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Research

Potential Impacts of Translocation of Neonicotinoid Insecticides to Cotton (*Gossypium hirsutum* (Malvales: Malvaceae)) Extrafloral Nectar on Parasitoids

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Abstract

Neonicotinoid seed treatments are frequently used in cotton (*Gossypium hirsutum* L. [Malvales: Malvaceae]) production to provide protection against early-season herbivory. However, there is little known about how these applications affect extrafloral nectar (EFN), an important food resource for arthropod natural enemies. Using enzyme-linked immunosorbent assays, we found that neonicotinoids were translocated to the EFN of clothianidin- and imidacloprid-treated, greenhouse-grown cotton plants at concentrations of 77.3 ± 17.3 and 122.6 ± 11.5 ppb, respectively. We did not find differences in the quantity of EFN produced by neonicotinoid-treated cotton plants compared to untreated controls, either constitutively or after mechanical damage. Metabolomic analysis of sugars and amino acids from treated and untreated plants did not detect differences in overall composition of EFN. In bioassays, female *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) parasitoid wasps that fed on EFN from untreated, clothianidin-treated, or imidacloprid-treated plants demonstrated no difference in mortality or parasitization success. We also conducted acute toxicity assays for *C. marginiventris* fed on honey spiked with clothianidin and imidacloprid and established LC_{50} values for male and female wasps. Although LC_{50} values were substantially higher than neonicotinoid concentrations detected in EFN, caution should be used when translating these results to the field where other stressors could alter the effects of neonicotinoids. Moreover, there are a wide range of possible sublethal impacts of neonicotinoids, none of which were explored here. Our results suggest that EFN is a potential route of exposure of neonicotinoids to beneficial insects and that further field-based studies are warranted.

Key words: seed treatment, parasitoid, natural enemy, biological control

Use of insecticidal seed treatments in production of field crops, such as corn (*Zea mays*), soybean (*Glycine max*), and cotton (*Gossypium hirsutum*), is widespread in the United States and worldwide (Jeschke et al. 2011, Stewart and Baute 2013, Douglas and Tooker 2015). Seeds are commonly coated with neonicotinoid insecticides, including thiamethoxam, clothianidin, and imidacloprid. Although these systemic chemicals can provide protection from herbivorous insect pests during early stages of plant growth (Jeschke et al. 2011, Goulson 2013), it has been suggested that the prophylactic use of neonicotinoid seed treatments (NSTs) violates principles of integrated pest management (IPM) because insecticides are used indiscriminately without regard for pest pressure (Tooker et al. 2017). Although overall insecticide application rates across the United States have decreased, recent analyses show that total land area receiving insecticides and the potency of insecticides have increased, leading to a greater risk of toxicity for beneficial insects (DiBartolomeis et al. 2019, Douglas et al. 2019). These trends are primarily due to an increase in adoption of NSTs (Douglas et al. 2019). Despite being applied to seeds, neonicotinoids can reach beneficial insects via a variety of routes, including soil (Zaller et al. 2016, Atwood et al. 2018), insecticidal dust associated with planting of coated seeds (Nuyttens et al. 2013, Krupke et al. 2017), translocation to guttation droplets (Girolami et al. 2009) and floral resources (Krupke et al. 2012, Botías et al. 2015), and by feeding on tainted prey (Douglas et al. 2015) or honeydew excreted by hemipterans (Calvo-Agudo et al. 2019).

Exposure to neonicotinoid residues can have both lethal and sublethal effects on non-target beneficial arthropods, including parasitoids and predators of herbivorous insects (Hopwood et al. 2013, Douglas and Tooker 2016). Sublethal doses of neonicotinoids can affect insect movement and orientation (Baines et al. 2017, Tappert et al. 2017), foraging behavior (Schneider et al. 2012), communication (Tappert et al. 2017), learning (Tan et al. 2015, Piiroinen and

Goulson 2016), immunity (Di Prisco et al. 2013, Brandt et al. 2017), and reproduction (Whitehorn et al. 2015, Straub et al. 2016). Such negative impacts could affect the role natural enemies play in biological control of plant pests and as components of IPM programs (De Bach and Rosen 1991, Orr 2009).

Extrafloral nectar (EFN) is another potential source of neonicotinoid exposure for beneficial insects (Stapel et al. 2000, Moscardini et al. 2014, Bredeson and Lundgren 2018). This sugary substance is secreted from nectaries located on non-floral parts of many plant species, including crops such as castor (*Ricinus communis*), bean (*Phaseolus vulgaris*), lima bean (*Phaseolus lunatus*), and cotton. In contrast to floral nectar that serves to attract pollinators, the primary function of EFN is thought to be a reward for predators and parasitoids (Röse et al. 2006, Lundgren 2009) that can deliver top–down control of herbivore pests (Heil 2008), possibly improving plant fitness (Cuautle and Rico-Gray 2003; Kost and Heil 2005, 2008). Although concern over pollinator declines has generated a multitude of studies on neonicotinoid translocation into floral nectar (Pisa et al. 2017), less attention has been given to the effects of neonicotinoid use on EFN (but see Stapel et al. 2000, Moscardini et al. 2014, Bredeson and Lundgren 2018). Recently, it has been shown that neonicotinoid active ingredients can be translocated to EFN from seed dressings (Bredeson and Lundgren 2018), indicating that EFN could be a route of exposure to natural enemies feeding on this resource.

