MILAINE FERNANDES DOS SANTOS

SUBLETHAL STRESS BY INSECTICIDES ON THE NEOTROPICAL BROWN STINK BUG *Euschistus heros*

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

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APROVADA: 31 de julho de 2017.

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“Todas as substâncias são venenosas. Não há nenhuma que não seja. A dose certa diferencia um veneno de um remédio”

Philippus Paracelsus.

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RESUMO

SANTOS, Milaine Fernandes, D.Sc., Universidade Federal de Viçosa, julho de 2017. **Estresse subletal causado por inseticidas sobre o Neotropical percevejo marrom *Euschistus heros***. Orientador: Raul Narciso Carvalho Guedes. Coorientadores: Eugenio Eduardo de Oliveira e Gerald Christopher Cutler.

O uso de inseticidas constitui-se no principal método utilizado para controlar os danos causados por pragas na agricultura, pois reduz efetiva e rapidamente as populações. Porém, o uso frequente de inseticidas pode potencialmente desencadear uma série de efeitos subletais sobre espécies não alvo. Nas últimas décadas o foco das pesquisas sobre o efeito de inseticidas tem-se concentrado no impacto negativo sobre inimigos naturais ou polinizadores, porém ainda faz-se necessário conhecer o efeito causado por inseticidas sobre espécies de pragas que não foram alvo inicial da aplicação. Nesse contexto, esta pesquisa avaliou o estresse causado por inseticidas na sobrevivência, comportamento de acasalamento e reprodução associados a demografia do percevejo marrom *Euschistus heros*. No primeiro trabalho avaliamos o estresse causado pelo neonicotinoide imidacloprido na sobrevivência e reprodução do percevejo marrom. No segundo trabalho verificamos o estresse secundário causado por clorantraniliprole, espinosade e piriproxifeno na sobrevivência e reprodução associada a demografia do percevejo marrom. E por fim, no terceiro trabalho testamos se clorantraniliprole, espinosade e piriproxifeno interferem no comportamento de acasalamento e consequentemente na demografia de *E. heros*. Imidacloprido reduziu a sobrevivência de fêmeas, por outro lado, provocou aumento no nível de dano celular, no comprimento de ovaríolos, na área total do folículo mais desenvolvido dos ovários e na reprodução. Clorantraniliprole causou médio impacto demográfico, enquanto espinosade e piriproxifeno apresentaram forte impacto demográfico, com supressão no caso do espinosade. Curiosamente clorantraniliprole causa efeito estimulatório sobre a primeira cópula do percevejo marrom. Fêmeas e/ou machos tratados com clorantraniliprole mostraram aumento da taxa líquida de reprodução (R_0) e taxa intrínseca de reprodução (r_m), contribuindo com aumento demográfico populacional. Os resultados mostram que surtos recentes de *E. heros* na cultura da soja estão relacionados ao efeito hormético ocasionado por baixas concentrações de imidacloprido e ao efeito de clorantraniliprole, que também desencadeia resposta hormética nesta espécie. Por outro lado, espinosade e piriproxifeno reduzem o crescimento populacional, e portanto, são inseticidas com potencial de uso nos programas de controle do percevejo marrom no Brasil.

ABSTRACT

SANTOS, Milaine Fernandes, D.Sc., Universidade Federal de Viçosa, July, 2017. **Sublethal stress by insecticides on the Neotropical brown stink bug *Euschistus heros***. Advisor: Raul Narciso Carvalho Guedes. Co-advisors: Eugenio Eduardo de Oliveira and Gerald Christopher Cutler.

The insecticide use is the mainly method to control pest species in the agriculture, because reduce the populations. However, the frequently insecticide use can potentially stimulate sublethal effects on species non-target. In the last decades the focus of the researches about the insecticide use has been concentrated about the negative impact on natural enemies and pollinators, but is necessary to know the potential effects caused by insecticides on pest species non-target. In this context, we evaluated the stress induced by insecticide in the survival, mating behavior and reproduction associated to demography of the Neotropical brown stink bug *Euschistus heros.* In the first experiment, we evaluated the stress by imidacloprid in the survival and reproduction of the stink bug. In the second experiment, we evaluated the secondary stress by chlorantraniliprole, spinosad and pyriproxyfen in the survival and reproduction associated to demography of the stink bug. Finally, in the third experiment we tested the effect by chlorantraniliprole, spinosad and pyriproxyfen in the mating behavior and consequently in the demography of the *E. heros*. Imidacloprid reduced the female survival compared with untreated females, on the other hand, induced higher levels of cell damage, greater ovariole length, larger area of the most developed follicle in their ovaries up to the 6th day of adulthood and higher reproduction. Chlorantraniliprole caused mild demographic impact on this pest population, while pyriproxyfen and especially spinosad showed strong demographic impacts. Curiously, chlorantraniliprole cause stimulatory effect on the first copulation of the stink bug. Treated females and/or males with chlorantraniliprole showed increase in the net reproductive rate (*R0*) and intrinsic rate of population growth (r_m). Our results shows that outbreaks of *E. heros* in the soybean are related to hormesis induced by low concentrations of imidacloprid and also by chlorantraniliprole. In other hand, spinosad and pyriproxyfen decreases the population growth and are potential insecticides for use in control programs of the Neotropical brown stink bug *E. heros* in Brazil.

INTRODUÇÃO GERAL

Historicamente a base da economia brasileira é sustentada pela produção de produtos primários, e atualmente cultivares como algodão, arroz, milho e soja estão em evidência (IBGE, 2007; SILVA e BOTELHO, 2014). Nesse sentido, o controle de artrópodes pragas em sistemas agrícolas é essencial para a manutenção e/ou aumento da produção nacional anual dessas commodities. Apesar de existirem estratégias de controle biológico de pragas, o uso de inseticidas ainda constitui-se no principal método utilizado para controlar os danos causados por pragas na agricultura, pois reduz efetiva e rapidamente as populações (BIANCHI et al. 2006; DESNEUX et al. 2007; GUEDES et al. 2016; PANIZZI, et al. 2013).

A eficácia de inseticidas convencionais contra insetos-praga é normalmente elevada, mas o uso destes compostos é sujeito a problemas. O uso frequente de inseticidas pode potencialmente desencadear efeitos letais (em curta duração) ou uma série de efeitos subletais sobre espécies não alvo (DESNEUX et al. 2007). Isso ocorre porque a degradação de pesticidas ao longo do tempo e a distribuição irregular de pesticidas na planta contribuem para que os insetos sejam expostos a baixas concentrações na maioria dos campos agrícolas (AYYANATH et al. 2013). Efeitos subletais ocorrem porque em alguns casos os inseticidas interagem com sítios secundários de ação e ao invés de levarem a morte, acabam interferindo com a reprodução ou sobrevivência do indivíduo (GUEDES et al. 2016).

Sabe-se que os inseticidas podem desencadear efeitos indiretos e diretos sobre espécies de artrópodes. Efeitos indiretos estão associados a alteração da qualidade de plantas hospedeiras devido a variações de umidade, nitrogênio disponível e fibras em suas sementes; redução de espécies competidoras ou mesmo a supressão de inimigos naturais (HARDIN et al. 1995; OJIAKO e KAYODE, 2014; WHITE, 1984). Em contraste, efeitos diretos estão associados à alterações fisiológicas (reprodução, condução nervosa, desenvolvimento, razão sexual, imunidade, longevidade) ou comportamentais (aprendizagem, acasalamento, predação, orientação e mobilidade) desencadeados pelo contato direto do organismo com o inseticida (HARDIN et al. 1995; HAYNES, 1988).

Nas últimas décadas o foco das pesquisas sobre o efeito de inseticidas concentrou-se no impacto negativo sobre inimigos naturais (AMARASEKARE et al., 2016;

GONTIJO et al. 2015; LIMA et al. 2015) ou polinizadores (BARBOSA et al. 2015; CUTLER e RIX, 2015; PERVEEN e AHMAD, 2017). No entanto, pouco se conhece sobre o efeito causado por inseticidas sobre espécies de pragas que não foram alvo inicial da aplicação. Especificamente sobre o efeito de inseticidas em espécies de pragas secundárias, existem relatos demonstrando aumento no número de cópulas e na fecundidade diária do percevejo marrom *E. heros* (F.) exposto a clorantraniliprole, principalmente pelo efeito do macho; e aumento demográfico do ácaro fitófago *Oligonychus ilicis* (McGregor, 1917) exposto a piretróides (CORDEIRO et al. 2013; TUELHER et al. 2017).

Atualmente um dos efeitos subletais mais peculiares, apesar de ainda relativamente pouco estudado, é a hormese. Esta caracteriza-se por uma resposta reversa entre altas e baixas concentrações para um indivíduo quando exposto à um mesmo estressor, como um inseticida por exemplo (CALABRESE e BALDWIN, 2001; GUEDES e CUTLER, 2014). A hormese torna-se uma resposta desvantajosa quando favorece o aumento populacional de espécies de pragas que potencialmente pode provocar a ressurgência destas espécies e/ou surtos de pragas secundárias (CUTLER, 2013; HARDIN et al. 1995; MORSE, 1998). Processos de ressurgência referem-se a um aumento na abundância de espécies de pragas a um nível que excede populações não tratadas após a aplicação de inseticida, enquanto surtos de pragas secundárias ocorrem quando a abundância de espécies não-alvo aumentam após a aplicação de inseticidas contra a praga primária foco do controle (HARDIN et al. 1995; METCAL, 1980).

Fenômenos horméticos induzidos por inseticidas são desencadeados após a realocação temporária de recursos energéticos pelo organismo, incorrendo por exemplo em aumento reprodutivo combinado com redução na sobrevivência de fêmeas, ou aumento no peso de adultos combinado com redução na reprodução (CUTLER, 2013; FORBES, 2000; SIAL e BRUNNER, 2010). Mesmo espécies de pragas resistentes a inseticidas podem induzir hormese sobre a fertilidade e consequentemente aumentar a taxa intrínseca de crescimento populacional (GUEDES et al. 2010).

Atualmente o percevejo marrom *Euschistus heros* (F.) é reconhecido como praga primária na cultura da soja no Brasil, devido ao seu aumento demográfico nos últimos anos. Diversos fatores podem estar envolvidos na mudança de status de *E. heros* na soja, como: grande capacidade de adaptação e exploração de recursos; mudanças de dominância ecológica com outras espécies de percevejos, *Nezara viridula* L. e

Piezodorus guildinii Westwood (PANIZZI, 2013; PANIZZI e LUCINI, 2016); e uso de variedades transgênicas de soja expressando toxina de *Bacillus thuringiensis* (Berliner, 1915), que são efetivas no controle de lagartas.

O controle de *E. heros* na soja é normalmente realizado com o uso de neonicotinoides (imidacloprido) (MAPA, 2017). Contudo, a degradação ambiental natural destes compostos permite a exposição do percevejo a efeitos subletais, que podem ser de natureza estimulatória e favorecer a sobrevivência e reprodução destes organismos. Neonicotinoides atuam em receptores pós-sinápticos de acetilcolina atuando como agonistas nestas moléculas, e provocando hiperatividade nervosa seguida de colapso do organismo (JESCHKE e NAUEN, 2008). Além disso, existe o estresse potencial causado por inseticidas usados para o controle de espécies de pragas associados ao percevejo marrom, como lagartas desfoliadoras e outras espécies de fitófagos. Na soja, clorantraniliprole e espinosade são normalmente utilizados para o controle das lagartas *Anticarsia gemmatalis* (Hübner), *Chrysodeixis includens* (Walker) e *Heliothis virescens* (Fabrícius, 1781), enquanto piriproxifeno é usado para o controle de mosca-branca (*Bemisia tabaci*, Gennadius) (MAPA, 2017).

Clorantraniliprole é uma diamida antranílica que atua como modulador de receptores de rianodina. Os receptores de rianodina estão presentes em canais de Ca++ do retículo endoplasmático de células musculares. Estimulam a liberação do conteúdo intracelular e contração muscular (CASIDA e DURKIN, 2013). Piriproxifeno é um regulador de crescimento que atua como um mímico do hormônio juvenil, enquanto espinosade é um inseticida neurotóxico que atua como modulador dos receptores de acetilcolina (nAChR), levando à abertura de canais iônicos na membrana pós-sináptica e à condução do estímulo nervoso (CASIDA e DURKIN, 2013; HATAKOSHI, 2013). Assim, o foco desse trabalho foi verificar os potenciais efeitos letal e subletal (sobre a sobrevivência, acasalamento e reprodução) de clorantraniliprole, espinosade, imidacloprido e piriproxifeno no percevejo marrom da soja. E fornecer subsídios para compreender o impacto destes na demografia desta espécie-praga, contribuindo para seu manejo adequado.

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CHAPTER 1

Imidacloprid-mediated effects on survival and fertility of the Neotropical brown stink bug *Euschistus heros*

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Abstract

Enhanced reproductive output after sublethal insecticide exposure, including neonicotinoid exposure, has been reported in a diversity of arthropods. Suspicions of such a phenomenon in the Neotropical brown stink bug, *Euschistus heros* (Hemiptera: Pentatomidae), were sparked by the increasing densities of naturally occurring populations of this insect pest species in Brazilian soybean fields. Here, we tested whether the sublethal exposure to imidacloprid would induce changes in the survival and reproductive performances of *E. heros* adult females. The imidacloprid estimated LC_{50} was 0.83 (0.60-1.25) µg a.i./cm^2, and the dose recommended for field applications (4.2 µg a.i./cm^2) was within the concentration range of the imidacloprid estimated LC_{80} (2.66 [1.65-5.49] µg a.i./cm^2). Newly emerged ($\leq$ 24 h) adult females were exposed for 48 h to dry imidacloprid residues (0.042 µg/cm^2, equivalent to 1% of the field rate dose), and exhibited higher levels of cell damage, greater ovariole length and a larger area of the most developed follicle in their ovaries up to the 6th day of adulthood. Furthermore, these females exhibited reduced rates of survival but higher fecundity and fertility rates compared with untreated females. Our results thus suggest that females of *E. heros* increased their reproductive output in response to the imidacloprid sublethal exposure. These findings suggest a potential involvement of sublethal exposure to neonicotinoids in the recent outbreaks of the Neotropical brown stink bug *E. heros* observed in Brazilian soybean-producing regions.

Key-words: reproductive responses, hormesis, insect ovaries, damaged cells, stink bugs

Key message:

- Insecticide-induced changes in *Euschistus heros* reproduction capacity has been sparked by the increasing densities of this pest in Brazilian soybean fields.
- Females of *E. heros* increased their reproductive output (fecundity and fertility rates) to overcome imidacloprid-induced sublethal stress (higher number of damaged ovarian cells and reduction on female's survival).
- These findings suggest a potential link between imidacloprid sublethal exposure and the recent outbreaks of *E. heros* observed in the Brazilian soybean fields.

Author Contribution Statement

EEO, GFM, HVVT and WFB conceived and designed the research. MFS and RLS conducted the experiments. RNCG, GFM and EEO contributed new reagents and/or analytical tools. EEO, GFM, RNCG, WFB and RLS analyzed the data. EEO, RNCG and MFS wrote the manuscript. All authors read, corrected and approved the manuscript.

1. Introduction

The use of insecticides in agroecosytems has enabled farmers to increase the yields of food and other agricultural products (Macfadyen et al. 2014). However, the sustainability of this practice is heavily dependent on its proper use. Indiscriminate insecticide applications can lead to substantial biodiversity loss in agricultural landscapes, selection for insecticide-resistant populations, resurgence and/or outbreaks of insect pests, and impairment of non-target organisms, including humans (Szczepaniec and Raupp 2013; Guedes and Cutler 2014; Macfadyen et al. 2014; Quarcoo et al. 2014).

Insect pest resurgence and secondary pest outbreak are broadly recognized ecological backlashes of insecticide use, but whose underlying causes have been the object of little attention. The occurrence of secondary pest outbreaks may be related to a reduction of the pest’s natural enemies, selection for resistant individuals or insecticide hormesis on the pest species (Desneux et al. 2007; Guedes and Cutler 2014; Roubos et al. 2014). Insecticide-induced hormesis refers to a biphasic dose-response phenomenon that is characterized by a low-dose stimulation and a high-dose inhibition achieved with a given compound (Calabrese and Baldwin 2003; Calabrese et al. 2008; Cutler 2013; Jager et al. 2013; Guedes and Cutler 2014). Indeed, insecticide-induced hormesis has been identified in a number of arthropod species (Guedes et al. 2010; Yu et al. 2010; Ayyanath et al. 2013; Cordeiro et al. 2013; Vilca Mallqui et al. 2014).

Neonicotinoid insecticides, with imidacloprid being the most notable example, have been registered as agricultural pesticides in more than one hundred countries. The market share for these compounds in 2011 corresponded to 28.5 % of the total global insecticide market, a likely consequence of their high efficacy against a broad range of arthropod pest species (Jeschke et al. 2013). In Brazil, the amount of neonicotinoid applications has increased considerably over the last few years because these compounds have been used to replace the applications of long-established insecticide classes (e.g., organophosphates, carbamates, and endosulfan). Neonicotinoids, for instance, have become the most common insecticides used to control the Neotropical brown stink bug *Euschistus heros* (F.) (Hemiptera: Pentatomidae) (Sosa-Gómez et al. 2009; Sosa-Goméz and Silva 2010). This species is currently the most abundant and prevalent arthropod pest species in Brazilian soybean fields (Silva et al. 2011; Farias et al. 2014; Panizzi et al. 2014).

