UNIVERSIDADE FEDERAL DE LAVRAS

VALÉRIA FONSECA MOSCARDINI

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LAVRAS – MG

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Entomologia, área de concentração em Entomologia, para a obtenção do título de Doutora.

Orientador

Dr. Geraldo Andrade Carvalho

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(EFEITOS SUBLETAIS DE TRATAMENTO DE SEMENTES DE
GIRASSOL COM INSETICIDAS SOBRE TRÊS ESPÉCIES DE
INIMIGOS NATURAIS DE AFÍDEOS)

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> de Lavras, como parte das exigências do
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> Doutora.

APROVADA em 12 de fevereiro de 2015.

Dr. J.P. Michaud - Kansas State University - EUA

Dr. Maurício Sérgio Zacarias – Embrapa - Café

Dr. Renê Luis de Oliveira Rigitano - UFLA

Dr. Ronald Zanetti Bonetti Filho - UFLA

Dr. Geraldo Andrade Carvalho
Orientador

LAVRAS – MG
2015

Aos meus pais Iara e Renato pelo amor, exemplo e incentivo,

DEDICO

AGRADECIMENTOS

A Deus pelo milagre da vida e por estar sempre presente em meu caminho.

À Universidade Federal de Lavras e ao Departamento de Entomologia pela oportunidade concedida para a realização do Doutorado.

À Fundação de Amparo à Pesquisa do estado de Minas Gerais (FAPEMIG), pela concessão da bolsa de estudos e pesquisa.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela concessão de bolsa de Doutorado sanduíche.

Ao professor Dr. Geraldo Andrade Carvalho, pela orientação, amizade, confiança e pelos ensinamentos que foram de grande importância para realização deste trabalho e para meu crescimento profissional. Meus agradecimentos também a sua esposa Ana Paula, pelo agradável convívio e amizade.

Ao professor Dr. J.P. Michaud, pela amizade, paciência, orientação e ensinamentos de grande importância durante o Doutorado sanduíche.

À Kansas State University, Agricultural Research Center - Hays, pela oportunidade para realização do Doutorado sanduíche.

Ao meu esposo Pablo Gontijo, pela amizade, companheirismo, paciência, amor e pela inestimável parceria nos trabalhos realizados.

Aos amigos do laboratório de Seletividade Rafaella, Rodrigo, Jader, Thaís, Brenda, Mariana, Andrea, Dejane, Wellington, Lucas, Dyrson e Frontino pela amizade.

Às amigas Lilian, Fernanda, Elisangela, Ana Luiza e Érika, pela amizade, companheirismo, longas conversas e momentos de alegria.

Aos professores do Departamento de Entomologia da UFLA, pelos ensinamentos e harmoniosa convivência durante o doutorado.

Aos funcionários do Departamento de Entomologia da UFLA Lisiane, Érica, Elaine, Nazaré, e em especial Léa, D. Irene e Julinho, pela valiosa colaboração e amizade.

Aos amigos do laboratório da Kansas State University, Clint e Mohamed, pela valiosa ajuda nos trabalhos realizados, amizade e harmoniosa convivência.

Às minhas amigas bibliotecárias americanas, Paula Elder e Jean Curtis-Neitz, por disponibilizarem tempo para ensinar inglês, pela amizade, momentos de alegria e diversão.

Aos professores Dr. Renê Luis de Oliveira Rigitano, Dr. Maurício Sérgio Zacarias, Dr. Ronald Zanetti Bonetti Filho por participarem da banca examinadora e pelas sugestões.

Aos meus pais, Iara e Renato, pelo amor incondicional e incentivo, meus irmãos Vinícius e Viviani, pela amizade e companheirismo, aos meus avós Hebe, Alaíde (*in memoriam*) e Djalma, pela torcida e incentivo, aos meus sobrinhos pelos momentos de alegria, descontração e carinho, aos meus cunhados Thiago e Rafaella, pela amizade e apoio e a todos meus familiares que contribuíram durante toda essa jornada.

Aos meus sogros Selma e João, pelo carinho, amizade e incentivo, ao meu cunhado Douglas e concunhada Larissa pelos momentos de alegria e amizade e a todos das famílias Costa e Gontijo por me receberem com muito carinho.

A todos, que direta ou indiretamente, contribuíram para o êxito deste trabalho, os meus sinceros agradecimentos.

GENERAL ABSTRACT

The extrafloral nectar (EFN) of sunflower, *Helianthus annuus* L., is an important resource for many insects and represents a potential route of exposure to systemic insecticides applied as seed treatments to cultivated varieties. This study examined whether chlorantraniliprole or thiamethoxam might negatively impact *Lysiphlebus testaceipes*, *Coleomegilla maculata* and *Hippodamia convergens* when they consumed the EFN of sunflowers grown from treated seed. Consumption of EFN by *L. testaceipes* caused no lethal effects, but reduced the numbers of *Schizaphis graminum* Rondani attacked and parasitized in Petri dish arenas. Whereas control females self-superparasitized every fourth host, those exposed to chlorantraniliprole did not. Thiamethoxam greatly reduced the proportion of female offspring, suggesting an impact on the egg fertilization process. Exposure of *C. maculata* larvae to chlorantraniliprole and thiamethoxam delayed adult emergence by prolonging the pupal period. When adults were exposed, thiamethoxam reduced the preoviposition period compared to chlorantraniliprole, whereas the latter treatment caused females to produce fewer clutches. Larvae of *C. maculata* did not appear to obtain sufficient hydration from the sunflower stems and their subsequent fecundity and fertility were compromised. Exposure of *H. convergens* larvae to thiamethoxam skewed the sex ratio in favor of females; both materials reduced the egg viability of resulting adult females and increased the period required for eclosion. Exposure of *H. convergens* adults to chlorantraniliprole reduced egg eclosion times compared to thiamethoxam and exposure to both insecticides reduced pupation times in progeny.

Keywords: Parasitoid. *Coleomegilla maculata*. *Hippodamia convergens*. Extrafloral nectar. Systemic insecticides.

RESUMO GERAL

Néctar extrafloral (NEF) de girassol, *Helianthus annuus* L. é uma importante fonte de alimento para muitos insetos e apresenta-se como potencial via de exposição a inseticidas sistêmicos utilizados no tratamento de sementes para diversas culturas. Este estudo teve como objetivo avaliar se clorantraniliprole ou tiametoxam podem afetar negativamente *Lysiphlebus testaceipes*, *Coleomegilla maculata* e *Hippodamia convergens* quando estes insetos alimentaram-se de NEF de girassol provenientes de sementes tratadas. O consumo de NEF por adultos de *L. testaceipes* não causou efeito letal, mas reduziu o número de *Schizaphis graminum* Rondani atacados e parasitados quando expostos em placa de Petri aos adultos do parasitoide. Fêmeas do tratamento controle auto-superparasitaram um a cada quatro hospedeiros encontrados quando comparadas com fêmeas expostas a clorantraniliprole. Tiametoxam reduziu consideravelmente a proporção de progênies fêmeas. A exposição de larvas de *C. maculata* a clorantraniliprole e tiametoxam prolongou o período de pupa. Quando adultos foram expostos, tiametoxam reduziu o período de pré-oviposição em comparação com clorantraniliprole. Fêmeas expostas a clorantraniliprole tiveram número reduzido de ovos. Larvas de *C. maculata* possivelmente não conseguiram se alimentar de forma adequada das hastes de girassol e adultos provenientes destas larvas expostas apresentaram baixa fecundidade e fertilidade. Adultos oriundos de larvas de *H. convergens* expostas a tiametoxam tiveram a razão sexual alterada; ambos inseticidas reduziram a viabilidade dos ovos da geração subsequente e aumentaram o período de eclosão dos ovos. Exposição de adultos de *H. convergens* a chlorantraniliprole reduziu o tempo de eclosão das larvas quando comparado com tiametoxam, e a exposição a ambos inseticidas reduziu o tempo de pupa na progênie.

Palavras-chave: Parasitoide. *Coleomegilla maculata*. *Hippodamia convergens*. Néctar extrafloral. Inseticidas sistêmicos.

SUMMARY

FIRST PART

GENERAL INTRODUCTION

Over the past several years, systemic insecticides have been used as seed treatments in sunflower (*Helianthus annuus* L.), cotton (*Gossypium* spp.), oilseed rape (*Brassica napus* L.), corn (*Zea mays* L.), soybean (*Glycine max* L.) and other cereals and crops (BRADSHAW; RICE; HILL, 2008; STRAUSBAUGH; EUJAYL; FOOTE, 2010) to control pests that cause damage early in the crop (HODGSON; KEMIS; GEISINGER, 2012; NUYTTENS et al., 2013). Systemic insecticides used in the treatment of seeds are characterized by low lipophilicity (CLOYD; BETHKE, 2011) which facilitates their translocation in plant tissues.

