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Thiamethoxam seed treatments reduce foliar predator and pollinator populations in sunflowers (*Helianthus annuus*), and extra-floral nectaries as a route of exposure for seed treatments to affect the predator, *Coleomegilla maculata* (Coleoptera: Coccinellidae)

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ABSTRACT

Neonicotinoid seed-treatments are used frequently in sunflower production to prevent early-season herbivory, and have been implicated in declines in non-target species. Over three site-years, both insecticide treated (Cruiser®, rate: 0.25 mg a.i. (thiamethoxam)/seed) and untreated sunflower (*Helianthus annuus* [Family: Asteraceae]) fields were planted in Eastern and Central South Dakota. Foliar and subterranean predatory arthropod communities and pollinator populations were compared among the two treatments. A greenhouse study was performed to collect and quantify toxins in extra-floral nectar from treated and untreated sunflowers. A laboratory assay was performed to examine the effect of an artificial nectar diet laced with thiamethoxam or clothianidin on survival, fecundity and mobility of *Coleomegilla maculata* (De Geer [Coleoptera: Coccinellidae]). Seed-treated fields had significantly fewer above-ground natural enemies and pollinators than untreated fields, while subterranean predators were unaffected. Beetle flip-over times increased as concentration of thiamethoxam in diet increased, but clothianidin showed no effect. Neither toxin affected number of eggs oviposited by beetles, or size of developing oocytes. However, there was a negative correlation between increasing thiamethoxam concentration and number of developing eggs. Extra-floral nectar (EFN) collected from treated greenhouse-grown plants contained thiamethoxam (range: 1.23 ± 0.09 ppb to 4.83 ± 0.63 ppb), but no clothianidin. Toxin-laden EFN was identified as a potential route of exposure between beneficial arthropods and seed-applied neonicotinoids. Risks of neonicotinoid seed treatment use are discussed in the light of additional exposure pathways being confirmed.

1. Introduction

Sunflower producers (*Helithanus annuus*) in the Upper Great Plains of North America frequently integrate insecticidal seed-treatments into their pest management strategies. With over 1.60 million acres of sunflowers planted across the United States in 2016 (NASS, 2016), opportunity exists for arthropod visitors of this highly entomophilous crop to become exposed to pesticides used to manage pests of sunflowers. Technical resources frequently list insecticidal seed treatments as a viable option for preventing damage from wireworms, cutworms, flea beetles, sunflower beetles and other herbivores (NDSU, 2015). Researchers have quantified toxins in neonicotinoid seed-treated sunflower's vegetative tissues (Bredeson and Lundgren, 2015b; Mogren and Lundgren, 2016), pollen, and floral nectar (Laurent and Rathahao, 2003; Mogren and Lundgren, 2016; Schmuck et al., 2001). These quantities are often below 5 ppb for most of the growing season, although one report found much higher concentrations in untreated feral sunflowers than in cultivated varieties (Mogren and Lundgren, 2016). In some cases, seed-applied neonicotinoid insecticides have been successful at limiting pest populations in other crops (Castle et al., 2005 (control of *Homalodisca coagulata* in citrus); Megalhaes et al., 2009 (control of *Aphis glycines* in *Glycine max*)). However, seed treatments have not improved pest management in sunflowers (Bredeson and Lundgren, 2015b).

In recent years, laboratory studies report that important predatory insects are detrimentally affected when exposed to neonicotinoid seed-

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treated sunflowers. An assay performed by Gontijo et al. (2015) found that minute pirate bug (*Orius insidiosus* (Say); [Hemiptera: Anthocoridae]) egg viability and nymph development were reduced when insects were caged on sunflowers whose seeds were treated with thiamethoxam compared to untreated plants. In a similar assay, adult *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) were confined to thiamethoxam seed-treated sunflower stems with an extrafloral nectary (EFN). As a result, lacewings on treated plants had reduced fecundity and higher mortality in comparison to insects on untreated plants (Gontijo et al., 2014). Two lady beetle species (*Coleomegilla maculata* De Geer, and *Hippodamia convergens* Guéren-Méneville [Coleoptera: Coccinellidae]) native to regions of intensive sunflower production also had slower development rates when exposed to the EFN of thiamethoxam seed-treated sunflower stems (Moscardini et al., 2015). To date, there have been no studies that examine the effects of seed treatments on sunflower predator or pollinator communities.

The documented fitness effects on natural enemies in the laboratory underscore the need for further field-based research on neonicotinoid seed treatments and their effects on non-target arthropod populations, especially in light of the lack of herbivore suppression in field trials (Bredeson and Lundgren, 2015b). This study monitored beneficial predator and pollinator communities over three site-years in thiamethoxam seed-treated and untreated sunflower fields in central and eastern South Dakota, USA. A supporting laboratory assay examined the effects of an artificial nectar diet spiked with neonicotinoid insecticides on adult female *C. maculata*. Finally, neonicotinoid concentrations within extra-floral nectar collected from seed-treated sunflowers was quantified using enzyme-linked immunosorbent assays (ELISAs).

