

Thiamethoxam Differentially Impacts the Survival of the Generalist Predators, *Orius insidiosus* (Hemiptera: Anthocoridae) and *Hippodamia convergens* (Coleoptera: Coccinellidae), When Exposed via the Food Chain

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Abstract

Insect predators are seldom considered during toxicological trophic assessments for insecticide product development. As a result, the ecological impact of novel insecticides on predators is not well understood, especially via the food chain, i.e., when their prey is exposed to insecticides. Neonicotinoids are systemic insecticides widely used in agriculture to control herbivorous insects, but their effects on predatory insects via the food chain have not been well characterized. In this study, we documented the time-course effects of the neonicotinoid thiamethoxam on the survival of two predators, the insidiosus flower bug *Orius insidiosus* (Say) and the convergent lady beetle *Hippodamia convergens* Guérin-Ménéville, when preying upon the aphids *Aphis glycines* Matsumura (Hemiptera: Aphididae), *Aphis gossypii* Glover (Hemiptera: Aphididae), and *Myzus persicae* (Sulzer) (Hemiptera: Aphididae). Aphids were exposed to thiamethoxam-treated or untreated plants every week over the course of 5 wk. After transferring aphids to Petri dishes, predators were allowed to feed on aphids. We found that the survival of the insidiosus flower bug, but not the convergent lady beetle, was reduced after consuming aphids reared on thiamethoxam-treated plants compared to untreated plants. Survival reduction of the insidiosus flower bug was observed only during the first weeks after thiamethoxam application; no reduction occurred 28 d after treatment or beyond. These results demonstrate that a systemic application of thiamethoxam could be compatible with convergent lady beetles and insidiosus flower bugs, if the time of predator release does not coincide with thiamethoxam activity. These findings are critical for the development of future pest control programs that integrate biological and chemical control.

Key words: natural enemy, biological control, neonicotinoid, aphid, insecticide

A frequent consequence of insecticide use is their detrimental effects on non-target organisms. Historically, older insecticides (e.g., DDT, dieldrin) have harmed non-target predatory organisms via the food chain through contaminated prey (Risebrough et al. 1967, Hargrave et al. 1992, Vos et al. 2000, Desneux et al. 2007). Currently, there are over 200 insecticide molecules registered in the United States (EPA 2017), of which we have limited to no information about the adverse effects on predators through the food chain. This list includes the neonicotinoids (Group 4A, Insecticide Resistance Action

Committee), one of the most important groups of insecticides worldwide (Casida and Durkin 2013, Simon-Delso et al. 2015). Despite their importance around the globe, the effects neonicotinoids may have on non-target organisms remain partially unexplored.

Thiamethoxam is a commonly used neonicotinoid in agriculture. It is implemented as either seed treatment, drench or spray for the control of various insect pests including sap feeders (e.g., aphids) (Maienfisch et al. 2001, Jeschke et al. 2011, North et al. 2016, Krupke et al. 2017). After application, thiamethoxam and its active metabolites are known

to move primarily through the xylem, but phloem translocation also occurs (Nauen et al. 2003, Bonmatin et al. 2015). Despite its systemic movement, previous research suggests that thiamethoxam can disrupt biological control agents, especially when predatory natural enemies feed on prey that may contain insecticide residues (Grafton-Cardwell and Gu 2003, Douglas et al. 2015). These studies imply that the prey functioned as a biological bridge for thiamethoxam to move from treated plants to predators up to 10 wk after application (Grafton-Cardwell and Gu 2003). Empirical evidence shows that thiamethoxam can remain bioactive against target pests for 5–25 wk (Castle et al. 2005, Díez-Rodríguez et al. 2006, Qureshi et al. 2014, Krupke et al. 2017, Esquivel et al. 2019), suggesting that the time period of thiamethoxam trophic toxicity could also last for several weeks. Nonetheless, the severity and length of trophic thiamethoxam toxicity on natural enemies is expected to change under different conditions (e.g., crop and its phenological stage, thiamethoxam dose, insect pest feeding on plants, susceptibility of the natural enemy to thiamethoxam, among others). Therefore, further research with various crop systems and natural enemies is essential to better understand the longevity of thiamethoxam toxicity via the food chain and the risks for biological control agents.

Protecting biological control agents is critical for pest management and to minimize economic losses in natural and agricultural ecosystems (Flint et al. 1998, Cardinale et al. 2003, Chagnon et al. 2015). Predatory natural enemies, such as lady beetles, consume insect pests in high numbers throughout their life cycle (Weber and Lundgren 2009, Hodek et al. 2012). One of the most abundant species within the U.S. is the convergent lady beetle *Hippodamia convergens* Guérin-Méneville, a natural enemy of soft-bodied insects, such as aphids (Gardiner et al. 2009). Another important group of predatory natural enemies are the anthocorid bugs. The insidious flower bug, *Orius insidiosus* (Say), is one of the most prevalent into the Anthocoridae family and an active predator of small pest insects such as thrips and aphids (McCaffrey and Horsburgh 1986). As part of an integrated pest management (IPM) program, lady beetles and insidious flower bugs mitigate the economic impact of pests, especially aphids, in various agricultural landscapes (Dixon et al. 1997, Rutledge and O'Neil 2005, Desneux et al. 2006, Harwood et al. 2007, Bahy El-Din et al. 2013).

Aphid control relies heavily on the conservation and/or release of natural enemies and the use of insecticides (especially systemic insecticides) (Jeschke et al. 2011). Without proper management, aphid populations can negatively impact crops due to their rapid asexual reproduction, transmission of plant viruses, and promoting fungal infections (e.g., sooty mold) (Ebert 1997, Radcliffe and Ragsdale 2002, Ng and Perry 2004, Tilmon et al. 2011). For instance, populations of the melon aphid *Aphis gossypii* Glover (Hemiptera: Aphididae) can exceed hundreds of individuals per plant in few days after infestation (O'Brien et al. 1993). Other aphid species, such as the soybean aphid *Aphis glycines* Matsumura (Hemiptera: Aphididae) and the green peach aphid *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) have several generations in a single growing season (Blackman and Eastop 2000, Ragsdale et al. 2004, Holman 2009). The destructive effects that these aphid species have on crops could be exacerbated by impairing natural enemy populations following insecticide applications (Wilson et al. 1999, Varenhorst and O'Neal 2012).

