), and in relation to Cry1F-resistant and heterozygous insects in the treatments when Bt cotton was the natal plant (Table 3.5).

Table 3.5 Mean (%) (\pm SE) of Cry1F-resistant, susceptible, and heterozygous genotypes of *Spodoptera frugiperda* that moved in response to artificial micro-landscapes tested

Treatment	Cry1F-resistant	Heterozygote	Susceptible
T1	74.14 \pm 07.80 A a	55.96 \pm 12.95 B b	43.45 \pm 06.14 B b
T2	64.61 \pm 10.14 A a	55.08 \pm 06.39 AB b	48.54 \pm 08.61 B b
T3	56.17 \pm 08.61 B b	57.43 \pm 08.72 B b	78.94 \pm 05.36 A a
T4	68.34 \pm 08.55 B a	69.05 \pm 05.18 B a	81.23 \pm 01.99 A a

Means within the same insect genotype column with the same lowercase letter or means between genotypes within of the same treatment (row) with the same capital letters are not significantly different when the confidence interval overlaps (95% CI). The 95% CI were estimated with a quasi-binomial generalized linear model. T1= natal plant was non-Bt and adjacent plants were non-Bt; T2= natal plant was non-Bt and adjacent plants were Bt; T3= natal plant was Bt and adjacent plants were non-Bt, and finally, T4= natal plant was Bt and adjacent plants were Bt.

Based on the *BCR* under choice conditions, the distribution of Cry1F-resistant neonates was more concentrated in the *NE* and *S* regions (Fig. 3.2), but on the treatment with pure non-Bt cotton plants (T1) this genotype explored practically all regions of the arenas with a lowest concentration on *NW* and *S* (Fig. 3.2). With pure Bt cotton plants (T4) there was no significant preference by the Cry1F-resistant genotype for any coordinate, and its distribution was not significantly different from a uniform circular distribution (*Watson's test value*= 0.1065; $P < 0.05$) (Fig. 3.2).

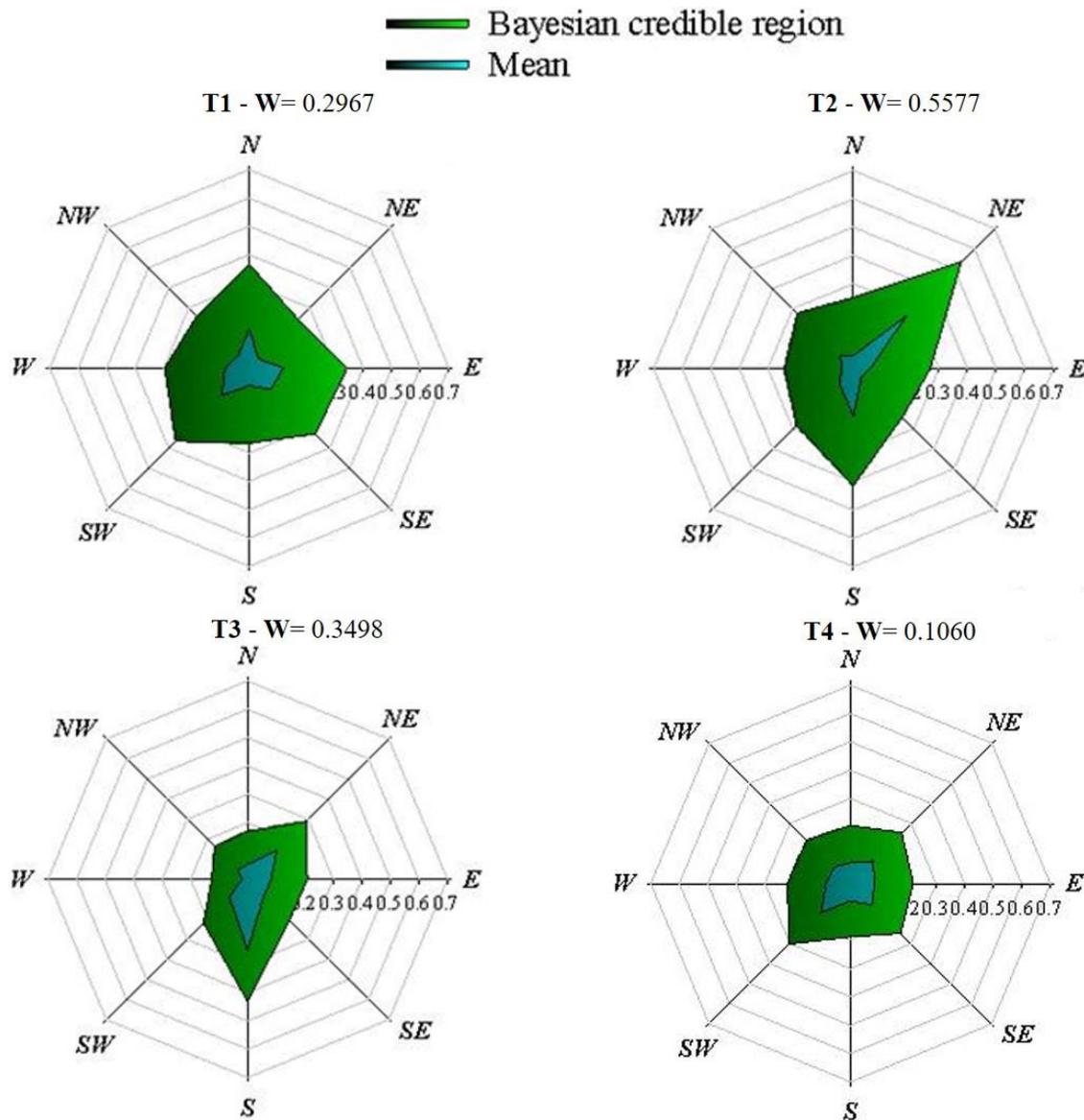


Fig. 3.2 Frequency distribution of Cry1F-resistant *Spodoptera frugiperda* paths per treatment on coordinates. The blue area refers to mean frequency of insects, while the green area refers to the Bayesian credible region. T1= natal plant was non-Bt and adjacent plants were non-Bt; T2= natal plant was non-Bt and adjacent plants were Bt; T3= natal plant was Bt and adjacent plants were non-Bt, and finally, T4= natal plant was Bt and adjacent plants were Bt.

The heterozygote distribution was not significantly different from a uniform circular distribution (*Watson's test value* = 0.1351; $P < 0.05$) on pure Bt cotton plants (T4). On other treatments the null hypothesis of a uniform circular distribution pattern was rejected. *BCR* showed that the distribution of the heterozygote was significantly higher in the regions *SE*, *E* and *NE* with pure non-Bt cotton plants (T1), *SE* when non-Bt was the natal plant and the adjacent plants were Bt cotton (T2) and *NW* when Bt cotton was the natal plant and the adjacent plants were non-Bt (T3) (Fig. 3.3).

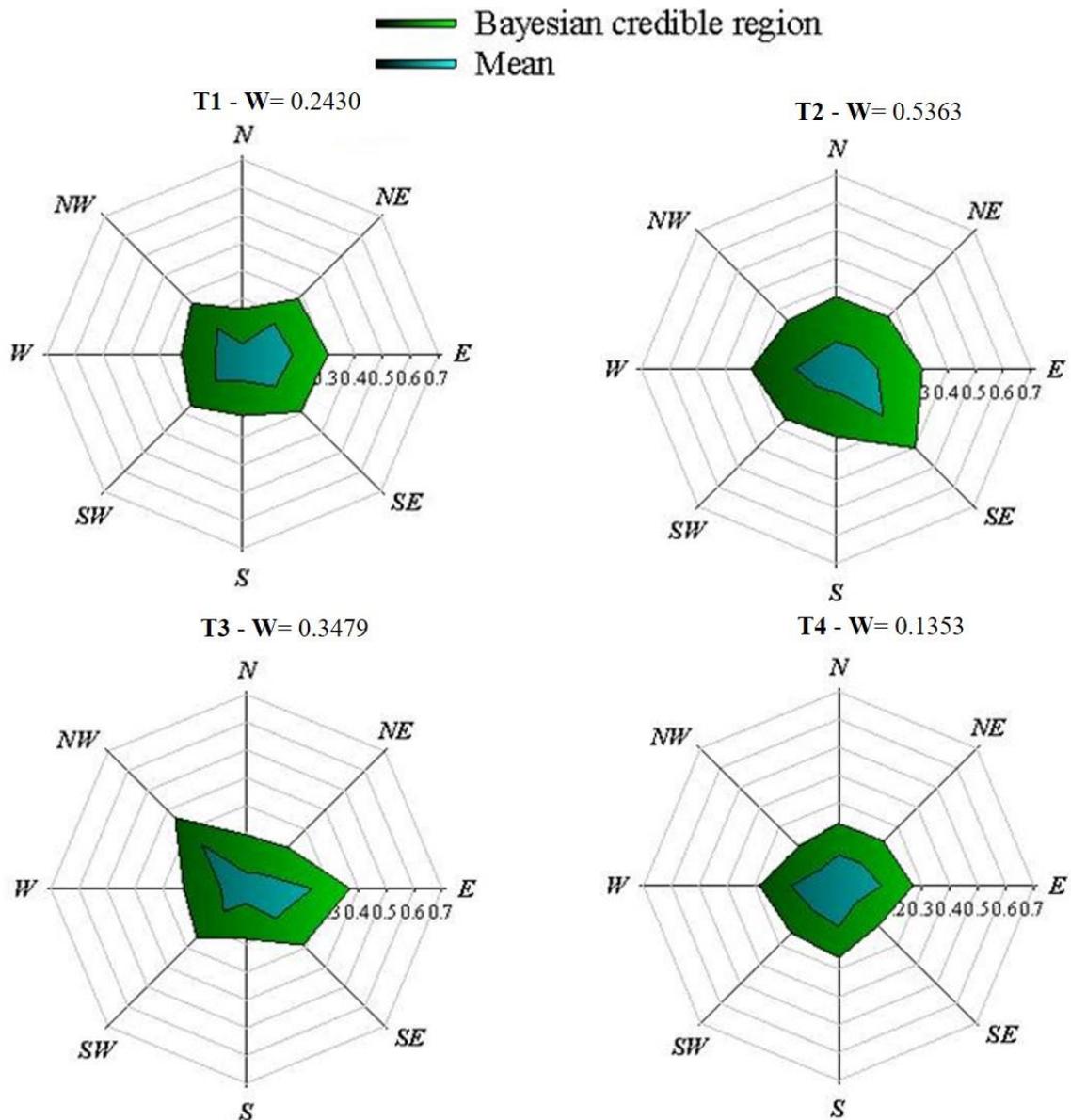


Fig. 3.3 Frequency distribution of heterozygote *Spodoptera frugiperda* paths per treatment on coordinates. The blue area refers to mean frequency of insects, while the green area refers to the Bayesian credible region. T1= natal plant was non-Bt and adjacent plants were non-Bt; T2= natal plant was non-Bt and adjacent plants were Bt; T3= natal plant was Bt and adjacent plants were non-Bt, and finally, T4= natal plant was Bt and adjacent plants were Bt.

The susceptible distribution was not significantly different from a uniform circular distribution (*Watson's test value*= 0.1133; $P < 0.05$) with pure Bt cotton plants (T4) and approximately uniformly distributed on non-Bt cotton as natal plant and adjacent plants were Bt cotton plants (T2) (*Watson's test value*= 0.0590; $P < 0.05$). The *BCR* revealed that there was a tendency of dispersal to *NW*, *E* and *NE* regions in susceptible insects on condition of pure non-Bt cotton plants (T1) and when Bt cotton was the natal plant and adjacent plants were non-Bt cotton (T3) there was a directed pattern to South region (*S*) (Fig. 3.4).

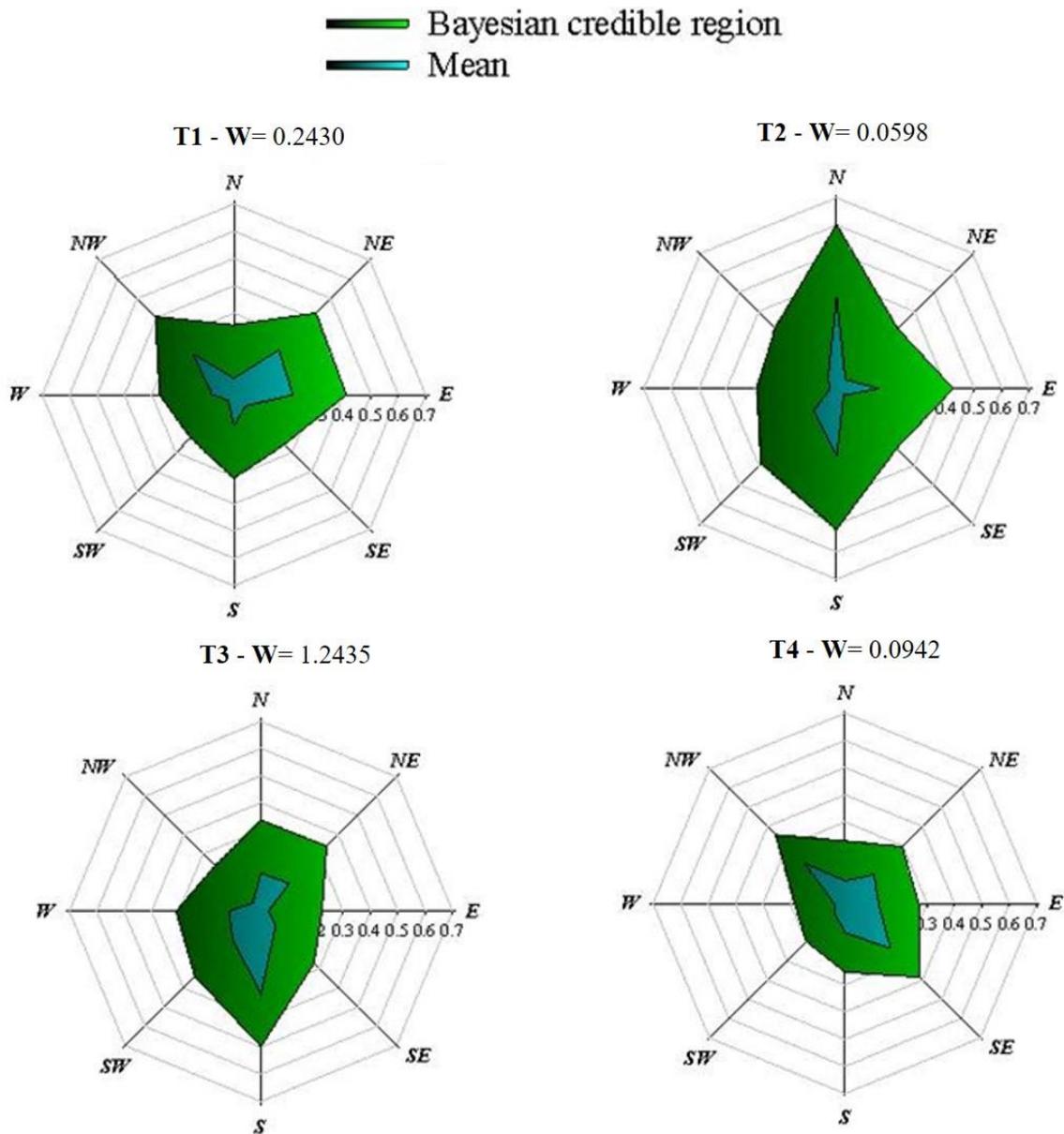


Fig. 3.4 Frequency distribution of susceptible *Spodoptera frugiperda* paths per treatment on coordinates. The blue area refers to mean frequency of insects, while the green area refers to the Bayesian credible region. T1= natal plant was non-Bt and adjacent plants were non-Bt; T2= natal plant was non-Bt and adjacent plants were Bt; T3= natal plant was Bt and adjacent plants were non-Bt, and finally, T4= natal plant was Bt and adjacent plants were Bt.

The interaction between treatments, sites and insect genotype on the survival rate of *S. frugiperda* was significant ($F_{6,171} = 20.86$, $P = 0.0490$). The highest survival rate for the Cry1F-resistant genotype was when this genotype was kept or had contact with Bt plants in multiple choice treatments (T2 and T3), in relation to survivorship of Cry1F-resistant genotype in the treatment with pure non-Bt cotton plants (T1) (Table 3.6).

Table 3.6 Larval survival (%) (mean \pm SE) of Cry1F-resistant, susceptible, and heterozygous genotypes of *Spodoptera frugiperda* when found at different sites according to artificial micro-landscapes tested

Treatment	Site	Cry1F-resistant	Heterozygote	Susceptible
T1	Natal plant	50.00 \pm 22.36 b B	69.23 \pm 13.32 a A	62.45 \pm 03.05 a A
	Adjacent plants	61.30 \pm 09.77 ab AB	69.56 \pm 9.98 a A	59.50 \pm 5.24 a B
T2	Natal plant	75.00 \pm 16.36 a A	68.96 \pm 08.74 a AB	57.95 \pm 03.73 a B
	Adjacent plants	71.41 \pm 08.85 a A	N/A *	N/A *
T3	Natal plant	66.66 \pm 33.33 ab A ¹	N/A *	N/A *
	Adjacent plants	74.44 \pm 9.52 a A	75.00 \pm 12.11 a A	57.44 \pm 5.74 a B
T4	Natal plant	57.14 \pm 20.20 ab A ¹	N/A *	N/A *
	Adjacent plants	56.61 \pm 9.98 ab A ¹	N/A *	N/A *

Means within the same insect genotype column with the same lowercase letter or means between genotypes within of the same sites with the same capital letters are not significantly different when the confidence interval overlaps (95% CI). The 95% CI were estimated with a binomial generalized linear model. An asterisk (*) indicates that confidence intervals were not estimated because no variability existed. T1= natal plant was non-Bt and adjacent plants were non-Bt; T2= natal plant was non-Bt and adjacent plants were Bt; T3= natal plant was Bt and adjacent plants were non-Bt, and finally, T4= natal plant was Bt and adjacent plants were Bt.