2. Methods

2.1. Sunflower production and experimental design

Many of the details for experimental design and methods are described in Bredeson and Lundgren (2015a&b). Six sunflower (Pioneer®, Variety: 63M80-N422) fields measuring 30.5 × 30.5 m were planted near Brookings, SD (44.3064° N, 96.7881° W) on June 14, 2013. Sorghum Sudan (*Sorghum* × *drummondii*, Millborn Seeds Inc., Variety: MS9000) borders were planted around the fields to separate them by a distance of 12 m and limit inter-plot movement of arthropods. Sorghum Sudan borders were occasionally mowed for weed control. Untreated seeds were planted in three fields, while seeds treated with thiamethoxam (Cruiser®, Syngenta, Greensborough, North Carolina, USA, 27409) at a rate of 0.25 mg a.i./seed were planted in the remaining three fields. No fungicidal seed treatments were applied to these fields. A seeding rate of 76,600 seeds/ha was used in both treatments. Fields were arranged in a line with insecticide-treated and untreated fields alternating.

On May 23, 2014, at the Brookings location, eight, 24.5 × 36.5 m sunflower (*Helianthus annuus*, Mycogen®, var 8H288CLDM) fields were established at a rate of 76,600 seeds/ha. Untreated seeds were used in half of the fields while the remaining fields were planted with a thiamethoxam seed dressing at a rate of 0.25 mg a.i./seed. Field margin size was unchanged from the 2013 field design, but untreated-soybeans (*Glycine max*) were used instead of Sorghum × Sudan to comply with crop rotation requirements for the field. On June 6, 2014 at Dakota Lakes Research Farm (Pierre, SD 44.3680° N, 100.3364° W) the same experimental conditions were established as in Brookings 2014, with the exception of narrower (6 m) untreated-soybean borders.

At the Brookings location in 2013, glyphosate (Roundup WeatherMAX® 2.34 L/ha) and sulfentrazone (Spartan®; 0.44 L/ha), were applied at sunflower planting. At Brookings in 2014 fields were subject to the same herbicide regime except the glyphosate rate was reduced to 1.61 L/ha. In the fall preceding sunflowers at Dakota Lakes fields were sprayed with sulfentrazone (0.29 L/ha). Shortly after spring planting the fields were, again, sprayed with a mixture of glyphosate (rate: 1.17 L/ha) and pendimethalin (Prowl® H2O; 2.92 L/ha). At the Brookings location, no fertilizer was added in either study year. Nitrogen was broadcasted at a rate of 84.06 kg/ha on June 1, 2014 at the Dakota Lakes location.

2.2. Insect collections

2.2.1. Foliar insects

Foliar arthropods were sampled eight times between the V-6 and R-7 sunflower stages (Schneiter and Miller, 1981) at the Brookings location in 2013. In 2014, foliar arthropods were sampled 10 and six times between sunflower growth stages V-2 and R-6 at the Brookings and Dakota Lakes locations, respectively. On each of the sampling dates, randomly selected plants from each field were visually examined. All beneficial arthropods found on the foliage, stems, and inflorescences were aspirated, then placed in 70% ethanol for identification and curation. During sunflower anthesis and high pollinator visitation insects on vegetative tissue were aspirated as usual, but plastic bags were placed over sunflower heads to capture highly mobile insects, including pollinators. Arthropod escape was rare and this method proved very effective. Pollinators were subsequently identified using the Apoidea key at www.discoverlife.org. At the Brookings 2013 location, 10 plants were sampled from each field on every sampling date. At the Brookings location in 2014, 20 plants per field were sampled on the first two dates, 15 plants on the third and fourth dates, and 10 plants per field on all remaining sampling dates. In 2014 at the Dakota Lakes location 15 plants per field were examined on the first date and 10 plants were examined per field on all the remaining dates. Time and labor constraints restricted the ability to sample a large number of plants late into the growing season when insect activity is high.

2.2.2. Soil arthropod community

Soil insect communities were assessed on six sampling dates between planting and the R-6 plant stage at Brookings in 2013. In 2014, at the Brookings and Dakota Lakes locations soil insects were assessed eight and six times, respectively, between the V-2 and R-6 plant stages. Soil cores (10 cm diam., 10 cm deep) were taken between adjacent sunflower plants within a row at random locations using a golf-hole cup cutter. Four soil cores from each field in 2013 were taken on every sampling date. During the 2014 field season, due to limited soil insect extraction infrastructure, three cores were taken from each field per sampling date at both the Brookings and Dakota Lakes locations. Berlese funnel systems were implemented for 7 d to extract arthropods from soil cores. Specimens were preserved in 70% aqueous ethanol until identification.