Chlorantraniliprole and thiamethoxam represent two different systemic groups, diamides and neonicotinoids, respectively. The anthranilic diamide class of insecticides activates ryanodine receptors, thus stimulating uncontrolled calcium ion release from muscle cells, causing insect paralysis (CORDOVA et al., 2006; LAHM et al., 2007). In contrast, neonicotinoids target nicotinic acetylcholine receptors in the insect central nervous system, causing receptor blockage, paralysis and death (TOMIZAWA; CASIDA, 2005). Furthermore, the conversion of thiamethoxam into highly toxic metabolites such as clothianidin (CASIDA, 2011), a process which can occur within both plant (CLOYD; BETHKE, 2011) and insect (NAUEN et al., 2003) tissues, may increase their toxicity to insects and their natural enemies. Nauen et al. (2003) demonstrated that clothianidin is one of the primary metabolites of thiamethoxam in true leaves of cotton plants treated via soil drench. However, its greater lipophilicity and lower solubility in water make clothianidin more likely than thiamethoxam to contaminate nectar.

Seed treatment may reduce the total amount of insecticide applied by confining it to the seed and thus confer environmental advantages compared with broadcast or soil or applications (TAYLOR; ECKENRODE; STRAUB, 2001) including reduced exposure of beneficial insects (CLOYD; BETHKE, 2011; MIZELL; SCONYERS, 1992). Despite these advantages, research has revealed lethal and sublethal effects of seed treatments on non-target organisms such as *Orius insidiosus* (Say) (Hemiptera: Anthocoridae) (GONTIJO et al., 2014a), *Chrysoperla carnea* (Neuroptera: Chrysopidae) (GONTIJO et al., 2014b) and *Anagrus nilaparvatae* (Pang and Wang) (Hymenoptera: Mymaridae) (LIU et al., 2010). Negative effects are due contamination of pollen and nectar, both floral and extrafloral (EFN), which are often important food sources for natural enemies (CHOATE; LUNDGREN, 2013; LUNDGREN, 2009; WÄCKERS; ROMEIS; VAN RIJN, 2007).

Non-target effects of systemic insecticides on beneficial species are a substantial risk because the majority of crops, including commercial sunflower, are now planted with seed treatments. On the High Plains of the USA, sunflower plants are an important source of EFN for beneficial insects during the hot, dry summers (ROYER; WALGENBACH, 1991). Sunflower extrafloral nectaries are extremely small and highly abundant along petioles and leaf veins; they secrete EFN continuously from the time the first true leaves expand until the plants senesce. The production of EFN by plants is associated with attraction of herbivore natural enemies and thus fosters mutualistic protection for the plant (MARAZZI; BRONSTEIN; KOPTUR, 2013). Intercropping plants with EFN-producing plants has even been proposed to supplement food for natural enemies early in the growing season before pests become abundant (JAMONT; CREPELLIERE; JALOUX, 2013). The generalist aphid parasitoid, *Lysiphlebus testaceipes* (Cresson) (Hymenoptera: Braconidae) and two species of predatory lady beetle, *Coleomegilla maculata* DeGeer and *Hippodamia convergens*

Guérin-Méneville (Coleoptera: Coccinellidae), are among the beneficial species attracted to sunflower EFNs on the High Plains of the USA.

Lysiphlebus testaceipes is a solitary endoparasitoid that attacks many aphid species (PIKE et al., 2000). It is an important natural enemy of the greenbug, *Schizaphis graminum* (Rondani) (Hemiptera: Aphididae) and contributes to biological control of this aphid in both sorghum (JONES, 2001) and wheat (GILES et al., 2003). Without access to nectar or some other sugar source, many parasitoids show a dramatic reduction in their ability to parasitize their hosts (LEWIS et al., 1998; STAPEL et al., 1997). Lady beetles such as *C. maculata* and *H. convergens* also consume pollen and nectar, both floral and EFN (PEMBERTON; VANDENBERG, 1993; SMITH; KRISCHIK, 1999). These species are two of the most abundant coccinellids in the central USA, and *H. convergens* is of particular importance in providing biological control of cereal aphids in wheat, sorghum and other grains (MICHAUD, 2013; NECHOLS; HARVEY, 1998; RICE; WILDE, 1988).

Considering the economic importance of sunflowers in grain and oil production and the widespread use of insecticidal seed treatments, the objective of the present study was to assess whether chlorantraniliprole or thiamethoxam could have any negative or sublethal impacts on *L. testaceipes*, *C. maculata* or *H. convergens* when various life stages fed on the EFN of sunflower seedlings grown from treated seed.

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SECOND PART – ARTICLES

ARTICLE 1

SUBLETHAL EFFECTS OF CHLORANTRANILIPROLE AND THIAMETHOXAM SEED TREATMENTS WHEN *Lysiphlebus testaceipes* FEED ON SUNFLOWER EXTRAFLORAL NECTAR

This article was written in accordance with the standards of BioControl, for which it was submitted, accepted and published (BioControl: Vol. 59, No. 5, p. 503-511, 2014).

Sublethal effects of chlorantraniliprole and thiamethoxam seed treatments when *Lysiphlebus testaceipes* feed on sunflower extrafloral nectar

Valéria F Moscardini,[a,b] Pablo C Gontijo,[a,b] JP Michaud[b]* and Geraldo A Carvalho[a]

[a]Department of Entomology, Federal University of Lavras, Lavras, Minas Gerais, Brazil.
[b]Department of Entomology, Kansas State University, Agricultural Research Center-Hays, Hays, Kansas, USA.
* Corresponding author: J.P. Michaud, 1232 240th Ave., Hays, KS, 67601, Email: jpmi@ksu.edu

Abstract   The extrafloral nectar (EFN) of sunflower, *Helianthus annuus* L., is an important-summer resource for many insects and represents a potential route of exposure to systemic insecticides applied as seed treatments to cultivated varieties. Among the many parasitoids that utilize sunflower EFN, *Lysiphlebus testaceipes* (Cresson) (Hymenoptera: Braconidae) is an important generalist parasitoid of cereal aphids in North America. This study evaluated the performance of adult wasps fed EFN of sunflower plants grown from seed treated with chlorantraniliprole and thiamethoxam. Consumption of EFN from treated sunflower seedings caused no lethal effects, but reduced the numbers of greenbug nymphs, *Schizaphis graminum* Rondani, attacked and parasitized when wasps foraged in Petri dish arenas. Whereas control females self-superparasitized every fourth hosts, those exposed to chlorantraniliprole did not. Offspring developmental time and adult emergence were unaffected by either treatment, but thiamethoxam greatly reduced the proportion of female offspring.

Introduction

Plants have evolved multiple strategies to defend themselves against herbivores. Aside from direct physical and chemical defences, nectar and pollen may attract natural enemies of herbivores in addition to pollinators (Pemberton and Lee 1996; Nicolson et al. 2007). Both floral and extrafloral nectar (EFN) are an important source of hydration and nutrition for parasitoids (Heimpel and Collier1996; Heimpel et al. 1997). The composition of EFN differs from that of floral nectar in most plant species (Baker et al.1978). Although both are mostly sugar by weight, EFN usually contains a full complement of essential amino acids, albeit in low concentrations, with sucrose the dominant sugar, rather than glucose or fructose (Baker and Baker1979; Rogers1985). Sugars can be essential for parasitoid survival (Lewis et al. 1998), longevity (Dyer and Landis 1996) and fecundity (Olson and Andow1998), and can improve parasitoid performance in biological control contexts (Gurr et al.2004). Another benefit of EFN is that it is available to natural enemies for a much longer period than floral nectar, beginning early in plant development and continuing up to, and beyond, flowering (Pacini et al. 2003; Rose et al. 2006).

On the High Plains of the USA, sunflower plants are an important source of EFN for beneficial insects during the hot, dry summers (Royer and Walgenbach 1991). Small nectaries that occur profusely along petioles and leaf veins secrete nectar from the time the first true leaves expand until the plants senesce. A great diversity of parasitoids feed on sunflower EFN (e.g., Charlet and Gavloski 2011) and the presence of sunflowers has been shown to improve

the area-wide survival of natural enemy species important in the biological control of key pests in neighboring crops (e.g., Brewer et al. 2008), probably by supplying critical resources for natural enemies (Gurr et al. 2004). Intercropping cultivated plants with EFN producing companion plants has even been proposed to supplementary food for natural enemies early in the growing season before pests become abundant (Jamont et al. 2013).

Like many other row crops cultivated on the High Plains, the majority of commercial sunflower seed is now planted with a systemic insecticide seed treatment which poses a potential hazard to natural enemies. Seed treatments have been promoted as more compatible with biological control than broadcast sprays of insecticides, primarily because seed treatment involves a smaller amount of pesticide applied in a more selective manner, with consequently reduced impacts on non-target organisms (Hull and Beers 1985; Albajes et al. 2003) and lower levels of environmental contamination (Taylor et al. 2001). Systemic insecticides typically show low lipophilicity and exhibit a low octanol-water partition coefficient (log Poct) (Cloyd and Bethke 2011). This property facilitates their translocation in plant tissues, and may lead to contamination of pollen, floral and EFN (Maienfisch et al. 2001; Lahm et al. 2009) that, in turn, can cause both lethal and sublethal effects on natural enemies over potentially prolonged periods (Lundgren 2009; Li et al. 2012). EFN is produced in much earlier stages of plant development than floral nectar and thus has the potential to bear higher concentrations of systemic insecticides applied as seed treatments. For example, residues of the neonicotinoid imidacloprid were 50 times more concentrated in cotyledons than in the first true leaves of sunflower, and 800 times more concentrated than in the apex of plant. Concentrations were typically reduced by half in each successive pair of leaves, reflecting progressive dilution as a function of plant growth (Laurent and Rathahao 2003).

