EDMAR DE SOUZA TUELHER

SUSCEPTIBILITY TO CHLORANTRANILIPROLE, COMPETITION ABILITIES AND STYLET PROBING BEHAVIOR OF HETEROPTERAN PESTS

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

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Contents

Abstract

TUELHER, Edmar de Souza, D.Sc., Universidade Federal de Viçosa, February, 2017. **Susceptibility to chlorantraniliprole, competition abilities and stylet probing behavior of heteropteran pests.** Advisor: Eugênio Eduardo de Oliveira. Co-Advisors: José Eduardo Serrão and Raul Narciso Carvalho Guedes.

Large losses in agricultural production worldwide have occurred due to insects in the order Heteroptera. Records of severe losses in agricultural commodities [eg, soybean, *Glycine max* (L.)] in Brazil have been attributed to the action of a stink bug complex, whose main insect species have changed both spatially and temporally in soybean producing regions. More recently, other stink bug pests without occurrence in Brazil have been the main cause of crop losses with great relevance for Brazilian agriculture. Therefore, the present research was divided into three parts, aiming not only to address the current situation of two of the main pests in Brazilian soybean crops, the brown stink bug, *Euschistus heros* (F.), and the red banded stink bug, *Piezodorus guildinii* (West.), but also to provide relevant information about heteropteran pests in other countries and likely to become pests in Brazil, like *Lygus lineolaris* (Palisot de Beauvois). In the first study, the susceptibility of *E. heros* to the chlorantraniliprole insecticide was evaluated, as well as the alteration in the reproductive fitness mediated by sublethal exposure to this insecticide. The susceptibility of *E. heros* was evaluated by assessing the mortality of newly emerged nymphs and adults. Alterations in reproductive fitness were evaluated by testing four combinations of treated and untreated couples: untreated male and treated female; treated male and untreated female; both male and female treated; both male and female untreated. Although chlorantraniliprole insecticide caused low mortality to *E. heros* during the developmental stages (3^{rd}, 4^{th} and 5^{th} instars and adults) treated, adults exposed to sublethal concentration of chlorantraniliprole had their reproductive fitness affected. There was an increase in survival time for untreated males coupled with treated females, and a higher oviposition peak for treated females coupled with untreated males, with shorter egg incubation time and delayed peak of fertility. Additionally, the untreated females coupled with treated males exhibited higher daily fecundity, potentially representing chlorantraniliprole-induced hormesis. In the second work, the competitiveness of *E. heros* and *P. guildinii* was assessed by direct competition experiments with mixed (adult) insect infestations in soybean plants. Fitness of each species was measured by quantifying the populational growth rate, and the soybean yield was accessed at harvesting. The results showed that *E. heros* was a better competitor than *P. guildinii*, and the competitive ability of *P. guildinii* was compromised by the increase in the amount of conspecific and heterospecific (i.e., *E. heros*). However, despite the fact that *P. guildinii* apparently lost the competition with *E. hero*s, no yield was observed in the plants infested with *P. guildinii*. In the third work, the stylet probing behavior of *L. lineolaris* (Palisot de Beauvois) on cotton terminal axillary leaves was studied. *L. lineolaris* was chosen because it is a pest of great importance in several countries, but has not been recorded in Brazil. However, due to international trade there is a probability that this pest would become a problem if introduced in Brazil. The relationship between characteristics of stylet probing behavior of *L. lineolaris* (3rd, 4th and 5th instars and adult males) and the amount of leaf damage was analyzed. *L. lineolaris* stylet probing behavior was measured and quantitatively described by electropenetrography (EPG). Cell rupturing (CR) events, composed of combined stylet movements and salivation during insect probing, are the main cause of damage on cotton axillary leaves. Strong correlation between total duration of CR with the amount of leaf damage was found. Differences between life stages and amount of damage on leaves were not found. Therefore, the CR feeding strategy was highly detrimental to leaves, even with low-duration events. These studies reinforce that the recent outbreaks and the higher abundance of *E. heros* observed in Brazilian soybean fields are related to sublethal effects of chlorantraniliprole, and probably others insecticides, by altering the sexual fitness of *E. heros*, associated with its higher competitive ability against its heterospecific competitor, *P. guildinii*. Additionally, EPG will be a important tool to understand stylet probing behavior of soybean stink bugs pests. EPG application, including for studies with insecticides and their sublethal effects, will be a valuable tool to development of strategies to reduce soybean crop lost.

Resumo

TUELHER, Edmar de Souza, D.Sc., Universidade Federal de Viçosa, fevereiro de 2017. **Suscetibilidade a clorantraniliprole, habilidades competitivas e comportamento alimentar de heterópteros pragas.** Orientador: Eugênio Eduardo de Oliveira. Coorientadores: José Eduardo Serrão e Raul Narciso Carvalho Guedes.

Grandes perdas da produção agrícola mundial têm ocorrido devido a ação de insetos da ordem Heteroptera. No Brasil, os registros de perdas severas na produção de commodities agrícolas [e.g., a soja, *Glycine max* (L.)] têm sido atribuídos a ação de um complexo de percevejos, cujo os principais representantes tem se alterado ao longo do tempo e nas diferentes nas regiões produtoras de soja. Mais recentemente, outros percevejos que ainda não existem no Brasil tem sido os principais agentes de perdas em culturas agrícolas com destacada relevância para agrícola brasileira. Por isto, a presente investigação foi dividida em três etapas, visando abordar não somente a situação atual de dois dos principais percevejos pragas em cultivos brasileiros de soja, o percevejo marrom da soja, *Euschistus heros* (F.) e o percevejo verde pequeno, *Piezodorus guildinii* (West.), mas também obter informações relevantes sobre percevejos que são pragas em outros países mas que tem extremo potencial de serem pragas também aqui no Brasil, como *Lygus lineolaris* (Palisot de Beauvois). No primeiro trabalho, foi avaliada a susceptibilidade de *E. heros* ao inseticida clorantraniliprole, bem como a alteração do potencial ou capacidade reprodutiva mediada por exposição subletal a este inseticida. A suscetibilidade de *E. heros* foi avaliada por meio da determinação de mortalidade de ninfas e adultos recém emergidos. A alteração do potencial reprodutivo foi avaliada testando quatro combinações de casais tratados e não tratados: macho não tratado e fêmea tratada; macho tratado e fêmea não tratada; ambos macho e fêmea tratados; ambos machos e fêmeas não tratados. Apesar do inseticida clorantraniliprole ter causado baixa mortalidade em *E. heros* às fases de desenvolvimento avaliadas (3º, 4º e 5º instares e adultos), a exposição subletal de adultos ao clorantraniliprole afetou o potencial reprodutivo da espécie. Ocorreram aumento no tempo de sobrevivência para machos não tratados acasalados com fêmeas tratadas, e maior pico de oviposição em fêmeas tratadas acasaladas com machos não tratados, com consequente menor tempo de incubação de ovos e atraso no pico de fertilidade. Além disso, fêmeas não tratadas acasaladas com machos tratados apresentaram maior fecundidade diária, o que pode indicar hormese induzida por clorantraniliprole. No segundo trabalho, foi estudado a capacidade competitiva entre as espécies *E. heros* e *P. guildinii* por meio de experimento de competição, com a infestação de adultos de ambas as espécies em plantas de soja. Para cada espécie foi determinada a taxa de crescimento populacional, e adicionalmente o efeito sobre a produção das plantas de soja. Os resultados mostraram que *E. heros* foi um melhor competidor do que *P. guildinii*, tendo a capacidade competitiva de *P. guildinii* sido comprometida pelo aumento na quantidade de coespecíficos e heteroespecíficos (isto é, *E. heros*). No entanto, apesar do fato de que *P. guildinii*, aparentemente, ser um pior competidor em relação a *E. heros*, nenhuma produção de grãos foi observada nas plantas infestadas com *P. guildinii*. No terceiro trabalho, foi verificado o comportamento alimentar de *Lygus lineolaris* (Palisot de Beauvois) em folhas da axila terminal de plantas de algodão do terminal axilar. *L. lineolaris* foi escolhido por ser um heteróptero praga de relevada importância em diversas culturas, mas que ainda não tem registro de ocorrência no Brasil. No entanto, devido ao comércio internacional existe a possibilidade de vir a ser praga caso sua presença seja detectada. Foi verificada a relação entre características do comportamento de prova de *L. lineolaris* (3º, 4º e 5º instares e adultos machos) e a quantidade de danos nas folhas resultante da alimentação dos insetos. O comportamento de prova foi caracterizado e mensurado por Eletropenetrografia (EPG). Os eventos de ruptura de células (CR), que são movimentos do estilete dos insetos combinados com salivação durante a prova estiletar, são a principal causa de danos nas folhas de algodão havendo uma forte correlação entre o tempo total de CR e a quantidade de dano foliar. No entanto, não houveram diferenças entre os instares na quantidade de danos nas folhas. Portanto, a estratégia de CR durante a alimentação por *L. lineolaris* foi altamente prejudicial às folhas, mesmo em eventos de baixa duração. Estes estudos reforçam a percepção de que os recentes surtos e a maior abundância de *E. heros* observados nos campos de soja podem ser devidos a efeitos subletais de clorantraniliprole, e provavelmente também de outros inseticidas, via alteração do potencial reprodutivo de *E. heros*, associada à sua maior capacidade competitiva contra seu competidor heteroespecífico *P. guildinii*. A técnica de EPG utilizada neste estudo virá a ser uma importante ferramenta no estudo do comportamento alimentar de insetos fitossuccívoros da cultura da soja. A utilização de EPG, inclusive para estudos com inseticidas e seus efeitos subletais, é uma valiosa ferramenta no desenvolvimento de estratégias para reduzir as perdas na produção de soja.

General introduction

The stink bug (Pentatomidae) complex is one of the most important pest groups on soybean *Glycine max* (L.), mainly because their damage occurs primarily during the reproductive stage of the plants (Corrêa-Ferreira et al. 2010; Panizzi et al. 2012; Panizzi and Slansky Jr 1985; Prado et al. 1982; Smaniotto and Panizzi 2015). This complex comprises three main species: the neotropical brown stink bug, *Euschistus heros* (F.), the redbanded stink bug, *Piezodorus guildinii* (West.), and the southern green stink bug, *Nezara viridula* (L.)(Panizzi et al. 2000; Panizzi and Slansky Jr 1985; Smaniotto and Panizzi 2015). In addition, the importance of these species as soybean pests has changed spatially and temporally, in the U.S. (Baur and Baldwin 2006; Kamminga et al. 2012; Temple et al. 2013; Vyavhare et al. 2014), especially in Brazil (Bueno et al. 2013; Panizzi 2000; Panizzi 2013; Smaniotto and Panizzi 2015), and also Argentina (Saluso et al. 2011). A clear example of this change in status is the increased abundance and distribution of *E. heros* and the green belly stink bug, *Dichelops furcatus* (F.), and *Dichelops melancanthus* (Dallas), and consequently decreasing importance of *N. viridula*, mainly in Central Brazil (Panizzi 2015; Panizzi and Lucini 2016).

Which factors could be triggering the changes in pest status of some species of the stink bug complex are not completely understood; however, based on some previous work certain hypotheses have been conjectured. For example, theses include differential susceptibility to insecticides (Snodgrass et al. 2005; Willrich et al. 2003), pesticide resistance (Sosa-Gomez and Silva 2010; Sosa-Gomez et al. 2009), ecological interactions between species of this stink bugs complex, e.g. inter- and intraspecific competition (Tuelher et al. 2016b), changes in agricultural practices (Panizzi 2013; Smaniotto and Panizzi 2015; Zerbino et al. 2015), multiple cropping systems (Quintela et al. 2006; Soria et al. 2010; Tomquelsky and Martins 2011), and global warming (Panizzi and Lucini 2016; Tougou et al. 2009).

Stink bug control is still accomplished mainly by application of insecticides, which can number as many as five applications per season (Bueno et al. 2015; Bueno et al.

2013; Panizzi 2013). This means that insecticide applications are one of the most important factors driving stink bug populations. Insecticides as a stressor could impair physiological responses and ecological impacts of many organisms, although newer application technologies have been used to achieve improved insect control practices. Inappropriate insecticide use could result, for example, in insect resistance or secondary pest outbreaks (Bueno et al. 2015; Sosa-Gomez et al. 2001; Sosa-Gomez and Silva 2010), or at sublethal concentrations, such use could result in a phenomen called hormesis, i.e., stimulation of one organism exposed to one sublethal concentration that is toxic at higher doses (Cutler 2013; Guedes and Cutler 2014; Guedes et al. 2016). Insecticidal sublethal effects upon *E. heros* have already been reported, and results include increased female fecundity (Haddi et al. 2016; Santos et al. 2015; Tuelher et al 2016a;), male survival (Tuelher et al 2016a), female fertility (Santos et al. 2015), and increased mating frequency (Haddi et al. 2016). Otherwise, they could alter the ecological relationships between conspecifics or heterospecifics in soybean fields, and influence herbivorous pentatomid populations.

One important ecological process in soybean fields is intra- and interspecific competition (Tuelher et al. 2016). Most of the important pentatomids feeding on soybean overlap their niches and ranges, besides having some differences in time they attack and plant structure they prefer to feed (Corrêa-Ferreira 2005; Kuss et al. 2012; Panizzi et al. 2012). In Brazil, *E. heros* are found feeding on soybean from December to April, and dispersing afterwards to alternate host plants (Panizzi and Niva 1994; Panizzi and Vivan 1997). *Piezodorus guildinii* tends to feed on soybean reproductive stages and it is more damaging to seeds (Depieri and Panizzi 2011; Panizzi et al. 2012; Silva et al. 2012). Its occurrence its variable and changes spatially and temporally from one year to another (Kuss et al. 2012). In general, these two species are the most damaging to soybean plants, because of their abundance and distribution (*E. heros*) or because of the degree of damage they impose to plants (*P. guildinii*).

The stink bugs that coexist in the soybean environment had their pest status changed during the last decades (Panizzi and Lucini 2016), and probably they influenced each other in abundance and occurrence. Stink bug are piercing-sucking insects that insert their stylets inside plant tissues to feed. Consequently, they damage its host, in the case of soybean, primarily seeds, causing severe losses in yield. Soybean stink bugs have an interesting feeding strategy that was recently described by using electropenetrography (EPG) technology. They shift between two different feeding strategies according to the plant tissue they are exploring. They act as cell rupturing feeders to ingest from endosperm or switch to the salivary sheath feeding strategy to ingest from xylem in soybean leaves and stems (Lucini and Panizzi 2016b; Lucini et al. 2016). This strategy could allow them to explore vegetative tissues of alternative plant hosts when the main host is not available and thereby increase their chance to establish a population at specific areas. Otherwise, the amounts of time spent in each kind of feeding strategy are different among species according to their preferred site of feeding (Lucini and Panizzi 2016a; Lucini and Panizzi 2016b; Lucini et al. 2016). Probably, how efficiently the insect species shift between strategies to explore better the available food source, the greater the probability of being a better competitor. Detailed studies about feeding behavior could bring more information about potentially successful competitors.

An important heteropteran pest of the Northern hemisphere studied here is the tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois). Species from the *Lygus* genus are economically important pests in western and southeastern USA and cause large damage to agricultural fields (Cooper and Spurgeon 2013). They are very polyphagous and could feed from hosts belonging to more than 35 families (Schwartz and Foottit 1998). Because lygus bugs are not recorded in Brazil yet, they are a potential invasive species, and because they are polyphagous they could establish and cause damage to an array of important crops in Brazil. So, detailed information about their feeding behavior could help to develop management strategies to prevent damage.