The overall objective of our study was to examine the survival of convergent lady beetles and insidious flower bug after consuming melon aphids, soybean aphids or green peach aphids. These aphids fed on untreated or thiamethoxam-treated plants at different time-points. We hypothesized that the convergent lady beetles and

the insidious flower bugs would exhibit reduced survival after preying on aphids from thiamethoxam-treated plants. We also hypothesized that aphids feeding on plants at earlier time points after thiamethoxam treatment would be more toxic to natural enemies than those feeding at later time points.

Materials and Methods

Plant Material

Soybean *Glycine max* (L.) Merr. seeds were Mycogen (a subsidiary of Corteva Agrisciences Indianapolis, IN) variety 5N248R2, treated with Cruiser Maxx (Syngenta, Greensboro, NC) containing thiamethoxam (56.3 g of active ingredient), and the fungicides mefenoxam (3.75 g of active ingredient) and fludioxonil (2.5 g of active ingredient), per 100 kg of seed. To obtain untreated seeds (without insecticide or fungicides), we removed the treatment following the serial washing protocol utilized by Esquivel et al. (2019). Three soybean seeds (treated or untreated) were planted into a plastic pot (Kord Regal, Toronto, Canada; 10.1 cm upper diameter, 7.6 cm lower diameter, 8.9 cm height) filled with soilless media Pro-Mix BX (Québec, Canada). Pots were placed in a greenhouse and maintained at 25°C, 16:8 (L:D) h cycle, using an Argus Control System—a Conviron Company (British Columbia, Canada). No difference in seed germination was observed between treated or untreated soybeans (data not shown). Soybeans were watered every day with the following drip irrigation schedule: 1) weeks 1–3, 34 ml per pot; 2) weeks 3–4, 60 ml per pot; 3) and weeks 4–5, 90 ml per pot. Fertilizer was applied via irrigation by diluting, in a 1:64 ratio, a solution of 121.13 g N, 52.49 g P₂O₅, and 121.13 g K₂O in 7.57 l of water.

Zinnia seeds, *Zinnia elegans* Jacq. Purity White variety (BFG Co., Harrisonburg, VA) were germinated at 25°C with a 16:8 (L:D) h cycle (note: zinnia seeds with insecticide or fungicide treatment were not available at the time of the experiment). At 10 d after germination, seedlings (one seedling per pot) were transplanted to a greenhouse under the same agronomic and environmental conditions used for soybean. Pots were placed in cages (47 cm height, 47 cm width, 89 cm length) made with fabric mesh U.S. #100 (Casa collection, South Korea). At 21 d after transplanting, plants were either drench-treated with thiamethoxam or remained untreated. A treated plant received a total of 100 ml of Flagship 25WG solution (a.i. thiamethoxam) (Syngenta, Greensboro, NC) at 170 g of a.i. per 100 liters of water. Plants were watered and fertilized following the same regimen for soybean.

Green peppers *Capsicum annuum* L. seeds were the hybrid Aristotle-X3R (Tomato Growers Supply Co., Myers, FL) (note: green pepper seeds with insecticide or fungicide treatment were not available at the time of the experiment). Seeds were germinated in organic soil at 25°C with a 12:12 (L:D) h cycle and grown at the Biological Control Department facilities of the Escuela Agrícola Panamericana (EAP), El Zamorano, Honduras. At 10 d after germination, individual seedlings were transplanted to black growing plastic bags with a 10 cm upper diameter, 10 cm lower diameter, 10 cm height, and filled with organic soil media. Plants were kept under greenhouse conditions (27°C, 12:12 (L:D) h cycle) throughout the experiment. Growing conditions of green peppers differed from those used with soybean and zinnias, because soilless media or artificial lights were not available at the time of the experiment. At 21 d after transplanting, plants were either drench-treated with thiamethoxam or remained untreated. Each treated plant received a total of 100 ml of Actara 25WG solution (a.i. thiamethoxam) (Syngenta, Greensboro,

NC) at 170 g of a.i. per 100 liters of water. Plants were watered and fertilized following the same regimen for soybean.

Soybean, zinnias, and green pepper plants were grown separately. All plants were arranged in a factorial complete randomized design. The experimental unit for soybean and zinnias was 'a planted pot', whereas for green pepper was a 'planted bag'. Factors were 1) insecticide treatment: 'treated and untreated', and 2) days after planting (for soybean) or days after drench treatments (for zinnias and green peppers) when aphid cohorts were placed on plants: '7, 14, 21, 28, and 35 d'. Treatments for all crops were replicated 10 times. Soybean plants were used to feed soybean aphids. Zinnias and green peppers were used to feed green peach aphids and melon aphids, respectively.

Aphid Colonies and Thiamethoxam Exposure

Soybean aphids 'biotype 1' were reared on Williams 82 soybean in the Michel Laboratory at the College of Food, Agriculture, and Environmental Sciences (CFAES) in Wooster Campus, The Ohio State University (OSU), Wooster, Ohio. Green peach aphids were reared on Waltham broccoli cultivar (BFG Supply Co., Harrisonburg, VA) in the Canas Laboratory, CFAES Wooster Campus, OSU, Wooster, Ohio. The melon aphid was obtained from field collections (14°00'29.81" N; 87°00'12.13" W, 782 m elevation) in May of 2017 and reared at the Biological Control Department facilities of the EAP. Melon aphids were reared on hybrid Aristotle-X3R green peppers. Soybean aphids and green peach aphids were kept at 25°C, with 16:8 (L:D) h cycle, whereas melon aphids were kept at 25°C, with 12:12 (L:D) h cycle (note: the rearing conditions used for melon aphid were the same used for growing green peppers). All aphid species were age-synchronized by transferring adults to insecticide-free detached leaves in customized Petri dishes. Adults were removed 48 h later, leaving nymphs behind. Nymphs remained on the leaf until they reached 7 d of age (e.g., adult stage) at which time they were used for experiments. Petri dishes for age synchronization were 100 × 25 mm in size and filled ~40% with DAP Plaster of Paris (DAP, Baltimore, MD) and 2320 ppm of activated charcoal (Sigma-Aldrich, St. Louis, MO). The plaster surface was covered with a 9-cm-diameter Whatman filter paper #1 (GE Healthcare, Chicago, IL). We added deionized water to the plaster and filter paper until saturation to preserve leaf freshness.