Chlorantraniliprole and thiamethoxam represent two different systemic insecticide groups used in seed treatment, diamides and neonicotinoids, respectively. Diamide insecticides have a novel mode of action that acts exclusively on the ryanodine receptor in insect neuromuscular junctions (Nauen 2006; Lahm et al. 2009). In contrast, thiamethoxam is a neonicotinoid insecticide that targets nicotinic acetylcholine receptors in the insect central nervous system (Tomizawa and Casida 2005) and can be converted within the plant into highly toxic metabolites such as clothianidin (Casida 2011) which can increase its toxicity.

Lysiphlebus testaceipes (Cresson) (Hymenoptera: Braconidae) is a solitary endoparasitoid that attacks more than 100 aphid species, mostly on grasses and herbaceous plants (Pike et al. 2000). It is an important natural enemy of the greenbug, *Schizaphis graminum* (Rondani) (Hemiptera: Aphididae) and helps to maintain populations of this aphid below economic levels in both sorghum (Jones 2001) and wheat (Giles et al. 2003). Without nectar, many parasitoids show a dramatic reduction in their ability to parasitize insect pests (Stapel et al. 1997; Lewis et al. 1998), and adult *L. testaceipes* can often be observed feeding on sunflower EFN in the High Plains environment (J.P.M. unpublished observations). Therefore, the objectives of this study were to assess whether either chlorantraniliprole or thiamethoxam would have any impacts on the survival of adult wasps, their foraging behavior, or the development of their offspring, when the adults fed on EFN of sunflower seedings grown from treated seed.

Materials and methods

Insect colonies

All insect colonies were held in growth chambers under the same physical conditions: 21 ± 1°C and a L:D 16:8 photoperiod. The colony of *S. graminum* was initiated from aphids naturally infesting sorghum in a greenhouse at the Agricultural Research Center-Hays, in Hays, KS, USA. The aphids were established on seedlings of a greenbug-susceptible sorghum variety (cv. P8500) planted in metal trays (8.0 cm x 51.0 cm x 36.0 cm) filled with a mixture of soil, peat moss and perlite (1:1:1) and germinated in a greenhouse at 25 ± 2 °C. A colony of *L. testaceipes* was established from material obtained from a laboratory culture that had been maintained on *Rhopalosiphum padi* (L.) (Hemiptera: Aphididae) at the Department of Entomology, University of Minnesota, MN, USA for many generations. The colony was reared on *S. graminum* for three generations prior to use in bioassays. For each generation, mated parasitoid females (2-3 days old, n = 20) were each provided ca. 50 2nd and 3rd instar aphids in a Petri dish (5.5 cm diam) for a period of 50 min tallied from the time the first aphid was stung. Following removal of wasps, the aphids were transferred to sorghum seedlings in plastic pots (15.0 cm diam x 14.0 cm ht), ca. 250 aphids per pot. The seedlings had been germinated in a mixture of soil, peat moss and perlite (1:1:1) in a greenhouse at 25 ± 2 °C. The pots were placed in a growth chamber and, once mummification of parasitized aphids occurred, mummies were removed from the plants and placed in a wax paper cup (8.5 cm diam x 12.5 ht) covered with a plastic Petri lid until adult emergence. Emergent adults were fed with a solution of diluted honey (50 %) streaked onto the plastic lid. Males and females were held together for ca. 48 h to permit mating before females were removed and used to produce the next generation.

Sunflower plants

Seeds of Pioneer 63N82 sunflower treated with chlorantraniliprole (1,800 mg a.i. 100 kg^{-1}) were provided by DuPont Crop Protection (division of E.I. du Pont de Nemours and Co., Wilmington, DE, USA). Seeds of Triumph Nusun cv. 810CL were obtained from Triumph Seed Corp. (division of Dow Agrosciences, Indianapolis, ID, USA) both with and without treatment with Cruiser 5FS$^{®}$ (thiamethoxam, 50 mg a.i. 100 kg^{-1} , Syngenta Crop Protection, Greensboro, NC, USA). Untreated Triumph seed served as the experimental control. Seeds for each treatment were planted in separate metal trays (8.0 cm x 51.0 cm x 36.0 cm) filled with a mixture of soil, peat moss and perlite (1:1:1) and germinated in a greenhouse at 25 ± 2 °C under natural light supplemented during daylight hours with metal halide lamps (L:D =12:12). Plants were watered daily, but sparingly, to avoid excessive leaching of insecticide. Sunflower stalks were harvested beginning at the V2 stage (two true leaves expanded) and every two days thereafter throughout each period of parasitoid exposure in experimental treatments. This growth stage corresponded to 14-15 days-old plants, post-emergence, under the prevailing temperature conditions. Sunflower plants begin secreting extrafloral nectar shortly about two weeks after germination, the first nectaries developing on the main stem between the cotyledons and the first true leaves. These small nectaries are invisible to the naked eye, but they occur profusely over the surface of stems, leaf petioles and leaf veins. The nectar is exuded in very small amounts from these tiny pores and it appears to require considerable feeding effort on the part of insects to obtain the EFN. For provisioning to insects, stem segments (ca. 10.0 cm long) were excised from seedlings and the cut ends dipped in liquid paraffin to seal vascular tissues and maintain turgor, while at the same time preventing the exudation of any resinous materials that might pose a hazard to the insects.

Experimental procedure

In order to test for lethal and sublethal effects of exposure to EFN form treated and untreated seedlings, mummies of *L. testaceipes* (n = 30) were placed in wax paper cups (n = 4 cups per treatment), each containing two sunflower stem segments and sealed with a plastic Petri dish lid. Upon emergence, the adult wasps were exposed to the sunflower stems as their only food source for a period of 48 h, with all stems replaced after 24 h. Mortality was then tallied after 48 h with cup as the experimental unit. Sublethal effects on behavior were assessed by aspirating individual females (n = 16 per treatment) into Petri dishes (5.5 cm diam), each containing 40 2nd and 3rd instar aphids. Following release of each female wasp, the time to first sting (attack latency) was recorded and the wasp was left to forage for 30 min, whereupon the female was removed. All aphids from each dish were then transferred with a fine brush to a single sorghum seedling (one per replicate) that had been germinated in a 16.0 cm plastic cone (Stuewe & Sons, Corvallis, OR, USA) filled with a mixture of soil, peat moss and perlite (1:1:1). Wasps that did not sting an aphid within 10 min of release were replaced. Each cone was then covered with a custom-made, clear plastic cylinder (30 cm length), sealed at the top with a plastic plug and ventilated on the sides by means of a series of screened holes. The cones were inserted into a supporting rack and transferred to a growth chamber. Plants were watered every 48 h by submerging the rack of cones in a water bath for 30 min. Four days after parasitism, a sample of aphids from each plant (n = 10) were dissected in saline under a stereo microscope (40x magnification) to verify the presence/absence of parasitoid larvae. The attack rate was estimated as the sum of parasitized aphids (those containing a parasitoid larvae) and pseudoparasitized aphids (those with teratocytes present and evident deterioration of aphid embryos, but no parasitoid larva). Aphids containing more

than one wasp larva were tallied as self-superparasitized. As mummies formed, they were carefully removed from plants and transferred to a wax paper cup (as above) until emergence of adults.

Data analysis

Data were subjected to one-way ANOVA and, when significant, means were separated using Fisher's LSD test ($\alpha = 0.05$) (PROC GLM; SAS Institute 2008). Exposure cups were considered replicates for analysis of mortality during exposure, individual females as replicates for analysis of attack behavior, and sorghum seedlings each bearing the aphids exposed to a single female as replicates for analysis of progeny fitness. Attack latency data were log(x + 1) transformed, whereas percentage data (percent hosts attacked, parasitized, and offspring emergence) were arcsine square root transformed to fit requirements for normality and homoscedasticity (PROC UNIVARIATE; SAS Institute 2008). Untransformed means are presented in figures and tables. Proportional data (self-superparasitism, mummification, and sex ratio ($\Sigma\female/\Sigma(\female + \male)$)) were analyzed using the χ^2 test ($\alpha = 0.05$) (PROC FREQ; SAS Institute 2008).

Results

Neither chlorantraniliprole nor thiamethoxam seed treatments produced mortality of *L. testaceipes* any higher than controls after 48 h when adults consumed the EFN of excised sunflower stem segments (percent mortality ± SE: control = 20.8 ± 5.0; chlorantraniliprole = 18.3 ± 7.5; thiamethoxam = 19.2 ± 6.4; $F_{2,9} = 0.04$, $P = 0.961$). However, both insecticide treatments reduced the latent period to first sting, number of aphids stung, and number of aphids

parasitized (Table1). Self-superparasitism was reduced by the chlorantraniliprole treatment, indicating lower levels of oviposition, and thiamethoxam reduced the offspring sex ratio (proportion female). Neither the proportion of remaining aphids forming mummies, adult emergence, nor development time from parasitism to emergence, were significantly affected by either treatment (Table2).