There was no survival of the susceptible and heterozygous genotypes when on Bt cotton as natal plant (T3), as adjacent plants (T2) or in the treatment with pure Bt cotton plants (T4); however, when these genotypes were exposed in a choice test involving Bt cotton as natal plant and adjacent plants were non-Bt (T3), the larval survival was higher and lower than 70% in heterozygote and susceptible genotypes, respectively (Table 3.6).

3.3.2 The impact of *S. frugiperda* adult genotype migration and contamination in refuge areas on resistance evolution

Results suggested that the time to resistance increased in the absence of refuge contamination (0 %) and when migration of susceptible genotypes from other areas to the refuge was simulated. However, with migration of the A_{RA_S} and A_{RA_R} genotypes, even with contamination-free refuges there was a tendency of time to resistance to decrease (Table 3.7).

Table 3.7 Effects of adult genotypic migration¹ and contamination of non-Bt cotton with Bt cotton on time (generations) to resistance of *Spodoptera frugiperda*

Migration Scenario ^a	CL (%) ^b		
	0.00	20.00	30.00
$A_S A_{S[1]} / A_R A_{S[1]} / A_R A_{R[1]}$	123	11	4
$A_S A_{S[1]} / A_R A_{S[1]} / A_R A_{R[0]}$	199	24	4
$A_S A_{S[1]} / A_R A_{S[0]} / A_R A_{R[0]}$	> 300	48	4
$A_S A_{S[0]} / A_R A_{S[0]} / A_R A_{R[0]}$	123	10	4
$A_S A_{S[0]} / A_R A_{S[0]} / A_R A_{R[1]}$	120	9	4
$A_S A_{S[0]} / A_R A_{S[1]} / A_R A_{R[1]}$	87	9	4
$A_S A_{S[1]} / A_R A_{S[0]} / A_R A_{R[1]}$	110	10	4
$A_S A_{S[0]} / A_R A_{S[1]} / A_R A_{R[0]}$	124	12	4

^a₀= no migration of the genotype. ₁= migration of the genotype. ^bCL= contamination level (%) in refuge (non-Bt cotton) areas.

Contamination-free non-Bt cotton fields, mainly in the presence of susceptible migration, delayed the evolution of Cry1F-resistance. The time to resistance increased under 20% contamination when there was susceptible migration, except the scenario with migration of all genotypes ($A_S A_{S[1]} / A_R A_{S[1]} / A_R A_{R[1]}$) (Table 3.7). Contamination of 20% and migration of the $A_R A_{R[1]}$ genotype with all combinations of migration with the other genotypes decreased the time to resistance to less than 13 generations (Table 3.7). Independent of all migration conditions, 30% contamination in non-Bt cotton fields with Bt cotton plants tended to increase dramatically the proportion of resistant genotype in refuge areas (Table 3.7).

In the contaminated refuge scenarios, the absolute and relative fitness of the susceptible insects was decreased under 20 and 30% contamination. The increase in the relative fitness of susceptible (94%) and heterozygous (88%) genotypes (Fig. 3.5) combined with differentiated reproductive capacity promoted a delay of the resistance genotypes under the contamination-free refuge scenario (Table 3.3). In addition, the inheritance of resistance to Bt cotton was not completely recessive ($h \neq 0$) for all simulated conditions. Furthermore, the dominance of

resistance to Bt cotton was significantly higher with 20% ($h = 0.5047$) or 30% ($h = 0.5000$) contaminated refuge than contamination-free refuge ($h = 0.3148$) (Fig. 3.6).

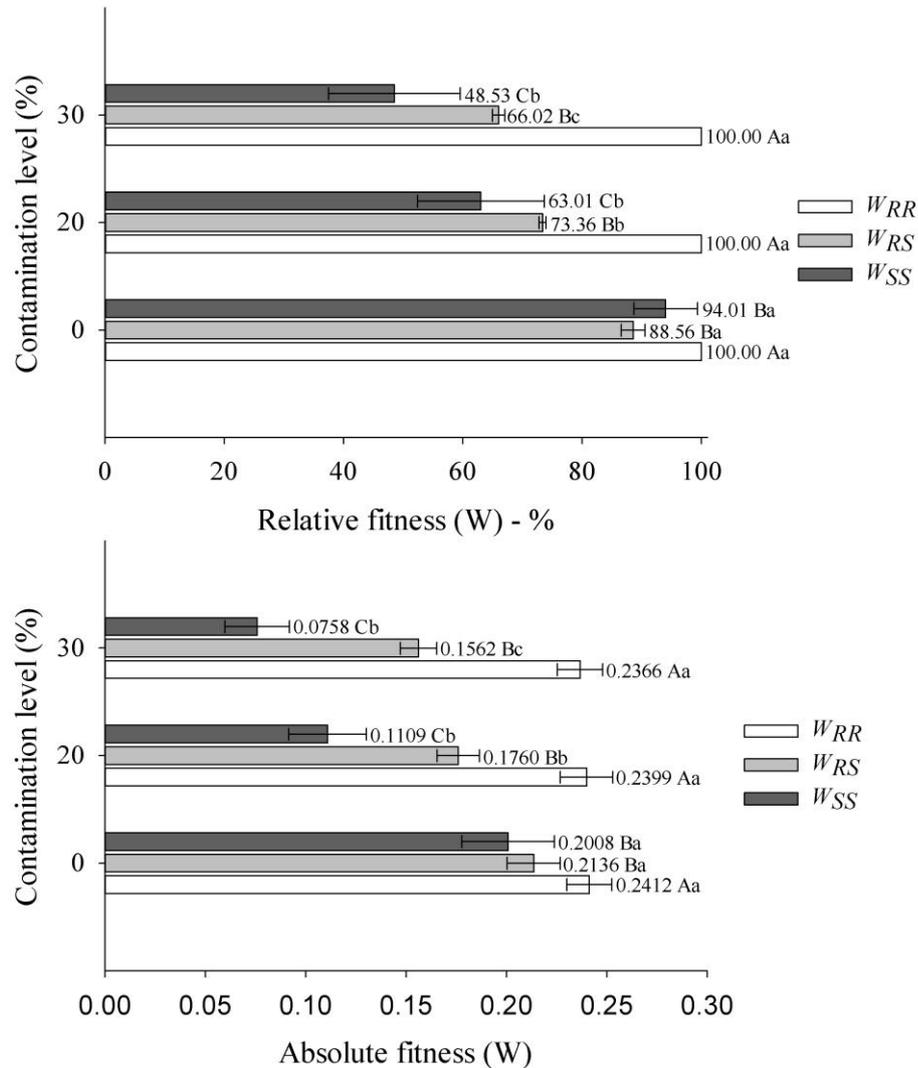


Fig. 3.5 Absolute and relative (%) fitness (W) estimated for Cry1F-resistant, heterozygote and susceptible populations of *S. frugiperda* under the contamination levels tested. W_{SS} , W_{RS} , and W_{RR} refer to fitness of SS , RS and RR , respectively. The relative fitness of the SS and RS is expressed in relation to RR as the standard of comparison, such as, $W_{SS} (\%) = (W_{SS}/W_{RR}) * 100$ and $W_{RS} (\%) = (W_{RS}/W_{RR}) * 100$. Bars (\pm SE) with the same lowercase letter (comparing bars of the different contamination levels in the same genotype) and capital letter (comparing bars of genotypes in the same contamination level) are not significantly different when confidence intervals overlap (95% CI). Average and 95% CI of fitness were estimated with a non-parametric bootstrap of the of the *S. frugiperda* genotypic samples.

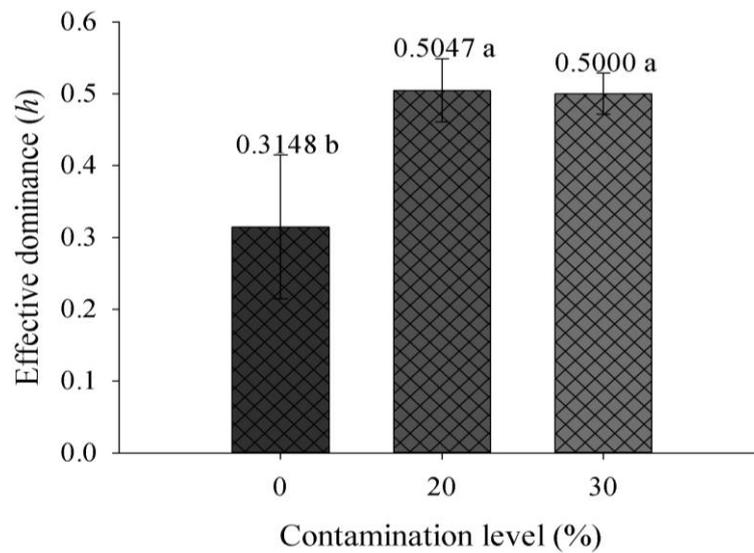


Fig. 3.6 Effective dominance of resistance (h) estimated in *S. frugiperda* by the contamination level tested. h was calculated as $= (W_{RS} - W_{SS}) / (W_{RR} - W_{SS})$. Bars (\pm SE) with the same letter are not significantly different when confidence intervals overlap (95% CI). Average and 95% CI of fitness simulated with a non-parametric bootstrap of the *S. frugiperda* genotypic samples.

3.4 Discussion

Our findings from artificial micro-landscapes showed that the dispersal pattern of *S. frugiperda* genotypes was influenced by contact with Cry toxins on Bt cotton plants or with non-Bt plants; therefore, the neighborhood structure affected the distribution pattern of *S. frugiperda* genotypes. The results suggest that Cry1F-resistant and susceptible genotypes avoided non-Bt cotton and Bt cotton plants, respectively. This is supported by adaptation of the Cry1F-resistant and non-adaptation of the susceptible insects to WideStrike cotton cultivars (Horikoshi et al. 2016) and the fitness cost associated with the mobility of the Cry1F-resistant genotype (Malaquias et al. 2017). Here we showed that the similarity in terms of neighbourhood dispersal behaviour between heterozygous and susceptible or Cry1F-resistant genotypes depends on the landscape. Susceptible and heterozygote genotypes had similar dispersal patterns when they were released in the centre of the arena with only non-Bt plants. Meanwhile, there was no survival of heterozygotes when the insects were found and kept on Bt plants, because WideStrike is effective for controlling heterozygous larvae (Horikoshi et al. 2016).

Different dispersal behaviour in the presence of choice and no-choice Bt cotton plants was found, because substantial variation in the distribution of *S. frugiperda* genotypes on adjacent plants was revealed among the micro-landscape structures. These results are supported by the hypothesis test of circular distributions that indicated random dispersal of Cry1F-resistant, susceptible and heterozygous genotypes when kept with only Bt cotton, and approximately uniformly distributed when susceptible insects were released on non-Bt plants with adjacent Bt cotton plants. It's possible that *S. frugiperda* genotypes have the capacity to detect the toxin in the neighborhood, perhaps contacting plants and ingesting tissues after walking dispersal, or the response to cultivars by *S. frugiperda* larvae may be due to metabolic differences between Bt and non-Bt cotton plants (Carbonari et al. 2016) that may be enough to promote contrasting attractiveness (Gonçalves de Jesus et al. 2014). Distinct feeding behaviour of *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) was also apparent as function of the resistance to Cry1Ab (Prasifka et al. 2009). Cry1Ab-resistant and heterozygote *O. nubilalis* had higher acceptance of diets with Cry1Ab than susceptible insects, at 10-50% levels on no choice test and 10 or 25% on multiple-choice bioassay, respectively (Prasifka et al. 2009).

Our results suggest that movement from non-Bt plants to Bt plants caused complete mortality of susceptible and heterozygote genotypes, but there was survival when both genotypes moved from Bt to non-Bt plants. The survival of both genotypes after dispersal from a Bt cotton natal plant may be due to the high pre-feeding dispersal, that is, a small proportion of *S. frugiperda* larvae feeds in the first hours of the exposure to Bt cotton and dispersal occurs without feeding on the plant tissues (Malaquias et al. 2017). This information can be extremely important for resistant management of lepidopteran pests to Bt cotton (Ramalho et al. 2014), in as much as it is possible to infer how dispersal capacity mediated the effects of heterogeneous landscapes of cotton involving Bt and non-Bt plants (Malaquias et al. 2017) and simulations of the evolution of resistance in these fields may be applied (Carroll et al. 2012; Caprio et al. 2016), because models suggest that the rate of resistance evolution depends on larval movement of some Lepidoptera species such as *Helicoverpa zea* (Lepidoptera: Noctuidae) (Caprio et al. 2016) and *S. frugiperda* (Garcia et al. 2016).

The results from the experiment motivated the following question: what are the consequences of our results of *S. frugiperda* dispersal behaviour and survival to resistance evolution in refuge areas with different contamination levels and different combinations of adult genotypic migrations? The simulation with a computer model using the results from our laboratory experiments confirmed the hypothesis that the levels of movement of the susceptible and heterozygous genotypes from non-Bt cotton to Bt cotton plants caused mortality of these individuals when dispersing, promoting rapid evolution of resistance of *S. frugiperda* within the contamination levels tested in the simulations. Whatever, the results from simulations suggest resistance evolution can be significantly impacted by differential genotypic dispersal. We found that the number of generations for resistance to evolve in areas with migration of susceptible insects in contamination-free refuge areas is greater than 300, but with 30% contamination in refuge areas with Bt cotton plants the time to resistance is only 4 generations. Naturally, the emigration from a resistant population may spread the resistance alleles in Bt crops (Miller and Sappington 2017). The technology life span was higher than 80 generations with at least resistant genotype migration from other areas to refuge areas. Some reasons could be listed to explain the dramatic differences on resistant allele frequency between contamination-free and contaminated refuges. First, confirming the third hypothesis of this study, in the absence of selection pressure, the fitness of the susceptible and heterozygous genotypes was higher than under selection pressure, leading to a higher growth rate when these genotypes were kept in contamination-free refuges contributing to the delay

of resistance. Second, when the Cry1F-resistant *S. frugiperda* had contact with both Bt and non-Bt cotton, it was shown that the survival rate was higher than when kept with only pure Bt or pure non-Bt cotton plants. Thus, contamination of refuge areas with Bt cotton always favors resistant populations. On the other hand, the higher fitness of the Cry1F-resistant genotype in contaminated refuges could result from other factors not addressed in the current study, such as greater food intake and utilization (Ramalho et al. 2011), metabolization of cotton cultivars by associated microbiota (Almeida et al. 2017), genomic differentiation (Gouin et al. 2017) and/or a transcript expression plasticity (Silva-Brandão et al. 2017) when in contact with the both hosts. However, further studies are necessary to investigate these potential mechanisms.

In the current study we used Cry1F-resistant *S. frugiperda* as a genotype model, and the simulations and scenarios used in this paper could explain the rapid field-evolved resistance to Cry1F in Brazilian agroecosystems (Farias et al. 2014a, b) and/or similar conditions. In addition, it is of utmost importance to emphasize that the Cry1F-resistant *S. frugiperda* genotype is able to survive on other cultivars of Bt cotton and maize such as Bollgard II, YieldGard VT PRO and PowerCore (Horikoshi et al. 2016). In addition, there is evidence of considerable gene flow between *S. frugiperda* populations from cotton and maize in Brazilian geographical regions (Martinelli et al. 2006). Therefore, our current study highlights the importance of the refuge areas on regional scales in Brazilian agroecosystems to promote the migration of susceptible insects, mainly where cotton fields are cultivated in sequence (Martinelli et al. 2006) or in adjacent areas (Horikoshi et al. 2016) with other crops such as maize.

In conclusion, all efforts to promote the migration of the susceptible genotype and to avoid contamination of refuge areas are important to prolong the life span of Bt technologies that are present in host crops for Cry1F-resistant *S. frugiperda*. In order of the limitations of the results from laboratory conditions, field experiments combining passive (such as ballooning) and active movement (such as walking movement) are encouraged. Both information from laboratory and field experiment generated may be useful in the design and implementation of resistance management strategies for *S. frugiperda* in South America and/or similar conditions and create ecological basis for understanding the reasons of the rapid resistance evolution in these agroecosystems.