2.3. Bioassay of lady beetles on a toxic nectar diet

To examine the effects of the neonicotinoids thiamethoxam and clothianidin in nectar on an ecologically relevant predator species, female adult *C. maculata* were fed diets of artificial nectar spiked with insecticide. For thiamethoxam, eight groups of 15 mated female beetles were separated into individual plastic cups (30 ml WNA Commet™, Chelmsford, MA, USA, 01824) with a water wick. Each group was fed a different concentration of thiamethoxam (100.0, 10.0, 5.0, 2.5, 1.0, 0.5, 0.1 or 0 ppb) in artificial nectar (2 g sucrose in 10 mL water). These doses were determined based on environmental levels observed in other studies. Diets were created by preparing thiamethoxam solutions with twice the desired concentration, and a sucrose solution that was also double the desired concentration. Combination of the two 2 × concentrations resulted in artificial nectar with the desired sucrose and insecticide concentrations. A second assay used the same experimental procedures, except new beetles were fed clothianidin. Three times weekly the beetles were placed in new cups with a fresh 20 µL nectar droplet. Assays for thiamethoxam and clothianidin nectar feeding were monitored for 33 and 34 d, respectively. On experimental days 7, 15

and 20 for thiamethoxam, and days 4 and 7 for clothianidin, approximately 20 *Ephestia kuehniella* eggs were given to each individual to encourage egg production.

2.3.1. Lady beetle fitness metrics

Individual beetles were placed on their backs and the time to right themselves was recorded (Lundgren and Wiedenmann, 2002). These flip times were conducted five times for thiamethoxam-fed beetles (on days 4, 8, 14, 20 and 33), and three times for clothianidin-fed beetles (on days 7, 22 and 34). The number of eggs laid per individual was recorded throughout the experiment. At cessation of the experiment, ovaries of all individuals were dissected, and the number of developing oocytes were counted per female. A developing oocyte was yellow in color and easily distinguished from clear or opaque ovarian fingers not possessing an oocyte. The length and width of the three largest oocytes on each ovary (six per beetle) were measured using a microscope optical micrometer. These measurements were converted into egg volume (formula for volume of an ellipsoid used in this calculation was: $V = 4/3 \times (\pi \times (\text{Length} \times \text{Width} \times \text{Width}))$.

2.4. Thiamethoxam quantification in field collected sunflower tissue

For a detailed account of the methods for tissue collection and insecticide quantification of field-collected sunflower tissue refer to Bredeson and Lundgren (2015b). Leaf discs (4.5 mm diameter) were removed from five randomly selected sunflower plants during seven dates in Brookings 2013, 11 in Brookings 2014 and six dates in Dakota Lakes during the 2014 season. Samples were stored at −20 °C until analysis via competitive enzyme-linked immunosorbent assays (Thiamethoxam HS plate kit, lot no. 13014E; Beacon Analytical Systems Inc., Saco, ME). Manufacturer instructions were followed, except standard curves were developed using untreated sunflower tissue to control for matrix effects.

2.5. Greenhouse sunflower production

Fifty-six plastic pots (11.4 L) were filled with potting soil mix (4:2:1 parts black topsoil, peat moss, and vermiculite). Sunflower seeds (Mycogen®, var 8H288CLDM) were planted untreated, or were treated with Cruiser Maxx ™ (Syngenta, Greensborough, North Carolina, USA, 27409) at the highest recommended label rate (0.25 mg a.i./seed) (n = 28 pots per treatment). No fungicides were applied in this experiment. Sunflowers were grown in a greenhouse at 22- 29 °C with a 16 h photophase (Greenhouse lights: GE® Lucalox LU1000, and GE® MVR1000/U, General Electric Company®, Fairfield, Ct, USA, 06828). Pans of tap water were kept between the plants to elevate greenhouse humidity and encourage pooling of nectar on plant nectaries. Plants were watered twice daily using an automatic watering system. Watering rate was adjusted as needed to accommodate for plant growth and greenhouse conditions to ensure soil reached near its full holding capacity. Larger plants transpired a greater amount of water necessitating the increase in irrigation as plants matured.

2.6. Extrafloral nectar collection

Sunflower EFN was collected on 20, 26, 29, 33, 36, 42, 47, 50, 54, 62, and 75 days after planting; plant stages ranged from the four-leafed vegetative growth stage (V4) until the completion of anthesis (R6). Dissecting needles were used to scrape partially or completely dried nectar from plant extrafloral nectaries. Each droplet was dried under a current of air to constant weight on a glass slide, and the quantity was weighed to the nearest 0.00001 g. The dried nectar was then placed into 1.5 mL microcentrifuge tubes, which were then stored at −20 °C until insecticide analysis.

