MAYRA CAROLINA VÉLEZ RUIZ

SUBLETHAL EFFECTS BY DELTAMETHRIN AND SPINOSAD ON THE GRAIN WEEVILS *Sitophilus zeamais* AND *S. granarius*

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

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Rosa Angelica Plata Rueda

Terezinha Maria Castro Della Lucia

Ricardo Siqueira da Silva

Wagner Faria Barbosa
(Coorientador)

Raul Narciso Carvalho Guedes
(Orientador)

Dedico este trabalho

A Deus, a minha família pelo amor incondicional, principalmente aos meus pais que me deram o apoio necessário para nunca desistir dos meus sonhos e a meu país Equador.

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CONTENTS

ABSTRACT

VÉLEZ RUIZ, Mayra Carolina, D.Sc., Universidade Federal de Viçosa, February, 2018. **Sublethal effects by deltamethrin and spinosad on the grain weevils *Sitophilus zeamais* and *S. granarius.*** Advisor: Raul Narciso Carvalho Guedes. Co-advisor: Wagner Faria Barbosa.

Insecticides are compounds widely used to prevent and control of pests, allowing the rapidly reduction of their population growth as well as their damage. However, insecticides not necessarily cause pest mortality, this may stimulate sublethal effects which may lead to harmful or even beneficial responses that may affect (or not) the behavior and sexual fitness of the exposed insects. One ongoing concern with pesticides use is the impact on non-target organisms, particularly natural enemies and pollinators. Little is known about such effects on grain weevils. In this context, we evaluated the sublethal effects by deltamethrin and spinosad on two species of grain weevils (*Sitophilus zeamais* e *S. granarius*). In the first experiment, we evaluated the insecticide effect of deltamethrin and spinosad on survival, walking activity, irritability and different feeding and drinking responses of *S. zeamais* and *S. granarius*. In the second experiment, we evaluated the sublethal effects of deltamethrin and spinosad on mating and reproductive output of *S. zeamais*. Deltamethrin and spinosad were able to control (100%) both species of grain weevils. Walking activity pattern changes, feeding and drinking responses of both species exposed to insecticides were also detected, but, deltamethrin exhibited a higher impact than spinosad. Couples of *S. zeamais* sublethally exposed to deltamethrin and spinosad exhibited altered reproductive behavior. A higher grain consumption and increased progeny emergence were observed in deltamethrin-exposed insects. These results suggest that deltamethrin elicit hormetic effects in *S. zeamais* that may compromise control efficacy by this compound. Although spinosad exhibited less impact on the evaluated behavior this also benefited weevil progeny emergence; this suggests caution using this and others compounds, particularly deltamethrin for controlling the weevil *S. zeamais*.

RESUMO

VÉLEZ RUIZ, Mayra Carolina, D.Sc., Universidade Federal de Viçosa, fevereiro de 2018. **Efeitos subletais causados por deltametrina e espinosade nos gorgulhos dos grãos *Sitophilus zeamais* e *S. granarius*.** Orientador: Raul Narciso Carvalho Guedes. Coorientador: Wagner Faria Barbosa.

Os inseticidas são compostos amplamente utilizados para a prevenção e controle de pragas, pois permitem reduzir rapidamente suas populações assim como os danos produzidos por estas. No entanto, os inseticidas podem não necessariamente chegar a causar a morte das pragas, desencadeando uma série de efeitos subletais com respostas nocivas ou mesmo benéficas que podem afetar (ou não) o comportamento e a aptidão sexual dos insetos expostos. Na atualidade, uma das preocupações com o uso de pesticidas tem sido o impacto em organismos não-alvo, particularmente inimigos naturais e polinizadores. Pouco se sabe sobre tais efeitos nos gorgulhos dos grãos. Nesse cenário, esta pesquisa avaliou os efeitos subletais causados pelos inseticidas deltametrina e espinosade nos gorgulhos dos grãos *Sitophilus zeamais* e *S. granarius*. No primeiro trabalho, avaliamos o efeito inseticida de deltametrina e espinosade na sobrevivência, atividade de caminhamento, irritabilidade e respostas no consumo de alimento e líquidos nas espécies *S. zeamais* e *S. granarius*. No segundo trabalho, foram avaliados os efeitos subletais causados por deltametrina e espinosade nos comportamentos de acasalamento e produção de progênie de *S. zeamais*. Deltametrina e espinosade foram capazes de controlar (100%) as duas espécies de gorgulhos. Mudanças no padrão de atividade de caminhamento, alimentação e ingestão de líquidos das duas espécies expostas aos dois inseticidas foram observadas, contudo deltametrina causou maior impacto que espinosade. Casais de *S. zeamais* expostos subletalmente a deltametrina e espinosade alteraram seu comportamento reprodutivo. Maior consumo de grãos de milho e aumento na progênie foi observado quando os parentais foram expostos a deltametrina. Estes resultados sugerem que deltametrina possui efeito hormético em *S. zeamais* o que pode comprometer a eficiência de seu controle com este composto. Embora espinosade apresentou impacto menor nos comportamentos avaliados, este foi capaz de aumentar a emergência de progênie, o que sugere cautela no uso deste e outros compostos, particularmente deltametrina no controle do gorgulho *S. zeamais*.

INTRODUÇÃO GERAL

O Brasil tem desenvolvido um sistema de agricultura comercial em grande escala, sendo as culturas de soja, milho, arroz, café e cana de açúcar as que apresentam maior destaque (Martinelli et al. 2010, IBGE 2017). Porém, entre os muitos fatores, os insetos pragas representam um dos principais limitantes nos rendimentos dessas culturas, e o controle delas tem se tornado essencial para redução de perdas (Moscardi e Sosa-Gómez 1992; Viana e Guimarães, 1997). Embora tenham sido relatados efeitos negativos em alguns cenários a utilização de inseticidas continua sendo o principal método para o controle de pragas na agricultura (Lee 2000; Wilson e Tisdell 2001; Bogorni e Vendramim 2003; Guedes et al. 2016).

A utilização de inseticidas tem sido destinada a causar mortalidade rápida de uma praga alvo, no entanto, a possibilidade de receberem exposições letais necessita da interação dinâmica de fatores abióticos e bióticos. As interações desses fatores podem minimizar o desempenho dos efeitos letais do inseticida levando-o para níveis subletais que podem não necessariamente causar a morte da praga (Lee 2000; Guedes et al. 2017b).

Os efeitos subletais dos inseticidas podem se manifestar na fisiologia do organismo (desenvolvimento, longevidade, imunidade e fecundidade) assim como em mudanças comportamentais (mobilidade, orientação, alimentação e acasalamento) (Haynes 1988; Lee 2000; Guedes et al. 2016). Nesta perspectiva, os inseticidas podem induzir estresse em insetos o que enfatiza a importância potencial dos efeitos subletais principalmente no manejo e controle das pragas, fatores frequentemente negligenciados (Guedes et al. 2016; 2017b).

Uma contínua preocupação do uso de inseticidas tem sido o impacto que causam em organismos não-alvo, particularmente inimigos naturais e polinizadores (Desneux et al. 2007; Tomé et al. 2012; Barbosa et al. 2015; Lima et al. 2016). No entanto, pouco se sabe sobre estes efeitos em pragas de grãos armazenados. No caso dos gorgulhos dos grãos (*Sitophilus granarius* e *S. zeamais*), efeitos subletais tem sido reportados na reprodução, atividade locomotora e aspectos fisiológicos como respiração e longevidade

(Bond e Upitis 1973; El- Nahal e El Halfawy 1973; Spratt 1979), contudo novos estudos são necessários.

O controle dos gorgulhos dos grãos e outras pragas de grãos armazenados depende do uso de inseticidas sintéticos (piretróides, organofosforados, fosfina) (Guedes 1991; Ribeiro et al. 2003; Li et al. 2010), no entanto o uso intensivo desses compostos no controle dessas pragas tem levado à resistência a inseticidas e frequentes falhas no controle (Guedes et al. 1995; Subramanyam e Hagstrum 1996; Guedes et al. 2009; Pereira et al. 2009; Guedes 2017a). Como consequência, a atenção a novos inseticidas, particularmente os de origem biológica, tem aumentado. Entre os bioinseticidas que têm apresentado potencial efetividade na proteção aos grãos armazenados encontramos o espinosade (Toews e Subramanyam 2003; Huang e Subramanyam 2007; Athanassiou et al. 2008).

Espinosade é um inseticida produto da fermentação da espécie de actynomyceto *Saccharopolyspora spinosa* Mertz & Yao (Thompson et al. 2000; Sparks et al. 2001), o qual ativa os receptores nicotínicos de acetilcolina e interfere nos receptores do ácido γ-aminobutírico (GABA) no sistema nervoso (Salgado 1998; Thompson et al. 2000; Sparks et al. 2001; Hertlein et al. 2011). No entanto, em contraste com o inseticida deltametrina (Spratt 1979; Salerno et al. 2002; Guedes et al. 2009; Guedes et al. 2009b) pouco se conhece sobre a resposta dos gorgulhos dos grãos à exposição subletal do espinosade, embora sua eficácia tem sido reconhecida (Athanassiou et al. 2004; Huang e Subramanyam 2007).

Dessa forma, os seguintes estudos tiveram como objetivos avaliar a sobrevivência dos gorgulhos dos grãos quando expostos aos inseticidas deltametrina e espinosade, assim como os efeitos subletais na atividade de caminhamento em grupo e individual, irritabilidade, preferências alimentícias e da ingestão de líquidos, respostas no acasalamento, fecundidade e a perda de grãos associada à exposição dos inseticidas.

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CHAPTER 1

Deltamethrin- and spinosad-mediated survival, activity and avoidance of the grain weevils *Sitophilus granarius* and *S. zeamais*

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M. Vélez [a], W.F. Barbosa [a], J. Quintero [a], M. Chediak [a, b], R.N.C. Guedes [a, c, *]

[a] Departamento de Entomologia, Universidade Federal de Viçosa, Viçosa, MG, Brazil.

[b] ENTO Soluçoes & Pesquisa, CENTEV-UFV, Viçosa, MG, Brazil.

[c] USDA-ARS San Joaquin Valley Agricultural Sciences Center, Parlier, CA, USA.

Abstract

The granary and maize weevils are serious worldwide threats to stored products and their control has heavily relied on synthetic insecticides, which are largely recommended based on studies with acute lethal doses neglecting the importance of potential sublethal effects of insecticides. Deltamethrin has been widely used for managing grain weevils and other stored grain pest species, but reported control failures with this insecticide sparked the search for alternative insecticidal compounds. The bioinsecticide spinosad is one of such alternatives whose use against stored grain insect pests is relatively recent, but encompasses the control of grain weevils. Nonetheless, little is known about the sublethal effects of spinosad on these insect pest species. Here we assessed the insecticidal effects of commercial formulations of spinosad and deltamethrin against the weevil species *Sitophilus granarius* and *S. zeamais*. Both spinosad and deltamethrin were able to effectively control the insects, although the latter caused a faster mortality than the former. Behavioral pattern changes were caused by both insecticides, especially deltamethrin, triggering irritability (i.e., avoidance after contact). Different feeding and drinking responses were also detected for both weevil species, which exhibited significant avoidance to deltamethrin and to spinosad, but with a milder response to the latter. Apparently spinosad is not as easily recognizable as deltamethrin by *S. zeamais* and *S. granarius*, remaining effective against both species without minimizing as much the potential exposure as deltamethrin.

Keywords: Stored cereal pest, Insecticide avoidance, Sublethal exposure, Biopesticide, Spinosyns

1. Introduction

The rather broad use of conventional insecticides as grain protectants remains a dominant pest management tactic against stored product insects particularly in warmer climates (White and Leesch, 1996; Zetler and Arthur, 2000; Obeng-Ofori, 2010). Such use and recommendations are largely based on acute lethal studies, which although valuable, neglect the likely occurrence and importance of potential sublethal effects of insecticides on these pest species and associated community (Haynes, 1988; Guedes et al., 2011, 2014, 2017). Nonetheless, sublethal effects of conventional grain protectants have been recorded in the granary and maize weevils (*Sitophilus granarius* and *S. zeamais*), but compounds of more recent used in stored product protection were not targeted in such studies, as the biopesticide spinosad, in contrast with pyrethroids (Bond and Upitis, 1973; El- Nahal and El Halfawy, 1973; Spratt, 1979; Salerno et al., 2002; Guedes et al., 2009a,b; Veloso et al., 2013).

