CLÉBSON DOS SANTOS TAVARES

SUSCETIBILIDADE DE ÍNSTARES LARVAIS DE POPULAÇÕES DE *Spodoptera frugiperda* A MILHOS Bt E O POTENCIAL DE UM MARCADOR PROTEICO PARA ESTUDAR A DISPERSÃO DE MARIPOSAS

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

VIÇOSA
MINAS GERAIS - BRASIL
2018

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

T
T231s
2018

Tavares, Clébson dos Santos, 1990-
Suscetibilidade de ínstares larvais de populações de *Spodoptera frugiperda* a milhos Bt e o potencial de um marcador proteico para estudar a dispersão de mariposas / Clébson dos Santos Tavares. – Viçosa, MG, 2018.
x, 60 f. : il. (algumas color.) ; 29 cm.

Orientador: Eliseu José Guedes Pereira.
Dissertação (mestrado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Lagarta-do-cartucho. 2. Toxinas. 3. Milho - Resistência a doenças e pragas. 4. Albumina. 5. *Helicoverpa zea*.
I. Universidade Federal de Viçosa. Departamento de Entomologia. Programa de Pós-Graduação em Entomologia.
II. Título.

CDD 22. ed. 632.78

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APROVADA: 20 de fevereiro de 2018.

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Eliseu José Guedes Pereira
(Orientador)

A Deus pela oportunidade a vida;

Aos meus pais pelo amor incondicional e exemplo de simplicidade e honestidade;

Aos meus irmãos pelo amor, carinho e apoio nos momentos difíceis;

Aos meus amigos do Instituto Federal de Alagoas, em especial aos professores Luciano de Holanda e Ângela Froehlich, por acreditarem em mim;

À família de Paula pelo apoio, carinho e companheirismo nestes últimos anos.

Dedico

O maior inimigo do conhecimento não é a ignorância, mas a ilusão do conhecimento

Stephen Hawking

AGRADECIMENTOS

À Universidade Federal de Viçosa e ao Departamento de Entomologia pelo suporte e oportunidade de concluir o mestrado.

Ao programa de Pós-Graduação em Entomologia pela oportunidade e infraestrutura, e ao Conselho Nacional de Desenvolvimento Científico (CNPq) pela concessão da bolsa.

Ao Professor Eliseu José Guedes Pereira pela orientação, dedicação, e confiança nestes últimos anos.

Aos meus coorientadores, Oscar Fernando Santos Amaya pelos ensinamentos e dedicação, e a Professora Silvana Vieira de Paula Moraes por aceitar e facilitar minha viagem para a *University of Nebraska* onde conduzi os experimentos relativos ao segundo capítulo desta dissertação.

À *University of Nebraska* e ao laboratório de toxicologia de inseticidas pela infraestrutura concedida.

Ao meu orientador nos Estados Unidos, Dr. Thomas Hunt pela oportunidade, confiança e apoio financeiro.

À professora Ana Maria Velez e ao Dr. Arnubio Valencia da *University of Nebraska* pelo suporte na condução dos experimentos e ensinamentos.

Aos meus amigos do Laboratório de Interação Inseto-Planta pela convivência, amizade e companheirismo.

Aos funcionários do Departamento de Entomologia responsáveis pela limpeza e manutenção do ambiente de trabalho e a secretaria de Pós-Graduação em Entomologia pela destreza nos serviços prestados.

SUMÁRIO

RESUMO

TAVARES, Clébson dos Santos, M.Sc., Universidade Federal de Viçosa, fevereiro de 2018. **Suscetibilidade de ínstares larvais de populações de *Spodoptera frugiperda* a milhos Bt e o potencial de um marcador proteico para estudar a dispersão de mariposas.** Orientador: Eliseu Jose Guedes Pereira. Coorientadores: Oscar Fernando Santos Amaya e Silvana Vieira de Paula Moraes.

A adoção de refúgio tem sido a principal estratégia para postergar a seleção de resistência de pragas em cultivos Bt. Dependendo do tipo de refúgio, a movimentação larval entre plantas e o padrão de dispersão dos adultos podem afetar a eficácia desta estratégia. Na modalidade de refúgio por mistura de sementes, a exposição a plantas Bt em diferentes idades larvais pode favorecer a seleção de resistência se a suscetibilidade for diminuída em ínstares avançados. Já na modalidade de refúgio em bloco, a distância entre as áreas Bt e não-Bt deve permitir o encontro de adultos provenientes do refúgio e da área Bt. Nesta dissertação de mestrado, determinou-se a suscetibilidade larval da lagarta-do-cartucho em diferentes ínstares a milhos Bt e testou-se o potencial da proteína albumina para estudos de marcação-recaptura de adultos de *S. frugiperda* e *Helicoverpa zea* (Lepidoptera: Noctuidae). Ao se expor larvas de populações de *S. frugiperda* com distintos perfis de resistência a toxinas Bt a folhagem de diferentes milhos Bt, a suscetibilidade variou com o perfil da resistência a Bt da população e com a idade larval (i.e., tempo de exposição) em dois dos três tipos de milho Bt testados. A sobrevivência larval da população resistente a toxina Cry1F aumentou de 8.33% (< 24h de idade) a 100% (larvas expostas com 10 dias) no milho piramidado Cry1A.105+Cry2Ab2. Em contrapartida, a população suscetível padrão apresentou alta mortalidade larval independentemente da idade de exposição ao milho Cry1A.105+Cry2Ab. Diferentemente do milho Cry1A.105+Cry2Ab, folhagem de milho Bt com a toxina Vip3Aa causou perto de 100% de mortalidade em larvas com até 10 dias de idade (máxima idade testada) independente do genótipo da população. Estes resultados indicam que a resistência prévia a certas toxinas Bt pode reduzir a suscetibilidade a alguns milhos Bt e que a toxina Vip3Aa tem grande potencial para o controle de populações de *S. frugiperda* resistentes a toxinas Cry, inclusive de larvas de último ínstar. No segundo estudo, a

pulverização de mariposas com albumina no laboratório resultou em 100% de indivíduos marcados de ambas as espécies e com cinco dias após a marcação a proteína foi adequadamente detectada. A maioria dos adultos pulverizados em gaiolas no campo também foram marcados pela proteína e esta foi detectada até cinco dias depois. Finalmente, a pulverização direta de albumina no campo resultou em 15% de adultos de *H. zea* marcados em 317 que foram coletados em armadilhas luminosas e de feromônio. Estes resultados demonstram que albumina é um potencial marcador que pode ser adotado para o estudo do comportamento de dispersão de *S. frugiperda* e *H. zea.* Em conjunto, os resultados desta dissertação devem auxiliar na tomada de decisões no manejo de populações da *S. frugiperda* e em futuras pesquisas relacionadas à adequada adoção de refúgio para manejo de resistência em culturas Bt.

ABSTRACT

TAVARES, Clébson dos Santos, M.Sc., Universidade Federal de Viçosa, February, 2018. **Susceptibility of larval instars of *Spodoptera frugiperda* populations to corn Bt hybrids and the potential of a protein marker to study moth dispersion.** Adviser: Eliseu José Guedes Pereira. Co-advisers: Oscar Fernando Santos Amaya and Silvana Vieira de Paula Moraes.

The adoption of refuge has been the main strategy to delay the evolution of pest resistance in Bt crops. Depending on the refuge configuration, the larval movement between plants and the dispersion pattern of adults may affect the efficiency of this strategy. In seed-mixture refuge, the exposure to Bt plants at different larval ages may favor the evolution of resistance if the susceptibility is decreased at advanced instars. In structured refuge (i.e., in blocks), the distance between Bt and non-Bt areas must allow the encounter between adults from the refuge and the Bt area. In this master thesis, we determined the susceptibility of fall armyworm at different larval stages to Bt corn hybrids and tested the potential of the protein albumin for marking-recapture studies of moths of *S. frugiperda* and *Helicoverpa zea* (Lepidoptera: Noctuidae). When exposing *S. frugiperda* larvae from populations of contrasting Bt resistance profile to different types of Bt corn, the susceptibility varied with the population and the age of exposure in some Bt transgenic events. Larval survival of the Cry1F-resistant population varied from 8.33% (< 24h old) to 100% (exposed at 10 days old) on Cry1A.105+Cry2Ab. In contrast, the standard susceptible population showed high mortality on Cry1A.105+Cry2Ab regardless of the age of exposure. Unlike Cry1A.105+Cry2Ab corn, Bt corn with Vip3Aa toxin effectively killed close of 100% of larvae up to 10 days old (the highest age tested) regardless of the population tested. These results indicate that previous development of resistance to Bt toxins can reduce the susceptibility to certain Bt corn hybrids and that the Vip3Aa toxin has high potential to control *S. frugiperda* populations that have developed resistance to other Cry toxins even if the larvae are close to pupation. In the second study, spraying *H. zea* and *S. frugiperda* moths with albumin in the laboratory resulted in 100% individuals marked of both species, and the protein was properly detected five days later. Most adults sprayed in field cages were also marked by the protein, and they

were detected up to five days later. In a broadcast application of albumin in the field, 15% of *H. zea* adults scored positive out of the 317 ones that were collected in light and pheromone traps. These results show that the protein albumin is a potential marker that can be used to study the dispersion behavior of *S. frugiperda* and *H. zea*. Taken together, the results of these studies should assist in decisions for pest management of *S. frugiperda* and in future research on the appropriate use of refuge for resistance management in Bt crops.

INTRODUÇÃO GERAL

Plantas transgênicas que produzem toxinas inseticidas de *Bacillus thuringiensis* (Bt) tem sido amplamente utilizada para o controle de insetos praga no mundo (James 2014). No entanto, a evolução de resistência de pragas a culturas Bt constitui a principal ameaça a eficácia e durabilidade desta tecnologia (McGaughey and Whalon 1992, Gould 1998, Bravo and Soberón 2008). Para retardar o desenvolvimento de resistência, a adoção de alta dose refúgio tem sido a principal estratégia recomendada (McGaughey and Whalon 1992, Gould 1998, Tabashnik 2008). O refúgio de plantas não Bt retarda a evolução de resistência ao manter indivíduos suscetíveis para acasalar com indivíduos resistentes da área Bt. Se a herança da resistência é recessiva e a planta expressa alta dose os filhos deste cruzamento morrem em plantas Bt (Gould 1998). Análises prévias de dados demonstram que o refúgio tem retardado a evolução de resistência em pragas alvo de culturas Bt (Tabashnik et al. 2009, 2013, Huang et al. 2011).

Além do tipo de herança da resistência, a abundância de refúgio e o comportamento de dispersão da praga alvo durante a fase larval e de adultos também podem afetar a eficácia desta estratégia (Gould 1998). A movimentação de adultos é especialmente relevante quando o plantio do refúgio é feito em bloco separado da área Bt. Nesta configuração, se a distância entre a área Bt e não Bt for suficientemente grande, adultos suscetíveis podem não mover do refúgio para acasalar com adultos resistentes da área Bt e vice-versa (Gould 1998). Deste modo, o acasalamento não-preferencial entre suscetíveis e resistentes comprometeria a efetividade desta estratégia. Apesar de muitos estudos reportarem que o refúgio pode retardar a evolução de resistência (Meihls et al. 2008, Tabashnik 2008, Carriere et al. 2012, Tabashnik et al. 2013, Jin et al. 2014), a maioria deles são baseados em modelagem que não incluem o padrão de dispersão da praga alvo especifica. A principal limitação para a avaliação da dinâmica da população praga em experimentos de dispersão é a dificuldade de marcar insetos adultos devido à baixa

disponibilidade de materiais adequados e o tamanho reduzido do organismo (Hagler and Jackson 2001). O uso de proteínas como marcador para insetos foi recentemente desenvolvido (Jones et al. 2006) e tem se mostrado efetivo para estudar a movimentação de alguns insetos no campo (Boina et al. 2009, Horton et al. 2009, Hagler et al. 2011, Klick et al. 2016, Blaauw et al. 2017). O uso de proteínas como marcadores tem a vantagem da ampla disponibilidade, baixo custo, fácil detecção e pode ser aplicado diretamente no campo (Jones et al. 2006). Apesar do potencial deste marcador para monitorar o padrão de dispersão de insetos, este método não tem sido testado em mariposas, cuja fase imatura é alvo de toxinas Bt, tais como *S. frugiperda* e *Helicoverpa zea* (Lepidoptera: Noctuidae).