References

- Agostinelli C, Lund U (2017) R package 'circular': Circular Statistics (version 0.4-93). <https://r-forge.r-project.org/projects/circular/>. Accessed 25 Dec 2017
- Agostinelli C (2012) CircStats: Circular Statistics, from "Topics in circular Statistics". R package version 0.2-4. <https://CRAN.R-project.org/package=CircStats>. Accessed 25 Dec 2017
- Almeida LG, Beraldo de Moraes LA, Trigo JR, Omoto C, Cônsoli FL (2017) The gut microbiota of insecticide-resistant insects houses insecticide-degrading bacteria: a potential source for biotechnological exploitation. *Plos One* 12:e0174754. doi.org/10.1371/journal.pone.0174754
- Andow D, Farias JRM, Horikoshi RJ, Bernardi D, Nascimento ARB, Omoto C (2015) Dynamics of cannibalism in equal-aged cohorts of *Spodoptera frugiperda*. *Ecol Entomol*, 40: 229–236.
- Bernardi D, Salmeron E, Horikoshi RJ, Bernardi O, Dourado PM, Martinelli S, Head G, Omoto C (2015) Cross-resistance between Cry1 proteins in fall armyworm (*Spodoptera frugiperda*) may affect the durability of current pyramided Bt maize hybrids in Brazil. *Plos One* 10:e0140130. doi:10.1371/journal.pone.0140130
- Brévault T, Tabashnik BE, Carrière Y (2015) A seed mixture increases dominance of resistance to Bt cotton in *Helicoverpa zea*. *Sci Rep* 5:09807. doi:10.1038/srep09807
- Carbonari CA, Latorre DO, Gomes GLGC, Velini ED, Owens DK, Pan Z, Dayan FE (2016) Resistance to glufosinate is proportional to phosphinothricin acetyltransferase expression and activity in LibertyLink® and WideStrike® cotton. *Planta* 243:925–933. doi:10.1007/s00425-015-2457-3
- Caprio MA, Martinez JC, Porter PA, Bynum E (2016) The impact of inter-kernel movement in the evolution of resistance to dual-toxin Bt-corn varieties in *Helicoverpa zea* (Lepidoptera: Noctuidae). *J Econ Entomol* 109:307–319. doi:10.1093/jee/fov295
- Carroll MW, Head G, Caprio MA (2012) When and where a seed mix refuge makes sense for managing insect resistance to Bt plants. *Crop Prot* 38:74–79. doi:10.1016/j.cropro.2012.02.015
- Common IFB (1990) *Moths of Australia*. Carlton, Victoria: Melbourne Univ. Press

- Demétrio CG, Hinde J, Moral RA (2014) Models for overdispersed data in entomology. In: Ferreira CP, Godoy WAC (eds.) Ecological modelling applied to entomology. Springer Cham Heidelberg, London, pp 219–259
- Farias JR, Horikoshi RJ, Santos AC, Omoto C (2014a) Geographical and temporal variability in susceptibility to Cry1F toxin from *Bacillus thuringiensis* in *Spodoptera frugiperda* (Lepidoptera: Noctuidae) populations in Brazil. *J Econ Entomol* 107:2182-2189. doi:10.1603/EC14190
- Farias JR, Andow DA, Horikoshi RJ, Sorgatto RJ, Fresia P, Cesar dos Santos A, Omoto C (2014b) Field-evolved resistance to Cry1F maize by *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Brazil. *Crop Prot* 64:150–15. doi.org/10.1016/j.cropro.2014.06.019
- Garcia AG, Cônsoli FL, Ferreira CP, Godoy WAC (2016) Predicting evolution of insect resistance to transgenic crops in within-field refuge configurations, based on larval movement. *Ecol Complex* 28:94–103. doi.org/10.1016/j.ecocom.2016.07.006
- Gonçalves de Jesus F, Boiça Júnior AL, Alves GCS, Busoli AC, Zanuncio JC (2014) Resistance of cotton varieties to *Spodoptera frugiperda* (Lepidoptera: Noctuidae). *Rev Colomb Entomol* 40:158-163
- Gouin A, Bretaudeau A, Nam K, Gimenez S, Aury JM, Duvic B, Fournier P (2017) Two genomes of highly polyphagous lepidopteran pests (*Spodoptera frugiperda*, Noctuidae) with different host-plant ranges. *Sci Rep* 7:11816. doi:10.1038/s41598-017-10461-4
- Han P, Velasco-Hernández MC, Ramirez-Romero R, Desneux N (2016) Behavioral effects of insect-resistant genetically modified crops on phytophagous and beneficial arthropods: a review. *J Pest Sci* 89:859–883. doi:10.1007/s10340-016-0791-2
- Head GP, Dennehy T (2010) Insect resistance management for transgenic Bt cotton. In: Zehr UB (ed) Cotton, Biotechnology in Agriculture and Forestry. Springer Cham Heidelberg, London, pp 113-125
- Head GP, Greenplate J (2012) The design and implementation of insect resistance management programs for Bt crops. *GM Crops and Food: Biotechnology in Agriculture and the Food Chain*, 3:144-153. doi:10.4161/gmcr.20743
- Horikoshi RJ, Bernardi O, Bernardi D, Okuma DM, Farias JR, Miraldo LL, Omoto C (2015) Near-isogenic Cry1F-resistant strain of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) to investigate fitness cost associated with resistance in Brazil. *J Econ Entomol* 109:1–6. doi:10.1093/jee/tov387
- Horikoshi RJ, Bernardi D, Bernardi O, Malaquias JB, Okuma DM, Miraldo LL, Omoto C (2016) Effective dominance of resistance of *Spodoptera frugiperda* to Bt maize and cotton

varieties: implications for resistance management. *Sci Rep* 6:34864. doi.org/10.1038/srep34864

- Lima EABF, Ferreira CP, Bernardes AM, Godoy WAC (2009) Neighborhood interactions and larval dispersal behavior in blowflies. *J Insect Behav* 22:245-255. doi:10.1007/s10905-008-9170-z
- Malaquias JB, Godoy WAC, Garcia AG, Ramalho FS, Omoto C (2017) Larval dispersal of *Spodoptera frugiperda* strains on Bt cotton: a model for understanding resistance evolution and consequences for its management. *Sci Rep* 7:16109. doi:10.1038/s41598-017-16094-x
- Mallet J, Porter P (1992) Preventing insect adaptation to insect-resistant crops: are seed mixtures or refugia the best strategy? *P R Soc B* 250:165–169. doi:10.1098/rspb.1992.0145
- Martinelli S, Barata RM, Zucchi MI, Silva-Filho MC, Omoto C (2006) Molecular variability of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) populations associated to maize and cotton crops in Brazil. *J Econ Entomol* 99:519-526. doi.org/10.1603/0022-0493-99.2.519
- Martinelli S, Carvalho RA, Dourado PM, Head GP (2017) Resistance of *Spodoptera frugiperda* to *Bacillus thuringiensis* proteins in the western hemisphere. In: Fiuza L, Polanczyk R, Crickmore N (eds.) *Bacillus thuringiensis* and *Lysinibacillus sphaericus*. Springer Cham Heidelberg, London, pp 273-288
- Moral RA, Hinde J, Demétrio CGB (2017) Half-normal plots and overdispersed models in R: the hnp package. *J Stat Softw* 81:1-23. doi: 10.18637/jss.v081.i10
- Miller NJ, Sappington TW (2017) Role of dispersal in resistance evolution and spread. *Curr Opin Insect Sci* 21:68-74. doi: 10.1016/j.cois.2017.04.005
- Omoto C, Bernardi O, Salmeron E, Sorgatto RJ, Dourado PM, Crivellari A, Head GP (2016) Field-evolved resistance to Cry1Ab maize by *Spodoptera frugiperda* in Brazil. *Pest Manag Sci* 72:1727-36. doi:10.1002/ps.4201
- Prasifka JR, Hellmich RL, Sumerford DV, Siegfried BD (2009) *Bacillus thuringiensis* resistance influences european corn borer (Lepidoptera: Crambidae) larval behavior after exposure to Cry1Ab. *J Econ Entomol* 102:781-787. doi:10.1603/029.102.0240
- Ramalho FS, Azeredo T, Bezerra de Nascimento AR, Fernandes FS, Nascimento Júnior JL, Malaquias JB, Zanuncio JC (2011) Feeding of fall armyworm, *Spodoptera frugiperda*, on Bt transgenic cotton and its isoline. *Entomol Exp Appl* 139:207–214. doi:10.1111/j.1570-7458.2011.01121.x
- Ramalho FS, Pachú JKS, Lira ACS, Malaquias JB, Zanuncio JC, Fernandes FS (2014) Feeding and dispersal behavior of the cotton leafworm, *Alabama argillacea* (Hübner)

- (Lepidoptera: Noctuidae), on Bt and non-Bt cotton: implications for evolution and resistance management. *Plos One* 9:e111588. doi.org/10.1371/journal.pone.0111588
- Ramalho FS, Pachú JKS, Lira ACS, Malaquias JB, Brito BDS, Zanuncio JC, Fernandes FS (2017) Effects of temperature on the feeding behavior of *Alabama argillacea* (Hübner) (Lepidoptera: Noctuidae) on Bt and non-Bt cotton plants. *An Acad Bras Cienc* 89:1-15. doi:10.1590/0001-3765201720150525
- R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. <http://www.R-project.org/>. Accessed 25 Dec 2017
- Silva-Brandão KL, Horikoshi RJ, Bernardi D, Omoto C, Figueira A, Brandão MM (2017) Transcript expression plasticity as a response to alternative larval host plants in the speciation process of corn and rice strains of *Spodoptera frugiperda*. *BMC Genomics* 18:792. doi.org/10.1186/s12864-017-4170-z
- Sturtz S, Ligges U, Gelman A (2005) R2WinBUGS: a package for running WinBUGS from R. *J Stat Softw* 12:1-16. doi.org/10.7916/D80C55HH
- Yang F, Kerns DL, Head GP, Leonard BR, Levy R, Niu Y, Huang F (2014) A challenge for the seed mixture refuge strategy in Bt maize: impact of cross-pollination on an ear-feeding pest, corn earworm. *Plos One* 9:e112962. doi:10.1371/journal.pone.0112962

4 Evolutionary Process Modelling with Bayesian Inference of *Spodoptera frugiperda* Ballooning and Walking Dispersal on Bt and non-Bt Cotton Plants Mixture

Abstract

Transgenic crops expressing *Bacillus thuringiensis* (Bt crops) (Berliner) have been promoted vigorously throughout the world as a great technological tool for developing of integrated pest management. We hypothesized in our research that neighborhood with Bt and non-Bt cotton plants in small scales could influence on the ballooning dispersal of *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae). We simulated also how the ballooning dispersal combined walking movement could impact the resistance evolution process in different scenarios of plant mixture involving non-Bt and Bt cotton plants with high and non-high dose. The ballooning frequency on Cry1F-resistant larvae found on a treatment with non-Bt cotton as natal plant and Bt cotton on neighborhood was twice low as when Bt cotton was the natal plant and non-Bt cotton was in the adjacent sites. There was a negative correlation between time to resistance with walking movement rate associated with ballooning in non-high dose event. Contamination in Bt cotton fields with non-Bt cotton plants in a high dose event showed the longest time to the resistance. However, in the case of non-high dose, high movement rates reversed the effect of contamination of Bt fields with non-Bt cotton plants to slow the resistance evolution. The current chapter was written following the manuscript submission guidelines of the journal Nature Biotechnology.

Keywords: Passive dispersal; Active dispersal; Fall armyworm; Resistance; Contamination; Bt fields; Refuge areas

4.1 Introduction

Genetically modified crops traits expressing *Bacillus thuringiensis* (Bt crops) (Berliner) toxin genes are grown in 28 countries by 18 million farmers¹. The bulk of Bt crops fields are grown in the U.S., Brazil, Argentina, India, Canada and China¹. Perhaps resistance evolution in the targeted insects to overcome the Bt crops is the greatest concern, because in that case if steps are not taken to manage the evolutionary process could evolve in few insect generations. The high-dose/structured refuge strategy is advised for preventing insect adaptation to Bt crops, and this strategy requires refuges of host plants that do not express Bt toxins near Bt crops to promote survival of susceptible insects, particularly if resistance is functionally recessive². In that context, rare resistant adults emerging from Bt plants mate with relatively abundant susceptible adults from refuges, and their heterozygotes progeny are killed by a high dose of toxin from Bt plants³.

Insect resistance can provide insights into basic evolutionary processes⁴. This process may be studied by using the same theoretical frameworks that have been applied to other types of evolutionary change. In that context, quantitative genetic models are fascinating tools to identify and analyze genetic and environmental factors that influence the rate and direction of evolution^{5,6}. In the recent years, many computer models in evolutionary ecology problems applied to insect resistance to Bt crops have been formulated at the population level towards individual-based models. However, the majority of the quantitative genetic models at the individual level are not analyzed with statistics inference and remain defined in terms of a deterministic process.

Models suggest that the presence of non-Bt plants in Bt fields with two or three Bt-pyramids toxin high-dose events may delay resistance evolution⁷. However, the contamination of refuges by Bt crops could undermine the high-dose/refuge strategy and accelerate pest resistance to Bt crops^{8,9}. Concerns about plants mixture have been applied on susceptible larvae movement from non-Bt plants to Bt plants. Larval mobility is an important behavioural factor influencing the selection for resistance in fields with plant mixtures, because if the susceptible larvae move from plant to plant the presence of non-Bt plants in Bt fields could hasten pest resistance compared with pure fields¹⁰. When there is a high movement rate of larvae, it is expected that plant mixtures increase the effective genetic dominance of resistance to Bt crops resulting in increased selection for resistance¹¹.

Spodoptera frugiperda (J. E. Smith) (Lepidoptera: Noctuidae) is a major pest target of Bt corn and Bt cotton in North and South America¹². Resistance of *S. frugiperda* has been documented in Puerto Rico¹³, Brazil^{14,15}, Argentina¹⁶ and the southeast of the United States of America^{17,18}. Ballooning is a process in which the neonate lowers itself on a strand of silk and is carried by the wind¹⁹. Knowledge about *S. frugiperda* larval movement by ballooning on Bt and non-Bt cotton plants mixture can provide interesting insights for understanding adaptation process in many scenarios and may be a helpful tool to design future resistance management programs, but information in this direction is yet unexplored.

In the current paper we investigated the impact of active and passive larval movement on resistance evolution of *S. frugiperda* to Bt cotton with different scenarios of contamination in Bt and on non-Bt fields. We report in the current paper the contamination condition due the inadvertent mixing seeds, pollen flow between the transgenic and non-transgenic cotton varieties, and volunteer plants that are plants that grow from seed or on its own that remained from the previous year's harvest. Walking movement was considered as active movement and the ballooning was the passive movement. An interplay between modelling and biological data is provided using a stochastic computer model with Bayesian inference to predict *S. frugiperda* resistance evolution considering cases of non-high dose and high dose genetically modified cotton.

4.2 Results

4.2.1 Ballooning behaviour in field experiment

The ballooning frequency varied significantly as a function of time after artificial infestation ($F= 6.39$; $P< 0,0001$), however, the time effect does not depend on the strain influence or micro-landscape. There is significant interaction only between insect genotype or micro-landscape ($F= 3.11$; $P= 0.0068$). Analyzing the ballooning behaviour of *S. frugiperda*, independent of strains and micro-landscape, it is possible to observe a clear asymptotic response (**Figure 4.1**).

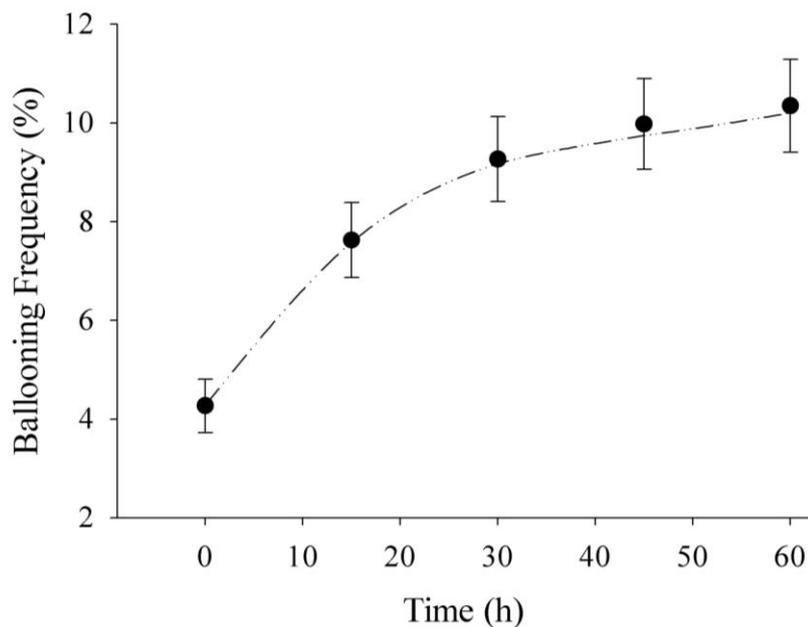


Figure 4.1 – Ballooning frequency (%) (mean±SE) in *Spodoptera frugiperda* neonate as a function of time. Observed data are represented by points, standard error is expressed by bars, while the lines are estimated data by a quasi-binomial model.

In relation to the effects of genotypes and treatments on no-choice, with only Bt cotton, or with only non-Bt cotton as natal plant and in the adjacent sites, there was no evidence of a difference in ballooning frequency between these two treatments after artificial infestation of the Cry1F-resistant *S. frugiperda*. On the other hand, in Cry1F-resistant *S. frugiperda*, the lowest ballooning frequency was found on the treatment with Bt cotton as natal plant and non-Bt cotton in the adjacent sites. The ballooning frequency of Cry1F-resistant larvae found with non-Bt cotton as natal plant and Bt cotton in the adjacent sites was 2-fold greater in relation to the treatment with Bt cotton as natal plant and non-Bt in the adjacent sites.

Therefore, these results consolidate evidence that some Cry1F-resistant individuals have a capacity to detect and avoid non-Bt cotton (**Figure 4.2**).