The sustained levels of pyrethroid use against grain weevils have been leading to problems of resistance to these compounds in *S. granarius* and *S. zeamais* (Guedes et al., 1994; 1995; Ribeiro et al., 2003, Kljajić and Perić, 2009; Corrêa et al., 2011). Therefore, new alternatives have been developed and launched in the market as grain protectants (Athanassiou and Kavallieratos, 2014). One of them is the biopesticide spinosad, which belongs to a class of naturally derived compounds currently available in many countries, the spinosyns (Fang et al., 2002; Mutambuki et al., 2002; Subramanyam et al., 2002; Subramanyam et al., 2006; Huang and Subramanyam, 2007; Chintzoglou et al., 2008; Hertlein et al., 2011; Athanassiou and Kavallieratos, 2014).

Spinosad is an insecticide based on fermentation products of the actinomycete *Saccharopolyspora spinosa* (Mertz and Yao) (Actinomycetales: Pseudonocardiaceae). The effects of this compound are consistent with the activation of nicotinic acetylcholine receptors and also interferes with receptors of γ-aminobutiric acid (GABA) in the nervous system (Salgado, 1998; Thompson, et al., 2000; Sparks et al., 2001; Hertlein et al., 2011). Spinosad was considered highly effective for the control of several species of stored product insect pests in different types of grains (Toews and Subramanyam, 2003; Huang and Subramanyam, 2007; Athanassiou et al., 2008; Vayias et al., 2010). However, few sublethal studies are available for spinosad among stored product insects and even less with the grain weevils *S. zeamais* and *S. granarius,* what may affect exposure and consequent field efficacy of this compound, as recognized for other insecticides (Pereira et al. 2009; Braga et al. 2011; Guedes et al., 2014, 2016).

Insecticide resistance and associated reports of control failure in populations of grain weevils emphasize the need of new insecticidal compounds aimed at their sustainable control. However, the impact of such compounds goes beyond mortality, as sublethal effects are frequently as important and even more important and are usually neglected particularly when biopesticides and stored grain species are considered (Guedes et al. 2014; Guedes et al. 2016). Thus, the present study aimed to assess the survival, activity and avoidance elicited by spinosad in *S. zeamais* and *S. granarius* against a pyrethroid used to control these insect pest species, the insecticide deltamethrin. While doing so, we first analyzed the consequences in the insect survival, then we explored the sublethal effects on overall and individual walking activity, irritability, feeding and drinking preferences.

2. Material and Methods

2.1. Insects and insecticides

An insecticide-susceptible population of each weevil species (*Sitophilus zeamais* and *S. granarius*) was used in this study. The population of *S. zeamais* (Sete Lagoas) was obtained from the Maize and Sorghum National Research Center of the Brazilian Agricultural Research Corporation (CNPMS/EMBRAPA, Sete Lagoas, MG, Brazil). The population of *S. granarius* was obtained from the Department of Grain Sciences and Industry of the Kansas State University (Manhattan, Kansas, USA). Both strains were reared on maize grains free of insecticide residue in glass containers (1 L) within growth chambers at 27 ± 2°C, 70 ± 10% of relative humidity (RH), and 12:12 h photoperiod (D:L).

Two commercial formulations of insecticides available in Brazil for controlling maize weevils were used at their recommended label rates: deltamethrin (K-Obiol 25 CE; emulsifiable concentrate at 25 g of active ingredient (a.i.)/L; Bayer CropScience Brasil, São Paulo, SP, Brazil) and spinosad (Tracer 480 SC; suspension concentrate at 480 g a.i./L, Dow AgroSciences, Mogi Mirim, SP, Brazil). The insecticides deltamethrin and spinosad were diluted in distilled and deionized water at the concentrations of 0.25 and 0.5 mg active ingredient (a.i.)/mL, respectively. Then, each water solution containing deltamethrin or spinosad was sprayed on 400 g of maize grains at the concentration of 0.5 mg and 1.0 mg active ingredient (a.i.)/kg of maize, respectively, using an artist air brush (Sagyma SW440A, Yamar Brasil, São Paulo (SP), Brazil) connected to an air compressor (model 131, type 2VC, Prismatec, Itu (SP), Brazil). The maize grains were placed inside a stainless steel bowl coupled to a revolving rotor to homogenize the grain coverage until the drying of residues. At the end of

every application the air brush was cleaned with acetone to prevent contamination between treatments. Distilled and deionized water was used as negative control.

2.2. Survival bioassay

Time-mortality bioassays were performed to assess the lethal toxicity of the insecticides deltamethrin and spinosad to unsexed weevil adults (3-7 days old). Both insecticides were used at their commercial recommended concentrations as above-mentioned. Distilled and deionized water was used as negative control. A single insect (*S. zeamais* or *S. granarius*) was exposed to ten grams (10 g) of treated maize grains placed in a small 30 mL glass vial comprising an experimental unit or replicate. Twenty (20) replicates were used for each treatment (i.e., deltamethrin, spinosad or water) and insect species (granary and maize weevils). The mortality of every single insect was recorded at regular time intervals. Deltamethrin-treated insects were monitored every 30 minutes during the first 2 hours and thereafter at every hour until all individuals were dead. Spinosad-treated insects were monitored every hour during the first 8 hours of the experiment and subsequently every 6 hour-intervals until all insects were dead. The control treatment was monitored as was carried out for spinosad. The insects were considered as dead when they did not respond to prodding with a fine hair brush, and the mortality was confirmed 1 h after recording the insect death and again at the following day allowing for the possibility of knock down recovery.

2.3. Overall group activity

Unsexed adults of *S. zeamais* and *S. granarius* (1-2 weeks old) were used to assess the overall group activity on each insecticide treatment. First, insects were exposed to 200 g of maize grains treated with the previously described concentrations of deltametrin, spinosad and water during sublethal periods of exposure corresponding to half of LT_{25} (the lethal time for 25% of the population) for each insecticide (deltamethrin = 30 min; spinosad 10 h; water = 10 h), as estimated through survival curves (Fig. 1A and Fig. 1B). Groups of ten adults were placed within an arena comprising a Petri dish (9 cm in diameter and 2 cm high) lined at the bottom with filter paper (9 cm diameter, 80g/m^2 density; Nalgon. Equipamentos Científicos Ltda. Itupeva, SP, Brazil) and coated with Teflon PTFE along the inner walls (DuPont, Wilmington, DE) to prevent insect escape. Each filter paper disc was treated with 1 mL of solution corresponding to 3.93 µg (a.i.)/cm^2 of deltamethrin and 7.86 µg (a.i.)/cm^2 of spinosad (Guedes et al., 2009b).

The overall group activity within the arena was recorded for 15 min and digitally transferred to a computer using the ViewPoint automated tracking system equipped with a charge-coupled device (CCD) camera (ViewPoint LifeSciences, Montreal, Canada), after 1-min acclimation. Overall activity was digitally determined as changes in captured pixels between two subsequent pictures taken every 10^{-2} s from the insect group inside the arena. The quantified pixels (Δ pixels/s x 10^{-2}) represented any change in position and posture of the individuals within the arena between two subsequent pictures registered in the video track system. The behavioral bioassays were carried out between 8:00 and 19:00 h in a room with artificial incandescent light and an average temperature of 25±3°C. Twenty replicates were used for each treatment in the assessments in which insects and arenas were treated with insecticides and water.

2.4. Walking bioassay in fully-treated arenas

The walking biossay of adults of *S. zeamais* and *S. granarius* were recorded using the same conditions, containers and equipment used to record the overall activity. However, walking activity was recorded for each individual insect released alone in the Petri dish area; the movement of each insect within the arena was recorded for 15 min using the ViewPoint tracking system. The variables recorded in the individual activity included: number of stops, resting time (s), distance walked (cm) and walking velocity (cm/s). Twenty replicates were used for each combination of insecticide (i.e. deltamethrin, spinosad) and surface (filter paper disc); every one included a control treatment where insects and surfaces were exposed to only water (distilled and deionized).

2.5. Walking bioassays in half-treated arenas

The behavioral bioassay carried in half-treated arenas was evaluated using the same conditions previously described. Each experimental unit was composed by insects exposed to water, deltamethrin or spinosad placed in a Petri dish comprising water treated filter paper (9 cm diameter, 80g/m^2 density; Nalgon Equipamentos Científicos Ltda. Itupeva, SP, Brazil) settled to the bottom of the Petri dish and half of an insecticide (deltamethrin or spinosad) treated disc was fixed over the control disc using glue resin. The bioassays carried in half-treated arena considered the proportion of time that a single individual spent in each half of arena. Insects that spend less than 1s on the insecticide-treated half of the arena were considered as repelled, while the ones remaining less than 50% of the time on the treated half one were considered as irritated. However, the statistical analysis was performed only for irritated insects due the low number of repelled insects found. Twenty replicates were used for each treatment. All the

behavioral bioassays were carried out following methods adapted from previous studies (Guedes et al., 2008, 2009b; Cordeiro et al., 2010; Morales et al., 2013).

2.6. Feeding preference

A free-choice test adapted from Guedes et al. (2009a) was also performed using white plastic trays (30 x 18 x 6 cm), which received dichotomous treatments (200 g of water- and insecticide-sprayed grains placed in opposite sides of the tray) as follows: water:deltamethrin, water:spinosad and spinosad:deltamethrin. The plastic trays were coated with Teflon PTFE (DuPont, Wilmington, DE) and covered with a fine stiff nylon fabric to prevent insect escape. Twenty-five unsexed adult insects (1-2 weeks-old) were released in the center of the tray and insect preference was assessed after 1 h by determining the proportion of insects in each side of the plastic tray. Five replicates were used for each dichotomous free-choice test. This experiment was carried out using both *S. zeamais* and *S. granarius* and they were compared within each dichotomous free-choice test.

2.7. Drinking preference

Sexed adults of *S. zeamais* and *S. granarius* (1-2 weeks-old) were used to assess the ability of both species to discriminate between uncontaminated and insecticide-contaminated water. Firstly, groups of 240 male and female weevils were placed in the growth chambers (27 ± 2°C, 70 ± 10% RH, 12 h:12 h photoperiod (D:L)) for 24 h without food (grains of maize). The insects were then dehydrated for 24 h within glass desiccators (3000 cm^3) at 30 to 40% R.H., which was achieved by using 3g drierite (anhydrous calcium sulfate) and monitored with digital thermohygrometers (IP-747RH, Impac, São Paulo, SP, Brazil). This low range of relative humidity was chosen to force the insects to drink during the test, as based on previous studies of water balance in insects (Wharton, 1985; Hadley, 1994; Guedes, et al., 2014; Malia et al., 2016). The dehydrated weevils were then provided with a dual choice of 50 µL of water droplets encompassing the following dichotomous treatments: uncontaminated- and deltamethrin-contaminated water, uncontaminated- and spinosad-contaminated water, and deltamethrin- and spinosad-contaminated water. The insecticides deltamethrin and spinosad were used at the concentrations of 0.25 and 0.5 mg a.i./mL, respectively; thus, the amount of deltamethrin and spinosad used per 50 µL of water-droplet was 0.0125 and 0.025 mg a.i., respectively. Each droplet was randomly stained with either artificial blue or red dye (Mix Industria, São Bernardo do Campo, SP, Brazil). The bioassay was performed in a Petri dish (9.0 cm diameter). The weevils were observed for 5 min and their choice for drinking and if they indeed drank the stained contaminated or uncontaminated water were duly recorded. To confirm the intake,

weevils were collected, dissected and examined under stereomicroscope (Stemi 2000, Zeiss, Göttingen, Germany) for evidence of either blue or red coloration in the gut diverticula (Benoit et al., 2005; Guedes et al., 2014).