Além do refúgio em bloco, o plantio aleatório de sementes Bt e não Bt (mistura de sementes) na mesma área foi implantado a partir de 2010 como uma estratégia de refúgio para o manejo da resistência nos Estados Unidos (Onstad et al. 2011, US EPA 2011). O principal benefício desta estratégia é resolver o problema com a não adoção do refúgio, o que tem contribuído para a evolução de resistência em campo (Huang et al. 2011, Tabashnik et al. 2013). No entanto, a movimentação entre plantas pode levar a exposição de larvas com diferentes idades a doses subletais de toxinas Bt (Gould 1998) visto que a suscetibilidade pode ser diminuída em larvas mais velhas (Lorence et al. 1995, Keller et al. 1996, Rausell et al. 2000, Walker et al. 2000, Gilliland et al. 2002, Head et al. 2014). Além disso, a exposição em diferentes idades pode favorecer a sobrevivência de indivíduos heterozigotos em detrimento da sobrevivência de homozigotos suscetíveis, aumentando a dominância da resistência (Mallet and Porter 1992, Brévault et al. 2015). Diante destas premissas, modelos matemáticos e estudos de campo tem concluído que mistura de semente pode acelerar a evolução de resistência (Mallet and Porter 1992, Tabashnik 1994, Yang et al. 2014, Brévault et al. 2015, Burkness et al. 2015). Apesar do decréscimo na suscetibilidade a toxinas Bt ser bem documentado na literatura (Lorence et al. 1995, Keller et al. 1996, Rausell et al. 2000, Walker et al. 2000, Gilliland et al. 2002, Head et al. 2014), não há relatos se a resistência prévia a eventos Bt afeta a suscetibilidade de pragas alvo ao

longo do desenvolvimento larval a outros eventos cuja (s) toxina (s) age (m) de forma diferente e/ou apresentam potencial resistência cruzada.

Este trabalho de dissertação visou o desenvolvimento de ferramentas e conhecimento para auxiliar no manejo de resistência de *Spodoptera frugiperda*, principal praga do milho no Brasil e grande desafio como espécie invasiva na África, a eventos de milho Bt. No primeiro capítulo, caracterizamos a suscetibilidade de populações de *S. frugiperda* a híbridos de milho Bt em diferentes idades larvais. No segundo capítulo, testamos a eficiência da toxina albumina para marcar adultos e larvas de *S. frugiperda* e *H. zea*. Os resultados destes trabalhos auxiliarão na escolha de estratégias adequadas para o manejo da resistência a eventos Bt além de fornecer um marcador com potencial de ser utilizado para estudar o padrão de dispersão de adultos de *S. frugiperda* e *H. zea*

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Chapter 1: Changes in susceptibility of fall armyworm populations to Bt corn hybrids and the implications for resistance management

ABSTRACT

Changes in susceptibility of larvae of lepidoptera to *Bacillus thuringiensis* (Bt) toxins during ontogeny may be a challenge for resistance management in Bt crops, especially in seed mixture refuge. Here we determined the larval susceptibility of fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae), at different ages to Bt corn hybrids. When exposing fall armyworm larvae of populations with contrasting Bt resistance profiles to different types of Bt corn, the susceptibility varied with the population and the age of exposure in some Bt corn events. Larval survival of the Cry1F-resistant population increased from 8.33% in neonates to 100 % in late-instars (10 days old) on Cry1A.105+Cry2Ab corn, and survival rates to adulthood ranged from 4.16% to 87%, respectively. Cry1F-resistant larvae exposed on Cry1A.105+Cry2Ab corn throughout larval development produced viable adults that successfully reproduced without any penalty. In contrast, the standard susceptible population had high larval mortality on Cry1A.105+Cry2Ab corn, and no adults emerged from the larvae exposed to the toxins no matter the larval age. Importantly, Vip3Aa corn killed all larvae of any age, except pre-pupa, regardless of the Bt resistance profile of the population. These results indicate that Cry1F resistance reduce the susceptibility to Cry1A.105+Cry2Ab corn, and that the Vip3Aa toxin has high potential to control field populations of fall armyworm, including those that have developed resistance to Cry toxins. Our findings warn about the risk of adopting seed-mixture refuge for resistant management, particularly if Cry1A.105+Cry2Ab is concurrently deployed with Cry1F, which should be taken into consideration for sustainable pest management of fall armyworm in Bt crops.

INTRODUCTION

Transgenic crops producing toxins from *Bacillus thuringiensis* (Bt) have been widely adopted to control insect pests around the world (James 2014). These crops have provided several benefits, such as reduced use of broad-spectrum insecticides, successful management of key pests, increasing yield and low impact to non-target invertebrates (Cattaneo et al. 2006, Marvier et al. 2007, Wolfenbarger et al. 2008, Carpenter 2010, Osteen and Fernandez-Cornejo 2013, Shi et al. 2013, Jin et al. 2014). However, the evolution of pest resistance to Bt toxins due to the high adaptability of pests and widespread use of Bt crops may compromise these benefits (McGaughey and Whalon 1992, Gould 1998, Tabashnik et al. 2008). In Brazil, the use of Bt transgenic corn to control its main pest, *Spodoptera frugiperda* (Lepidoptera: Noctuidea) (Fall armyworm), has been challenged by the great ability of this pest to adapt to Bt toxins. In fact, several cases of field-evolved resistance to single corn event producing Cry toxin has been documented in Brazil (Farias et al. 2014, Omoto et al. 2016), Porto Rico (Storer et al. 2010) and the United States (Huang et al. 2014, Li et al. 2016). In addition, a previous study has shown that fall armyworm has high potential for developing resistance to a pyramided corn currently deployed in Brazil (Santos-Amaya et al. 2015).

To delay the evolution of resistance to Bt crops, the high-dose/refuge has been the primary recommended strategy (McGaughey and Whalon 1992, Gould 1998, Tabashnik et al. 2008). The refuge of non-Bt plants postpone the evolution of resistance by allow survival of susceptible individuals to mate with rarely potential resistant individuals that can emerge from Bt area (Gould 1998). If the frequency of resistance is low and the inheritance is recessive most of offspring is heterozygous and will be killed by using high-dose plants (Gould 1998, Huang et al. 2011). The historical use of Bt cultivars since 1996 has demonstrated that the refuge strategy can delay the evolution of resistance in target pests of Bt crops (Tabashnik et al. 2009, Huang et al. 2011). However, the major concern is the grower compliance with block refuge requirements. Most cases of field evolved resistance to Bt crops is associated

with the low abundance of refuge near Bt crops (Tabashnik et al. 2009, Huang et al. 2011). For instance, the rapid field evolved resistance of fall armyworm for Cry corn in Porto Rico (Cry1F) and Brazil (Cry1F and Cry1Ab) is in part because of the low compliance with refuge recommendations (Storer et al. 2010, Farias et al. 2014, Omoto et al. 2016). Also, the same explicative hypothesis may explain the faster evolution of pink bollworm (*Pectinophora gossypiella*) resistance to Cry1Ac cotton in India and china (Dhurua and Gujar 2011, Wan et al. 2012).

To provide an alternative refuge configuration to manage resistance, growing random mixture of Bt and non-Bt seed (seed mixture) was recently authorized to delay the evolution of resistance to pyramided corn in the United States (US EPA 2011). Such strategy is known as refuge in the bag and ensures that compliance with refuge requirements by farmers would be no longer a problem (Onstad et al. 2011). However, while in structured refuge the dispersal behavior of adults is the main concern, in seed mixture the larval movement among plants is the main risk factor (Gould 1998). For instance, larval movement from non-Bt to Bt plants at later instars can lead to sublethal exposure (Gould 1998) because the susceptibility to Bt toxins may decrease at larval age (Lorence et al. 1995, Keller et al. 1996, Rausell et al. 2000, Walker et al. 2000, Gilliland et al. 2002, Head et al. 2014). In this context, survival of heterozygous larvae could be increased relative to susceptible larvae. As consequence, the dominance of resistance is increased, and less susceptible adults would be produced, which would decrease the effective refuge size. These concerns were early addressed using mathematical models (Mallet and Porter 1992, Tabashnik 1994) and recently tested in field experiments (Brévault et al. 2015, Burkness et al. 2015). That susceptibility decrease throughout larval development is well documented (Lorence et al. 1995, Keller et al. 1996, Rausell et al. 2000, Walker et al. 2000, Gilliland et al. 2002, Head et al. 2014) but may depends on the type of Bt toxin and insect species. Furthermore, no reports have investigated whether previous resistance to Bt events affects the susceptibility to other events throughout larval on which the toxin (s) act differently and/or exhibit potential cross-resistance.

The fall armyworm is a major polyphagous pest in corn and other crops in Brazil (Cruz et al. 1999), and recently has spread throughout Africa (Prasanna et al. 2018). The fall armyworm larvae have great ability to disperse among plants (Pannuti et al. 2016) and has field-evolved resistance to Cry corn (specially Cry1F) in Brazil (Farias et al. 2014). Although seed mixture is not yet adopted in Brazil, knowledge on susceptibility of this pest to Bt corn events at different age may help to predict the risks in adopting this strategy. We showed that susceptibility varied with the population and the age of exposure to Bt events which support some of the previous assumptions. The scientific knowledge acquired in this study should assist in decisions for pest management of fall armyworm and for appropriate adoption of refuge strategy for resistance management in Bt corn.

METHODS

Fall armyworm populations

We used three fall armyworm populations in this experiment. A susceptible population (LabSS) was obtained from the laboratory of insect Ecology and Management of Embrapa Maize & Sorghum (Sete Lagoas, MG, Brazil). This population has been kept without exposure to any pesticide for over 16 years. All bioassays performed here used LabSS as susceptible population. A Cry1F-resistant population (MTHX) was originated from collections on non-Bt corn field in four Brazilian states and selected for resistance by exposure to Cry1F corn as described by Santos-Amaya et al. 2016. The third population, a pyramided-resistant (Bahia Bt), was obtained from larvae collection on Cry1F corn field in the country of Luiz Eduardo Magalhães, BA, Brazil. The selection for resistance was made by exposure to Bt corn producing Cry1A.105+Cry2Ab as described by Santos-Amaya et al. 2015. These populations have been maintained in laboratory at the Federal University of Viçosa (UFV), MG, Brazil, in artificial diet (Kasten et al. 1978).

Corn plants

This study was performed by using four corn hybrids currently planted in Brazil: a non-Bt corn hybrid 30F53 (Dupont Pioneer, Santa Cruz do Sul, RS, Brazil); a single-protein Bt corn event (TC1507) producing Cry1F (30F53H; Dupont Pioneer, Santa Cruz do Sul, RS, Brazil); a pyramided Bt corn event (MON89034) producing Cry1A.105+Cry2Ab (DKB390PRO2, Monsanto do Brasil, Sao Paulo, SP) & a pyramided Bt corn event (Bt11/MIR162) producing Cry1Ab+Vip3Aa20 (Status Viptera, Syngenta, Sao Paulo, SP, Brazil). The plants were cultivated in a field at Universidade Federal de Viçosa experiment station. The cultivation practices followed the recommended for growing corn in the region. During the experiment, pesticide was not applied and the weed control was managed manually. All bioassays were performed with leaves taken from plants in V4-V5 stage.

Experiment design

We determined the performance of fall armyworm populations (LabSS, MTHX & Bahia Bt) to Bt corn hybrids currently planted in Brazil throughout larval development. We tested the hypothesis that susceptibility to Bt toxin may decrease at later ages and the previous resistance to Bt toxin may lead to a better performance on Bt corn hybrids which the fall armyworm populations are susceptible. To test these hypotheses, fall armyworm larvae from each population were fed with Bt corn hybrids at different larval age. Two generations before the experiment take place, all fall armyworm populations were fed with the Bt hybrid to which they ware resistant.

Overall, fall armyworm neonates (< 24 hours age) were placed in 16-well PVC trays (Advento do Brasil, Diadema, SP) containing leaf pieces of non-Bt, Cry1F, Cry1A.105+Cry2Ab or Cry1Ab+Vip3Aa corn. Also, we maintained fall armyworm neonates fed only with leaves of not-Bt corn to provide larvae to be exposed to Bt corn (Cry1F, Cry1A.105+Cry2Ab and Cry1Ab+Vip3Aa) at different larval age (2, 4, 6, 8, 10 days). Larvae transferred from non-Bt to Bt corn were fed only on non-Bt, Cry1F, Cry1A.105+Cry2Ab or Cry1Ab+Vip3Aa corn for their entire larval development (**Fig 1**). In each larval age 48 individuals were tested (12 replicates of 4 larvae) and placed individually into the well tray. The corn leaves were excise from the V4-V5 stage, brought to the laboratory, cut in small pieces (~4 cm^2) and placed in each well of 16-well PVC tray. Larvae were provided with the appropriate fresh leaf pieces every two days until pupation. The bioassay trays were kept in a chamber under controlled conditions (27 ± 2 °C, 70 ± 15% r.h., and 14L: 10D photoperiod).

Life-history traits

To evaluate the performance of fall armyworm populations to Bt corn hybrids at different larval age, we recorded the larval survival every single day throughout the larval development until pupation (~14 days). Also, we recorded the total rate

survival from neonates to adult, pupal weight and the development time from neonate to adult.