Discussion

Although no direct wasp mortality was observed in this study, the consumption of EFN from sunflower seedlings treated with chlorantraniliprole and thiamethoxam caused various sublethal effects on *L. testaceipes* behavior and biological performance. Female wasps exposed to either material were faster to attack their first aphid compared to control females, although they attacked fewer aphids within the test period. These females displayed various combinations of erratic behavior, disorientated movements, and extended periods of antennation and ovipositor probing on the same host, suggesting impaired host acceptance behavior. We suspect that the time to first host encounter was faster for treated females because they moved randomly within the dish from time of introduction, whereas control wasps spent an initial period evaluating sensory cues associated with their surrounding environment prior to initiating search behavior. For example, Hopkinson et al. (2013) observed that female *L. testaceipes* foraging for *Aphis gossypii* Glover and *Aphis craccivora* (Koch) (Hemiptera: Aphididae) on cotton leaf discs required a mean of 14.7 and 16.4 min, respectively, to attack their first host, illustrating how much time may be expended by these insects exploring a leaf surface prior to an initial host encounter. Therefore, it is reasonable to expect that other behavioral impairments might be evident in a more complex, natural environment, that

would not be observed in the experimental arenas. The relatively short latent periods observed in the present study are likely the result of presenting hosts in a small arena in the absence of plant material or other cues such as honeydew. Residues of various insecticides are known to trigger changes in parasitoid mobility, orientation, feeding and parasitism ability (Desneux et al. 2007; Garcia 2011). Changes in foraging behavior and rates of parasitism were observed after *Anagrus nilaparvatae* (Pang and Wang) (Hymenoptera: Mymaridae) fed on a mixture of honey and imidacloprid (Liu et al. 2010) and subsequent work found similar effects of chlorantraniliprole on the same species (Liu et al. 2012). Both a braconid and an encyrtid parasitoid that fed on floral nectar of *Eucalyptus* trees treated with an imidacloprid soil drench suffered reduced survival and reproductive performance (Paine et al. 2011), although in this case the concentrations detected in nectar were more than double the LC50 values for these species. In a three year field study, cotton seeds treated with either imidicloprid or thiamethoxam reduced the season-long rate of whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), parasitism by a complex of three aphelinid parasitoids (Naveed et al. 2010).

The similar reductions in parasitoid performance caused by the chlorantraniliprole and thiamethoxam treatments likely result from entirely different effects on the insects, as the former material interferes with muscle contraction, and the latter with neurotransmission. Chlorantraniliprole induces the ryanodine receptors to release stored calcium ions from the sarcoplasmic reticulum, causing impaired regulation of muscle contraction, paralysis and ultimately death in sensitive species (Cordova et al. 2006; Lahm et al. 2007). In contrast, thiamethoxam targets nicotinic acetylcholine receptors on the post-synaptic membranes of nerve cell junctions producing both lethal and sublethal neurological effects (Tomizawa and Casida 2005). Both materials appeared to lower the overall mobility of females, resulting in fewer host encounters during

the test period, and consequently less parasitism. This can be construed to result entirely from impaired muscle function in the chlorantraniliprole treatment. However, the neurotoxic activity of thiamethoxam has the potential to both impede wasp motor functions and their sensory perception of cues involved in host evaluation and acceptance. Disruption of parasitoid sensory perception and motor function as a function of sublethal exposure to neurotoxic insecticides is well documented (Haynes 1988; Garcia 2011). For example, Komeza et al. (2001) studied *Leptopilina boulardi* (Hymenoptera: Figitidae) females exposed to the LD20 of the organophosphate chlorpyrifos and found them to be less efficient in finding host patches marked with kairomones, and exhibit longer patch residence times. In contrast, Delpuech et al. (2005) found that the congeneric *Leptopilina heterotoma* Thomson females responded to kairomones of *Drosophila* with greater arrestment following contact with chlorpyrifos and deltamethrin at LD20. Thus, either inhibitive or stimulative (hormoligosis) effects of neurotoxic insecticides may be observed at sublethal doses, depending on the insecticide and species of insect.

Self-superparasitism occurs when a female lays eggs in a host that she has previously parasitized (Waage 1986). In contrast to control females that laid an additional egg in almost every fourth host, *L. testaceipes* females exposed to chlorantraniliprole did not engage in this behavior (Table 1). Although *L. testaceipes* is a solitary parasitoid and only one offspring can survive per host, there exist several potential payoffs for self-superparasitism. Aphid parasitoids such as *L. testaceipes* emerge with hundreds of eggs ready to lay and laying an additional egg in some hosts can improve a female's probability of host possession for her offspring if there is a risk of superparasitism by conspecific females (Michaud and Mackauer 1995). Although supernumerary larvae are eliminated, upon hatching, additional eggs release additional teratocytes critical to selective digestion of host tissues (Falabella et al. 2000; Caccia et al. 2012)

and these may improve the survival of neonate parasitoids in their physiological struggle against host resistance mechanisms. For example, Bai and Mackauer (1992) found that *Aphidius ervi* Halliday developing in superparasitized pea aphids, *Acyrthosiphum pisum* Harris, gained 14 % more dry mass compared to those in singly-parasitized hosts, with no cost in additional developmental time. Similarly, Zhang et al. (2010) reported that oviposition of multiple eggs increased parasitism success of the solitary parasitoid *Campoletis chlorideae* Uchida (Hymenoptera: Ichneumonidae) in larvae of *Mythimna separate* Walker (Noctuidae: Lepidoptera). In keeping with the inference that self-superparasitism of some fraction of hosts is an adaptive 'insurance' strategy for solitary parasitoids, its absence in females exposed to chlorantraniliprole can be considered an abnormality likely to have adverse effects on female fitness, for example if the behavior is adaptive for improving offspring survival in hosts of marginal suitability.

The lack of any significant difference among treatments in the proportion of aphids mummified appears somewhat inconsistent with the results obtained from dissected subsamples, and likely reflects a bias toward sampling parasitized aphids during removal of these subsamples. In order to minimize disturbance of the remaining aphids, subsamples were removed from sorghum seedlings by gently tapping them over a sheet of paper and collecting the first ten aphids so dislodged. Many factors can potentially influence the dropping propensity of aphids from a plant, including parasitism (e.g. McAllister and Roitberg 1987). Thus, we suspect that parasitized aphids had greater dropping propensity than unparasitized aphids and were thus over-represented in the dissected samples and under-represented in the remaining fraction that was reared to mummification.

Neither offspring survival nor their developmental time was affected by either seed treatment (Table 2), but the sex ratio (proportion female) was

significantly reduced by the thiamethoxam treatment. In arrhenotokous Hymenoptera such as *L. testaceipes*, unfertilized eggs become males, so egg fertilization (and thus sex ratio) is actively controlled, and females may even selectively place fertilized eggs in better hosts (e.g. Srivastava and Singh 1995a). In aphidiine wasps, sex ratio can be diminished by various stressors such as high temperature (Deng and Tsai 1998; Matin et al. 2009), low temperature (Sigsgaard 2000; Mahi et al. 2014), multiple matings (Kant et al. 2012) and aging (Srivastava and Singh 1995b; Kant et al. 2013). Since the rate of mummification among aphids not harvested for dissection was similar across treatments, the lower sex ratio in the thiamethoxam treatment is most likely attributable to neurologic impairment of females that interfered to some extent with their ability to fertilize eggs. Since both parasitoid population growth and biological control efficacy hinge on numbers of parasitoid females, a reduction in sex ratio to only 11 % female can be considered a highly adverse result.

In summary, many studies have emphasized that systemic insecticides used in seed treatments pose risks to beneficial species such as parasitoids (Prabhaker et al. 2011), predators (Al-Deeb et al. 2001; Moser and Obrycki 2009), and pollinators (Girolami et al. 2009) due to their translocation within plants and their potential contamination of pollen and nectar (Cloyd and Bethke 2011). The treatment of seed with such insecticides is now a widespread practice in production agriculture because it can improve stand establishment in row crops by protecting germinating seeds and young seedlings. However, neonicotinoids are notoriously incompatible with integrated pest management (IPM) (Hutchins 2010), can accumulate in soils, and are prone to leaching into waterways (Goulson 2013). Furthermore, there are risks to pollinators associated with improper handling or disposal of residues during planting operations (Nuyttens et al. 2013). Work in soybean has questioned whether producers gain any significant benefits from seed treatments (Seagraves and Lundgren 2012).

Producers buying factory-treated seed incur a control cost prior to suffering any pest infestation, an expense that is not recouped if economically damaging pests do not attack plants in early growth stages. Because seed treatments constitute a prophylactic control, they are not consistent with IPM principles that require assessment of pest numbers and projection of an economic impact prior to resorting to pesticide application (Stern et al. 1959). Further studies are warranted to determine just how compatible seed treatments are with IPM programs that rely on conservation biological control to any significant extent.