2.8. Statistical analyses

The data from the time-mortality (survival) bioassays were subjected to non-parametric survival analysis using Kaplan Meyer estimators to obtain the survival curves and estimates of the median survival time (LT_{50}) (PROC LIFETEST; SAS Institute, 2008). The insects still alive at the end of the bioassays were treated as censored data. The overall similarity among survival curves was tested by the χ^2 Log-Rank test, and the dichotomous comparisons between curves were tested using the Bonferroni's method. Data from the walking bioassay were subjected to different analyses. The data of the individual and overall group activities were firstly subjected to deviance analysis (R version 3.3.2, R Core Team, 2016) with general linear model to adjust the residues to an adequate family distribution which was based on parsimony considering low overdispersions and subdispersions. Subsequently, the treatments were contrasted using χ^2 test according to the presence (or not) of significances showed in the tested model (PROC GENMOD statement; SAS Institute, 2008). The data from insecticide irritability, feeding free-choice and drinking preference were analyzed using a general linear model based on binomial distribution and the treatments were contrasted by χ^2 test (PROC GENMOD; SAS Institute, 2008). No statistical analysis was possible for the repellence data since the number of repelled insects was negligible.

3. Results

3.1. Survival time

The survival analysis of *S. zeamai*s and *S. granarius* exposed to deltamethrin, spinosad and water (control) indicated significant differences among insecticidal treatments for both species, *S. zeamais* (Log-rank test: $\chi^2 = 409.37$, df = 2, $p < 0.001$, Fig. 1A) and *S. granarius* (Log-rank test: $\chi^2 = 421.21$, df = 2, $p < 0.001$, Fig. 1B). Insects of species exposed to deltamethrin died within 24 hours (Figs. 1A and 1B). In contrast, all insects of both species treated with spinosad died between 12 and 13 days after exposure. The mean survival times (LT_{50}) of *S. zeamais* and *S. granarius* were, respectively, 3.55 and 5.33 hours for the insects treated with deltamethrin and 76.59 and 124.03 hours for the ones treated with spinosad (Fig. 1C, 1D).

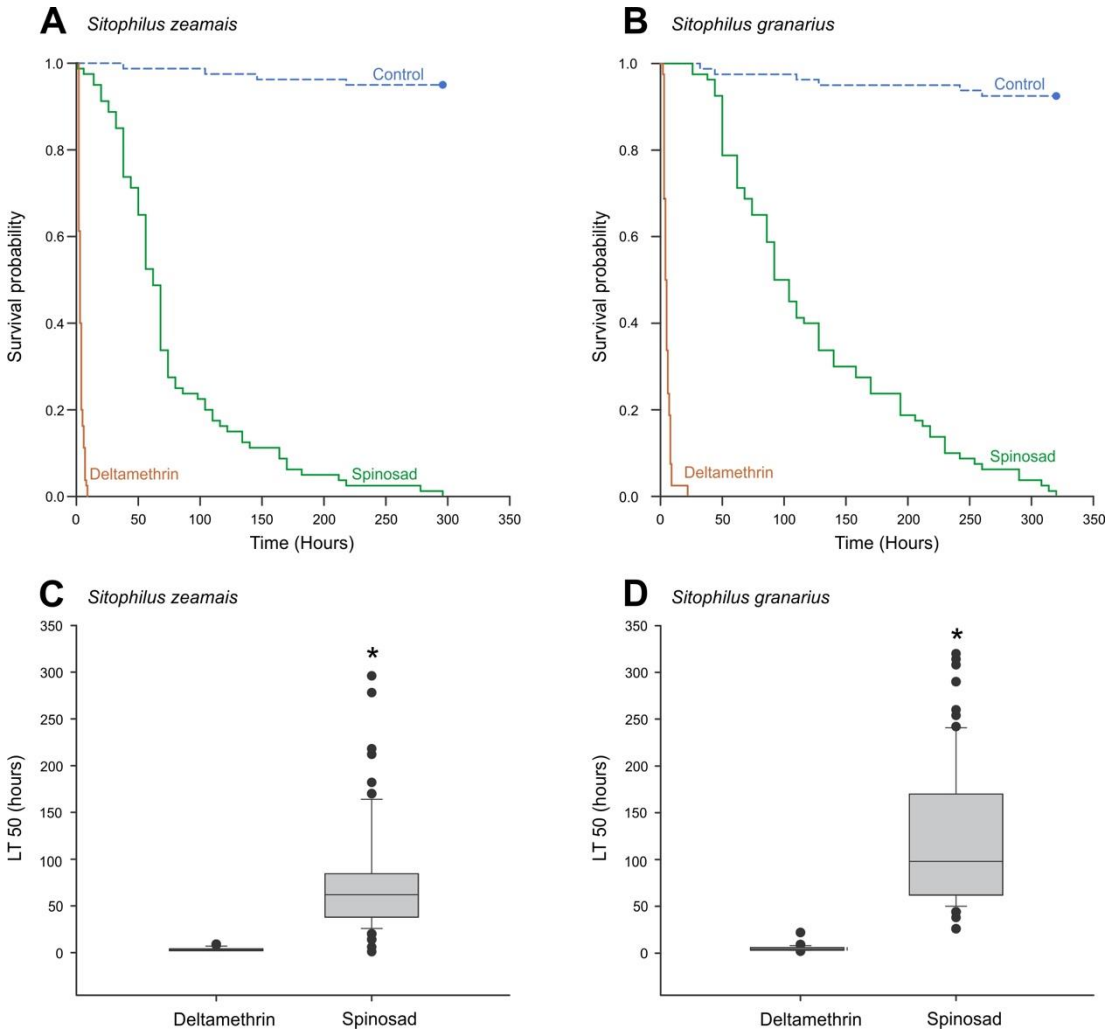

Fig. 1. Survival curves of *Sitophilus zeamais* (A) and *Sitophilus granarius* (B) exposed to deltamethrin-, spinosad- and water- (control) treated grains. Median survival times (LT_{50}: lethal time 50%) of exposed insects of *Sitophilus zeamais* (C) and *Sitophilus granarius* (D). The box plots indicate the median (solid line) and dispersion (lower and upper quartiles, and outliers) of the median survival times (LT_{50}). Asterisks indicate significant differences between weevil species using the Bonferroni's method ($p < 0.05$).

3.2. Overall group activity of Sitophilus zeamais and Sitophilus granarius

The overall activity of *S. zeamais* and *S. granarius* exposed to water (control), deltamethrin or spinosad indicated significant effect for the triple interaction among the factors species, insect treatment (exposure via treated grains) and arena treatment (exposure via treated bottom surfaces of the video-tracking arenas) ($\chi^2 = 51.80$, d.f. = 4, $p < 0.001$). The profile of

overall group activity indicated that *S. zeamais* was less active than *S. granarius* (Fig. 2). Lower activity levels were also observed in both species when insects from deltamethrin treated-grains were placed on untreated and deltamethrin-treated surfaces, but no differences were observed when the weevils were exposed to spinosad-treated surfaces (Fig. 2). In both species, spinosad-treated arenas led to lower overall activity on weevils from unexposed or deltamethrin-treated grains (Fig. 2).

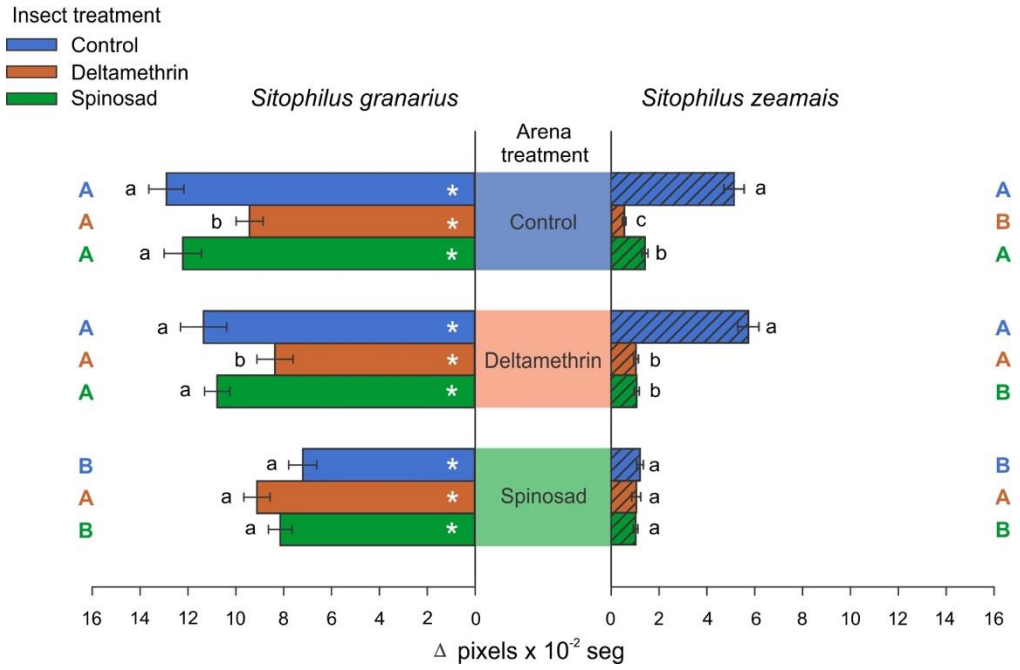

Fig. 2. Average of the overall activity (±SE) of *Sitophilus zeamais* (hachured bar) and *S. granarius* (plain bar) expressed as changes in registered pixels per second (Δ pixels x 10^{-2} seg), representing the study of the interaction among three factors: species, arena treatment, and insect treatment. Asterisk indicates significant differences of the insect activity in contrasts stablished for the factor species within every combination of the factors arena treatment and insect treatment. Small case letters indicate significant differences of the insect activity in contrasts stablished for the factor insect treatment (i.e., weevils exposed to deltamethrin, spinosad or control via treated-grains) within every combination of the factors arena treatment and species. Capital letters indicate significant differences on insect activity in contrasts stablished for the factor arena treatment within every combination of the factors insect treatment and species. The significances of all contrasts within every combination of factors were tested by chi-square test ($p < 0.05$).

3.3. Walking activity in fully-treared arenas

As far as individual performance of *S. zeamais* and *S. granarius*, significant differences were only detected for the insect treatment (i.e., insects exposed to water-, deltamethrin- or spinosad-treated grains) (χ^2 = 13.04, df = 1, p = 0.0015, Fig. 3A) and species (χ^2 = 6.48, df = 1, p = 0.0109, Fig. 3B) in the number of stops; no triple nor double interactions among insect, arena and species treatments were significant for this endpoint ($p > 0.05$). The number of stops was significantly higher for deltamethrin-treated insects than water- (χ^2 = 13.19, df = 1, p = 0.0003) or spinosad-treated ones (χ^2 = 4.26, df = 1, p = 0.0389) (Fig. 3A). The number of stops was higher for *S. zeamais* than *S. granarius* (χ^2 = 6.48, df = 1, p = 0.0109, Fig. 3B).

The insects exposed to treated arenas led to significant differences in the resting time (χ^2 =7.29, df = 2, p = 0.0262) (Fig. 3C), as well as the interaction between species and insect treatment (insect exposed to insecticides) (χ^2 = 14.68, df = 2, p = 0.0006) (Fig. 3D). *Sitopohilus zemais* exhibited higher resting time than *S. granarius* and resting time was higher with deltamethrin exposure (Fig. 3D). The distance walked was affected by the arena treatment (χ^2 = 6.87, df = 2, p = 0.0321) (Fig. 3E) with higher values for control and deltamethrin treated arenas (p <0.05) than the spinosad treated arena. The species-insecticide interaction was also significant for distance walked (χ^2 =22.16, df = 2, $p < 0.0001$, Fig. 3F) with higher values when both *S. granarius* and *S. zeamais,* were exposed (via treated grains) to water (control) and spinosad, although the general mean was higher for *S. granarius* in these treatments.