Neonicotinoids may impact insect populations through both lethal and sublethal effects. However, the results obtained thus far in insect populations sublethally exposed to these compounds are contradictory. Although imidacloprid seems to reduce the progeny production or survival rates in some insect species (Bao et al. 2009; Tan et al. 2012; Pan et al. 2014), a few studies have associated sublethal imidacloprid exposure with enhanced reproductive outputs in arthropod species (James and Price 2002; Wang et al. 2008; Yin et al. 2008; Cutler et al. 2009; Yu et al. 2010; Szczepaniec and Raupp 2013). Although the mechanisms regulating the positive and/or negative sublethal effects of insecticides are not well understood (Calabrese and Baldwin 2003; Guedes and Cutler 2014), it is plausible that the endocrine, antioxidant and/or detoxification systems may be disrupted when insects are under insecticidal stress (Flatt and Kawecki 2007; Rattan 2008; Ge et al. 2009; Mattson and Calabrese 2010; Yu et al. 2010), which affects their expected performance.

Immediately following the ban of organophosphate and endosulfan, the organophosphate-based control strategies used to control *E. heros* in Brazilian soybean fields were rapidly replaced by the utilization of neonicotinoid compounds, which initially did not result in control failures. However, the density of naturally occurring populations of *E. heros* has increased over the last few years in Brazil (Panizzi et al. 2014), reinforcing the suspicion of a potential involvement of sublethal exposure to neonicotinoids in these recent *E. heros* outbreaks. Thus, by combining toxicological and morphological procedures, the present investigation was carried out to evaluate whether the sublethal exposure to the neonicotinoid imidacloprid would affect the survival and reproductive performances of *E. heros* adult females.

2. Materials and Methods

2.1. Insects

The initial colony of *E. heros* was established from eggs kindly provided by Dr. Raúl Laumann (Laboratório de Semioquímicos, Embrapa Recursos Genéticos e Biotecnologia, Brasília, Distrito Federal, Brasil). The colony was started with approximately 1000 individuals and was multiplied and reared under controlled conditions (27 ± 2°C, 75 ± 5% relative humidity, with an L:D photoperiod of 14:10 h) to prevent diapause. Artificial lighting was maintained between 08:00 and 22:00 h. All of the developmental stages of *E. heros* were mass reared following methods previously described elsewhere (Borges et al. 2008; Silva et al. 2008). To increase the genetic variability of the insects used in the experiments, field-collected individuals from soybean farms in the Tangará da Serra region (State of Mato Grosso, Brazil) and from the experimental soybean fields at the Federal University of Viçosa (Viçosa, State of Minas Gerais State, Brazil) were routinely introduced into the laboratory colony.

2.2. Concentration-mortality bioassays

Insecticide bioassays followed methods that were adapted from toxicological studies on *E. heros* performed with glass vials (Willrich et al. 2003, Snodgrass et al. 2005). The neonicotinoid insecticide imidacloprid (water-dispersible granules at 700 g active ingredient (a.i.)/L; Bayer CropScience, São Paulo, SP, Brazil) was used to coat the inner walls of 250 mL transparent glass-vials (EME Equipment, Pauliceia, SP, Brazil). Water (distilled and deionized) was used as a carrier for the commercial insecticide formulation, and 2 mL aliquots were added to each glass-vial. A control treatment (distilled and deionized water only) and seven different insecticide concentrations (calculated as μg a.i./cm^2 of treated surface and ranging from 0.0042 to 12.6 μg a.i./cm^2, i.e. the equivalent of 0.001- to 3-fold the field label rate, which corresponds to 4.2 μg a.i./cm^2) were used to estimate the concentration-mortality curve. Ten replicates, each with 10 newly emerged 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. Mortality data were corrected by the mortality in the control treatments.

2.3. Reproduction and survival bioassays

For these experiments, groups of newly emerged adult females of *E. heros* were exposed to dry imidacloprid residues (at 0.042 μg a.i./cm^2, the equivalent of 1% of the field rate dose, which was the highest concentration tested that did not induce mortality in our concentration-response bioassays), or to deionized water (control). After the exposure period (48 h), the females were placed individually in plastic cups (250 mL). The bottoms of the cups were covered with paper towels to absorb moisture, and their tops were covered with a piece of organza veil and a rubber band to prevent the insects from escaping. These imidacloprid-exposed and unexposed females were fed *ad libitum* with a mixture of the fresh pods of green beans (*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, with the supplies replenished at four day intervals. All of the females were coupled with virgin and water-exposed males after 13 days of (adult) emergence. These couples were kept together in plastic cups (with the aforementioned conditions of feeding and maintenance) for the next three days, allowing sufficient time for mating, after which the male were removed from the cups. Twenty imidacloprid-exposed females and 20 water-exposed females were used in the bioassay, and the number of laid eggs per female, the egg viability and the survival rates were recorded daily. Insects were recognized as dead when they were unable to walk after being prodded with a fine hair brush.

2.4. Ovary morphometry

Female reproductive tracts were dissected in a saline solution for insects (0.1 M NaCl, 20 mM KH_2PO_4, 20 mM Na_2HP_4), and fixed and stored (4°C) in Zamboni's fixative solution (paraformaldehyde + picric acid). Adult insects at 2, 4, 6, 8, 10, 12 and 14 days after emergence were used, and the following morphometric parameters were determined on the median ovariole: length (mm), number of follicles, and the area of the most developed follicle (mm^2). The parameters were determined in one ovariole from each ovary. These parameters were measured using the Image Pro-Plus 4.5 software (Media Cybernetics, Rockville, MD, USA). A minimum of seven reproductive tracts were assessed in each treatment (i.e., deionized water or imidacloprid), at each age. The follicles were most often absent or were very small in 2 and 4 day old adults, which did not allow for precise measurements. After being fixed, the samples were washed in PBS (phosphate buffered saline, 0.1 M), dehydrated in an ascending ethanol dilution series (70-95%), and embedded overnight in Leica historesin (Leica Biosystems, Heidelberg, Germany). The samples were subsequently embedded in historesin with hardener and subjected to microtomy. Sections of 3 μm were obtained and subsequently stained with hematoxylin and eosin (HE) and mounted with Eukitt mounting medium (Sigma-Aldrich St. Louis, MO, USA). Microscopic images were obtained using a Zeiss Primo Star light microscope (Zeiss, Oberkochen, Germany) fitted with an AxioCam ERC5s digital camera (Zeiss).

2.5. *Cell damage*

The fixed and washed ovaries of each adult age were transferred to 1.5 mL microtubes and treated with proteinase K [10 µM/mL of Tris-HCl (10 mM, pH 7.4)] for 1 h at 37°C. Fragmented DNA was labeled with the In Situ Cell Death Detection Fluorescein kit (Roche Applied Science, São Paulo, SP, Brazil). The samples were washed three times in 0.1 M phosphate buffer (pH 7.4) and incubated in the reaction buffer for 45 min at 37°C, according to the manufacturer's instructions. The samples were subsequently washed, covered with Mowiol solution (Sigma-Aldrich), analyzed, and photographed using a fluorescence microscope (Olympus BX53, Olympus, Tokyo, Japan). As a negative control for the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) reaction, one histological section of ovary of each stage was incubated without the transferase enzyme, according to the manufacturer's protocol.

2.6. Statistical analyses

Concentration-mortality curves were estimated by probit analyses using the PROC PROBIT procedure (SAS Institute 2008). The results of the survival bioassays were subjected to survival analysis performed using the Kaplan-Meier estimators (Log-rank method) with SigmaPlot 12.0 (Systat Software, San Jose, CA, USA). The ovary morphometric results were subjected to univariate analyses of variance, and Tukey's HSD test ($P < 0.05$) whenever necessary (PROC UNIVARIATE, SAS Institute, 2008). When appropriate, regression analysis was performed using the curve fitting procedure of SigmaPlot 12.0. Regression analyses were performed to recognize the trends of the daily fecundity and fertility results in each treatment over time. The assumptions of normality and homogeneity of variance were checked, and no data transformation was necessary (PROC UNIVARIATE, SAS Institute, 2008). Unless otherwise stated, all data were presented as the mean ± S.E.

3. Results

3.1 Concentration-mortality and survival bioassays

The probit model was suitable for the concentration-mortality results because the goodness-of-fit test provided low χ^2-values (< 2.0) and high P-values (> 0.05). The lethal concentrations (in µg a.i/cm^2) of imidacloprid, which were estimated based on concentration-mortality bioassays, are shown in Fig. 1A. The estimated LC_{50} was 0.83 (0.60-1.25) µg a.i./cm^2, and the dose recommended for field applications (4.2 µg a.i/cm^2) was within the concentration range of the estimated LD_{80} (2.66 [1.65-5.49] µg a.i./cm^2) according to the probit analysis (slope [± SE] = 3.13 ± 0.33; n = 350, df = 5, $\chi2$ = 0.98, $P = 0.91$). As shown in Fig. 1B, sublethal exposure to imidacloprid (at 0.042 µg/cm^2, equivalent to 1% of the field rate dose) significantly reduced the survival of *E. heros* females (log-rank test: $\chi^2 = 8.61$, df = 1, $P = 0.003$). The median survival time (LT_{50}) for the unexposed females was 46.7 days, whereas the LT_{50} for imidacloprid-exposed females was 40.9 days (Fig. 1B). These differences increased overtime and 60 days after the exposure, adult survival was above 41% for unexposed females and approximately 24% for imidacloprid-exposed females (Fig. 1B).

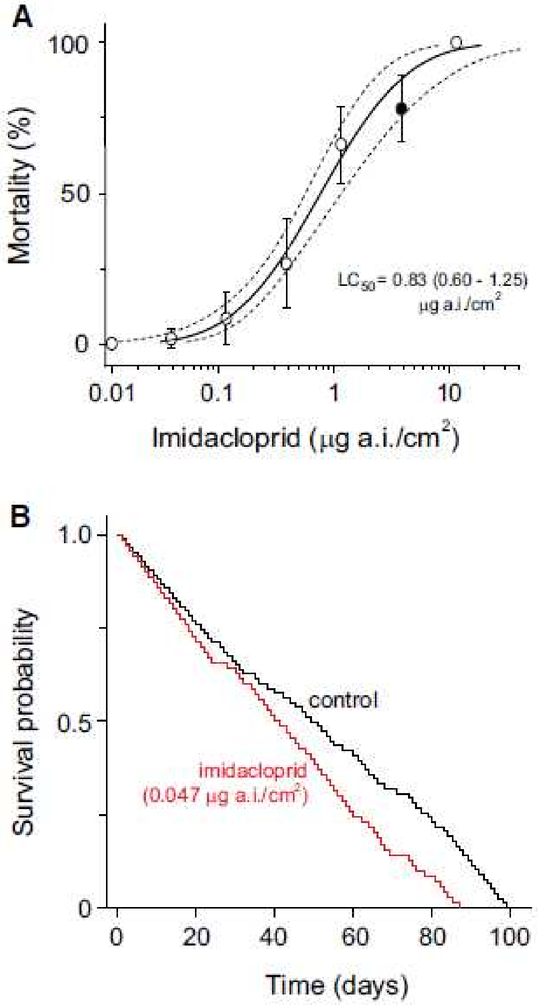


Fig. 1. Toxicity of imidacloprid to the stink bugs *Euschistus heros*. ***A.*** Lethal concentration (LC) values were estimated based on concentration-mortality bioassays using probit analyses (LC_{50} = 0.83 [0.60-1.25] µg a.i./cm^2, slope [± SE] = 3.13 ± 0.33, n = 350, df = 5, $\chi2$ = 0.97, $P = 0.91$). Dotted lines denote 95% confidence intervals. Filled circle denotes the results obtained with the application of

imidacloprid at a concentration of 4.2 μg a.i./cm^2 (equivalent to the field rate). ***B.*** Survival curves of *E. heros* after the 48-h exposure to imidacloprid (0.042 μg a.i./cm^2). The survival curves were significantly different by Holm-Sidak's test ($P < 0.05$); the median survival time (LT_{50}) for the unexposed females was 46.7, whereas the LT_{50} for imidacloprid-exposed females was 40.9 days.

3.2 Ovary morphometry

Females of *E. heros* exhibited clear and intense yellowish ovaries with seven ovarioles per ovary and two lateral oviducts connecting to a common oviduct (Fig. 2). The ovarioles of each ovary fused at the proximal end, forming the lateral oviduct. The lateral oviducts combine to form a common oviduct with a diameter similar to that of the lateral oviducts. In general, the ovarioles are long tubes, and terminal filaments join the ovarioles, forming a compact bunch-shaped structure (Fig. 2B).

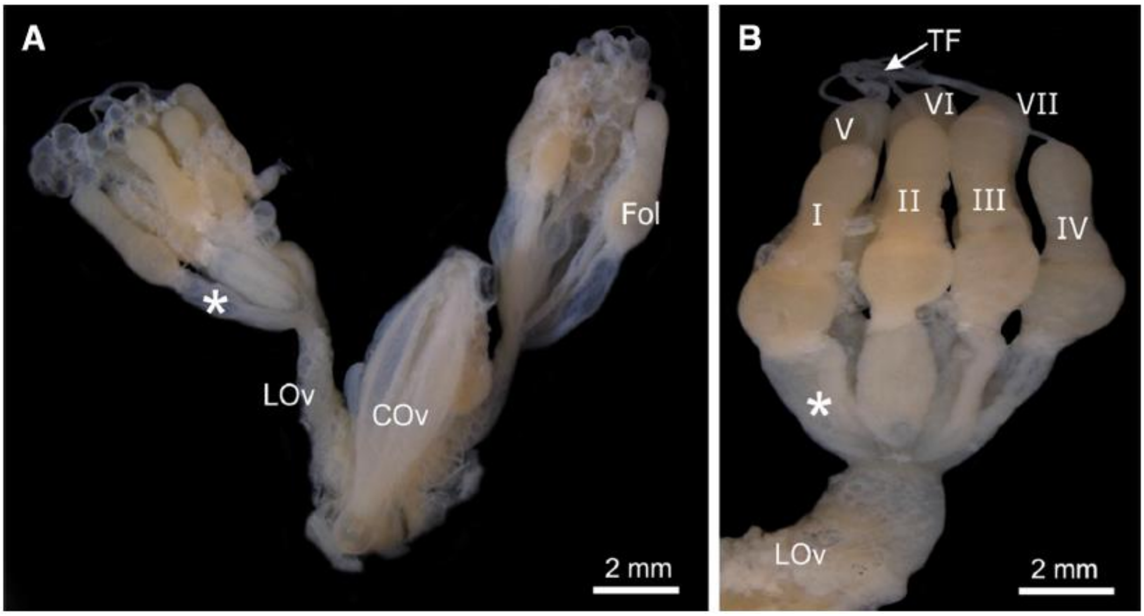
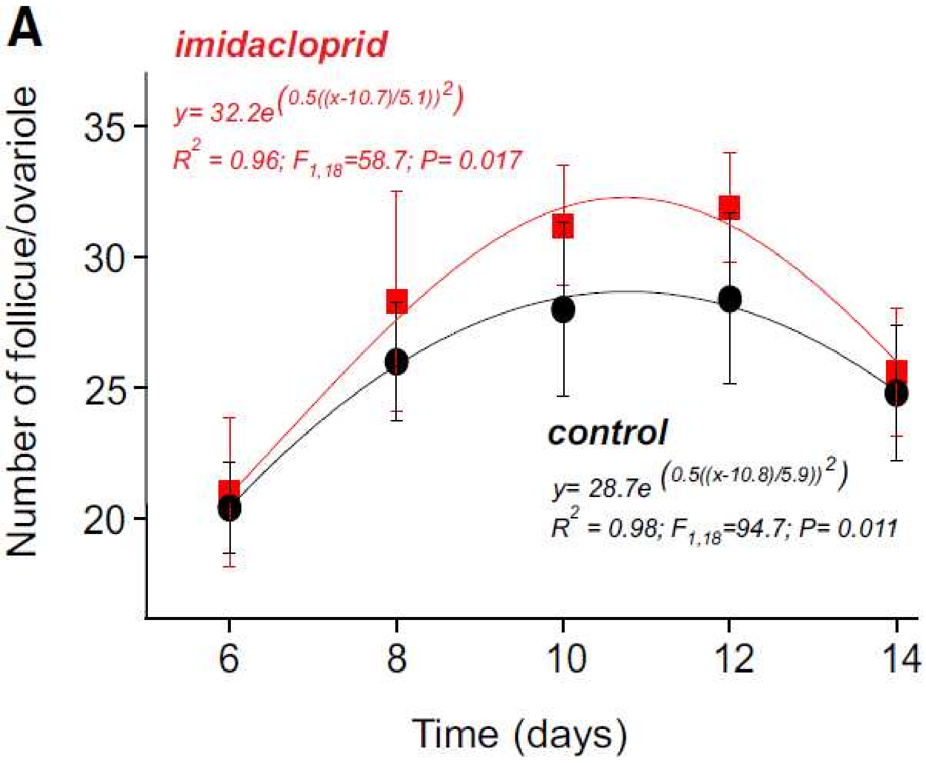


Fig. 2. General view of the *E. heros* female reproductive tract at six days after adult emergence. The central (Cov) and lateral (Lov) ovarioles as well as the follicles (Fol) and calyx (asterisk) are shown in (***A***). The terminal filament (TF) and the seven ovarioles (I to VII) are shown in (***B***).