2.7. ELISA of nectar for insecticide

Competitive ELISAs were used to quantify insecticide in nectar. Dried nectar was resuspended using laboratory grade water to a 20% (g/mL) sucrose solution, a ratio used by researchers in other feeding trials examining nectar (Singaravelan et al., 2005; Tadmor-Melamed et al., 2004; Lundgren, 2009). ELISA is a colorimetric assay that saturates unless observed value fall below a certain detection threshold; based on preliminary assays, the nectar solutions were diluted to 5% solution using laboratory grade water to ensure that resulting data fell within the discernible range of the ELISA analysis. Two kits, one sensitive to thiamethoxam (Thiamethoxam HS plate kit, lot no. 1113F; Beacon Analytical Systems Inc., Saco, ME) and one to clothianidin (Product No. 500800, Abraxis LLC®, 54 Steamwhistle Drive, Warminster, PA, USA, 18974) (clothianidin is an insecticidal metabolite of thiamethoxam), were used to quantify toxins in nectars. Standard curves for the two insecticides were created using the same sucrose dilution as mentioned above, except purified sucrose (Sucrose, Product code: 1001640636, Sigma-Aldrich, St. Louis, MO) was used instead of dried nectar. Standard curves for the two ELISAs were generated with thiamethoxam (Product number 37924, Sigma-Aldrich) and clothianidin (Product number 33589, Sigma-Aldrich) respectively, at concentrations of 2.0, 1.0, 0.5, 0.25, 0.13, 0.06, 0.03 and 0.0 ppb. Manufacturer instructions were followed when performing the assays.

2.8. Statistics

Treatment comparisons were all performed with the statistical software SYSTAT® 13 (Systat Software, Inc., San Jose, CA). Linear mixed models were used to determine how site-year, treatment, and plant growth stage affected predator and pollinator populations per plant and per m^2 soil (log transformed). The same approach was used to determine the effects of treatment and site-year on seasonal Coccinellidae adult abundance. All factors were fixed in the initial model; randomizing site-year and growth stage did not affect the outcome of the model, so they were included as fixed factors. In the case of pollinators, plant growth stage included only the reproductive stages when pollen was present (R4-R5.9), which were pooled and not included as a factor in the analysis.

One-way ANOVAs were used to compare fecundity, and number of developing eggs and size of oocytes from lady beetles fed thiamethoxam and clothianidin or untreated nectar. A Mantel non-parametric survival analysis was used to determine if beetles survived equally well among the different toxin doses. Rm-ANOVAs were used to compare flip times between treatments across sampling dates. Linear regressions were used to examine the relationships between nectar toxin concentration and the number of developing eggs in the ovaries. Significance level $\alpha = 0.05$ was set for all statistical tests.

3. Results

3.1. Field collected arthropods

A complete listing of species taxa collected in this study can be found in Bredeson and Lundgren (2015a).

3.1.1. Predators on sunflower foliage

A diverse arthropod community was collected from South Dakota sunflowers. Linear mixed models with all factors fixed revealed significant effects of treatment ($F_{1, 146} = 7.86$, $P < .01$), site-year ($F_{2, 146} = 10.65$, $P < .01$), and growth stage of the sunflower ($F_{12, 146} = 40.06$, $P < .01$) on foliar predators. The significant effects of treatment on predator densities did not vary when the site-year and growth stage were made random effects. Thiamethoxam treated sunflowers had significantly fewer predators than the untreated fields. Total populations were reduced by approximately 19%, from 4.16 ± 0.63 predators