The walking velocity for weevils exposed to water (control), deltamethrin or spinosad indicated significant effect for the double interaction between the factors arena treatment and insect treatment (χ^2 = 10.74, d.f. = 4, p =0.0296) (Fig. 3G); interactions were also significant between insect treatment and species (χ^2 = 16.08, d.f. = 2, $p < 0.0003$) (Fig. 3H). Insects were not significantly affected when exposed (via treated grains) to the insecticides and evaluated over control arena ($p > 0.05$), however the velocity was lower than in the control for the insects treated with deltamethrin (χ^2 =17.60, df = 2, p = 0.0002) and spinosad (χ^2 =8.12, df = 2, p = 0.004) over the arena treated with deltamethrin. Reduced velocity was also detected in insects exposed to deltamethrin over the arena treated with spinosad (χ^2 =14.54, df = 2, p = 0.0001) (Fig. 3G). Only spinosad-treated arena impaired the velocity when the insects were exposed to different treatments via treated grains (χ^2 = 8.38, d.f. = 2, p = 0.003, Fig. 3G). *Sitophilus granarius* exhibited higher walking velocity than *S. zeamais* in control treatment (χ^2 = 34.34, d.f. = 2, $p < 0.0001$, Fig. 3H). No significant differences in walking velocity were found among treatments on *S. zeamais* ($p > 0.05$, Fig. 3H), however both deltamethrin and spinosad affected the velocity of *S granarius* ($p < 0.05$, Fig. 3H).

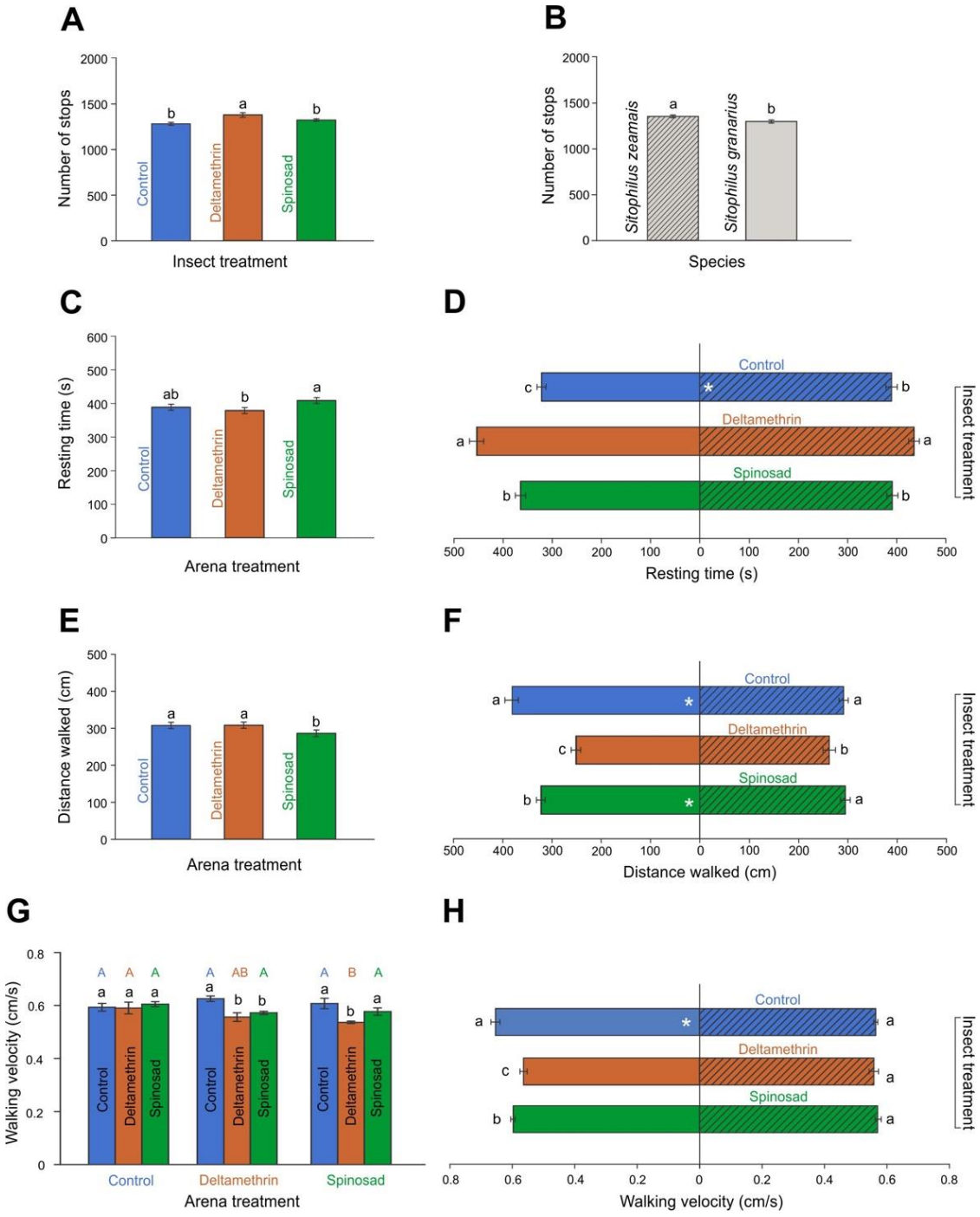

Fig. 3. Individual walking activity (mean ± SE) of *Sitophilus granarius* (plain bars) and *Sitophilus zeamais* (hachured bars) exposed to deltamethrin, spinosad or water (control). (A) number of stops of the insects exposed to control or insecticides via treated-grains, (B) number of stops between species, (C) resting time of insects exposed to control or insecticides via arena treatment, (D) resting time of insects within the interaction between species and insect treatment (i.e., insects exposed via treated-grains), (E) distance walked of insects exposed to control or insecticides via arena treatment, (F) distance walked of insects within the interaction between

species and insect treatment, (G) walking velocity of insects exposed to control or insecticides via arena and insect treatment, (H) walking velocity of insects within the interaction between species and insect treatment. In A, B, C and E, letters indicate significant differences among a single factor (insect treatment, arena treatment or species). In G, lowercase letters indicate significant differences on walking velocity in contrasts stablished for the factor insect treatment within every level of the factor arena treatment and capital letters indicate significant differences in contrasts stablished for arena treatment within every level of the factor insect treatment. In D, F and H, lower case letters indicate significant differences in contrasts stablished for insect treatment in each level of the factor species (i.e., *S. granarius* or *S. zeamais*) and, asterisks indicate significant differences between species in each level of the factor insect treatment (i.e., control, deltamethrin or spinosad treatments). The significances of all contrasts within every combination of factors were tested by chi-square test ($p < 0.05$).

3.4. Activity in half-treated arenas

Behavioral avoidance of *S. zeamais* and *S. granarius* exposed to deltamethrin and spinosad (via either by exposure to treated grains or half-treated bottom arena surfaces) were detected only for the species ($\chi^2 = 16.33$, df = 1, $p < 0.0001$) and arena treatment ($\chi^2 = 4.73$, df = 1, $p = 0.0297$). Irritability was higher for *S. granarius* than *S. zeamais* ($\chi^2 = 15.52$, df = 1, $p < 0.0001$) although no significant difference was observed between non-irritated and irritated insects for *S. granarius* ($\chi^2 = 0.18$, df = 1, $p = 0.671$) (Fig. 4A). In contrast, deltamethrin contaminated arenas sparked about 42% avoidance, which was higher than spinosad ($\chi^2 = 4.65$, df = 1, $p = 0.0310$) (Fig. 4B). Insecticide repellence (when the insect stays less than 1 s on the half-treated area of the arena) was only detected for three insects of *S. granarius* in all the experiments; thus no statistical analyses were performed.

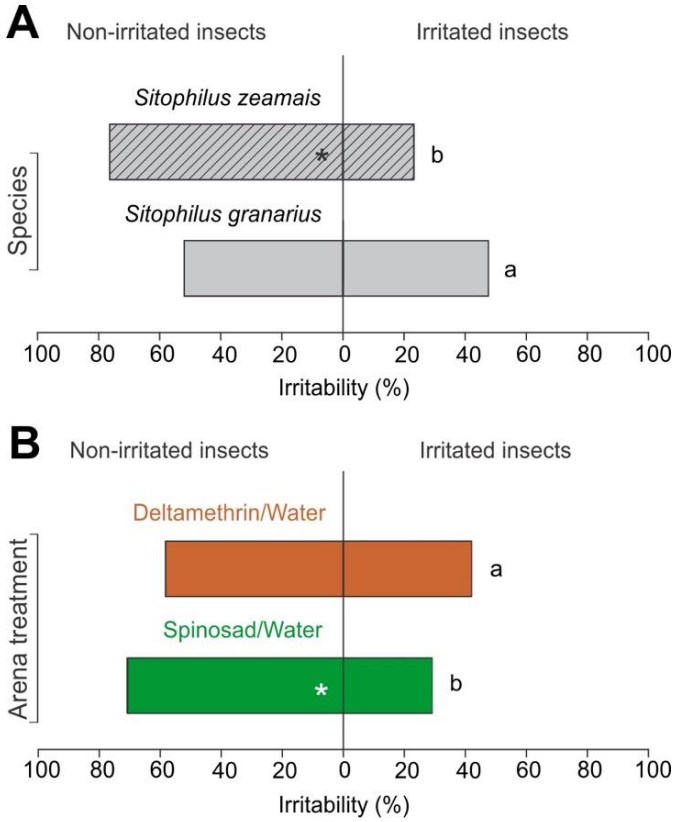

Fig. 4. Insecticide irritability (%) of *Sitophilus zeamais* and *Sitophilus granarius*. A) The bars represent the levels of irritability for each species (A) and arena treatment (B) (i.e., the half-treated arenas encompassing the dichotomous treatments control/deltamethrin and control/spinosad). In A and B different letters indicate significant differences between treatments (i.e., levels within the factor). Asterisk indicates significant difference between the proportion of irritated and non-irritated insects. All differences were obtained by contrasts with chi-square test ($p < 0.05$).

3.5. Free-choice test of preference

The linear model for the proportion of insects of *S. zeamais* and *S. granarius* selecting deltamethrin- or water-treated grains indicated significant differences between species with *S. granarius* exhibiting higher avoidance to deltamethrin-treated grains ($\chi^2 = 12.35$, df = 1, $p = 0.0004$), as well as between deltamethrin-contaminated and non-contaminated grains for both *S. zeamais* ($\chi^2 = 25.53$, df = 1, $p < 0.0001$) and *S. granarius* ($\chi^2 = 54.55$, df = 1, $p < 0.0001$) (Fig. 5A). In contrast, no significant preference was observed between species when they were offered both spinosad- and water-sprayed grains ($\chi^2 = 1.66$, df = 1, $p = 0.1979$), despite significant avoidance of *S. granarius* to spinosad-treated grains ($\chi^2 = 8.50$, df = 1, $p = 0.0035$) (Fig. 5B). Finally, both weevil species exhibited significant preference for spinosad-treated

grains than for deltamethrin treated-ones (*S. zeamais*: χ^2 = 6.60, df = 1, p = 0.0102; *S. granarius*: χ^2 = 16.78, df = 1, p < 0.0001) with no significant difference between species (χ^2 = 1.43, df = 1, p=0.2318) (Fig. 5C).

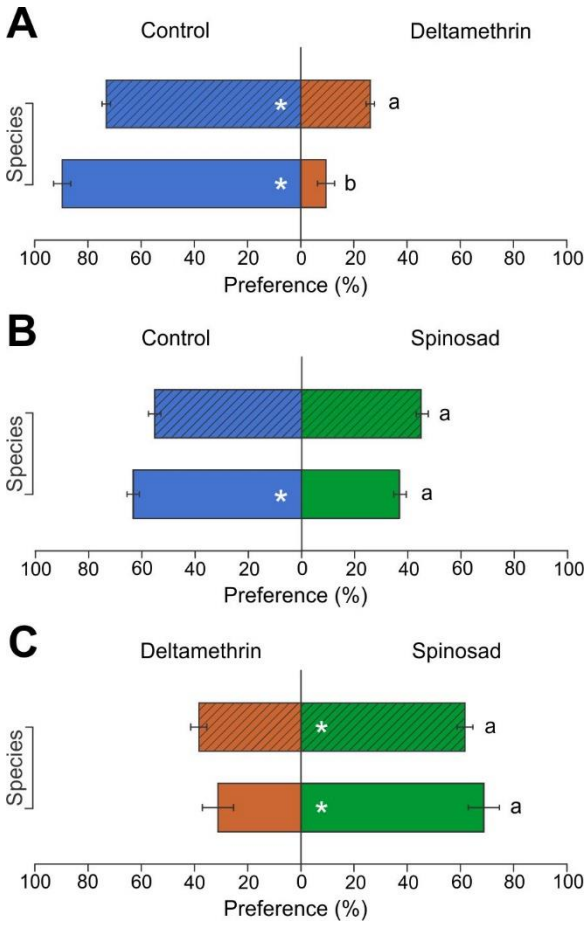

Fig. 5. Proportion (±SE) of adults of *Sitophilus zeamais* (hachured bars) and *Sitophilus granarius* (plain bars) showing their preference in dichotomous free-choice tests: (A) deltamethrin- and water- sprayed, (B) spinosad- and water-sprayed and (C) deltamethrin- and spinosad-sprayed grains. Different letters indicate significant differences between species (i.e., *S. granarius* and *S. zeamais*) and asterisk indicates significant difference between treated or untreated grains by chi-square test (p < 0.05).