The number of follicles per ovariole increased over time up to the 14th day of adult emergence, and sublethal exposure to imidacloprid did not affect this parameter ($F_{1,124} = 0.67$, $P = 0.417$) (Fig. 3A). Intriguingly, when the females were exposed to imidacloprid, a mild acceleration of ovarian development was observed for the length of the median ovariole and for the area of the most developed follicle in the ovariole (Fig. 3B,C). Imidacloprid-exposed females exhibited larger areas for the most developed follicles ($F_{1,17} = 9.33$, $P = 0.008$) compared with unexposed females six days after adult emergence, but their development was subsequently equivalent on the 14th day of emergence (Fig. 3B). Similar responses were observed for the length of the median ovariole, as imidacloprid exposure resulted in larger ovarioles in younger females ($F_{1,17} = 10.82$, $P = 0.004$) (Fig. 3C).



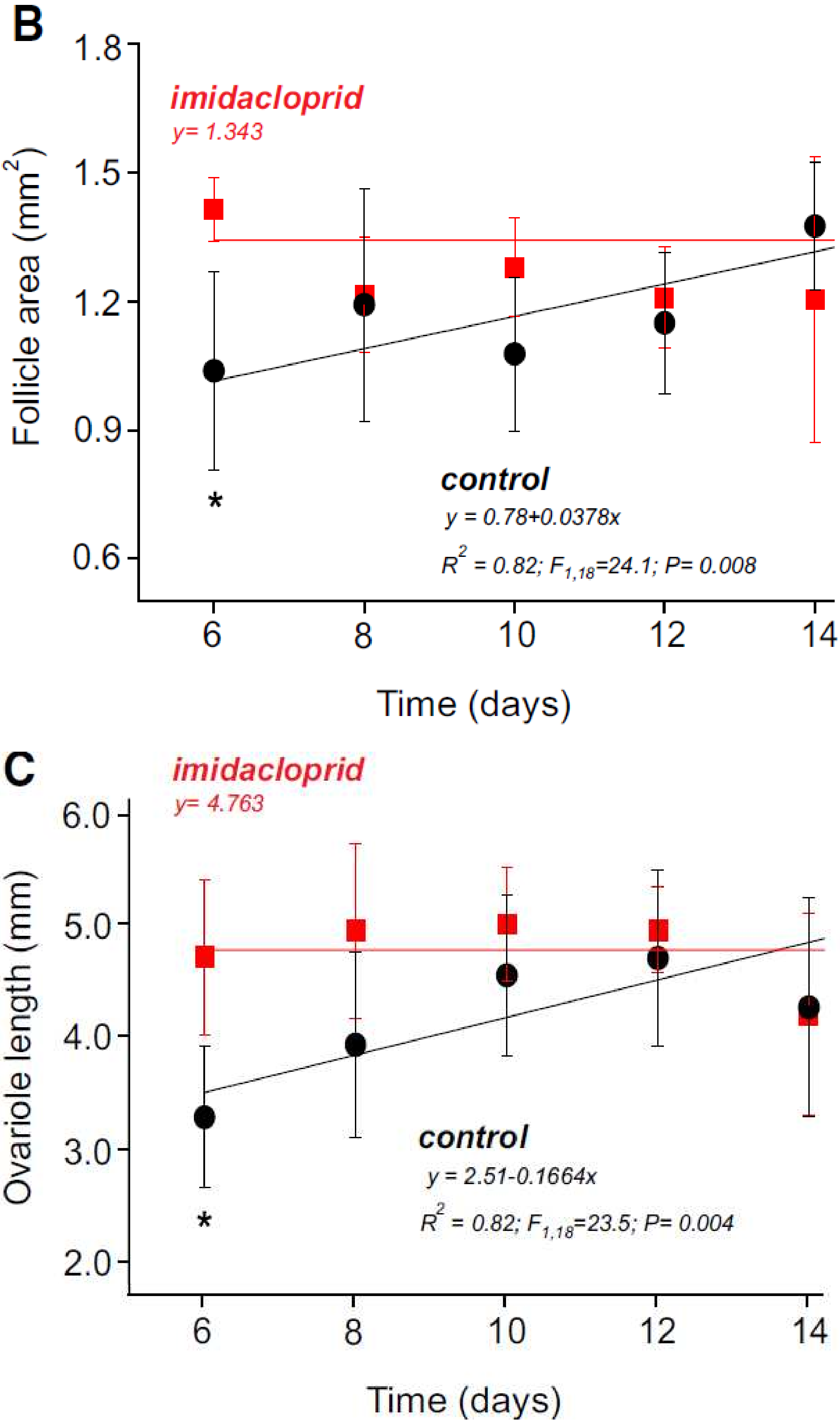


Fig. 3. Effects of imidacloprid on *E. heros* ovary morphometry. The number of follicles per ovary (***A***), the follicle area (***B***) and the length of median ovariole (***C***) were measured in unexposed and imidacloprid-exposed females. The imidacloprid concentration used was 0.042 μg a.i./cm^2, and the females were exposed to imidacloprid for 48 h. The symbols represent the average of at least seven

replicates and the vertical bars represent the SD. Asterisks indicate significant differences between unexposed and imidacloprid-exposed females (Tukey's HSD test; $P < 0.05$).

3.3 Cell damage

The general appearance of the ovarian cells of unexposed and imidacloprid-exposed *E. heros* females stained with HE is shown in Fig. 4. No morphological changes were observed in the prefollicular tissue (including the tropharium, the prefollicular tissues, and the previtellogenic oocytes) or in the vitellogenic follicles of the ovaries from imidacloprid-exposed females. The arrangement of the cells was the same for the ovaries of both unexposed and exposed females. Although the visual inspection of the hematoxylin-eosin (HE) stained sections did not reveal evidence of any morphological changes, the ovaries from exposed females exhibited a significantly higher number of damaged cells (with fragmented nuclear DNA) up to the 4th day after emergence compared with the ovaries from unexposed females ($F_{1,26} = 7.32$, $P = 0.013$) (Fig. 5).

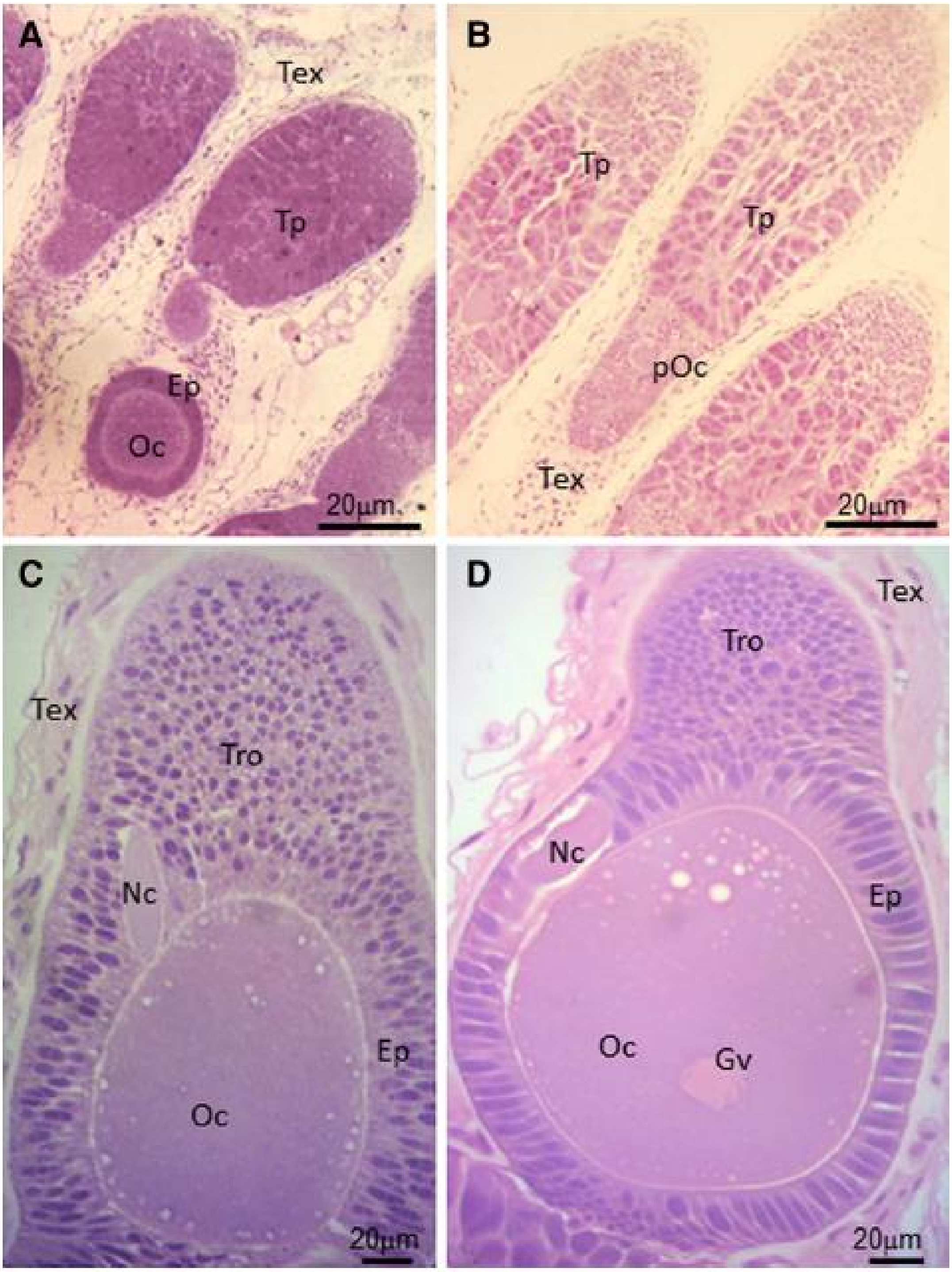


Fig. 4. Hematoxylin and eosin (HE) staining of ovarian tissues in unexposed (***A*** and ***C***) and imidacloprid-exposed *E. heros* females (***B*** and ***D***). ***A*** and ***B*** depict the distal portion of the ovarioles of 8-day-old females; ***C*** and ***D*** depict the proximal portion of the ovarioles of 10-day-old females. Ep- follicular epithelium; Gv- germinal vesicle; Nc- Nutritive cord; pOc- previtellogenic oocyte; Tex- External tunic; Tp- tropharium; Oc- oocyte.

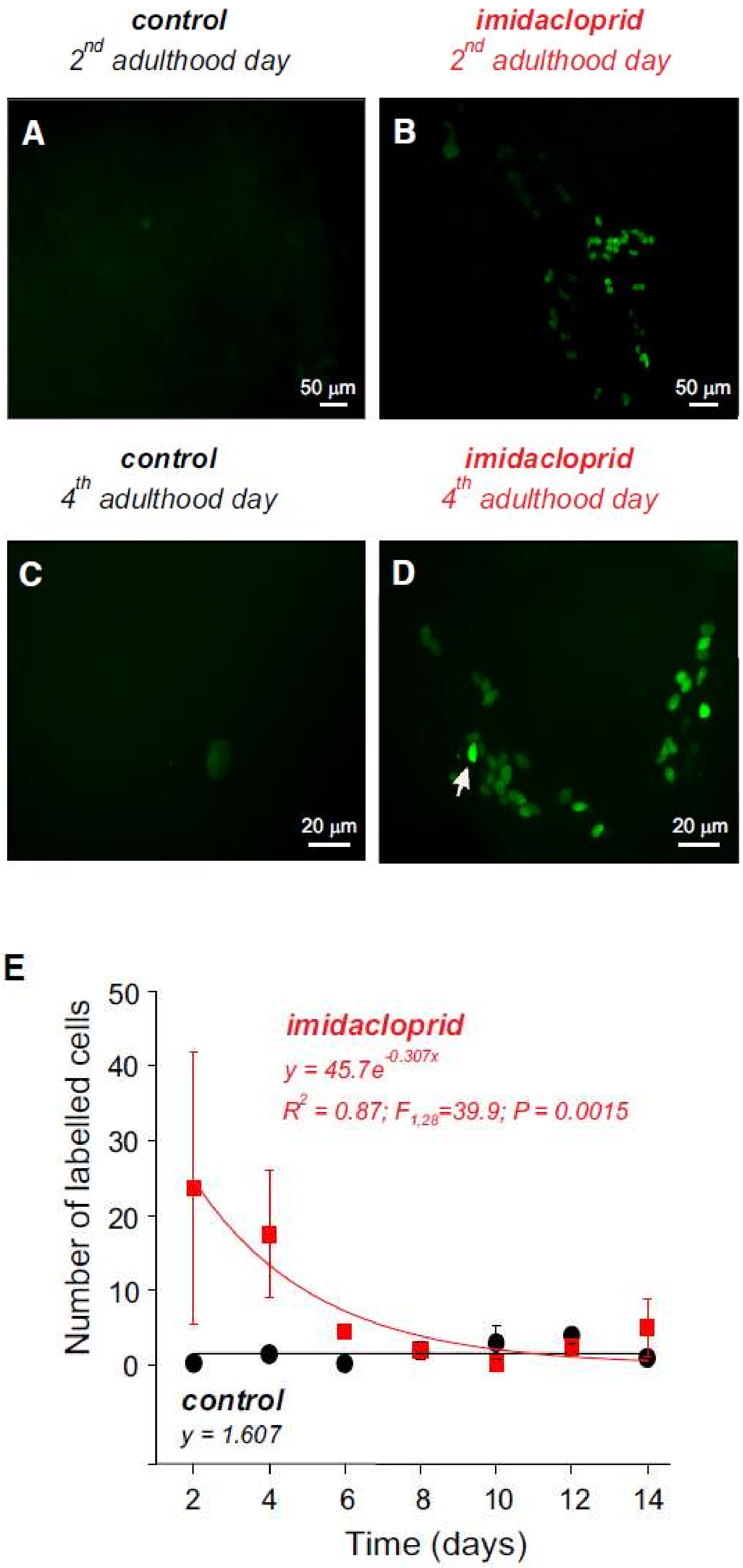


Fig. 5. Labeling of *E. heros* ovarian cell nuclei with fragmented DNA. ***A-D***. The ovarian structure of unexposed (***A*** and ***C***) and imidacloprid-exposed females (***B*** and ***D***). The arrow in (***D***) indicates an ovarian cell nucleus with fragmented DNA. ***E.*** Quantitative determination of ovarian cells with

fragmented nuclear DNA up to 14 days after emergence. For the imidacloprid-exposed females, the exposure time was 48 h, and the imidacloprid concentration used was 0.042 µg a.i./cm^2. The symbols represent the average of at least seven replicates, and the vertical bars represent the SD.

3.4 Fecundity and fertility responses

The results of the regression analyses for each treatment showed that imidacloprid exposure positively stimulated the fecundity and fertility rates of *E. heros* females (Fig. 6). Imidacloprid-exposed females showed higher daily fecundity rates from the beginning of the oviposition period compared with unexposed females (Fig. 6A). Such stimulatory effects on the fertility rate of imidacloprid-exposed females began at approximately 10 days after the start of the oviposition period (i.e., approximately 25 days after adult emergence), but the trend remained throughout the life of the female (Fig. 6B).

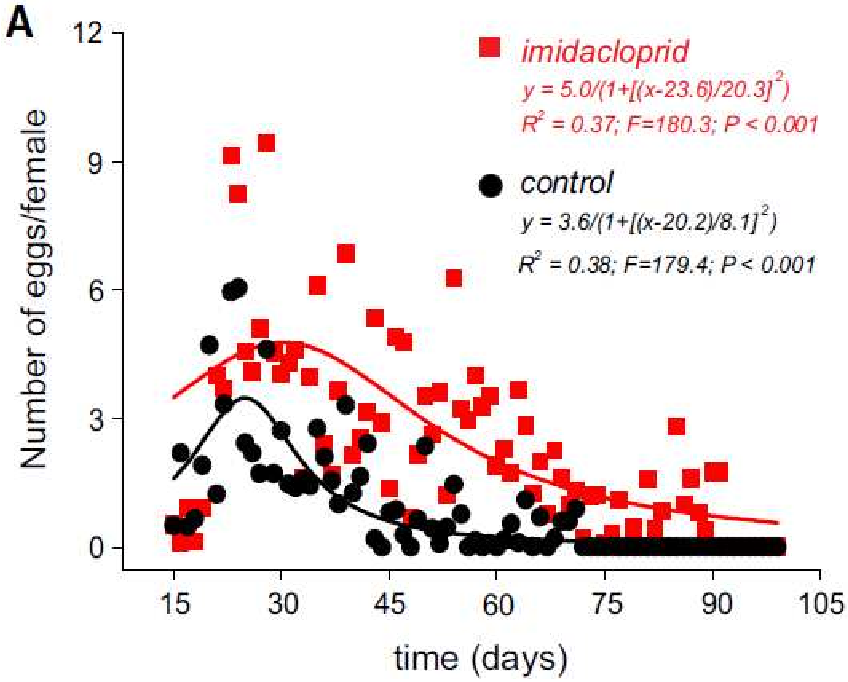


Fig. 6. Effects of imidacloprid on fecundity (***A***). The daily fecundity result was registered up to 95 days after 48 h of exposure to imidacloprid (0.042 µg a.i./cm^2). Lines represent the fit of daily fecundity (***A***) result. Symbols represent the mean observed results.