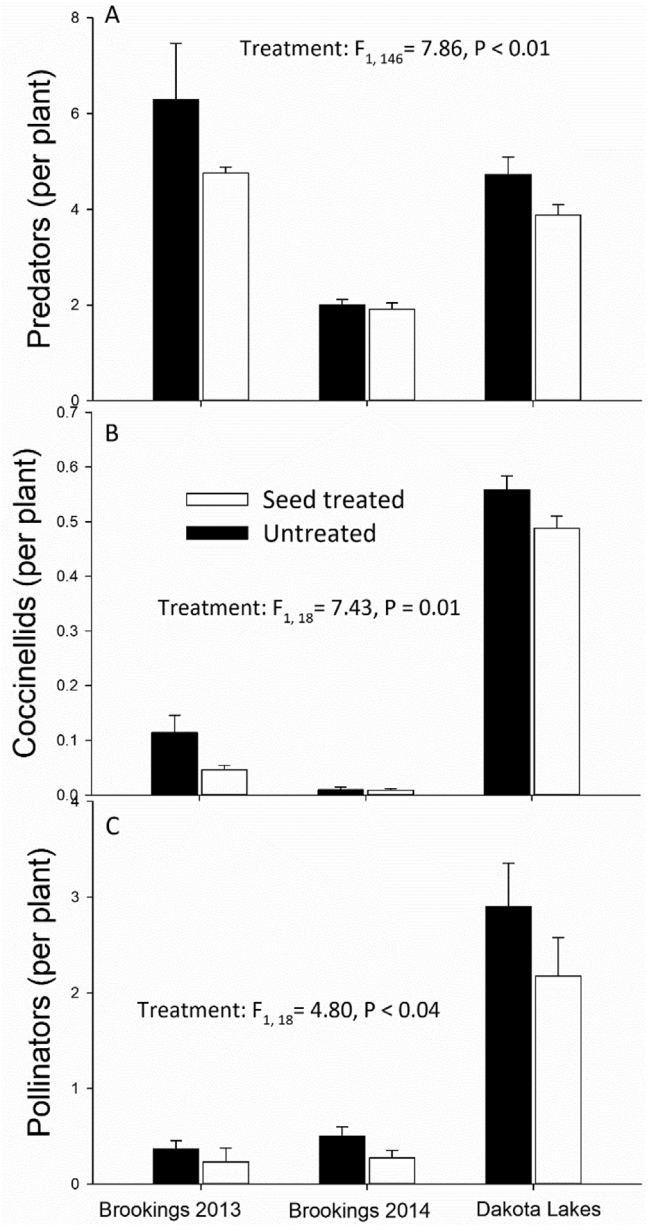

Fig. 1. Effects of thiamethoxam seed treatments on beneficial insect communities in sunflowers of eastern South Dakota; A) total predator populations, B) Coccinellidae populations, C) pollinator populations. Values presented are mean ± SEM insects per plant (seasonal means), and sample sizes are presented above the bars. Pollinator values were recorded during anthesis anthesis (R4-5.9 plant stage) in sunflower fields, and sample sizes are indicated above each set of bars.

per plant on untreated sunflowers to 3.40 ± 0.38 in fields treated with a thiamethoxam seed treatment (Fig. 1; for presentation purposes, these values represent seasonal means pooled across sample dates and site-years). During the pre-reproductive period of the plant, predatory insects per plot were pooled across plant growth stages and compared to the maximum average thiamethoxam concentration recorded for a particular plot, there was a negative correlation found between predator populations and thiamethoxam concentrations ($F_{1, 9}$ = 5.33, P = .046; r^2 = 0.37; thiamethoxam = 12.63–0.35(predators)). Interactions between treatment and plant stage were observed at the Brookings 2013 and Dakota Lakes locations ($F_{6, 24}$ = 2.59, P = .05, and $F_{5, 30}$ = 4.93, P < .01, respectively), due to predator populations being significantly reduced during only a portion of the plant stages sampled. The significant reduction in total foliar predators was not due to substantial reductions in a few dominant predators, but rather was a result of accumulated non-significant decreases across several predatory taxa. Of all predatory taxa examined, 64.14% of species had non-significantly lower abundances in treated sunflower fields compared to untreated controls. The only taxon that was significantly reduced in treated fields across site-years was Coccinellidae (Treatment: $F_{1, 18}$ = 7.43, P = .01, Site: $F_{2, 18}$ = 407.56, P < .001) (Fig. 1).

3.1.2. Predators below the ground

The subterranean predatory arthropod community was unaffected by the thiamethoxam seed treatment (Treatment: $F_{1, 133}$ = 1.09, P = .30, Site: $F_{2, 133}$ = 0.03, P = .97, Plant growth stage: $F_{11, 133}$ = 1.48, P = .15). Predators per m^2 in untreated fields were 202.94 ± 29.15 and in fields with seed treatments were 252.96 ± 51.85.

3.1.3. Pollinators

During sunflower anthesis (plant stages included were between R4 and R5.9), there were 24% fewer pollinators in treated fields compared to untreated fields (Treatment: $F_{1, 18}$ = 4.80, P = .04, Site: $F_{2, 18}$ = 53.69, P < .001). Untreated sunflowers were visited by 0.78 ± 0.25 (mean ± SEM) pollinators per plant at the moment of sampling during this phenological stage, while pollinators in the treated sunflowers were only 0.60 ± 0.19 (Fig. 1). Insect families pooled into the functional guild 'pollinators' were from the families Apidae, Andrenidae, Colletidae and Halictidae. Honey bees (*Apis mellifera*) were uncommonly collected and did not differ significantly between treatments (Treatment: $F_{1, 16}$ = 3.02, P = .10, Site: $F_{2, 16}$ = 16.03, P < .01, Interaction: $F_{2, 16}$ = 1.24, P = .32).

3.2. Nectar feeding trial

There were no significant differences in survival among the different thiamethoxam doses (Mantel χ^2 = 11.59, *P* = .12), or for those fed different clothianidin doses (Mantel χ^2 = 11.43, *P* = .12). All lady beetles survived in the control groups, and mortality per treatment was 10.47 ± 3.52% and 14.28 ± 3.96% for clothianidin and thiamethoxam assays, respectively.

3.2.1. Flip times

Average time for lady beetles to right themselves increased significantly as the dose of thiamethoxam increased ($F_{1, 114}$ = 4.85, P = .03; r^2 = 0.04) (Fig. 2), but clothianidin had no effect on the beetle's ability to right themselves ($F_{1, 116}$ = 0.03, P = .86).