3.6. Drinking preference

Neither sex, species, nor the interaction among these factors affected drinking preference in the three ditochomous treatments of drinking preference (control-deltamethrin, control spinosad and deltamethrin-spinosad treatments) (chi-square test: p > 0.05). Nonetheless, weevils from both species were able to discriminate between deltamethrin-contaminated and

uncontaminated water with preference for the latter (χ^2 = 63.65, df = 1, p <0.0001; Fig. 6A); most intakes were confirmed only for uncontaminated water-droplets (χ^2 = 39.32, df = 1, p < 0.0001) compared with those treated with deltamethrin (χ^2 = 25.23, df = 1, p < 0.0001). Spinosad did not elicit avoidance in the drinking preference (χ^2 = 2.91, df = 1, p < 0.0879), however the number of confirmed intakes was higher for *S. granarius* than *S. zeamais* (χ^2 = 5,88, df = 1, p = 0.0153; Fig. 6B)**.** Lastly, weevils chose spinosad- rather than deltamethrin-contaminated water (χ^2 = 60.05, df = 1, p < 0.0001; Fig. 6C); in addition, the number of ingestions were higher for spinosad- (χ^2 = 46.12, df = 1, p < 0.0001; Fig. 6C) than for deltamethrin-contaminated water droplets (χ^2 = 22.02, df = 1, p < 0.0001; Fig. 6C).

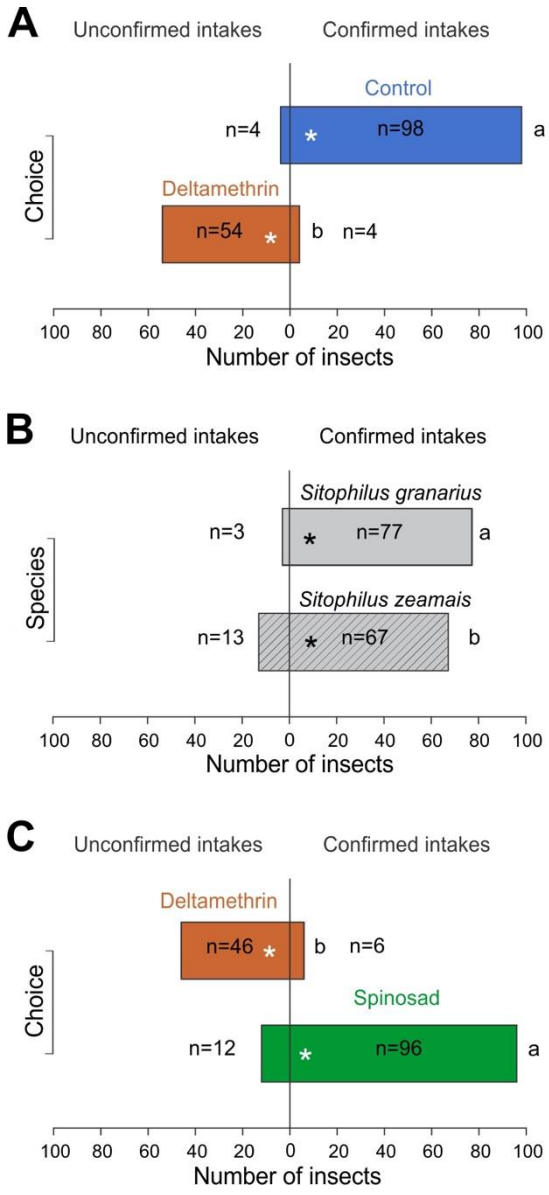

Fig. 6. Drinking preference of adults of *Sitophilus zeamais* and *Sitophilus granarius* weevils in dichotomous free-choice tests. (A) represents the test between deltamethrin-contaminated and

uncontaminated water, (B) represents deltamethrin-contaminated and spinosad-contaminated and, (C) represents spinosad-contaminated and uncontaminated water. Different letters indicate significant difference in the insect choice (A and C) or species (B) and asterisk indicates significant difference in the confirmation of the drinking by the insect (chi-square test: $p < 0.05$).

4. Discussion

Spinosad use has been increasing for the control of insect pest species, but there is sparse information available regarding its sublethal effects, in contrast with what is available for pyrethroid insecticides such as deltamethrin. Deltamethrin and spinosad at the concentrations of 0.5 mg (a.i.) /kg maize and 1 mg (a.i.) /kg maize respectively, were able to controll *S. zeamais* and *S. granarius*, although spinosad killed 100% of the insects after almost 12 days longer than deltamethrin. As corroborated by other authors, spinosad seems to be less effective than deltamethrin for short (24 h) exposure periods (Sanon et al., 2010). Despite the methodological differences presented in the literature, our findings contradict the perception that *S. granarius* is more susceptible to deltamethrin than *S. zeamais* (Williams et al., 1978; Kljajić and Perić, 2009). Spinosad also led to high mortality of *S. zeamais* and *S. granarius* at its label rate and was as effective as deltamethrin.

The adverse lethal and sublethal effects of spinosad and deltamethrin have been observed in diverse insect species (Elliot et al., 1978; Casida et al., 1983; Desneux et al., 2004; Huang and Subramanyam, 2007; Guedes et al., 2009a,b; Barbosa et al., 2015). The present study showed that deltamethrin and spinosad compromised walking activity, but deltamethrin exhibited a higher impact than spinosad possibly due to its fast mode of action, which paralyzes the nervous system leading to a quick knock down effect, loss of co-ordination and subsequent death (Ananware, et al., 2014; Velki et al. 2014; Paudyal et al., 2016).

Spinosad and deltamethrin reduced the overall group activity in *S. zeamais* and *S. granarius*. However, *S. zeamais* exhibited a higher suppression in activity when treated with deltamethrin and spinosad, while in *S. granarius* this reduction was observed only when the insects were treated with deltamethrin. The low activity level detected with sublethal deltamethrin and spinosad exposure was due to a reduced rate of activity with insects remaining inactive or under low levels of activity for longer. The exposed insects also frequently changed the pattern of activity from higher to lower levels likely induced by the mechanism of action of these insecticides (Salerno et al., 2002; Castro et al., 2013; Barbosa et al., 2015).

Two different types of insecticide avoidance have been observed in insects: repellence (i.e., avoidance with very brief or non-contact with insecticide) and irritability (i.e., avoidance after contact with insecticide) (Metcalf, 1955; Gould, 1984; Hodge and Longley, 2000; Sungvornyothin et al., 2001; Cordeiro et al., 2010). Insecticide repellence was not observed in *S. zeamais* and *S. granarius* although insecticide irritability was observed. *Sitophilus granarius* exhibited higher irritability than *S. zeamais*, and deltamethrin led to higher irritability than spinosad. Irritability to pyrethroids have also been reported among mosquitoes, flies, predatory stink bugs, and grain weevils (Quisenberry et al., 1984; Vatandoost, 2001; Vatandoost and Borhani, 2004; Mongkalangoon et al., 2009; Silva et al., 2013). In contrast, spinosad avoidance was only reported in the predatory stink bugs *Podisus nigrispinus* and *Supputius cincticeps* (Castro et al., 2013).

Different feeding responses were also detected for *S. zeamais* and *S. granarius* weevils in free-choice tests. Our results showed a preference for water-sprayed maize grains and a clear avoidance of deltamethrin-treated maize grains indicating that both species can detect the insecticide residue on the grain surface. The avoidance to spinosad-treated grains was lower than deltamethrin-treated ones. Similar response of both species was also observed in the drinking preference test. In this case, most of the insects walked towards uncontaminated rather than to deltamethrin-contaminated water. In addition, the insects avoided to drink when they chose the deltamethrin-contaminated water, unlike when uncontaminated and spinosad-contaminated water were reached. This result may be possible because the neurotoxic activity of pyrethroids has been also associated to avoidance behavioral responses among insects and other arthropods (Gammon, 1978; Lockwood et al., 1984; Pekar and Haddad, 2005). In addition, spinosad exhibits better safety profile than deltamethrin (Castro et al., 2013). Our results also provide support for the earlier reports of deltamethrin-induced behavioral avoidance among populations of the maize weevil (Guedes et al., 2009a; Guedes et al., 2009b, 2014; Corrêa et al., 2011; Morales et al., 2013).

Previous studies on labial and maxillary palpi of *S. zeamais* and *S. granarius* indicated the presence of sensilla on the distal apex of each maxillary and labial palp that function as gustatory chemoreceptors (Farazmand and Chaika, 2008; Moon, 2015; Fouda et al., 2016). Some studies pointed out that anti-feeding response to antifeedants is likely caused by compromising the activity of these chemoreceptors (Luo, et al., 1995; Li, 1999). Therefore, the different levels of avoidance of *S. zeamais* and *S. granarius* to insecticides may be related to the presence of the chemoreceptors and the presence of sensilla governing the avoidance.

In summary, this study assessed the survival time, and the walking, feeding and drinking behavioral responses of *S. zeamais* and *S. granarius* to two insecticides, deltamethrin and spinosad. Both weevil species exhibited abrupt behavioral changes when exposed to deltamethrin and showed a faster mortality than adult weevils exposed to spinosad, although spinosad also led to changes in all the behaviors recorded. Other relevant behaviors are also likely affected by insecticide exposure, including exposure to the biopesticide spinosad, what remains to be assessed. The current study suggests that spinosad is not as easily recognizable as deltamethrin by both grain weevil species. Such a fact encourages spinosad use as exposure this insecticide will not be subjected to the levels of avoidance elicited by deltamethrin. Thus, despite the lower toxicity of spinosad than deltamethrin to *S. zeamais* and *S. granarius*, this biopesticide is still very effective against both species and it is less likely to spark behavioral avoidance than deltamethrin, favoring the weevil exposure to this insecticide enhancing this pest control.

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CHAPTER 2

Spinosad- and deltamethrin-induced impact on mating and reproductive output of the maize weevil *Sitophilus zeamais*

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M. Vélez[1], L. Botina[1], L.M. Turchen[1], W.F. Barbosa[1], R.N.C. Guedes[1,2]

[1]*Departamento de Entomologia, Universidade Federal de Viçosa, Viçosa, MG, Brazil*

[2]*USDA-ARS San Joaquin Valley Agricultural Sciences Center, Parlier, CA, USA*

Abstract

Assessments of acute insecticide toxicity frequently focus on the lethal effects on individual arthropod pest species and populations neglecting the impacts and consequences of sublethal exposure. However, the sublethal effects of insecticides may lead to harmful, neutral, or even beneficial responses that may affect (or not) the behavior and sexual fitness of the exposed insects. Intriguingly, little is known about such effects on stored product insect pests in general and the maize weevil in particular. Thus, we assessed the sublethal effects of spinosad and deltamethrin on female mate-searching, mating behavior, progeny emergence and grain consumption by maize weevils. Insecticide exposure did not affect the resting time, number of stops and duration of mate-searching by female weevils, but their walking velocity was compromised. Maize weevil couples sublethally exposed to deltamethrin and spinosad exhibited altered reproductive behavior (walking, interacting, mounting and copulating), but deltamethrin caused greater impairment. Curiously, higher grain consumption and increased progeny emergence were observed in deltamethrin-exposed insects, suggesting that this pyrethroid insecticide elicits hormesis in maize weevils that may compromise control efficacy by this compound. Although spinosad has less of an impact on weevil reproductive behavior than deltamethrin, this bioinsecticide also benefited weevil progeny emergence, but did not affect grain consumption. Therefore, our findings suggest caution using either compound, and particularly deltamethrin, for controlling the maize weevil, as they may actually favor this species population growth when in sublethal exposure requiring further assessments. The same concern may be valid for other insecticides as well, what deserves future attention.