In addition, we investigated if adults from larvae exposed to Cry1A.105+Cry2Ab corn at different larval age could reproduce and leave offspring. To test this hypothesis, pupae were separated by sex (Capinera 2000) and couples were formed after the emergence of the adults. The couples were placed individually in a polyvinyl chloride (PVC) small cage (8 cm height x 8 cm diameter) for mating. The adults were fed with a solution of 10% sugar and 5% ascorbic acid soaked in cotton (Kasten et al. 1978). The cages were lined with paper sheet to provide an oviposition substrate. Eggs were collected every day, placed in a 200 ml plastic cup and the number of neonates recorded just after hatching. Data were used to determine the life table format as described by Birch 1948 using SAS statistical package (Maia et al. 2000). The life table estimates were the net reproductive rate (R_o, female offspring per parental female), intrinsic rate of population increase (r_m, daily female offspring production per parental female) and generation time (T) (Maia et al. 2000).

Data analysis

The survival analysis of the fall armyworm populations exposed to Bt corn hybrids were analyzed using Kaplan-Meier estimators. The overall difference among survival curves were statistically compared by the log-rank test, and if needed, multiple comparisons were conducted using the Holm-Sidak test ($\alpha = 0.05$). The statistical analyses as well as the survival curves were performed using SigmaPlot 12.5. To determine if the larval age of exposure to Bt corn affects the performance during pupa and adult stages, we tested the relationship between larval age and life-history traits (survival, developmental time and pupae weight) by performing linear regression (PROC REG) (SAS Inc. 2011). Finally, the population growth potential was estimated by calculating life-table parameters (r_m, R_o and T) as reported by Maia et al. 2000 using the Jackknife technique to estimate variance in SAS (SAS Inc.

2011). Pairwise comparisons for each combination of larval age versus population were performed using one-tailed *t*-tests.

RESULTS

The larval survival is influenced by the insect population and larval age

Survival analysis of fall armyworm populations exposed to Bt corn hybrids throughout larval development are shown in **Fig 2.** The susceptibility of fall armyworm to Bt corn varied with the population and the age of exposure ($P < 0.05$). On leaves of Cry1F corn, most of the larvae of the standard susceptible population (LabSS) died in three days of exposure and showed a low survival probability (<10%) regardless of the larval age (**Fig 2a**). In contrast, high larval survival was observed for Cry1F-resistant (MTHX) and Cry1A.105+Cry2Ab-resistant (Bahia Bt) populations on Cry1F corn, confirming the resistance of these populations to the Cry1F toxin. The survival of the Cry1F-resistant population on leaves of the Cry1A.105+Cry2Ab corn ranged from 8.33% (< 24 h old) to 100% (exposed at 10 days) (**Fig 2b**). After the fourth day of age, the Cry1F-resistant population showed similar survival to Cry1A.105+Cry2Ab-resistant population ($P > 0.05$). Larvae of the LabSS population had high mortality regardless of the larval age of exposure. There was a gradual increase in larval survival of the Cry1F-resistant population and differential survival on Cry1A.105+Cry2Ab corn, indicating that susceptibility to this Bt corn decreases throughout larval development and that such response is dependent on the Bt-resistance profile of the population. Finally, on Vip3A corn leaves high larval mortality was observed for all populations tested regardless of the age of exposure (**Fig 2c**), although 50% of late-instar larvae of the Bahia Bt population survived on this Bt corn and produced adults (**Fig S2**)**.**

Cry1F-resistance increase the performance of fall armyworm in Cry1A.105+Cry2Ab corn

In addition to survival analysis, we also determined the effects of the larval exposure to Cry1A.105+Cry2Ab corn in pupae and adults by measuring life-history traits related to growth and development. There was no relationship between larval exposure age and pupal weight in both of populations (**Fig 3a**). In contrast, we found a linear positive relationship ($P < 0.05$) between larval exposure age and adult survival to the Cry1F-resistant population exposed to Cry1A.105+Cry2Ab. As expected, the survival in this population was significantly increased throughout the larval exposure age (**Fig 3b**). The survival was not affected by the larval exposure age in the Cry1A.105+Cry2Ab-resistant population ($P = 0.94$) and no adults were obtained from the susceptible population LabSS on the same pyramided event (**Fig 3b**). Likewise, the developmental time (from neonate to adult) was influenced by the larval exposure age in both of fall armyworm populations: MTHX and Bahia Bt ($P < 0.05$). The results showed a decreasing of the developmental time throughout the larval exposure age and the Cry1F-resistant population took longer for emergence of adults than the Cry1A.105+Cry2Ab-resistant population (**Fig 3c**). In addition, adults of Cry1A.105+Cry2Ab-resistant population exposed to Cry1Ab+Vip3Aa at later instar were recorded (**Fig S2**) and successfully reproduced (**Fig S3**), showing that this population may be less susceptible to this corn event compared with the others at 10 days of age. Taken together, our results demonstrate that the exposure to the pyramided corn Cry1A.105+Cry2Ab during the larval stage seems not affect the viability of adults. Additionally, as older the larvae, higher the survival and faster was the development time.

Reproductive success is affected by larval exposure to Cry1A.105+Cry2Ab

There was significant difference between the Cry1F-resistant and Cry1A.105+Cry2Ab-resistant populations for the fertility life table parameters ($P < 0.05$). The Intrinsic rate of population increase (r_m) only reduced for 2 days old larvae although the reproductive net reproductive rate (r_o) decreased for 2 and 8 days larvae (Fig 3a,b). The generation time increased for larvae of 2, 4 and 6 days old (Fig 4c). Although we have obtained adults from the Cry1F-resistant population exposed at neonates (0 day) to Cry1A.105+Cry2Ab corn, reproductive output could not be recorded because of the low survival of adults.

DISCUSSION

In this study we assessed the susceptibility of fall armyworm populations with contrasting Bt resistance to different types of Bt corn hybrids at different larval age. The results demonstrated that the susceptibility varied with the population and the age of exposure in some Bt corn events. The Cry1F-resistant population showed high survival on leaves of the Cry1A.105+Cry2Ab pyramided corn throughout larval development relative to a standard susceptible population (LabSS) and the exposure at early age did not compromise the reproductive success of the adults. Unlike Cry1A.105+Cry2Ab corn, Cry1Ab+Vip3Aa corn kill all the larvae even if they were in late instars (up to 10 days of age) and previous resistance to Cry toxins did not affect its efficacy. These findings corroborate those reported for survival increases at later instars in fall armyworm and other species (Halcomb et al. 1996, Wierenga et al. 1996, Walker et al. 2000, Head et al. 2014, Miraldo et al. 2016), but for the first time, we demonstrated that previous resistance to Cry toxins may affect this response.

The high survival of the Cry1F-resistant population on Cry1A.105+Cry2Ab pyramided corn (8.33%) at neonates (< 24 h old) relative to a susceptible population is consistent with the hypothesis of cross-resistance among Cry toxins, especially between Cry1F and Cry1A.105. Competition binding assays with Cry toxins in fall armyworm showed that Cry1F and Cry1A.105 compete with high affinity for the same binding site (Hernández-Rodríguez et al. 2013). Also, experiments with fall armyworm using corn plants and concentration-response bioassays with pure toxin have provided evidence of cross-resistance between these toxins (Huang et al. 2014, Bernardi et al. 2015, Santos-Amaya et al. 2015). In addition, we have demonstrated that the survival on Cry1A.105+Cry2Ab2 increased throughout larval development. The susceptibility decrease with increasing age may occur due to ontogenetic changes related to reduction in affinity of the binding sites affinity (Rausell et al. 2000), reactivity of gut proteases (Keller et al. 1996), and decrease of toxin receptor density (i.e. Aminopeptidase N) (Gilliland et al. 2002). Furthermore, considering that

Cry2Ab is the only active toxin against Cry1F-resistant population because of cross-resistance with Cry1A.105, the level of toxicity of this protein to fall armyworm could also explain the susceptibility decrease at larvae age. Although we did not test this assumption in this study, it has been already documented that Cry2Ab exhibit a little effective LC50 against fall armyworm (Hernández-Rodríguez et al. 2013), which suggest that fall armyworm has low inherent susceptibility to Cry2Ab toxin.

Our results also showed that Vip3Aa was the most effective toxin, causing high mortality up to 10 days of larval age (maximum age of exposure). The survival of fall armyworm throughout larval development on Vip3Aa was reported by Miraldo et al. 2016. Studying functional dominance of different age, the authors showed that a susceptible population died up to the fourth instar which is in agreement to our findings. Bioassays and midgut binding studies showed that Vip3Aa is highly toxic to fall armyworm relative to Cry1 toxins and no competition was found between Vip3Aa and Cry toxins, suggesting distinct mechanism of action (Sena et al. 2009, Hernández-Rodríguez et al. 2013). In fact, Vip3A toxins have no structural homology with Cry toxin (Maagd et al. 2003). The interaction with distinct receptor in insect midgut may explain results found in this study, in which, we demonstrated that previous resistance to Cry toxins did not change the survival response of fall armyworm for Vip3Aa. This is consistent with recent findings reported by (Santos-Amaya et al. 2015). The low Survival of Cry1-resistant populations on Vip3Aa have key implication for resistance management because Vip3Aa has high potential to control fall armyworm that have field-evolved resistance to Cry toxins.

Despite the benefits of seed mixture, our results warn about the risk of adopting this strategy in Brazil. Considering that larval movement is the main concern regarding its adoption (Mallet and Porter 1992, Onstad and Gould 1998) and fall armyworm has great ability to move among corn plants (Pannuti et al. 2016), our findings suggest the following scenarios. High survival is expected if Cry1F-*resistant* larvae move from non-Bt to Cry1A.105+Cry2Ab corn, which could hasten the

resistance to Cry1A.105+Cry2Ab and no considerable gain would be obtained to control Cry1F-resistant populations. Conversely, if Cry1F-resistant larvae move from non-Bt to Vip3A corn all die, such that, this pyramid can reduce the frequency of alleles conferring resistance to Cry1F in the field. However, the movement of susceptible larval from non-Bt to any of the corn hybrids used here leads to high larval mortality, which could reduce the population of susceptible individuals and thereby the effective refuge size. Therefore, larval movement and distinct survival of fall armyworm on Cry1A.105+Cry2Ab is expected to hasten the resistance to this pyramided event, specially where allele frequency of Cry1F resistance is high (Farias et al. 2014, Huang et al. 2014, Santos-Amaya et al. 2017).

In conclusion, this study showed that the susceptibility of fall armyworm to Bt corn hybrids was dependent on the Bt resistance profile of the population and on the larval age of exposure to Bt toxins. The survival rate of Cry1F-resistant larvae was high and increased on Cry1A.105+Cry2Ab corn throughout larval development, further providing evidence for the previously reported cross-resistance between Cry1A.105 and Cry1F. In contrast, Vip3Aa corn was effective to kill late-instar larvae (up to 10 days old) regardless of the previous resistance to Cry toxins, indicating that Bt corn producing Vip3Aa has high potential to control field populations of fall armyworm that have evolved resistance to Cry toxins. To better support decisions about seed mixture strategy further studies may be conducted to determine the functional dominance of resistance and investigate the larval movement in the field. Overall, our findings warn for the risk of adopting seed mixture strategy for resistance management and should be taken into recommendations for sustainable pest management of fall armyworm.

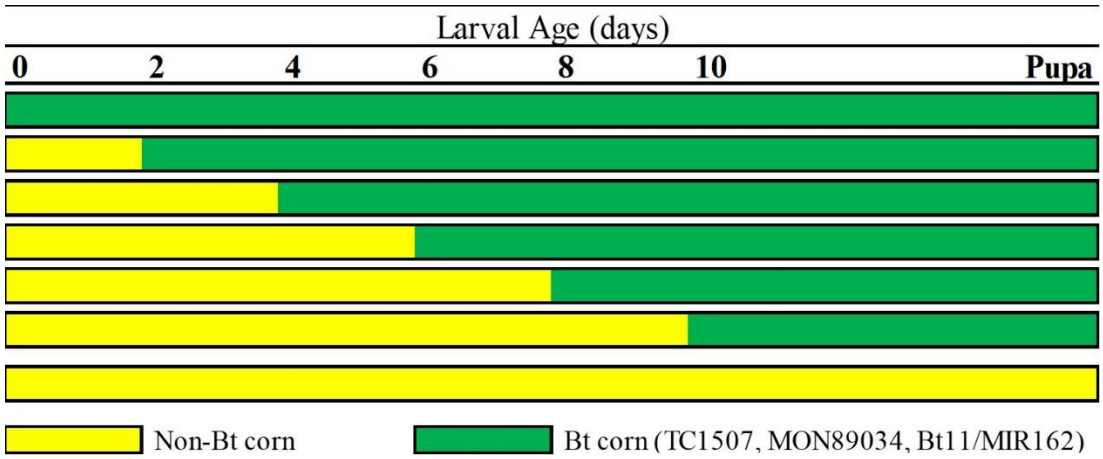

Figure 1. Layout of the experiment design. Each fall armyworm population (LabSS, MTHX and Bahia Bt) was tested in each corn hybrid and each larval age as showed above.