This study aimed to provide information about increasing reports of outbreaks of *E. heros*. The first chapter discusses the insecticidal toxicity of chlorantraniliprole and its sublethal effects on sexual fitness of the Neotropical stink bug, *E. heros*. *E. heros* is a non-target species of chlorantraniliprole, which is registered to control lepidopteran pests in soybean (MAPA 2015). However, the sublethal effects of pesticides on soybean stink bugs have not received attention. The second chapter discusses the competitive abilities of *E. heros* against *P. guildinii* on soybean plants. Because the composition of insect pests of soybean has changed both spatially and temporally in neotropical soybean production areas, the competitiveness of each species was assessed in direct competition experiments. The third chapter discusses the stylet probing behavior of *L. lineolaris* on cotton leaf terminals, aiming to quantitatively describe its behavior in relation to damage caused on plants over time. This chapter is a result from data collected at the United States Department of Agriculture, Agriculture Research Service (USDA-ARS) facility in Parlier, CA , facilitated by a scholarship provided by the Minas Gerais State Foundation for Research Aid (FAPEMIG) as part of its PhD Sandwich Program. The EPG technology used in this experiment is the most rigorous way to study stink bugs feeding behavior and it is an important tool to be applied for Brazilian stink bug research, especially the soybean complex.

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Chlorantraniliprole-mediated toxicity and changes in sexual fitness of the Neotropical brown stink bug *Euschistus heros*

Chlorantraniliprole-mediated toxicity and changes in sexual fitness of the Neotropical brown stink bug *Euschistus heros*[1]

Abstract

Chlorantraniliprole is a diamide insecticide that has been widely used against lepidopteran pests. However, the actions of this compound on non-targeted insect pests such as the Neotropical brown stink bug *Euschistus heros* (Hemiptera: Pentatomidae) have not received attention. Here, we assessed the susceptibility of *E. heros* to chlorantraniliprole as well as the potential chlorantraniliprole mediated changes in the sexual fitness of *E. heros*. Newly emerged (<24 h old) individuals were exposed for 48 h to dry residues of chlorantraniliprole, and mortality was recorded. To assess the sexual fitness of *E. heros*, we tested four couple combinations: untreated male and female; untreated male and treated female; treated male and untreated female; and treated male and female. Chlorantraniliprole caused low mortality regardless of the developmental stage assessed (e.g., 3^{rd}, 4^{th}, 5^{th}, and adults). However, sublethal exposure (i.e., at the concentration of 0.56 μg/cm^2 to chlorantraniliprole affected the sexual fitness of stink bug couples. While treated males coupled with untreated females exhibited a higher number of matings, untreated males coupled with treated females exhibited a higher mean survival time. These treated females coupled with untreated males showed a higher oviposition peak and shorter egg incubation time but a delay in their peak of fertility. Curiously, the untreated females coupled with treated males exhibited higher daily fecundity, which potentially represents chlorantraniliprole induced hormesis which may have contributed to the recent outbreaks of *E. heros* observed in Brazilian soybean fields.

Key words: Anthranilic diamides, Phytophagous pentatomids, Population growth, Hormesis, Reproductive fitness gain

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1.1 Introduction

Insecticides are still an influential tool used to control agricultural insect pests (Ghimire and Woodward 2013; Guedes et al. 2016). However, the inappropriate use of insecticides is a threat to sustainable pest control, especially in crops that are cultivated over extensive areas and subjected to a broad array of insect pests. For instance, despite the already known benefits of using integrated and more sustainable practices, control of soybean insect pests still relies on five insecticide applications per season (Bueno et al. 2015; Panizzi 2013; Song and Swinton 2009). Consequently, pest resistance as a result of increased insecticide usages, and potential insecticide secondary pest outbreaks occur (Bueno et al. 2015; Santos et al. 2016; Sosa-Gómez et al. 2001; Sosa-Gómez and Silva 2010), both of which are unintended complications that soybean farmers must cope with.

Chlorantraniliprole is an anthranilic diamide insecticide that has provided efficient control of a range of lepidopteran pests (Cordova et al. 2006; Jeanguenat 2013; Lahm et al. 2009) as well as to other insect groups (Jiang et al. 2012; Lai and Su 2011; Teixeira et al. 2009; Yeoh and Lee 2007) including sucking insect pests (Caballero et al. 2013). These diamides activate the ryanodine receptors in insect muscle cells, which stimulate the release and depletion of calcium from the internal stores in the sarcoplasmic reticulum, causing impaired regulation of muscle contraction leading to feeding cessation, paralysis, and ultimately death (Cordova et al. 2006; Jeanguenat 2013; Lahm et al. 2009).

Chlorantraniliprole is registered for use in Neotropical soybean fields to control key lepidopteran pests such as the velvetbean caterpillar *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) and the soybean looper *Chrysodeixis includens* (MAPA 2015). However, the extensive use of insecticides may unintentionally expose non-targeted pests, which may lead to insecticide-induced stress. The Neotropical brown stink bug, *Euschistus heros* (Hemiptera: Pentatomidae), is certainly one of these potential non-target soybean pests that suffer unintended chlorantraniliprole exposure. As recently described for other insect pests (Guedes et al. 2016), such unintended exposure of *E. heros* to chlorantraniliprole may result in either detrimental or beneficial impacts on their sexual fitness (e.g., increasing or reducing their survival, mating, reproductive, or competitive abilities). Sexual fitness gain mediated by sublethal exposure to chlorantraniliprole has not yet been reported, but several studies have reported detrimental impacts of chlorantraniliprole on reproduction (Gontijo et al. 2014, 2015; Guo et al. 2013; Han et al. 2012; Liu et al. 2012a, b; Moscardini et al. 2015; Smagghe et al. 2013; Teixeira et al. 2009), immature and adult longevity (Lai and Su 2011; Zhang et al. 2013), and mating behavior (Knight and Flexner 2007; Zhang et al. 2013) of diverse insect species.

There are a restricted number of chemicals registered to control *E. heros*, but toxicological studies investigating the chlorantraniliprole action against this important soybean pest species still are absent. The relatively low plant systemicity of chlorantraniliprole means this insecticide is of limited use against sucking insect pests; therefore, its use with *E. heros* will most probably result in sublethal effects. However, recent studies provided evidence that *E. heros* females are able to increase their fecundity and fertility rates to overcome insecticide-induced sublethal stress (Santos et al. 2016). Thus, by combining toxicological and behavioral procedures, the present investigation was carried out to evaluate the toxicity and potential chlorantraniliprole-mediated changes in the sexual fitness of *E. heros*.

1.2 Materials e methods

1.2.1 *Insects*

The colony of *E. heros* was established from eggs obtained from the Semiochemical Laboratory at the Natural Resources and Biotechnology Center of the Brazilian Agricultural Research Corporation (EMBRAPA; Brasília, DF, Brazil). The colony was multiplied and reared under controlled conditions (27 ± 2 C, 60 ± 20 % relative humidity, L:D photoperiod of 14:10 h) to prevent diapause. *Euschistus heros* at all developmental stages were mass reared in plastic boxes (25 x 20 x 20 cm) following the methods previously described (Borges et al. 2008; Silva et al. 2008, 2011). Briefly, the insects were fed ad libitum with a mixture of fresh green bean pods, *Phaseolus vulgaris* (L.), dry soybean seeds, *Glycine max* (L.), raw shelled peanuts, *Arachis hypogaea* (L.), and sunflower seeds, *Helianthus annuus* (L.), in addition to water. The plastic boxes were cleaned and supplies replenished at 2 days intervals. Eggs were removed from pieces of cheesecloth inside the mass rearing boxes and transferred to plastic Petri dishes with a piece (ca. 3 cm) of green bean pod inside. When nymphs reached the second instar, they were moved to plastic boxes and reared there until reaching the adult stage.

1.2.2 *Concentration-mortality bioassays*

Insecticide bioassays followed methods adapted from previous studies on *E. heros* performed with glass vials (Santos et al. 2016; Snodgrass et al. 2005; Willrich et al. 2003). Briefly, chlorantraniliprole (concentrate suspension at 200 g a.i./L; Du Pont do Brasil S.A., Barra Mansa, RJ, Brazil) was used to coat the inner walls of 250 mL transparent glass vials (EME Equipment, Paulicéia, SP, Brazil). Deionized water was used as a carrier for the commercial insecticide formulation; 2 mL aliquots were added to each glass vial. Four different insecticide concentrations were used (0.056; 0.56; 2.8 and 5.61 $\mu g/cm^2$ of treated surface, which are the equivalent of 0.1-; 1-; 5- and 10-fold the field label rate to control *C. includens* Walker (Lepidoptera: Noctuidae) in Brazilian soybean fields). These concentrations were used to assess the insect mortality of newly emerged (i.e., <24 h) 3^{rd}, 4^{th}, and 5^{th} instar nymphs, as well as newly emerged adults of *E. heros*. In the control treatments, the insects were exposed to glass vials coated with deionized water subsequently left to dry. Nine to twelve replicates, each with 10 newly emerged nymphs or adults (<24 h), were used at each concentration. The inner part of the top of each vial was coated with Teflon PTFE (DuPont, Wilmington, DE, USA) and closed with a piece of organza veil and a rubber band to prevent the insects from escaping. Insects were counted as dead if they were unable to walk the length of their body when prodded with a fine-hair brush after a 48-h exposure to the insecticide.

1.2.3 *Mating behavior, survival, and reproductive bioassays*

To assess the sublethal effects on mating behavior, newly emerged (i.e., <24 h) adult females and males were sexed and individually exposed for 48 h to dried chlorantraniliprole residues at a single concentration corresponding to the insecticide field label rate (0.56 μg a.i./ cm^2). This period of exposure was chosen because this insecticide exhibits fast activity after ingestion (<40 min), as reported for other insect pest species (Hannig et al. 2009). The adult males and females that survived the chlorantraniliprole exposure were individually placed into covered plastic Petri dishes (90 mm diameter and 10 mm high) with food ad libitum up to the 13th adulthood day (i.e., 10 days after insecticide exposure). The choice of adult age (i.e., 13 days) was made to allow sexual maturation of the insects (Costa et al. 1998) and was preliminarily tested with chlorantraniliprole-exposed and unexposed couples exhibiting latency for egg laying of 10.4 ± 0.38 and 10.7 ± 0.89 days, respectively. Thus, virgin female and male couples (13 days old) in four different combinations (untreated female and male; treated female and treated male; treated female and untreated male; and untreated female and treated male) were allowed to mate and digitally recorded (HDR-XR520V, Sony, Tokyo, Japan) for 13 h. Twenty-one replicates (i.e., couples) were used per treatment. The number of mating attempts, number of matings, the latency to the first mating, and the total mating time were recorded.

After mating, the *E. heros* couples were kept inside a 9-cm-diameter plastic Petri dish (covered with the lid) with food, and their longevity recorded. The food supplies consisted of a 2-cm-long pod of green bean and two seeds of each grain type (i.e., dry soybean, raw shelled peanuts, and sunflower seeds). The green pods were replenished at 3-day intervals and the grains at 6-day intervals. A 2 x 1 cm piece of orange 100 % polypropylene non-woven fabric was placed inside each Petri dish to serve as oviposition site. Eggs of each couple were collected daily and transferred to cubette mini ice cube trays (26.9 x 1.2 x 8.9 cm; Melida Comércio e Indústria Ltda, Sorocaba, SP, Brazil) until they hatched and the nymphs emerged. Number of eggs laid per female, egg viability, and female and male survival were recorded daily.

1.2.4 *Statistical analysis*

The mortality caused by chlorantraniliprole was subjected to analyses of covariance with the life stages (i.e., 3rd instar, 4th instar, 5th instar, and adults) as the independent variable and the insecticide concentration as a covariate (PROC GLM; SAS Institute

2008). Complementary linear regression analyses were performed when necessary (PROC REG; SAS Institute 2008). The assumptions of normality and homogeneity of variance were checked, and no data transformation was necessary (UNIVARIATE procedure, SAS Institute 2008). Mating behavior parameters and mean time of egg incubation were submitted to univariate analysis of variance (ANOVA) or Kruskal-Wallis one-way ANOVA on ranks when assumptions of normality and homoscedasticity were not satisfied. All pairwise multiple comparisons, whenever necessary, were performed by applying Tukey's HSD test ($P<0.05$) using SigmaPlot 12.5 (Systat Software, San Jose, CA, USA). Female and male survival analyses were performed using Kaplan–Meier estimators (Log-rank method) again using SigmaPlot 12.5. Daily fecundity and fertility were subjected to regression analysis, using time after adult emergence as an independent variable and the curve fitting procedure of Table Curve 2D 5.01 (Systat Software, San Jose, CA, USA). The regression model was chosen based on parsimony, lower standard errors, and steep increases in R^2 with model complexity. The regression models for each treatment were considered different from each other if the interval confidence limits of their parameters did not overlap.

1.3 Results

1.3.1 *Concentration-mortality bioassays*

The model of analyses of covariance for the mortality caused by chlorantraniliprole demonstrated significant effect of the sources of variations [i.e., the life stages (3^{rd} instar, 4^{th} instar, 5^{th} instar, and adults) and the insecticide concentration] as well as their interaction (Table 1.1). As shown in Fig. 1.1, the highest mortality levels were achieved at the highest chlorantraniliprole concentration (i.e., 5.61 μg/cm^2, but that level never killed more than 45 % of the insects tested. Interestingly, increased insecticide concentrations did not result in higher mortality for individuals of the 3^{rd} and 5^{th} instar nymphs as well as adults of *E. heros* (Fig. 1.1). However, *E. heros* adults were significantly less susceptible than the individuals of 3^{rd} and 5^{th} instar nymphs (Fig. 1.1). For individuals of the 4^{th} instar, increases in the insecticide concentration resulted in higher mortalities (Fig. 1.1).

Table 1.1: Analyses of covariance for the mortality caused by chlorantraniliprole on the 3rd, 4th, and 5th instar nymphs as well as adults of *E. heros*

Sources of variation	*df*	Mortality	
		F	*P*
Model	7	11.9	<0.0001*
Error	112	-	-
Insecticide concentration	4	10.8	<0.0001*
Life stage	3	39.0	<0.0001*
Interaction	12	6.7	0.0003*

Asterisks indicate significant difference at $P<0.05$

1.3.2 *Mating behavior, survival, and reproductive bioassays*

Sublethal exposure to chlorantraniliprole (i.e., 0.56 $\mu g/cm^2$) differentially affected the sexual fitness of *E. heros* couples. Treated males coupled with untreated females exhibited a significantly higher number of mating attempts than couples where only females were treated (H = 8.43, df = 3, *P* = 0.038; Fig 1.2a). The higher number of mating attempts resulted in significantly higher numbers of matings for the couples where only males were treated (H = 7.55, df = 3, *P* = 0.048; Fig. 1.2b). No significant differences were found between treatments for the other parameters, i.e., time elapsed to first mating (H = 0.82, df = 3, *P* = 0.85; Fig. 1.2c) and total mating duration ($F_{3,78}$ = 0.66, *P* = 0.66; Fig. 1.2d).