We exposed aphids to untreated or treated plants by using a modified bioassay with excised leaves from Nauen (1995). In brief, we collected leaves from treated or untreated plants at 7, 14, 21, 28, and 35 d after treatment/planting. After placing the leaves in the Petri dishes, we used a fine-haired paintbrush to transfer a total of 50, 7-d-old adult aphids on the underside of the detached leaf. Soybean aphids, green peach aphids, and melon aphids fed on leaves of soybean, zinnia, and green pepper, respectively. Detached leaves with aphids were kept in the Petri dishes for 24 h. Aphids were then randomly selected and transferred to insecticide-free leaves (see below) to serve as prey of the natural enemies used on the feeding bioassay. Immediately after collecting leaves from plants, we transferred a total of 10, 7-d-old adult soybean aphids, green peach aphids or melon aphids to soybean, zinnia or green pepper plants, respectively, to estimate the toxicity of thiamethoxam present in plants. Aphids on plants were confined with clip cages as described in Esquivel et al. 2019. Mortality of soybean aphids, green peach aphids, and melon aphids was recorded 72 h after plant infestation (note: soybean aphid mortality values in the experiment with insidiosus flower bug, also include the mortality caused by the predator and it was not performed using clip cages). Aphid survival between treatments

(i.e., aphids fed on thiamethoxam-treated or untreated plants) at each time point (7, 14, 21, 28, and 35 d after planting/treatment) was compared using factorial ANOVA with PROC GLM. For mean separation we used Tukey HSD post-hoc test with 95% family-wise confidence for mean separation of main effects, and LSMEANS for interactions (SAS version 9.4). For details about thiamethoxam residues in soybean and zinnias after planting/treatment under similar experimental conditions, please refer to Esquivel et al. (2019) and Derksen et al. (2015).

Natural Enemy Colonies

Experiments performed at CFAES Wooster Campus used adults of convergent lady beetles and insidiosus flower bugs purchased from ARBICO-Organics (Tucson, AZ) and Rincon-Vitova (Ventura, CA), respectively. Upon arrival to CFAES Wooster Campus, convergent lady beetles and insidiosus flower bugs fed on green peach aphids from an unexposed colony until needed for the experiment. Experiments at the EAP in Honduras used adults from a colony of insidiosus flower bugs reared on eggs of the Mediterranean flour moth *Ephesia kuehniella* (Zeller) (Lepidoptera: Pyralidae), kept at controlled conditions (21°C, 12:12 (L:D) h cycle). All colonies were constantly supplied with 10% sucrose-soaked cotton wicks.

Predation of Aphids by Natural Enemies

The sugar solution and prey were removed from the predatory natural enemies 24 h prior to the experiment. Sexing of convergent lady beetles and insidiosus flower bugs adults was initiated by collecting adults with mouth-operated aspirators (BioQuip, Rancho Dominguez, CA). Adults were anesthetized with CO₂ gas and ice for 10 min. While stunned, males and females were sorted by observing their abdominal dimorphism distinctions (note: no sexing of insidiosus flower bugs was performed for the experiment at the EAP). For the predation trials, each customized Petri dish contained a soybean, zinnia, or green pepper insecticide-free leaf, on which we transferred either a female insidiosus flower bug with 20 randomly selected aphids or a female convergent lady beetle with 30 randomly selected aphids. The number of aphids offered to insidiosus flower bugs and lady beetles was based on reports from previous experiments (see Boiça Junior et al. (2004), Camargo et al. (2017), and Elliott et al. (2000) for more information). Convergent lady beetles preyed on either soybean aphids or green peach aphids, whereas insidiosus flower bugs preyed on soybean aphids or melon aphids.

After 24 h of convergent lady beetles feeding, the remaining aphids were counted to estimate predation and then removed from the Petri dish. No molecular gut analysis was performed with convergent lady beetles, since aphid consumption could be confirmed and quantified based on the number of aphids eaten per convergent lady beetle. To compare the number of aphids eaten per convergent lady beetle, we used a factorial ANOVA analysis with PROC GLM, Tukey HSD post-hoc test with 95% family-wise confidence for mean separation of main effects, and LSMEANS for interactions (SAS version 9.4). Similarly, after 24 h of insidiosus flower bugs preying, alive and dead aphids were removed from the Petri dish. Aphid predation by insidiosus flower bugs was confirmed via molecular gut analysis (see *Molecular Gut Analysis of Insidiosus Flower Bug* below) since the physical aperture left by its piercing mouthparts in the soft exoskeleton of aphids was not consistently visible. Molecular gut analysis was performed only for the experiment at the CFAES Wooster Campus (soybean–soybean aphid) because facilities at EAP did not have the equipment needed to perform this test at the time of the

experiment. Mean comparison between treatments (i.e., aphids fed on thiamethoxam-treated or untreated plants) of the molecular gut analysis of insidiosus flower bugs (i.e., soybean aphid predation) was done using the Student's T-test (SAS version 9.4).