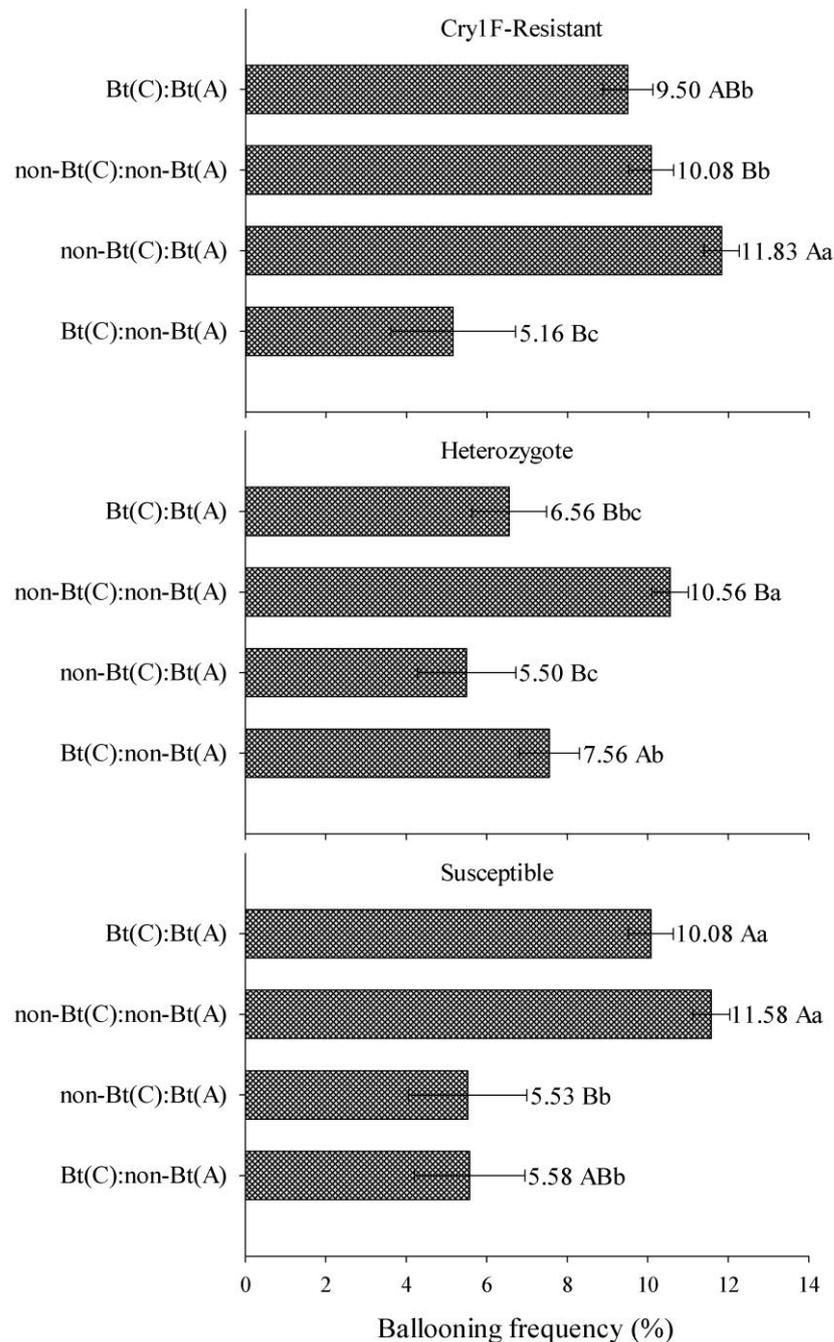


Figure 4.2– Ballooning frequency (mean±SE) of *Spodoptera frugiperda* strains kept in four micro-landscapes conditions (treatments) of Bt and non-Bt cotton. Capital letters compare the same treatments within each larvae genotype, while lower case letters compare insect strains within the same treatment by the confidence intervals overlapping from a quasi-binomial linear generalized model. Bt: WideStrike cotton. non-Bt: FM 930 (non-transgenic cotton). C: center or natal plant. A: adjacent plants.

The highest ballooning frequency of heterozygous larvae was observed with non-Bt cotton as natal plant and non-Bt in the adjacent sites, while the lowest occurred when the insects were released on non-Bt cotton as natal plant and Bt cotton in the adjacent sites. Comparing the heterozygote ballooning behaviour with other genotypes, it was found that the similarity depends on the choice condition, in other words, there was no difference on ballooning behaviour between heterozygote and resistant genotypes when in the no-choice condition; and in choice condition the ballooning frequency of susceptible and heterozygote is not different (**Figure 4.2**).

The highest and lowest ballooning frequencies on susceptible insects were found, respectively, when in the choice and no-choice condition. On the other hand, within each choice condition the susceptible ballooning frequency was not affected, that is between pure non-Bt and Bt cotton plants or between mixed plants conditions. In this way, the choice condition was more important to ballooning dispersal to susceptible insects than landscape configuration properly (**Figure 4.2**).

4.2.2 Population genetic models - The impact of *S. frugiperda* ballooning and plant mixture on Bt cotton fields and on structured refuge areas on insect resistance evolution

4.2.2.1 First case

The results from the model suggest that the ballooning data combined with walking movement between plants impacted the technology durability on contaminated Bt cotton with non-Bt cotton plants, mainly under high walking movement combined with ballooning (**Table 4.1**).

On contaminated Bt cotton with non-Bt cotton plants, the results suggest a decreasing on the time to resistance between 4-5 generations, on structured refuge of 10%, when the walking movement rate were 0.40 or 0.60 in relation to absence of larvae walking movement. This result is supported on hypothesis that the fitness of the susceptible and heterozygote insects will be lower when there is active movement, therefore it will increase the probability of these strains move from non-Bt cotton to Bt cotton (**Table 4.1**).

Table 4.1– The evolution of resistance (in generations) of *Spodoptera frugiperda* in Bt cotton fields contaminated¹ with non-Bt cotton plants with structured refuges of 20%

Movement condition ²	Contamination level (%)	
	0	10
1	37.32 (34.99-39.59)	45.36 (44.51 – 46.18)
2	37.32 (34.99-39.59)	44.05 (43.26 – 44.83)
3	37.32 (34.99-39.59)	40.96 (40.10 – 41.80)
4	37.32 (34.99-39.59)	39.99 (38.58 – 40.19)

¹The plants used in the contamination condition are free of Bt-toxin.

The size of the structured refuge was of 20% in relation to the size of the Bt field.

Movement conditions are given by the sum between Ballooning (*BL*) data + Walking Movement (*WM*) rate.

²The *BL* rates are the collected data expressed on Figure 2 and the *WM* rates are theoretical.

Movement conditions: 1= only *BL* movement data and absence of *WM* (*WM* of 0.00). 2= *BL* data + *WM* of 0.20. 3= *BL* data + *WM* of 0.40. 4= *BL* data + *WM* of 0.60.

In relation to contamination on refuge areas, when we considered only ballooning movement (without walking movement) the durability of the technology was impacted by the simulated contamination level, mainly under contamination degree greater than 10% on structured refuge of 10 and 20%. In terms of decreasing time to resistance, equivalent results were obtained with ballooning combined with walking movement rate of 0.40 in all refuge patches, with exception of 5% of contamination on structured refuge of 10% (**Table 4.2**).

With absence of seed contamination, the time to resistance is about 26-27, 37-39 and 42-43 generations on 10, 20 and 30% of structured refuge, respectively. The highest impact of 5% of contaminated refuge was under consideration of ballooning combined with walking movement on structured refuge of 30%, reducing the average life span in approximately 5 generations (**Table 4.2**).

Table 4.2 – Mean time (Bayesian credible intervals) (in generations) to evolution of resistance of *Spodoptera frugiperda* in structured refuge areas contaminated with Bt cotton plants

Movement condition	Structured refuge (%)	Contamination level (%)				
		0	5	10	15	20
1	10	26.29 (24.31-28.22)	25.25 (23.33-27.12)	22.93 (21.12-24.67)	20.29 (18.63-21.91)	19.52 (17.88-21.09)
1	20	37.32 (34.99-39.59)	37.38 (35.05-39.65)	34.50 (32.28-36.66)	30.01 (27.96-32.00)	26.60 (24.96-28.47)
1	30	43.01 (40.45-45.53)	39.54 (37.12-41.91)	38.94 (36.54-38.95)	39.27 (36.86-41.60)	34.39 (32.17-36.55)
2	10	27.30 (25.35-29.19)	27.30 (25.35-29.19)	21.63 (19.94-23.29)	19.88 (18.28-21.45)	16.60 (15.15-18.01)
2	20	39.84 (37.41-42.22)	33.56 (31.37-35.69)	27.40 (25.45-29.29)	25.00 (23.15-26.80)	23.06 (21.30-24.78)
2	30	42.01 (39.00-45.06)	36.98 (34.23-39.69)	33.58 (31.39-35.72)	29.80 (27.75-31.79)	25.62 (23.74-27.44)

Structured refuge area size used was with non-Bt cotton plants. The size of the structured refuge was defined in relation to the size of the Bt field. Movement conditions are given by the sum between Ballooning (*BL*) data + Walking Movement (*WM*) rate. The *BL* rates are the collected data expressed on Figure 2 and the *WM* rates are theoretical.

Movement conditions:

1= only *BL* movement data and absence of *WM* (*WM* of 0.00).

2= *BL* data + *WM* of 0.40.

4.2.2.2 Second case

To examine the impact of contaminated landscapes on evolution of *S. frugiperda* resistance to Bt cotton, we compared the resistance frequency according to time using logistic regression. The curves were plotted based on estimated credible intervals of the alpha and beta parameters from logistic equation: $[1 / 1 + \exp(\alpha + \beta x)]$.

With the dispersal by ballooning combined with walking rates of 0.60 and 0.40, the resistance occurs fastest in contaminated refuge areas (**Figures 4.3C and 4.3D**). This result was more evident with a walking movement rate of 0.60. Under these conditions the time to resistance was 56 and 41% less when in the absence of contamination or of contamination only in Bt cotton fields, respectively (**Figures 4.3C and 4.3D**).

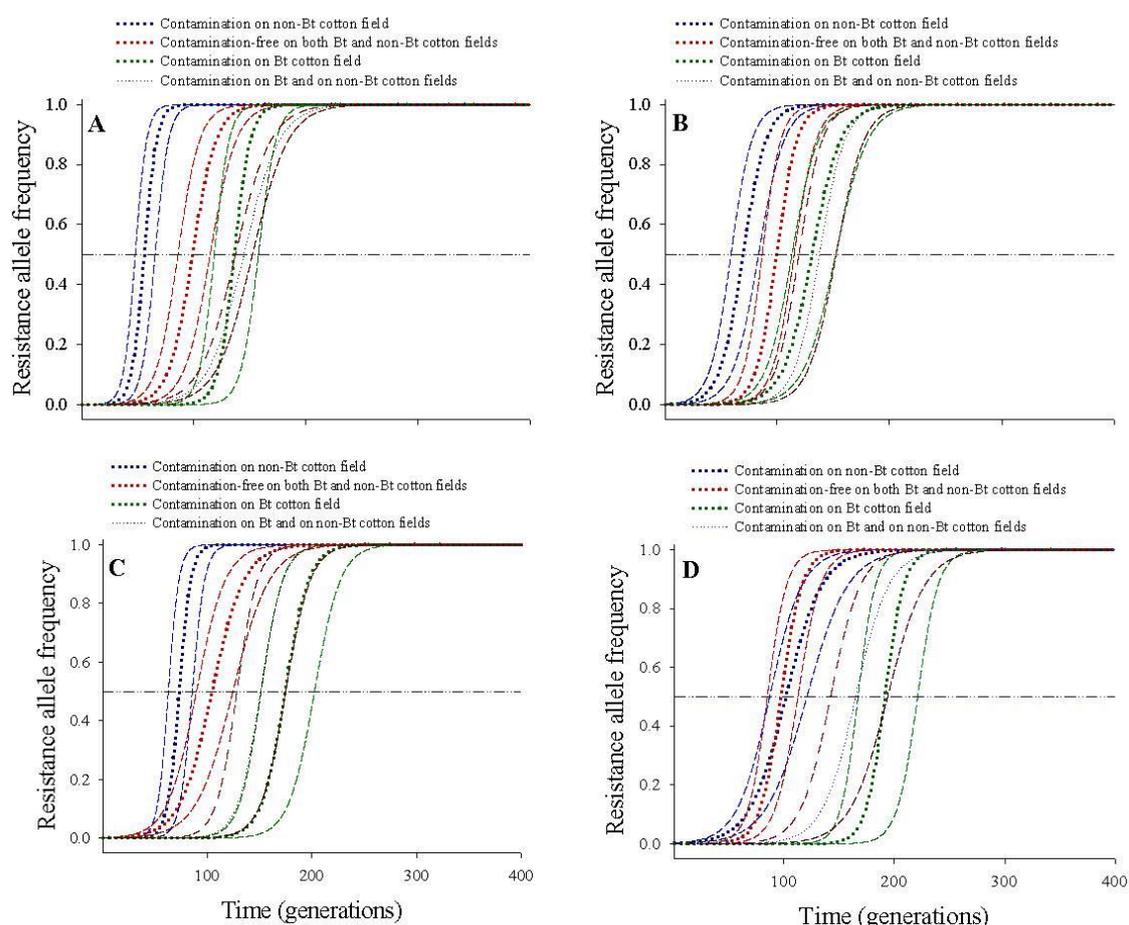


Figure 4.3 – Mean (dots) and Bayesian credible intervals (dashed lines) values of resistance alleles predicted by Bayesian logistic regression in the two-locus two toxin model with stochastic values of ballooning dispersal combined with walking movement (*WM*) rate of 0.00 (**D**) (only ballooning dispersal), 0.20 (**C**), 0.40 (**B**) and 0.60 (**A**). The scenarios assessed consisted of four conditions: contamination on refuge (non-Bt cotton) and contamination-free Bt cotton field (*i*), contamination-free

refuge and contamination on Bt cotton field (ii), contamination on both areas (Bt and non-Bt fields) (iii), and both contamination-free areas (iv).

When the ballooning was combined with walking rate was 0.2, even with the low initial resistance allele frequency, the rate of resistance was greater in the absence of contamination only in the early stages of resistance evolution. The two curves overlapped only during the first 70 generations. After this time, the resistance allele frequency was significantly greater in the contaminated refuge scenario (**Figure 4.3B**).

In the ballooning dispersal with absence of walking movement, there was no difference in terms of resistance evolution between contaminated refuge in relation to contamination-free on Bt cotton and refuge fields (**Figure 4.3A**).

According to biplot (**Figure 4.4**), the highest time to resistance – that corresponds to the relation of the coefficients $-\alpha/\beta$, and the heterozygote fitness on Bt cotton were found on the conditions with contamination of Bt cotton field with non-Bt plants. The results from the vector arrangement in the biplots [correlation = cosine θ], show that a high negative correlation [cosine $\theta = -0.8815$] was evidenced between the variables time to resistance ($-\alpha/\beta$) and ratio W_{RS}/W_{SS} on Bt cotton (**Figure 4.4**).

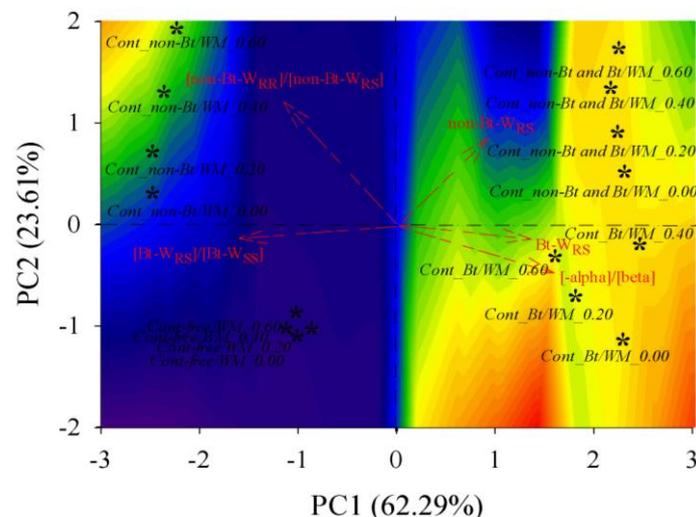


Figure 4.4 – Biplot for the fall armyworm (*Spodoptera frugiperda*) variables on simulated scenarios of contamination on Bt and non-cotton Bt fields and four walking movement rates. *Cont* = contamination; *WM* = walking movement (0.60, 0.40, 0.20 and 0.00); *Bt* = Bt cotton field; *non-Bt* = non-Bt cotton field. The contour area was defined by eigenvectors values map.

Two components were selected based on the presence of components with eigenvalues of the correlation matrix greater than 1. Besides the accumulated sum of the two first components to both cases explains more than 85% of the total data variation, with more than 60% being explained by the first component. According to eigenvector

(v) values the first component is mainly represented by the contrast between the ratios $[W_{RS}]/[W_{SS}]$ on Bt cotton ($v= -0.5514$) and $[-\alpha]/[\beta]$ ($v= -0.5306$) (**Figure 4.4**).

The second component explains 23.61% of the total data variation and is described, highly, by the weighted average of W_{RR}/W_{RS} ($v= 0.8055$) and of heterozygote fitness ($v= 0.5337$) on non-Bt cotton. The highest ratios W_{RR}/W_{RS} on Bt cotton and W_{RS}/W_{SS} on non-Bt cotton were found on scenarios of contamination-free on both Bt and non-Bt cotton fields and contamination on non-Bt cotton field, respectively (**Figure 4.4**).