Key-words: grain consumption, progeny production, insecticides, biopesticide, hormesis

Introduction

Insecticides are basic tools used to prevent and control the population growth of arthropod pest species (Cooper and Dobson 2007, Rabczenko et al. 2011, Maksymiv 2015), but their benefits and hazards remain contentious, at least in some scenarios (Matsumura 2004, Maksymiv 2015). One ongoing concern with insecticide use is the impact on non-target organisms, particularly natural enemies and pollinators (Desneux et al. 2007, Tomé et al. 2012, Barbosa et al. 2015, Lima et al. 2016). Curiously, while concerns over sublethal insecticide exposure in non-target organisms have increased (Hoy et al. 1998, Lee 2000, Jallow and Hoy 2005, Guedes et al. 2009a), lethal effects remain prevalent when the focus is pest species (Guedes et al. 2017b). However, insecticides are used to control insect pests, not necessarily cause pest mortality, which emphasizes the potential importance of sublethal insecticide stress in either enhancing or compromising control, possibilities that are frequently neglected (Guedes et al. 2016, 2017b).

The effects of sublethal insecticide exposure may manifest as physiological (i.e., development, longevity, immunology, and fecundity) and behavioral changes (i.e., mobility, navigation, orientation, feeding, mating and oviposition behavior) (Haynes 1988, Lee 2000, Guedes et al. 2016). From this perspective, insecticides may induce stress in insects (Guedes et al. 2016, 2017b), resulting in a range of potential responses among the sublethally exposed individuals that may affect their longevity and reproduction as well as the genetic make-up of subsequent generations potentially leading to community-level effects (Lee 2000, Wingfield 2003, Guedes et al. 2009b, 2016, 2017a). Nevertheless, relatively few species of insect pests of stored products have been scrutinized regarding sublethal insecticide stress, and only to a limited extent and to few compounds. The species studied included flour beetles (*Tribolium confusum* and *T castaneum*, *Latheticus oryzae*, and *Gnatocerus maxillosus*), grain weevils (*Sitophilus oryzae* and *S. zeamais*), the sawtoothed grain beetle (*Oryzaephilus surinamensis*), and the lesser grain borer (*Rhyzopertha dominica*) (Faragalla et al. 1985, Hodges and Meik 1986, Hobbs and Bond 1989, Bell 1991, Lorini and Galley 1998, Hagstrum and Subramanyam 2006, Pimentel et al. 2012). Despite the frequent problems of irregular insecticide coverage and/or unsuitable application in storage facilities, in addition to environmental degradation, that make sublethal exposure as important as lethal exposure (Guedes et al. 2016, 2017b).

The maize weevil, *Sitophilus zeamais* Motsch. (Coleoptera: Curculionidae), is a key arthropod pest species of stored cereals, particularly maize, throughout the warm regions of the world, and it frequently reaches economically damaging population sizes that require control measures to minimize grain losses (Rees 1996, White and Leesch 1996, Pereira et al. 2009,

Pimentel et al. 2009, Makundi et al. 2010). Maize weevil control relies heavily on the use of synthetic insecticides (i.e., pyrethroids and organophosphates) and fumigants (i.e., phosphine) (Guedes 1991, Ribeiro et al. 2003, Li et al. 2010), which are commonly used in a variety of stored commodities (Fragoso et al. 2005, Pereira et al. 2009, Pimentel et al. 2009, Kavallieratos et al. 2015). However, over-reliance on insecticides and fumigants to control this pest species has led to insecticide resistance and frequent control failures (Guedes et al. 1995, Subramanyam and Hagstrum 1996, Guedes et al. 2009a, Pereira et al. 2009, Guedes 2017).

The search for novel insecticides is one of the consequences of growing concerns over insecticide resistance and control failure (Boukouvala et al 2016 ab, 2017). Among novel insecticidal compounds, those of natural origin are drawing increased attention due to the perception of their greater safety, which is not always justified since origin is not a determinant of toxicity or safety. Few biopesticides, of which spinosad is the main example, exhibit potential for the protection of stored products. Spinosad is a bioinsecticide made from a mixture of spinosyn A and D, which are fermentation products of the actynomycete species *Saccharopolyspora spinosa* Mertz & Yao (Thompson et al. 2000, Sparks et al. 2001), and it is used against stored grain pest species, including the maize weevil (Toews and Subramanyam 2003, Huang and Subramanyam 2007, Athanassiou et al. 2008, Kavallieratos et al. 2010, Athanassiou and Kavallieratos 2014). However, unlike other insecticides such as the pyrethroid deltamethrin (Spratt 1979, Salerno et al. 2002, Guedes et al. 2009a, Guedes et al. 2009b, Guedes et al. 2017a), there is sparse information available regarding the response of insect pests to sublethal exposure to spinosad, although its lethal efficacy has been broadly recognized (e.g., Toews and Subramanyam 2003, Athanassiou et al. 2004, Huang and Subramanyam 2007, Kavallieratos et al. 2015).

Our study aimed to explore the mating responses of the maize weevil to sublethal exposure to spinosad and deltamethrin (used as positive control) and the reproductive consequences of such exposure as well as the associated grain loss. We expected significant behavioral effects from both insecticides based on their neurotoxic activity (Sparks et al. 2001, Velki et al. 2014). Deltamethrin impairs different maize weevil behaviors, and we expected the same with spinosad. However, the latter is a slower-acting compound that has not yet been the target of sublethal assessments in this group of pest species.

Materials and Methods

Insects and insecticides

An insecticide-susceptible strain of the maize weevil was used in the present investigation. It was collected in Sete Lagoas County and provided by the National Center of Maize and Sorghum of the Brazilian Agricultural Research Corporation (EMBRAPA Milho and Sorgo). This strain has been maintained in whole maize kernels (var. BG 7049) free of insecticide residues since the mid-1980s under controlled temperature (27 ± 2°C), relative humidity (70 ± 10 %) and photoperiod (L:D 14:10 h) (Guedes et al. 1995, Morales et al. 2013, Ribeiro et al. 2003, Carvalho et al. 2014). These same conditions were employed in every bioassay.

The insecticides used in this study were the pyrethroid deltamethrin (K-Obiol 25 CE (emulsifiable concentrate at 25 g of active ingredient (a.i.)/L), Bayer CropScience Brasil, São Paulo, SP, Brazil) and the bioinsecticide spinosad (Tracer 480 SC (suspension concentrate at 480 g a.i./L), Dow AgroSciences, Mogi Mirim, SP, Brazil). Water (distilled and deionized) was used as a carrier for the commercial formulations of deltamethrin and spinosad, which were used at concentrations of 0.25 and 0.5 mg active ingredient (a.i.)/mL, respectively. A volume of 0.8 mL of insecticide solution, either deltamethrin or spinosad, was sprayed in 400-g batches of maize grains at concentrations of 0.5 and 1.0 mg a.i./kg of maize, respectively, using an air brush connected to an air compressor (Prismatec, model 131, type 2 VC) at a pressure of 3 bar. These concentrations correspond to the label rates for these insecticides against this pest species in Brazil (MAPA, 2017). The air brush was cleaned with acetone to prevent residual contamination, and distilled and deionized water was used as a negative control treatment.

Female mate-searching behavior

Virgin adults of *S. zeamais* were obtained by daily monitoring the emergence of the insects from maize kernels and removing the newly-emerged individuals before reaching sexual maturity, what takes a few days (Walgenbach et al. 1983, Walgenbach and Burkholder 1987). This procedure allowed gathering three groups of twenty-one virgin adult *S. zeamais* couples (one-week-old), which were sexed using their patterns of rostrum length, texture, and punctuation (Halstead 1963, Tolpo and Morrison 1965). Each group of virgin females was sublethally exposed to 400-g of maize grains (var. BG 7049) treated with either deltamethrin or spinosad at the previously described concentrations (or water as a negative control). The exposure lasted for half the lethal time for 25 % of the population (LT_{25}) for each insecticide (i.e., 30 min for deltamethrin and 10 h for spinosad), as previously estimated from time-

mortality survival bioassays (Vélez et al. 2017). After exposure, each virgin female was individually transferred with a fine hair brush to an arena comprising a Petri dish (9 cm in diameter and 2 cm high) lined at the bottom with filter paper (9 cm in diameter with a density of 80 g/m^2; Nalgon Equip. Cient., Itupeva, SP, Brazil) and whose inner walls were coated with Teflon PTFE (DuPont, Wilmington, DE, USA) to prevent the insects from escaping.

A perforated and transparente plastic cage (2cm in diameter and 2 cm high) was placed in the center of each arena and contained an unexposed virgin male, and each cage was covered at the top with a circular piece of paper (2.5 cm) to hide the male, preventing its activity from being recorded. The male-contained cage allowed visualization of the male by the female and also diffusion of the male pheromone allowing female attraction. The searching activity of the virgin females within the arena was recorded until the female reached the plastic cage or up to 2 h; this walking activity was digitally transferred to a computer using an automated video tracking system equipped with a CCD camera (ViewPoint Life Sciences Inc., Montreal, Canada). The parameters recorded in each arena were mate-searching time (s), velocity (cm/s), proportion of time spent resting (%), and number of stops per minute (Guedes et al. 2009b, Morales et al. 2013, Carvalho et al. 2014, Pereira et al. 2014, Lima et al 2015).

Mating behavior

Females and males (one day old) were sexed and isolated in 30-mL transparent glass tubes (60 mm high x 25 mm diameter) containing 4 g of maize (var. BG 7049) and maintained for 7 d until they reached sexual maturity (Walgenbach and Burkholder 1987). The upper portions of the containers were closed with a piece of organza and a rubber band, and one week-old insects were then exposed to deltamethrin, spinosad, or water, as detailed previously. Male insects were code-marked on the thorax using white ink (Acrilex S.A., São Bernardo do Campo, SP, Brazil) to allow easy recognition. Insects were then placed in the center of an arena consisting of a Petri dish (5.5 cm in diameter and 1 cm high) lined at the bottom with filter paper and whose inner walls were coated with Teflon PTFE (DuPont, Wilmington, DE, USA).

Exposed females and males were paired according to their treatment (deltamethrin, spinosad, or water) and allowed to mate in the Petri dish arenas. Twenty couples from each treatment were observed and digitally recorded (HDR-XR520V, Sony, Tokyo, Japan) for 12 h or until the end of the first mating, the point when the focal couple separated. The fertility of these mated couples was not recorded because the stress of the required starvation (more than 12 h without food) may affect progeny production (Walgenbach and Burkholder 1987). Therefore, mediation of fertility by insecticide exposure was assessed in an additional

experiment described below.

The assessed behavioral traits were adapted from previous studies (Walgenbach and Burkholder 1987, Guedes et al. 2017a), and the mating behavior and its transitions were recorded using the software JWatcher (Blumstein and Daniel 2007). The sequence of mating behaviors included walking (i.e., walking and exploratory movements), interacting (i.e., initial and subsequent contacts via rostral probing or antennation), mounting (i.e., mounting attempts and male mounting of a quiescent female for less than 1 h), dismounting (i.e., male gives up of mounting and/or female rejects the mounting male and dislodged male) and copulating (i.e., female becomes quiescent for over 1 h thus allowing fertilization). These mating bioassays were performed under the same controlled environmental conditions used for rearing the weevils.

Progeny emergence and grain consumption

Three groups of thirty-five virgin adult maize weevil couples (1-week-old) were sexed and sublethally exposed to 400-g batches of maize grains maize (var. BG 7049) treated with deltamethrin or spinosad (or water, as a negative control), as detailed above. Each couple was individually released into 140-mL plastic jars containing 50 g of whole maize and removed after 30 days following the methods of Trematerra et al. (1996), Fragoso et al. (2005) and Carvalho et al. (2014). The daily and cumulative progeny emergences were recorded every other day and evaluated until 5 days after the last insect emerged. Grain consumption was determined by contrasting the difference in grain weight at the beginning and at the end of the experiment (after insect removal and when no further progeny emerged, i.e. 60 days) with eventual correction for humidity change, if necessary.