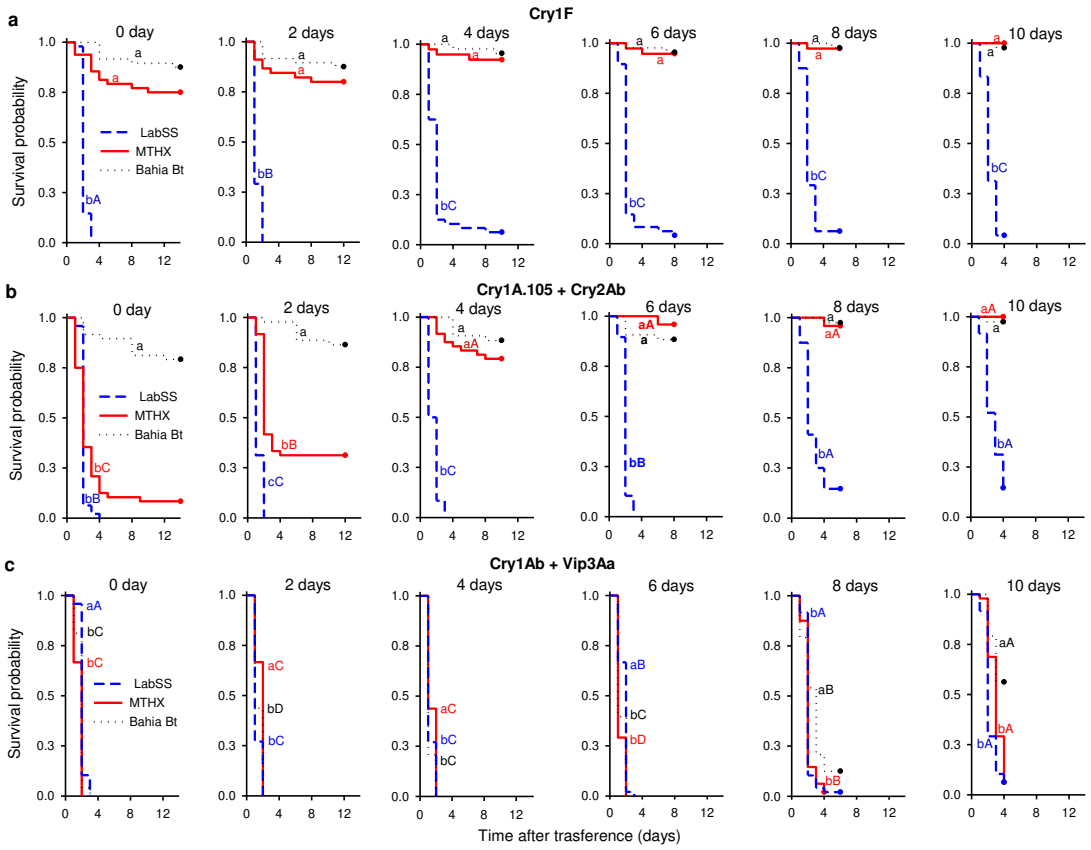

Figure 2: Survival plots for larvae of three fall armyworm populations exposed to Bt corn hybrids at different larval ages. The insects are a Cry1F-resistant (MTHX), Cry1A.105+Cry2Ab2-resistant (Bahia Bt) and a susceptible (LabSS) populations. The Bt corn hybrids are**: a**) Cry1F, event TC1507 (Pioneer 30F53H); **b)** Cry1A.105+Cry2Ab2, event MON89034 (DKB390PRO2) & c) Cry1Ab+Vip3Aa, event Bt11/MIR162 (Syngenta Status Viptera). Ages on the top of each graph represent the exposure larval age. Survival curves that do not significantly differ (P > 0.05) were marked with the same letter. Comparison within a larval age are represented by small letter and within a population by capital letter.

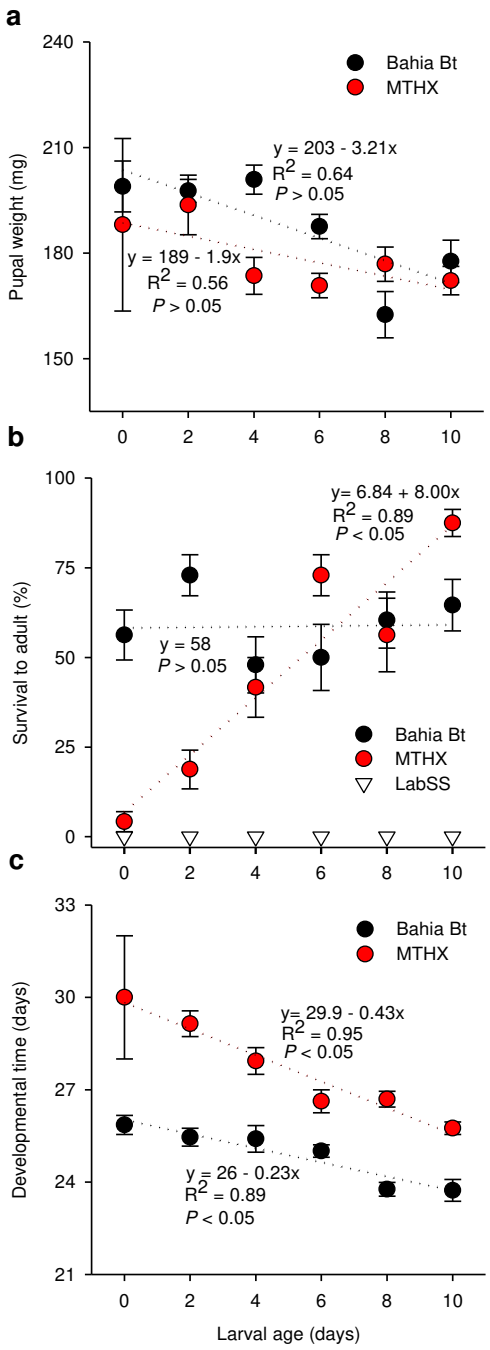

Figure 3: Life-history traits of fall armyworm populations feeding on leaves from Cry1A.105+Cry2Ab corn exposed at different larval age. The Fall armyworm populations are represented by a Cry1F-resistant (MTHX), Cry1A.105+Cry2Ab2-resistant (Bahia Bt) and a susceptible (LabSS) populations. Data are mean ± SE. Red and black lines show the relationship between larval age and the life-history traits.

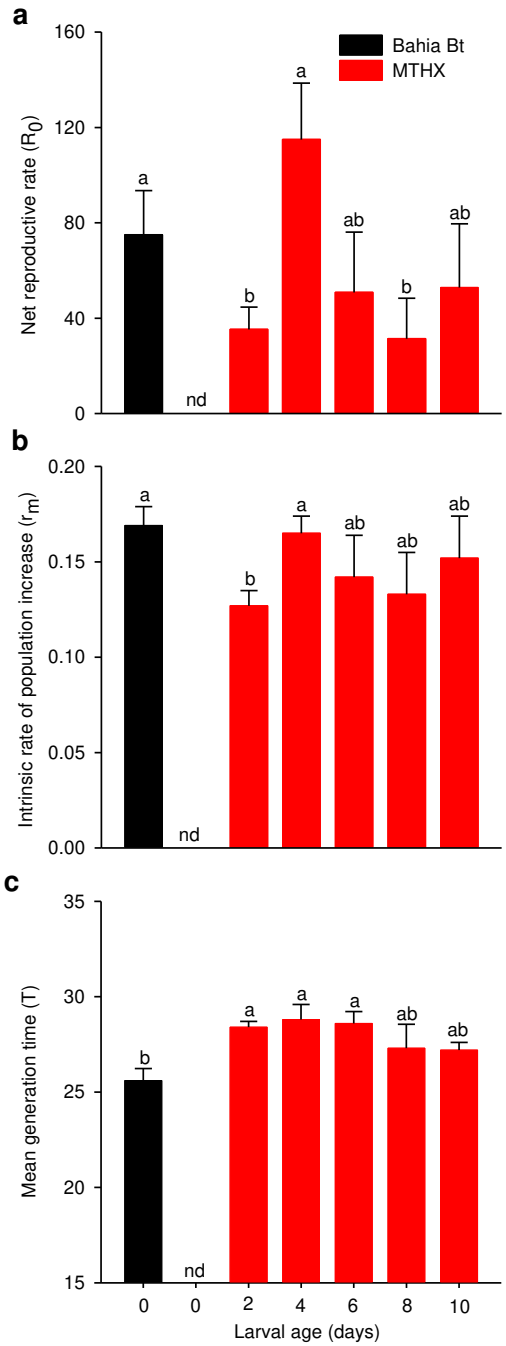

Figure 4: Life table statistics of Bt-resistant fall armyworm population exposed on Cry1A.105+Cry2Ab Bt corn at different larval age. R_0, net reproductive rate (**a**); **r_m,** intrinsic rate of population increases (**b**); **T,** mean generation time (**c**). Colum with the same letter are not significantly different ($P > 0.05$) by one-tailed *t*-test with jackknife-estimated variances. Data are means (± SE).

Supplementary Information

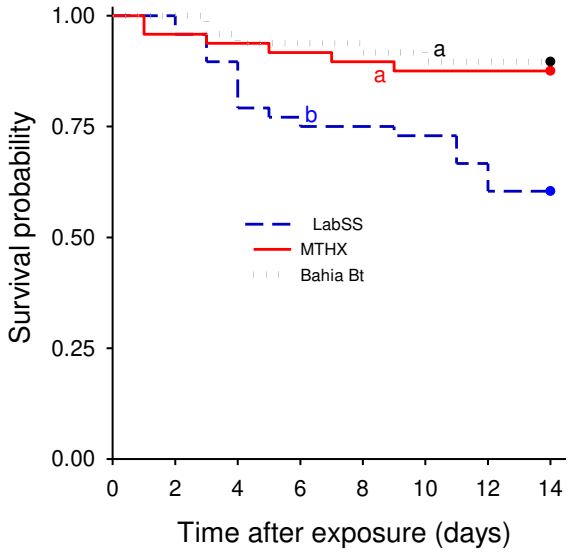

Figure S1: **Survival plots of fall armyworm populations exposed at neonates on non-Bt corn**. The insects are represented by a Cry1F-resistant (MTHX), Cry1A.105+Cry2Ab2-resistant (Bahia Bt) and a susceptible (LabSS) populations. Survival plots that not significantly differ ($P < 0.05$) according to Long-Rank test are represented with the same small letter.

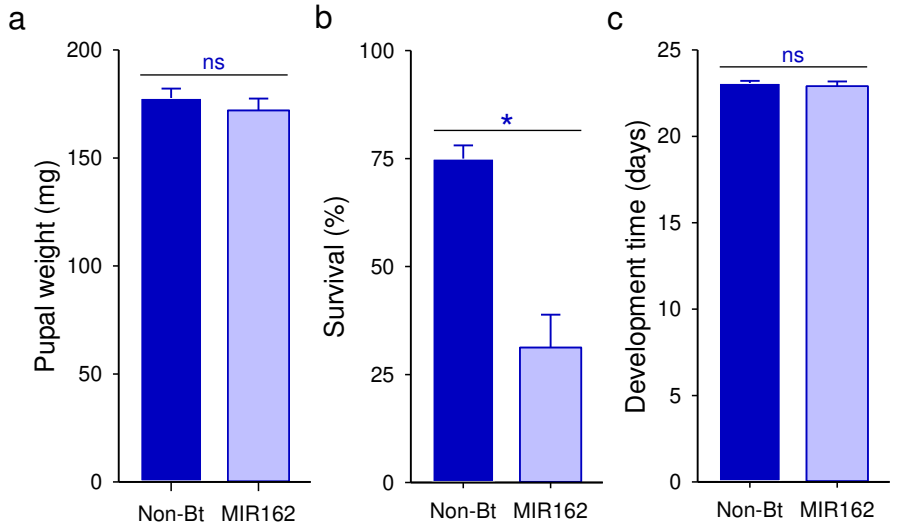

Figure S2: Life-history traits of the Bahia Bt population exposed at 10 days of age on Cry1Ab + Vip3Aa corn. Data are mean (± SE). Asterisk indicates significant difference by ANOVA analysis ($P < 0.05$).