4.3 Discussion

Here, we tested the hypothesis that the neighborhood could be detected by *S. frugiperda* neonates and this may be enough to discourage ballooning when the natal environment and neighborhood are favorable and unfavorable, respectively, to the larvae; and inverse happened in the opposite condition, mainly for Cry1F-resistant *S. frugiperda*. Based on previous study it was possible to show that non-cotton leaves are not attractive to *S. frugiperda* neonates, probably promoting a fitness cost²⁰. In our research the ballooning frequency on Cry1F-resistant larvae found on a treatment with non-Bt cotton as natal plant and Bt cotton on neighborhood was twice low as when Bt cotton was the natal plant and non-Bt cotton was in the adjacent sites. The host-plant selection by *S. frugiperda* Cry1F-resistant and susceptible population based on ballooning dispersal to avoid non-Bt and Bt cotton plants, respectively, may be attributed to differences in terms of biochemical or electrical physiological differences between non-Bt and Bt cotton plants. In spiders, Morley & Robert²¹ observed the capacity to detect electric fields, and some atmospheric electrostatics provided enough force for dispersal by ballooning.

In simulations with contaminated refuge areas with size of 10 and 20% and with a natural (low) dose of Cry1F and Cry1Ac to *S. frugiperda* on WideStrike, it was revealed that considering only the ballooning movement data, the time to resistance was greatly impacted by the contamination level of 15 and 20%. However, with exception contamination degree greater than 10% on structured refuge of 10 and 20%, our simulations suggested that to the absence of walking movement in a high dose event the ballooning data did not affect strongly the time to resistance. Overall, the time to resistance increased non-linearly in all scenarios, regardless of contamination scenarios. In cases in which the time to resistance is affected by the larval movement, it is expected that the effective dominance may be increased in the movement of heterozygous larvae between Bt to non-Bt plants²². Brévault *et al.*¹¹, showed this response in *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae), with significantly higher dominance of resistance in a seed mixture relative to a structured refuge in block.

We found evidence for the hypothesis that contamination of the refuge by Bt plants speeded resistance evolution; hence, the contamination of the Bt fields by non-Bt plants might be predicted to promote opposite effect and reduce the rate of resistance.

But in both cases of non-high dose and high-dose the rate of resistance was strongly affected by the larval movement. There was a negative relation between time to resistance with walking movement rate associated with ballooning in non-high dose event. Therefore, high movement rates reversed the effect of contamination of Bt fields to slow the resistance evolution⁹.

When we considered a high dose event in our model, results from simulations suggest that the use of a high dose event instead a low dose event could delay resistance evolution even when in situations where there is contamination on Bt or non-Bt cotton fields. Previous research also supported the evidence that the resistance has shown to evolve faster in high movement rates, because the increased likelihood of the heterozygote larvae to move from non-Bt plant to Bt plants, and with survival of some heterozygote larvae in the first case on low dose event and to die in a high frequency in the second case with high dose event⁷.

In the case of presence of non-Bt plants in Bt fields, our findings also are supported by previous modelling results indicating that in low movement rates in both cases (high and non-high dose) there was a significant increased technology life span, because when the movement rate of susceptible larvae off non-Bt plants onto Bt plants is null, the effect of non-Bt plants on Bt fields increases the number of susceptible insects produced by non-Bt plants⁷.

Contamination in Bt cotton fields with non-Bt cotton plants in a high dose event showed the longest time to the resistance. May & Dobson²³ showed that the relation W_{RS}/W_{SS} is approximately equal to proportion resistance frequency at time $\text{step}_{[time+1]}/\text{time step}_{[time]}$. Besides, the time to resistance may be logarithmically inversely proportional to W_{RS}/W_{SS} . We could show with our simulations that the approximations made by May & Dobson²³ are consistent, because there was a negative relation between time to resistance and fitness relation of the heterozygote and susceptible genotypes W_{RS}/W_{SS} . The highest and lowest values of W_{RS}/W_{SS} were observed when there was no contamination and in contaminated refuges, respectively.

The results from this paper are important for implementation of resistance management programs mainly in developing countries, because many farmers that use Bt technology do not have enough infrastructure and personnel to implement and

enforce efficient resistance management practices^{24,9}. In many cases, to eliminate the cost of purchasing seed, they harvest and save their seed in the next year, thereby increasing the contamination probability in both Bt and non-Bt fields. The main concern about the given contamination on refuge areas to low doses events is strong impact on small structured refuge fields. Therefore, our results are within expected with the suggestions made by Farias *et al.*²⁵ in terms of implementation of resistance management strategies, such as the emergencial replacement of single-trait Bt with Bt events with high dose that produce multiple proteins targeting *S. frugiperda*.

4.4 Methods

Spodoptera frugiperda rearing was performed at the Insect Ecology and Forestry Entomology Laboratory (ESALQ/USP), Piracicaba, São Paulo, Brazil. Larvae rearing stock were kept in a phytotron controlled chamber at $26\pm 1^{\circ}\text{C}$, with a relative humidity of $70\pm 10\%$ and 12-h photophase.

4.4.1 Ballooning behaviour in field experiment

Three *S. frugiperda* genotypes were used in the study: a susceptible (*SS*), a Cry1F-resistant (*RR*) and a heterozygote (*RS*). The heterozygote was obtained from a reciprocal cross of resistant with susceptible insects²⁶. Two cotton varieties were used in the study, one a Bt cotton expressing the genes for the Bt proteins Cry1Ac/Cry1F [variety FM 975 (WideStrike[®])] and its non-Bt isoline [variety FM 993]. The study was performed at ESALQ's cotton field. The experiment design consisted of a factorial 3 (insect genotypes) \times 4 (cotton landscapes) \times 5 (time exposure) factorial in randomized blocks, with four replications, each represented by a plot measuring 0.05 ha. We used the following 4 treatments: non-Bt cotton plant in the center, and non-Bt cotton plants in the adjacent sites to center (treatment 1); non-Bt cotton plant in the center and Bt cotton plants in the adjacency sites (treatment 2); Bt cotton plant in the center and non-Bt cotton plants in the adjacency sites (treatment 3) and Bt cotton plant in the center and Bt cotton plants in the adjacent sites (treatment 4).

Each treatment was assessed in five periods of plant exposure to neonate, i.e. 0 (immediately after insect release), 15, 30, 45 and 60 min after artificial infestation. The field plots were planted in the last week of August 2015 and first week of September 2016. The cotton plot consisted of rows spaced 0.20 apart with 0.20 m between the plants in the row. There were no recorded incidences of natural occurrences of any lepidopteran pests, included *S. frugiperda*, in the cotton plants.

To assess the effect of central plant and the neighborhood with Bt and non-Bt cotton plants on ballooning behaviour of genotypes over time, plants that reached the eight to ten-leaf stage were infested with recently hatched neonate ≤ 24 h old *S. frugiperda*. According to each time interval, the plants were inspected in relation to ballooning frequency in each central plant. During the experiment time we measured the speed of the wind using a digital anemometer (HYELEC MS6252A Multifunction Digital Anemometer Handheld LCD). Based on the variation of the wind speed, we

conducted an additional experiment about the artificial wind effects on dispersal distance by ballooning as described in the **Supplementary Material I**.

4.4.2 Statistical analysis

Generalized linear models with binomial, beta binomial and quasibinomial with logit, cauchit and log-log complement linkage functions were tested to fit the ballooning data with a linear interaction predictor (insect genotypes *vs* cotton landscapes *vs* time exposure). The goodness-of-fit was evaluated using half-normal plot with simulated envelope²⁷ employing hnp package²⁸ in R program²⁹. An analysis of deviance was performed to assess the significance of the interaction between the factors ($P = 0.05$). Nested models were assessed and compared with *lmtest* package³⁰ in R program²⁹, we used AIC values and likelihood-ratio test to assess the nested models³¹.

4.4.3 Population genetic models - The impact of *S. frugiperda* ballooning and seed contamination on Bt cotton fields and on structured refuge areas on insect resistance evolution

We performed a stochastic computer model using a population-level approach. The model was written in R²⁹. We used the model in two cases, the first case we used the biological data collected of *S. frugiperda* in the described experiments before. In the second case we used the same data ballooning, but we simulated the evolution dynamics in a situation of high dose event. We used a “two-patch” model that contains Bt and refuge fields. Both models can investigate the impact on resistance evolution of larval dispersal in scenarios of contamination in Bt fields with non-Bt cotton plants and non-Bt areas (refuge areas) with Bt cotton plants.

The probability functions used in the current report were defined by Mallet and Porter¹⁰. Here we assumed that the movement rate (M) is the result of the sum between ballooning (BL) and walking movement (WM), and we are assuming that BL may vary according to micro-landscape and genotype. Then, different values were given to BL , and consequently, also M . The key assumption is that the model does not address questions arising from the ballooning movement between the patches (Bt and non-Bt fields). Because the speed wind during the experiment was not enough to promote long distance passive movement (please see **Supplementary Material I**).

Individual movement was modeled as a two-stage life cycle. The first stage is laid on a free-toxin plant with probability F or Bt plant with probability $F-I$, thereafter it may move or not move with probability M or $M-I$, respectively, in which case it ends up on a non-Bt plant or Bt plant again in the second stage with probability F or $F-I$, respectively. The probabilities functions used in the simulations are summarized in **Figure 4.5**.

In case 1 and 2, we considered two Bt toxins, and resistance to each of the two Bt-toxins was governed by diallelic, independently segregating loci. The model focused on the change in frequency of a single resistance locus for each toxin. We are assuming that genes conferring resistance are already present in the population and that resistance to each toxin is coded by a single gene. Therefore, there is one single locus associated with resistance for each of the two toxins (toxin 1 and toxin 2). Each locus is characterized by two alleles. We assumed in both cases (1 and 2) that there was no linkage, and denoted R_1 and R_2 corresponding to alleles conferring resistance to Bt-toxin 1 and Bt-toxin 2, respectively. The same denotation was applied to alleles that promote susceptibility (S_1 and S_2).

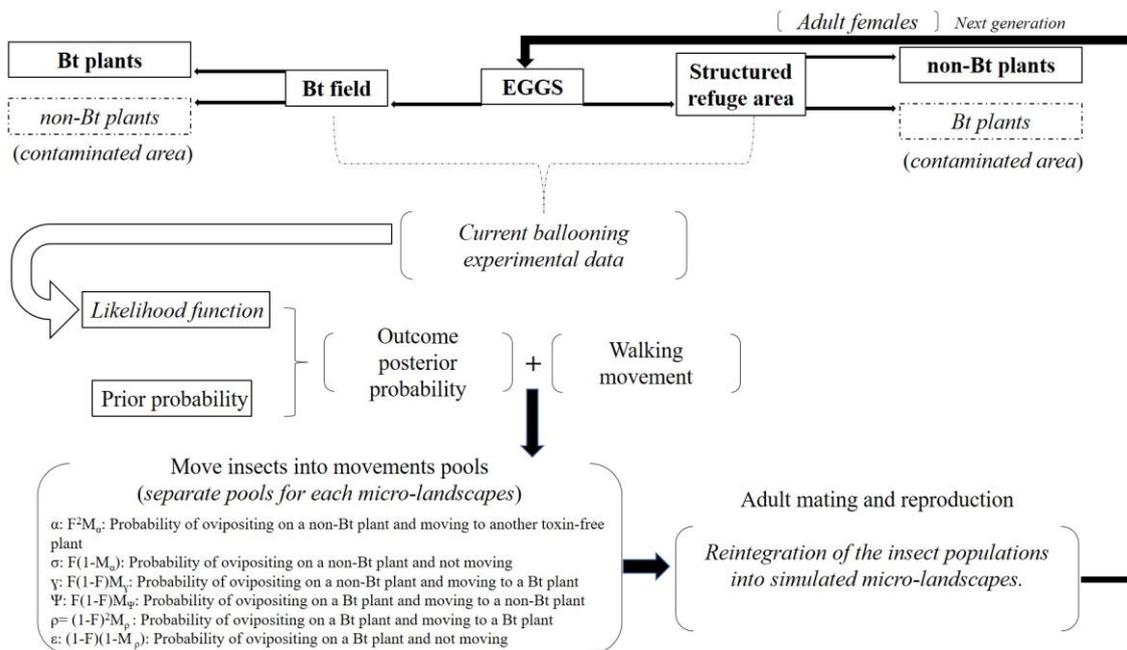


Figure 4.5. Sketch for the selection method of the insect in a given micro-landscape. The patches include Bt fields contaminated with non-Bt plants and structured artificial refuge contaminated with Bt plants.

In both cases (1 and 2) we assumed that there is fitness cost on the susceptible (A_1B_1) and resistant homozygous (A_2B_2) and heterozygous (A_1B_2) or (A_2B_1) on Bt or non-Bt cotton plants. The parameters $c_{1[Bt]}$ and $c_{2[Bt]}$ are the fitness cost on Bt cotton, $c_{1[nBt]}$ and $c_{2[nBt]}$ are the fitness costs on non-Bt cotton, while d_1 and d_2 are the dominances of fitness cost associated with locus 1 and 2, respectively. More precisely, the fitness cost of homozygous and heterozygous are given by $1-c_{i[plant]}$ and $1-d_i \times c_{i[plant]}$, respectively, on Bt cotton or on non-Bt cotton. The selection coefficient is given by s_i that is mortality associated with Bt toxin i . The parameter I_i is the incomplete resistance associated with the resistant allele R_i ³². In overall the homozygous and heterozygous ($i = 1,2$) fitness associated with both loci is the product (multiplicative) of the fitness of the two one-locus genotypes, as follows:

$$WA_{ij}B_{kl} = WA_{ij} \times B_{kl}, i, j, k, l = 1, 2 \quad (\text{eqn 1})$$

The sum of all probabilities, each multiplied by its respective fitness in each condition, gives the survival of each genotype in the agroecosystem. Information about the probabilities (α , σ , γ , Ψ , ρ and ε) are summarized in **Figure 4.5**. In the case of heterozygotes for the resistance gene, the survival individuals when exposed to Bt only on the natal plant and disperse to non-Bt plants (Ψ , ρ) or exposed to Bt plants only after dispersal from non-Bt plant (γ) will be given by: $(1-s_{i[Bt]})+h_i[(1-c_{i[Bt]})-(1-s_{i[Bt]})]$, where h is genetic dominance of resistance. When the natal plant was Bt cotton and there is no dispersal (ε) or when the insects dispersal from Bt plants to other Bt plants the survival is: $[(1-s_{i[Bt]})+h_i(1-c_{i[Bt]})-(1-s_{i[Bt]})]^2$. A similar procedure was adopted to homozygous, but with different coefficients and respective values varying according to the cotton variety, as summarized in **Table 4.3**. Therefore, we denoted the fitness of the susceptible, heterozygous and resistant genotypes associated with Bt toxins expressed in WideStrike are as follows:

$$W(SiSi) = \alpha(Fit_{[SiSi]}) + \sigma(Fit_{[SiSi]}) + \gamma(Fit_{[SiSi]}) + \Psi(Fit_{[SiSi]}) + \rho(Fit_{[SiSi]}) + \varepsilon_n(Fit_{[SiSi]}) \quad (\text{eqn 2})$$

$$W(SiRi) = \alpha(Fit_{[SiRi]}) + \sigma(Fit_{[SiRi]}) + \gamma(Fit_{[SiRi]}) + \Psi(Fit_{[SiRi]}) + \rho(Fit_{[SiRi]}) + \varepsilon_n(Fit_{[SiRi]}) \quad (\text{eqn 3})$$

$$W(RiRi) = \alpha(Fit_{[RiRi]}) + \sigma(Fit_{[RiRi]}) + \gamma(Fit_{[RiRi]}) + \Psi(Fit_{[RiRi]}) + \rho(Fit_{[RiRi]}) + \varepsilon_n(Fit_{[RiRi]}) \quad (\text{eqn 4})$$

Table 4.3 - Fitness of genotypes on Bt and non-Bt cotton plants

Cultivar	$Fit_{[SiSi]}$	$Fit_{[SiRi]}$	$Fit_{[RiRi]}$
Bt cotton	$1-s_{i[Bt]}$	$(1-s_{i[Bt]})+h_i[1-c_{i[Bt]}-(1-s_{i[Bt]})]$	$I_i \times (1-c_{i[Bt]})$
non-Bt cotton	$1-s_{i[nBt]}$	$1-d_i c_{i[nBt]}$	$1-c_{i[nBt]}$

Based on a paper published recently by Martinez et al.³³, we incorporated the density dependence effects on resistance evolution. The maximum population growth rate, R_{max} , represented the maximum insect growth. We studied the effects of density dependence using the Hassell equation³⁴.

4.4.3.1 First case

Model scenarios examined the resistance evolution with contamination levels (%) of 0 and 10% of non-Bt plants in Bt cotton fields. We assumed that the insect can move by ballooning and by walking, but in this case, we used 4 fixed walking movement rates: 0.00, 0.20, 0.40 and 0.60. We simulated contamination in refuge areas with four relative rates: 0.00, 5.00, 10.00, 15.00 and 20.00% contamination with Bt plants. In the last case we varied the walking movement rate, as follows: 0.00 and 0.40.

In the simulations we used a uniform distribution to determine the proportion of individuals that would leave the plants by passive movement, thus new ballooning values were randomly selected. We used 95% Bayesian credible intervals of the ballooning experimental data to provide a measure of uncertainty by evaluating a range of values. Therefore, the passive dispersal range by ballooning varied according to the micro-landscape structure (**Table 4.4**).