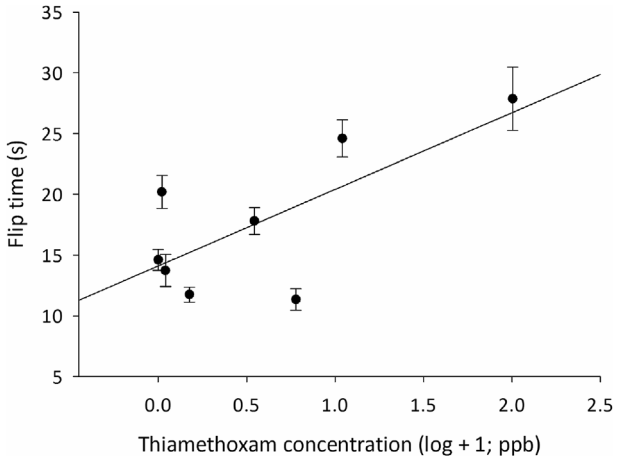

Fig. 2. Effect of thiamethoxam-laced artificial diet on the ability of *Coleomegilla maculata* (De Geer) to right themselves after being flipped. Data points represent mean ± SEM flip-over times for each group of beetles consuming an artificial diet with a particular concentration of thiamethoxam (100.0, 10.0, 5.0, 2.5, 1.0, 0.5, 0.1 or 0 ppb).

3.2.2. Lady beetle egg development

Insecticide concentration had no significant effect on the number of eggs laid in either the thiamethoxam or clothianidin-fed beetles ($F_{7, 112} = 1.55$, $P = .16$ and $F_{7, 112} = 1.34$, $P = .24$, respectively). Of the 120 beetles in the thiamethoxam-fed experiment a total of 233 eggs was oviposited, while in the clothianidin-fed treatment, 118 eggs were laid during the 34 day experimental duration. Number of developing eggs within ovaries varied between treatments in thiamethoxam-fed beetles ($F_{7, 97} = 2.08$, $P = .05$), with the highest concentration treatment (100 ppb) yielding the fewest number of developing eggs (using Duncan's post hoc comparison). There was a significant negative correlation between increasing thiamethoxam concentration and the number of developing eggs ($F_{1, 103} = 6.92$, $P = .01$). This pattern did not translate to clothianidin-fed beetles where there were no treatment differences in the number of developing eggs ($F_{7, 96} = 0.46$, $P = .86$), and no correlation between toxin concentration and number of developing eggs within ovaries ($F_{1, 102} = 0.79$, $P = .38$). Volumes of the largest developing eggs did not vary between treatments in either thiamethoxam or clothianidin-fed beetles ($F_{7, 97} = 1.76$, $P = .10$ and $F_{7, 96} = 1.58$, $P = .15$, respectively), and there was no correlation between insecticide concentration and egg size (thiamethoxam: $F_{1, 103} = 2.80$, $P = .10$, clothianidin: $F_{1, 102} = 1.07$, $P = .30$).

3.3. Neonicotinoids in extra-floral nectar

Thiamethoxam was detected in samples from all 11 nectar collection dates. Concentrations ranged from a minimum of 1.23 ± 0.09 ppb, to a maximum of 4.83 ± 0.63 ppb in nectar resuspended to a consistent solution (20% sucrose by weight). There was no trend of high insecticide concentration in plant tissues immediately post-planting then gradually tapering off throughout the growing season (as was observed in field-grown sunflower leaf tissues by Bredeson and Lundgren, 2015b). Insecticide concentration in extra-floral nectar collected from greenhouse-grown plants in this study did not follow a discernable pattern throughout plant development (Fig. 3). Clothianidin was not detected in sunflower EFN on any sampling date.

4. Discussion

Although previous studies have observed individual species being harmed by a neonicotinoid seed treatment in sunflowers (Gontijo et al., 2014, 2015; Moscardini et al., 2015), the current study is the first of its kind to describe a reduction in overall predator community in sunflower fields treated with a neonicotinoid seed-treatment (Fig. 1). Fewer predators within treated fields in this study were not driven by large reductions in a few dominant predator taxa (total Coccinellidae was the only taxon significantly reduced across site-years), but rather from the numerous aggregated small, non-statistically significant reductions, combined. Results presented here provide evidence that neonicotinoid seed treatments, designed to limit contact with non-target organisms (Jeschke and Nauen, 2008), might adversely affect beneficial insect communities in agroecosystems; the low-level reductions in predator communities we observed are consistent with many other studies on neonicotinoids and predators (Douglas and Tooker, 2016).

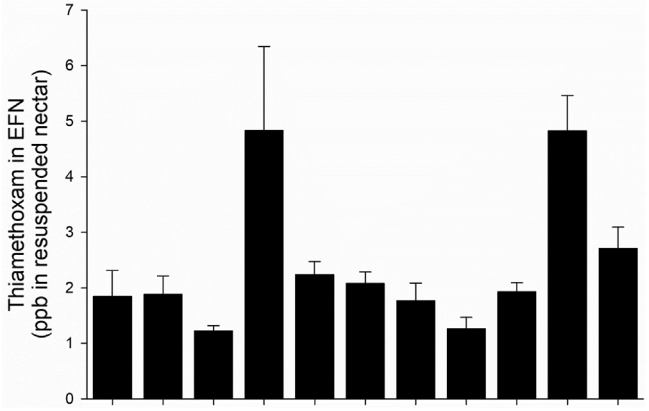

Fig. 3. Thiamethoxam residues in the extra-floral nectar of seed-treated sunflowers grown in the greenhouse. Bars represent mean ± SEM ppb thiamethoxam in resuspended (2:10, dried nectar: water) sunflower extra-floral nectar collected off of seed-treated plants growing in the greenhouse.