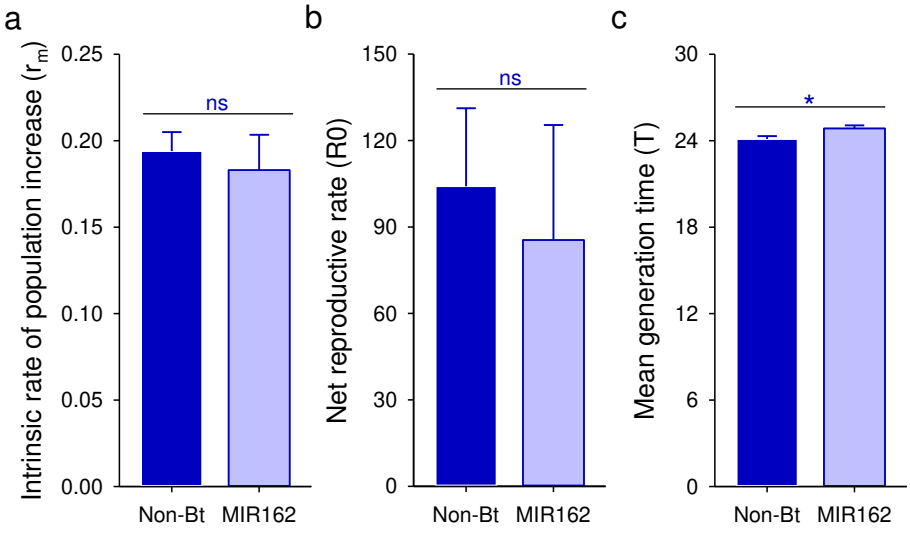

Figure S3: **Life table statistics of Bt-resistant fall armyworm population exposed on Cry1Ab + Vip3A corn from 10 days until pupation.** Data are means (± SE). **R_0,** net reproductive rate (**a**); **r_m,** intrinsic rate of population increases (**b**); **T,** mean generation time (**c**). Colum with the same letter are not significantly different ($P > 0.05$) by one-tailed t-test with Jackknife estimated variances.

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Chapter 2. Egg albumin as suitable protein marker to study dispersal of Noctuidae in the agroecosystem

ABSTRACT

Knowledge about the dispersal and spatial dynamics of pest populations is fundamental for implementation of integrated pest and insect resistance management. Tracking the movement of insects, however, constitute a major challenge because of the small size and low availability of suitable materials and methods. Here, we evaluated the effectiveness of the albumin protein from egg whites to mark larvae and adults of *Spodoptera frugiperda* (Fall armyworm) and *Helicoverpa zea* (Corn earworm) (Lepidoptera: Noctuidae), two polyphagous and high mobile pests. We performed a series of laboratory and field experiments using egg albumin as a protein marking. The detection of egg albumin was performed by two Enzyme-linked Immunosorbent assays using microplate (ELISA) and Polyvinylidene Diflhoride membrane (dot blot ELISA). In the laboratory, 100% of the moths sprayed with 20% egg white solution acquired the albumin marker and it was detected at high levels by both detection assays up to five days after the application. In contrast, egg albumin was not effective to mark larvae, being only detected at the first larval instar. Field application using a backpack sprayer in cages resulted in high percentage of moths with positive albumin presence after 24 hour and five days. The efficacy of the albumin marking was high for both fall armyworm and corn earworm. Egg albumin applied directly in the field resulted in 15% of corn earworm moths marked, most of them collected near the sprayed area, although some were caught up to 1600 m from the sprayed site. Dot blot ELISA was as effective to detected egg albumin as was plate ELISA. In summary, our findings showed that egg albumin is a suitable marker to study the dispersion pattern of fall armyworm and corn earworm moths, which could assist current methods used to describe patterns of dispersal of these pests in the agroecosystem.

INTRODUCTION

Dispersal is defined as any movement of organisms away from their parent source (Nathan 2001). This behavior is a fundamental biological process that has important ecological and evolutionary consequences (Kokko 2006, Ronce 2007). By dispersion, insects look for food, mating, and favorable environment conditions which affects the survival, growth and reproduction. As consequence, insect dispersal has enormous implications, including losses of crops, the spread of pests and diseases, the provision of essential ecosystem services such as crop pollination (Holland et al. 2006) and gene flow (Kokko 2006, Ronce 2007). Understanding and measuring the patterns of insect dispersal is valuable for manage pest populations since the dispersal movements affects the natural population dynamics as well as the distribution of genetic diversity throughout the space (Ronce 2007).

Spodoptera frugiperda (J.E. smith) (Fall armyworm) and *Helicoverpa zea* (Boddie) (Corn earworm) are high mobile and economic important pests of crops throughout the western hemisphere (Sparks 1979, Capinera 2008, Luttrell and Jackson 2012, Olmstead et al. 2016) and recently fall armyworm was introduced and spread over 30 countries in the Africa continent (Prasanna et al. 2018). These pests are known by the great ability to migrate long distance annually, causing infestation and economic losses. The dispersal behavior associated with the wide range of crops utilized by these species constitute a challenge for the effective local management. Although long-distance movement of fall armyworm and corn earworm is well documented (Westbrook and López 2010, Nagoshi et al. 2012, Westbrook et al. 2016) little is known about the dispersal behavior of these pests throughout the agricultural landscape. Developing a method to access the local movement pattern could provide important information about the range of cultivated and wild hosts utilized by these pests. Thus, understanding the dispersal pattern as well as the sequence and distribution of hosts around the agricultural landscapes can contribute to provide an effective management of the population in the agricultural landscape (Luttrell and Jackson 2012).

In addition, knowledge on the dispersal pattern of adults of fall armyworm and corn earworm could provide valuable information for the primary strategy indicated to delay the evolution of pest resistance to crops producing toxins from *Bacillus thuringiensis* (Bt). Transgenic corn expressing toxins from bacillus thuringiensis has been widely adopted to control these pests (James 2014). However, because of the large-scale exposure, field-evolved resistance to Bt toxin has been recorded worldwide (Tabashnik et al. 2009, 2013, Storer et al. 2010, Dhurua and Gujar 2011, Wan et al. 2012, Farias et al. 2014, Omoto et al. 2016). The High-dose/refuge is the current strategy used to delay the evolution of pest resistance to Bt crops (McGaughey and Whalon 1992, Gould 1998). This strategy embraces the cultivation of non-Bt plants to provide susceptible moths to mate with resistant pests emerging from Bt area (Gould 1998). However, the effectiveness of this strategy depends on a fundamental assumption: the movement of adults in the field must be random, otherwise the strategy would be compromised, since a key factor to success of the refuge is the mating between resistant and susceptible individuals (Gould 1998). Although several studies reported that refuge strategy can delay the evolution of pest resistance (Liu and Tabashnik 1997, Tabashnik 2008, Huang et al. 2011, Carriere et al. 2012, Tabashnik et al. 2013, Jin et al. 2014), most of them are based in mathematical models, in which, the dispersal pattern of specific pests are not taken into account.

Despite the several benefits of the knowledge of pests dispersal pattern, tracking the movement of insects is a major challenge due to the relatively small size and cryptic behavior (Hagler and Jackson 2001). According to Hagler and Jackson 2001, an effective marker should be “durable, inexpensive, non-toxic (safe to the insect and environment), easily applied and clearly identifiable. Also, it is required that the marker does not irritate the insect or affects its normal dispersal behavior, growth, reproduction and life span”. Although several materials and methods have been used to mark insects, some of them failure to one or more of the effective characteristics. A recently technique using protein developed to mark insect has proved to be effective, easy, safe and stable (Hagler et al. 1992, Hagler 1997). Initially, specific vertebrate

protein (Rabbit or chicken IgG) was applied externally as a spray or incorporated into the insect diet (Hagler 1997). The IgG could be easily detected by applying an anti-IgG sandwich enzyme-immunosorbent assay (ELISA). IgG is a very sensitive technique (Hagler 1997, Hagler and Miller 2001), but the major limitation as a marker is the high cost of the purified protein mark, which makes it impractical to apply directly in the field for mark-capture type studies (Jones et al. 2006).

In face of this limitation, Jones et al. 2006 developed an inexpensive immunomarking alternative to mark insects in the field using easily available food proteins, such as, chicken egg albumin (as egg white), bovine casein (as cow`s milk) and soy protein (as soy milk). Over the past years, several studies have been using this technique to study insect dispersal patterns (Jones et al. 2006, Boina et al. 2009, Hagler and Jones 2010, Hagler et al. 2014, Klick et al. 2016). Despite the undoubted effectiveness of protein to study insect dispersal, the detection method of proteins currently performed in microplates can be made easier. An alternative to plate ELISA is applying the protein (antigen) directly onto a membrane for antibody detection (Hawkes et al. 1982). This method is known as dot blot ELISA the protein detection has shown to be as sensitivity as plate ELISA or greater, the results are easily interpreted, a large samples volume can be screened at the same time, and does not require ELISA reader/computer to detect positive and negative samples (Hawkes et al. 1982).

In this study, it was tested the effectiveness and persistence of albumin (egg white) as a foreign protein marking of larvae and adults of fall armyworm and corn earworm to document the movement of adults in the agricultural landscape. It was also assessed the efficacy of dot blot ELISA to detect albumin compared to ELISA plate. Laboratory tests were performed to demonstrate the efficacy for mark-release recapture type studies and we also assessed the acquisition of egg albumin by moths in field conditions for mark-capture type studies. The results indicate that adults of both fall armyworm and corn earworm sprayed with egg albumin solution in laboratory or field

condition acquired the marker and it persisted throughout the time. Finally, dot blot ELISA showed to be a good alternative to ELISA plate to detect egg albumin based on its effectiveness.

MATERIAL AND METHODS

Lepidoptera stock colony

Eggs and pupae of fall armyworm and corn earworm were obtained from Benzon Research Inc. (Carlisle, NE, USA). Eggs were placed in plastic bag until hatching. Neonates larvae (<24h old) of both species were sprayed to measure the acquisition and retention of the protein chicken egg albumin. Pupae were placed in containers containing vermiculite and stored in small cages (25 x 25 x 25 cm) until the emergence of the adults. Adults were sprayed with chicken egg albumin in laboratory and field conditions. Larvae, pupae and adults were kept at controlled temperature of 27 ± 2 °C, relative humidity 70 ± 10 % and 12L: 12D photoperiod.

Protein Marker

Albumin marking from chicken white egg were tested as a potential marker for larvae and adults of fall armyworm and corn earworm. The source of the albumin was from Egg Beaters Original Real Egg© (ConAgra foods, Omaha, NE). The insects were marked by spraying with 20% (vol/vol) egg white solution. The presence of egg albumin on insect body was tested by Enzyme-linked Immunosorbent Assay (Elisa) and Dot Blot Enzyme-linked Immunosorbent Assay (Dot Blot Elisa) (described below).

Effectiveness and persistence of egg albumin in larvae and moths in the laboratory

The experiments were conducted in the laboratory of toxicology located at the University of Nebraska-Lincoln`s Plant Science Hall, Lincoln, NE. Larvae and adults of both species were kept in a chamber at 27 ± 2 °C, relative humidity 70 ± 10 % and 12L: 12D photoperiod.

Larval study. Neonates (< 24 hours old) of fall armyworm and corn earworm were separated in four groups (n= 60 per group) and placed inside a petri dish (90 mm diameter and 15 mm height) containing a filter paper to cover the surface (one group per petri dish). Three groups were sprayed with 1ml of a 20% egg albumin solution each and one group was not sprayed (negative control). All treatments were applied using a trigger sprayer bottle (skilcraft applicator spray). One hour after spray, larvae were placed to a small container (8.5 cm diameter and 3.5 cm height) with artificial diet and held in a growth chamber. Four days after treatment, each group of sprayed and non-sprayed larvae was individualized in small cups (30 ml) containing artificial diet. In each larval instar were sampled three larvae from each sprayed group (n=9) and eight larvae from negative control group. Individual samples were placed into a clean 1.0 ml microtube and frozen at -10 °C to be later tested for the presence of egg albumin. The larval manipulation was carry out using gloves and tweezers to avoid contamination and transference of egg albumin between marked and unmarked larvae.

Moth study. Before the emergence of the adults, pupae of fall armyworm and corn earworm were separated by sex Capinera (2000). Male and female pupae were placed separately inside cages (25 x 25 x 25 cm) until the emergence of the moths. Three days after the emergence of the moths, three small cages from each species were prepared and twelve females and twelve males were placed in each cage. Immediately after the placement of the moths in each cage, 10 ml of the egg albumin solution was sprayed using a trigger sprayer bottle. For negative control a fourth cage was not sprayed with egg albumin solution and held in a growth chamber at 27 ± 2 °C, relative humidity 70 ± 10 % and 12L: 12D photoperiod. Two couples were sampled from each cage (sprayed and non-sprayed cages) every other day starting on the day of the egg albumin spray. The moth samplings were concluded at the fifth day after the protein application. Each moth was placed individually into a clean 2 ml microtube and frozen at -10 °C to be later tested for the presence of egg albumin by ELISA and dot blot ELISA assays. To avoid contamination and occurrence of false

positive, the samples were collected directly using the same microtube where each sample was placed.

Acquisition and persistence of egg albumin in adults in the field

Study Site. The experiments were conducted during 2016 crop season in a corn field located in the Haskell Agricultural Laboratory at University of Nebraska, Concord, NE (42°22`50.4``N and 96°57`17``W). The corn was cultivated following the recommended agronomic practices for the region.