After aphid removal from the leaves, the survival of both the lady beetles and the insidiosus flower bugs was recorded every day for the following 7 d. Natural enemies were considered dead if no movement was observed within 10 min after gently touched with a fine-haired paintbrush. The number of dead convergent lady beetles or insidiosus flower bugs within the 7 d after aphid exposure was used to estimate their 'survival time' (survival length of natural enemies after feeding on aphids). We compared the survival of natural enemies preying on aphids from thiamethoxam-treated or untreated plants using the Cochran-Mantel-Haenszel (CMH) categorical analysis ($s \times r$ table) (Mantel 1963) with PROC FREQ under SAS platform, version 9.4. The independent variables for the CMH analysis were 'aphid species eaten by natural enemy' (3), 'days after planting or treating' (5) and 'insecticide treatment' (2). The categorical dependent variable was 'survival length of natural enemies after feeding on aphids', expressed in days (0–7 d). We used the ORDER=DATA option for SAS, as the dependent variable was considered ordinal. Survival of natural enemies feeding on aphids from thiamethoxam-treated or untreated plants were statistically different if the Cochran-Mantel-Haenszel χ^2 had a $P < 0.05$.

Molecular Gut Analysis of Insidiosus Flower Bug

Insidiosus flower bugs were stored in 1.5 ml microcentrifuge tubes at -80°C when found dead or collected after surviving 7 d. Soybean aphid predation was confirmed by a molecular gut analysis detecting the 'soybean aphid *cytochrome c oxidase 1*' (CO1) gene in insidiosus flower bugs. The primers for the insidiosus flower bug and soybean aphid CO1 genes (accession numbers KR036545.1 and AY842503.1, respectively) were designed and purchased from Thermo Fisher Scientific Inc. (Waltham, MA). Species specificity of the primers was confirmed in silico with a standalone tBLASTx v2.2.31 (e -value $< 10^{-6}$) and in vitro via PCR/electrophoresis using genomic DNA from soybean aphids and insidiosus flower bugs. Insidiosus flower bug CO1 primers generated amplicons of 230 base pairs (bp), whereas CO1 of soybean aphid primers generated amplicons of 450 bp. Samples without insidiosus flower bug CO1 amplification were discarded. Primer reverse and forward sequences are included in Supp Table 1 (online only). Genomic DNA was extracted using the QIAGEN DNease Blood & Tissue Kit (Germantown, MD) following the manufacturer's protocol. Conventional PCR was performed by using 12.5 μl Promega GoTaq Green Master Mix (Madison, WI), 2 μl of 10 μM primer (forward and reverse), 2 μl of DNA, and 8.5 μl of nuclease-free water per reaction. The thermal cycler protocol was obtained from Harwood et al. (2007): denaturation for 4 min at 95°C ; 35 cycles of 10 s at 95°C , 30 sec at 55°C , 30 s at 72°C ; and 5 min at 72°C . A total of 5 μl of the PCR reaction was electrophoresed on a 1.5% agarose gel, including 0.5X TAE and GelRed (Biotium Inc., Fremont, CA). Electrophoresis occurred for 35 min at 75 mV. Soybean aphid CO1 amplification in insidiosus flower bug gut was confirmed by band visualization with MYECL Imager (Thermo Fisher Scientific Inc.).

Results

Mortality of Aphids Caused by Insecticide in Plants

We observed consistency in mortality values among aphid species when exposed to plants from different treatments (Fig. 1A–D). We

found that soybean aphid mortality was significantly higher at 7 and 14 DAP when fed on soybean from treated soybeans (plants used for the bioassay with convergent lady beetles) ($P = 0.0001, 0.0001$, Fig. 1A) and at 14, 21 and 28 DAP (plants used for the bioassay with insidiosus flower bugs) ($P = 0.0044, 0.0002, 0.0022$, Fig. 1C). We observed higher mortality when green peach aphid (Fig. 1B) and melon aphid (Fig. 1D) fed on thiamethoxam-treated zinnias and green peppers, respectively, in comparison to feeding on untreated plants. Mortality of green peach aphid was significantly higher on zinnias at 7, 14, 21, 28 d after thiamethoxam treatment ($P = 0.005, 0.0006, 0.0001, 0.0005$, Fig. 1B), whereas mortality of melon aphid on green peppers was significantly higher at all times points (7, 14, 21, 28, and 35 d after thiamethoxam treatment) ($P = 0.0001, 0.0001, 0.0001, 0.0001, 0.0001$, Fig. 1D).

Predation and Survival of Lady Beetles

We found no significant difference in the number of soybean aphids consumed by convergent lady beetles that had fed on thiamethoxam-treated or untreated plants (treatment as main effect) ($F = 0.92$; $df = 1, 90$; $P = 0.34$). The main effect 'days after planting' ($F = 6.05$; $df = 4, 90$; $P = 0.0002$) and the interaction 'treatment \times days after planting' ($F = 2.59$; $df = 4, 90$; $P = 0.04$) were both significant. The significance in the interaction was due to differences between treatments that had no relevance for our hypothesis testing; e.g., treated plants at 14 DAP in comparison to 28 DAP ($P = 0.0001$), or untreated plants at 14 DAP in comparison to treated plants at 28 DAP ($P = 0.0001$). However, when we compared the predation of soybean aphids between treated or untreated soybeans at each time point (7, 14, 21, 28, and 35 DAP), none of them were statistically different ($P = 0.94, 0.99, 0.97, 0.76, 0.50$) (Fig. 2A). Similarly, no difference was detected in convergent lady beetle's predation of green peach aphids that had fed on thiamethoxam-treated or untreated plants (treatment as main effect) ($F = 2.26$; $df = 1, 90$; $P = 0.13$) (Fig. 2B). The main effect 'days after planting' was significant ($F = 8.67$; $df = 4, 90$; $P < 0.0001$), but not the interaction 'treatment \times days after planting' ($F = 0.48$; $df = 4, 90$; $P = 0.74$).

Moreover, we found no significant difference in survivorship time of lady beetles when they consumed soybean aphids from thiamethoxam-treated or untreated soybeans across all time points (7, 14, 21, 28, and 35 DAP) ($F = 0.89, 2.19, 2.09, 3.51, 0.005$; $df = 1$; $P = 0.34, 0.13, 0.14, 0.06, 0.93$) (Fig. 3A). Similarly, no significant difference was observed in survival time of lady beetles feeding on green peach aphids from thiamethoxam-treated or untreated zinnia plants across all time points (7, 14, 21, 28, and 35 DAP) ($F = 1.05, 0.35, 1.80, 0.29, 1.77$; $df = 1$; $P = 0.30, 0.55, 0.17, 0.58, 0.18$) (Fig. 3B).