Suppression of predatory insects observed in the present study has implications for pest management and arthropod community stability. Natural enemy communities have the ability to regulate and prevent pest insect outbreaks in agroecosystems (Landis et al., 2000; Naranjo et al., 2015), and this is based largely on the diversity and evenness of these communities (Klapwijk et al., 2016; Lundgren and Fergen, 2014; Lundgren and Fausti, 2015). Non-target effects of systemic insecticides in these agroecosystems might disrupt predator communities, resulting in cropland having reduced biotic resistance to pest outbreaks (Douglas et al., 2015). Reduced natural control of herbivores often segues to agricultural land managers' increased dependence on insecticides in hopes of preventing crop damage, a phenomenon termed the "insecticide treadmill" (Pimentel, 1995; Wilson and Tisdell, 2001). In sunflowers, we found that neonicotinoids had no effect on pest populations, although we found few pests in our experimental fields (Bredeson and Lundgren, 2015b). We found a similar lack of pest populations in corn and soybean fields (Seagraves and Lundgren, 2012; Lundgren et al., 2015). Pest responses to the treatments in sunflower may differ when pest populations are high; data supports the contention that insect abundance and diversity (and particularly predators) are important in maintaining these sub-economic pest populations (Lundgren and Fergen, 2014; Lundgren and Fausti, 2015).

In this study, Coccinellidae (lady beetle) populations within thiamethoxam-treated sunflower fields were significantly reduced (Fig. 1). Additionally, the number of developing eggs within lady beetle ovaries was negatively correlated with increasing thiamethoxam concentration in an artificial diet. Planting sunflowers in the spring is frequently followed by a period of low prey abundance (Bredeson and Lundgren, 2015b). Correspondingly, this portion of the growing season is when systemic insecticide concentrations in treated plant-tissues are at their highest (Bredeson and Lundgren, 2015b). During this period of low prey availability, and high toxin concentrations, lady beetles consume non-prey foods such as vegetative tissue and EFN to subsidize their diets (Choate and Lundgren, 2012; Moser and Obrycki, 2009; Lundgren, 2009). Neonicotinoids can be present in all of these plant materials in a seed-treated sunflower [vegetative tissue (Bredeson and Lundgren, 2015b) and EFN (Fig. 3)], and each of these tissues may be a route of exposure for lady beetles to these toxins. In a study conducted by Smith and Krischik (1999), adult *C. maculata* were confined to inflorescences of sunflowers treated systemically with imidacloprid. After 7 d of exposure, beetles on treated plants had significantly higher mortality, slower walking speeds, took longer to right themselves when flipped, and produced fewer offspring than beetles exposed to untreated sunflowers. In addition to being affected by toxic non-prey food items, lady beetle fitness can also be adversely affected by systemic neonicotinoids tri-trophically through consumption of insecticide-fed prey (Bredeson et al., 2015). It is perhaps due to consuming toxic prey or non-prey food that resulted in a reduction of Coccinellidae populations within sunflower fields treated with a systemic insecticide.

To our knowledge, this is the first occurrence that a neonicotinoid has been quantified in EFN. Though treated plants were grown in a greenhouse setting, translocation of thiamethoxam into EFN is confirmed. We detected 1–5 ppb of thiamethoxam in the EFN of sunflowers in the greenhouse; this quantity might be expected given the range of nectar contamination reported in other studies (Botias et al., 2015; Mogren and Lundgren, 2016). But these quantities are substantially lower compared to floral nectar in milkweed flowers grown in a

greenhouse (Cowles and Eitzer, 2017), and in guttation fluids of seed-treated maize from the field (Girolami et al., 2009). Greenhouse conditions provided a consistent and dependable environment for nectar collection, whereas variations in temperature, humidity, wind and other abiotic factors makes field-collecting challenging. A pattern of high toxin-concentration in plant tissues early in a growing season, then gradually tapering, was not observed in this study. This same lack of reduction in the quantities of seed-applied imidacloprid was shown by Bonmatin et al. (2003). One reason for this might be that seed-treated plants were grown in confined pots. In field-conditions, the percentage of neonicotinoid insecticide seed dressing that is taken up by the plant systemically ranges from 2 to 20% (Sur and Stork, 2003); the remaining 80–98% is either metabolized in soil (Sarkar et al., 2001) or is unavailable for uptake due to the highly water-soluble chemical being transported away from a treated plant's zone of uptake by roots (Main et al., 2014; Schaafsma et al., 2016). Greenhouse-grown treated plants in containers were likely able to internalize a greater percentage of toxins and for a longer period of time since insecticide leaching may have been reduced in the pots compared to field conditions. A surprising result is that the thiamethoxam was not metabolized into clothianidin in this study. Our work shows that thiamethoxam is readily converted into clothianidin in other sunflower tissues, which has been reported similarly in other plant species as well (Nauen et al 2003; Bredeson and Lundgren, 2015b). Whatever enzymatic pathways that support this conversion is apparently absent in the tissues and organs that give rise to the EFN of sunflower, which may be of interest as future studies examine the environmental fate of neonicotinoid seed treatments.