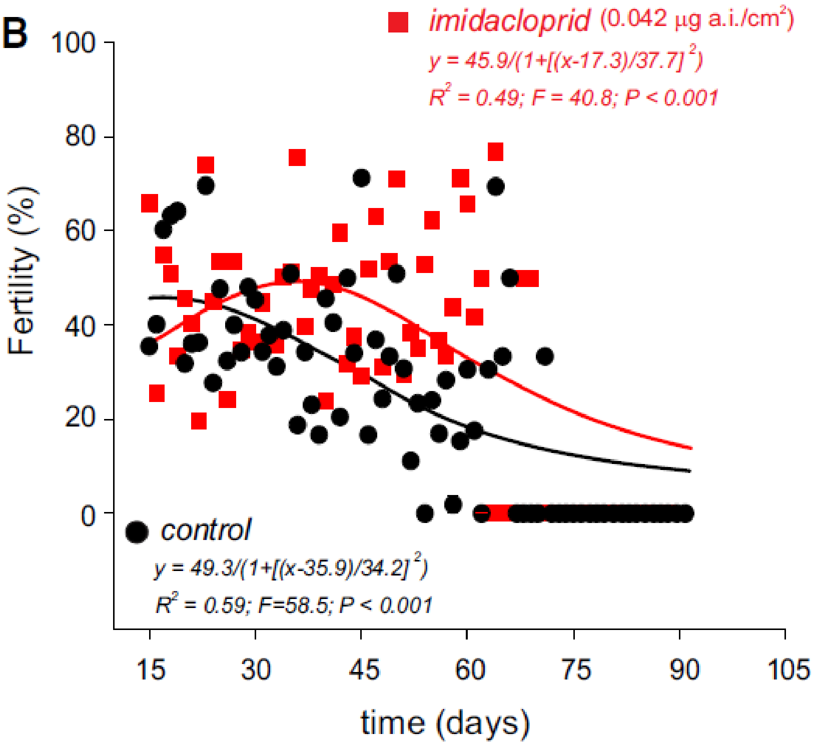


Fig. 6. Effects of imidacloprid on fertility (***B***) of *E. heros*. The daily fertility result was registered up to 95 days after 48 h of exposure to imidacloprid (0.042 µg a.i./cm^2). Lines represent the fit of daily fertility (***B***) result. Symbols represent the mean observed results.

4. Discussion

Here, we showed that sublethal exposure to imidacloprid affected the survival-fertility trade-off in *E. heros* females. Sublethal exposure of the newly emerged females to the neonicotinoid insecticide reduced their survival but favored the earlier development of their reproductive tract and enhanced their reproductive output.

Although imidacloprid does reduce natural insect populations through lethal means, sublethal exposure to this compound has been linked to increased reproductive responses in insects and mites (James and Price 2002, Wang et al. 2008, Yu et al. 2008, Cutler et al. 2009, Yu et al. 2010, Szczepaniec and Raupp 2013). Such a biphasic dose response of a chemical is termed insecticide hormesis, or insecticide-induced hormesis (Calabrese and Baldwin 2003, Calabrese et al. 2008, Cutler 2013, Jager et al. 2013, Guedes and Cutler 2014). The secondary actions of imidacloprid on oogenesis, on the muscle fibers of the reproductive tract, an on the hormonal status of females have been suggested as possible causes of changes in oviposition (Charpentier et al. 2010), which can result in increased reproductive output. Here, we observed not only a faster ovarian development (larger follicle area and longer ovariole length) on imidacloprid-exposed females but also higher fecundity and fertility of imidacloprid-exposed females. Such changes in the reproductive responses might reflect changes in hormonal status, as hypothesized by Charpentier et al. (2010).

Alternatively, increases in *E. heros* reproduction might be the result of compensatory effects for the reduced survival caused by imidacloprid side-effects. A shorter life-span has been reported in insect pests sublethally exposed to insecticides (Lee et al. 1998, Lee 2000, Tan et al. 2012, Vilca Mallqui et al. 2014). In addition to its distinct pharmacological actions on insect nicotinic receptors (nAChRs), imidacloprid causes cell damage in the insect midgut, salivary glands, mushroom bodies and ovaries (Benzidane et al. 2011, Gregorc and Ellis 2011, Tomé et al. 2012, Rossi et al. 2013). These effects of imidacloprid have also been reported in the reproductive tracts of vertebrates (Kapoor et al. 2011, Bal et al. 2012, Cardone 2014, Hoshi et al. 2014). The sublethal concentrations of insecticides can also cause changes in insect cell morphology (Braeckman et al. 1997), which in *Spodoptera litura* (Lepidoptera: Noctuidae) resulted in reductions of oocyte size and follicular epithelium thickness (Perveen 2000).

The higher number of damaged cells observed during the initial ovarian development of insecticide-exposed *E. heros* females might indicate that imidacloprid side-effects compromised the survival of *E. heros* but also elicited reproductive compensatory effects. However, such trade-offs might not always be present because ovary development in other insects may be somewhat resilient to pesticide intoxication (Laycock et al. 2012). In bumble bees, environmentally realistic doses of imidacloprid (1 µg of a.i./L) significantly reduced brood production without any impairment in ovary development (Laycock et al. 2012). Furthermore, sublethal insecticide exposure can also reduce ovarian weight, the number of mature eggs, and insect pest fertility (Lee 2000, Perveen 2000, Liu et al. 2013), which are all negative effects.

Thus, our results showed that *E. heros* females increased their reproductive output to overcome imidacloprid-induced sublethal stress. These findings suggest a potential link between imidacloprid sublethal exposure and the recent outbreaks of *E. heros* observed in Brazilian soybean fields. Although not addressed here, further investigations attempting to elucidate the physiological mechanisms that elicit such changes in the survival-fertility trade-off of *E. heros* females need to be performed. The effects of imidacloprid on ovary contents (e.g., protein, lipids, and carbohydrates), and in the endocrine control of the ovarian development, will certainly provide new insights to explain these processes.

Acknowledgments

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CHAPTER 2

Non-targeted insecticidal stress on the Neotropical brown stink bug *Euschistus heros*

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ABSTRACT

Lethal and sublethal insecticide effects on non-targeted pest species are frequently neglected but have potential consequences for pest management and secondary pest outbreaks. Here, the lethal and demographic effects of four soybean insecticides (chlorantraniliprole, deltamethrin, pyriproxyfen, and spinosad, which are used against caterpillars, whiteflies, and green stink bugs) on the brown stink bug *Euschistus heros* (F.), the main stink bug species currently attacking Neotropical soybean fields, were assessed. Deltamethrin exhibited drastic acute mortality in adult females of *E. heros* with a median lethal time (LT_{50}) of 0.6 days, whereas pyriproxyfen exhibited a survival similar to that of water-treated insects (LT_{50} of 89 and 67 days, respectively). Chlorantraniliprole and spinosad-treated females exhibited intermediate survival with a TL_{50} of 54 and 47 days, respectively. Further scrutiny of the three most selective insecticides (i.e., chlorantraniliprole, pyriproxyfen, and spinosad) using age-structured matrices to assess the demographic impact of these compounds under earlier exposure indicated that both pyriproxyfen and spinosad significantly compromised the population growth of the pest species by compromising the survival of eggs (< 17% hatching) and 1st nymphs (LT_{50} of 3 and 2 days, respectively) and fertility; these results were unlike those obtained for chlorantraniliprole- and water-treated controls, which exhibited less than 50% mortality during development. However, chlorantraniliprole significantly compromised the fertility of adult insects (> 50% reduction compared with water-treated insects) to a greater extent than pyriproxyfen and spinosad, but without impairing as much the insect population growth. Therefore, although pyriproxyfen and spinosad, besides deltamethrin, are not used to target the Neotropical brown stink bug, these insecticides particularly compromised this pest species at the dose recommended for application on the label, thereby preventing its outbreak under such conditions.

Keywords: Non-target effects; secondary pest outbreaks; median lethal time; insecticide selectivity; soybean stink bug

1. Introduction

Insecticides are crop protection tools that are used to reduce the damage caused by particular pest species, usually by reducing their local population while minimizing the impact on non-targeted species (Desneux et al., 2007; Guedes et al., 2016). Nonetheless, non-targeted species will be exposed to potentially lethal and, more frequently, sublethal concentrations of the insecticide applied, and these species can be affected by such exposure. These responses are the subject of continuous attention (Casida and Durkin, 2013; Desneux et al., 2007), but this concern tends to be focused on natural enemies (Casida and Durkin, 2013; Desneux et al., 2007; He et al., 2013) and pollinators (Arena and Sgolastra, 2014; Barbosa et al., 2015a; Godfray et al., 2014), rather than on non-target pest species (Guedes et al., 2016).

Label rates of insecticide compounds, while ideally reducing the population and damage caused by a target pest species, can have lethal and/or sublethal effects on non-targeted species and can either compromise or favor them (Cutler and Rix, 2015; Guedes et al., 2016; Guedes and Cutler, 2014). The consequences of these non-targeted exposures range from the suppression of natural enemies and pollinators to pesticide-induced hormesis, including unintended selection for insecticide resistance and shifts in pest dominance (Cordeiro et al., 2014, 2013; Haddi et al., 2015; Hardin et al., 1995). Eventual shifts in ecological dominance that lead to outbreaks or even more persistent changes in pest status are of particular concern and are rarely anticipated; the underlying causes of such changes remain debatable due to an important knowledge gap regarding the unintended consequences of pesticide use and pest management (Cordeiro et al., 2014; Guedes et al., 2016; Hardin et al., 1995).

The arthropod species assemblage that is associated with Neotropical soybean crop fields provides an interesting scenario to assess non-targeted insecticidal stress. This is true because this crop production system continues to undergo changes in this region, which currently exhibits prevalent no-tillage cultivation and the use of cultivars with pyramided transgenic events, including stacked genes expressing *Bacillus thuringiensis* toxins (Bernardi et al., 2014a, 2014b). One result of such changes is the steady loss of the velvetbean caterpillar (*Anticarsia gemmatalis* Hübner) importance as a soybean (key) pest species; this loss began in mid-Brazil and spread southwards (Gallo et al., 1988, 2002; Panizzi, 2013). Other defoliating caterpillar species, whitefly (*Bemisia* *tabaci* (Gennadius)) and stink bugs are currently the focus of attention as the main soybean pest species in the Neotropics (Panizzi and Corrêa-Ferreira, 1997; Gallo et al., 2002; Panizzi 2013).

In addition to the soybean looper (*Chrysodeixis includens* Walker) and budworms (*Heliothis virescens* (F.) and *Helicoverpa armigera* (Hübner)), the velvetbean caterpillar appears to have been strongly affected by Bt toxins expressed in soybean cultivars that are currently used in the Neotropics. These toxins do not strongly affect armyworms (Bernardi et al., 2012, Bernardi et al., 2014), allowing a shift in the ecological dominance towards the latter (Bernardi et al., 2014). Whitefly and stink bugs are not directly affected by the Bt toxins currently expressed in soybeans, and their importance has been steadily growing as pest species of soybean in recent decades (Bueno et al., 2015; Panizzi, 2013; Silva et al., 2014). Even among stink bugs in soybean, the earlier prevalence of the southern and the small green stink bugs (*Nezara viridula* (L.) and *Piezodorus guildinii* (Westwood), respectively) has been replaced by that of the Neotropical brown stink bug *Euschistus heros* (F.) (Panizzi and Corrêa-Ferreira, 1997; Gallo et al., 2002; Panizzi 2013; Saluso et al., 2011). Consequently, insecticide use accrued against these pest species; different profiles of use targeted whiteflies and caterpillars earlier in the soybean phenology, and an alternate set of compounds targeted stink bugs later during the plant reproductive period (MAPA 2015).

Insecticide use in soybean against caterpillars, whitefly, and even green stink bugs are likely to affect the Neotropical brown stink bug and its later infestation, leading to seed losses; this is a more recent species that is currently spreading and gaining importance from mid-Brazil to southern Brazil and Argentina (Panizzi and Corrêa-Ferreira, 1997; Panizzi 2013; Saluso et al., 2011). Curiously, the impact of insecticides that are used against other soybean pest species on Neotropical brown stink bug remains unknown, and this lack of data is aggravated by the lack of compounds registered against this more recent (key) soybean pest species in the region. Thus, the present study was carried out to assess the potential lethal and sublethal effects, as well as the demographic consequences of the following insecticides, registered against other species, on the Neotropical brown stink bug: chlorantraniliprole, deltamethrin, pyriproxyfen and spinosad (MAPA, 2015). Because deltamethrin is broadly used against stink bugs in soybean, a drastic acute lethal effect of this pyrethroid was expected. In contrast, chlorantraniliprole and even spinosad were thought to be more selective because these well-known insecticides are used against soybean caterpillars with potentially more important sublethal effects on the Neotropical brown stink bug (Salgado, 1998; Sattelle et al., 2008; Sparks et al., 2001; Casida and

Durkin, 2013). Pyriproxyfen targets sucking insects, particularly whiteflies, and is likely to exhibit non-target effects on bugs; however, these effects are more likely to be sublethal (Hatakoshi, 2013).

2. Material and methods

2.1. Insects

The laboratory colony of the Neotropical brown stink bug was initially established from 1,000 individuals obtained from a colony maintained at EMBRAPA Genetic Resources and Biotechnology (Brasília, DF, Brazil). Field-collected individuals from soybean farms located in the county of Tangará da Serra (Mato Grosso, Brazil) and from soybean experimental fields of the Federal University of Viçosa (Viçosa, Minas Gerais, Brazil) were periodically introduced into the colony to enhance the genetic variability of the insects used in the bioassays. The colony was reared following previously described methods (Borges et al., 2006; Silva et al., 2008). The colony was maintained under the same conditions used for the experiments: 27 ± 2°C, 75 ± 5% relative humidity, and 14:10 (L:D)-h photoperiod.

2.2. Insecticides

Four insecticides registered for soybean use in Brazil were used in the experiments at their maximum field recommended rates and appropriate spraying volumes for this crop in the country (MAPA, 2015). The following insecticides were used at the indicated concentrations and spraying volumes: chlorantraniliprole at 0.68 µg a.i./cm^2 (200 g a.i./L at the spraying volume of 150 L/ha; suspendable concentrate; DuPont, Paulínia, SP, Brazil); deltamethrin at 0.95 µg a.i./cm^2 (25 g a.i./L at the spraying volume of 80 L/ha; emulsifiable concentrate; Bayer CropScience, São Paulo, SP, Brazil); pyriproxyfen at 1.02 µg a.i./cm^2 (100 g a.i./L at the spraying volume of 300 L/ha, emulsifiable concentrate, Sumitomo Chemical, São Paulo, SP, Brazil); and spinosad at 2.44 µg a.i./cm^2 (480 g a.i./L at the spraying volume of 100 L/ha; suspendable concentrate; Dow AgroSciences, São Paulo, SP, Brazil).

2.3. Time-mortality bioassays

Insecticide exposure was achieved by exposure to a glass surface that was impregnated with dried insecticide residue, as previously described (Santos et al., 2015). Briefly, the inner walls of 250-mL transparent glass containers (EME Equipamentos, Paulicéia, SP, Brazil) were impregnated with 2 mL of insecticide solution using water as the solvent (distilled and deionized). The inner portion of the top of each container was coated with Teflon® PTFE (DuPont, Wilmington, DE, USA) to prevent the insects from escaping, and the containers were closed with a piece of organza and a rubber band. The inner container walls were coated with insecticide using a heavy-duty rotator (Roto-Torque model 7637, ColeParmer, Vernon Hills, IL, USA) until the coating had dried. A control treatment (only water was applied) was used to assess natural mortality. Ten female adults (≤ 24 h old) were placed in each container and exposed for 24 h to the dried insecticide residues; three replicates were used for each treatment and the water-treated control**.** After exposure, the females were removed and individually placed in 250-mL plastic containers with *ad libitum* provision of a mixture of soybean, peanut and sunflower seeds together with bean pods, which were replenished every other day. Mortality was assessed at 20-min intervals when testing deltamethrin and at 24-h intervals when testing the other insecticides and the water-treated control; the assessment was terminated when all of the insects had died. The insects were recognized as dead if they were unable to walk the length of their body when prodded with a fine-haired brush.