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Table 1 Mean (± SE) parameters of *Lysiphlebus testaceipes* parasitism behavior when females were subjected to three treatments (wasps fed for 48h on extrafloral nectar of sunflower stems excised from seedlings grown from seed treated with either chlorantraniliprole or thiamethoxam, or controls) prior to foraging as individuals on 40 second and third instar nymphs of *Schizaphis graminum* in a plastic Petri dish for 30 min.

Seed treatment	Attack latency[a] (min)	Percent attacked[a]	Percent parasitized[a]	Self-superparasitism[b,c]
Control	3.0 ± 0.7 a	43.1 ± 7.4 a	38.8 ± 7.9 a	0.24 a
Chlorantraniliprole	1.5 ± 0.4 b	21.9 ± 6.0 b	19.4 ± 5.7 b	0.00 b
Thiamethoxam	1.1 ± 0.3 b	24.4 ± 5.2 b	20.6 ± 5.0 b	0.09 ab
F or χ^2	4.55	4.39	3.56	10.86
df	2,45	2,45	2,45	2
P	0.016	0.018	0.037	0.004

Ten aphids were dissected from each replicate to determine percentages attacked and parasitized, and the proportion self-superparasitized.

Means (± SE) and proportions followed by different letters were significantly different within columns (Fisher's LSD, or $\chi^2 \alpha = 0.05$).

[a] Analysis by one-way ANOVA, or [b] χ^2

[c] Proportion of dissected hosts that contained two larvae.

Table 2 Mean (± SE) developmental parameters of *Lysiphlebus testaceipes* progeny whose mothers were subjected to three treatments (wasps fed for 48h on extrafloral nectar of sunflower stems excised from seedlings grown from seed treated with chlorantraniliprole or thiamethoxam, or controls) prior to parasitizing second and third instar nymphs of *Schizaphis graminum* in a plastic Petri dish.

Seed treatment	Proportion aphids Mummified[b]	Adult emergence[a] (%)	Development time[a] (days)	Sex ratio[b] (proportion female)
Control	0.25 ± 0.02 a	82.2 ± 5.2 a	15.7 ± 0.2 a	0.41 ± 0.05 a
Chlorantraniliprole	0.19 ± 0.02 a	79.3 ± 7.9 a	15.8 ± 0.2 a	0.40 ± 0.06 a
Thiamethoxam	0.21 ± 0.02 a	72.6 ± 4.2 a	15.5 ± 0.1 a	0.11 ± 0.04 b
F or χ^2	4.19	0.71	0.74	20.15
df	2	2,35	2,35	2
P	0.123	0.498	0.485	< 0.001

Values followed by different letters were significantly different within columns (χ^2, $\alpha = 0.05$).

[a] Data were analyzed by one-way ANOVA, or [b] χ^2.

ARTICLE 2

SUBLETHAL EFFECTS OF INSECTICIDE SEED TREATMENTS ON TWO NEARCTIC LADY BEETLES (COLEOPTERA: COCCINELLIDAE)

This article was written in accordance with the standards of Ecotoxicology, for which it was submitted.

Sublethal effects of insecticide seed treatments on two nearctic lady beetles (Coleoptera: Coccinellidae)

Running head: Toxicity of seed treatments to lady beetles

Valéria Fonseca Moscardini,[a,b] Pablo Costa Gontijo,[a,b] JP Michaud[b]* and Geraldo Andrade Carvalho[a]

[a]Department of Entomology, Federal University of Lavras, Lavras, Minas Gerais, Brazil.
[b]Department of Entomology, Kansas State University, Agricultural Research Center-Hays, Hays, Kansas, USA.
*Corresponding author: J.P. Michaud, 1232 240*th* Ave., Hays, KS, 67601, Email: jpmi@ksu.edu*

Abstract Predatory insects often feed on plants or use plant products to supplement their diet, creating a potential route of exposure to systemic insecticides used as seed treatments. This study examined whether chlorantraniliprole or thiamethoxam might negatively impact *Coleomegilla maculata* and *Hippodamia convergens* when the beetles consumed the extrafloral nectar of sunflowers grown from treated seed. We reared both species on eggs of *Ephestia kuehniella* and then switched adult *H. convergens* to a diet of greenbugs, *Schizaphis graminum*, in order to induce oviposition in this species. Excised sunflower stems, either treated or control and refreshed every 48 h, were provided throughout larval development, or for the first week of adult life. Exposure of *C. maculata* larvae to chlorantraniliprole and thiamethoxam applied as seed treatments delayed adult emergence by prolonging the pupal period. When adults were exposed, thiamethoxam reduced the preoviposition

period compared to chlorantraniliprole, whereas the latter treatment cause females to produce fewer clutches during the observation period. Larvae of *C. maculata* did not appear to obtain sufficient hydration from the sunflower stems and their subsequent fecundity and fertility were compromised in comparison to the adult exposure experiment where larvae received supplemental water during development. Exposure of *H. convergens* larvae to thiamethoxam skewed the sex ratio in favor of females; both materials reduced the egg viability of resulting adults and increased the period required for eclosion. Exposure of *H. convergens* adults to chlorantraniliprole reduced egg eclosion times compared to thiamethoxam and exposure to both insecticides reduced pupation times in progeny.

Introduction

Many beneficial arthropods are omnivorous, consuming both prey and plant material (Coll and Guershon 2002). Natural enemies of insect pests may utilize various plant resources, nibbling tender shoots or consuming pollen and nectar, both floral and extrafloral (Wackers et al. 2007; Lundgren 2009a; Choate and Lundgren 2013). Although floral and extrafloral nectar (EFN) are both rich in sugar, the latter contains sucrose as the dominant sugar, rather than glucose or fructose (Baker and Baker 1979; Rogers 1985). Sugars can be an essential dietary component for coccinellids, improving their survival and reproductive capabilities, and providing metabolic fuel for flight and other behaviors (Lundgren 2009b; Hodek and Evans 2012). Unlike floral nectar which is

available only during flowering, EFN can be available to natural enemies for a much longer period (Pacini et al. 2003; Rose et al. 2006).

On the High Plains of the USA, the EFN secreted by annual sunflowers, *Helianthus annuus* L., (Asteraceae) is an important source of both sugar and hydration for beneficial insects during the hot, dry summers. Although extremely small, the nectaries are highly abundant along petioles and leaf veins and secrete nectar continuously from the time the first true leaves expand until the plants senesce. The production of EFN by plants is associated with attraction of herbivore natural enemies and thus fosters mutualistic protection for the plant (Marazzi et al. 2013). More than 40 species of Coccinellidae are known to utilize EFN in 15 plant families (Pemberton and Vandenburg 1993). A wide variety of insects, beneficial and otherwise, can be directly observed utilizing sunflower EFN a source of hydration during summer months (Charlet and Gavloski 2011) and likely accounts for the great diversity of insects associated with this plant (Royer and Walgenbach 1991).

Coleomegilla maculata DeGeer and *Hippodamia convergens* Guérin-Méneville (Coleoptera: Coccinellidae) are two of the most abundant lady beetles in the central USA, the latter species being of particular importance for the biological control of cereal aphids in wheat, sorghum and other grains (Rice and Wilde 1988; Nechols and Harvey 1998; Michaud 2013). Both species consume pollen and nectar, both floral and EFN (Pemberton and Vandenberg 1993; Smith and Krischik 1999). These species breed only during periods of high aphid populations, which are usually limited to several weeks in both spring and fall. Aphids and alternative insect prey are rare during summer months, forcing most species into a reproductive diapause (Michaud and Qureshi 2005). Hydration is critical to survival during this period when the beetles must survive on alternative food sources such as pollen and non-aphid prey that have lower water content than aphids, and sunflower EFN can be a key moisture source (Michaud

and Qureshi 2006). Although *C. maculata* can complete development on an exclusive diet of pollen, it has a relatively high water demand when feeding on non-aphid food sources and is sensitive to desiccation stress during development (Michaud and Grant 2005). In contrast, laboratory observations (JP Michaud unpublished) indicate that the daily water consumption of diapausing adult *H. convergen*s is only 20-25% that of diapausing *C. maculata*, and the drought tolerance of the former species is likely key to its success in this arid environment.

Recently, both floral nectar and EFN have been recognized as potential routes of exposure to systemic insecticides applied to soil or seeds. Both lethal and sublethal effects have been observed in honey bees (van der Sluijs et al. 2013), predators such as *C. maculata* (Smith and Krischik 1999), *Orius insidiosus* (Say) (Hemiptera: Anthocoridae) (Seagraves and Lundgren 2012; Gontijo et al. 2014a) and *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) (Rogers et al. 2007; Gontijo et al. 2014b), and parasitoids such as *Anagyrus pseudococci* (Girault) (Hymenoptera: Encyrtidae) (Krischik et al. 2007) and *Lysiphlebus testaceipes* (Cresson) (Hymenoptera: Braconidae) (Moscardini et al. 2014). Seed treatment with systemic insecticides has been widely adopted as a means of early-season pest control in row crops (Hodgson et al. 2012; Nuyttens et al. 2013). However, some studies in soybean, corn and canola have questioned the economic benefit of prophylactic seed treatments, aside from the potential non-target hazards they present (Royer et al. 2005; Wilde et al. 2007; Seagraves and Lundgren 2012). Like most other row crops, the majority of commercial sunflowers are now planted with a systemic insecticide seed treatment, usually thiamethoxam.