Statistical analyses

The female mate-searching time was subjected to survival analysis using Kaplan–Meier estimators to obtain time-response curves (i.e., time failure or survival analyses), and the overall similarity among the curves was tested using the χ^2 log-rank test ($P < 0.05$). The female mate-searching parameters (i.e., velocity (cm/s), rate of insect inactivity (%) and number of stops per minute) were subjected to analysis of variance (ANOVA) and contrasted with Tukey's HSD test ($P < 0.05$) when appropriate (R Core Team 2015). The assumptions of normality and homoscedasticity were checked for each variable using Bartlett's and Shapiro-Wilk's tests, respectively, but no data transformation was required.

The mating behavior of *S. zeamais* was represented as simplified ethograms based on 1st-

order behavioral transitions. The frequencies of the behavioral transitions of each couple exposed to each insecticide were tested using χ^2 contingency tables (3 x 4; $P < 0.05$; PROC FREQ; SAS software) (SAS Institute 2002), and the time budget of every mating behavior was subjected to analysis of variance and Tukey's HSD test ($P < 0.05$) after ascertaining the required assumptions, as previously indicated. The daily and cumulative emergence results were subjected to non-linear regression analysis using the curve-fitting procedure of TableCurve 2D (Systat, San Jose, CA, USA); the significant regression models ($P < 0.05$) were tested from the simplest (linear and quadratic) to more complex peak models. Model selection was based on parsimony, high F-values (and mean squares), and a steep increase in R^2 with model complexity. The overall grain consumption by the emerging progeny was subjected to ANOVA and Tukey's HSD test ($P < 0.05$) after checking the normality and homoscedasticity assumptions.

Results

Female mate-searching behavior

The searching activity of virgin *S. zeamais* females exposed to water, spinosad or deltamethrin to find virgin males did not lead to significant differences in the proportion of time spent inactive or resting (%) (water: 9.83 ± 0.47 %; spinosad: 9.42 ± 0.5 %; deltamethrin: 11.49 ± 0.64 % [$F_{2,55} = 0.898$; $P = 0.413$]) or in the number of stops per minute (water: 10.00 ± 0.48; spinosad: 9.50±0.50; deltamethrin: 10.25±0.57 [$F_{2,55} = 3.971$; $P = 0.054$]). Additionally, insecticide exposure did not elicit significant differences in female mate-searching time (log-rank test: $\chi^2 = 2.21$, df = 2, $P = 0.346$), but significant differences were observed during mate-searching in walking velocity, with unexposed females exhibiting higher searching velocity (Fig. 1).

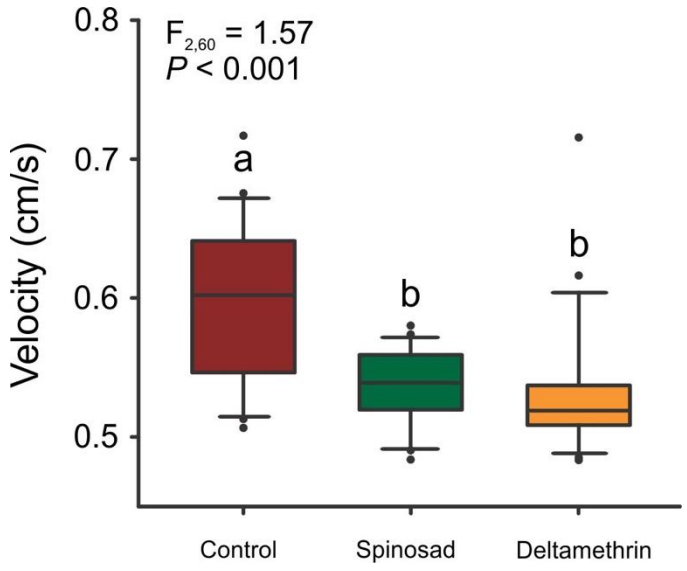

Fig. 1. Walking velocity of adult female maize weevils (*Sitophilus zeamais*) exposed to water (negative control), spinosad or deltamethrin while searching for an unexposed mate. Different letters at the top of the box plot indicate significant differences by Tukey's HSD test ($P < 0.05$).

Mating behavior

The simplified ethograms of the 1st-order maize weevil behavioral transitions were represented as general diagrams (Fig. 2), and their overall frequency varied significantly among treatments ($\chi^2 = 256.72$; df = 10; $P < 0.001$). Remarkable differences in behavioral transitions were observed when spinosad- and deltamethrin-exposed couples were compared with water-exposed couples, mainly in the transition between mounting to copulating, whose frequency was much higher in water-exposed couples. In contrast, the transition from mounting to walking was exhibited at a lower frequency in water-exposed couples.

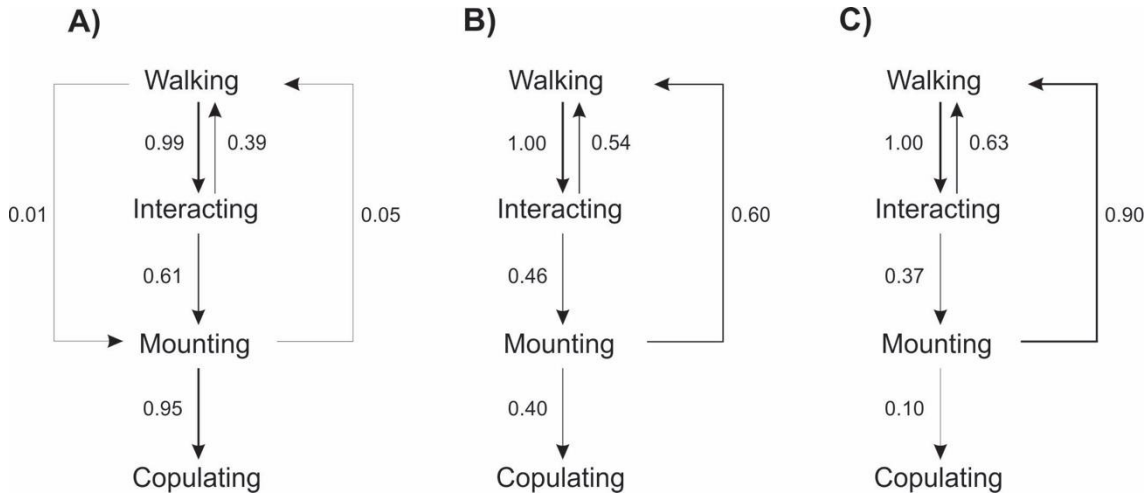

Fig. 2. Ethograms of mating behavior by the maize weevil (*Sitophilus zeamais*) exposed to water (negative control) (A), spinosad (B) and deltamethrin (C) represented as 1st-order transition diagrams. The solid arrows indicate each behavioral transition, and the relative thickness of each arrow represents the frequency of each behavioral transition (A, B, C: $n = 20$).

The duration of each behavior during mating significantly differed among treatments (Fig. 3A-D). Unexposed weevil couples spent less time walking and interacting than the insecticide-exposed couples (Fig. 3A,B). In contrast, the same unexposed couples spent longer mounting and copulating, particularly compared to deltamethrin-exposed couples, although spinosad-exposed weevils also exhibited lower coupling time than unexposed insects (Fig. 3C,D).

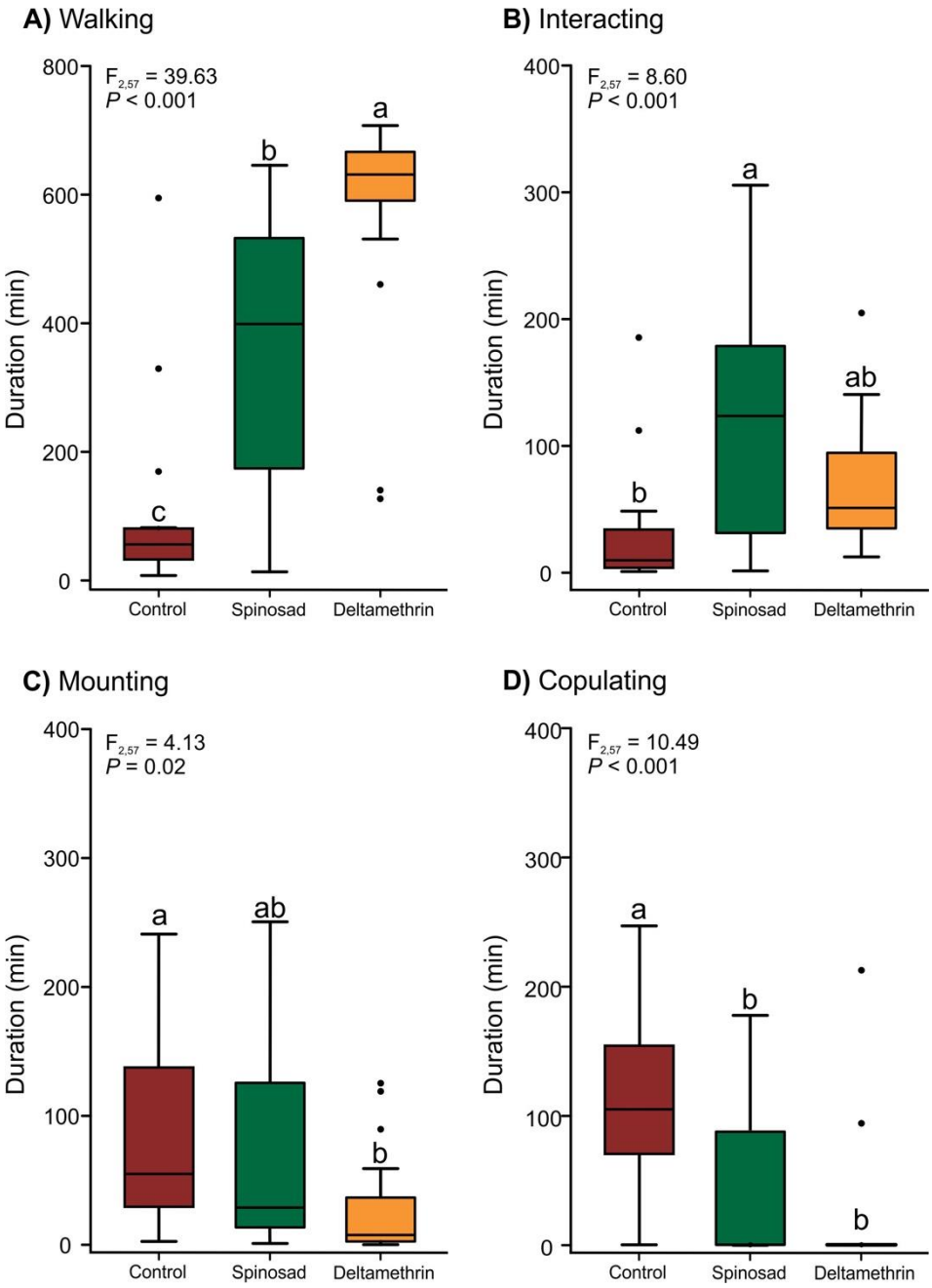

Fig. 3. Duration (± SE) of walking (A), interaction (B), mounting (C) and copulating (D) among couples of the maize weevil, *Sitophilus zeamais*, exposed to water (red box plot), spinosad (green box plot) and deltamethrin (orange box plot). Box plots indicate the median (solid line) and dispersal (lower and upper quartiles and outliers) of the duration values. Different letters at the top of the box plot indicate significant differences by Tukey's HSD test ($P < 0.05$).

Progeny emergence and grain consumption

The daily and cumulative emergence of adult progeny differed among treatments based on the non-overlapping of standard erros with the respective equation parameters of the alternative treatments fitted with the same model (Table 1). The peak adult emergence was slightly lower in unexposed than insecticide-exposed insects and took place slightly earlier for the progeny of deltamethrin-exposed insects followed by that of spinosad-exposed weevils (Fig. 4A, Table 1). The cumulative emergence profiles of parental *S. zeamais* weevils are a direct consequence of the daily emergence, and cumulative emergence was slightly higher for the progeny of spinosad-exposed weevils followed by the deltamethrin-exposed insects (Fig. 4B).