Field Cages. A V9 corn stage field were inspected for natural infestation from noctuid eggs and larvae and six big cages (1.6 x 3.2 x 1.8 m) and six small cages (1.6 x 1.6 x 1.8 m) were placed and spaced three meters from each other. Pupae of fall armyworm and corn earworm were placed in small containers (13 x 13 x 5 cm) with vermiculite. One day after the emergence of the moths, at least 200 pupae of each species were placed in each big cage (three big cages received with fall armyworm and three big cages with corn earworm. The emergence of the moths was observed and after 90% of the adults emerged (3 days after), the containers containing the unmerged pupae were removed. Immediately after, the plants inside the cages were sprayed with 2.5 L of a 20% egg albumin solution using a backpack sprayer. The negative control (no marker application) for each specie were placed in two small cages with at least 100 pupae of each species. The first sampling was performed in the day after the albumin marker application. We also transferred approximately 50 adults of each species from sprayed big cages to the other three available small cages 24 hours after spray. This transference was done to ensure that the moths did not pick up the marker by residual contact after the marker dried on the corn leaves surface. The second sample was collected five days after the egg albumin application. The moths were placed into a clean 2.0 ml microtube and frozen at -10 °C to be tested for the presence of egg albumin by Elisa and Dot Blot Elisa assay.

Field. Before egg albumin spraying, at least 1000 pupae of corn earworm were placed in six containers (26 cm diameter and 8 cm height) with vermiculite and held in a growth chamber until the emergence of moths. The day after starting the emergence of moths these containers were placed in the middle of the sprayed area spaced three meters from each other. Three days after, when 80 % of the adults had emerged, the containers with unmerged pupae were removed. Immediately after, 17.5 L of a 20% egg albumin solution was sprayed within a single block (8 x 21 m) of a research corn field with V9 plant stage using a backpack sprayer. Ten light and four pheromone traps were placed throughout the area for recapture adults of corn earworm. Light traps were kept illuminated from sunset to sunrise during the recapture period and the moths were capture in small cages following Paula-Moraes et al. 2013. The small cages were checked for moths of corn earworm each other day after the egg albumin spraying in the corn field during an interval of six days. Pheromone traps were used to recapture corn earworm males. Pheromone traps were also checked daily during six days after marker application. Moths captured in the light traps and in the liners from pheromone traps were transported to laboratory and the samples were screened and the specimens of corn earworm were placed into a clean 2.0 ml microtube and frozen at -10 °C to be later tested for the presence of egg albumin. The date and trap location of each specimen of corn earworm were recorded to allow the determination of the percentage of recapture marked moths of corn earworm.

Enzyme-linked Immunosorbent Assay (ELISA)

Sample preparation. Microtubes containing collected insect samples were removed from the freezer and 1 ml of Tris-buffered saline (TBS, 50 mM Tris-Cl, pH 7.4. 150 mM NaCl) was added in each one. The samples were soaked at room temperature for 1 h on a laboratory shaker (Reliable Scientific, Inc.) at 50 rocking motions per minute. After that, the moths were discarded and the remaining solution was centrifuged at 12,000 rpm for 15 min at 4 °C and the supernatant was used for ELISA and dot immunoblotting assays.

ELISA sensitivity. The detection limit of egg albumin by ELISA assay was performed by preparing a triple serial dilution from a chicken egg white (Cat. A5503, Sigma Chemical Company, St. Louis, MO, USA) solution starting at 3.33 ppm and ending at 1.52 ppb. Tris-buffered saline (TBS) solution and samples from non-sprayed adults were used as negative controls. Each serial dilution and blank samples were evaluated four times following the ELISA procedure as described below. The assay sensitivity was determined as the concentration of protein giving a mean value higher than the mean plus 3 times the SD value of the TBS buffer and of the negative control blank.

ELISA procedure. Egg albumin was quantified in all samples by an indirect ELISA assay following the protocol described by Hagler and Jones 2010 with some modifications. Briefly, 96-well plates containing 80 µL of each supernatant were incubated for 1.5 h at 37 °C and then blocked for 2 h using 300 µL of blocking solution. After adding TMB substrate, 50 µL of 2 N sulfuric acid solution were used to stop the enzymatic reaction before reading. Color development was measured using a 96-well microplate reader (BioTek instruments, INC, Highland Park, Winooski, VT) at 490nm. The standard curve (R^2 = 0.97) established using serial dilutions was saved and used in quantification of egg albumin. Negative controls were also used in ELISA assays. Each sample was evaluated by triplicate.

Dot blot Enzyme-linked Immunosorbent Assay (dot blot ELISA)

Sensitivity of the assay. A triple serial dilution was prepared using albumin from chicken egg white (Cat. A5503, Sigma Chemical Company, St. Louis, MO, USA) in order to evaluate the sensitivity to detect egg albumin using the dot blot ELISA approach. The serial dilution was performed with eight chicken egg white concentrations diluted in TBS (pH 7.4) starting at 10 ppm and ending at 4.5 ppb. The limit of detection for each concentration was tested using seven different aliquot volumes (1, 3, 5, 7, 10, 15 and 20µL). Non-spray and sprayed samples from the laboratory experiment were used as negative and positive control, respectively.

Aliquots from each concentration were manually transferred to a Polyvinylidene Diflhoride (PVDF) membrane (Bio rad, Cat. #162-0175) as described below.

Assay procedure and sample transfer. The PVDF membrane was cut with a clean scissor in rectangular portions according to the number of samples assayed. The membrane was then wetted in methanol 100% for 5 seconds, until the entire membrane become translucent and then placed in a container with pure water for 3 min for equilibration. To prevent the membrane from drying out before applying protein samples, the membrane was placed on the top of three sheet of paper filter wetted in pure water. An aliquot of 5 µL from each individual protein sample was manually spotted on the PDVF membrane. After the samples dried, the membrane was blocked for 2 hours at room temperature using 300 µl of a non-fat milk solution (3%) per each cm^2 of membrane diluted in phosphate buffer saline (PBS) (Bio Rad, Cat.1610780). The primary antibody, Rabbit anti-chicken egg albumin (Sigma Aldrich, Cat. C6534), was diluted 1:5000 in 3% non-fat milk/PBS buffer, previous described. After washing the membrane three times, 10 minutes each, with phosphate buffer saline solution (0,5 % tween, pH 7.4) (Sigma-Aldrich, Cat. P3563), the secondary antibody, goat anti-rabbit IgG (Sigma Aldrich, Cat. 6154), conjugated to horseradish and diluted 1:5000 in the 3% non-fat milk/PBS buffer was added and kept at room temperature for 1 h. After washing steps, the horseradish peroxidase substrate (Opti-4CN™ substrate kit) (Bio Rad, Cat. 170-8235) was added following the manufacture recommendations. After 30 min of incubation time at room temperature, the reaction was stopped by washing the membrane with pure water, dried and the image recorded.

Data Analysis

Unmarked samples were used to calculate the ELISA critical threshold values. For those experiments performed in laboratory conditions, the threshold values were determined by the mean of the ELISA optical density (OD) from negative control greater three times the standard deviation (Crowther 2001). For the experiments

performed in field conditions the presence of false positive could lead to inflated estimates of long-distance dispersal (Sivakoff et al. 2011). To provide more protection against the incidence of false positive, the threshold for those experiments was calculated by a new procedure based in the criteria described by Sivakoff et al. (2011). Thus, samples were scored positive for egg albumin marker if the ELISA OD were above the threshold previously calculated.

Each PVDF membrane assayed in the dot blot ELISA study included negative control (unmarked), positive control (known marked samples) and samples in which the presence of egg albumin would be confirmed. The reaction between the enzyme peroxidase conjugated with the secondary antibody and the Opti-4CN substrate results in the production of a black/gray solid product in the blotting where the sample aliquot was placed. Thus, a sample was considered positive when a visual and well defined black/gray spot was observed on the PVDF membrane.

RESULTS

Sensitivity of the detection assay

The ELISA assay detected egg albumin up to 4.5 ppb with a positive threshold value of 0.039 from TBS buffer blank and 41.1 ppb with a positive threshold value of 0.109 from negative control blank (non-spray adults). These results indicate that the ELISA Sensitivity is lower when calculated with the ELISA optical density from non-spray adults.

Dot blot ELISA Sensitivity

The limit of detection for egg albumin by dot blot ELISA was 41.0 ppb (**Fig. 1**). The sensitivity was not influenced by the aliquot volume and the spot color intensity was basically depending of the antigen (egg albumin) concentration.

Acquisition and persistence of egg albumin in larvae

Egg albumin was detected in larvae of fall armyworm and corn earworm in the first instar by using both ELISA and dot blot ELISAs (**Fig. 2)**. However, egg albumin does not persistence throughout the larval stage. Eight larvae of fall armyworm and corn earworm in each larval instar were assayed (**Fig. 2a**). The samples from negative control yielded a mean (± SEM) ELISA OD of 0.046 ± 0.0036 (n = 42) for fall armyworm and 0.047 ± 0.0025 (n = 46) for corn earworm. That resulted in ELISA threshold values of 0.1162 and 0.0985, respectively. Like ELISA assay, the dot blot ELISA was able to detect egg albumin marker of larvae sprayed with egg white solution (**Fig. 2b**). The dot blot ELISA was assayed only for those samples of the first instar since egg albumin does not persist in older instars. Similar results were obtained in both immunoassays tests.

Acquisition and persistence of egg albumin in adults in laboratory

The presence of egg albumin was confirmed by ELISA and dot blot ELISA assays (**Fig. 3**). All the samples assayed in each species and throughout the collect

period were scored positive (**Fig. 3a**). Samples from negative control yield a mean (± SE) ELISA OD of 0.060 ± 0.0106 for fall armyworm and 0.052 ± 0.0061 for corn earworm. That resulted in ELISA threshold values of 0.241 and 0.052 for fall armyworm and corn earworm, respectively. The number of samples assayed for the presence of egg albumin by dot blot ELISA (n=8) was lesser than those assayed by ELISA assay (n = 12). Importantly, dot blot ELISA detected egg albumin in all samples scored positive in the regular plate ELISA (**Fig. 3 b-c**).

Acquisition and persistence of egg albumin in adults in field cages

Adults of fall armyworm and corn earworm sprayed with a 20% egg white solution in field cages were marked and egg albumin was detectable up to five days after spray (**Fig. 4**). The negative samples tested by ELISA assay (**Fig. 4 a**) yield a mean (± SEM) ELISA OD of 0.219 ± 0.029 and 0.052 ± 0.008 for fall armyworm and ELISA OD of 0.058 ± 0.021 and 0.061 ± 0.017 for corn earworm one and five days after white egg spraying, respectively. The calculated threshold values from those mean was 0.308 / 0.078 for fall armyworm and 0.122 / 0.133 for corn earworm one and five days after spray with an egg white solution, respectively. Likewise, dot blot ELISA detected egg albumin in moths from samples sprayed in field cages of both species throughout the collecting time (**Fig. 4 b-e**). Despite the ability of both methods to detect egg albumin, a small percentage of positive samples was verified between the methods assayed as showed in the summary **Table 1**.

Acquisition of albumin in adults sprayed in corn field

A total of 126 and 191 moths of corn earworm were captured during six days in light and pheromone trap, respectively. Of those samples from light traps, 38 (30.16%) and 46 (36.51%) were marked with albumin according to ELISA and dot blot ELISA assays results, respectively. Of the 191 moths caught from the pheromone trap, 8 (4.19%) and 9 (4.71%) scored positive for egg albumin according to ELISA and dot blot ELISA essays (**Table 2**). Overall, there was difference in the percentage of moths collected throughout the time and a larger number of marked moths were

caught in light traps which were located nearest from the area sprayed with egg albumin solution (**Fig. 5**). In addition, the number of marked samples scored positive by dot blot ELISA was very close with ELISA, which confirm its efficiency and sensitivity (**Table 2**). Our results clearly showed that corn earworm presents a great ability to move throughout the area being collected up to 1600 m from the sprayed site.

DISCUSSION

The potential of egg albumin to mark larvae and moths of fall armyworm and corn earworm and its persistence throughout time were tested. Egg albumin is a suitable protein mark to tracking and monitoring the movement pattern of moths in the field. This conclusion is supported by the high percentage of moths with positive score when sprayed with egg albumin solution in laboratory and field conditions. In addition, our findings demonstrated that egg albumin can be detected by an easier approach using polyvinylidene Diflhoride membrane (dot blot ELISA) instead of being performed on plates (ELISA), the common method mostly used in previous studies (Jones et al. 2006, 2011, Boina et al. 2009, Hagler and Jones 2010, Klick et al. 2016, Blaauw et al. 2017).