The mean survival time (LT_{50}) of males varied significantly among the *E. heros* couples (Log-rank test: χ^2 = 11.4, df = 3, *P* = 0.010; Fig. 1.3a). Untreated males that coupled with treated females showed the highest LT_{50} value (54.8 days) and differed significantly from treated males that coupled with treated (35.0 days) or untreated females (35.4 days), but they did not differ significantly from untreated males that coupled with untreated females (44.6 days). The mean survival times of untreated or treated females that coupled with untreated or treated males were not significantly different from each other (Log-rank test: χ^2 = 1.9, df = 3, *P* = 0.59; Fig. 1.3b). The LT_{50} for females varied from 35.2 days for treated females coupled untreated males up to 40.4 days for untreated females coupled with treated males (Fig. 1.3b).

Regarding the fecundity and fertility parameters, it is worthwhile to note that egg incubation time was significantly reduced when only females were treated (H = 20.73,

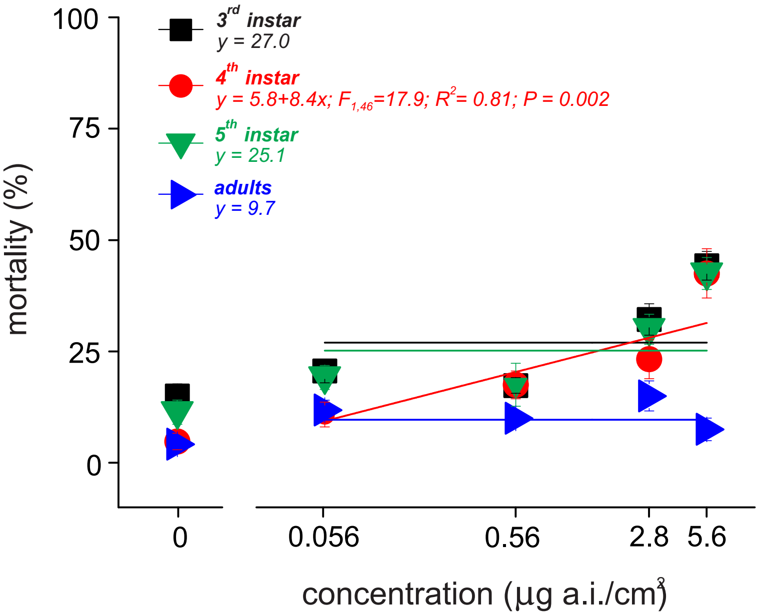

Figure 1.1: Toxicity of dried residues of chlorantraniliprole to 3rd, 4th, 5th instar nymphs, and adult of *E. heros*. Symbols show the averaged mortality (±SE) for each *E. heros* nymph (e.g., 3rd, 4th, 5th) and adults studied. The symbols represent the mean of 12 replicates, and the vertical bars represent the standard error of the average (SE)

df = 3, *P*<0.001; Fig. 1.4a). For both daily fecundity and fertility results (Fig. 1.4b,c), a three-parameter log-normal model (i.e., $Y = a \times \exp(-0.5 \times (\ln(X/c)/b)^2)$ was suitable because it showed a strong non-linear relationship with time, demonstrating a bell-shaped curve skewed to the left with a long tail to the right. In terms of daily fecundity, while treated females coupled with untreated males showed the highest peak of oviposition (Fig. 1.4b; Table 1.3.2), higher fecundity rates in untreated females coupled with treated males persisted for longer than all the other females (Fig. 1.4b; Table 1.3.2). The only significant difference observed in fertility rates was a significant (although small) delay in the emergence peak observed in treated females coupled with untreated males (Fig. 1.4c; Table 1.3.2).

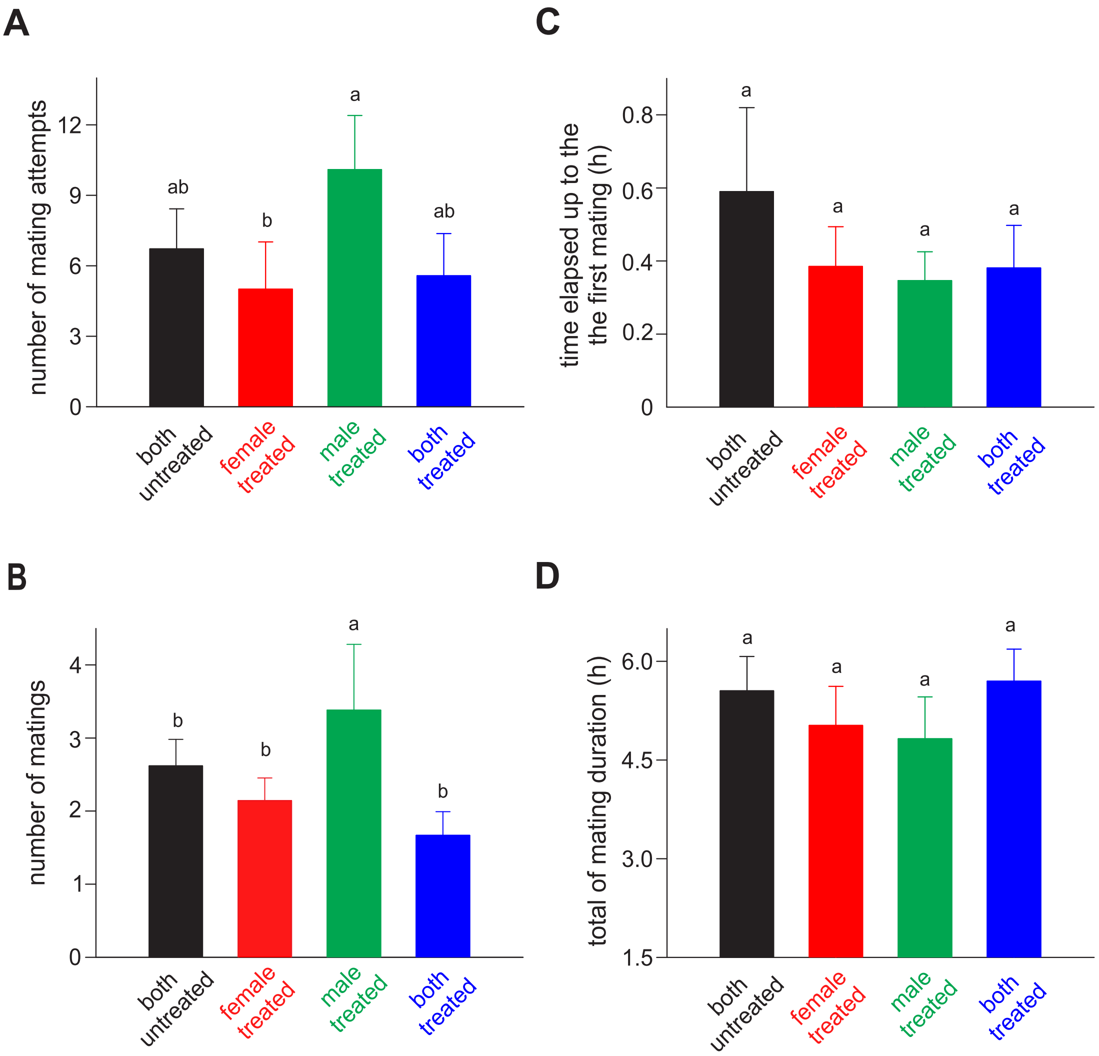

Figure 1.2: Mating behavior of *E. heros* after sublethal exposure to dried residues of chlorantraniliprole or distilled water. Bars with the same letter indicate that no significant differences were noted (Tukey's HSD test; *P*<0.05)

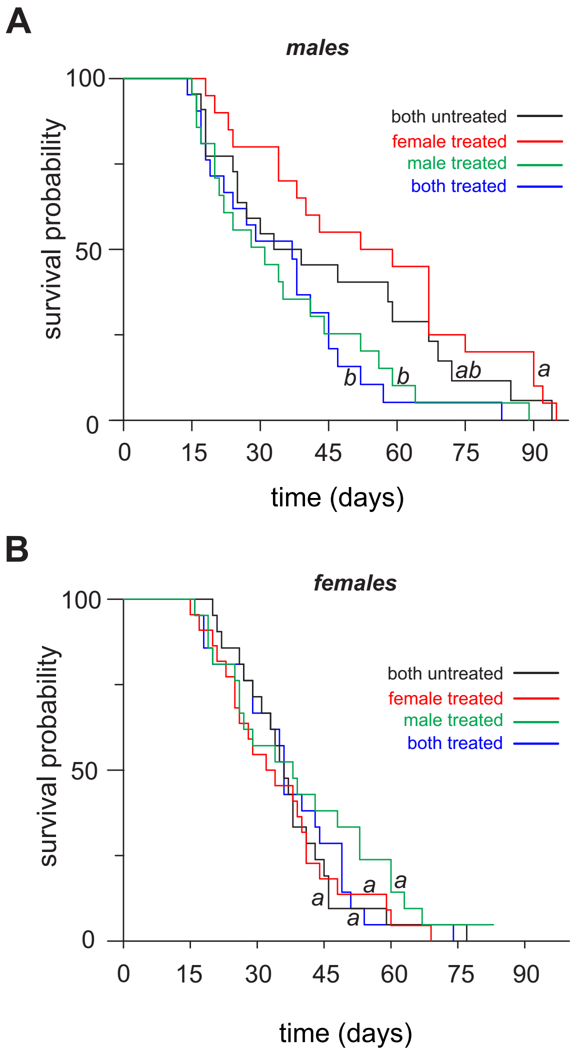

Figure 1.3: Survival curves of *E. heros* males (a) and females (b) after a 48-h exposure to dried residues of chlorantraniliprole or distilled water and coupled with insecticide-treated or insecticide-untreated partners. The survival curves with different letters are significantly different according to the Holm-Sidak test ($P<0.05$)

Table 1.2. Summary of the non-linear regression analyses of the curves shown in Fig. 4b, c

Parameters[a]	Treatment	Eggs/female/day			Daily emergence/female (%)		
		Value (95% C.L.)	*t*-value	*P*	Value (95% C.L.)	*t*-value	*P*
a	Both untreated	9.2 (8.3- 10.1) ab	20.8	<0.001	78.1 (68.5- 87.8) a	16.3	<0.001
	Female treated	10.1 (9.5- 10.9) a	29.3	<0.001	84.7 (75.1- 94.3) a	17.8	<0.001
	Male treated	9.0 (8.0 - 9.9) ab	19.1	<0.001	85.2 (78.8- 91.6) a	26.6	<0.001
	Both treated	8.3 (7.5- 9.0) b	21.6	<0.001	80.6 (74.4- 86.8) a	26.5	<0.001
b	Both untreated	19.8 (16.9- 22.6) a	13.79	<0.001	19.5 (13.6- 25.3) ab	6.7	<0.001
	Female treated	22.5 (21.3- 23.6) a	39.74	<0.001	27.5 (24.8- 30.3) a	20.0	<0.001
	Male treated	20.0 (15.9- 24.1) a	9.67	<0.001	22.7 (21.1- 24.3) b	28.1	<0.001
	Both treated	22.5 (20.6- 24.4) a	23.54	<0.001	21.9 (19.1- 24.8) ab	15.7	<0.001
c	Both untreated	0.61 (0.49- 0.72) ab	10.79	<0.001	0.79 (0.53- 1.05) a	6.0	<0.001
	Female treated	0.46 (0.41- 0.50) b	19.71	<0.001	0.61 (0.47- 0.74) a	9.0	<0.001
	Male treated	0.72 (0.56- 0.88) a	9.05	<0.001	0.53 (0.47- 0.60) a	16.5	<0.001
	Both treated	0.51 (0.44- 0.59) ab	13.55	<0.001	0.72 (0.53- 0.92) a	7.7	<0.001

Confidence limits of each parameter value are shown in parentheses

[a] Coefficients from a three model log-normal regression: $Y = a \text{ x } \exp(-0.5 \text{ x } (\ln(X/c)/b)^2)$. The three parameters characterize different attributes of the curves, where *a* is the maximum value of the dependent variable, *b* is the location of the peak response value on the time axis, and *c* is the skewness (or rate of change) of the response as a function of time. Parameter values followed by different letters in the columns were significantly different (based on non-overlapping of confidence limits)

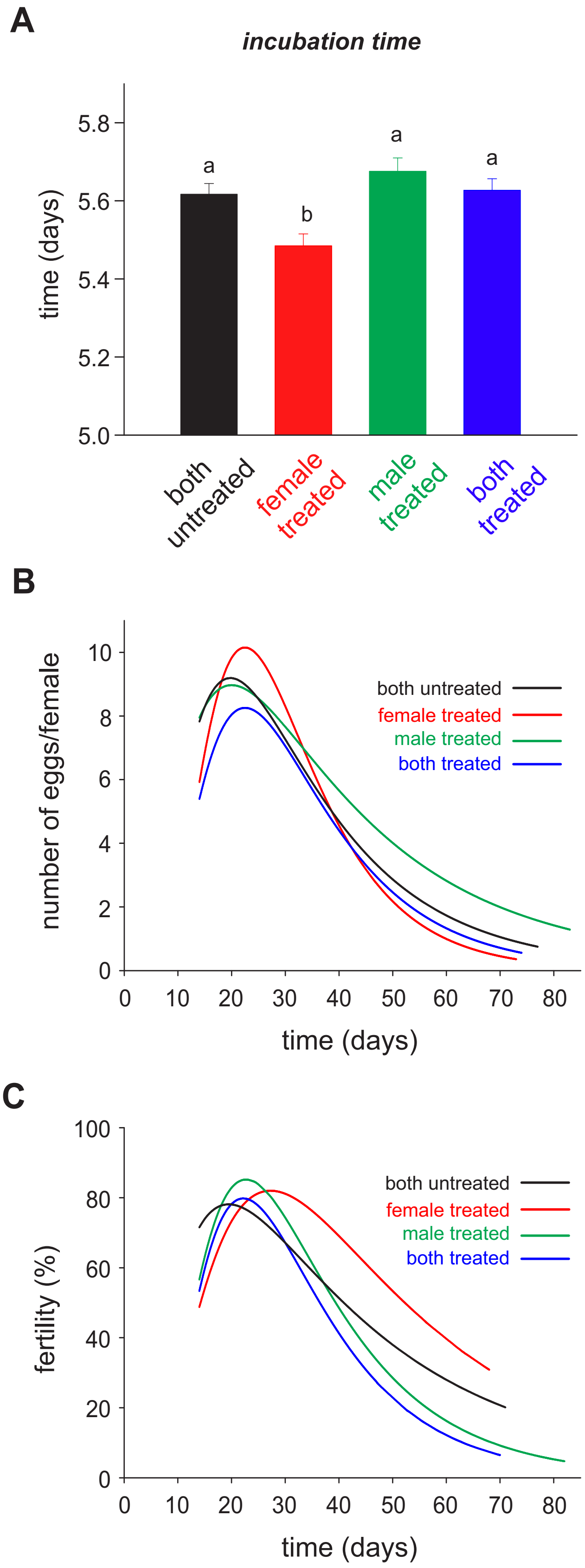

Figure 1.4: Egg incubation time (**a**), fecundity (**b**), and fertility (**c**) of *E. heros* females that were sublethally exposed to dried residues of chlorantraniliprole or to distilled water and coupled with insecticide treated or insecticide-untreated partners. **a** Bars with the same letter indicate that no significant differences were noted (Tukey's HSD test; $P<0.05$). **b**, **c** Lines represent the fit of daily fecundity (**b**) and fertility (**c**) results. The mean observed results for both fecundity and fertility rates are shown, respectively, in supplementary Figs. 1, 2

1.4 Discussion

In this study, we investigated lethal and sublethal effects of chlorantraniliprole on *E. heros*. Although chlorantraniliprole caused only very low mortality in nymphs (<45 %) and adults (<25 %) of *E. heros*, sublethal exposures of adult males and females were sufficient to alter their sexual fitness, which may result in a hormesis-like phenomenon (i.e., sublethal stimulation by a compound that is toxic at higher doses) (Cutler 2013; Guedes and Cutler 2014; Guedes et al. 2016).