Damage to plants from herbivory or mechanical wounding can lead to greater production of EFN (Heil 2015) and enhanced attraction of natural enemies (Ness 2003). EFN production is thought to be primarily regulated by the plant hormone jasmonic acid (JA; Heil et al. 2001, Schmitt et al. 2018). On the other hand, neonicotinoids have been shown to upregulate the salicylic acid (SA) pathway in some plants (Ford et al. 2010). Because the JA and SA signaling pathways often exhibit negative cross-talk (Thaler et al. 2012), application of neonicotinoids could affect plant regulation of EFN and alter the quality or quantity of this resource for natural enemies.

NSTs are commonly used in cotton-growing operations in the United States. Between 2010 and 2013, approximately 52–77% of cotton produced in the United States was grown from neonicotinoid-treated seeds (Douglas and Tooker 2015). Although seed treatments can protect cotton yield (North et al. 2017), there is concern about non-target effects (Tooker et al. 2017, Hladik et al. 2018) and plant pests developing resistance to neonicotinoids (Herron and Wilson 2011, Huseth et al. 2018). Parasitoids and predators including wasps, flies, ants, coccinellids, and spiders can be found in cotton fields and can provide biological control of plant pests (Wu and Guo 2004, Luo et al. 2014, Ali et al. 2016). NSTs can reduce the abundance of natural enemies associated with cotton, particularly when applied at higher than the recommended dose (Saeed et al. 2016), although the contribution of EFN to such declines has not been addressed in cotton.

In this study, we examined the hypotheses that NSTs alter the quantity or composition of EFN in cotton and negatively influenced natural enemies that consume EFN. Using cotton grown from seeds treated with either clothianidin or imidacloprid, we measured the amount of EFN produced constitutively and in response to mechanical damage. We also used untargeted metabolomic analysis of sugars and amino acids to determine the composition of cotton EFN grown from treated and untreated seeds. We ran enzyme-linked immunosorbent assays (ELISAs) to determine the concentration of neonicotinoid residues present in the EFN. To investigate potential impacts on natural enemies feeding on this resource, we used laboratory bioassays to examine lethal and sublethal responses of the parasitoid wasp *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) feeding on EFN. We also conducted acute oral toxicity assays with *C. marginiventris* to establish LC_{50} values for both clothianidin and imidacloprid.

Methods

Plants and Insects

Cotton (Malvales: Malvaceae) 'UA222' (Bourland and Jones 2012) seeds were left untreated or were treated with the neonicotinoids clothianidin or imidacloprid at a rate of 0.375 mg of active ingredient per seed. Treatments were provided by Bayer Crop Science (Durham, NC). No fungicides were applied to the seeds. We planted seeds in potting soil (Sunshine Mix4 Aggregate Plus, Sungrow Horticulture) and added 2.5 g of Osmocote (ICL Specialty Fertilizers, Dublin, OH) fertilizer when two true leaves were visible. We first grew plants in a temperature-controlled growth chamber (16:8 (L:D) h, 27°C, 50–60% humidity) to minimize damage by greenhouse pests. Three days prior to experiments, we relocated plants to a greenhouse at Pennsylvania State University (University Park, PA) with natural sunlight augmented with high-pressure sodium and metal halide lights (400 W).

For bioassays, we used the solitary endoparasitoid wasp *C. marginiventris*, a generalist parasitoid of noctuid larvae. This wasp species is often collected in cotton fields (Carpenter and Jewett 2003) and is known to feed on cotton EFN (Röse et al. 2006). The colony was established in 2016 with individuals obtained from Ted Turlings (University of Neuchâtel) and has since been maintained in our laboratory on fall armyworm (*Spodoptera frugiperda* (J. E. Smith); Lepidoptera: Noctuidae). To maintain the colony, we offered healthy second-instar fall armyworm larvae to mated female wasps for 3 h to allow parasitization. We reared parasitized caterpillars on artificial diet in a growth chamber (26 ± 1°C, 16:8 (L:D) h) until parasitoid larval egression and cocoon formation. After parasitoid cocoons formed and hardened, we transferred cocoons to glass test tubes closed with cotton wool. Upon adult eclosion, we transferred wasps to large plastic containers at a female: male ratio of 1:2. Wasps had ad libitum access to water and a 20% honey solution.