Table 4.4. Parameter values obtained for the three genotypes using Bayesian credible intervals (CRI 95%)

Condition	Genotype		
	RR	RS	SS
Bt _(C) :Bt _(A)	0.0804 – 0.1617	0.0497 – 0.1076	0.0910 – 0.1758
non-Bt _(C) :non-Bt _(A)	0.0981 – 0.1852	0.0920 – 0.1645	0.0875 – 0.1711
non-Bt _(C) :Bt _(A)	0.1161 – 0.2084	0.0472 – 0.1039	0.0400 – 0.1034
Bt _(C) : non-Bt _(A)	0.0400 – 0.1034	0.0600 – 0.1221	0.0531 – 0.1232

We calculated the Bayesian credible intervals in two steps, the first step was using the observations of the treatment as described before. The second step was to determine credible intervals of the time when the resistance allele frequency is at 0.50. When the

mean survivorship in the fields (Bt or refuge areas) becomes 50% is assumed that the resistance has evolved. For both analysis on both steps, we used 30,000 iterations using a Monte Carlo and Markov chains (MCMC) process with three strings for each parameter and with a burn-in of 5,000 samples. The convergence of the chains was checked by means of graphical analysis (data not included). The parameters were estimated using the R2jags package³⁵.

The sum of resistant and susceptible alleles equal 1 at the start and the end of each generation in the model. We used logistic regression to estimate the time to resistance and its respective credible intervals. Bayesian credible intervals from ballooning data and resistance allele frequency were calculated as follows:

$$\mu_1 \pm t_{n-1} \sigma_1 \quad (\text{eqn 1})$$

where

$$\mu_1 = \frac{1/\sigma_0^2}{n/\sigma^2 + 1/\sigma_0^2} \mu_0 + \frac{n/\sigma^2}{n/\sigma^2 + 1/\sigma_0^2} \bar{x} \quad (\text{eqn 2})$$

and

$$\sigma_1^2 = \frac{\sigma^2 \sigma_0^2}{\sigma^2 + n \sigma_0^2} \quad (\text{eqn 3})$$

where σ^2 is the population variance, μ_0 is the prior mean and σ_0^2 is the prior variance.

4.4.3.2 Second case

In the second case, we used the same procedure mentioned before and ballooning data to simulate the resistance evolution of *S. frugiperda*, but in this case we simulated how long the technology would be lost with a high dose event. From the operational viewpoint, we are using the concept recommended by Caprio & Sumerford³, that a high dose should be one that exceeds, by 50-fold, the toxin concentration needed to kill 99% of susceptible individuals at an identical stage. Therefore, we used a low value of the dominance of the resistance to both allele (h_1 and h_2). The complete information about default values of both cases (1 and 2) is available in **Table 4.5**.

We simulated the mean frequency resistance alleles of *S. frugiperda* to Bt cotton with four walking movement rates: 0.00; 0.20; 0.40 and 0.60 on the following cotton conditions: contaminated refuge (*i*) and Bt (*ii*) areas, contamination (*iii*) and free-contamination (*iv*) on both areas. The contamination rate used was 5% for these

simulations. The refuge size simulated was defined in relation to the size of the Bt field, we used refuge size of 30% of the Bt field. We also used Bayesian credible intervals to estimate the resistance frequency.

Table 4.5. Default values for parameters used in model simulations

Parameter	Default values	
	Case 1	Case 2
Initial population	10^{12}	10^{12}
Initial allele frequency (R_1)	10^{-11}	10^{-11}
Initial allele frequency (R_2)	10^{-11}	10^{-11}
μ_1	0.99999	0.99999
μ_2	0.99999	0.99999
I_1	0.90000	0.90000
I_2	0.90000	0.90000
h_1	0.50000	0.00100
h_2	0.50000	0.00100
cBt_1	0.00001	0.00001
cBt_2	0.00001	0.00001
$cnBt_1$	0.35000	0.35000
$cnBt_2$	0.35000	0.35000
d_1	0.40000	0.40000
d_2	0.40000	0.40000

i = allele, toxin or locus 1 or 2. μ_i = mortality associated with Bt toxin i . I_i = incomplete fitness of the resistance allele i . h_i = dominance of the resistance allele i . cBt_i = fitness cost of the resistance to Bt cotton associated with locus i . $cnBt_i$ = fitness cost on non-Bt cotton associated with locus i . d_i = dominance of the fitness cost with associated with locus i .

References

- ¹ISAAA. Global status of commercialized biotech/GM crops: 2016. ISAAA: Ithaca, New York. ISAAA Brief No. 52. 135p. (2016).
- ²Roush, R. T. Managing pests and their resistance to *Bacillus thuringiensis*: Can transgenic crops be better than sprays? *Journal Biocontrol Science and Technology*, **4**: 501-516. (1994).
- ³Caprio, M. A. & Sumerford, D. V. "Evaluating transgenic plants for suitability in pest and resistance management programs." Field manual of techniques in invertebrate pathology. Springer, Dordrecht. 769-789 (2007).
- ⁴Rosenheim, J. A. & Tabashnik, B. E. Influence of generation time on the rate of response to selection. *American Naturalist* **137**, 527-541 (1991).
- ⁵Via S. Quantitative genetic models and the evolution of pesticide resistance. In: Pesticide resistance. Strategies and tactics for management. Washington, DC: National Academy Press; p. 222–35. 10.1021/bk-2017-1249.ch005. (1986).
- ⁶Campagne, P., Smouse, P. E., Pasquet, R., Silvain, J. F., Lu, B. L. & Van den Berg, J. Impact of violated high-dose refuge assumptions on evolution of Bt resistance. *Evolutionary Applications*, 596–607 (2016).
- ⁷Carroll, M. W., Head, G., & Caprio, M. A. When and where a seed mix refuge makes sense for managing insect resistance to Bt plants. *Crop Protection* **38**, 74–79 (2012)
- ⁸Chilcutt, C. F. & Tabashnik, B. E. Contamination of refuges by *Bacillus thuringiensis* toxin genes from transgenic maize. *Proceedings of the National Academy of Sciences of the United States of America National* **101**, 7526-9 (2004).
- ⁹Glaum, P. R., Ives, A. R. & Andow, D. A. Contamination and management of resistance evolution to high-dose transgenic insecticidal crops. *Theoretical Ecology* **5**, 195–209 (2012).
- ¹⁰Mallet, J. & Porter, P. Preventing insect adaptation to insect – resistant crops: are seed mixes or refugia the best strategy? *Proceedings Royal Society of London Service B* **250**, 165-169 (1992).
- ¹¹Brévault, T., Tabashnik, B. E. & Carrière, Y. A seed mixture increases dominance of resistance to Bt cotton in *Helicoverpa zea*. *Scientific Reports* **5**, 9807 (2015).
- ¹²Yang, F., Kerns, D. L., Head, G. P., Price, P. & Huang, F. Cross-resistance to purified Bt proteins, Bt corn and Bt cotton in a Cry2Ab2-corn resistant strain of *Spodoptera frugiperda*. *Pest Management Science*, **73**, 2495-2503 (2017).

- ¹³Storer, N. P., Babcock, J. M., Schlenz, M., Meade, T., Thompson, G. D., Bing, J. W. & Huckaba, R. M. Discovery and characterization of field resistance to Bt maize: *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Puerto Rico. *Journal of Economic Entomology* **103**, 1031-8 (2010).
- ¹⁴Farias, J. R., Andow, D. A., Horikoshi, R. J., Sorgatto, R. J., Fresia, P., Santos, A. C. & Omoto, C. Field-evolved resistance to Cry1F maize by *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Brazil. *Crop Protection* **64**: 150-158 (2014).
- ¹⁵Omoto, C., Bernardi, O., Salmeron, E., Sorgatto, R. J., Dourado, P. M., Crivellari, A. & Head, G. P. Field-evolved resistance to Cry1Ab maize by *Spodoptera frugiperda* in Brazil. *Pest Management Science* **72**, 1727-36 (2016).
- ¹⁶Chandrasena, D. I., Signorini, A. M., Abratti, G., Storer, N. P., Olaciregui, M. L., Alves, A. P. & Pilcher, C. D. Characterization of field-evolved resistance to *Bacillus thuringiensis*-derived Cry1F δ -endotoxin in *Spodoptera frugiperda* populations from Argentina. *Pest Management Science* **74**, 746-754 (2018).
- ¹⁷Huang, 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., Yang, F. & Dangal, V. Cry1F resistance in fall armyworm *Spodoptera frugiperda*: single gene versus pyramided Bt maize. *PLoS One* **9**, e0112958 (2014).
- ¹⁸Li, G., Reisig, D., Miao, J., Gould, F., Huang, F. & Feng, H. Frequency of Cry1F non-recessive resistance alleles in North Carolina field populations of *Spodoptera frugiperda* (Lepidoptera: Noctuidae). *PLoS One* **11**: e 0154492 (2016).
- ¹⁹Common, I. F. B. Moths of Australia. Carlton, Victoria: Melbourne Univ. Press. (1990).
- ²⁰Malaquias, J. B., Godoy, W. A. C., Garcia, A. G., Ramalho, F. S. & Omoto, C. Larval dispersal of *Spodoptera frugiperda* strains on Bt cotton: a model for understanding resistance evolution and consequences for its management. *Scientific Reports* **7**:16109 (2017).
- ²¹Morley, EL & Robert, D. Electric fields elicit ballooning in spiders. *Current Biology*, <https://doi.org/10.1016/j.cub.2018.05.057> (2018).
- ²²Ramalho, F. S., Pachú, J. K. S., Lira, A. C. S., Malaquias, J. B. & Zanuncio, J. C. Feeding and dispersal behavior of the cotton leafworm, *Alabama argillacea* (Hübner) (Lepidoptera: Noctuidae), on Bt and non-Bt cotton: implications for evolution and resistance management. *PLoS ONE*. **9**, e111588 (2014).

- ²³May, R. M. & Dobson, A. P. Population dynamics and the rate of evolution of pesticide resistance, pp. 170-193. In National Research Council, Pesticide resistance. National Academy of Sciences, Washington. (1986).
- ²⁴Ellstrand, N. C. Dangerous liaisons? When cultivated plants mate with their wild relatives. Johns Hopkins University Press, Baltimore. (2003).
- ²⁵Farias, J. R., Andow, D. A., Horikoshi, R. J., Sorgatto, R. J., Santos, A. C. & Omoto, C. Dominance of Cry1F resistance in *Spodoptera frugiperda* (Lepidoptera: Noctuidae) on TC1507 Bt maize in Brazil. *Pest Management Science*. **72**, 974–979 (2016).
- ²⁶Horikoshi, R. J., Bernardi, D., Bernardi, O., Malaquias, J. B., Okuma, D. M., Miraldo, L. L. & Omoto, C. Effective dominance of resistance of *Spodoptera frugiperda* to Bt maize and cotton varieties: implications for resistance management. *Scientific Reports*, **6**, 34864 (2016).
- ²⁷Demétrio, C. G., Hinde, J. & Moral, R. A. Models for overdispersed data in entomology. In C.P. Ferreira & W.A.C Godoy (Eds.), *Ecological modelling applied to entomology* (pp. 219–259). London, Springer Cham Heidelberg. (2014).
- ²⁸Moral, R. A., Hinde, J. & Demétrio, C. G. B. Half-normal plots and overdispersed models in R: the hnp package. *Journal of Statistical Software*, **81**, 1-23 (2017).
- ²⁹R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. <http://www.R-project.org/> (2017).
- ³⁰Zeileis, A., Hothorn, T. Diagnostic checking in regression relationships. *R News* 2(3), 7-10. URL <https://CRAN.R-project.org/doc/Rnews/> (2002).
- ³¹Thomas, R. Data Analysis with R statistical software: A guidebook for scientists. Eco-explore. 166 p.
- ³²Tabashnik, B. E., Gassmann, A. J., Crowder, D. W. & Carrière, Y. Insect resistance to Bt crops: evidence versus theory. *Nature Biotechnology* **26**, 199–202 (2008).
- ³³Martinez, J. C., Caprio, M. A., & Friedenberg, N. A. Density dependence and growth rate: evolutionary effects on resistance development to Bt (*Bacillus thuringiensis*). *Journal of Economic Entomology* **111**, 382-390 (2018).
- ³⁴Hassell, M. P. Density-dependence in single species populations. *Journal of Animal Ecology* **44**, 283–295 (1975).
- ³⁵Su, Y-S, Yajima, M. R2jags: Using R to Run 'JAGS'. R package version 0.5-7. <https://CRAN.R-project.org/package=R2jags> (2015).

5 Behavioural Ecological and Evolutionary Traits of Cry1F-Resistant and -Susceptible Genotypes of *Spodoptera frugiperda* Mediated by Temperature in Refuge Patches of Bt Cotton

Abstract

The knowledge about how the refuge patches, combined with environment factors such as temperature, would influence on population genotypes fitness and on ecological interactions between the genotypes on these patches has been unexplored. In our study, we characterized the movement behaviour of susceptible and Cry1F-resistant genotypes of *S. frugiperda* on Bt cotton expressing Cry1Ac/Cry1F and its non-Bt isoline. In a computer model we tested the hypothesis if one of the genotypes could persist and would lead the other to the exclusion in refuge areas. Our results about movement behaviour of Cry1F-resistant *S. frugiperda* suggested that there was response variability according to the temperature and cotton variety. It was possible to show that the greatest distance moved was Cry1F-resistant *S. frugiperda* on non-Bt cotton at 28°C. The temperature or varieties did not affect the time of resting and continuous mobility of the *S. frugiperda* genotypes. There was no difference in the Cry1F concentration between Bt cotton plants kept at 28°C and 32°C. And finally, the capacity of population expansion of the Cry1F-resistant genotype is similar on patches with 28°C and 32°C. There is therefore little risk of competitive exclusion among the genotypes in refuge areas. The current chapter was written following the manuscript submission guidelines of the journal *Entomologia Experimentalis et Applicata*.

Keywords: Competitive dynamics; Fall armyworm; Resistance; Spatial pattern; Non-Bt areas

5.1 Introduction

Arthropod movement behaviour in a context of decision-making for exploitation of nutritional sources may be an important factor associated with physiological responses from toxic or nutritionally inadequate substances. From that point of view, insect feeding behaviour, which includes plant tissue ingestion in the feeding test stage, and mobility behaviour may be strongly influenced by microclimatic conditions, and several factors may interact and generate additive or antagonistic effects on host acceptance, food intake, and consequent mortality (Zalucki et al., 2002).

The thermal stress may be a stimulus in the dispersal behaviour of Lepidoptera neonates. Genetically modified plants expressing insecticidal proteins from *Bacillus thuringiensis* Berliner and high temperatures may have several consequences on host acceptability and consequent dispersal behaviour as well as survival of the target lepidopteran pest species of Bt crops, such as *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae). The expression of *B. thuringiensis* proteins could be modified by temperature, thus stimulating larval dispersal (Ramalho et al., 2017).

High temperature may partially inhibit the production of enzymes of both insect and Bt plants. Some insect species can distinguish and selectively feed on some parts of cotton plants with low expression of *B. thuringiensis* proteins (Gore et al., 2002; Bommireddy et al., 2007; Jackson et al., 2010). It is likely that the higher intake of Bt cotton plant tissue at high temperatures is related to the synthesis of insecticidal protein, because the expression of the Bt toxin may be affected by many factors, such as the genetic constitution of the cotton varieties, but also by the environmental conditions (Zhang et al., 2001; Mahon et al., 2002; Shen et al., 2010), such as light, temperature, water availability and rainfall (Cui & Xia, 1999; Xing et al., 2001).

Refuge areas of Bt crops are plants that do not express Bt toxins and serve as hosts for the pests and allow the survival of toxin-susceptible individuals (Jin et al., 2015). Functionally, the nearby refuge can contribute to production of relatively abundant susceptible insects in relation to the rare resistant individuals emerging from Bt fields. Insects from both areas will likely mate. It is expected that early in the evolution process, the proportion of susceptible insects will be higher than resistant insects in refuge areas. Based in the fact that there is difference in mobility capacity between susceptible and resistant genotypes on non-Bt plants (Malaquias et al., 2017), it would

be important to model the coexistence of these genotypes in patches that could be favorable for the survival of these genotypes, for example the refuge areas of Bt fields with non-Bt cotton plants. Information about how larval dispersal determines the establishment of a refuge of Bt crops is well recognized (Razze & Mason, 2012); Nonetheless, knowledge of how refuge patches combine with environment factors such as temperature might influence genotypic fitness and on ecological interactions between the genotypes on these patches has been unexplored. Therefore, in our research we studied the movement behaviour of susceptible and Cry1F-resistant genotypes of *S. frugiperda* on Bt and non-Bt cotton in a context of effects of different temperatures.

Currently, the importance of spatial explicit approaches has been well recognized in theoretical ecology, although the space has been neglected by theoretical models in entomology in recent decades (Rodrigues et al., 2014). We used computer modelling with spatial explicit as a tool for understanding the behavioural process. We simulated with individual-based model the differential genotypic dispersal capacity in different scenarios and tested the hypothesis if at least one genotype could persist and would lead to the competitive exclusion of other genotypes in refuge areas with non-Bt cotton plants. Additionally, we quantified the Cry1F production under two temperatures (28°C and 32°C) (**Supplementary Material II**) on Bt cotton plant tissues.