The low chlorantraniliprole toxicity to *E. heros* was expected because this compound has been shown to have higher efficacy when ingested (Ioriatti et al. 2009), which, due to its limited systemic activity (Lahm et al. 2009; Reding and Ranger 2011), compromises its efficacy against stink bugs. Furthermore, the affinity of chlorantraniliprole for its target site (i.e., ryanodine receptors) appears to be significantly higher in lepidopteran insects than in other insect groups (Cordova et al. 2006; Lahm et al. 2009). However, the possibility that sublethal exposure to chlorantraniliprole would affect the reproductive success of *E. heros*, as it was recently demonstrated for the neonicotinoid insecticide imidacloprid (Santos et al. 2016), should not be neglected.

Differently that observed for mating duration of other insects of the gender *Euschistus* (Krupke et al. 2008), the *E. heros* never mated for periods longer than 8 h, indicating that mating behavior might be specie-specific with this gender or may reflect the climatic difference between the Neotropical region (where *E. heros* is most prevalent) and temperate regions where *E. conspersus* is normally found. In the present investigation, males sublethally exposed to chlorantraniliprole and coupled with untreated females exhibited an increased number of matings, which has been proposed as an indicator of male fitness gain (Arnqvist and Nilsson 2000; Harano 2015; Vahed 2007). Such gains in male fitness may result from improved male abilities to entice the females to multiple matings and therefore increase their number of opportunities to sire offspring (Arnqvist and Nilsson 2000; Droge-Young et al. 2015; Duffield et al. 2015; Gwynne 2007; Harano 2015; Vahed 2007). Although controversial, because multiple matings involves time and energy expenditures, these multiply mated females may experience an increase in fitness by benefiting from the receipt of male-contributed materials (Droge-Young et al. 2015; Harano 2015; Vahed 2007).

Our results did not suggest clear benefits for treated females coupled with untreated males. By delaying the peak of daily fertility for females coupled with untreated males, the sublethal exposure to chlorantraniliprole negatively affected the reproductive biology of *E. heros*. The negative impacts of sublethal chlorantraniliprole exposure would be expected to compromise fecundity/fertility rates, as reported in several groups of phytophagous insects (Gontijo et al. 2014, 2015; Guo et al. 2013; Han et al. 2012; Knight and Flexner 2007; Lanka et al. 2013; Moscardini et al. 2015; Smagghe et al. 2013; Teixeira et al. 2009). However, because the sublethal exposure to chlorantraniliprole did also positively alter the female reproductive output by shortening egg incubation time and increasing the fecundity peak, further investigation is required to elucidate the physiological mechanisms associated with the reproductive biology of chlorantraniliprole treated *E. heros* females. In a recent investigation, Santos et al. (2016) demonstrated that females of *E. heros* increased their reproductive output (e.g., fecundity and fertility rates) to overcome stresses (i.e., higher number of damaged ovarian cells and reduction in female survival) induced by sublethal exposure to the neonicotinoid insecticide imidacloprid. However, the potential reproductive fitness gains in this study were observed only when the male partner was sublethally exposed to chlorantraniliprole, and these gains disappeared when the both couple partners were sublethally exposed to the insecticide.

Although further investigation is required before conclusive statements can be made, our findings suggest that chlorantraniliprole may enhance the sexual fitness of *E. heros*, particularly when only males are exposed, and may potentially have contributed to the recent outbreaks of this stink bug species observed in Brazilian soybean fields. In these agroecosystems, the applications of chlorantraniliprole targeted at controlling lepidopteran pests occur prior to infestation by *E. heros* (Bueno et al. 2013; Moscardi et al. 2014; Panizzi et al. 2014), which would favor insecticide degradation prior the arrival of the stink bugs and most probably lead these insects to face sublethal exposure. Curiously, we detected enhanced fitness only when the male insects were exposed, which may seem unusual at first, but in truth is likely to take place considering the individual stink bug possibility of escaping insecticide exposure due to its within and between field dispersal behavior, and relatively broad host range (Borges et al. 2011; Smaniotto and Panizzi 2015).

The potentially different gender effect of chlorantraniliprole in stink bugs is also of interest since the morphophysiological action of chlorantraniliprole on male and female reproductive tracts may differ. Their locomotory (e.g., walking and flying) capacities may also differ requiring further studies to assess the potential links between the reproductive success (e.g., anticipation of sexual maturity, changes on acceptance/rejection of sexual partners) and dispersal activities (e.g., inside the crop fields or between crop fields/alternative hosts) of these insects. Further experiments aiming to evaluate the potential benefits of multiple mating in unexposed and sublethal exposed *E. heros* females adults still are needed.

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Competition between the phytophagous stink bugs *Euschistus heros* and *Piezodorus guildinii* in soybeans

Competition between the phytophagous stink bugs *Euschistus heros* and *Piezodorus guildinii* in soybeans[1]

Abstract

BACKGROUND: The abundance and contribution of the neotropical brown stink bug, *Euschistus heros* (F.), and the redbanded stink bug, *Piezodorus guildinii* (West.), to the composition of insect pests of soybean, Glycine max (L.), fields have changed both spatially and temporally in neotropical soybean production areas. Therefore,we assessed the competitiveness of each species in direct competition experiments following an additive series.We performed mixed (adult) insect infestations in soybean plants and evaluated the fitness of each species and the soybean yield.

RESULTS: While the competitive ability of *E. heros* was significantly compromised by increments in conspecifics and heterospecifics (i.e. *P. guildinii*), the competitive ability of *P. guildinii* was compromised by the presence of heterospecifics (i.e. *E. heros*). The reproductive output of *P. guildinii* remained unaffected by increments in E.heros or of *P. guildinii*. Intriguingly, despite the fact that *P. guildinii* apparently lost the competition with *E. heros*, almost no pod production was observed in any plant colonised by the former.

CONCLUSIONS: The higher abundance of *E. heros* in neotropical soybean fields seems to result from higher competitive ability than its heterospecific competitor *P. guildinii*, which may prevent the higher losses caused by *P. guildinii*.

Key words: phytophagous pentatomids; population growth; interspecific competition; intraspecific competition

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2.1 Introduction

Intra- and interspecific competition dynamics aremajor ecological processes that shape the patterns of insect abundance, distribution and diversity.[1–3] However, despite the strong evidence that herbivore competition is ubiquitous, the vast majority of investigations focus on leaf-chewing or grain-feeding insects,[4–9] leaving the competitive interactions between vascular-feeding – and especially, seed-sucking insects (e.g. pentatomids) – as a largely unexplored subject.

Several species of these phytophagous pentatomids overlap in both niche and range. [10–14] In commercial fields of soybeans, *Glycine max* (L.), the competition among herbivorous pentatomids will not only unbalance the stink bug assemblages but also determine the integrated pest management actions adopted to reduce economic losses by these key pest species. For instance, the annual impact of the stink bug complex on Brazilian soybean yield and quality is a combination of economic losses from reduced seed quality, direct yield losses and chemical control costs.[15,16] In neotropical savannah-like areas, which are prevalent in mid-South America, the current pentatomid complex attacking soybean encompasses three main species: the neotropical brown stink bug *Euschistus heros* (F.), the southern green stink bug *Nezara viridula* (L.) and the redbanded stink bug *Piezodorus guildinii* (West.).[10,12,17] However, the abundance and contribution of each of these stink bug species to the soybean yield and quality have changed spatially and temporally in these neotropical soybean production areas.

While *E. heros* is now more widespread and occurs in greater numbers than *P. guildinii* and *N. viridula*, it rarely occurred in the region until the 1970s.[12,18,19] Although *P. guildinii* currently exhibits lower abundance and distribution, it usually causes more seed injury per insect than *E. heros* and N. viridula,[20] and has been responsible for the green bean syndrome observed in Brazilian soybeans.[21] Interestingly, *P. guildinii* was of secondary importance in the US soybean producing areas for many years, but it has recently become a major pest of soybean in both Louisiana and Texas.[22–25]

The number of recorded host plants used by *E. heros* in neotropical regions is lower than the number recorded for *P. guildinii* or *N. viridula*,[11,12] which mainly restricts the populations of the former to soybean fields. Other factors such as the widespread adoption of no-tillage cultivation systems, the introduction of multiple cropping and higher insecticide tolerance have certainly contributed to the expansion of *E. heros* in Brazilian territory and into adjacent countries such as Argentina.[11,12,15,26,27] In the present study, we investigated the role of competition as a contributing factor for the increased incidence and importance of *E. heros* in neotropical soybean fields over time.We expected that the current prevalence of *E. heros* over other stink bug species would be due to its higher competitiveness.

2.2 Materials e Methods

2.2.1 *Insects*

A colony of *E. heros* was established, starting from insects maintained at Embrapa Genetic Resources and Biotechnology (Brasília, DF, Brazil). The colony of *P. guildinii* was established from nymphs and adults collected in soybean fields near Embrapa Rice and Beans (Santo Antônio de Goiás, GO, Brazil – geographic coordinates 16° 29´ 13.15´´ S, 49° 17´ 54.55´´ W). The colonies were reared under controlled conditions (27 ±2 °C, 60±20% relative humidity, with an L:D photoperiod of 14:10 h). All the developmental stages of the stink bugs were reared following methods previously described elsewhere.[28–30] Field-collected individuals from soybean farms in the regions of Tangará da Serra (State of Mato Grosso, Brazil) and from the experimental soybean fields at the Federal University of Viçosa (Viçosa, State of Minas Gerais, Brazil) and at Embrapa Rice and Beans (Santo Antônio de Goiás, GO, Brazil) were routinely introduced into the laboratory colonies to increase the genetic variability of insects used in the experiments.

2.2.2 *Cultivation conditions and experimental plots*

The indeterminate soybean variety NA 8015 RR (Nidera Sementes, São Paulo, Brazil), maturity group 8.0, was sowed under no-tillage at the experimental farm of Embrapa Rice and Beans (16^o 29´ 30.8´´ S, 49^o 17´ 41.7´´ W) on 12 December 2014. Agronomic practices used were those recommended for soybean production in Brazilian savannah ('Cerrado'). Seeds were inoculated with *Bradyrhizobium elkanii* (6 mL kg^{-1} seeds) (Nitragin®; Novozymes, São Paulo, SP, Brazil) and sowed at a rate of 16 seeds m^{-1} with a 0.45m row spacing (∼355 000 plants ha^{-1}). When soybeans reached the R3 stage, field cages (1.0 × 1.0 × 1.0m) were placed over one row of soybean plants. Plants inside the cages were manually weeded, leaving only four soybean plants per cage. To prevent any damage caused by other insects and disease, 4 days before the stink bug infestation the plants inside the cages were sprayed with beta-cyfluthrin (125 g AI L^{-1}; Bayer CropScience Ltda, São Paulo, Brazil) at a rate of 0.25 g AI ha^{-1} and a fungicide mixture of azoxystrobin and cyproconazole at rates of 60 and 24 g AI L^{-1} respectively (azoxystrobin 200 g AI L^{-1}, cyproconazole 80 g AI L^{-1}; Syngenta Proteção de Cultivos Ltda., São Paulo, Brazil).

2.2.3 *Competition experiments*

The assays were implemented following an additive series expanded to a bivariate factorial design replicated in four soybean fields, each one containing each treatment. The experiment was carried out in a single season because the varying densities of both insect species encompass the species composition and outcome to be expected in different years. Prior to infestation, the plants were inspected to ascertain the absence of insects. Sixty-seven days after planting, sexually mature adults (16–18 days after emergence) of *E. heros* and *P. guildinii* were confined inside cages in a sex ratio of 1:1 (male : female). Although insect mortality was never higher than five insects per experimental unit, 3 days after the infestation the cages were checked and dead insects were replaced to maintain the initial population. The control treatment consisted of cages without insects.

Competition experiments were designed for *E. heros* and its heterospecific competitor *P. guildinii* using an additive series, as suggested by Snaydon.[31] Mixed infestations were established on four soybean plants; the initial number of insects of one species was fixed at 10, whereas the other species had an increasing number of insects varying from 0 to 10. Therefore, each species with a variable number of insects started the competition at initial proportions of 0, 0.17, 0.29, 0.38 and 0.50 against the second species, respectively, which exhibited a fixed total number of insects. The insects remained under competition with evaluations at 15, 30 and 45 days after initial infestation (DAI). At each evaluation, we recorded the total number of insects (nymphs and adults) of each species per experimental unit (i.e. a cage with four plants). The instantaneous rate of population increase (r_i) for each species in each experimental unit was calculated at 45 DAI using the formula $r_i = [\ln(N_f/N_i)]/\Delta T$, where N_f and N_i are the final and initial number of live insects, respectively, and ΔT is the duration of the experiment in days.[32,33] At maturity, all four plants from each cage were harvested, and yield parameters such as number of empty pods (no observable seeds), 100 seeds weight, total seed yield and number of seeds per pod were assessed.

2.2.4 *Statistical analysis*

The number of live insects per experimental unit was subjected to repeated-measures (multivariate) analysis of variance because determination of insect numbers per plant was carried out on the same plots at each evaluation date,[34,35] thereby avoiding the problem of pseudoreplication in time.[35–37] This analysis was carried out using the PROC MANOVA procedure with the PROFILE statement, as suggested by von Ende.[38] When necessary, post hoc Tukey's HSD tests (α=0.05) were performed to compare treatment means. The instantaneous rate of increase in each species at 45 DAI was subjected to analyses of covariance with the species presence as the independent variable and the proportional increase in density as a covariate (PROC GLM procedure). Complementary regression analyses were performed when necessary (PROC REG procedure). The assumptions of normality and homogeneity of variance were checked, and no data transformation was necessary (UNIVARIATE procedure). Comparisons of soybean yields among the treatments were made by the PROC ANOVA procedure, and post hoc Tukey's HSD tests (α=0.05) were performed to compare treatment means. All statistical procedures above were performed using SAS/STAT software for Windows (SAS Institute, Cary, NC).[39]

2.3 Results

2.3.1 *Population increase through time*

Repeated-measures ANOVA for the number of live *E. heros* indicated significant effects of the interaction of the presence and increasing proportion of heterospecifics (P <0.05) (Table 2.1). Significant effects were also found for evaluation day (time) and for the interactions between time and the initial proportion of the species that had its initial proportion changed (P <0.05) (Table 2.1). As shown in Fig. 2.1A, 15 days after the infestation, the number of live *E. heros* was unaffected by increments in *P. guildinii*, but in the other two assessments (i.e. 30 and 45 DAI) the quantities of live *E. heros* were significantly reduced when the competition began with higher proportions of *P. guildinii*. Increments in the initial proportion of *E. heros* caused significant increments in the number of live *E. heros* at the beginning of the competition period (i.e. 15 days after infestation) but not in the other evaluations (Fig. 2.1B).