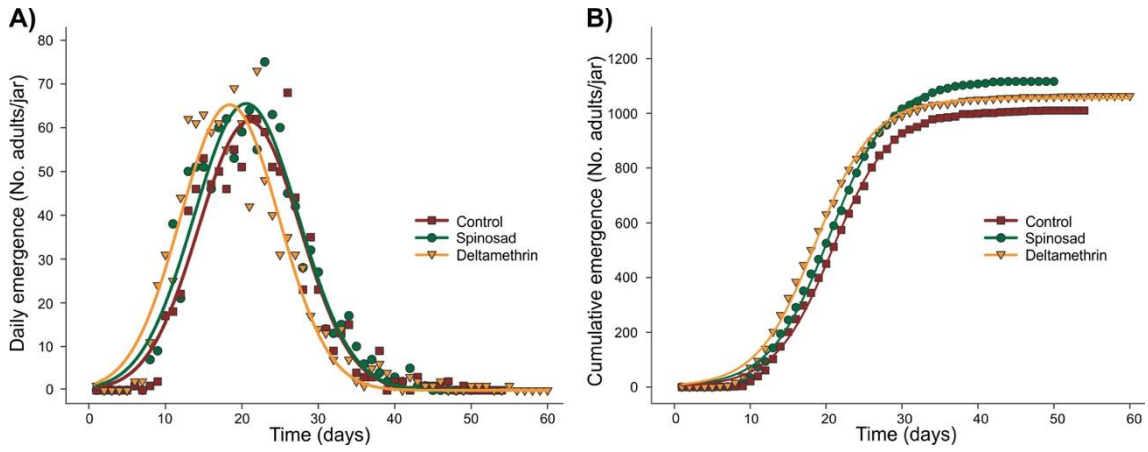

Fig. 4. Daily emergence of F_1 progeny (A) and the cumulative emergence (B) of maize weevils (*Sitophilus zeamais*) exposed to water (negative control), spinosad or deltamethrin. The equation parameters are exhibited in Table 1.

Table 1. Summary of the regression analyses of the daily emergence (Fig. 2A) and cumulative emergence (Fig. 2B) of the F_1 progeny of adult maize weevils (*Sitophilus zeamais*) exposed to water (negative control), spinosad or deltamethrin.

	Model	Treatment	Parameter estimates (± SE)			df_{error}	F	*P*	R^2 Adj
			a	*b*	*c*				
Daily emergence	Gaussian (3-parameter) $y = a \exp(-0.5((x-b)/c)^2)$	Control	61.48 ± 2.08	21.00 ± 0.26	6.62 ± 0.26	51	377.61	< 0.001	0.93
		Spinosad	65.50 ± 2.06	20.58 ± 0.25	6.84 ± 0.25	47	395.05	< 0.001	0.94
		Deltamethrin	65.22 ± 2.12	18.42 ± 0.24	6.43 ± 0.24	57	435.22	< 0.001	0.94
Cumulative emergence	Sigmoid (3-parameter) $y = a/(1+ \exp(-(x-b)/c))$	Control	1006.61 ± 3.30	20.84 ± 0.08	3.80 ± 0.07	51	21410.90	< 0.001	0.99
		Spinosad	1112.83 ± 4.13	20.50 ± 0.08	3.96 ± 0.07	47	19358.00	< 0.001	0.99
		Deltamethrin	1053.83 ± 3.36	18.61 ± 0.09	3.83 ± 0.08	57	16183.40	< 0.001	0.99

Grain consumption by the progeny of parental *S. zeamais* weevils exposed to water, spinosad or deltamethrin varied among treatments (Fig. 5). Weevils exposed to deltamethrin consumed more grain (2.82 ± 0.11 g) in contrast with the progeny from parental weevils exposed to spinosad (2.40 ± 0.09 g) or water (2.13 ± 0.06 g) (Fig. 5).

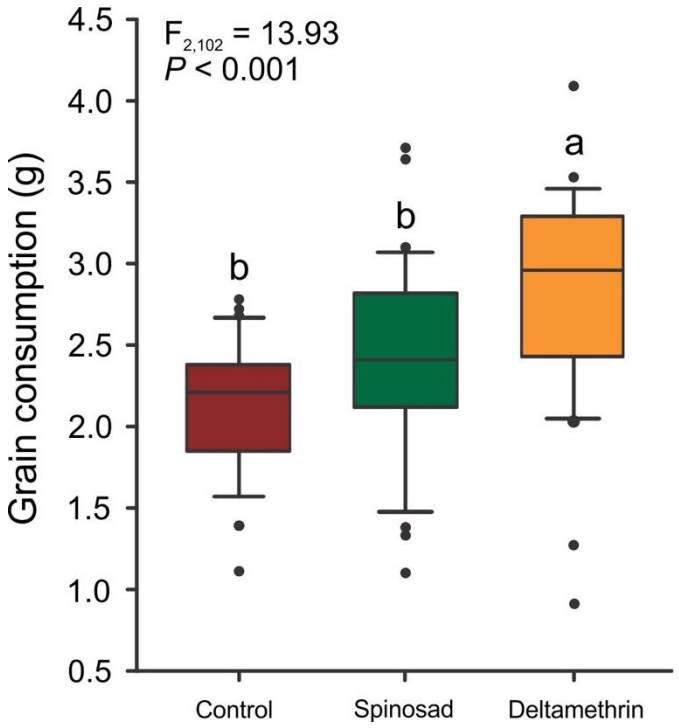

Fig. 5. Grain consumption by F_1 progeny of maize weevils (*Sitophilus zeamais*) exposed to water (negative control), spinosad or deltamethrin. Means followed by the same letter in the box plot are not significantly different by Tukey's HSD test ($P < 0.05$).

Discussion

Insecticide stress in arthropods is not restricted to lethal effects; sublethal effects are also important because insects are continually exposed to sublethal concentrations for longer periods (Pereira et al. 2014, Guedes et al. 2016, 2017b). While lethal effects are the usual focus, the impact and consequences of insecticidal stress by sublethal exposure have long been recognized as important, but they are frequently neglected (Metcalf 1980, Hardin et al. 1995, Guedes et al. 2016). Among stored product insect pests, the concern over sublethal insecticide stress is also lagging, particularly for compounds that have come into use relatively recently (Guedes et al. 2014), such as spinosad.

Deltamethrin and spinosad have been shown to be effective against a broad range of stored product insect pests, including the maize weevil, *S. zeamais* (Evans 1985, Toews and Subramanyam 2003, Hagstrum and Subramanyam 2006, Huang and Subramanyam 2007). However, studies of the sublethal effects of these insecticides, especially spinosad, on their targeted pest species remain scarce. Our study indicated that sublethal exposure to both deltamethrin and spinosad at their respective label rates significantly affected the maize weevil, but deltamethrin elicited a stronger response. Such divergence in the responses to sublethal doses of deltamethrin and spinosad is likely due to the different modes of action of these insecticides. While deltamethrin paralyzes the nervous system of insects, yielding a quick knockdown effect and the loss of co-ordination (Velki et al. 2014, Paudyal et al. 2016), spinosad disrupts the nicotinic acetylcholine receptors (nAChRs) and aminobutyric acid (GABA) receptors in the insect nervous system (Sparks et al. 2001).

Insecticides may also interfere with pheromonal systems and affect sexual communication, which may disrupt mate choice and decrease mating success (Haynes and Baker 1985, Park et al. 2001, Knight and Flexner 2007, Lürling and Scheffer 2007). The present study showed that communication between *S. zeamais* couples did not seem to be affected when females were exposed to insecticides because both unexposed and insecticide-exposed females were attracted by the males. This response was probably due to aggregation pheromone emission by (unexposed) males that is responsible for female attraction (Walgenbach et al. 1983, Phillips et al. 1985, Landolt 1997).

Although sublethal insecticide exposure did not affect the communication between insecticide-exposed females and untreated males, exposure compromised female walking velocity, which is consistent with the findings of previous laboratory studies in which

deltamethrin and spinosad altered the walking behavior of *S. zeamais* (Vélez et al. 2017). Furthermore, reproductive behavior (walking, interacting, mounting and copulating) was altered in maize weevil couples that were sublethally exposed to deltamethrin and spinosad, but these results were expected because insecticides can interfere with normal reproductive imperatives (Haynes 1988). The mode of action of the insecticides was likely responsible of this disturbance as both spinosad and deltamethrin are neurotoxic compounds, for which such secondary effects on motor activity have been reported in different insect species (Salerno et al., 2002; Guedes et al., 2009a; Pereira et al., 2014; Barbosa et al., 2015). Deltamethrin elicited a stronger behavioral response in maize weevils, likely due to its fast action in the axon Na+ channels of neurons, in contrast to the slower-acting (synaptic) effects of spinosad (Sparks et al. 2001; Casida and Durkin 2013).

The reported behavioral differences among insecticide-exposed and unexposed maize weevils led to differences in the daily and cumulative production of progeny, and deltamethrin-exposed weevils also exhibited higher grain consumption. This resulted in more abundant and earlier-emerging progeny, which is an apparent expression of insecticide-induced hormesis (i.e., a beneficial effect from low doses of compounds that are toxic at higher doses) (Cutler 2013, Guedes and Cutler 2014, Guedes et al. 2016). The impacts of such responses may lead to failures to control agricultural pests, thus favoring subsequent pest resurgence and/or outbreaks (Hardin et al. 1995, Cutler et al. 2009, Cordeiro et al. 2013, Guedes et al. 2010). The mechanisms regulating stimulatory responses (i.e., hormesis) to traditional insecticides are not well understood (Yu et al. 2010, Guedes and Cutler 2014), but the observed responses of maize weevils to (sublethal) insecticide exposure appears to result from compensatory biological processes by which exposed couples redirect their energy resources to offspring production rather than self-maintenance (Calabrese 1999, Guedes and Cutler 2014).

Although further investigation is required before any conclusive statements can be made, the current study suggests that sublethal insecticide exposure induces hormesis in maize weevils that compromises the control efficacy of these compounds, and even more so deltamethrin. Furthermore, this phenomenon is also a potential concern when managing pyrethroid-resistant maize weevil populations (Guedes et al. 2010). Although spinosad also elicit similar response, albeit somewhat milder regarding the behavioral responses and negligible regarding grain consumption, spinosad resistance is not yet a problem in this pest species. However, attention is also necessary when using this compound for managing this stored grain pest species and requires further assessments.

Acknowledgements

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CONSIDERAÇÕES FINAIS

Deltametrina e espinosade tem se mostrado eficazes contra uma ampla gama de insetos, incluindo pragas de grãos armazenados, tais como os gorgulhos do gênero *Sitophilus*. No entanto, muito pouco se conhece sobre os efeitos subletais dos inseticidas nesses insetos. No presente trabalho demostramos que deltametrina e espinosade, nas concentrações de 0,5 mg (i.a.) / kg de milho e 1 mg (i.a.) / kg de milho, respectivamente, foram capazes de controlar (100%) *S. zeamais* e *S. granarius* e que exposições subletais a esses inseticidas apresentaram mudanças comportamentais e fisiológicas no organismo dessas pragas.

A exposição subletal de deltametrina e espinosade aos gorgulhos dos grãos causou alterações nas respostas comportamentais de caminhamento e escolha de alimento e ingestão de líquidos dos insetos. Na avaliação do caminhamento, indivíduos expostos a deltametrina apresentaram mudanças abruptas comparados com aqueles provocadas pelo inseticida espinosade. Possivelmente pelo modo de ação do inseticida deltametrina o qual paralisa o sistema nervoso levando-o a uma rápida perda de coordenação. Nas avaliações de escolha de alimento e ingestão de líquidos, os resultados mostraram que insetos são capazes de evitar produtos que estejam contaminados, principalmente por deltametrina. Nossos resultados sugerem que espinosade é menos perceptível pelos insetos que deltametrina.

Na espécie *S. zeamais*, a procura da fêmea ao parceiro, comportamento de acasalamento, emergência da prole e consumo de grãos também foram avaliados. Em todos os casos, mudanças foram encontradas quando os gorgulhos foram expostos a deltametrina e ao espinosade, contudo insetos expostos a deltametrina mostraram

maior suscetibilidade. A maior emergência da prole e consumo de grãos detectados em insetos que foram expostos a deltametrina sugere que este inseticida desencadeia uma resposta hormética o qual pode comprometer a eficácia no controle desta praga.

O presente estudo recomenda cuidado na utilização dos dois inseticidas avaliados particularmente de deltametrina em *S. zeamais*, pois favorecem o crescimento da população dessa espécie o que pode prejudicar seu controle.