The effectiveness of vertebrate protein to mark insects in the field has been reported by several studies in different species and contributed to assess the movement pattern of important agricultural pests (Boina et al. 2009, Sivakoff et al. 2012, Reisig et al. 2013, Lewis-Rosenblum et al. 2015, Klick et al. 2016, Blaauw et al. 2017), natural enemies (Horton et al. 2009, Hagler and Jones 2010, Sivakoff et al. 2012) and pollinators (Hagler et al. 2011, Biddinger et al. 2013). Our findings demonstrate that egg albumin is also a suitable protein source to mark moths of fall armyworm and corn earworm. However, this study showed that egg albumin does not persist throughout larval development, being only detectable at the first larval instar. Because insects growth and development depends on the molting process, the residue of egg albumin absorbed on the first instar integument after spayed is likely discarded

during the subsequent ecdysis. The marking persistence is compromised during the larval stage in Lepidoptera. On the other hand, our results revealed that egg albumin has enormous potential to be used for mark release recapture and for mark-capture studies. All adults assayed in the laboratory acquired egg albumin, confirming the efficacy of this protein to be used for mark-release-recapture study. In addition, egg albumin applied in field cages and directly in the field resulted in a significant percentage of positive samples which means that direct spray in the field can mark natural population of corn earworm and therefore, be used for mark-capture study. Compared with previous studies (Jones et al. 2006, Boina et al. 2009, Hagler and Jones 2010) the percentage of corn earworm adults positive scored for egg albumin from the field experiment was significantly lower. However, some points should be considered. The percentage of positive samples was calculated based on the total of insect collected and some of them are likely from natural population, since the study were performed when occurs the migration of corn earworm to North of U.S. In addition, egg albumin was sprayed when at least 80% of adults had emerged, which suggests that some of them had already moved away from the spraying area.

The advantages of using protein as marker include its availability, low cost and possibility to be applied directly in the field (Jones et al. 2006). Also, ELISA assay is a simple, rapid, sensitive and relatively inexpensive method of protein detection (Jones et al. 2006). In fact, ELISA performed on plates showed to be effective to detect egg albumin in this current study. However, we also demonstrated that an alternative immunoassay (dot blot ELISA), in which protein detection is performed on PVDF membrane is equal or greater sensitive than plate ELISA. According to our results the limit of detection of egg albumin by both ELISA and dot blot ELISA was 41 ppb which indicate that both methods are equal sensitive. However, we should consider that ELISA was performed with 16 times the volume applied on the PVDF membrane (80 µl for plate ELISA against 5 µl for dot blot ELISA). This suggest that whether 80 µl would be applied on the PVDF membrane the sensitive level for egg albumin detection would be higher. Volume higher than 5

µl would be applied on PVDF membrane but according to our results no gain was obtained increasing the volume applied because as volume increased the diameter of the drop also increased and the ratio protein/area did not change significantly. An alternative to enable the application of larger volumes is to perform the protein detection on a microfiltration apparatus device, in which protein in solution are bound onto membrane by filtration using a vacuum. Besides the great sensitivity, dot blot ELISA is also recognized by the easy interpretation of the results, many samples can be screened at the same time and microplate reader attached to computer are not required (Hawkes et al. 1982).

A major benefit of having an effective and easily detectable marker is use it to study the dispersion pattern of agricultural pests with expectation to assist in studies on their ecology. Our laboratory and field studies clearly demonstrated that egg albumin is a suitable marker and can be used to measure the movement of fall armyworm and corn earworm. With a protein marker would be possible to evaluate the range of cultivated and wild host plants colonized by these pests as sink and source sites. The movement of *Diahorina citri*, vector of the citrus greening disease has been documented using egg albumin and milk proteins. Boina et al. 2009 found that this pest moved between managed and unmanaged area suggesting that abandoned groves may function as pest source in the landscape for cultivated areas. Klick et al. 2016 using egg albumin demonstrated that *Drosophila suzukii* utilize a wild alternative host, which may play a role as a source of infestation of *D. sizukii* in cultivated fruiting crops. In addition, egg albumin could be used to investigate some assumptions involved in the adoption of refuge to slow the evolution of resistance to Bt crops targeting fall armyworm and corn earworm. An assumption of the refuge is that non-Bt plants must be cultivated concomitantly with Bt plants to provide susceptible individuals to mating with eventual resistant individuals coming from Bt area (Gould 1998). However, the effectiveness of this strategy is conditional on the random adult movement and the distance between non-Bt and Bt field (Gould 1998). Despite many studies have reported that refuge can delay the evolution of resistance

(Tabashnik 2008), most of them are based on mathematical models which does consider the pest movement pattern in the field. Therefore, egg albumin could be effective marker for tracking and monitoring movement of adults of fall armyworm and corn earworm between Bt and non-Bt crops and, consequently, better assist research to improve resistance management. These results could also be considered a template to be used in the study of the dispersion of other noctuids.

Although protein marker has overcome several disadvantages presented by materials and methods previously adopted to study the movement pattern of insects (Hagler and Jackson 2001, Jones et al. 2006), some points deserve attention about its application. Especially in migration studies, concerns have arisen about the risk of underestimate the false positive, which can alter dispersal estimates (Sivakoff et al. 2011). Also, the protein persistence seems to be affected by abiotic factors, such as rain, humidity and wind (Jones et al. 2006, Boina et al. 2009), which should be better investigated. Finally, individuals externally marked may pass the marker to unmarked individuals. This concern appears to be relevant because collected moths are kept together in a small cage until be caught and contact among them may pass the protein marker from marked to unmarked individuals. Further studies to evaluate the marker transference are encouraged to be conducted in order to avoid the occurrence of false positive especially in long-dispersal studies.

In conclusion, the results of the present work demonstrate that fall armyworm and corn earworm acquired egg albumin sprayed at laboratory and field conditions, and this protein persisted in moths of both species. These results indicate how suitable egg albumin marker is for the study of dispersion pattern of these pests throughout the landscape. Tracking and monitoring the movement behavior of these species would provide useful information about the range of dispersion in the landscape and the size of landscape should be considered when recommending IPM management tactics, especially considering the dynamics of pest sink and source.

Also, it provides a method to validate the recommendation of the design of refuge areas in Bt crops.

Table 1. Comparative summary results between ELISA and dot blot ELISA for the egg albumin detection.

Studies	Time after spray	Insect	No. assayed	% of positive scored samples	
			ELISA/dot blot ELISA	**ELISA**	**dot blot ELISA**
Larvae (1st instar)	1st Instar	FAW	8/8	100	100
	1st Instar	CEW	8/8	100	100
Adults - laboratory	(1-5 days)	FAW	57/40	100	100
	(1-5 days)	CEW	57/40	100	100
Adults - field cages	1 day	FAW	45/45	98	100
	1 day	CEW	45/45	100	100
	5 days	FAW	39/39	95	89
	5 days	CEW	39/39	79	100
Adults – field dispersion	(1-6 days)	CEW	317/317	15	17

Table 2. Dispersion result and comparison between ELISA and dot blot ELISA to detect egg albumin in field open.

Trap	Collected samples	Positive to ELISA	Positive to blot dot ELISA	% ELISA	% dot blot ELISA
light	126	38	46	30.16	36.51
pheromone	191	8	9	4.19	4.71
Total	317	46	55	14.51	17.35

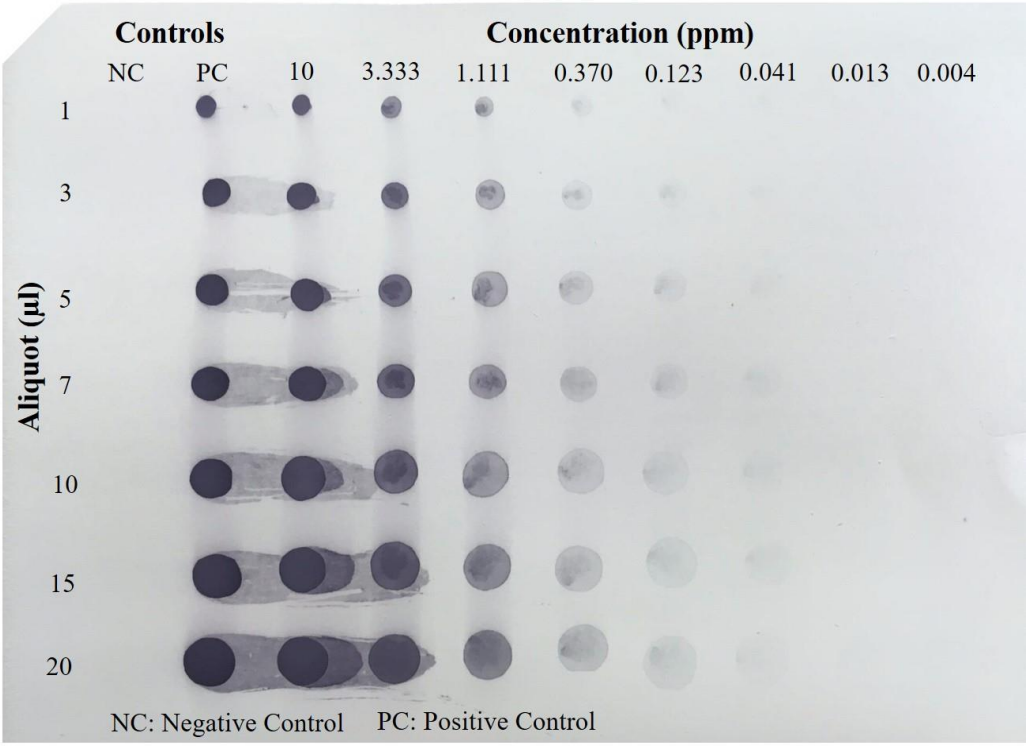

Figure 2. Sensitivity of dot blot ELISA to detect egg albumin. In the first and second column is represented the negative control (NC) and the positive control (PC). The next columns show the tested concentrations starting at 10 ppm and ending at 4 ppb. Each antigen concentration and the negative and positive controls were applied on the PVDF membrane in seven different aliquots. Black/grey spots mean samples scored positive for the antigen (albumin).

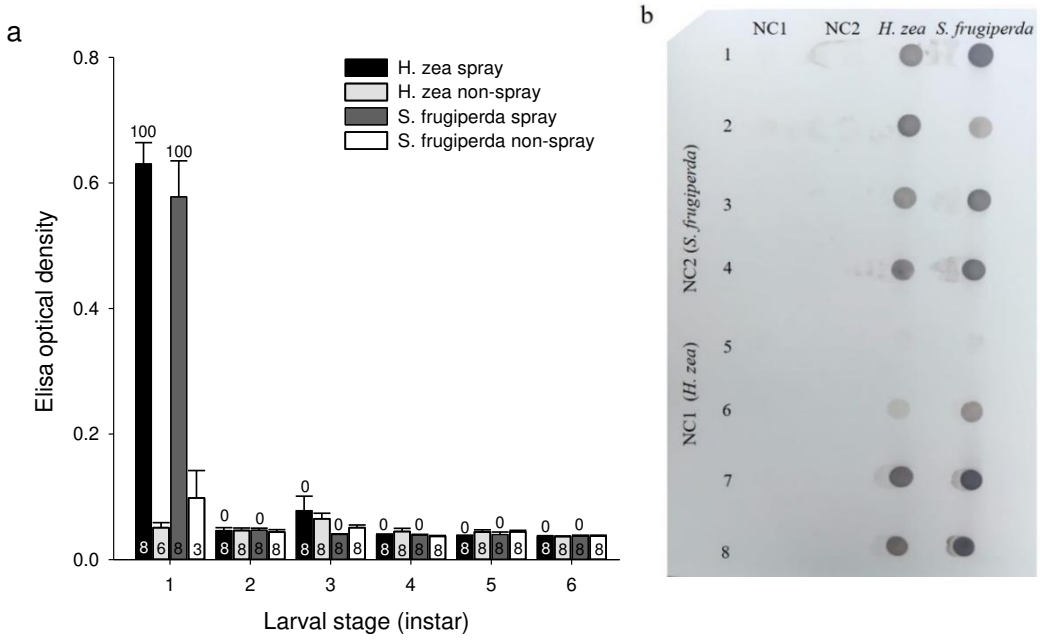

Figure 2: Immunoassay results for the presence of egg albumin in larvae of corn earworm and fall armyworm sprayed with a 20% egg whites solution. a) ELISA optical density values (mean ± SE) and the percentage of those samples scored positive for egg albumin. Indicated above the error bars is the % positive samples and inside the columns is the number of samples tested. b) dot blot ELISA assay for the detection of egg albumin in the first larval instar. The numbers before the first column indicates the dot lines. In the first and the second column (NC1, NC2) is represented the negative control for corn earworm and fall armyworm, respectively. The next two columns represent the results from samples sprayed with egg white solution. Black/gray spots are the samples that scored positive for albumin.