Predation and Survival of the Insidiosus Flower Bug

Across all treatments (i.e., aphids fed on thiamethoxam-treated or untreated plants) and time points (14, 21, 28, and 35 DAP), the percentage of insidiosus flower bugs detected with the soybean aphid CO1 ranged between 60 and 100%, with a mean \pm SEM of $80\% \pm 5.34$ (Fig. 4). Due to poor quality of DNA extracted, samples from 7 DAP were not available for analysis. We did not observe statistical differences in the percentage of insidiosus flower bugs detected with soybean aphid CO1 between treatments at any of the time points ($t = -0.4399$; $df = 5.9$; $P = 0.67$).

The survival time of insidiosus flower bugs on soybean aphid across treatments (i.e., soybean aphids fed on thiamethoxam-treated or untreated soybeans) ranged between 0 and 7 d, with a mean \pm SEM of 3.49 ± 0.22 d (Fig. 5A). In contrast to lady beetles,

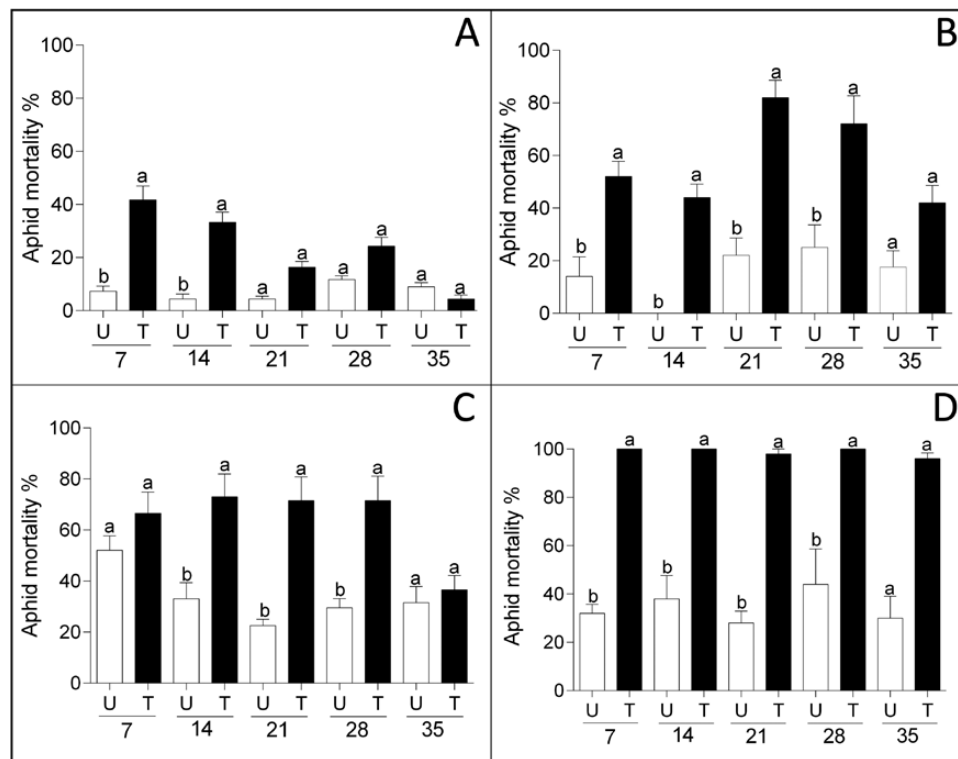


Fig. 1. Percentage of mortality of soybean aphids (A and C), green peach aphids (B) and melon aphids (C) (\pm SEM) when fed on untreated (U, white bars) soybeans (A and C), zinnias (B) or green peppers (D) or when fed on thiamethoxam-treated plants (T, black bars). The 7, 14, 21, 28, and 35 at the 'x-axis' are the time points, in days after planting for soybean (A and C) or after treatment for zinnias (B) and green peppers (D). Plants used to develop A and B were used for the tri-trophic toxicity bioassay with convergent lady beetles, whereas plants used for C and D were used for the tri-trophic bioassay with insidiosus flower bugs. Bars within the same time point followed by the same letter are not significantly different in percentage of mortality of aphids (Tukey HSD post-hoc test with a 95% family-wise confidence level, $P \geq 0.05$) (note: soybean aphid mortality values in C also include the mortality caused by insidiosus flower bugs).

we observed that insidiosus flower bugs feeding on aphids from treated plants lived fewer days in comparison to insidiosus flower bugs feeding on aphids from untreated plants. Significant survival time reductions of 3.6 d occurred after insidiosus flower bugs fed on soybean aphids from 21 DAP treated soybean ($F = 14.28$; $df = 1$; $P = 0.0002$) (Fig. 5A). We also observed survival time reductions of 2.1 and 1.3 d when insidiosus flower bugs fed on soybean aphids from 14 DAP ($F = 3.20$; $df = 1$; $P = 0.07$) and 28 DAP ($F = 2.74$; $df = 1$; $P = 0.09$) treated soybean; however, those values were not significant based on the CMH analysis (Fig. 5A). Similar results were observed when insidiosus flower bugs fed on melon aphids. Significant survival reductions of 1.1, 1.1, and 1.6 d were observed after insidiosus flower bugs fed on melon aphid from green peppers at 7, 14, and 21 d after thiamethoxam-drench treatment, respectively ($F = 6.04, 5.29, 9.27$; $df = 1$; $P = 0.01, 0.01, 0.002$) (Fig. 5B). We also observed survival reductions of 0.6 d when insidiosus flower bugs fed on melon aphids from green peppers 28 d after thiamethoxam-drench treatment, however that reduction was not significant based on the CMH analysis ($F = 2.01$; $df = 1$; $P = 0.15$) (Fig. 5B).