Repeated-measures ANOVA for the number of live *P. guildinii*, however, found no significant effects from the species that had their initial proportion changed or the initial proportion of each species or from their interactions (P >0.05) (Table 2.1). The abundance of *P. guildinii* was significantly affected by time and its interaction with the species that had their initial proportion changed (P <0.05) (Table 2.1). For the abundance of *P. guildinii*, while the number of live *P. guildinii* was significantly reduced over time when *E. heros* was the species with a variable number of insects (Fig. 2.2A), variations in the initial proportion of *P. guildinii* did not affect conspecific abundance over the study time (Fig. 2.2B).

2.3.2 *Competition and the instantaneous rate of population increase* (ri)

The models of the analyses of covariance for the total number of live insects of each species after 45 days of competition were significant (Table 2.1). However, while the growth rate of *E. heros* was significantly affected by increasing the level of competition (i.e. increasing the initial number of insects)with their conspecifics or with *P. guildinii* (Fig. 2.3A), the growth rate of *P. guildinii* was unaffected by increasing the initial proportion of conspecifics or of *E. heros* (Fig. 2.3B).

Table 2.1: Repeated-measures ANOVA for the total number of live insects from competition experiments between *E. heros* and *P. guildinii*[a]

Sources of variation		*E. heros*			*P. guildinii*		
	df	F		*P*	F	*P*	
Between samples							
Specie (S)	1	29.61		<0.0001*	0.18	0.67	
Initial proportion (P)	4	0.79		0.54	1.15	0.35	
S X P	4	4.71		0.0046*	0.73	0.57	
Error	30	-		-	-	-	
Sources of variation	df_{den}/den_{num}	Wilk's lambda	F	*P*	Wilk's lambda	F	*P*
Within samples							
Time (T)	2/29	0.6104	9.26	0.0008*	0.6000	9.67	0.0006*
T X S	2/29	0.8484	2.59	0.09	0.7624	4.52	0.0196*
T X P	8/58	0.3489	5.02	<0.0001*	0.7839	0.94	0.49
T X S X P	8/58	0.6243	1.93	0.07	0.8221	0.75	0.65

[a] * Significant at *P* <0.05.

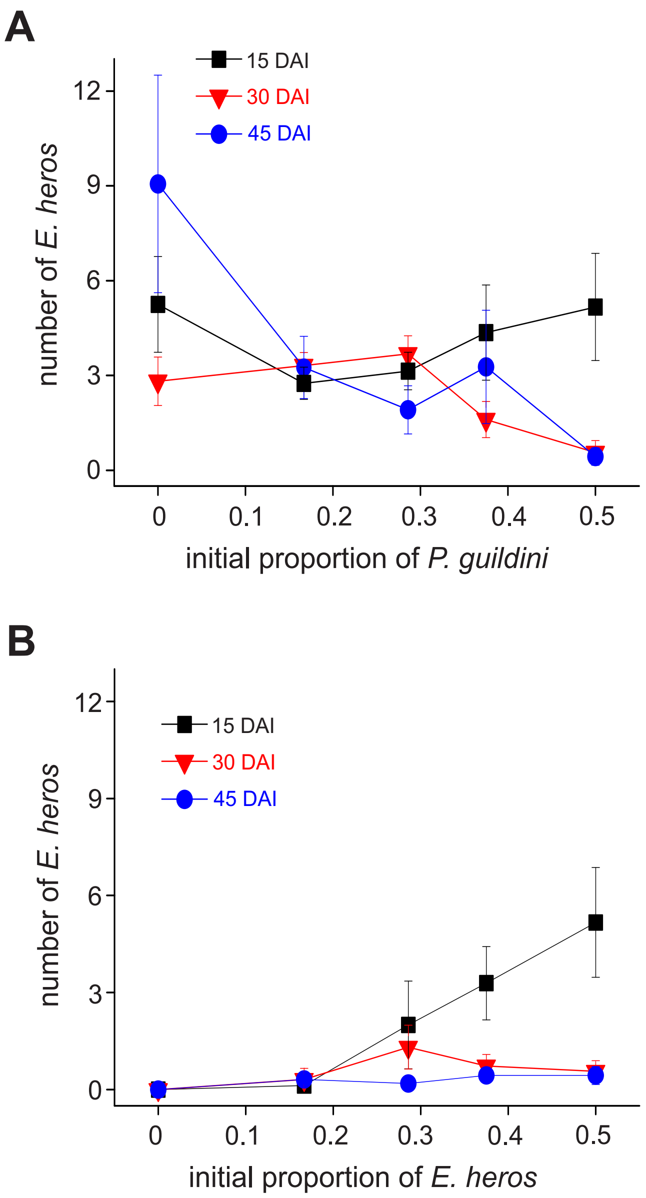

Figure 2.1: Total number of living *E. heros* obtained through 45 days of competition between *E. heros* and *P. guildinii*. (A) Although not affected at the beginning of the competition (i.e. 15 DAI), E. heros abundance was significantly reduced in the other two assessments (i.e. 30 and 45 DAI) in the experimental units that had higher initial proportions of *P. guildinii*. (B) Increasing the initial proportion of *E. heros* caused significant increments in the number of *E. heros* at the beginning of the competition period (i.e. 15 DAI) but not in the other evaluations. The box plots indicate the range of data (lower and upper quartiles and extreme values) and the median values.

2.3.3 *Yield parameters*

The production of empty pods was significantly ($F_{9,39}$ =65.9, P <0.001) increased by the presence of the stink bugs (Fig. 2.4). For the treatment where *E. heros* was the only stink bug present, the percentage of empty pods was significantly lower than in all the treatments containing *P. guildinii*, but it was higher than in the treatment

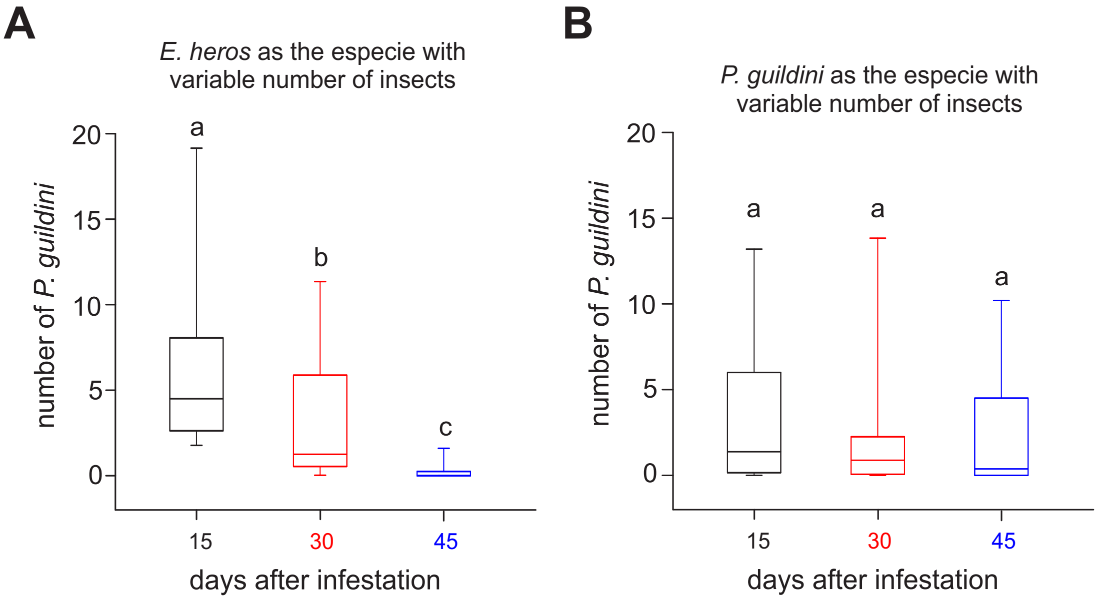

Figure 2.2: Total number of living *P. guildinii* obtained through 45 days of competition between *E. heros* and *P. guildinii*. The abundance of *P. guildinii* was significantly reduced over time when *E. heros* (A) was the species with a variable number of insects, but not when *P. guildinii* (B) was the species with a variable number of insects at the beginning of the competition period. The box plots indicate the range of data (lower and upper quartiles and extreme values) and the median values.

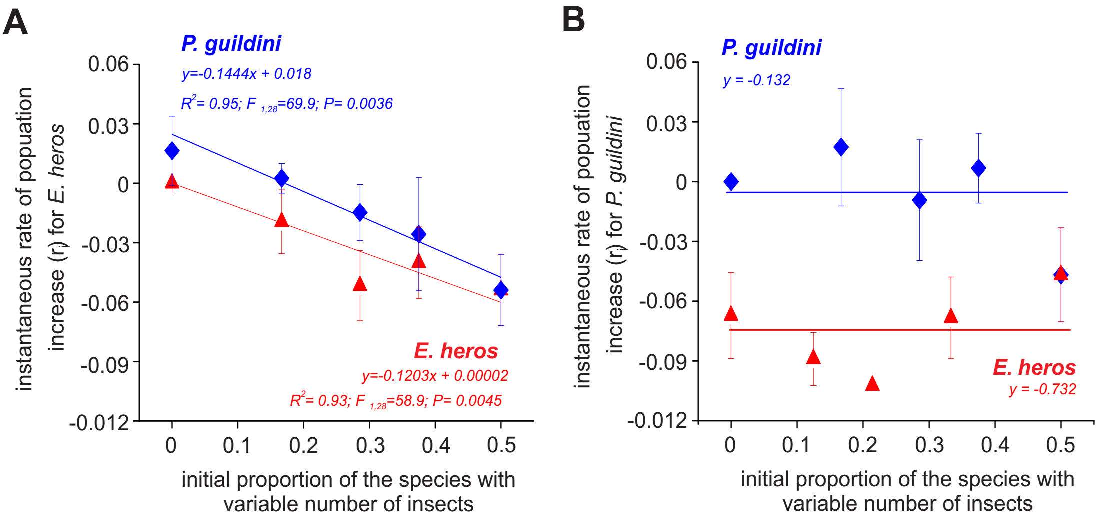

Figure 2.3: Instantaneous rate of population increase (r_i) of *E. heros* (A) and *P. guildinii* (B) obtained through 45 days of competition between *E. heros* and *P. guildinii*. The curves refer to the species with a fixed number of insects and the species with a variable number of insects, as indicated on each curve. The symbols represent the mean of four replicates and the vertical bars represent the standard error.

Table 2.2: Analyses of covariance for the total number of live insects after 45 days (one generation) of competition between *E. heros* and *P. guildinii*[a]

Sources of variation	*df*	*E. heros*		*P. guildinii*	
		F	*P*	F	*P*
Model	3	7.00	0.0008*	9.80	<0.0001*
Error	36	-	-	-	-
Species with increasing density	1	1.87	0.18	17.17	0.0002*
Initial proportion	1	17.62	0.0002*	0.28	0.60
Interaction	1	0.23	0.63	2.56	0.12

[a] *Significant difference at P<0.05

without stink bug infestation (Fig. 2.4A). The presence of stink bugs also significantly ($F_{9,39}$ =19.6, P <0.001) reduced the production of seeds (Fig. 2.4B). While *E. heros* alone significantly reduced the seed production, the production of marketable seeds was completely absent (except in one experimental unit) in all the treatments containing *P. guildinii* (Fig. 2.4B). For the treatments that produced marketable seeds (i.e. treatments with no stink bug infestations and those infested only by *E. heros*), we observed that the presence of stink bugs did not affect the 100 seeds weight ($F_{1,7}$ =0.75, P =0.49) but significantly reduced ($F_{1,7}$ =6.29, P =0.046) the number of seeds per pod (no stink bug infestation: 2.5±0.19 seeds pod^{-1}; infested only by *E. heros*: 1.9±0.15 seeds pod^{-1}).

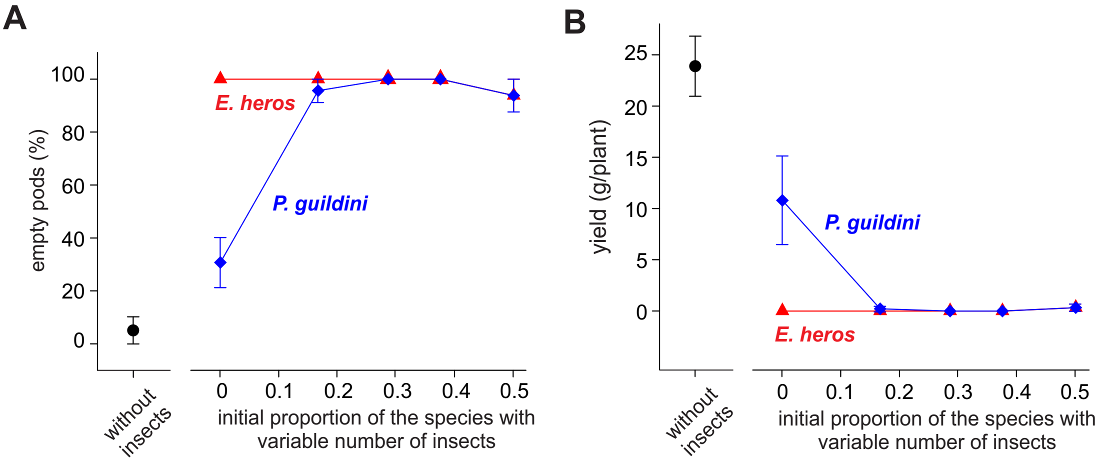

Figure 2.4: Evaluation of the number of empty pods (A) and productivity (B) of soybean plants in the presence and absence of stink bug infestations. The yield parameters of non-infested plants are indicated in black symbols. Linked symbols represent the yield of infested plants that had *E. heros* (red) or *P. guildinii* (blue) as the species with a fixed number of insects at the beginning of the competition period.

2.4 Discussion

Here, we assessed for the first time the competitiveness of two co-occurring stink bug pests (*P. guildinii* and *E. heros*) of neotropical soybean fields cultivated under Brazilian savannah conditions. The study was performed in four soybean fields, allowing spatial extrapolation, and in a single crop season. The varying densities simultaneously used of both species allow for their likely field variation in different seasons and years without the need to replicate such complex design through time, unless the underlying mechanisms determining the competition outcome are the target of attention, which was not the study objective.

The coexistence of such phytophagous stink bugs in neotropical soybean fields has been reported since the 1970s.[10,19] However, while *E. heros* was of rare occurrence in this region at that time,[18] it overtook the economic importance of *P. guildinii* and of the southern green stink bug *N. viridula*, which previously had higher abundance and wider territorial distribution,[12,15,40–42] becoming the most abundant stink bug pest of soybeans in Brazil.[12,15]

Although the mechanisms determining the competitive outcome among phytophagous stink bug species in soybean fields have not been investigated, we had the expectation that *E. heros* would present higher competitiveness in neotropical soybean fields than their heterospecific stink bug competitors. Such suspicion derived from the fact that the number of recorded host plants used by *E. heros* in the region is smaller than that recorded for *P. guildinii* and *N. viridula*.[11,12,17] Our findings confirmed such expectations and demonstrated that the actual prevalence of *E. heros* over other stink bug species is at least partially due to its higher competitiveness.