The focal insecticides in the present study were chlorantraniliprole and thiamethoxam, examples of two very different insecticide groups, diamides and neonicotinoids, respectively. Both insecticides exhibit systemic activity within

plant vascular tissues which facilitates their use as seed treatments, and their potential to contaminate plant products, including floral and extrafloral nectar (Maienfisch et al. 2001; Lahm et al. 2009; Li et al. 2012). Chlorantraniliprole acts as a ryanodine receptor modulator to block insect muscle contraction; once ingested by an insect, Ca^{++} depletion in muscle cells leads to feeding cessation, lethargy, muscle paralysis and death (Lahm et al. 2007). In contrast, thiamethoxam targets nicotinic acetylcholine receptors in the central nervous system of insects, producing both lethal and sublethal neurological effects (Tomizawa and Casida 2005). The objective of the present study was to assess the sensitivity of *C. maculata* and *H. convergens* to traces of these materials in sunflower EFN and test whether development or reproduction would be impacted when larvae or adults were exposed to sunflower seedlings grown from treated seed.

Materials and methods

Insect colonies

Adults of *C. maculata* and *H. convergens* were collected from fields of sorghum and corn at the Agricultural Research Center in Hays, Kansas, USA (38°51'31.14"N 99°20'10.86"W). Adults of each species were placed in 1-L glass mason jars (ca. 150 per jar) covered with an organdy mesh screen and filled with shredded wax paper as harborage. Water was provided on a cotton wick and approximately 50 mg of frozen *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae) eggs, obtained from a commercial supplier (Beneficial Insectary, Oak Run, CA, USA), were provided daily to each jar. Both species were held in a growth chamber at 24 ± 1 °C, 42 ± 5% RH, and a photoperiod of

16:8 (L:D). Under these crowded conditions with limited food, the beetles remain in reproductive diapause for many months.

For each experiment, a series of female beetles (n = 30) of each species were removed from the jar and isolated, *C. maculata* in plastic Petri dishes (5.5 cm diam) and *H. convergens* in ventilated plexiglass cylinders (5.0 cm diam x 10.0 cm ht) under the same physical conditions as the colony. Females of *C. maculata* were fed with frozen *E. kueniella* eggs daily with water provided on a small sponge, whereas females of *H. convergens* were fed with an *ad libitum* diet of greenbugs, *Schizaphis graminum* (Rondani) (Hemiptera: Aphididae), because aphids are required to induce oviposition in this species once it has entered diapause (Michaud and Qureshi 2006). The plexiglass cylinders facilitated the provisioning of aphids on excised sorghum seedlings and provided more secure containment of aphids than did the petri dishes. The aphids were obtained from colonies reared on sorghum seedlings in a growth chamber under the same physical conditions as the beetles. Food and water were refreshed daily and eggs, mostly laid on the inner surfaces of the containers, were collected by transferring the beetles to new containers. Upon eclosion, larvae of both species were reared on frozen eggs of *E. kuehniella* in Petri dishes (5.5 cm diam), five per dish, with water provided on a sponge cube, refreshed every 48h, until they emerged as adults. The first laboratory generation was used for larval exposure experiments and the second generation for adult exposure experiments for each species.

Sunflower plants

Triumph Nusun cv. 810CL sunflower seeds were obtained from Triumph Seed Corp. (Dow Agrosciences, Indianapolis, ID) both with and without treatment with Cruiser 5FS® (thiamethoxam, 50 mg a.i. 100 kg⁻¹, Syngenta Crop

Protection, Greensboro, NC); untreated Triumph seed served as the experimental control. Sunflower seeds cv. Pioneer 63N82 were obtained from DuPont Crop Protection (E.I. du Pont de Nemours and Co., Wilmington, DE) treated with chlorantraniliprole (1800 mg a.i. 100 kg^{-1}). All seeds were planted 2.0 cm deep in metal trays (8.0 cm x 51.0 cm x 36.0 cm) filled with a mixture of soil, peat moss and perlite (1:1:1) and germinated in a greenhouse at 25 ± 2 ºC under natural light supplemented during daylight hours with metal halide lamps (L:D = 12:12). Plants were watered daily, but sparingly, to avoid excessive leaching of insecticide. Sunflower stalks were harvested beginning at the V2 stage (14-15 d-old plants with two true leaves expanded) and every two days thereafter throughout each period of insect exposure, so that insects were exposed to progessively older plant tissues as material was replaced in the experiments. All stem segments were harvested before 9:00 a.m. and constituted the bulk of the upper portion of the main stem. For provisioning to insects, stem segments (ca. 4.0 - 5.0 cm in length) were excised from seedlings and the cut ends dipped in liquid paraffin to seal vascular tissues and maintain turgor, while at the same time preventing the exudation of any resinous materials that might pose a hazard to the insects.

Exposure of larvae

Experiments with both species were conducted under the same physical conditions used for rearing the beetle colonies. Each replicate (n = 8 per treatment) consisted of five first instar larvae held in a Petri dish (5.5 cm diam), their parentage recorded to prevent any subsequent pairing of related beetles. Larvae were fed frozen eggs of *E. kuehniella* ad libitum, refreshed every 48 h. Each Petri dish was supplied with a sunflower stem segment as the only source of hydration, either grown from untreated seed (controls) or from seed treated

with one of the two insecticides. The stem segments were replaced every 48 h until larvae pupated.

Data were recorded daily for all insects throughout the experiment. Larval developmental time was tallied the number of days from the beginning of the experiment until the formation of pupae and pupation time as the number of days from pupal formation to adult emergence. Immature survival was calculated as the percentage of neonate larvae placed in the treatment that successfully emerged as adults. Emergent adults were sexed and, when insects were 7-8 days old, the maximum possible number of pairs were established by confining each female with a male from the same treatment group, checking parentage to prevent the pairing of siblings. After 48 h, males were removed and females were isolated, *C. maculata* in Petri dishes (as above) provisioned with ad libitum frozen eggs of *E. kuehniella* and water on a cube of sponge, *H. convergens* in plexiglass vials (as above) with ad libitum *S. graminum* provided on excised sorghum seedlings. Food and water was refreshed every 48 h.

Eggs were harvested daily by transferring females to new containers and the preoviposition period of each female was calculated as the number of days from adult emergence until first oviposition. A series of 10 clutches were collected from each *H. convergens* female and days required to produce them, recorded. Because oviposition by *C. maculata* was much slower, female fecundity was assessed for a 21 d period post-copula, during which period not all females produced 10 clutches.

Exposure of adults

Pairs of adult beetles (ca. 24 h old) of each species (*C. maculata*, n = 18 per treatment, *H. convergens*, n = 14 per treatment) were established in their respective containers (as above), with parentage checked to prevent the pairing

of siblings. Each container contained a sunflower stem segment corresponding to one of the three treatments and was provisioned with ad libitum frozen eggs of *E. kuehniella*. The sunflower stems were refreshed every 48 h for a total exposure period of 10 days, whereupon males were removed and females isolated for oviposition, those of *C. maculata* receiving eggs of *E. kuehniella* and those of *H. convergens* receiving *S. graminum*. Procedures and data collection were thereafter the same as described above for insects exposed as larvae.

A series of ten neonate larvae hatching from the first clutch of each female were isolated in Petri dishes (5.5 cm diam), one per dish and fed ad libitum frozen eggs of *E. kuehniella* with water provided on a sponge cube, both refreshed every 48h. All insects were observed daily and all developmental data collected until they emerged as adults.

Data analysis

Data were subjected to Kolmogorov-Smirnov and Levine tests ($\alpha = 0.05$) for verification of normality and homoscedasticity, respectively (PROC UNIVARIATE, SAS Institute 2008). For each species and exposure phase (larvae or adults), data that passed these tests were subjected to one-way ANOVA followed and, when means were significant, to a Bonferroni test ($\alpha = 0.05$) to separate means (PROC GLM; SAS Institute 2008). The preoviposition periods of *C. maculata* in the adult exposure test were transformed to log (x + 1) before being subjected to one-way ANOVA. Untransformed means are presented in all tables. Data that were not normally distributed or failed a Levine test for equality of variances were analyzed using the Kruskal-Wallis test ($\alpha = 0.05$) (PROC NPAR1WAY, SAS Institute 2008). Sex ratio ($\Sigma\female/\Sigma(\female + \male)$) was

analyzed using the Chi-square Goodness of Fit test (α = 0.05) (PROC FREQ; SAS Institute 2008).

Results

Larvae of *C. maculata* exposed to sunflower stems grown from treated seeds spent about half a day longer in the pupal stage than did controls, but no other treatment effects were significant (Table 1). Survival of pupae was 100% in all three treatments, and the reproductive performance of the resulting females did not differ among treatments, although egg fertility was below normal values in all three. When *C. maculata* were exposed to sunflower stems as adults, there was no mortality in the three week observation period, but preoviposition periods were reduced by the thiamethoxam treatment relative to chlorantraniliprole, although neither was different from controls. However, females in the chlorantraniliprole treatment laid fewer clutches, although overall fecundity and egg viability did not vary significantly among treatments (Table 2). There were no significant treatment effects on any parameter of progeny development.