Discussion

Neonicotinoid insecticides could be compatible with biological control agents (Mizell and Sconyers 1992, Ohnesorg et al. 2009, Gontijo et al. 2014). Its application mode (e.g., seed coating, root drench) and movement within the plant typically limit insecticide exposure to insects that consume plant tissue or plant products (Mizell and Sconyers 1992, Pisa et al. 2015). Nonetheless, natural enemies (i.e.,

predatory insects) might be impaired when they feed upon prey that consumed thiamethoxam-treated plants (i.e., trophic food chain) (Grafton-Cardwell and Gu 2003, Douglas et al. 2015).

The effects of thiamethoxam on natural enemies via the food chain are likely stronger at early time points after plant treatment (Grafton-Cardwell and Gu 2003, Douglas et al. 2015), when presumably the insecticide concentration within plants is still high (Castle et al. 2005, Krupke et al. 2017, Esquivel et al. 2019). However, in some systems thiamethoxam has several weeks of insecticidal bioactivity after treatment (Castle et al. 2005, Diez-Rodríguez et al. 2006, McCornack and Ragsdale 2006, Seagraves and Lundgren 2012, Krupke et al. 2017, Esquivel et al. 2019) which might facilitate toxicity to predatory natural enemies via the food chain at later time points. Yet, evidence of thiamethoxam toxicity via the food chain is limited to few predatory natural enemy species, and available mostly for early time points after thiamethoxam treatment.

To expand our understanding of thiamethoxam's effects on natural enemies via the food chain, we used aphids fed on thiamethoxam-treated plants at weekly intervals for 5 wk as prey for the convergent lady beetle and the insidiosus flower bug. We found that the response depends on the natural enemy. For instance, aphid predation and survival time of lady beetles were not affected by consuming aphids from thiamethoxam-treated plants at any time point. Although the percentage of insidiosus flower bugs feeding on aphids did not differ, we observed significant changes in survival time of insidiosus flower bugs when fed on aphids from early-treated plants.

We observed significant higher mortality of soybean aphids and green peach aphids when fed on treated plants for the first 28 d after planting/treatment, in comparison to feeding on untreated

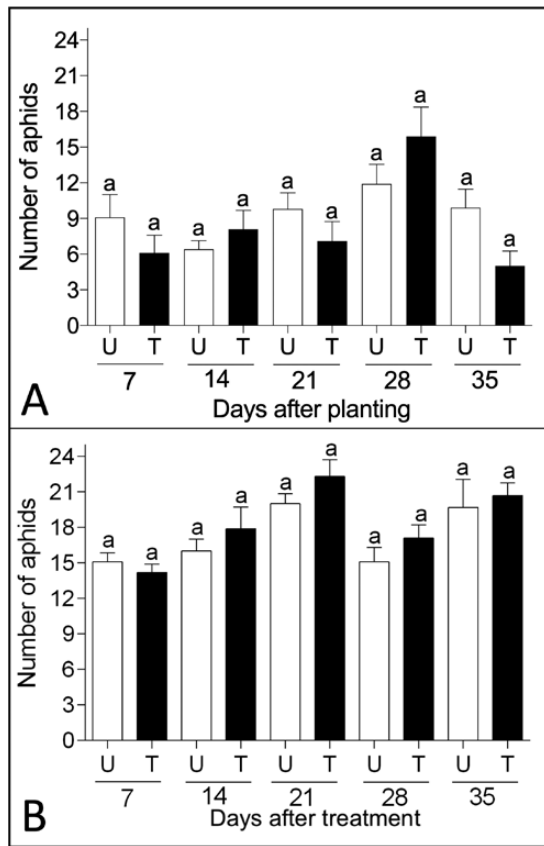


Fig. 2. Number of soybean aphids (A) or green peach aphids (B) eaten (\pm SEM) by convergent lady beetles within the first 24 h after feeding on untreated (U, white bar) or thiamethoxam-treated plants (T, black bar). The 7, 14, 21, 28, and 35 at the 'x-axis' are the time points, in days after planting for soybean (A) or after treatment for zinnias (B), when plant tissue was collected for aphid feeding. Bars within the same time point followed by the same letter are not significantly different in number of aphids eaten by convergent lady beetles (Tukey HSD post-hoc test with a 95% family-wise confidence level, $P \geq 0.05$).

plants. Higher mortality suggests that aphids exposed to treated plants also ingested insecticide present in foliage. After 28 d of planting/treatment, no significant difference in aphid mortality between treatments was observed, perhaps, due to the reduction of thiamethoxam in plants over time as reported in previous studies (Esquivel et al. 2019). Notably, soybean aphid and green peach aphid predation by convergent lady beetles did not vary significantly across treatments (i.e., aphids fed on thiamethoxam-treated or untreated plants), suggesting that lady beetles do not discriminate between thiamethoxam-exposed or unexposed aphids. Moreover, the survival length of convergent lady beetles showed no significant difference when fed on thiamethoxam-exposed or unexposed aphids. The lack of difference in survival time among treatments could be due to 1) limited ingestion of thiamethoxam by aphids as a result of reduced sap feeding on treated plants (Mowry 2005), 2) insufficient consumption of aphids to reach a disruptive dose under bioassay conditions, or 3) adult convergent lady beetles are tolerant to thiamethoxam. Our data did not support the first nor the second possibility, since aphid sap feeding was not measured, thiamethoxam residues in aphids were not quantified (despite the fact we observed higher aphid mortality on treated plants), nor we provided a constant supply of aphids with insecticide along the lifespan of convergent lady beetles. Though, thiamethoxam seems to have limited effects on the survival time of adult convergent lady