However, other factors such as favoured biology (i.e. higher number of generations per year), the widespread adoption of no-tillage cultivation and the introduction of multiple cropping have certainly contributed to the expansion of *E. heros* in Brazilian territory and into Argentina.[12,15,27] The climate conditions in the Brazilian savannah (mid-west Brazil), where soybean production has increased considerably since the 1970s, may also represent a major factor for *E. heros* expansion in the neotropical region.[11,15,43] Furthermore, despite the limited amount of information regarding the differential susceptibility to insecticides among these stink bug species in Brazil,[44–46]

Euschistus stink bugs seem to be more tolerant to insecticides than *P. guildinii* or *N. viridula*,[47,48] which might also have contributed to the prevalence of *E. heros*.

Interestingly, other investigations in southern Brazil[49,50] and recent surveys of stink bug species in some US states (e.g. Louisiana and Texas) have reported *P. guildinii* as the major pest of soybean fields,[22–25] prevailing inclusive over of *Euschistus* stink bugs. Such shifts in the composition and relative abundance of the stink bug complex in soybean fields have raised concerns about the current economic thresholds used for these insects,[26,41,51] pointing towards the adoption of multispecies action thresholds rather than single-species-based thresholds. In Brazil, the current economic threshold used for stink bugs is a multispecies threshold of two stink bugs bigger than 0.5 cm per m, which is regarded as a safe level regardless of the soybean cultivars.[26,52]

Such multispecies economic thresholds have to be accurately determined, and there is a need to evaluate the damage potential of the different stink bug species. For instance, several investigations have reported that the damage caused by *P. guildinii* in Brazilian soybean fields is higher than that cause by other phytophagous stink bugs.[20,21,53,54] The higher damage potential of *P. guildinii* has been attributed to their more deleterious salivary enzymes.[55]

Although the insect colonies used in our experiment were from different sources, which might lead to potential bias due to inadvertent selection on the insect competitive abilities while in the laboratory, this is unlikely to have taken place because even the laboratory population used was subjected to periodic introduction of field insects from representative soybean fields, maintaining proper genetic diversity in the population. This investigation did not attempt to elucidate the mechanisms determining the outcome of competition between *E. heros* and *P. guildinii*, but it aimed to assess their competitiveness, coexistence and potential dominance. Further investigations aiming to evaluate the simultaneous contribution of multiple factors such as susceptibility to pesticides and to naturally occurring biological control agents (e.g. parasitoids of stink bug eggs) are needed. Additionally, our results indicate that, although *P. guildinii* is a weaker competitor than *E. heros*, it exhibits higher damage severity that completely suppresses marketable seed production whenever it is present. Thus, future research also needs to evaluate the relative impact of different stink bugs relative to damage potential, and to determine whether the current multispecies economic threshold really reflects the needs of the neotropical soybean producing region.

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Quantifying *Lygus lineolaris* stylet probing behavior in relation to its damage to cotton leaf terminals

Quantifying *Lygus lineolaris* stylet probing behavior in relation to its damage to cotton leaf terminals[1]

Abstract

Electropenetrography (EPG) is the most accurate way to study the feeding behavior of piercing-sucking insects. In this study, EPG was used to study *Lygus lineolaris* stylet probing behavior on cotton leaf terminals. We hypothesized that: (1) damage resulting from *L. lineolaris* feeding is related to the characteristics of stylet probing behavior and (2) these characteristics and related damage are different among insect life stages. Stylet probing behavior was quantified for 3^{rd}, 4^{th}, and 5^{th} instars plus adult males. Insects were wired, allowed to feed for 16 hours on cotton leaf terminals with simultaneous data acquisition by EPG. EPG waveforms were quantified for non-probing (NP) and probing behaviors (cell rupturing [CR], transition [T] and ingestion [I]). Insect foliar damage was measured with image analysis software from pictures that were taken at regular intervals up to seven days after insect feeding. Overall, *L. lineolaris* spent most of their time in non-probing (NP) behavior, with longest duration by adults. For probing behavior waveforms, the longest duration was CR, especially for 4^{th} instars followed by the remaining immature insects. Interestingly, most of the adults did not perform any T waveform during probing. Additionally, a strong correlation between the amount of damage and total time spent in CR, which represent combined stylet movements and salivation during insect probing, is the main cause of damage on cotton axillary leaves. Despite the finding that the numerical amount of foliar damage increased greatly over time, there were no significant relationships between life stages and amount of damage. Thus, our first hypothesis was supported, but our second hypothesis was not. That said, even small amounts of cell rupture feeding by *L. lineolaris* is damaging to cotton leaf terminals. These results help to understand the cause of damage to *L. lineolaris* hosts, and consequently aid in developing strategies to reduce crop loss.

Key words: Electropenetrography, feeding behavior, cell rupturing feeding, stylet penetration

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3.1 Introduction

Tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois), is an important pest of cotton in the mid-southern United States, causing severe damage to plants. *L. lineolaris* is polyphagous and is known to have more than 300 plant species as host plants (Young 2014). This insect is known to prefer reproductive plant organs over vegetative structures (Cooper and Spurgeon 2013), and their feeding can result in square abscission, deformed fruits, site necrosis, and reduced plant growth (Strong 2014). However, *L. lineolaris* also are reported to feed on vegetative structures and plant terminals, causing terminal abortion and deformed leaves, reducing plant growth and cause excessive branching (Hanny et al. 2014).

All *L. lineolaris* life stages cause damage to plants, especially 5^{th} instars and adults, and the degree of damage varies among *Lygus* spp. instars. However, results about more severe life stages to cotton plants are controversial (Cooper and Spurgeon 2013; Gutierrez et al. 1977; Jubb and Carruth 2014; Rosenheim et al. 2006; Zink and Rosenheim 2005). Otherwise, most of the work comparing damage among life stages has been done with the congeneric pest species, *Lygus hesperus* (Knight), also a pest of cotton. Insect preference for a specific plant structure also varies depending on life stage. Younger instars prefer flower buds and later instars and adults prefer plant terminals (Cooper and Spurgeon 2013).

Damage caused by *L. lineolaris* to its host is caused by its cell rupturing feeding strategy. *L. lineolaris* is a piercing-sucking insect that inserts its mouthparts, the stylets, inside plant tissue. Stylet movements in plant cells cause mechanical damage simultaneously with injection of highly enzymatic saliva that causes cell maceration (Backus et al. 2005). The liquefied cell contents are then ingested through the stylets (Backus et al. 2005). Consequently, the area where the insect fed develops tissue damage whose specific symptoms are related to the type of structure on which the insect fed. Cotton reproductive structures seem to be more important, and feeding there result in higher direct damage. However, vegetative organs, especially leaf terminals, also are sites of lygus-related damage that can lead to great reduction in plant development or compromise of plant morphology (Hanny et al. 2014).

The most rigorous way to study stylet probing behavior of *L. lineolaris*, a piercing-sucking insect, is by electropenetrography (EPG) (Backus and Bennett 2009; Van Helden and Tjallingii 2000). By applying this technology, stylet probing behaviors of different *L. lineolaris* life stages were compared and quantitatively characterized, and the damage caused by each stage's feeding was correlated with the amount and time course of *L. lineolaris* damage at cotton leaf terminals.

3.2 Materials and Methods

3.2.1 *Insects and plants*

L. lineolaris egg packs were obtained from a rearing facility at the United States Department of Agriculture, Agriculture Research Service (USDA-ARS) facility in Stoneville, MS. Egg packs were placed on shredded paper inside semitransparent plastic cages (33 x 20 x 11 cm) for rearing. After 2 days, fresh, organic green beans pods were transferred to each rearing cage as food. Green bean pods were added and replaced every other day until nymphs became adults. Adults were transferred to a 30 cm cubic and collapsible plastic cage ("BugDorm1", BioQuip, Rancho Dominguez, CA) containing shredded paper and were fed raw sunflower seeds and organic green bean pods. Every other day, green bean pods were replaced; old pods with eggs were transferred to new nymph cages and, after eggs hatched, nymphs were fed until they reached the adult stage. To each immature and adult cage it was added one 2.5 cm cotton round inside a plastic Petri dish that was kept wet by addition of water. Insects were kept in a growth room at 27 ºC, 60 % RH and 16:8 h (L:D) photoperiod. For EPG recordings, insects were from 3^{rd} to 5^{th} instar nymphal stages and pre-reproductive adult males. Only newly eclosed (<24-h) insects of each stage were studied. To standardize insect age, one day before EPG recordings, insects from each life stage preceding the experi-mental stages (i.e, 2nd, 3^{rd}, 4^{th} and 5^{th} instars) were separated by instar and transferred to transparent vials containing one piece of green bean pod as food, and only the in-sects that had molted to the next stage one day later were used for experiments.

Cotton seeds cv. 'Coker' were obtained from Monsanto (St. Louis, MO). Plants were grown in a greenhouse using the commercial substrate Sun Gro Horticulture Professional Growing Mix (Sun Gro Horticulture, Agawam, MA) and fertilized with Jack's

LX 15:5:15 Ca-Mg (JR Peters INC, Allentown, PA). Plants were grown under photoperiod 16:8 (L:D) with a temperature ranging between 18 and 30 ^oC. Cotton plants were used for experiments when six to nine complete true leaves were present.

3.2.2 *Experimental design*

Four insects were simultaneously EPG-recorded on terminal axillary cotton leaves, one insect per plant, in a randomized complete block, each day being one block. Twenty insects per stage, for a total of 80 insects, were recorded. Channels were set at a plant voltage of 50 mV AC and an Ri level of 10^8 Ω for 3^{rd} and 4^{th} instars, and at 25 mV AC with Ri levels of 10^7 Ω for 5^{th} instars and adults. Undisturbed, continuous waveforms were recorded for a 16 h access period per block.

For *Lygus* damage quantification, we randomly sampled certain replicates used for EPG recordings. Four plants per replicate (block) were used simultaneously for EPG recordings, with one wired insect from each insect stage, i.e., 3^{rd}, 4^{th}, 5^{th} instar and adult male, fed during all 16 hours access period on each terminal axillary leaf; one leaf per insect. A fifth plant was kept clean without insect, as a control plant. Twelve plants per life stage plus controls were photographed, for a total of 60 plants. *Lygus* damage was identified and foliar area and insect damage was digitally measured. A timecourse of *Lygus* damage was followed whereupon each of the 60 plants was repeatedly photographed at days 1, 2, 3, 4 and 7 days after beginning of EPG recordings.

3.2.3 *Insect wiring and EPG instrument*

Insects were removed from their tube vials and anesthetized with CO_2 for 60 s then immobilized by low suction under a stereomicroscope (Leica MZ12.5, Leica Microsystems Ltd., Heerbrugg, Switzerland) during wiring. A 38.1 μm (in diameter; sold as 0.0015 in., Sigmund Cohn Corporation, Mt. Vernon, NY) gold wire (1-2 cm in length) with a small loop at its tip was sunk into a drop of conductive silver glue (water: silver flakes [Sigma-Aldrich, Saint Louis, MO, USA]: white glue [Elmer´s GlueAll, Westerville, OH, USA]) (1:1:1; v:w:w) dropped onto the insect pronotum. The insects were kept dangling before they were placed on the cotton terminal axillary leaf and connected to the EPG monitor, reaching a total starvation time of 3 h. To record the non-probing and the stylet probing behaviors of *L. lineolaris*, a four-channel AC-DC EPG monitor (Backus and Bennett 2009; EPG Equipment Co., Otterville, MO) was used inside a Faraday cage. Cotton plants were placed in a plastic pot tray inside the Faraday cage and each terminal (axillary) leaf was laid down on a Plexiglas stage (15 x 7 cm). The nearest partially developed axillary leaf was held in place using strips of Parafilm (Pechiney Plastic Packaging, Menasha, WI) on the plant main stem to ensure the correct leaf position during EPG recordings. The Plexiglas stage was held horizontally by an alligator clip connected to a "helping hand" holder (van Sickle Electronics, St. Louis, MO). EPG data acquisition were made by WinDaq Pro^{+} software (DATAQ Instruments, Akron, OH) at a sample rate of 100 Hz per channel, and the signal was digitized using a WinDaq DI-720 analog-to-digital (A-D) board. The monitor gain for all the recordings was 6,000X and WinDaq gain ranged according to specifications in figure captions (Fig. 3.1).

3.2.4 Lygus *damage quantification*

To quantify damage to cotton leaves resulting from stylet probing behaviors by different *L. lineolaris* life stages, each plant terminal was held on a plexiglass support with a non-glare glass on the leaf and under a circular LED light source. Pictures were taken of each terminal axial leaf using a standard digital camera (Sony Cyber-shot DSC-HX20V, Sony Corporation, Tokyo, Japan). The camera was positioned over the leaf held by upper surface of the LED light source apparatus, ensuring that pictures were taken at a fixed distance from the plexiglass stage. On the plexiglass stage, a ruler was affixed to calibrate the relationship between digital and real length (pixels to millimeters). Pictures were taken before and after EPG data acquisition, i.e, day 1 (before *Lygus* feeding), and 2, 3, 4, and 7 days afterwards. Pictures were transferred to a computer and edited with Adobe Photoshop CC version 2017 (Adobe Systems Incorporated, San Jose, CA), changing the color pattern to individual, false colors allowing easier application of the measuring tool in Adobe Photoshop. The color pattern had the following five colors and their meaning related to *Lygus* damage: green (undamaged area); red (early identifiable damage or beginning of the damage); blue (middle damage stage where the damage by feeding showed necrotic lesions); pink (damage by insect feeding that resulted in holes or losses at leaf area, and yellow (damage by insect feeding that resulted later in chlorotic areas near damaged areas). For the analysis, we considered the total damage to be the pooling together of red, blue, yellow and pink areas.

3.2.5 *Statistical analysis*

Quantification of **L. lineolaris** *waveforms*

Waveforms were recognized and named according to Cervantes et al. (2016). "Probing" or stylet penetration includes all behaviors occurring between stylet insertion into plant tissue until stylet withdrawal, and "Non-probing" represents all other behaviors that do not involve stylet penetration. Here, the three non-probing waveform families previously described by Cervantes et al. (2016) were pooled together for analysis. They were antennal tapping on plant surface (A), walking (W) and standing still (S).; all were coded together as Non-Probing (NP) events. Probing behavior comprised three waveform families, including cell rupturing (CR), transition (T), and ingestion (I) waveforms. Excerpts of waveforms are shown in Figure 3.1.

Waveform categories were based on stereotypical patterns while they were observed with WinDaq Waveform Browser software (Serrano et al. 2000). Waveform quantification was calculated using three non-sequential variables to describe the *L. lineolaris* feeding behavior, as shown in Table 3.1. The non-sequential variables analyzed were: waveform duration per insect (WDI), waveform duration per event per insect (WDEI), and number of waveform events per insects (NWEI). Descriptive and analytical statistics were performed using the Backus 2.0 program (Backus et al. 2007; Ebert et al. 2015) for Statistical Analysis Software (SAS Institute. SAS/STAT® 9.2 User's Guide 2008), providing mixed model analysis of variance (PROC GLIMMIX) and pairwise comparison by Least Significant Difference, with $\alpha = 0.05$. To achieve the assumptions of normality and homoscedasticity, data of WDI and WDEI were log-transformed and NWEI was square-root transformed prior analyses.