Discovery of neonicotinoids in EFN provides a possible explanation for adverse fitness effects previously observed in natural enemies exposed to systemically-treated sunflower EFN [described in the introduction: Gontijo et al. (2014) and Moscardini et al. (2015)]. In this study, we did not find that neonicotinoid exposure resulted in increased mortality in *C. maculata* adults; however the mobility and reproductive fitness of the thiamethoxam-exposed lady beetles were reduced. These sublethal effects on this native lady beetle species may be one explanation for the lower abundance of lady beetles in seed-treated sunflowers. Extra-floral nectaries are attractive to beneficial insects and predators frequently search them out as a food source (Choate and Lundgren, 2012; Kost and Hein, 2008; Lundgren, 2009). Attraction of predatory insects to a food source tainted with an insecticide could have wide-reaching consequences (Stapel et al., 2000), depending on the relative lethal and sublethal toxicities and context of the insecticide in question. Plants offering energy-rich nectar as a reward for protection is a trait that has coevolved between plants and natural enemies (Grasso et al., 2015), and is likely an important component in supporting beneficial insect communities within an agroecosystem (Choate and Lundgren, 2012; Gillespie et al., 2016; Landis et al., 2005; Lundgren, 2009). Presence of thiamethoxam in EFN is a possible cause for why fewer predatory insects were found in treated fields compared to untreated controls (Fig. 1). For this to be the case, the reductions in abundance would have to be linked to sublethal or behavioral effects of the toxin on lady beetle population rate of increase, since we did not observe direct mortality to *C. maculata* in our laboratory assay. To confirm this hypothesis, toxins in EFN should be quantified under field conditions. The linkage between consumption of toxic EFN and low predator fitness (reproductive and mobility) might lead to economically important pests being left unchecked by biological antagonists.

Numerous studies have measured the effects of neonicotinoids on native (Pecenka and Lundgren, 2015; Rundlof et al., 2015; Whitehorn et al., 2012) and non-native pollinating insects (Botias et al., 2015; Cresswell, 2010; Mogren and Lundgren, 2016; Van der Sluijs et al., 2013), and seed treatments are regarded as a contributing factor to colony collapse disorder seen in honey bee hives (Douglas and Tooker, 2016; Maus et al., 2003; Suchail et al., 2000). As mentioned above, pollinator food resources such as nectar and pollen from sunflower plants treated with a neonicotinoid seed treatment can contain measureable amounts of insecticide (Laurent and Rathahao, 2003). Previously described evidence of bee toxic forage, in addition to reduced in-field pollinator densities observed here (Fig. 1), may indicate that the behavior of these pollinators were affected by the seed treatment, or possibly that the seed treatment reduced the survival of the bees. Previous work has shown that bees do not distinguish between treated and untreated nectar sources, or they may even prefer feeding on neonicotinoid-contaminated nectar (Kessler et al., 2015). If neonicotinoid-susceptible bees were more attracted to seed-treated sunflowers, then this could feasibly produce the pattern in the results that we observed here. Despite reductions in pollinators, we did not see a significant reduction in sunflower yields (although at Dakota Lakes, yields were marginally lower in the treated fields) (Bredeson and Lundgren, 2015b).

Neonicotinoid insecticides are widely applied as seed treatments to the landscape (Jeschke et al., 2011), and these insecticides are implicated in negatively affecting biological communities (Cycoń et al., 2013; Douglas et al., 2015; Mason et al., 2013). Persistence and insecticidal fate assays demonstrate that organisms encounter biologically relevant amounts of neonicotinoids not just in the fields where the pesticide is applied, but also in habitats polluted by agrochemicals through various forms of runoff and drift (Krupke et al., 2012; Main et al., 2014; Mason et al., 2013; Pecenka and Lundgren, 2015; Xue et al., 2015). Multiple routes of exposure, and observed negative effects of neonicotinoid seed-treatments on non-targeted organisms highlights a need for further study of this class of insecticides and method of application. Taken together with Bredeson and Lundgren (2015a&b), our research program not only challenges the agronomic benefit of neonicotinoid seed treatments in sunflowers, but also suggests that this technology may be hurting sunflower producers by reducing beneficial species.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cropro.2017.12.019.

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