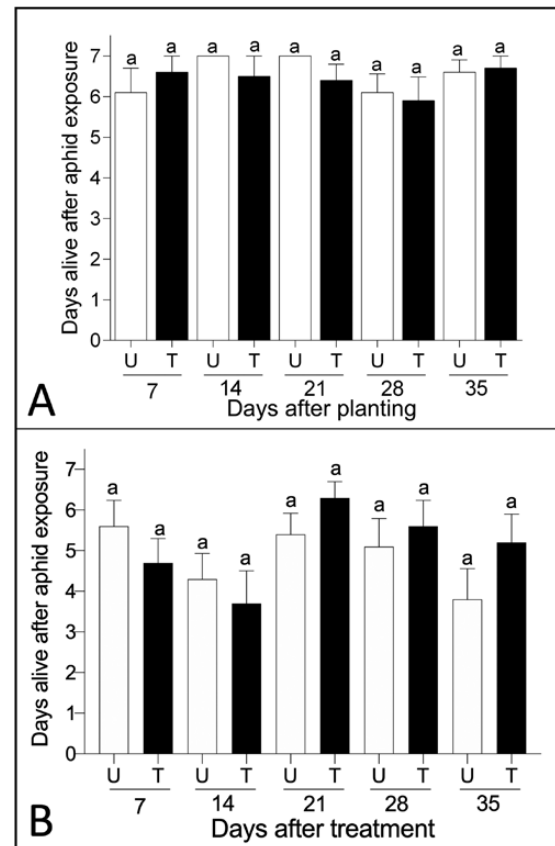


Fig. 3. Survival time of convergent lady beetles (in days, \pm SEM) when they preyed upon soybean aphids (A) or green peach aphids (B) that fed on untreated plants (U, white bar) or on thiamethoxam-treated plants (T, black bar). The 7, 14, 21, 28, and 35 at the 'x-axis' are the time points, in days after planting soybean (A) or days after treating zinnias (B), when plant tissue was collected for aphid feeding. Bars within the same time point followed by the same letter are not significantly different in survival time of convergent lady beetles (Cochran-Mantel-Haenszel χ^2 , $P \geq 0.05$).

beetles, supporting the third possibility. Empirical evidence suggests that adults of other lady beetle species, including the multi-colored Asian lady beetle *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) and the spotless lady beetle *Cycloneda sanguinea* (L.) (Coleoptera: Coccinellidae) have a tolerance to thiamethoxam (Youn et al. 2003, Fernandes et al. 2016, Wang et al. 2018). If convergent lady beetles are indeed tolerant, systemic applications of thiamethoxam might have minor negative effects on their ability to control aphid populations. However, to fully examine whether the convergent lady beetle and thiamethoxam are compatible when used simultaneously (as shown by Wright and Verkerk 1995, Abraham et al. 2013), it is necessary to further develop toxicological experiments through various exposure routes (e.g., topical applications, treated surfaces, injection of insecticide, releases in a treated area). It is also important to evaluate the toxicity of thiamethoxam on immature stages (such as egg or larva instars), as their susceptibility to this insecticide might be different than adults (Youn et al. 2003, Moser and Obrycki 2009, Bredeson et al. 2015). Lastly, the convergent lady beetle has at least two genetically distinctive populations within the United States (Sethuraman et al. 2015); therefore, toxicological trials should also include convergent lady beetle populations from different geographical sources, as their variation in genetics, selection pressure or exposure to thiamethoxam could provide distinctive degrees of tolerance to thiamethoxam.

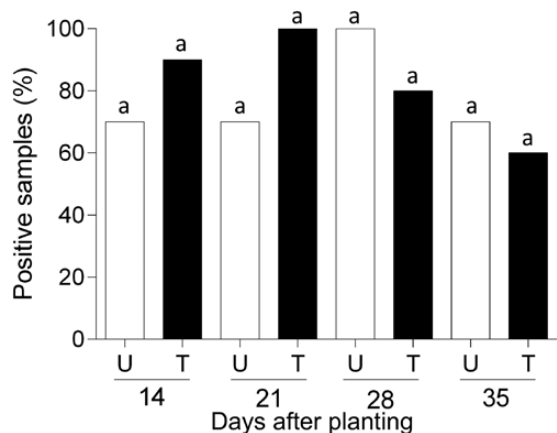


Fig. 4. Percentages of insidiosus flower bugs within each treatment detected positive by molecular gut analysis for 'soybean aphid CO1' when fed on soybean aphids from untreated (U, white bars) or thiamethoxam-treated soybean (T, black bars). The 14, 21, 28, and 35 at the 'x-axis' are the time points, in days after planting (DAP), when the soybean leaflets were collected for aphid feeding. No SEM ranges are shown, since values of each bar represents the percentage of positive insidiosus flower bugs, based on the total number of insidiosus flower bugs (10 in total) used for each treatment (U or T) at each time point (14, 21, 28, and 35 DAP). Bars within the same time point followed by the same letter are not significantly different in number of positive samples (Student's T-test, $P \geq 0.05$).

In the experiments with insidiosus flower bugs, we also observed that soybean aphids and melon aphids had significantly higher mortality feeding on treated plants than on untreated plants, at nearly all time points (7, 14, 21, 28, and 35 d after planting/treatment). Higher mortality levels suggest that aphids exposed on treated plants ingested insecticide present in foliage. Analogously to convergent lady beetles, the percentage of insidiosus flower bugs that consumed soybean aphids did not differ among treatments, suggesting that exposure of soybean aphids to thiamethoxam-treated soybean does not induce avoidance by insidiosus flower bugs. Gut analysis was not performed for insidiosus flower bugs feeding on melon aphids; however, similar non-discriminatory predation as with soybean aphid could be expected. Unlike lady beetles, we observed significant shorter survival time for insidiosus flower bugs when they preyed on melon aphids and soybean aphids from treated plants. Not all times were significant, but they occurred early and in accordance with the timing of decreasing thiamethoxam activity and an increase of survival of soybean aphids (McCarville and O'Neal 2013, Krupke et al. 2017, Esquivel et al. 2019). Moreover, our survival time results are in accordance with other tri-trophic toxicity tests, where insidiosus flower bugs preyed on herbivore insects that fed on thiamethoxam-treated plants (Al-Deeb et al. 2001, Camargo et al. 2017).