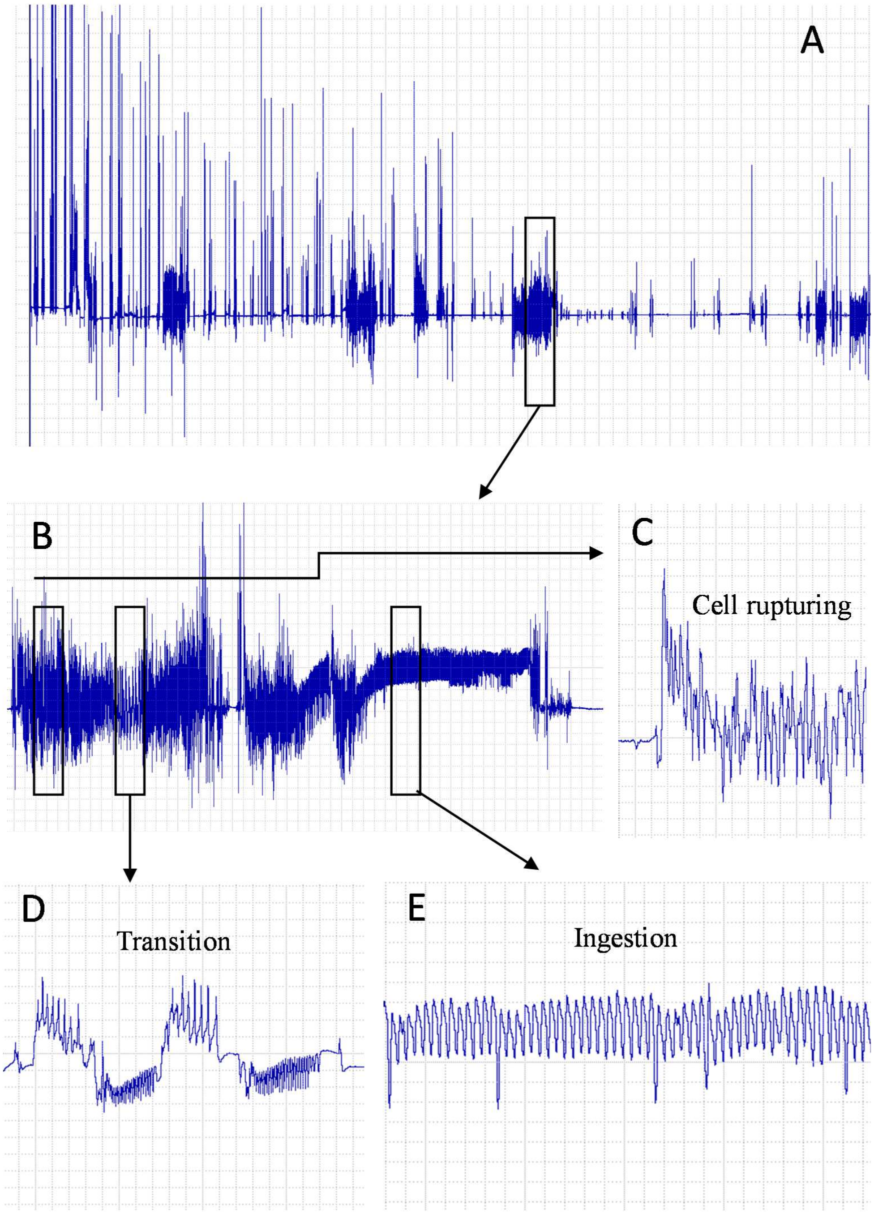

Figure 3.1: Overview of *Lygus lineolaris* waveforms recorded from insects of different life stages that fed on cotton terminals (cv. Coker) with AC applied signal. A) Complete, compressed view of 16 hours of EPG recording, B) Details of two consecutive probes (Windaq compression 100 [20 s/vertical div]), (C), (D) and (E) Detailed CR, T and I waveforms, respectively (Windaq compression 2 [0.4 s/vertical div]). Monitor gain was set at 6,000X and Windaq gain 128X for figures (C), (C) and (C). Boxed waveforms are enlarged in inset boxes.

L. lineolaris *damage on cotton leaves*

Measurements of foliar damage by *L. lineolaris* life stages were submitted to repeated measures (multivariate) analysis of variance because measurements were carried out on the same plants at each evaluation date (Green 1993; Paine 1996), thereby avoiding the problem of pseudoreplication in time (Hurlbert 1984; Paine 1996; Stewart-Oaten et al. 1986). This analysis was carried out using the PROC MANOVA procedure with the

PROFILE statement, as suggested by von Ende (1993). Data were transformed by log (x+1) to fit the assumptions of normality and homogeneity of variance (UNIVARIATE procedure). Comparisons of foliar damage among the treatments (life stages) were made by using PROC ANOVA procedure. In order to identify the probing behavior family that is the main cause of damage on cotton leaves, correlation analyses were performed between total time spent by each insect in each probing waveform (CR, T or I) and the amount of foliar damage caused on each plant using PROC CORR procedure. Measurements and correlations of foliar damage were done at day 1 (the day when insects were transferred to each plant), day 2 (just after the insects were taken off of leaf), and days 3, 4 and 7. These statistical procedures were performed using SAS/STAT of Statistical Analysis Software (SAS Institute. SAS/STAT® 9.2 User's Guide 2008)

3.3 Results

3.3.1 *Quantification of* L. lineolaris *waveforms*

Non-probing behavior.

L. lineolaris spent most of their time (93.6%) in non-probing behaviors, herein comprising standing still (S), walking (W) and antennation (A) behaviors. Waveform duration per insect (WDI) was highest for adults, lowest for 4^{th} and 5^{th} instars, with 3^{rd} instar being intermediate (Fig. 3.2a, Table 3.1). Adults spent 97.5% of total recording time in this behavior. Perhaps not surprisingly, the waveform duration per event per insect (WDEI) and the number of waveform events per insect (NWEI) were not significantly different among insect life stages (Table 3.1).

Probing behaviors.

The three probing waveforms families are described in order of their occurrence in a typical insect probe.

Family CR (Cell rupturing). Cell rupturing was performed for the longest overall duration (WDI) among waveform families. The immature stages spent numerically more time than adults performing this waveform family, with 3^{rd} and 4^{th} instars presenting the highest duration per insect (WDI) (Fig. 23.2b). The average duration of each event per insect (WDEI) differed only between 3^{rd} instar and the other stages; 3^{rd} instars performed longer events than others. No significant differences in WDEI were found among 4^{th}, 5^{th} instar and adults (Fig. 2 3.2c). Additionally, no significant differences in the average number of waveform events per insect (NWEI) were found among all insect life stages (Table 3.1). Therefore, the overall duration of CR was primarily due to increased per-event durations, not number of events.

Family T (Transition). Transition had the shortest event duration of any probing behaviors performed by *L. lineolaris*. No differences were found for any calculated variable among immature stages related to waveform duration per insect (WDI), waveform duration of each event per insect (WDEI) or number of waveform events per insect (NWEI) (Table 3.1). However, only one adult insect performed one event of T waveform, therefore, we were unable to do any calculation for this variable at this insect stage (Table 3.1).

Family I (Ingestion). This event had an intermediate duration among probing events. No differences were found for any calculated variable among all stages related to waveform duration of per insect (WDI), waveform duration of each event per insect (WDEI) or number of waveform events per insect (NWEI) (Table 3.1). However, overall duration (WDI) and number of events performed (NWEI) were numerically lowest for adults and highest for 5^{th} instars (Table 3.1).

Table 3.1: Calculated waveform duration per insect (WDI) (mean ± std error), duration of waveform events per insect (WDEI) (mean ± st. error), and number of waveform events per insect (NWEI) (mean ± std error). Data from recordings with AC voltage for 3^{rd}, 4^{th}, 5^{th} instars and adults of *L. lineolaris*. Durations are in seconds.

Waveform	Insect Stage	WDI	WDEI	NWEI
Non-probing (NP)	3^{rd} instar	53,551.4 ± 729.70 ab	986.19 ± 221.85	98.27 ± 20.08
	4^{th} instar	52,683.3 ± 1089.92 b	1032.75 ± 342.61	170.65 ± 39.71
	5^{th} instar	53,350.9 ± 1152.18 b	1712.82 ± 1101.16	192.17 ± 47.05
	Adult (males)	56,147.0 ± 261.59 a	1275.30 ± 341.68	94.00 ± 22.03
		$F_{(3,62)}=2.82$, $P=0.046$*	$F_{(3,62)}=0.98$, $P=0.408$	$F_{(3,62)}=1.68$, $P=0.179$
Cell rupturing (CR)	3^{rd} instar	2,317.4 ± 413.18 a	23.09 ± 3.32 a	114.53 ± 21.91
	4^{th} instar	2,837.2 ± 699.06 a	14.19 ± 1.93 b	180.82 ± 40.55
	5^{th} instar	2,246.5 ± 652.08 ab	13.13 ± 1.92 b	168.00 ± 48.19
	Adult (males)	652.2 ± 165.81 b	9.29 ± 1.52 b	78.25 ± 14.10
		$F_{(3,61)}=2.72$, $P=0.05$*	$F_{(3,61)}= 6.65$, $P=0.0006$*	$F_{(3,61)}= 0.60$, $P=0.199$
Transition (T)	3^{rd} instar	833.08 ± 265.27	35.27 ± 7.74	20.10 ± 4.63
	4^{th} instar	1182.91 ± 344.15	38.96 ± 7.83	30.58 ± 8.69
	5^{th} instar	832.78 ± 291.15	33.99 ± 4.14	22.55 ± 7.80
	Adult (males)	104.48 ± -	104.48 ± -	1.00 ± -
		$F_{(3,30)}=0.73$, $P=0.544$	$F_{(3,30)}=1.13$, $P=0.354$	$F_{(3,53)}=1.18$, $P=0.334$
Ingestion (I)	3^{rd} instar	1203.7 ± 160.37	362.60 ± 95.78	5.08 ± 1.02
	4^{th} instar	1241.0 ± 192.87	257.90 ± 34.25	6.07 ± 1.06
	5^{th} instar	1622.4 ± 338.18	359.86 ± 92.60	6.20 ± 1.20
	Adult (males)	987.9 ± 179.69	366.99 ± 60.86	3.36 ± 0.49
		$F_{(3,53)}=0.85$, $P=0.471$	$F_{(3,53)}=0.37$, $P=0.775$	$F_{(3,61)}=1.76$, $P = 0.166$

Asterisks indicate significant difference at $P<0.05$

Variables values followed by different letters in the columns at same waveform were significantly different (LSD Tukey test, $P<0.05$)

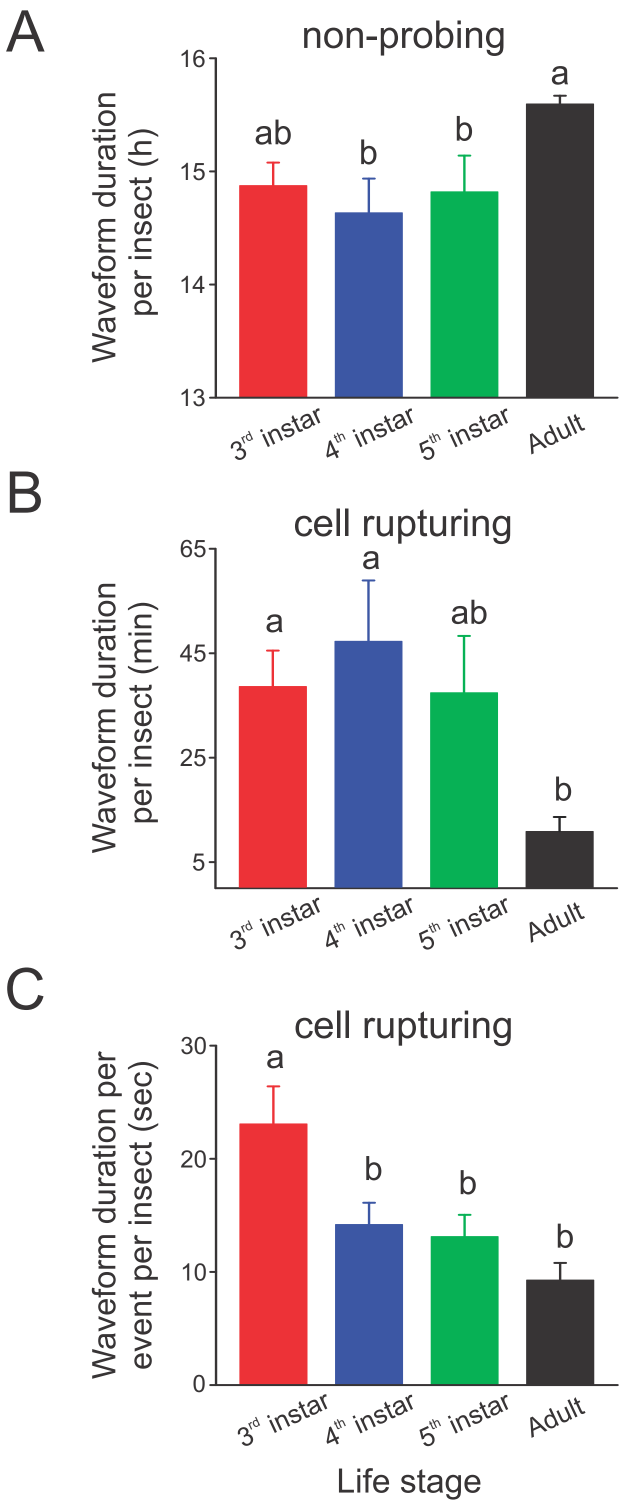

Figure 3.2: Calculated waveform duration per insect (WDI) (mean ± std. error) for (A) non-probing (NP), and (B) cell rupturing (CR); and waveform duration per event per insect (WDEI) (mean ± std. error) (C) for cell rupturing (CR). Data from recordings with AC voltage applied signal for *L. lineolaris* to 3^{rd}, 4^{th}, 5^{th} immature stages and adult.

3.3.2 **L. lineolaris** *damage on cotton leaves*

The appearance of damage imposed by *L. lineolaris* to cotton leaves can be seen in Figure 3.3. The repeated measures ANOVA showed that no differences were found among any life stages for overall amount of damage on cotton leaves resulting from *L. lineolaris* stylet probing behavior (Table 3.2, Figure 3.4a). That said, the amount of damage increased over time, with significant differences between consecutive days and a large increase in damage at seven days after the beginning of the insect feeding (Figure 3.4b)

Correlation analysis showed, in general, that higher waveform duration per insect (WDI) of probing waveform families resulted in higher amount of damage on cotton leaves (Table 3.3).

Family CR

The effect of cell rupturing (WDI) was found to be significantly correlated with damage for all insects stages and all dates analyzed, with correlation coefficients (r) tending to increase as the insect age increased (r varies from 0.63 [P=0.027] for 3^{rd} instar at day 2 to 0.97 [P<0.001] for adults at day 7).

Family T

The effect of transition (WDI) was significant and increased with insect age (r varies from 0.54 [P=0.05] for 4rd instar at day 4 to 0.92 [P<0.001] for 5^{th} instar at day 2). However, only one T event for one adult insect was observed, therefore it was not possible to perform a correlation analysis.

Family I

A significant correlation was found between ingestion (WDI) and foliar damage only for 4^{th} and 5^{th} instars, with higher coefficients for 5^{th} than 4^{th} (r varies from 0.59 [P=0.035] for 4^{th} instar at day 1 to 0.91 [P<0.001] for 5^{th} instar at day 2).

Table 3.2: Repeated measures ANOVA for the total amount of damage caused by *L. lineolaris* life stages to terminal axillary cotton leaves over time.