5.2 Material and Methods

5.2.1 Populations

The susceptible and resistant populations of *S. frugiperda* to Cry1F were obtained from Arthropod Resistance Laboratory, Entomology Department – ESALQ/USP, Piracicaba, São Paulo, Brazil. The susceptible population has been maintained in the laboratory for > 15 yr, free of exposure to any pesticides (insecticides or Bt proteins). *S. frugiperda* rearing was performed at Insect Ecology and Forestry Entomology Laboratory (ESALQ/USP). Larvae rearing stock were kept in a phytotron controlled chamber at $26\pm 1^{\circ}\text{C}$, with a relative humidity of $70\pm 10\%$ and 12-h photophase.

5.2.2 Behavioural ecology analyzed by capture images bioassay

Two genotypes of *S. frugiperda* were used in the study: (i) susceptible (*SS*) and (ii) Cry1F-resistant (*RR*). Two cotton varieties were used in the study, one Bt cotton expressing the genes for the Bt proteins Cry1Ac/Cry1F [variety FM 975 (WideStrike®)] and other is its non-Bt isoline [variety FM 993]. Both cotton varieties were planted in plastic pots 25 cm in diameter and 40 cm in height. Bt cotton and non-Bt cotton plants were grown in the Phytotron, and the leaf tissues were removed at plant stage with 6-8 leaves.

The movement behaviour of the caterpillars was recorded with infrared EthoVision® XT automated video capture system (Noldus et al., 2001) during a 24 h period. The following variables were estimated by the program: distance moved – the distance traveled by a body point from the previous sample to the current one; immobile state – indicating the time which there was no change in the animal movement, and time of continuous mobility – that calculates the mobility for which the complete area detected as animal is changing, even if the center point remains at the same place.

The experiment design consisted of a factorial with 3 treatments \times 2 temperatures in a split plot design with randomized blocks. The factors temperature and blocks were random. *S. frugiperda* susceptible larvae were kept on non-Bt cotton leaves (T_1), and Cry1F-resistant larvae were kept on Bt cotton (T_2) and non-Bt cotton leaves (T_3) under temperatures of 28°C and 32°C . For each treatment, 5 replicates (blocks) were used.

The experimental unit consisted of 5 caterpillars, and each insect was placed individually on a Petri dish containing a cotton leaf of Bt or non-Bt cotton plants. The insects were kept in an air-conditioned room regulated under the climatic conditions mentioned above.

5.2.3 Data analysis

We carried out all statistical analyzes using R (version 3.4.3; R Foundation for Statistical Computing, Vienna, AT, 2017). Mixed model ANOVA was used to explore the variance components. For the variables distance moved, immobile state and continuous mobility, the treatment is the fixed effect. Whereas, "block" and "block \times temperature" are the random effects. Tukey's multiple comparison analysis method available in the package ExpDes (Ferreira et al., 2018) was used to compare the experimental groups. The equality between the results from the determination of Cry1F protein content was tested by using the Tukey test ($P = 0.05$). We verified the normality and homogeneity of variance of the results from the both experiments. The residual distribution was checked by using a half normal plot (Moral et al., 2017).

5.2.4 Simulations – Coexistence genotypes resulting from spatially explicit density dependence

In that individual-based model we considered individuals that have alleles conferring resistance and susceptibility to Cry1F toxins. We modelled the coexistence of these genotypes in refuge environment with non-Bt cotton plants, the code was programmed in R program (version 3.4.1; R Foundation for Statistical Computing, Vienna, AT, 2017). Our model examined cannibalism behaviour between immature insects of *S. frugiperda*. Therefore, each patch was represented by one non-Bt cotton plant that could be occupied by only one larva of *S. frugiperda*. We tested the hypothesis if one of the genotypes could persist and would lead to the competitive exclusion of another genotype. Based on our experimental results the mobility of the susceptible genotype is greater than the resistant genotype in Bt cotton fields at 32°C (see **Table 5.1**).

We used genotypic-specific parameter values of reproductive rates (Horikoshi et al. 2014), death rate of adults (Horikoshi et al., 2014), and the threshold number (TN) of genotypes above which recruitment cannot occur. We used the biological data from

Table 5.1, to estimate the TN parameter for our model considering the neighborhood of 8 patches for each central patch. Therefore, it was assumed that each resistant larva had TN of 4 patches at 28°C and 3 patches at 32°C. We introduced, additionally, the effect of local neighborhood density dependence and we tested the hypothesis if the neighborhood could prevent competitive exclusion and allow coexistence of genotypes in refuge areas with only non-Bt cotton patches.

In that model for individuals in the patches located at the boundary of the habitat, we defined that the particular movement rule was given by the periodic or cyclic boundary condition. In that case we assumed that the left-hand edge had the right edge as its left neighbor (and vice versa). In the same way, we assumed that the bottom edge as its neighbor above (and vice versa). The corners were assumed to be reciprocal diagonal neighbours. All patches had the same numbers of neighbors.

We used a universe of 100×100 patches, with total of 10,000 patches. The initial conditions filled the frequency of 10^{-3} of the total patches with resistant genotype and the rest of the patches were filled up with susceptible and heterozygous genotypes in the first-time step. To calculate the initial genotypic frequency, we used the Hardy-Weinberg equilibrium. We assumed that the heterozygous larvae have the same mobility behaviour than susceptible individuals. We wrote a function to define the margins for patches on the top, bottom and edge of universe, U , and which determined all the neighbours of the four corner patches. We wrote also a function to count the number of individuals with both alleles in the eight neighbouring patches (or plants), for any patch i, j . We estimated the total area occupied by each genotype within each time step and the speed of population expansion per time step. The expansion coefficient (β_1) per time step was estimated with the Bayesian linear regression coefficients ($\beta_0 + \beta_1 x$).

5.3 Results

Temperature and the treatments resulting from the combinations of *S. frugiperda* genotypes and varieties showed significant evidence of an effect on the distance moved by the larvae, because there was significantly interaction between these two factors ($F_{16,2} = 4.91$; $P = 0.0217$). The highest distance moved was with the resistant genotypes kept on non-Bt cotton at 28°C. There was no difference on distance moved in this condition in relation to all treatments within 28°C.

The distance moved by the Cry1F-resistant genotype on non-Bt cotton at 32°C was lower than Cry1F-resistant genotype at 28°C. In addition, the distance moved by the Cry1F-resistant genotype was lower on Bt cotton than the susceptible genotype at 32°C (**Table 5.1**).

Table 5.1 – Distance moved (mean±SE) (cm) of Cry1F-resistant and susceptible genotypes of *Spodoptera frugiperda* on Bt and non-Bt cotton kept in two temperatures

Treatments (Variety - Genotype)	Temperature (°C)	
	28	32
Bt cotton – Resistant	21.32±3.87 Aa	15.29±1.85 bA
non-Bt cotton – Resistant	27.28±2.56 aA	20.15±2.76 abB
non-Bt cotton – Susceptible	20.13±3.18 aA	25.88±4.56 aA

Means followed by the same capital letter (comparing temperature in the same treatment) and lower case letter (comparing treatment in the same temperature) are not significantly different as determined by Tukey's test ($P = 0.05$).

The heat maps were generated to investigate the influence of the variety in combination with temperature on larval mobility pattern. This analysis from the heat map shows the average distribution of all replicates/treatment in a Petri dish in the accumulated recording time. We observed that the temperature influenced mobility patterns for all treatments. Comparing all conditions, the susceptible genotype kept on non-Bt cotton at 28°C had the most uniform mobility pattern. On the other hand, the most aggregated mobility pattern was found with Cry1F-resistant individuals with Bt cotton at both 28°C and 32°C (**Figure 5.1**).

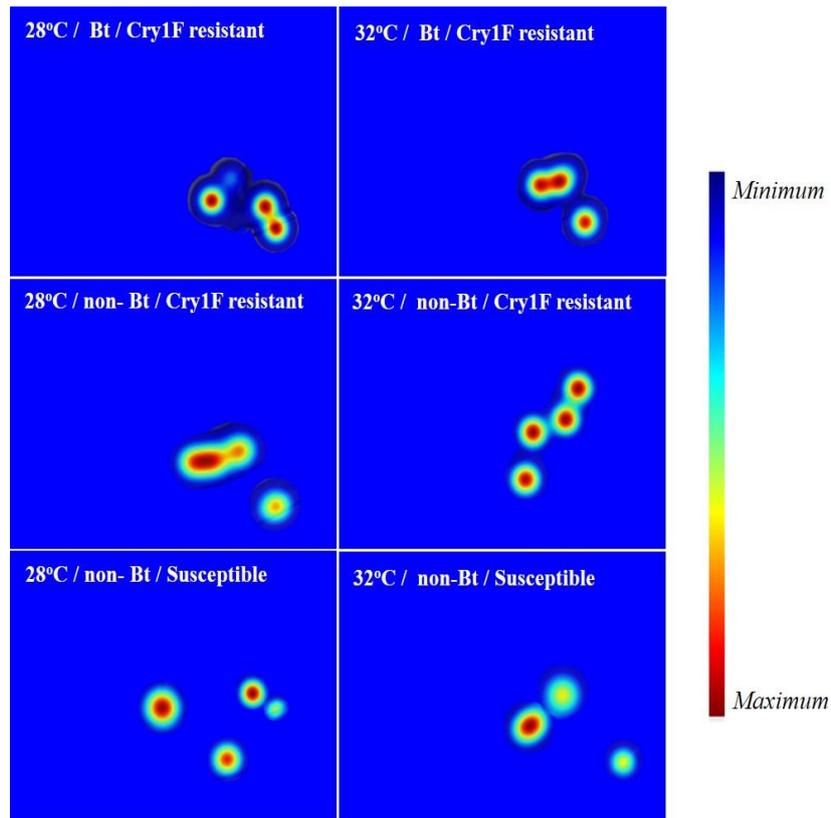


Figure 5.1 – Heat maps of activity movement of Cry1F-resistant and susceptible genotypes of *Spodoptera frugiperda* on Bt and non-Bt cotton leaves at 28°C and 32°C. Red points show the maximum movement, while blue points reveal the minimum movement.

There was no effect of temperature or treatment and the interaction between them ($P > 0.05$) on mean immobile state and on mean, maximum and total (accumulated) continuous mobility time (**Table 5.2**).

Table 5.2 – Immobile and continuous mobility time (mean±SE) of Cry1F-resistant and susceptible genotypes of *Spodoptera frugiperda* in Bt and non-Bt cotton leaves kept in two temperatures

Temperature (°C)	Treatment (Variety / Genotype)	Mean immobile time (s)	Continuous mobility time (s)		
			Mean	Maximum	Total
28	Bt cotton/Resistant	2542.28±650.86	1.36±0.28	44.41±22.78	9532.85±1996.52
	non-Bt cotton /Resistant	2573.15±663.55	1.25±0.25	42.89±21.19	8682.14±1778.96
	non-Bt cotton /Susceptible	2542.20±650.89	1.32±0.13	42.23±23.58	9187.92±921.31
32	Bt cotton/Resistant	1221.58±618.99	2.24±0.53	67.33±20.28	10842.88±2280.00
	non-Bt cotton /Resistant	1860.40±757.49	2.04±0.59	51.98±17.84	14252.91±4119.17
	non-Bt cotton /Susceptible	2598.25±634.56	1.32±0.12	40.28±21.92	9220.19±869.45
Temperature effect		$F_{16,1}= 0.664; P= 0.461$	$F_{16,1}= 2.277; P= 0.161$	$F_{16,1}= 0.116; P= 0.751$	$F_{16,1}= 1.502; P= 0.288$
Treatment effect		$F_{16,2}= 0.600; P= 0.561$	$F_{16,2}= 1.189; P= 0.428$	$F_{16,2}= 0.401; P= 0.676$	$F_{16,2}= 0.465; P= 0.637$
Interaction effect between Temperature vs Treatment		$F_{16,2}= 0.655; P= 0.533$	$F_{16,2}= 0.879; P= 0.434$	$F_{16,2}= 0.289; P= 0.753$	$F_{16,2}= 0.758; P= 0.485$

$F= F$ -value. $P= P$ -value

Based on Bayesian credibility intervals of 95% (95% CrI), we compared the expansion area (m^2) average per hectare between landscapes with mean temperatures of 28°C and 32°C . There was evidence that the posterior density overlapped zero, therefore showing no statistically significant difference between groups (**Figure 5.2**). Additionally, we observed that the population expansion of the resistant individuals per time step is greater than zero (**Figure 5.2**), therefore excluding the hypothesis of extinction risk of this genotype by the susceptible or heterozygous larvae.

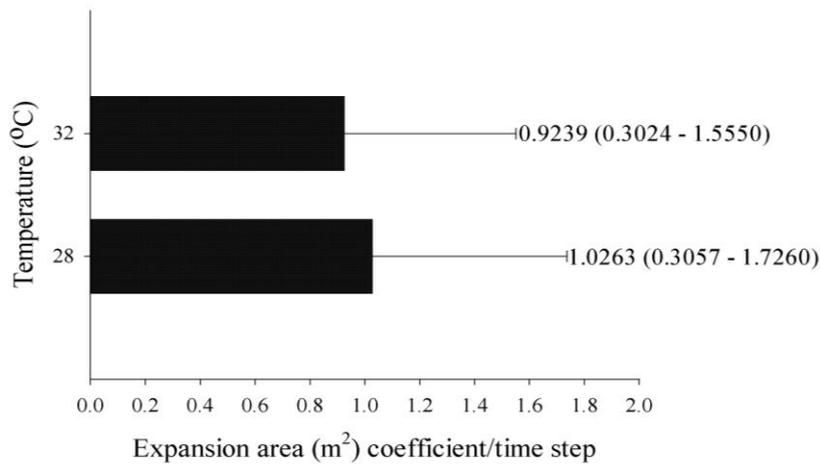


Figure 5.2 – Expansion area (m^2) average (Bayesian credibility intervals 95%) per hectare of the Cry1F-resistant on refuge area with non-Bt cotton plants during 200-time steps simulations.

5.4 Discussion

Our study shows differences on movement behaviour and probably on feeding behaviour of *S. frugiperda* larvae exposed to Bt and non-Bt cotton leaves, in two different temperatures. At 28°C with non-Bt cotton, Cry1F-resistant individuals had a higher movement rate and more aggregated dispersal than the individuals of the same genotype kept at 32°C with non-Bt cotton. In several caterpillar species, the biological activity pattern is associated with the body temperature, and in response to changes in environmental conditions (Casey et al., 1988). Few studies have examined whether rising temperature influences larval mobility on Bt plants. In *Alabama argillacea* (Hübner) (Lepidoptera: Noctuidae), the increased temperature (31°C and 34°C) affected detection capacity and plant abandonment by the larvae and thus resulted in lower ingestion of vegetal tissue on non-Bt cotton plants in relation to 28°C (Ramalho et al., 2017).

The results from our experiment highlight the response of *S. frugiperda* genotypes to temperature and exposure to Bt and non-Bt cotton. In the video tracking assays and using the same genotypes of *S. frugiperda* that we used in the current study, Malaquias et al. (2017) observed that the least distance moved was by larvae Cry1F-resistant on non-Bt cotton under temperature of 26°C, however, in this study the lowest movement rates occurred with Cry1F-resistant *S. frugiperda* kept on Bt cotton at 32°C. Therefore, the fitness cost of *S. frugiperda* on non-Bt found in mobility behaviour by Malaquias et al. (2017) could not be generalized and extrapolated to other conditions, but it varies from the environment condition.

Climate change could alter the strength and sign of plant–insect interactions. Excluding the supposition that rising temperature would affect the toxin production of WideStrike on vegetative growth, the temperature-driven impacts on *S. frugiperda* movement could be influenced by the interaction between host plant and insect genotype. Specially, in some cases, rising temperatures influenced insect feeding behaviour promoting alteration of plant nutritional and chemical traits (Lemoine et al., 2013). On other hand, studies have reported that increased temperatures (36-40°C) may cause reduction in protein expression in Bt plant tissue, resulting in reduced efficiency of Bt plants during cotton square formation (Chen et al., 2005, 2012); This hypothesis was rejected to the conditions of our experiment, because there was no difference on Cry1F protein amount in Bt cotton plants between 28°C and 32°C (please see Supplementary Material II). The same was observed during vegetative growth or during flowering of plants in other studies (Chen et al., 2005, 2012).

In our simulations of the spatiotemporal dynamics of *S. frugiperda* showed that coexistence between individuals with resistant and susceptible alleles in the investigated environment is possible. Some researchers have been drawing attention to the possible consequences of rising temperature for *S. frugiperda* dynamics (Garcia et al., 2018a). Garcia et al. (2018b) investigated the spatio-temporal dynamics of *S. frugiperda* in landscapes with Bt and non-Bt fields and observed that a rise of 1°C in the weekly mean temperature could almost double the size of *S. frugiperda* populations. Even the reduced mobility capacity observed in our experiment under 32°C in relation to 28°C of the Cry1F-resistant *S. frugiperda* on non-Bt cotton, the rate of area expansion was not significantly different between the environment with these two temperature conditions.

According to our results, it was possible to conclude that the greatest distance moved was on Cry1F-resistant *S. frugiperda* with non-Bt cotton at 28°C. The temperature or varieties did not affect the time of resting and continuous mobility of the *S. frugiperda* genotypes. There was no difference on Cry1F concentration on Bt cotton plants that were kept at 28°C and 32°C. And finally, the capacity of population expansion area of Cry1F-resistant genotype is similar on patches at 28°C and 32°C. Besides, there is no risk of competitive exclusion between resistant and susceptible individuals on refuge area.