2.4. Adult longevity and fertility bioassays

The insecticides exhibiting only low acute mortality in the time-mortality bioassays were subjected to further scrutiny that assessed female longevity and fertility; a water-treated control was used to assess natural mortality. For this purpose, between 40 and 56 3rd instar insects (≤ 24 h old) were subjected to insecticide treatment as previously described and were separately placed in 250-mL plastic containers and provided with the same diet under the same conditions as those previously described. Third instar insects were used because during this phase, Neotropical brown stink bugs begin to feed on seeds in the field. Upon reaching the adult stage, insects of both sexes were paired within their respective treatments and their longevity, fecundity and fertility were assessed daily until eventual death. Between 20 and 25 couples were used per treatment to evaluate the reproductive parameters. The number of eggs laid was daily recorded for each female, and egg hatching was also subsequently recorded to allow fertility estimation (i.e., nymphs produced/couple/day).

2.5. Age-structured matrix bioassays

An age-structured matrix model was used for four insecticidal treatments (chlorantraniliprole, pyriproxyfen, spinosad, and a water-treated control). All developmental stages of the Neotropical brown stink bug were included (i.e., egg, nymph, and adult). The assessment of each stage started with between 20 and 30 individuals. Individual eggs (24-hs old) were treated by immersion in the appropriate insecticide solution, and nymphs (1^{st} instar) and adults (females) were treated as previously described via 24-h contact with a glass surface coated with dried insecticide residue. The survival and duration of each developmental stage was recorded, as was the fertility of each couple that reached the adult stage. The data obtained was used to build age-structure matrices using the software PopTools 3.2.2 (Hood, 2010).

The resulting projection matrices comprise a set of upper diagonal elements (P_i), which refer to the probability of an individual surviving and remaining in that life stage, and a set of lower diagonal elements (G_i), which refer to the probability of an individual surviving and passing to the next life stage (Keyfitz and Caswell, 2005). These probabilities are calculated based on the survival (σ_i) and development (γ_i) of each life stage (i) in relation to the time step of the matrix projection (one day), such that $P_i = \sigma_I (1 - \gamma_i)$ and $G_i = \sigma_I \gamma_I$ (Celestino et al., 2014; Vilca Mallqui et al., 2014). This procedure allows estimation of the generation time (T), net reproductive rate (R_o), rate of population increase (r), and population growth for each treatment.

2.6. Statistical analyses

The survival results of the time-mortality bioassays were subjected to survival analysis using Kaplan-Meier estimators (PROC LIFETEST; SAS Institute 2008). The insects that remained alive at the end of the bioassay were censored for the analyses. Overall similarity among the survival and median survival times (LT_{50} values) was tested using the χ^2 log-Rank test, and pairwise comparisons among the curves were tested using Holm-Sidak's test ($P < 0.05$). The dichotomous egg hatching results (i.e., hatched or not) under each insecticidal exposure were subjected to logistic regression (PROC LOGIST; SAS Institute, 2008). Egg incubation period, longevity, fecundity and fertility data were tested for normality and homoscedasticity, and no data transformation was necessary. Therefore, the results were subjected to analyses of variance and Tukey's HSD test ($P < 0.05$), when necessary (PROC GLM; SAS Institute, 2008).

3. Results

3.1. Adult survival

The survival curves obtained from the time-mortality bioassays with female adults of the Neotropical brown stink bugs that were exposed to dried insecticide residues indicated significant differences among the treatments (log-rank test: χ^2 = 150.81, df = 4, P < 0.001) (Fig. 1A). Deltamethrin caused early mortality with a median survival time (LT$_{50}$) of 14.4 hours (95% CL: 14.0-15.0 hours), a value that was significantly different from that obtained in all other treatments (Holm-Sidak's statistics > 6.86, P < 0.03) (Fig. 1B). In contrast, pyriproxyfen-treated females exhibited a survival time that was similar to that of water-treated females (LT$_{50}$ values [95% CL] of 89.0 [58.0-120.0] days and 67.0 [40.6-93.4] days, respectively). Chlorantraniliprole and spinosad exhibited intermediate survival times (LT$_{50}$ values [95% CL] of 54.0 [47.2-60.7] days and 47.0 [37.8-56.2] days, respectively), although these values were not significantly different from obtained for pyryproxyfen and water (Fig. 1B).

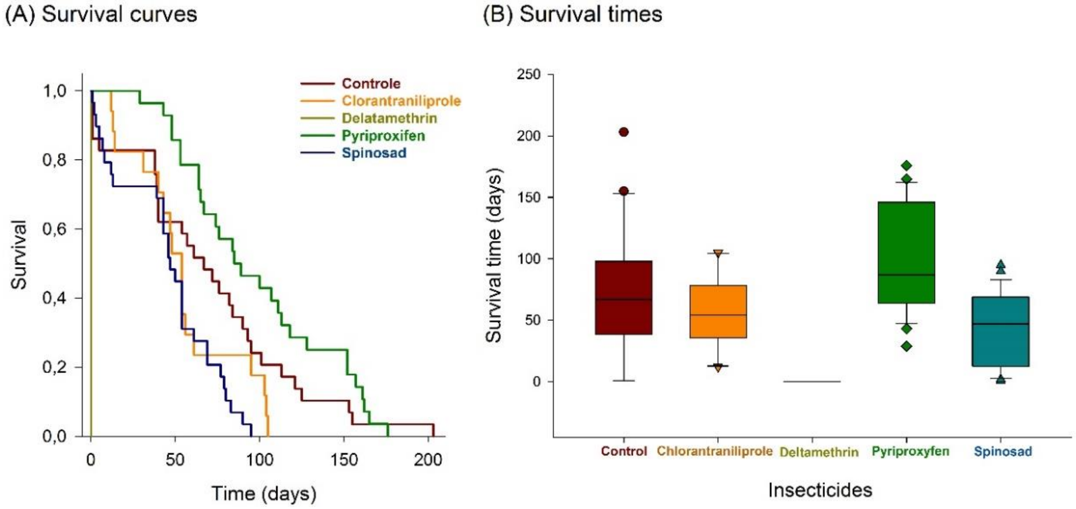

Fig. 1. Survival curves (A) and box plots of the median survival times (LT50 values) (B) of female adults of the Neotropical brown stink bug (*Euschistus heros*) after 24-h contact exposure to dried insecticide residues. Box plots indicate the median and range of dispersion (lower and upper quartiles, and outliers) of the LT50 values. The survival curve and box plot shown for deltamethrin are significantly different from those shown for the other treatments according to Holm-Sidak's test (P < 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Adult longevity and fertility

Mild-to-negligible acute mortality of adult brown stink bug females was caused by chlorantraniliprole, pyriproxyfen and spinosad, but not by deltamethrin. Thus, we subjected these three insecticides to further scrutiny; under sublethal exposure, the female longevity, fecundity and fertility of 3rd instar nymphs were assessed. No insecticide affected longevity significantly, compared with water-treated nymphs ($F_{3,89} = 1.41$, $P = 0.25$); the overall average longevity of the nymphs was 64.70 ± 3.00 days. In contrast, daily fecundity and, in particular, daily fertility were significantly affected by insecticide exposure ($F_{3,89} = 8.42$, $P < 0.001$ and $F_{3,89} = 10.76$, $P < 0.001$, respectively); chlorantraniliprole always caused the greatest reproductive impairment (Figs. 2A and 2B). Although pyriproxyfen and spinosad did not significantly affect daily fecundity, which resembled that of water-treated females (Fig. 2A), both compounds significantly compromised female fertility (Fig. 2B).

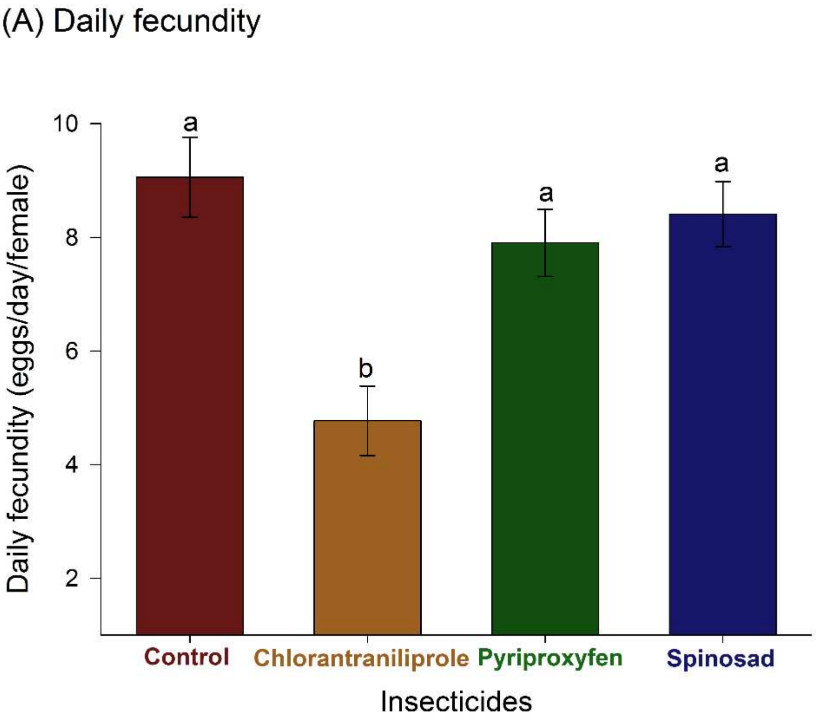

Fig. 2. Daily fecundity (A) (mean ± SE) of the Neotropical brown stink bug (*Euschistus heros*) after exposure to dried insecticide residues. Histogram bars with the same lower case letters are not significantly different according to Tukey's HSD test (P < 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

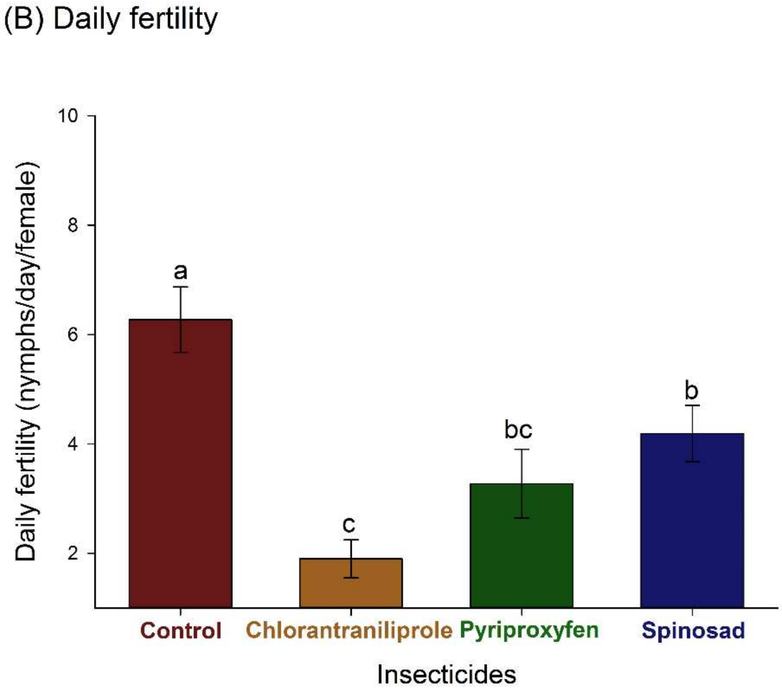

Fig. 2. Daily fertility (B) (mean ± SE) of the Neotropical brown stink bug (*Euschistus heros*) after exposure to dried insecticide residues. Histogram bars with the same lower case letters are not significantly different according to Tukey's HSD test ($P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. Survival and population growth

The three insecticides that were used in the adult longevity and fertility bioassays were also used in age-structured matrices bioassays to assess the demographic consequences of their exposure. Egg survival, unlike adult longevity, was significantly affected by insecticide exposure ($\chi^2 = 55.95$, df = 3, $P < 0.001$); pyriproxyfen and spinosad significantly compromised egg hatching when compared with the water-treated eggs ($\chi^2 > 8.29$, df = 1, $P < 0.004$) (Fig. 3A). Incubation time also differed significantly among the surviving eggs ($F_{3,36} = 12.00$, $P < 0.001$); however, in this case, only pyriproxyfen caused a significant delay in egg hatching (Fig. 3B). Insecticidal exposure of 1st instar nymphs also led to significant differences in survival (log-rank test: $\chi^2 = 22.07$, df = 3, $P < 0.001$); the results showed a median survival time (LT_{50}) of 2.0 hours (95% CL: 0.6 -3.4 hours) for pyriproxyfen-treated insects and of 3.0 hours (95% CL: 2.2 -3.8 hours) for spinosad; these values were not significantly different from each other (Holm-Sidak's statistics > 14.66, $P < 0.001$) but differed from the values obtained for the water-treated and chlorantraniliprole-treated insects (Holm-Sidak's statistics < 3.59, $P > 0.17$). In the latter two treatments, mortality was less than 50% (not allowing estimation of the respective LT_{50} values, Fig. 4).

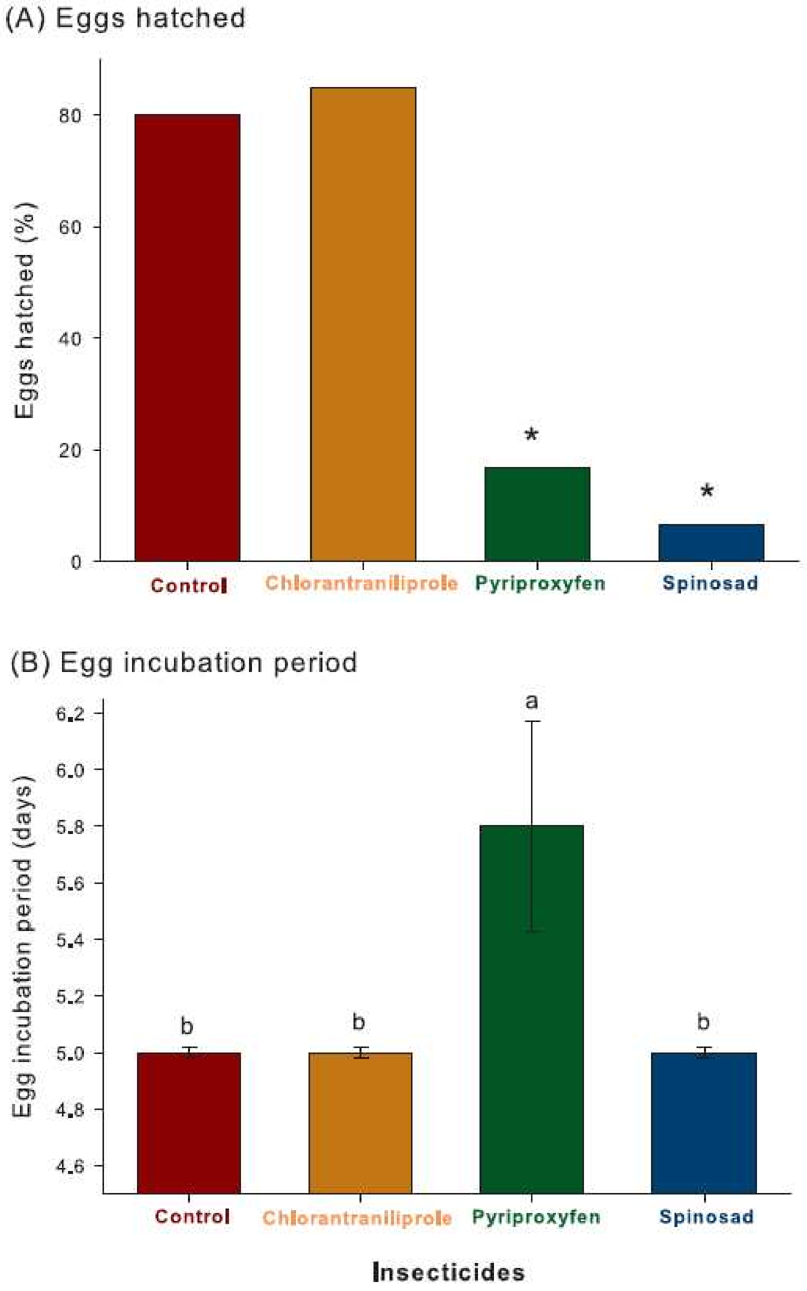

Fig. 3. Egg hatching (%) (A) and egg incubation period (B) of the Neotropical brown stink bug (*Euschistus heros*) after exposure to dried insecticide residues. An asterisk above the histogram bars representing the percent of eggs hatched (A) indicates a significant difference from the water-treated insects (control) according to a c2 test. Histogram bars representing the egg incubation period (mean ± SE) (B) with the same lower case letters are not significantly different according to Tukey's HSD test ($P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