The similarity of our results from the two aphid systems (soybean aphid–soybean, and melon aphid–green peppers) suggests that the initial 21–28 d after planting/treatment could present a toxic window for prey that transfers thiamethoxam obtained from the plant to the insidiosus flower bug. Incidentally, this toxic window also resembles the duration of thiamethoxam residues in plants after treatment in various agronomic scenarios (McCornack and Ragsdale 2006, McCarville and O'Neal 2013, Krupke et al. 2017, Esquivel et al. 2019). Toxicity of thiamethoxam to insidiosus flower bugs via the food chain has also been documented under other experimental conditions. For instance, insidiosus flower bugs that preyed on greenbugs *Schizaphis graminum* (Rondani) (Hemiptera: Aphididae) fed on thiamethoxam-treated corn had higher mortality than greenbugs

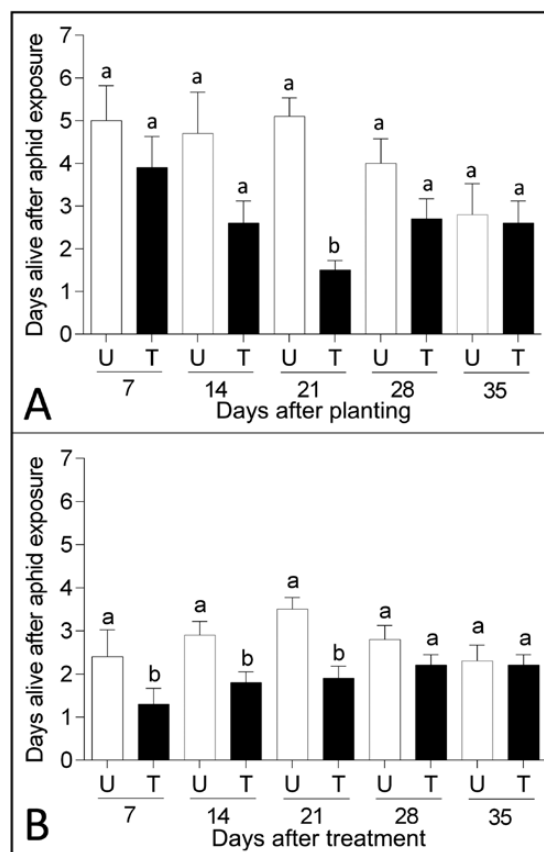


Fig. 5. Survival length of insidiosus flower bugs (in days, \pm SEM) when they preyed upon soybean aphids (A) or melon aphids (B) that fed on plants from untreated-seeds (U, white bar), or from thiamethoxam-treated seeds (T, black bar). The 7, 14, 21, 28, and 35 at the 'x-axis' are the time points, in days after planting for soybean (A) or days after treatment for green peppers (B), when plant tissue was collected for aphid feeding. Bars within the same time point followed by the same letter are not significantly different in survival time of insidiosus flower bugs (Cochran-Mantel-Haenszel χ^2 , $P \geq 0.05$).

without thiamethoxam exposure (Al-Deeb et al. 2001). Moreover, insidiosus flower bugs had higher mortality when preyed on soybean aphids that fed on soybean leaves where the petiole was immersed in thiamethoxam solutions at 5 and 10 ng/ml of thiamethoxam for 24 h (Camargo et al. 2017). Our study complements the aforementioned studies by providing evidence of a time window when thiamethoxam can be toxic to insidiosus flower bugs via the food chain.

We also observed slight differences in survival time between our experiments (soybean aphid–soybean; melon aphid–green peppers) that might reflect system-specific outcomes. For example, the reduction in survival time of insidiosus flower bugs feeding on melon aphids from thiamethoxam-treated green peppers was greater than the reduction in survival time of insidiosus flower bugs feeding on soybean aphids from thiamethoxam-treated soybean (in comparison to insidiosus flower bugs feeding on aphids from untreated plants). This difference in survival time reduction between systems might be associated with the higher dose applied per plant via drench to green peppers (170 mg a.i./plant) compared to the dose in seed treated soybean (~0.091 mg a.i./seed). A greater thiamethoxam dose per plant might also be associated with longer periods of insecticide activity that could affect natural enemies via the food chain (Grafton-Cardwell and Gu 2003). However, we did not observe that plants treated with a higher thiamethoxam dose (i.e., green peppers) caused longer negative effects in insidiosus flower bugs via the food chain than soybean plants. Why

a higher dose of thiamethoxam in green peppers did not translate into longer negative effects on insidiosus flower bugs is unclear, but it might be related to physiological attributes in green peppers that promote faster breakdown of thiamethoxam into less toxic compounds for this natural enemy (Nauen et al. 2003).

Lastly, our results suggest that convergent lady beetles and insidiosus flower bugs could be integrated with systemic applications of thiamethoxam to complement aphid control. We suspect that early releases of convergent lady beetles, within the first 21 d after a thiamethoxam application, could complement aphid management with minimal impacts on their control services. At 28 d or later after thiamethoxam application, insidiosus flower bugs could be released to complement pest control, when presumably aphids no longer have lethal residues of thiamethoxam. Therefore, we consider that the timing of releases for these natural enemy after insecticide application is key to combine the benefits of these predators and thiamethoxam for pest control. The integration of pest management strategies, such as biological and chemical control, has been one of the fundamental objectives of integrated pest management (Stern et al. 1959, van den Bosch and Stern 1962). However, thiamethoxam applications and the release of biological control agents should be carefully implemented and evaluated, since thiamethoxam and other systemic insecticides have been found to disrupt populations of predatory natural enemies (Szczepaniak et al. 2011, Seagraves and Lundgren 2012). Other practices, such as the use of untreated refugia, a low pesticide dose, spatially targeted insecticide applications, and insecticide-tolerant natural enemies, can minimize pesticide exposure or effects to natural enemies (Roubos et al. 2014). Conserving the invertebrate predator-prey associations is critical for the balance and sustainability of agricultural ecosystem services (Schlapfer et al. 1999, Cardinale et al. 2003, Chagnon et al. 2015), and disturbances caused by insecticides could lead to losses in yield, aesthetics, profits and pest outbreaks (Shepard et al. 1977, Riley 1988, Hardin et al. 1995, Dutcher 2007, Bommarco et al. 2011, Guedes et al. 2016, Hill et al. 2017).

Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

Supplementary Table 1. Forward and reverse oligo primers to amplify 'cytochrome c oxidase 1' (CO1) of insidiosus flower bug and soybean aphid.

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