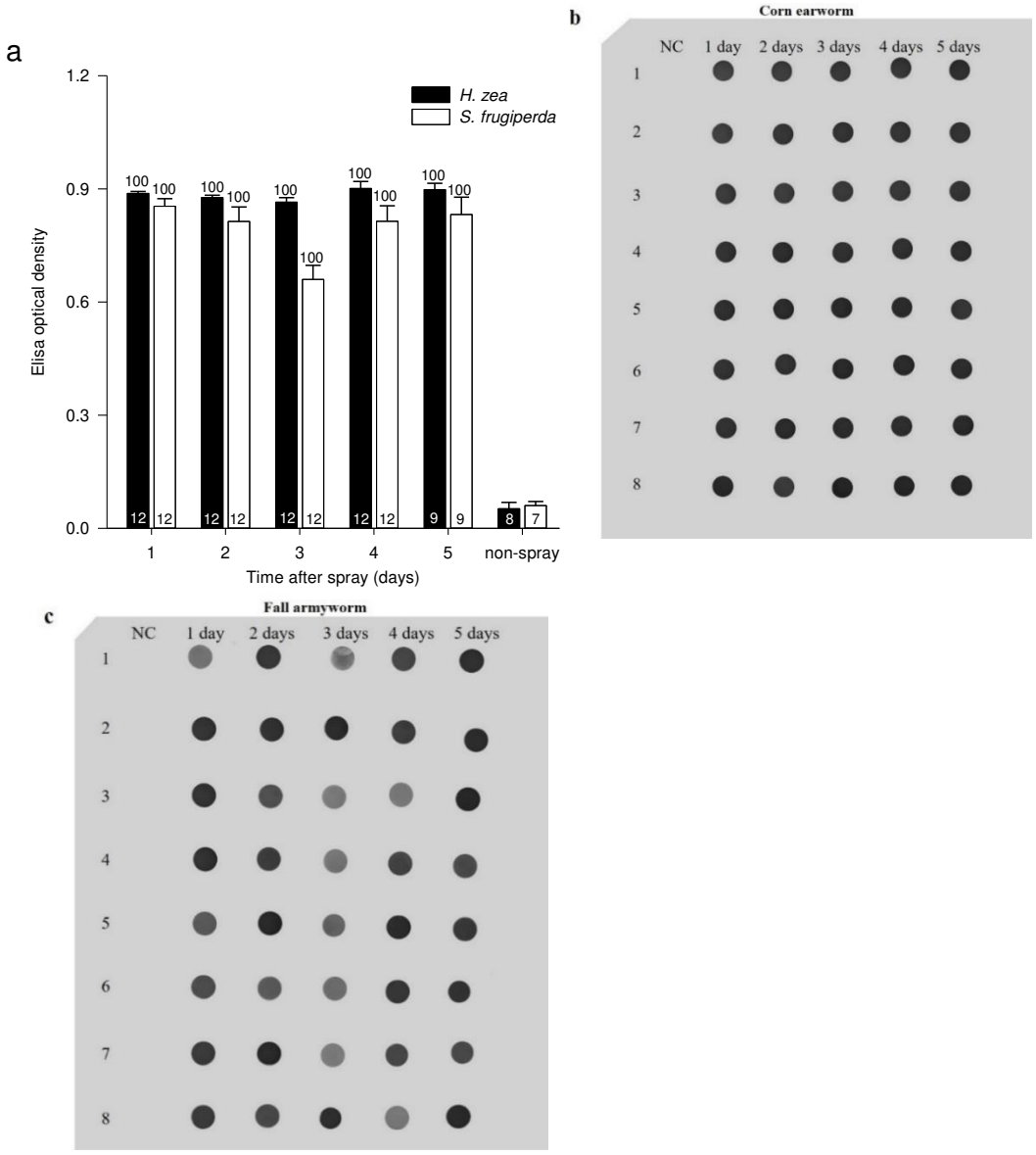

Figure 3. Immunoassay results for the presence of egg albumin in adults of corn earworm and fall armyworm sprayed with egg whites under laboratory conditions. A) ELISA Optical density values (Mean ± SE) and the percentage of the samples that scored positive for the presence of egg albumin (number above the bars). Inside the bar is the number of samples tested. B1 & B2) dot blot ELISA assay for egg album detection in corn earworm and fall armyworm, respectively. In the first column is represented the negative control followed by samples collected in different days after spraying the egg albumin. Black/gray spots were the samples that scored positive for the marker.

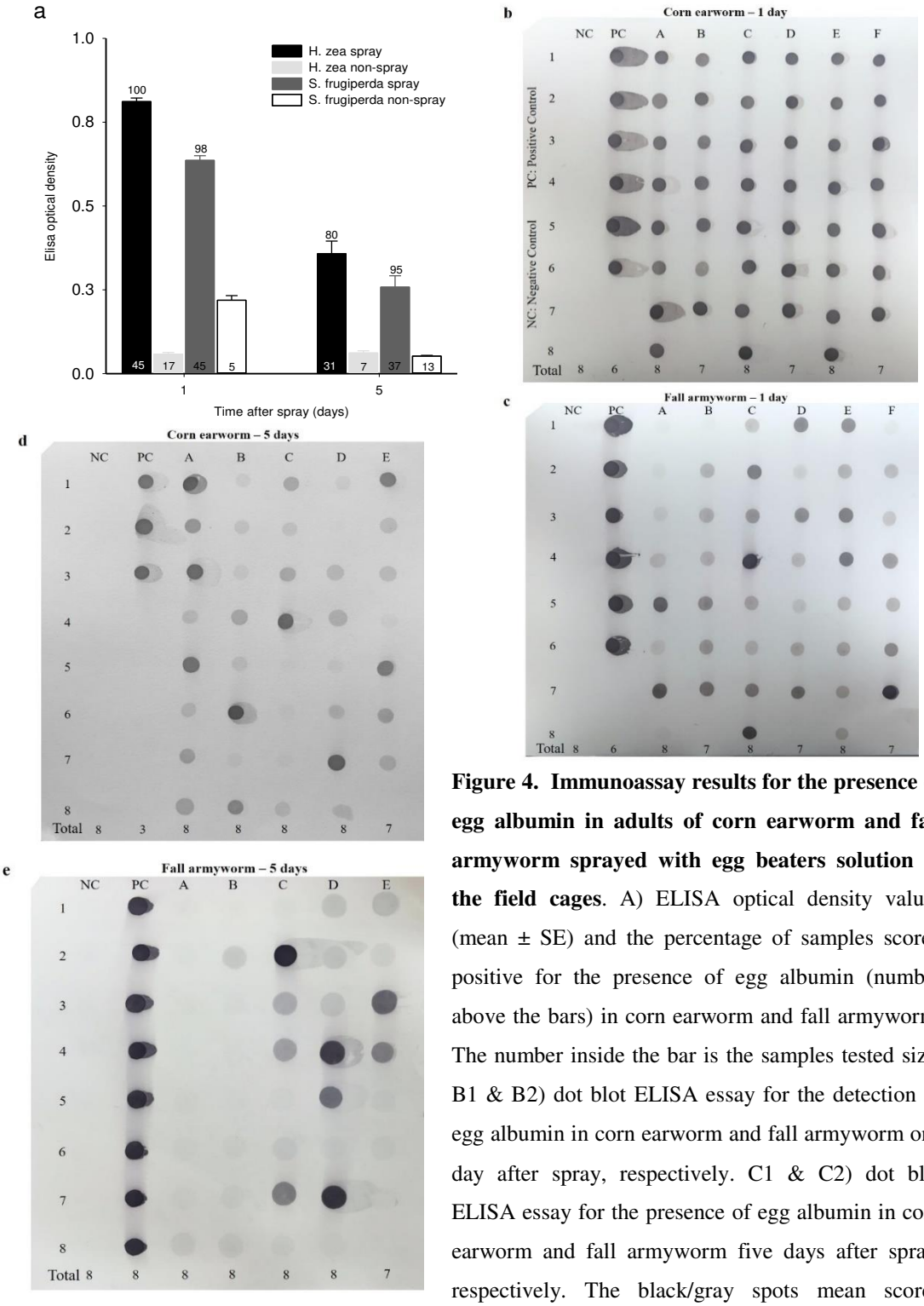

Figure 4. Immunoassay results for the presence of egg albumin in adults of corn earworm and fall armyworm sprayed with egg beaters solution in the field cages. A) ELISA optical density values (mean ± SE) and the percentage of samples scored positive for the presence of egg albumin (number above the bars) in corn earworm and fall armyworm. The number inside the bar is the samples tested size. B1 & B2) dot blot ELISA essay for the detection of egg albumin in corn earworm and fall armyworm one day after spray, respectively. C1 & C2) dot blot ELISA essay for the presence of egg albumin in corn earworm and fall armyworm five days after spray, respectively. The black/gray spots mean scored positive samples for the marker.

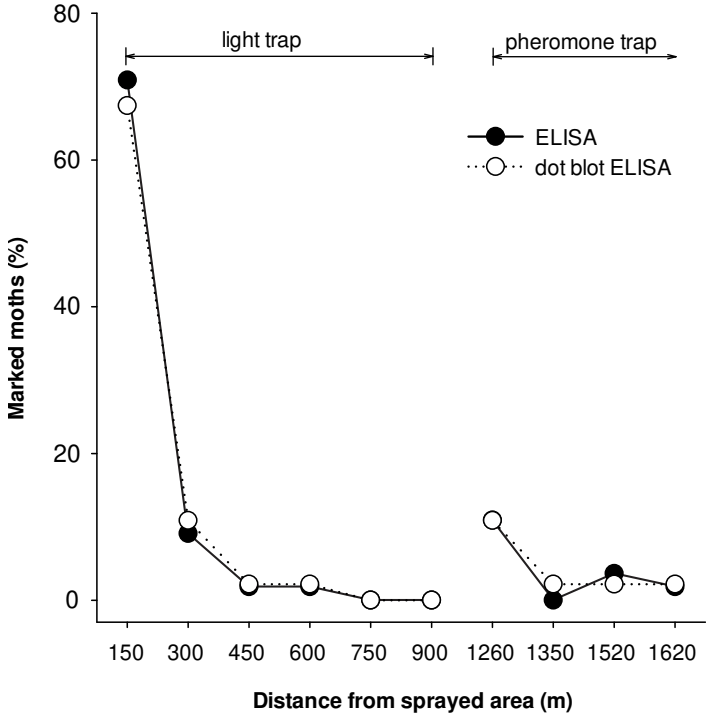

Figure 5. Percentage of marked moths of corn earworm detected by ELISA and dot blot ELISA. The marker was sprayed in an open corn field in Nebraska ant the moths were collected in light and pheromone traps placed at different distances around the area sprayed with egg white albumin.

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CONCLUSÕES GERAIS E CONSIDERAÇÕES FINAIS

No primeiro capítulo desta dissertação foi mostrado que a suscetibilidade de *S. frugiperda* a eventos de milho Bt durante o desenvolvimento larval foi influenciada pelo perfil de resistência Bt da população testada e pela idade de exposição. A população resistente a toxina Cry1F apresentou alta sobrevivência no milho piramidado Cry1A.105+Cry2Ab a partir do quarto dia de idade comparado com a população padrão de suscetibilidade. Diferente padrão de resposta foi observado para as populações suscetíveis aos milhos Cry1F e Cry1Ab+Vip3Aa. Marcadamente, o milho Cry1Ab+Vip3Aa mostrou-se efetivo no controle de larvas de *S. frugiperda* com até 10 dias de idade (máxima idade testada) independentemente da resistência prévia a toxinas Bt, demostrando a importância desta toxina para o controle de *S. frugiperda*. Juntos, estes resultados sugerem que a resistência prévia a Cry1F afeta a suscetibilidade ao milho piramidado Cry1A.105+Cry2Ab ao longo do desenvolvimento larval e que eventos Bt que produzem a toxina Vip3Aa pode ser uma alternativa para o controle e manejo de populações de *S. frugiperda* resistentes a toxinas Cry.

O segundo estudo mostrou que a proteína albumina encontrada em ovo de galinha funciona como marcador em adultos de *S. frugiperda* e *H. zea*. Ensaios realizados em laboratório e em campo comprovaram a efetividade de aquisição e persistência de albumina em mariposas de ambas as espécies. Os resultados também demonstraram que albumina pode ser detectada por um método relativamente mais simples (dot blot Elisa) comparado com ELISA, geralmente adotado pela maioria dos trabalhos usando marcadores proteicos. A disponibilidade de um marcador facilmente aplicável e detectável constitui uma importante ferramenta para o estudo do padrão de dispersão destas espécies em pequena e até mesmo larga escala. Conhecer a dinâmica de dispersão da população da praga auxiliará no manejo adequado, fornecendo informações relevantes aos métodos de controle vigentes.

Os resultados da investigação aqui realizada podem ajudar na escolha da melhor configuração de refúgio para manejar a resistência. A perda de suscetibilidade ao logo da idade larval aqui relatada aliado a grande capacidade de movimentação larval de *S. frugiperda* e a alta frequência de alelo de resistência a Cry1Fa no campo são fatores de risco a adoção de mistura de sementes concomitante com ao milho piramidado Cry1A.105+Cry2Ab. Além dos resultados aqui apresentados, estudos futuros com indivíduos heterozigotos do cruzamento entre as populações aqui testadas podem auxiliar na adoção de mistura de semente pela determinação da dominância funcional da resistência, importante variável a ser considerada. Por fim, a proteína albumina, primeira vez testada em *S. frugiperda* e *H. zea*, constitui um potencial marcador para uma abordagem que vise decifrar o padrão de dispersão destas pragas, cujo conhecimento pode auxiliar no manejo destas pragas no campo.