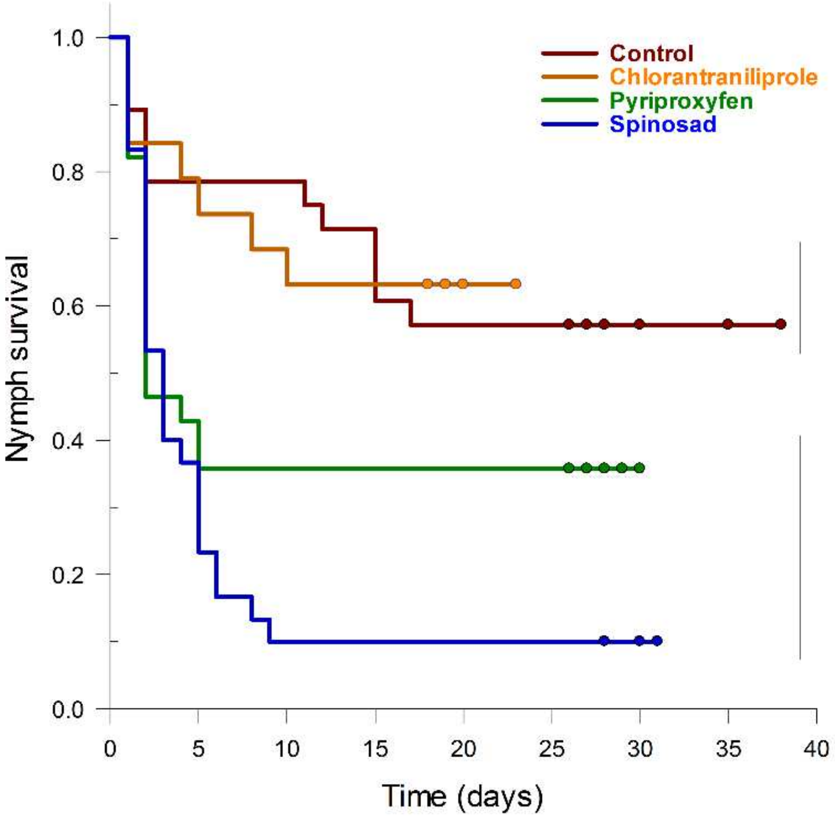

Fig. 4. Survival curves of the Neotropical brown stink bug (*Euschistus heros*) after exposure to dry insecticide residues as 1st instar nymphs. The symbols at the end of each curve indicate censored data. The curves encompassed by the same vertical bar at the left side of the plot are not significantly different according to Holm-Sidak's test ($P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The consequences of survival and fertility were expressed in the age-structured matrices; each insecticidal treatment led to a distinct outcome. The generation time (T) ranged from 17.15 days for the chlorantraniliprole-treated insects to 30.19 days for the pyriproxyfen-treated insects, while spinosad resembled the generation time of the water-treated insects (18.27 and 17.89 days, respectively). The range of variation was even higher for the net reproductive rate (R_o): 0.47, 1.28, 4.17 and 5.80 for the spinosad-, pyriproxyfen-, chlorantraniliprole- and water-treated insects, respectively. These differences in T and R_o led to rates of population growth (r) that varied from -0.043 and 0.008 for spinosad- and pyriproxyfen-treated insects, respectively, to 0.083 and 0.096 for chlorantraniliprole- and water-treated insects, respectively. Consequently, the population growth projection was steeper for the water-treated insects, followed by the chlorantraniliprole-treated insects. In contrast, only marginal population growth was observed for pyriproxyfen-treated insects, and population suppression was observed for spinosad-treated insects (Fig. 5).

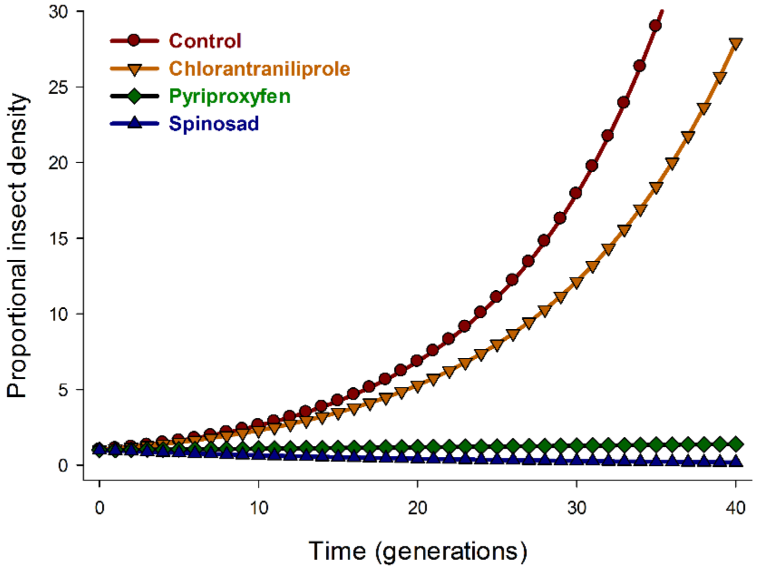

Fig. 5. Population growth projections for insecticide-treated Neotropical brown stink bugs (*Euschistus heros*) based on an age-structured matrix model. Population density includes all developmental stages of the pest species (egg, nymph, and adult). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

We hypothesized that among the four insecticides tested, the pyrethroid deltamethrin was the most likely to cause significant acute mortality in the Neotropical brown stink bug because this insecticide is already registered for use against other stink bugs, particularly the southern and the small green stink bugs (MAPA, 2015). The remaining three insecticides tested, namely chlorantraniliprole, pyriproxyfen, and spinosad, were expected to be more selective, but might exhibit sublethal effects on the brown stink bug based on their activity profiles and use recommendations in Brazil (Crouse et al., 2013; Hatakoshi, 2013; Lahm et al., 2013; Sparks et al., 2001). The results obtained here support these expectations; however, the sublethal effects observed were stronger than anticipated, particularly for pyriproxyfen and spinosad.

Deltamethrin exposure led to rapid death of all exposed insects within 24 hours. This result is consistent with the efficacy and recommendation of this insecticide for use against the southern and small green stink bugs, which remain important soybean pests although they have lost their status of key pest to the Neotropical brown stink bug in recent years (Panizzi and Corrêa-Ferreira, 1997; Gallo et al., 2002; Panizzi 2013). The rapid activity exhibited by deltamethrin is also consistent with its mode of action: modifying the gating kinetics of sodium channels and thereby disrupting normal electrical signaling in nerve cells (Casida and Durkin, 2013). In contrast, the other three tested insecticides exhibited negligible acute mortality effects on the treated adults, thus warranting the study of their potential delayed and/or sublethal effects because such effects can occur and would have important consequences for pest populations and their management (Hardin et al., 1995; Guedes and Cutler, 2014; Guedes et al., 2016).

The effects of chlorantraniliprole, pyriproxyfen, and spinosad on the Neotropical brown stink bug were diverse. The insecticides did not compromise adult survival and longevity under acute short-term exposure but did compromise female fecundity when 3rd instar nymphs were exposed; this effect was particularly strong for chlorantraniliprole. In contrast, pyriproxyfen and spinosad led to high mortality in eggs and exposed 1st instar nymphs, and their longer generation time coupled with a low net reproductive rate caused very low or negative population growth rates. Thus, pyriproxyfen almost completely prevents population growth in the Neotropical brown stink bug, and spinosad suppresses the population of this species; subsequent generations were subjected to a higher control efficacy than anticipated, although this effect was delayed when compared with that of deltamethrin.

Chlorantraniliprole, pyriproxyfen, and spinosad are recommended for caterpillars, whiteflies control (MAPA, 2015) the first pest species to colonize soybean plants with infestations potentially lasting until the plant reproductive period overlapping with incidence of stink bugs (Bueno et al., 2013; Corrêa-Ferreira, 2005). This scenario allows simultaneous control of caterpillars and/or whiteflies, in addition to preventing stink bug outbreaks in soybean fields.

Chlorantraniliprole is an anthranilic diamide that acts by directly activating ryanodine receptors; these Ca^{2+}-activated calcium channels are located in the sarcoplasmic reticulum of muscle cells and stimulate massive receptor-mediated Ca^{2+} release from intracellular stores, leading to muscle contraction (Casida and Durkin, 2013; Lahm et al., 2013). Chlorantraniliprole targets mainly include caterpillars, although the compound also exhibits efficacy against sucking insect pests; however, the relatively low plant systemicity of this insecticide limits its use against the latter pest type, unlike the related insecticide cyantraniliprole (Lahm et al., 2013). In the Neotropical brown stink bug, chlorantraniliprole exerted a stronger effect on daily fertility, possibly due to its muscle activity and/or a secondary, as yet unreported action. However, the demographic effect of this insecticide on the Neotropical brown stink bug was mild and did not significantly impact population growth and control, in contrast with pyriproxyfen and spinosad. We speculate that cyantraniliprole, given its improved plant systemicity (Lahm et al., 2013), might exhibit better control of the Neotropical brown stink bug.

Demographic parameters, particularly estimates of population growth, are more robust and are thus preferable toxicological endpoints because they provide a more comprehensive indication of pesticide stress on exposed populations (Forbes and Calow, 1999; Guedes et al., 2016; Stark and Banks, 2003). Therefore, the significant impairment of population growth in the Neotropical brown stink bug caused by pyriproxyfen and spinosad are clear indications of the impact of these insecticides on this non-targeted pest species; the former compound prevents population growth, and the latter suppresses the pest population. Such effects however, are likely achieved differently by each compound. The modes of action of these insecticides are distinct, and whereas the bioinsecticide spinosad exhibits a broad range of activity against arthropods, a source of recent controversy regarding its alleged selectivity for non-target species (Barbosa et al., 2015a; Barbosa et al., 2015b; Biondi et al., 2012; Tomé et al., 2015), pyriproxyfen exhibits a more restricted range of targets with an emphasis on whiteflies, scales, thrips and psyllids (Hatakoshi, 2013).

Spinosad is a neurotoxic insecticide that primarily acts as an allosteric modulator of (non-desensitizing) nicotinic acetylcholine receptors (nAChR), whereas pyriproxyfen is a growth regulator that acts as a juvenile hormone mimic (Casida and Durkin, 2013; Crouse et al., 2013; Hatakoshi, 2013; Salgado and Saar, 2004; Sparks et al., 2001). The delayed activity of spinosad on the brown stink bug, and only under the exposure of eggs and 1st instar nymphs, is therefore surprising because neurotoxic action is usually quicker and not as selective as other types of action. However, recent evidence that spinosad appears to be selective for specific nAChR subtypes and also exhibits (secondary) activity on certain types of γ-aminobutyric acid (GABA) receptors in nervous synapses indicates that in addition to differential exposure/penetration and metabolic detoxification, such secondary sites might be important for its pesticidal activity (Crouse et al., 2013) (particularly on non-targeted species such as the brown stink bug) if these target sites prevail in this species at the mentioned developmental stage.

The delayed activity of pyriproxyfen is easier to understand because this compound exhibits activity towards sucking insects that varies with the species (Hatakoshi, 2013). Regardless, pyriproxyfen activity is usually stronger and compromises egg hatching, metamorphosis, and adult fertility while interfering with mimic JH activity and interfering with prothoracicotropic hormone (PTTH) and ecdysteroid release (Hatakoshi, 2013). Egg hatching and adult fertility of the Neotropical stink bug were indeed compromised after pyriproxyfen exposure. In addition, the exposure of early nymphs to pyriproxyfen is known to prevent further development (Hatakoshi et al., 2013), as observed here with 1st instar nymphs of the Neotropical brown stink bug, thus enhancing the negative impact of the insecticide on this pest species.

In summary, deltamethrin led to significant mortality and rapid control of the Neotropical brown stink bug, whereas chlorantraniliprole exhibited only a mild demographic impact on this pest population. Curiously, pyriproxyfen and especially spinosad exhibit strong and delayed demographic impacts on the Neotropical brown stink bug, thereby preventing population growth and even causing population suppression in subsequent generations. These effects were observed at sublethal acute exposure levels, which were strong enough to prevent outbreaks of this species. However, it is worth assessing a broader range of sublethal concentrations because the insects will be exposed to these concentrations during the course of insecticide degradation in the environment. Sublethal concentrations can induce the population growth of pest species, a phenomenon that is termed insecticide-induced hormesis (Cutler, 2013; Guedes and Cutler, 2014) and that potentially leads to pest outbreaks (Cordeiro et al., 2013; Guedes et al., 2016; Hardin et al., 1995). Understanding the hormetic range of concentrations and the rate of insecticide (environmental) degradation will allow the risk of undesirable outbreaks to be minimized.

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CHAPTER 3

Non-targeted insecticidal stress in a pest species: insecticides, sexual fitness, and hormesis in the Neotropical brown stink bug *Euschistus heros*

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Short running title: Non-targeted insecticidal stress on *Euschistus heros.*

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Abstract

An understudied aspect of insecticides is their stress on non-targeted pest species. Sublethal insecticidal stress may elicit a range of protective and non-protective responses that may affect behavior and sexual fitness of the exposed insects, which may lead to negative, neutral, or stimulatory (i.e., hormetic) responses. We assessed the behavioral response of the Neotropical brown stink bug, *Euschistus heros*, a soybean pest in the Neotropical region with increasing pest status, following exposure to chlorantraniliprole, pyriproxyfen, and spinosad, insecticides commonly used against soybean caterpillars and whiteflies. Both individuals, or only the male or female of each mating pair, were exposed. Reproductive behavior and output were measured to determine insecticide- and gender-mediated fitness. We found that treatment scenario significantly affected mating behavior, and that the duration of some behaviors were significantly affected. Chlorantraniliprole and pyriproxyfen reduced latency to mate, while spinosad increased this behavior. Insecticide exposure also decreased the interacting time of each couple and male antennation of the female. Fertility table analyses of exposed couples indicated negligible effect of pyriproxyfen exposure, while spinosad extended generation time and reduced net reproductive rate, leading to lower rates of population growth of the brown stink bug. In contrast, chlorantraniliprole led to only a slight extension on the generation time, but enhanced net reproductive rate of the stink bug leading to higher rates of population growth; no effects on sexual fitness were observed. Latency to mate correlated significantly with the population growth rate. The positive response to chlorantraniliprole exposure reinforces the notion that sublethal exposure of the brown stink bug to this insecticide may lead to stimulatory (hormetic) response favoring its outbreaks in soybean fields.

Keywords

Soybean stink bug; non-target stress; secondary pest outbreak; insecticide selectivity; insecticide hormesis.

Introduction

The usefulness of insecticides as a pest management tool is widely recognized (Metcalf, 1980; Cooper & Dobson, 2007; Matthews, 2008), as are the potential backlashes associated with such a use (Hardin et al., 1995; Matsumura, 2004; Köhler et al., 2013). Among these backlashes, insecticide resistance and impacts on non-target species are the usual focus. The latter concern is usually centered on natural enemies and pollinators (Desneux et al., 2007; Barbosa et al., 2015; Lima et al., 2016), but insecticides also stress detritivores and decomposers, as well as non-target arthropod pest species (Guedes et al., 2016, 2017).

Sublethal insecticidal stress on non-target arthropod pest species is seldom studied, although such impacts and potential consequences have been recognized since the 1950's (Ripper, 1956; Metcalf, 1980; Hardin et al., 1995; Guedes et al., 2016). Such exposure will often elicit behavioral and/or physiological responses on non-target pests. Behavioral responses are noteworthy since arthropod behavior is basically an integrated result of the changes in the organism's physiology when interacting with its environment, which is useful as an early-warning signal due to its high sensitivity to environmental perturbations (Hellou, 2011; Guedes et al., 2016). These behavioral responses may vary with the mode of action of the insecticide, or may be mediated by the individual or alterations in the environment. As a consequence, insecticide exposure may be reduced or enhanced (Haynes, 1988; Guedes et al., 2016a), with differing effects on each sex and subsequent consequences on sexual fitness, as recently observed in stink bug pests (Haddi et al., 2016; Tuelher et al., 2017a).

Sublethal insecticide exposure may also 'prime' exposed insects to protectively respond to subsequent insecticidal exposure favoring their survival, which is another concern for secondary pests (Guedes et al., 2017). Selection for insecticide resistance may also take place, as well as insecticide-induced hormesis (Guedes & Cutler, 2014; Cutler & Guedes, 2017; Guedes et al., 2017). Hormesis is a biphasic dose-response phenomenon characterized by a reversion in response between low and high doses of a compound allowing a stimulatory effect under low dose exposure of a compound toxic at higher doses (Cutler, 2013; Guedes & Cutler, 2014; Cutler & Guedes, 2017). Again, secondary pest outbreak is one of the potential consequences of insecticide-induced hormesis (e.g., Cordeiro et al., 2013)(Guedes & Cutler, 2014; Cutler & Guedes, 2017).

The current increase in importance of the Neotropical brown stink bug, *Euschistus heros* (F.), in soybean fields extending from central Brazil to Argentina presents an interesting model to explore potential effects of sublethal insecticide exposure on a non-target arthropod pest. Changes in soybean production in the region reflected in increased prevalence of no-tillage cultivation and use of pyramided transgenic cultivars stacked with genes expressing insecticidal proteins of *Bacillus thuringiensis* (i.e., Bt proteins) (Bernardi et al. 2014; Santos et al., 2016a). A consequence of this shift in management practices was the reduced pest status of the velvetbean caterpillar, *Anticarsia gemmatalis* (Hübner), and increased importance of more Bt-tolerant pest species such as armyworms, whiteflies, and particularly stink bugs, resulting in increased insecticide use against what were previously secondary pests (Panizzi, 2013; Bueno et al., 2015).