Sources of variation	*df*	Mortality		
		F		*P*
Between samples				
Life Stage	3	0.573		0.173
Error	45	-		-
Sources of variation	df_{den}/df_{num}	Wilk's lambda	F	*P*
Within samples	3/43	0.406	20.26	<0.0001*
Time (T)	9/104.8	0.776	1.29	0.25

Asterisks indicate signicant difference at $P<0.05$

Table 3.3: Correlation table over time between total of a specific waveform performed by each insect (Waveform Duration per insect: WDI) and amount of damage caused by *L. lineolaris* to cotton terminal axillary leaves. Waveform durations obtained from EPG recordings with AC voltage for 3^{rd}, 4^{th}, 5^{th} and adults of *L. lineolaris*.

Waveform	Day	3^{rd} instar		4^{th} instar		5^{th} instar		Adult (males)	
		r	*P*	r	*P*	r	*P*	r	*P*
Cell rupturing (CR)	2	0.63	0.028*	0.74	0.004*	0.83	<0.001*	0.94	<.0001*
	3	0.70	0.011*	0.75	0.003*	0.86	0.0001*	0.86	0.001*
	4	0.65	0.023*	0.73	0.004*	0.89	<0.001*	0.97	<.0001*
	7	0.63	0.027*	0.73	0.005*	0.72	0.006*	0.97	<.0001*
Transition (T)	2	0.66	0.019*	0.68	0.010*	0.90	<0.0001*	-	-
	3	0.71	0.010*	0.57	0.042*	0.92	<0.0001*	-	-
	4	0.66	0.019*	0.54	0.058	0.88	<0.001*	-	-
	7	0.57	0.050*	0.58	0.040*	0.63	0.021*	-	-
Ingestion (T)	2	0.26	0.407	0.59	0.035*	0.90	<0.0001*	0.09	0.794
	3	0.28	0.387	0.62	0.023*	0.91	<0.0001*	0.29	0.391
	4	0.31	0.333	0.61	0.028*	0.86	<0.001*	0.21	0.526
	7	0.23	0.474	0.66	0.015*	0.56	0.048*	0.28	0.410

Asterisks indicate significant correlation ($P<0.05$)

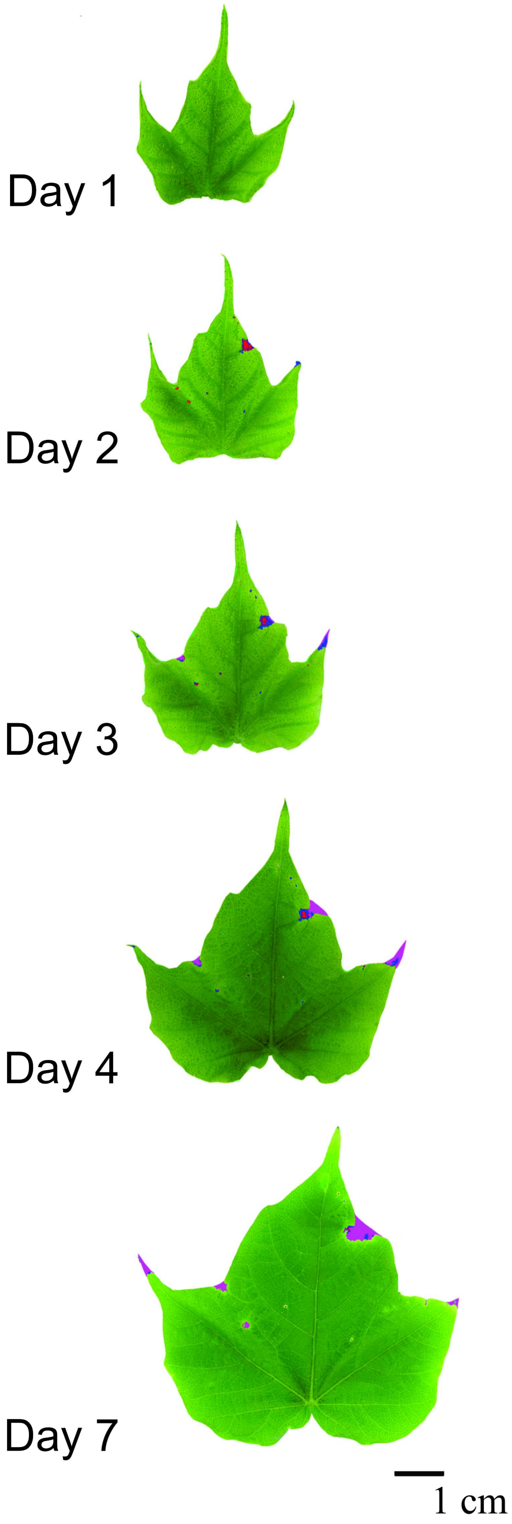

Figure 3.3: Damage caused by *L. lineolaris* life stages to terminal axial cotton leaves over time. Edited pictures with the color pattern changed prior to image analysis: green (undamaged area); red (early identifiable damage or beginning of the damage); blue (intermediate damage stage: necrotic lesions); pink (damage that resulted in holes or losses at leaf area, and yellow (damage resulted later in chlorotic areas near damaged areas).

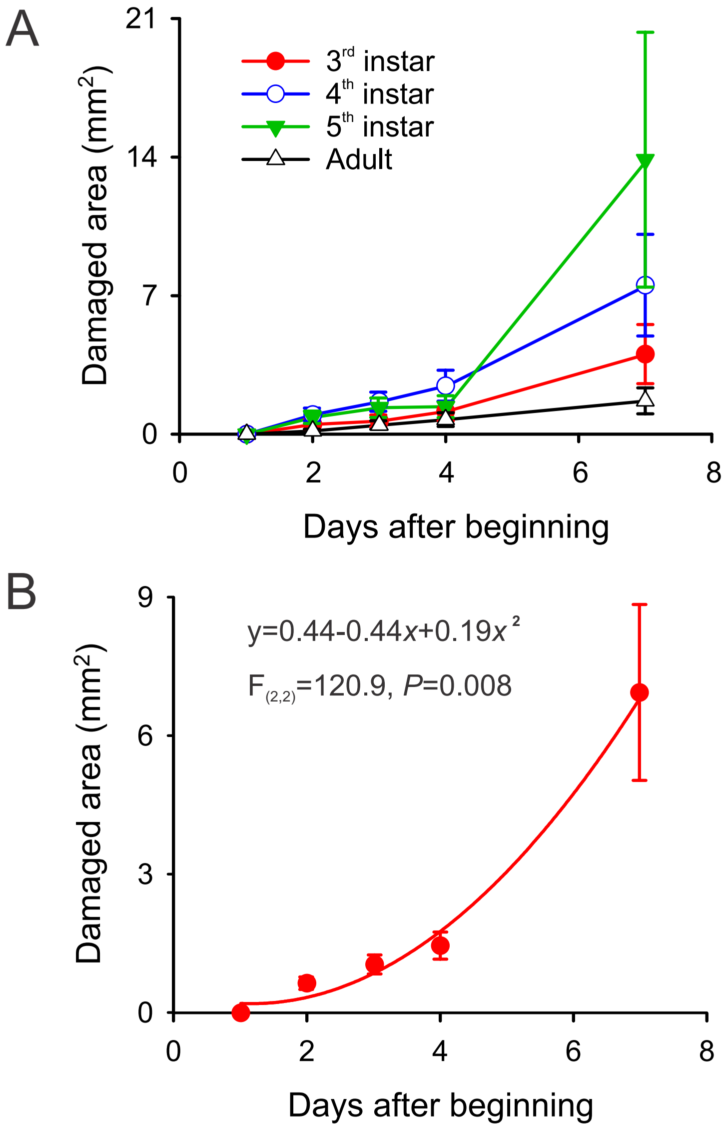

Figure 3.4: Damage caused by *L. lineolaris* life stages to terminal axillary cotton leaves over time. (a) damage caused by each *L. lineolaris* life stage over time; (b) overall *L. lineolaris* damage imposed to cotton leaves regardless of life stage. Symbols and vertical bars average ± standard errors.

3.4 Discussion

The objective of this study was to quantitatively compare stylet probing behavior between immatures and pre-reproductive males of *L. lineolaris*, by analysis of EPG waveforms and correlating their probing waveform durations with a time course of insect damage on cotton terminal leaves.

L. lineolaris stylet probing behavior was described earlier using EPG technology (Backus et al. 2007; Cervantes et al. 2016; Cline and Backus 2002). The authors, however, did not quantitatively correlate the amount of probing behavior with the damage caused to the plants and/or make comparisons among life stages. Our results using the new AC-DC EPG monitor (Backus and Bennett 2009) showed that *L. lineolaris* immatures and adults show differences in stylet probing behavior, mainly with respect to the variables of waveform duration per insect (WDI) and waveform duration per event per insect (WDEI), when they fed on cotton terminal leaves .

Quantitative measurements of EPG showed that *L. lineolaris* insects are predominantly motionless or spend almost all of their access time in non-probing behaviors on cotton axillary leaf terminals. On average, 93.4% of total recording time the insects were not probing. This rate of time spent in NP waveforms was longer than observed on cotton squares (Cervantes et al. 2016), probably because the terminals are less preferred than reproductive structures. Even on cotton squares, *L. lineolaris* spent only 20.4 - 30.1% of time performing probing behaviors (Cervantes et al. 2016). The average duration per insect (WDI) of non-probing behaviors (NP), i.e., including all behaviors outside of stylet penetration, was higher for pre-reproductive males than immature stages. The longest NP duration for adults could be because they have less probability of choosing leaf terminals than do younger instars, similar to *L. hesperus* (Cooper and Spurgeon 2013), or because they have a tendency to leave the plants, and spend more time walking, explaining the higher NP WDI compared to the others stages.

For *L. lineolaris* probing behaviors, our findings showed that adults spent less time than immature instars in cell rupturing (CR) and that CR events were significantly longer for 3^{rd} instars than older stages. However, this difference was not great enough to result in a significant difference in foliar damage up to seven days after the beginning of insect feeding, by which time the leaves were almost completely developed. Additionally, we observed a delayed effect on the timecourse of *L. lineolaris* damage to leaves, and a trend that damage could be higher for 4^{th} and 5^{th} instars after 7 days with an overall higher damage average on the last evaluated date. Although we did not find differences in damage between life stages, other authors found opposite results in reproductive structures for *L. hesperus*, for example, where *Lygus* adult probing results in more damage than immature stages (Gutierrez et al. 1977; Zink and Rosenheim 2005) or similar damage (Rosenheim et al. 2006).

Characteristics of stylet probing behavior could lead to different amounts of insect damage, as was verified by the correlation analysis table. Our results showed significant correlation coefficients between total time spent by each insect in CR and T waveforms and total damage inflicted on each plant. They were significant for all insect ages and evaluation dates except for adult stage, which did not show identifiable T waveform. Furthermore, correlation tended to increase as the insect age increased. We noted absence of significant correlation at 3^{rd} and adult stages for I waveform. These results together suggest that the main cause of damage to cotton leaves was the cell rupturing behavior. In fact, for pre-reproductive adult correlations, the duration of ingestion events was not correlated with the amount of damage and there was absence of T waveforms. Also, there were no significant differences in total damage on leaves by adults.

The amount of damage from multiple CR events is primarily caused by *L. lineolaris* feeding strategy. As a cell rupture feeder, they use their stylets and watery saliva to cause mechanical and chemical damage to the cells (Cervantes et al. 2016; Cline and Backus 2002; Miles 1972). The combination of events of stylet movements and saliva injection results in tissue breakdown and cell liquefaction prior to insect ingestion. After events of cell rupturing, development of the damaged tissue is compromised leading to necrotic lesions that can increase with foliar development or result in reduced leaf area. The absence of correlation between time spent in I waveform and total damage on leaves could be a result of ingestion, and thus removal, of the slurry of combined saliva and macerated cell contents. The removal of saliva from the site of insect puncture by ingestion could thus result in less salivary action over time in the cells near probing site. However, if the insect did not ingest the saliva, or part of it, the remaining injected enzymes could continue to dissolve the nearby cells and increase size of the damaged areas. *Lygus* spp. saliva has several enzymes exhibiting high activity that cause damage in leaf tissues (Celorio-Mancera et al. 2009). Similar results were found with *L. lineolaris* fed on pin-head cotton squares, where the removal of injected saliva reduces the amount of tannins that are secreted as a plant defense in response to lygus feeding on cotton squares (Cervantes et al, unpublished data). In our case, because we did not find differences for I waveforms in any quantitative variable, and there was lack of correlation between ingestion duration and foliar damage, but correlation between CR and foliar damage, we argue that cell rupturing waveform could be more important to cause damage on cotton leaf terminals. In the case of T waveforms, they are believed to represent tasting, testing and acceptance behaviors (Cervantes et al. 2016), functioning similarly to X-waves in sharpshooter leafhoppers (Backus et al. 2009). So, when an insect was performing T waveform it was tasting and swallowing small amount of slurry and the damage at this time was not higher than damage resulted from maceration during CR waveforms.

The timecourse of damage of *L. lineolaris* on cotton leaves frequently showed that the damage area developing into holes after 3-4 days from beginning of insect feeding (Fig. 3). Additionally, when the insect fed on a leaf's border, the amount of foliar area that was lost tended to be higher when the duration of a probe on that site was higher, reducing the growth rate of the leaf. For practical purposes, it was not possible to limit the area that the insect would feed upon, to prevent the deformed area on leaf's border. In this case, measurements that reduce general insect feeding could result in less damage on these young leaves.

The EPG technology used here allowed comparison of damage among instars, as well as correlation with amount of damage on leaves. As cell rupturing feeders, *L. lineolaris* did not produce a salivary sheath; thus, histological correlations between waveforms and site of feeding were not possible. However, by quantitative analysis, we provide some insights about the cause of damage, and suggest some important stylet probing behaviors related to damage on cotton leaves. Because *L. lineolaris* stages have preference for cotton reproductive organs, they could cause more damage to meristematic regions than developing leaves. Additionally, they could feed on incompletely developed structures and their damage could reduce total leaf area, thus compromising plant development.

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Final considerations

For this dissertation, I studied the susceptibility to insecticide chlorantraniliprole and the potential mediating changes in the sexual fitness of *Euschistus heros*, as well as the fitness of *E. heros* and *Piezodorus guildinii* in direct competition in soybean plants. Additionally, the stylet probing behavior of another heteropteran pest, *Lygus lineolaris*, was studied via electropenetrography (EPG) on terminal axillary cotton leaves and the amount of leaf damage was related to its stylet probing behavior, as well as insect life stages.

The exposure to sublethal concentrations of chlorantraniliprole changed the sexual fitness of *E. heros* in a hormesis-like phenomenon. This could be a serious problem, especially because the same phenomenon has occurred with other insecticides and probably with other species in a complex of stink bugs in soybean fields. Because competitive abilities of stink bugs of this complex are different, with some species showing better competitiveness that others, sublethal effects upon these species could shift the balance among them, as well as change species distribution and abundance. Thus, it will require better practices of monitoring and management to keep stink bugs at low-level infestations at soybean fields.

Electropenetrography technology is a very important tool to study insect stylet probing behavior. Studies with stink bugs that attack soybean fields are still incipient but are promising to help to understand insect-plant interaction, as well as inter- and intraspecific species relationship. This understanding will contribute to achieving better insect control by minimizing undesirable effects of diverse agronomic practices, especially insecticides applications.