A higher proportion of emergent adults were female when larvae of *H. convergens* were exposed to stems in the thiamethoxam treatment, but no other developmental parameters differed among treatments (Table 3). Pupal survival was 100% in all three treatments. However, the viability of eggs was significantly reduced for female adults in both the chlorantraniliprole and thiamethoxam treatments and the time required for eclosion of their eggs was slightly increased. Exposure of adults revealed no significant treatment effects on reproductive parameters, but some transgenerational effects were evident in the progeny. Chlorantraniliprole reduced egg eclosion time compared to

thiamethoxam, although controls were not different from either, and both treatments reduced pupation time (Table 4). There was no mortality of adults during the period of reproductive observations.

Discussion

Subtle, but significant, negative effects on development and reproductive biology were observed when *C. maculata* and *H. convergens* fed on EFN presumably contaminated with residues of chlorantraniliprole and thiamethoxam. Systemic insecticides become distributed throughout the plant and may contaminate the pollen, floral and extrafloral nectar (Cloyd and Bethke 2011). EFN can be an important food source for many beneficial organisms, especially coccinellids, because it is rich in sugars that are easily digested (Lundgren 2009b). Consumption of EFN may provide energy and increase fitness, especially when prey is scarce. Lundgren and Seagraves (2011) observed that *C. maculata* adult consuming EFN of *Vicia faba* (Fabaceae) in the absence of prey improved their survival, nutrient reserves and reproductive capacity.

Both seed treatments prolonged *C. maculata* pupation time following larval exposure, possibly because intoxicated larvae had lower mobility and feeding rates, which could have resulted in nutrient limitation and consequent prolongation of the pupal stage or because the materials impaired neural processes controlling pupation. For example, Vargas et al. (2013) showed that *C. maculata* larvae permitted to feed on *E. kuehniella* eggs for only 30 min daily had their total developmental time extended by 10-12 d compared to those permitted ad libitum access to food. Sublethal effects of neonicotinoid insecticides on the foraging behavior and predation rate of beneficial organisms have been previously reported (e.g., Desneux et al. 2007). Smith and Krischik (1999) confined *C. maculata* adults with inflorescences of sunflower plants

treated with imidacloprid via soil and observed significantly reduced motor activity. Imidacloprid reduced the functional response *Serangium japonicum* Chapin (Coleoptera: Coccinellidae) to whitefly *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) eggs, when applied at a sublethal rate (5 ppm) via egg immersion (He et al. 2012). Thiacloprid applied to tomato at the rate recommended for control of the tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) was shown to cause reductions in the foraging behavior and predation rate of fifth instar nymphs of *Macrolophus pygmaeus* (Hemiptera: Miridae) (Martinou et al. 2014). Thiamethoxam and clothianidin both caused neurotoxic symptoms (e.g., trembling, paralysis, and loss of coordination) in larvae of *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae) exposed for six hours to corn plants grown from treated seeds (Moser and Obrycki 2009). Neonicotinoids also causes negative effects on the motor functions of adult worker honeybees, *Apis mellifera* L. (Hymenoptera: Apidae) (Williamson et al. 2014). Lethargic behavior and feeding inhibition are thus sublethal effects often associated with chlorantraniliprole intoxication. Smagghe et al. (2013) found that workers of *Bombus terrestris* (L.) (Hymenoptera: Apidae) showed lethargic behavior and reduced food consumption following chronic oral exposure to chlorantraniliprole via contaminated pollen.

Chlorantraniliprole has been reported to reduce larval feeding in herbivores such aas *Plutella xylostella* L. (Lepidoptera: Plutellidae), *Trichoplusia ni* (Hubner), *Spodoptera exigua* (Hubner) and *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) (Hanning et al. 2009). Oral exposure of neonate *S. exigua* larvae to a sublethal concentration (LC_{30}) of chlorantraniliprole prolonged larval development and increased the appearance of supernumerary instars (Lai and Su 2011). Notwithstanding, the exposure of predatory bugs to surface residues of chlorantraniliprole did not seem to affect foraging behavior in any measurable way. Examples include *Amphiareus*

constrictus (Stal), *Blaptostethus pallescens* Poppius, *Orius tristicolor* (White) (Hemiptera: Anthocoridae) (Pereira et al. 2014), *Podisus nigrispinus* (Dallas) and *Supputius cincticeps* (Stal) (Heteroptera: Pentatomidae) (Castro et al. 2013). Sunflower EFN contaminated with chlorantraniliprole and thiamethoxam caused no lethal effects when consumed by *L. testaceipes* adults, but female foraging behavior was impaired and fewer greenbug nymphs were attacked and parasitized in each bout of foraging (Moscardini et al. 2014). Imidacloprid and chlorantraniliprole also impeded the parasitism of *Nilaparvata lugens* (Stal) (Heteroptera: Delphacidae) by *Anagrus nilaparvatae* (Pang and Wang) (Hymenoptera: Mymaridae) when the wasps consumed honey contaminated with these insecticides (Liu et al. 2010, 2012). Parasitism of aphids by *Aphelinus certus* Yasnosh (Hymenoptera: Aphelinidae) was reduced when the wasps host-fed on aphids that, in turn, fed on imidacloprid- and thiamethoxam-treated soybean plants (Frewin et al. 2014).

Effects of chlorantraniliprole on reproductive biology, similar to those on *C. maculata* in this study, have been reported in other insect groups. For example, Gontijo et al. (2014a) found that female *O. insidiosus* exposed as nymphs to chlorantraniliprole via sunflower EFN suffered extended preoviposition periods. When newly eclosed workers of *B. terrestris* consumed sugar water supplemented with chlorantraniliprole at 40 mg L^{-1}, or pollen sprayed with the insecticide, fewer drones were produced per nest (Smagghe et al. 2013). Negative effects of both insecticides on *H. convergens* biology were delayed and only observed later in life history (reduced egg viability and longer embryonic development when larvae were exposed) or in the next generation (faster pupation time in progeny of exposed adults). In the case of thiamethoxam, increased toxicity is associated with its metabolism into clothianidin, a process which can occur within both plant (Cloyd and Bethke 2011) and insect (Nauen et al. 2003) tissues, and might account for some

delayed impact. Benzidane et al. (2010) reported low toxicity of thiamethoxam to *Periplaneta americana* (L.) (Blattodea: Blattidae) adults was associated with a lack of breakdown into clothianidin within 24 h after its ingestion. In contrast, the primary metabolites of chlorantraniliprole are thought to have low toxicity (FAO, 2008).

Exposure of *H. convergens* larvae to thiamethoxam resulted in a sex ratio skewed toward females when compared to the chlorantraniliprole and control treatments. In contrast, Gontijo et al. (2014b) observed a reduced sex ratio in *C. carnea* when larvae were exposed to sunflower stems grown from seed treated with thiamethoxam as compared to chlorantraniliprole. Thus, patterns of gender-specific larval susceptibility to this material appear to vary among insect groups.

Both larvae and adults of *Coleomegilla maculata* have a high water demand and require supplementary water when feeding on non-aphid foods (Michaud and Grant 2005). The viability of *C. maculata* eggs was abnormally low in the larval exposure experiment, regardless of treatment, where the only moisture available was EFN from the exised stalks, but normal in the adult exposure experiment where larvae were reared with access to water. We infer that the sunflower stalks alone did not provide sufficient hydration for normal larval development in this species, resulting in compromised adult reproduction (reduced fecundity and egg fertility), even though larval survival was relatively good. It is also possible that treatment effects on egg viability, such as those evident in the analogous *H. convergens* experiment, were obscured in these drought-stressed larvae. In *H. convergens*, preoviposition periods averaged two days longer for beetles exposed as adults compared to those exposed as larvae, likely due to the fact the latter received aphid prey two to three days earlier in adult life. Interestingly, both insecticides altered egg eclosion times in *H. convergens* whether the beetles were exposed as larvae or adults, but only the

progeny of *H. convergens* exposed as adults to either insecticide had faster pupation times, with no such effect evident in larvae exposed directly. Thus, the transgenerational effects of these materials do not necessarily mimic the effects of direct exposure.

Transgenerational effects of neonicotinoids have been reported in some coccinellid species. Yu et al. (2014) observed that exposure of *Coccinella septempunctata* L. (Coleoptera: Coccinellidae) larvae to imidacloprid in laboratory microcosms reduced fecundity and egg viability in the next generation. Similar results were reported for topical exposure of *Eriopis connexa* (Gemar) (Coleoptera: Coccinellidae) larvae to acetamiprid that reduced subsequent egg viability (Fogel et al. 2013). Transgenerational effects of systemic insecticides have also been reported for other insect groups, for example thiamethoxam in *Bemisia tabaci* Gennadius (biotype B) and *Trialeurodes vaporariorum* Westwood (Homoptera: Aleyrodidae) (Liang et al. 2012) and chlorantraniliprole in *P. xylostella* (Guo et al. 2013).