Insecticides used early in soybean production against caterpillars and whiteflies may subsequently interact with Neotropical brown stink bugs that develop later in the season, potentially affecting populations of this pest population and consequently soybean losses. The neonicotinoid imidacloprid affects the brown stink bug, while sublethal chlorantraniliprole exposure can stimulate the fitness of this insect (Haddi et al., 2016; Tuelher et al., 2017a). Pyriproxyfen and spinosad can also affect brown stink bugs (Santos et al., 2016). Here we explored the potential behavioral effects of chlorantraniliprole, pyriproxyfen, and spinosad on the gender-based fitness (or sexual fitness) of the Neotropical brown stink bug and its potential demographic consequences. This was done by recording behavior and fertility table parameters of exposed and unexposed mating pairs, and when only one member of the mating pair was exposed to insecticide. We predicted there would be significant behavioral effects caused by spinosad and chlorantraniliprole due to the neural and muscle activities of these compounds (Qi & Casida, 2013; Casida & Durkin, 2013), but not to the juvenile hormone (JH) mimic pyriproxyfen. Spinosad compromises the population growth rate of the brown stink bug (Santos et al., 2016a), what is doubtful for chlorantraniliprole since its effects were not as clear and hormesis may take place (Tuelher et al., 2017a).

Material and methods

Insects

The laboratory colony of the Neotropical brown stink bug used in the study was obtained from EMBRAPA Genetic Resources and Biotechnology (Brasília, DF, Brazil). This colony received periodic introduction of field-collected insects from soybean farms in Tangará da Serra (state of Mato Grosso, Brazil) and Viçosa (state of Minas Gerais, Brazil) in order to maintain genetic variability. The insect colony was reared with a mixture of fresh green bean pods, dry soybean seeds, raw shelled peanuts and sunflower seeds, as described elsewhere (Borges et al., 2006; Silva et al., 2008). The colony and experiments were all maintained at the same environmental conditions of 27 ± 2°C temperature, 75 ± 5% relative humidity, and 14:10 (L:D) h photoperiod.

Insecticides

Commercial formulations of the diamide insecticide chlorantraniliprole, the pyridine-based JH mimic pyriproxyfen, and the spinosyn bioinsecticide spinosad were used in this study. We used spray volumes and maximum label rates for soybean for control of caterpillars (chlorantraniliprole and spinosad) or whiteflies (pyriproxyfen), as follows (MAPA 2017): chlorantraniliprole at 0.68 µg a.i./cm^2 (200 g a.i./L at the spraying volume of 150 L/ha; suspendable concentrate, DuPont, Paulínia, SP, Brazil); pyriproxyfen at 1.02 µg a.i./cm^2 (100 g a.i./L at 300 L/ha; emulsifiable concentrate, Sumitomo, São Paulo, SP, Brazil); and spinosad at 2.44 µg a.i./cm^2 (480 g a.i./L at 100 L/ha; suspendable concentrate, Dow AgroSciences, São Paulo, SP, Brazil). Distilled water was used as control in all experiments.

Insecticide exposure

Insecticide exposure was achieved by contact with glass surface impregnated with dried residues (Santos et al., 2016b,a). Briefly, 2 mL of insecticide solution of distilled water were applied to 250 mL transparent glass jars (EME Equip., Paulicéia, SP, Brazil), which were maintained under rotation until drying using a heavy-duty rotator (Roto-Torque mdel 7637, ColeParmer, Vernon Hills, IL, USA) to coat the inner walls of the jars with insecticide residue. The upper portion of each container was coated with Teflon PTFE (DuPont, Wilmington, DE, USA) to prevent insects from escaping. Ten newly emerged adults (≤ 24 h old) of a given sex were released and maintained for 24 h in the insecticide-contaminated jars, which were closed with a piece of organza tissue and rubber band; three independent replicates for each sex and exposure scenario were used. After exposure, insects were removed from the contaminated jars and placed in 1000 mL plastic jars until reaching reproductive maturity (12-14 days after emergence). During this period, insects received *ad libitum* provision of the mixed seed diet described above. Females and males were maintained in different jars and eventually mated according to desired mating treatments upon reaching sexual maturity.

Mating behavior

Exposed virgin females and males were arranged in four different couple combinations for each insecticide: unexposed couple, exposed female, exposed male, exposed couple. The couples were placed in Petri dish arenas (9 cm diameter) and allowed to mate. Mating behavior was digitally recorded until the end of the first copulation using a digital video camcorder (HDR-XR520V, Sony, Tokyo, Japan). Thirty replicate couples were used for each combination of mating treatment and insecticide. Behaviors recorded were based on preliminary observations and included: latency to mate (the start of recording with search time and approach until initial contact), interaction (from initial contact until male tremulates body), male body tremulation, male antennation of female abdomen, abdomen raising by the female, and copulation. The sequence and duration of the behaviors were recorded using the software JWatcher (Blumstein & Daniel, 2007).

Fertility tables

Upon completion of the first copulation, each couple was retrieved from the arena, males were discarded and females were placed in 250 mL transparent plastic jars with *ad libitum* food provision, as previously described. Female longevity and lifetime fertility, together with previous daily immature survival data, were daily recorded and used to construct fertility tables where generation time (T), net reproductive rate (Ro; female progeny produced per female), and intrinsic rate of population growth (r_m; daily female production per female) were estimated as described by Maia et al. (2000).

Statistical analyses

The sequence and frequency of mating behavioral events were depicted as simplified ethograms constructed based on first order behavioral transitions. The frequencies of behavioral transitions for each exposure scenario were tested using χ^2 contingency tables (4 x 5; $P < 0.05$); PROC FREQ; SAS software, SAS Institute, Cary, NC, USA). Eventual pointed differences in the proportion of behavioral transitions between couples for any given insecticide were compared with the transitions observed in the unexposed couples using χ^2 test with Yates' correction for continuity ($P < 0.05$).

Time budget data of each behavior were subjected to time failure analyses (i.e., survival analyses) using Cox regression to test the effect of insecticide and gender, and eventually using Kaplan-Meier estimators to obtain the median duration of each behavior (PROC LIFETEST; SAS); pairwise comparisons among insecticides and between unexposed and exposed couples were performed using Holm-Sidak tests ($P < 0.05$).

The jackknife method was used to estimate the fertility table parameters from the life tables constructed using the SAS protocols developed by Maia et al. (2000). This procedure allows the computation of confidence intervals for estimated life table parameters and *P*-values associated with *t*-tests to perform the desired comparisons. The duration of the significant behavioral transitions were subsequently correlated with population growth rate to test if these behavioral traits are associated with population growth, using the procedure PROC CORR from SAS ($P < 0.05$).

Results

Mortality and longevity

No mortality was observed among the adult insects exposed to dried residue of the tested insecticides. Furthermore, insecticide exposure did not impair longevity of the adult insects, regardless of the insecticide and exposure scenario for couple ($\chi^2 \geq 3.55$, df = 3, $P \geq 0.31$). The overall average adult longevity (± SE) was 28.10 ± 0.94 days.

Mating behavior

Sequential analyses

The simplified ethograms representing the first order behavioral transitions of brown stink bug mating are displayed as a general diagram of mating between unexposed insects (Fig. 1A). The overall frequency of these transitions varied significantly among exposure scenarios (i.e., unexposed or insecticide-exposed couples, exposed female, and exposed male) for every insecticide ($\chi^2 \geq 67.27$, df = 36, $P \geq 0.001$). However, the differences in behavioral transitions were only noticeable when insecticide-exposed couples were compared with unexposed couples, mainly in the transition between interacting and the female raising of her abdomen; the frequency of this behavior was significantly greater with insecticide-exposed couples (regardless of the insecticide and whether only one member of the pair, or both were exposed). Differences in the transition between male body tremulation and antennation, and between male antennation and couple interaction were greater with some of the insecticides and couple treatments (Fig. 1B).

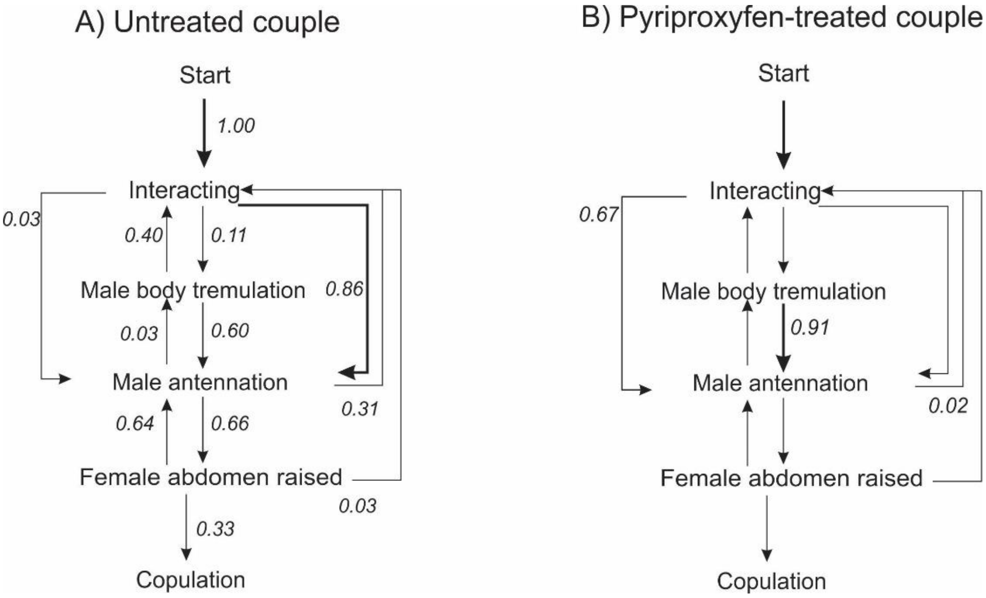


Fig. 1. Ethogram of the mating behavior of the Neotropical brown stink bug *Euschistus heros* subjected or not to insecticide exposure represented as first order transition diagrams; only the results of pyriproxyfen-treated couples were displayed as representative results of all insecticide treatments. The solid arrows indicate each behavioral transition. The relative thickness of each arrow represents the frequency of each behavioral transition, which is also indicated in italic (n = 30). Only the significantly differing transitions in (B), as compared with (A), are indicated (χ^2 test at $P < 0.05$).

Time budgets

Insecticide treatments and exposed sex had significant and differing effects on the duration of latency to mate, interaction, and male antennation during mating (Insecticide: $\chi^2 \geq 15.97$, df = 3, $P \geq 0.001$; sex: $\chi^2 \geq 12.35$, df = 3, $P \geq 0.006$). In contrast, the duration of male body tremulation (3.13 ± 0.33 min), female raising of her abdomen (0.29 ± 0.07 min), and copulation (7.09 ± 0.12 h) were not significantly affected by insecticide treatment ($\chi^2 \geq 2.67$, df = 3, $P \geq 0.44$). Relative to the controls, latency to mate was extended for spinosad-exposed insects, but reduced for chlorantraniliprole- and pyriproxyfen-exposed insects (Fig. 2A). Duration of initial interaction and male antennation were reduced irrespective of insecticide treatment (Figs. 2B and 2C), and did not differ whether one or both members of a mating pair were exposed (Fig. 3).

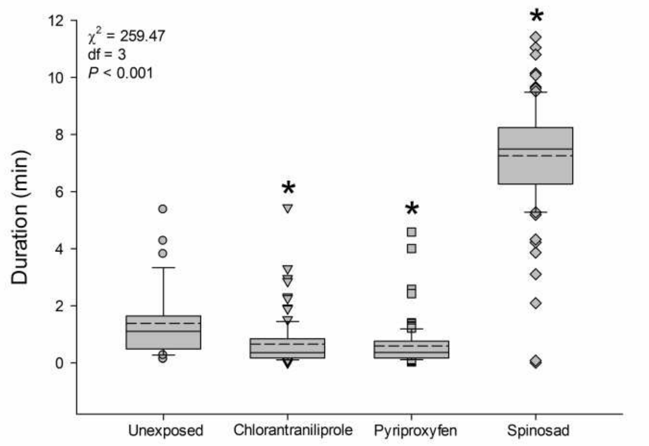
Fertility table parameters

The life table parameters estimated with jackknife and tested using student's *t* test ($P < 0.05$) indicated lack of effect of pyriproxyfen, with parameter estimates for this treatment very similar that of the control. However, generation time increased with chlorantraniliprole and especially with spinosad exposure (Figs. 4A and 4B). In addition, the net reproductive rate of chlorantraniliprole-exposed insects – especially couples with exposed males – was significantly greater than that of control insects (Fig. 4C), whereas spinosad exposure significantly reduced net reproductive rate (Fig. 4D). In line with these results, the intrinsic rate of population growth for couples exposed to chlorantraniliprole was greater than that of control insects (Fig. 5A), whereas spinosad exposure gave the opposite effect (Fig. 5B), and pyriproxyfen had no effect on the intrinsic rate of population growth.

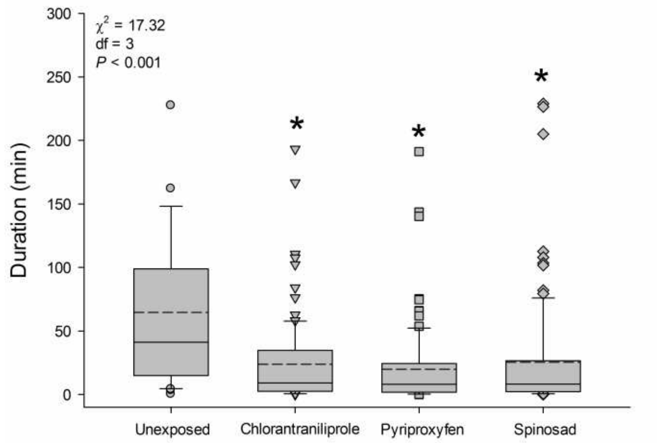
Latency to mate and population growth

Among the significant behaviors recorded during mating, only the pooled data of latency to mate was significantly correlated with the rate of population growth, irrespective of the insecticide and sex(es) exposed ($r = -0.57$, $P = 0.05$, $n = 12$). Extended latency to mate led to reduced population growth rates indicating that while chlorantraniliprole-exposed insects exhibited lower latency and higher population growth rates, the opposite occurred for spinosad-exposed insects.

(A) Latency to mate



(B) Interacting



(C) Antennation

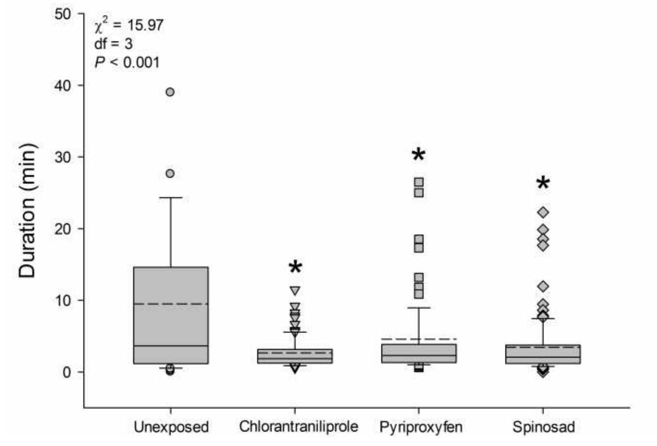
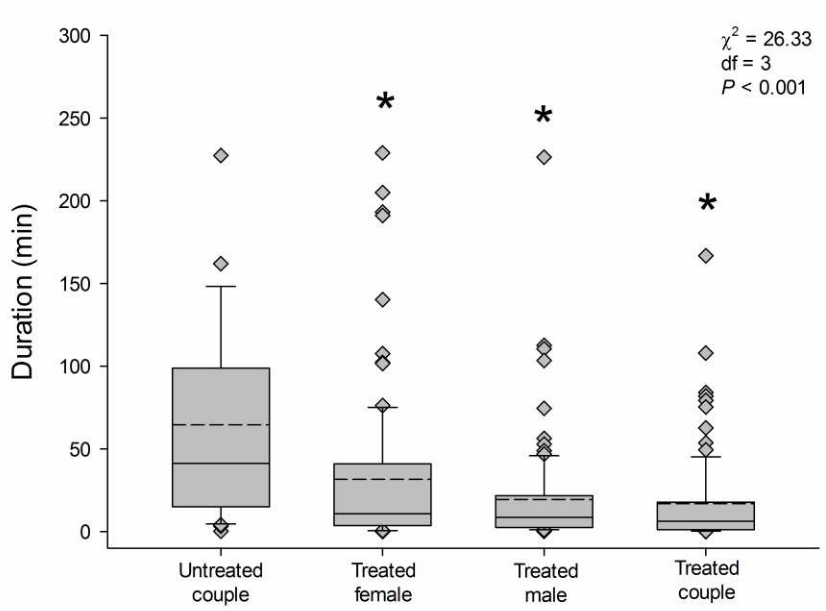
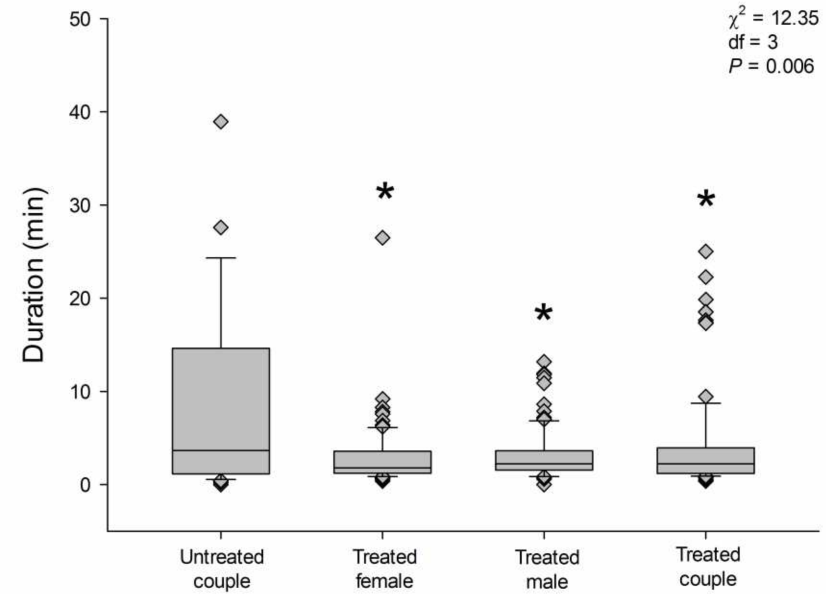


Fig. 2. Duration (± SE) of the latency to mate (A), couple interaction (B), and male antennation of female (C) in couples of the Neotropical brown stink bug *Euschistus heros* subjected to exposure of different insecticides, regardless of which sex was exposed to insecticide. Box plots indicate the median (solid line), mean (dashed line), and dispersal (lower and upper quartiles, and outliers) of the duration values. The asterisk indicates significant difference from unexposed couples by Holm-Sidak test ($P < 0.05$).