References

- Bernardi O, Bernardi D, Horikoshi R, Okuma DM, Miraldo L, Fatoretto J, Medeiros FCL, Burdc T & Omoto C (2016) Selection and characterization of resistance to the Vip3Aa20 protein from *Bacillus thuringiensis* in *Spodoptera frugiperda*. *Pest Management Science*. doi: 10.1002/ps.4223
- Bommireddy PL, Leonard BR & Emfinger K (2007) Heliothine larval behavior on transgenic cotton expressing a *Bacillus thuringiensis* insecticidal exotoxin, Vip3A. *The Journal of Cotton Science* 11:199–207.
- Casey TM, Joos B, Fitzgerald TD & Young PA (1988) Synchronized group foraging, thermoregulation, and growth of eastern tent caterpillars in relation to microclimate. *Physiological Zoology* 61 (4): 372-377.
- Chen D, Ye G, Yang C, Chen Y & Wu Y (2005) The effect of high temperature on the insecticidal properties of Bt cotton. *Environmental and Experimental Botany* 53: 333–342.
- Chen Y, Wen Y, Zhang X & Chen D (2012) The effects of the relative humidity on the insecticidal expression level of Bt cotton during bolling period under high temperature. *Field Crops Research* 137: 141–147.
- Cui J & Xia J (1999) The spatio-temporal distribution of Bt (*Bacillus thuringiensis*) insecticidal protein in Bt cotton. *Cotton Science* 11(3):141–146.
- Farias JR, Horikoshi RJ, Santos AC & Omoto C (2014) Geographical and temporal variability in susceptibility to Cry1F toxin from *Bacillus thuringiensis* in *Spodoptera frugiperda* (Lepidoptera: Noctuidae) populations in Brazil. *Journal of Economic Entomology* 107(6): 2182–2189.
- Ferreira EB, Cavalcanti PP & Nogueira DA (2018). *ExpDes: Experimental Designs*. R package version 1.2.0. <https://CRAN.R-project.org/package=ExpDes>.
- Garcia A, Cônsoli FL, Ferreira CP & Godoy WAC (2016) Predicting evolution of insect resistance to transgenic crops in agricultural landscapes, based on larval movement. *Ecological Complexity* (submitted/Under review).

- Garcia AG, Godoy WAC, Thomas JMG, Nagoshi RN & Meagher RL (2018a) Delimiting strategic zones for the development of fall armyworm (Lepidoptera: Noctuidae) on corn in the State of Florida. *Journal of Economic Entomology* 111:120–126
- Garcia AG, Ferreira CP, Godoy WAC & Meagher RL (2018b) A computational model to predict the population dynamics of *Spodoptera frugiperda*. *Journal of Pest Science*. doi: 10.1007/s10340-018-1051-4.
- Gore J, Leonard BR, Church GE & Cook DR (2002) Behavior of bollworm (Lepidoptera: Noctuidae) larvae on genetically engineered cotton. *Journal of Economic Entomology* 95(4): 763–769.
- Jackson RE, Gore J & Abel C (2010) Bollworm (Lepidoptera: Noctuidae) behavior on transgenic cotton expressing Cry1Ac and Cry1F proteins. *Journal of Entomological Science* 45(3): 252–261.
- Jin L, Zhang H, Lu Y, Yang Y, Wu K, Tabashnik BE & Wu Y (2015). Large-scale test of the natural refuge strategy for delaying insect resistance to transgenic Bt crops. *Nature Biotechnology* 33(2): 169-178.
- Lemoine NP, Drews WA, Burkepille DE & Parker JD (2013) Increased temperature alters feeding behavior of a generalist herbivore. *Oikos* 122(12): 1669-1678.
- Malaquias JB, Godoy WAC, Garcia AG, Ramalho FS & Omoto C (2017) Larval dispersal of *Spodoptera frugiperda* strains on Bt cotton: a model for understanding resistance evolution and consequences for its management. *Scientific Reports* doi:10.1038/s41598-017-16094.x
- Mahon R, Finnergan J, Olsen K & Lawrence L (2002) Environmental stress and the efficacy of Bt cotton. *The Australian Cottongrower* 22:18–21
- Moral RA, Hinde J & Demétrio CGB (2017) Half-normal plots and overdispersed models in R: the hnp package. *Journal of Statistical Software* 81, 1-23.
- Noldus LP, Spink AJ & Tegelenbosch RA (2001) EthoVision: a versatile video tracking system for automation of behavioral experiments. *Behavior Research Methods, Instruments & Computers* 33(3): 398-414.

- Noldus LP, Spink AJ & Tegelenbosch RA (2002) Computerised video tracking, movement analysis and behaviour recognition in insects. *Computers and Electronics in Agriculture* 35(2–3): 201–227.
- Omoto C, Bernardi O, Salmeron E, Sorgatto RJ, Dourado PM, Crivellari A, Carvalho RA, Willse A, Martinelli S & Head G (2016) Field-evolved resistance to Cry1Ab maize by *Spodoptera frugiperda* in Brazil. *Pest Management Science* doi: 10.1002/ps.4201.
- R Core Team. R (2017) A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. <http://www.R-project.org/>
- Ramalho FS, Pachú JKS, Lira ACS, Malaquias JB, Brito BDS, Zanuncio JC & Fernandes FS (2017) Effects of temperature on the feeding behavior of *Alabama argillacea* (Hübner) (Lepidoptera: Noctuidae) on Bt and non-Bt cotton plants. *Anais da Academia Brasileira de Ciências* 89 (4): 2955-2969.
- Razze JM, Mason CE & Pizzolato TD (2011) Feeding behavior of neonate *Ostrinia nubilalis* (Lepidoptera: Crambidae) on Cry1Ab Bt corn: Implications for resistance management. *Journal of Economic Entomology* 104: 806–813.
- Rodrigues LAD, Varriale MA, Godoy, WAC & Mistro DC (2014) Coupled Map Lattice Model for Insects and Spreadable Substances. *Ecological Modelling Applied to Entomology* (ed. by CP Ferreira & WAC Godoy), Springer Nature, Switzerland, pp. 141-169.
- SAS Institute (2005) SAS/STAT 9.1 user's guide - the MIXED procedure. Cary: SAS Institute.
- Shen P, Lin KJ, Zhang YJ, Wu KM & Guo YY (2010) Seasonal expression of *Bacillus thuringiensis* insecticidal protein and the control to cotton boll worm in different varieties of transgenic cotton. *Cotton Science* 5: 393–397.
- Vélez AM, Alves AP, Blankenship EE & Siegfried BD (2016) Effect of Cry1F maize on the behavior of susceptible and resistant *Spodoptera frugiperda* and *Ostrinia nubilalis*. *Entomologia Experimentalis et Applicata*, 159(1): 37–45.

Xing C, Jing S, Cui X, Guo L, Wang H & Yuan Y (2001) The spatio-temporal distribution of Bt (*Bacillus thuringiensis*) insecticidal protein and the effect of transgenic Bt cotton on bollworm resistance. *Cotton Science* 13(1): 11–15.

Zhang Y, Wu K & Guo Y (2001) On the spatio-temporal expression of the contents of Bt insecticidal protein and the resistance of Bt transgenic cotton to cotton bollworm. *Acta Phytopathologica Sinica* 8(1):1–6.

Zalucki. MP, Clarke AR & Malcolm SB (2002) Ecology and behavior of first instar larval Lepidoptera. *Annual Review of Entomology* 47: 361–393.

6. General Conclusions

Here, in the results it was found the first findings concerning the fitness cost of larval behaviour traits of *S. frugiperda* associated with Cry1F resistance in Brazilian populations, but that result was observed at 25°C. In addition, the effects on spatial distribution of the resistant individuals in Bt and non-Bt cotton fields indicated differences in mobility capacity between Bt and non-Bt cotton.

In the analysis about the consequences to resistance evolution of the dispersal pattern of genotypes of *S. frugiperda* in pure and contaminated landscapes, it was evidenced that Cry1F-resistant genotype avoided non-Bt cotton. The heterozygote had similar dispersal behaviour as the susceptible genotype when non-Bt cotton was the natal plant. Based on computer simulations, the evolution of resistance may be > 75-fold faster in relation to a contamination-free refuge.

In the assessment about the impact of the dispersal by ballooning combined with walking dispersal on resistance evolution in areas with plant mixture in events with high and non-high dose, we concluded that the ballooning frequency on Cry1F-resistant larvae found on non-Bt cotton as natal plant and Bt cotton in the adjacent sites was lower than when Bt cotton was the natal plant and non-Bt cotton was in the adjacent sites. The presence of non-Bt cotton plants on Bt cotton fields delayed the time to the resistance. However, in the case of non-high dose, high movement rates reversed the effect of contamination of Bt fields to delay the resistance progress.

In the comparison of the movement dynamics of *S. frugiperda* genotypes between 28°C and 32°C, the greatest distance moved was Cry1F-resistant *S. frugiperda* on non-Bt cotton at 28°C, therefore this result is different in relation to findings concerning the fitness cost of larval behaviour traits of *S. frugiperda* at 25°C. There was no difference between the temperature or varieties on the time of resting and continuous mobility of the *S. frugiperda* genotypes. And finally, when we used an individual-based model, the capacity of population expansion of the Cry1F-resistant genotype is similar on patches with both temperatures.

All information generated in this thesis could contribute in the optimization of resistance management practices under neotropical conditions. Based on importance of implementation of strategies to avoid contamination through inadequate agronomic practices,

it is strongly recommended future studies considering the impact of cross-contamination on larval dispersal of Lepidopteran pests.

Supplementary Material I

Wind influence on ballooning dispersal in laboratory assay - Methods

Three artificial speeds were adopted to assess their influences on passive dispersal distance during ballooning process of *S. frugiperda*. We used a ventilator to produce the wind speed, with the following treatments: 10, 13 and 16 km/h. We used recently hatched neonate ≤ 24 h old. In that experiment a *S. frugiperda* population from laboratory rearing was utilized. The insects were released in cotton plants with eight-leaf stage. The ventilators were turned on when the caterpillars were releasing a silk line as they were leaving the plant. After passive dispersal the moved distance was collected from the natal plant (origin point), that is the same place where the ventilator was installed. But the distance was measured only when the dispersal from natal plant to other sites occurred. The experiment was performed in a closed room at $26 \pm 1^\circ\text{C}$, with a relative humidity of $70 \pm 10\%$ and 12-h photophase. The experiment design consisted of a completely randomized design, with 20 replications per treatment.

Generalized linear models with binomial with logit linkage functions was used analyze the wind influence on the engagement of ballooning (passive dispersal). The goodness-of-fit was evaluated using half-normal plot with simulated envelope (Demétrio *et al.* 2014) employing hnp package (Moral *et al.* 2017) in R program (version 3.4.1; R Foundation for Statistical Computing, Vienna, AT, 2017). We compared the proportions of passive dispersal by contrasts using the *glht* function. The moved distance among the treatments (wind speed) were compared by Tukey test.

Wind influence on ballooning dispersal in laboratory assay - Results

In a simulation of wind impact on passive dispersal, we found that the proportion of passive dispersal differed among the wind speed ($Deviance = 21.50$; $P > \chi^2 = 0.00002$). In only 50% of the larvae in the 10 Km/h treatment engaged in passive dispersal from the source. This value differed significantly from the percentages of observed on 13 and 16 km/h ($P < 0.05$), with 95 and 100%, respectively. There was difference among the speeds used in the current experiment ($F = 3.32$; $P > F = 0.0446$). The unique statistical difference among the treatments was reported between the wind speeds of 13 and 10 km/h ($P > F = 0.0345$). There was no difference between 16 and 10 km/h ($P > F = 0.1914$) or between 16 and 13 km/h ($P > F = 0.5764$). 50% of the events (moved distance) were concentrated at 47.00; 22.00 and 27.50 cm

(**Figure 5.3**), and the average were 48.80; 25.89 and 32.67 (data not expressed at box plot), when we used 10; 13 and 16 Km/h, respectively.

On the wind speed of 10 km/h the values of *S. frugiperda* distance moved ranged from 3.00 to 106 cm from the source. On wind speed of 16 km these values ranged from 7.50 cm (minimum) to 71.00 cm (maximum), similar results were reported when we used the wind speed of 13 km/h, with maximum distance of 72.00 cm, but in that case that value is an outlier (**Figure 5.3**).

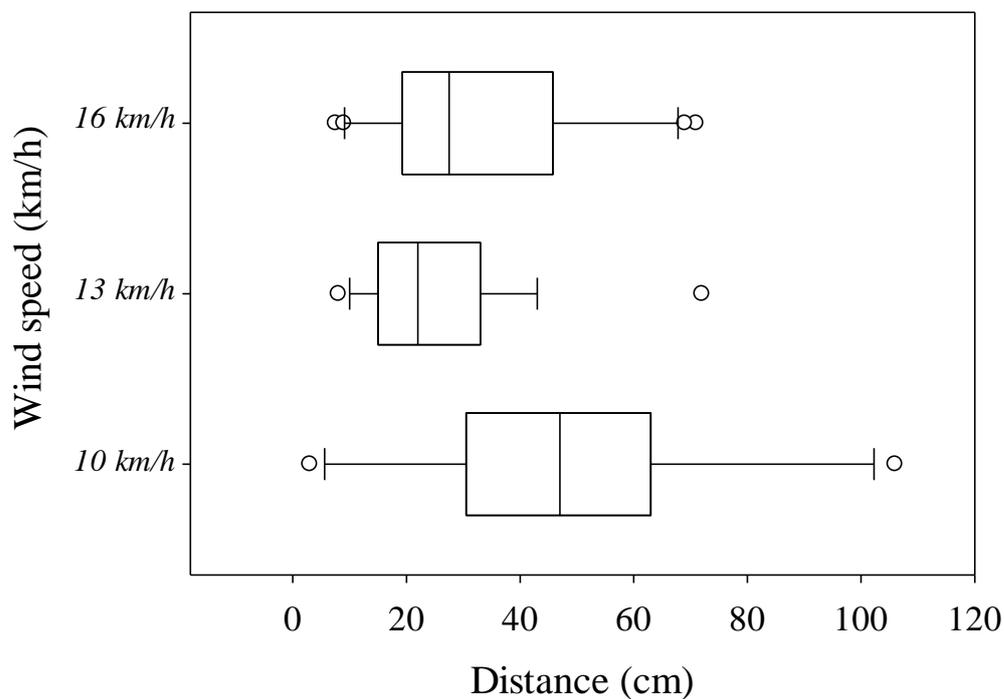


Figure 5.3 Box plot of the moved distance by the artificial wind speed (km/h) simulated in laboratory.

References

Demétrio, C. G., Hinde, J. & Moral, R. A. Models for overdispersed data in entomology in *Ecological Modelling Applied to Entomology*, (ed. Ferreira C. P. & Godoy W. A.) pp. 219–259 (Springer, 2014).

Moral RA, Hinde J, Demétrio CGB (2017) Half-normal plots and overdispersed models in R: the hnp package. *J Stat Softw* 81:1-23. doi: 10.18637/jss.v081.i10

Supplementary Material II

Determination of Cry1F protein content - Methods

The Cry1F protein concentrations in cotton leaves extracts were determined by immunological analysis (ELISA). The subsamples of leaves were prepared by homogenizing the tissue in 2 ml extraction buffer with Sodium chloride 8.0 g., Sodium phosphate, dibasic (anhydrous) 1.15 g, Potassium phosphate, monobasic (anhydrous) 0.2 g, Potassium chloride 0.2 g, Tween-20 0.5 g, dissolved in 1000 ml distilled water. The buffer is a standard part of the used Bt-Cry1F ELISA Kit (Quantitative DAS ELISA for the detection of the Bt-Cry1F transgenic protein, Catalog number: PSP 11700) provided by the company Agdia®.

We rubbed one leaf of each treatment with a marker to completely crush the sample and to mix the contents uniformly. We added the appropriate volume of buffer to glass tube containing the crushed leaf and massaged the samples to ensure good extraction. The extract was sat for 3 minutes before transferring sample to the test wells of the ELISA plate. The supernatant was collected after centrifugation at 11,180×g at 4°C for 20 min, passed through a C18 Sep-Pak Cartridge (Waters, Milford, MA, United States).

Enzyme conjugate (100 µl) were dispensed per well, and the same quantity of each prepared sample, positive control, negative control and PBST buffer were dispensed in their respective wells. With the standard Cry1F insecticidal proteins and samples, the plates were sat inside the humid box and incubated at 28°C for 60 minutes. The absorbance was recorded at 650 nm using Gen5 2.05 Software. Calculation of Cry1Ac protein concentrations from the ELISA data was performed as described on recommendations by the Company.

Determination of Cry1F protein content - Results

In relation to Cry1F concentration on leaves of Bt cotton, we did not find difference between the treatments control with infested ($F_{8,1} = 3.009$; $P = 0.1210$), between temperature ($F_{8,1} = 0.042$; $P = 0.8430$), and the interaction between the factors ($F_{8,1} = 0.016$; $P = 0.9040$) (Table 5.3).

Table 5.3 – Cry1F concentration (ng/ml) on non-infested (control) WideStrike cotton plant and infested with *S. frugiperda* under 28°C and 32°C

Temperature (°C)	Treatment	Cry1F concentration (ng/ml)
28	Control	4.38±0.67
	<i>S. frugiperda</i>	5.70±0.64
32	Control	4.61±0.88
	<i>S. frugiperda</i>	5.75±0.60

There was no difference between the treatments ($P > 0.05$).