Overall, treatment of sunflower seeds with chlorantraniliprole and thiamethoxam caused few negative effects in *C. maculata* and *H. convergens* compared to other beneficial species that have been similarly examined (Gontijo et al. 2014a,b; Moscardini et al. 2014). Because the majority of commercial crops on the High Plains (e.g., sunflower, sorghum, soybeans and corn) are all planted with seed treatments, the beetles colonizing these crops may be regularly exposed to sublethal doses of these insecticides, especially thiamethoxam which has been widely used for more than ten years. Increasing insecticide tolerance in natural enemies, including lady beetles, has been reported as a function of chronic insecticide exposure (Head et al. 1977; Ruberson et al. 2007; Rodrigues et al. 2013a). Rodrigues et al. (2013b) investigated lambda-cyhalothrin susceptibility in 31 populations of lady beetles (Coleoptera: Coccinellidae), focusing on seven species common in cotton fields, and found significant

variation between species and among populations of a given species which they inferred to reflect historical field exposure of the beetles to this insecticide. Thus, the relatively robust responses of both coccinellid species in these experiments may not be representative of other geographic populations that may have different insecticide exposure histories.

In summary, our results suggest that both chlorantraniliprole and thiamethoxam have subtle negative effects on the developmental and reproductive biology of these important predators with the potential for cumulative impacts on population dynamics. Other authors have suggested that the widespread use of systemic insecticides poses a risk to biodiversity and ecosystem services (Biondi et al. 2012; Chagnon et al. 2014; van der Sluijs et al. 2014). The integrated approach to pest management with insecticides is predicated on economic justification and various studies have now failed to identify economic benefits of prophylactic seed treatments in wheat (Royer et al. 2005) corn (Wilde et al. 2007) or soybeans (Seagraves and Lundgren 2012) and others have found them to be ineffective in reducing pests populations (Vernon et al. 2011). Field studies examining a range of nontarget arthropods, conducted over longer time frames, are therefore warranted to determine whether or not these materials are truly compatible with IPM in field crops.

Conflict of interest The authors declare that they have no conflicts of interest.

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Table 1 Mean (± SE) developmental and reproductive parameters of *Coleomegilla maculata* exposed as larvae to sunflower stems grown from treated seeds.

Parameter	Seed treatment			F, H or χ^2	df	P
	Untreated	Chlorantraniliprole	Thiamethoxam			
Larval survival (%)	72.5 ± 8.4	70.0 ± 7.6	87.5 ± 6.5	3.06*	2	0.216
Larval development time (d)	11.7 ± 0.2	11.7 ± 0.3	11.9 ± 0.2	0.13	2,21	0.881
Pupation time (d)	3.5 ± 0.1 b	3.9 ± 0.1 a	3.9 ± 0.1 a	8.89	2,21	0.002
Sex ratio[1] (n)	0.55 ± 0.09 (29)	0.50 ± 0.09 (28)	0.40 ± 0.08 (35)	1.54**	2	0.463
No. pairs mated	9	10	12			
Preoviposition period (d)	14.8 ± 2.1	18.1 ± 2.1	16.0 ± 1.6	0.72	2,28	0.494
Fecundity (eggs female^{-1})	102.0 ± 29.1	94.6 ± 31.6	122.1 ± 24.9	0.78*	2	0.677
No. clutches laid in 21 d	8.3 ± 2.1	7.3 ± 1.9	9.5 ± 1.1	0.44	2,28	0.650
Egg viability (% hatching)	50.7 ± 7.1	36.9 ± 6.3	37.5 ± 6.7	1.23	2,28	0.307
Eclosion time (d)	3.0 ± 0.0	3.0 ± 0.0	3.1 ± 0.1	2.55	2,25	0.098

Analysis by one-way ANOVA, Kruskal-Wallis (*) or Chi-square (**).

Means followed by different letters were significantly different within rows (Bonferroni test, α = 0.05).

[1]proportion female.

Table 2 Mean (± SE) reproductive parameters of *Coleomegilla maculata* adults exposed to sunflower stems grown from treated seed and the developmental parameters of their offspring.

Parameter	Seed treatment			F, H or χ^2	df	P
	Untreated	Chlorantraniliprole	Thiamethoxam			
No. pairs established	18	18	18			
Preoviposition period (d)	14.3 ± 1.1 ab	16.9 ± 1.6 a	12.4 ± 0.6 b	3.62	2,51	0.034
Fecundity (eggs female^{-1})	188.1 ± 21.8	152.8 ± 29.6	210.8 ± 26.4	1.25	2,51	0.294
No. clutches laid in 21 d	14.1 ± 1.1 a	10.4 ± 1.6 b	15.1 ± 1.0 a	3.79	2,51	0.029
Egg viability (% hatching)	92.1 ± 2.3	88.9 ± 1.8	83.9 ± 5.5	1.30	2,51	0.283
Eclosion time (d)	3.0 ± 0.0	3.1 ± 0.0	3.0 ± 0.1	0.79*	2	0.673
Larval development time (d)	11.7 ± 0.1	12.0 ± 0.1	11.9 ± 0.1	2.28	2,51	0.113
Pupation time (d)	3.1 ± 0.0	3.1 ± 0.1	3.1 ± 0.1	0.19	2,51	0.828
Immature survival (%)	96.1 ± 1.2	96.7 ± 1.9	94.4 ± 1.8	1.97*	2	0.373
Sex ratio[1] (n)	0.57 ± 0.04 (173)	0.53 ± 0.04 (171)	0.55 ± 0.04 (170)	0.74**	2	0.692

Analysis by one-way ANOVA, Kruskal-Wallis (*) or Chi-square (**).

Means followed by different letters were significantly different within rows (Bonferroni test, α = 0.05).

[1]proportion female.

Table 3 Mean (± SE) developmental and reproductive parameters of *Hippodamia convergens* exposed as larvae to sunflower stems grown from treated seeds.

Parameter	Seed treatment			F or χ^2	df	P
	Untreated	Chlorantraniliprole	Thiamethoxam			
Larval survival (%)	67.5 ± 6.5	67.5 ± 6.4	57.5 ± 10.9	0.49	2,21	0.620
Larval development time (d)	11.7 ± 0.2	12.0 ± 0.2	11.9 ± 0.3	0.55	2,20	0.587
Pupation time (d)	4.8 ± 0.1	4.6 ± 0.1	4.8 ± 0.1	0.86	2,20	0.448
Sex ratio[1] (n)	0.33 ± 0.09 (27) b	0.31 ± 0.09 (26) b	0.61 ± 0.10 (23) a	6.54*	2	0.043
No. pairs mated	9	7	8			
Preoviposition period (d)	9.6 ± 1.1	10.3 ± 1.3	8.9 ± 1.5	0.27	2,21	0.767
Fecundity (eggs female[-1])	251.3 ± 19.4	262.1 ± 23.0	254.0 ± 27.7	0.05	2,21	0.947
No. days for 10 clutches	10.6 ± 0.2	11.3 ± 0.5	11.5 ± 0.9	0.76	2,21	0.479
Egg viability (% hatching)	93.2 ± 1.9 a	79.9 ± 4.1 b	84.1 ± 3.4 b	4.87	2,21	0.018
Eclosion time (d)	3.1 ± 0.0 b	3.3 ± 0.0 a	3.2 ± 0.0 a	4.56	2,21	0.023

Analysis by one-way ANOVA or Chi-square (*).

Means followed by different letters were significantly different within rows (Bonferroni test or Chi-square, $\alpha = 0.05$).

[1]proportion female.

Table 4 Mean (± SE) reproductive parameters of *Hippodamia convergens* adults exposed to sunflower stems grown from treated seed and the developmental parameters of their offspring.

Parameter	Seed treatment			F, H or χ^2	df	P
	Untreated	Chlorantraniliprole	Thiamethoxam			
No. pairs mated	14	14	14			
Preoviposition period (d)	12.1 ± 0.7	12.4 ± 0.6	13.2 ± 0.3	1.17	2,39	0.332
Fecundity (eggs female^{-1})	247.6 ± 16.7	287.7 ± 18.8	264.1 ± 18.4	1.27	2,39	0.294
No. days for 10 clutches	13.6 ± 0.6	12.1 ± 0.6	12.5 ± 0.4	2.38	2,39	0.106
Egg viability (% hataching)	86.7 ± 2.8	82.8 ± 2.8	79.3 ± 5.7	1.02*	2	0.602
Eclosion time (d)	3.2 ± 0.0 ab	3.1 ± 0.0 b	3.3 ± 0.0 a	3.27	2,39	0.049
Larval development time (d)	11.5 ± 0.1	11.7 ± 0.1	11.7 ± 0.1	1.22	2,38	0.308
Pupation time (d)	4.9 ± 0.1 a	4.7 ± 0.1 b	4.7 ± 0.1 b	4.55	2,38	0.017
Immature survival (%)	86.4 ± 2.9	85.0 ± 4.5	83.8 ± 2.9	0.77*	2	0.682
Sex ratio[1] (n)	0.53 ± 0.05 (121)	0.45 ± 0.05 (119)	0.46 ± 0.05 (109)	2.09**	2	0.353

Analysis by one-way ANOVA, Kruskal-Wallis (*) or Chi-square (**).

Means followed by different letters were significantly different within rows (Bonferroni test, $\alpha = 0.05$).

[1]proportion female.