(A) Interacting



(B) Antennation

Fig. 3. Duration (± SE) of couple interaction (B) and male antennation of female (C) in couples of the Neotropical brown stink bug *Euschistus heros* subjected to insecticide exposure, and regardless of the insecticide. Box plots indicate the median (solid line), mean (dashed line), and dispersal (lower and upper quartiles, and outliers) of the duration values. The asterisk indicates significant difference from unexposed couples by Holm-Sidak test ($P < 0.05$).

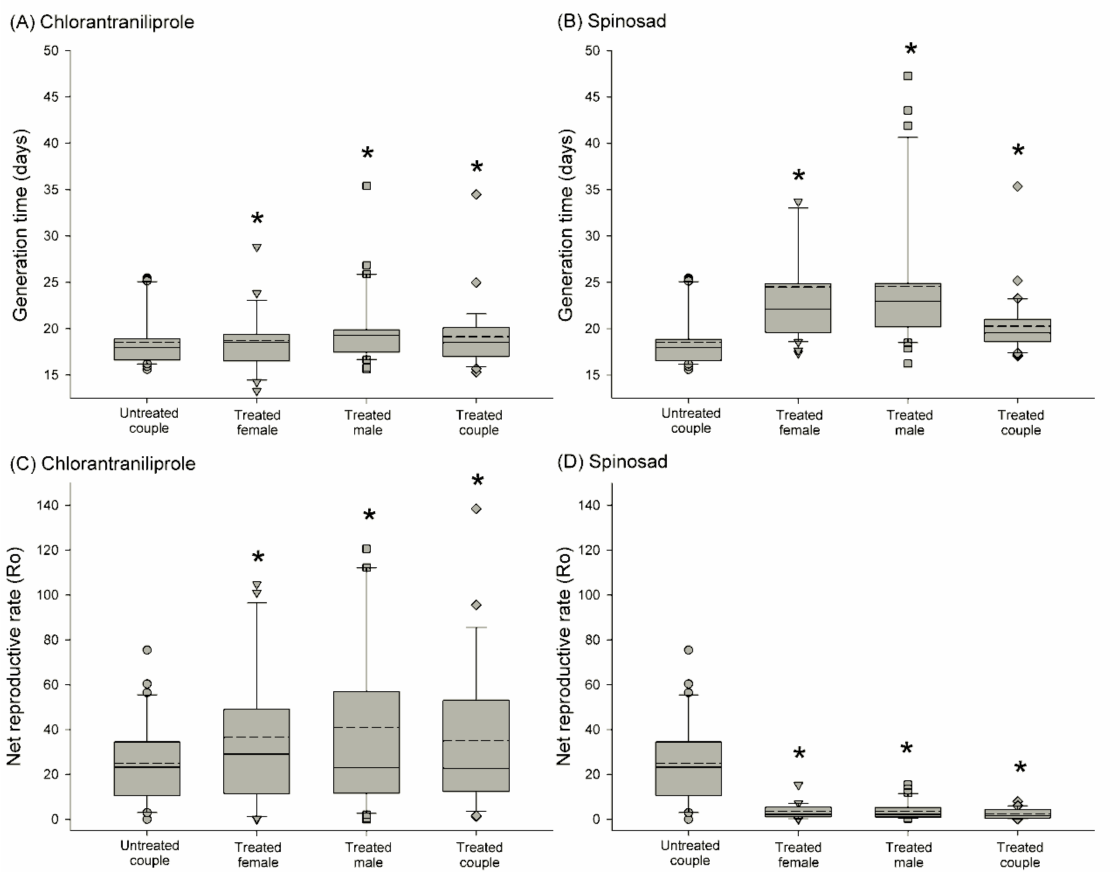


Fig. 4. Generation time (A and B) and net reproductive rate (C and D) (± SE) of couples of the Neotropical brown stink bug *Euschistus heros* subjected either chlorantraniliprole (A and C) or spinosad (B and D) exposure. Box plots indicate the median (solid line), mean (dashed line), and dispersal (lower and upper quartiles, and outliers) of the duration values. The asterisk indicates significant difference from unexposed couples by Student's *t* test ($P < 0.05$).

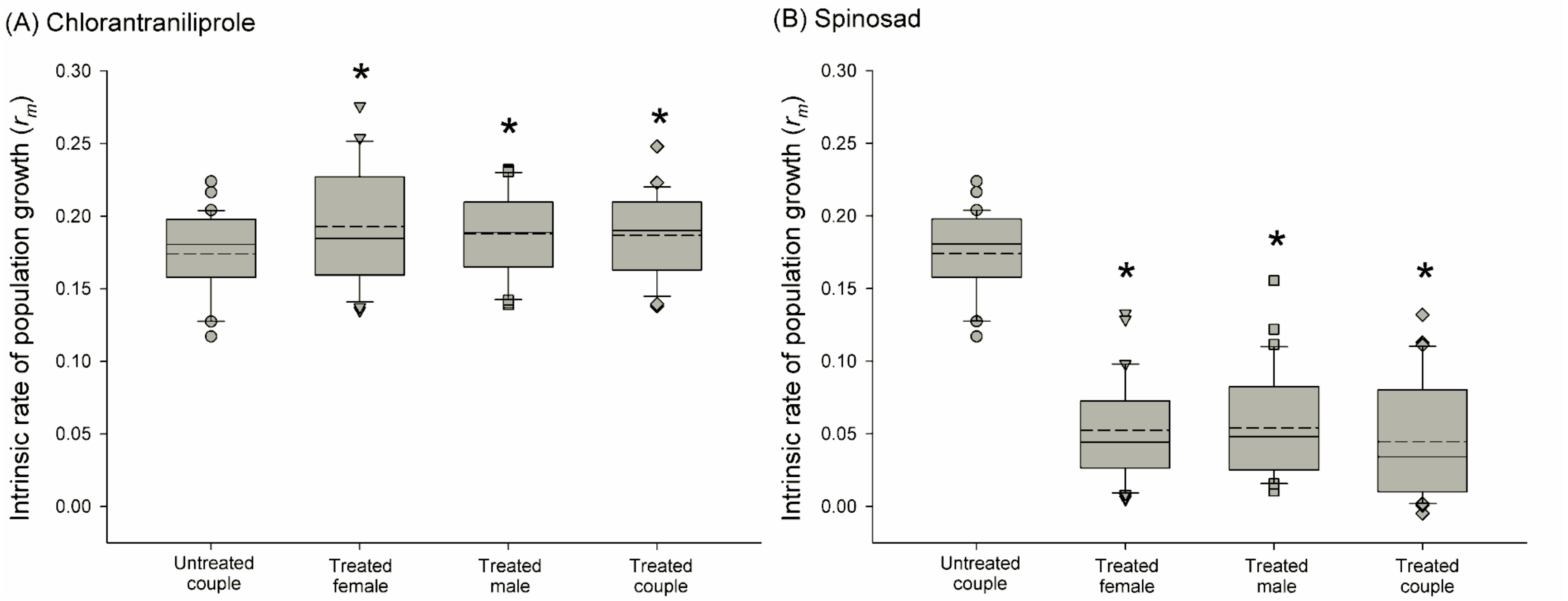


Fig. 5. Intrinsic rate of population growth (± SE) of couples of the Neotropical brown stink bug *Euschistus heros* subjected either chlorantraniliprole (A) or spinosad (B) exposure. Box plots indicate the median (solid line), mean (dashed line), and dispersal (lower and upper quartiles, and outliers) of the duration values. The asterisk indicates significant difference from unexposed couples by Student's *t* test ($P < 0.05$).

Discussion

Insecticides are prevalent stress agents in agroecosystems. The suite of insecticides to which insects are exposed can be complex and the studied consequences of insecticide exposure usually do not go beyond the mortality or suppression of the targeted pest species, and non-target effects on natural enemies and pollinators (Guedes et al., 2016, 2017). Examination of insecticidal stress on non-targeted pest species is lacking, despite its potential practical management consequences (Ripper, 1956; Hardin et al., 1995; Guedes & Cutler, 2014). We therefore explored effects of insecticides used in soybean for controlling caterpillars and whiteflies on the mating behavior and sexual fitness of the Neotropical brown stink bug, a pest of increasing status in Argentina and Brazil (Panizzi, 2013; Bueno et al., 2015; Panizzi & Lucini, 2016; Tuelher et al., 2017b).

We hypothesized that chlorantraniliprole and spinosad – insecticides exhibiting muscle and neural activity respectively – were more likely to affect mating behavior than pyriproxyfen, a JH analog that prevent insect larvae from maturing. We also predicted that these effects would be reflected through effects on fitness parameters, which may be gender-oriented given that females and males perform different behaviors during mating. All three insecticides affected the sequence of mating behavioral transitions. There were clear inter-treatment differences, but these were secondary to the effects on the duration of the behavioral events. Latency to mate was particularly important leading to consequences in stink bug population growth, but there were negligible effects of gender, except for a marginal effect on chlorantraniliprole-exposed males.

All of the insecticides led to what could be considered a simplification of the mating behavior favoring a more frequent direct transition from the initial meeting and interaction of the couple, to the female raising of its abdomen and coupling. This change in behavioral transition did not increase the transition to coupling, nor its duration. In contrast, latency to mating increased with insecticide exposure, regardless of the insecticide and exposed sex. Therefore, the insecticides seem to have impaired mate searching and finding in the brown stink bug, which has been reported in other pest species, including predatory bugs (Haynes 1988; Claver et al., 2003; Oliveira et al. 2012).

Chlorantraniliprole acts as a muscle poison that directly activates ryanodine receptors in the sarcoplasmic reticulum of muscle cells (Cordova et al., 2006; Casida & Durkin, 2013; Qi & Casida, 2013). This effect in Ca^{++}-activated receptors stimulates the massive release of Ca^{++} from intracellular stores leading to muscle contraction and subsequent paralysis due to the Ca^{++} depletion in the system.

Thus, either the muscle contraction or subsequent paralysis may have contributed delaying mate searching and finding among exposed stink bug couples. A similar mechanism is possible for spinosad, which acts as an allosteric modulator of (non-desensitizing) nicotinic acetylcholine receptors (nAChR) leading to hyperexcitation of neurons until eventual collapse of synaptic transmission, paralysis and death (Orr et al., 2009; Casida & Durkin, 2013). Slight impairment of coordination may delay mate searching and finding. This relatively mild effect may be due to the prevalence of different cholinergic currents activated by nAChR subtypes in the brown stink bug, as demonstrated in other insects (Salagado & Saar, 2004; Oliveira et al., 2010, 2011), or due to secondary spinosad activity on certain types of γ-aminobutiric acid (GABA) receptors in nervous synapses (Orr et al., 2009; Casida & Durkin, 2013).

Insect growth regulators like pyriproxyfen are slower in eliciting their toxic effects compared with neurotoxic compounds. Effects from pyriproxyfen nonetheless acted rapidly in delaying mating. Unlike chlorantraniliprole and spinosad, pyriproxyfen is particularly active against sucking insects with potential secondary effects on adult fertility. It can also interfere with JH activity and the release of prothoracicotropic and ecdisteroid hormones (Hatakoshi, 2013). Pyriproxyfen does sterilize Neotropical stink bugs (Santos et al., 2016a), but potential effect on mating behavior and its fitness consequences were not previously reported.

Among the behaviors measured, only latency to mate seemed to translate into fitness consequences in the Neotropical brown stink bug. Pyriproxyfen did not have any effect on fertility table fitness parameters. In contrast, chlorantraniliprole and spinosad extended the generation time of stink bugs, which would potentially compromise the rate of population growth for insects exposed to these insecticides. However, spinosad reduced fertility and chlorantraniliprole enhanced it, resulting in a significantly reduced rate of population growth under spinosad exposure, and the opposite effect under chlorantraniliprole exposure.

We previously demonstrated significant impairment of population growth in spinosad-exposed brown stink bugs, leading to the eventual suppression of this pest (Santos et al., 2016a). In the current study we associate this effect with the extension in latency to mate, regardless of the exposed sex, reinforcing its usefulness against this Neotropical brown stink bug when applied for caterpillar management in soybeans. Curiously, chlorantraniliprole exposure sparked contrasting response along the lines recently reported by Tuelher et al. (2017a).

Rather than impair population growth of the Neotropical brown stink bug, chlorantraniliprole exposure enhanced the species fertility and population growth. This insecticide was recently reported to enhance mating attempts and number of matings among males of the brown stink bug with a marginal increase in fertility resulting from this enhanced male performance (Tuelher et al., 2017a). Here the focus was on mating behavioral transitions and how they may affect sexual fitness. No evidence of difference in sexual fitness was observed in our study, although multiple matings were not followed, what may affect the male reproductive performance among stink bugs (Tuelher et al., 2017a). That chlorantraniliprole significantly enhanced fertility and population growth of the brown stink bug reinforces the notion that the use of this compound against soybean caterpillars may favor outbreaks of the stink bug (Santos et al., 2016a; Tuelher et al., 2017a).

Chlorantraniliprole does exhibit insecticidal activity against the Neotropical brown stink bug, but only at higher doses (Santos et al., 2016a). The opposite action occurs at low (sublethal) doses, leading to higher population growth. This biphasic response is the expected outcome of insecticide-induced hormesis (Calabrese & Baldwin, 1997; Cutler, 2013; Guedes & Cutler, 2014; Cutler & Guedes, 2017), which occurs with this insecticide in this pest species. Consequently, increased rate of population growth in this pest species under chlorantraniliprole use against soybean caterpillars can occur, likely favoring stink bug outbreaks contributing for its currently perceived importance as a soybean pest in Neotropical America. This deserves further attention.

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CONSIDERAÇÕES FINAIS

Espécies de artrópodes são expostas frequentemente a baixas concentrações de inseticidas devido a degradação ambiental e distribuição irregular destes em plantas. Processos de ressurgência de pragas são frequentemente associados ao efeito subletal de inseticidas, e hormese induzida por exposição subletal a estes compostos é uma das causas potenciais deste fenômeno.

Neonicotinoides são inseticidas neurotóxicos introduzidos recentemente para o controle de uma ampla variedade de pragas, como é o caso do percevejo marrom *E. heros*. Aqui demonstramos que 1% da concentração de imidacloprido recomendada para uso a campo (equivalendo a 0,047 µg i.a./cm^2) induziu o aumento no comprimento de ovaríolos e na área folicular dos ovários de fêmeas tratadas. Além disso, essas fêmeas exibiram redução na sobrevivência seguido de aumento na produção de ovos e eclosão, indicando realocação de recursos energéticos e melhoria da performance reprodutiva de fêmeas a exposições ao imidacloprido, uma evidência de hormese induzida por este composto.

Clorantraniliprole, espinosade e piriproxifeno são inseticidas normalmente usados para controlar outras espécies de pragas associadas ao percevejo marrom. No entanto, exposições à esses três inseticidas causaram alterações no comportamento de acasalamento e reprodução, com impacto sobre a demografia de *E. heros*. Clorantraniliprole provocou médio impacto sobre o crescimento populacional, enquanto que piriproxifeno e espinosade causaram forte impacto negativo com supressão populacional no caso do espinosade. Curiosamente existe um efeito estimulatório de clorantraniliprole sobre a primeira cópula do percevejo marrom. Fêmeas e/ou machos tratados com clorantraniliprole mostraram aumento da taxa líquida de reprodução (R_0) e taxa intrínseca de reprodução (r_m), contribuindo com aumento demográfico populacional.

Em resumo, surtos recentes de *E. heros* na cultura da soja podem estar associados ao efeito hormético ocasionado por baixas concentrações de imidacloprido e também ao efeito de clorantraniliprole, que também desencadeia resposta hormética nesta espécie. Por outro lado, espinosade e piriproxifeno reduzem o crescimento populacional do percevejo marrom, e portanto, são inseticidas com potencial de uso nos programas de controle do percevejo marrom no Brasil.