EFN Quantification

To characterize the influence of NSTs on production of EFN, we quantified the volume of EFN produced by undamaged and damaged plants grown from untreated seeds or those treated with clothianidin or imidacloprid. For these experiments, we used cotton plants that were approximately 24 d old with four fully expanded leaves. We used four to eight plants for each treatment combination and repeated the experiment on two separate dates. We washed extrafloral nectaries with Milli-Q (Millipore, Bedford, MA) water prior to the experiment to remove residual EFN that had accumulated. Using a custom tool that creates many small holes over a circular area (1 cm diameter), we produced the damage treatment by mechanically wounding leaves along the midvein of the second, third, and fourth leaves. For the undamaged treatment, we did not damage leaves. We excluded the first leaf of each plant in these experiments because in this variety of cotton the first leaf is highly variable in size and shape, with some leaves that are particularly small and have very large nectaries, which could skew the results. Despite some variation in the first leaf, the remaining leaves of all plants were similar in size and shape.

To prevent water stress, which can affect EFN secretion (Newman and Wagner 2013), we watered plants twice daily once they were moved to the greenhouse. To determine volume per nectary, we

collected and measured nectar for each individual leaf using 5-µl microcapillary tubes approximately 48 h after damage. We applied 1–2 µl of Milli-Q water to the nectary and collected it into the same microcapillary tube to obtain the total volume of EFN and water. Water was added for three reasons: to ensure that all sugars were collected from the nectary, to dilute the nectar so that it fell within the limits of the refractometer (0–50% Brix), and to increase the total volume of some samples up to ≥1 µl to meet the minimum volume requirements of the refractometer. We measured the Brix value of the nectar–water solution using an Eclipse 0–50% Brix low-volume refractometer (Bellingham & Stanley, Suwanee, GA) and adjusted this value based on ambient temperature of the greenhouse, according to manufacturer instructions. Using the total volume of the nectar–water solution and adjusted Brix value, we then calculated the total soluble sugars for each leaf.

EFN Metabolomics

To compare EFN metabolites between untreated and neonicotinoid-treated cotton plants, we obtained seven samples from each plant treatment for metabolomics analysis. For each sample, we pooled EFN from 4 to 6 plants over several days to meet the minimum volume requirement of 60 µl. On each individual plant, we damaged all fully expanded leaves along the midvein using the wounding tool described above; 48 h later we collected nectar using microcapillary tubes and froze immediately at –80°C. After collection, we wounded the leaf again and collected nectar 48 h later. From each plant within a batch, we collected EFN 2–4 times.

We expelled thawed nectar from the capillary tubes into Eppendorf tubes and weighed with a microbalance (Metter Toledo, Columbus, OH) to the nearest 0.001 mg. For each sample, we diluted the nectar 1:2 with HPLC-grade water, vortexed to mix, transferred 60 µl to a 2 ml Eppendorf tube, and refroze at –80°C. We sent frozen samples for analysis at the West Coast Metabolomics Center (University of California; Davis, CA). Metabolites included in the analysis were carbohydrates and sugar phosphates, amino acids, hydroxyl acids, free fatty acids, purines, pyrimidines, and aromatics.

Metabolite samples were analyzed following published methods (Fiehn et al. 2008). In short, metabolites were separated and identified using gas chromatography coupled with time-of-flight mass spectrometry (GC-TOF MS) with an Agilent 6890 gas chromatograph. The GC was equipped with an Rtx-5Sil MS column (30 m long × 0.25 mm internal diameter, 0.25 µm film, 95% dimethyl / 5% diphenylpolysiloxane; Restek Corp, Bellefonte, PA). Helium was used as the mobile phase at a flow rate of 1 ml per min. A 0.5 µl aliquot of each sample was injected into a multi-baffled glass liner using splitless injector mode with 25 s purge time. Injection temperature was 50°C ramped to 250°C at a rate of 12°C per second. The oven temperature program was 50°C for 1 min, then ramped at 20°C per min to 330°C, then held constant for 5 min. The GC was coupled to a Leco Pegasus IV mass spectrometer used with unit mass resolution at 17 spectra per second from 80 to 500 Da at –70 eV ionization energy and 1800 V detector voltage with a 230°C transfer line and a 250°C ion source.

Analytes were identified using retention index and mass spectral similarity. ChromaTOF version 2.32 was used for data pre-processing using previously described criteria (Fiehn et al. 2008). Absolute spectra intensities were exported and further processed using the BinBase algorithm (Fiehn et al. 2008). Quantification of analytes is reported as peak height for the quantification ion (mz value) at the specific retention index. Peak height is reported to be more precise than peak area for low-abundant metabolites due to the larger influence of baseline determinations on areas compared to heights. Furthermore, it is more difficult to deconvolute co-eluting ions on peak areas.

Neonicotinoid Residue Quantification in EFN Using ELISA

To quantify neonicotinoid residues present in EFN, we used ELISAs for imidacloprid and clothianidin (Abraxis, Warminster, PA). We damaged cotton plants on each leaf as previously described and after 48 h collected EFN using 5-µl microcapillary tubes. Each sample comprised the nectar pooled from all leaves on an individual plant. We collected 4–6 samples for untreated plants, and 10–12 samples for clothianidin- and imidacloprid-treated plants. We diluted neonicotinoid-treated samples 1:150 using the sample diluent provided in the kit, so that samples fell within the limits of detection of the assay (0.06 to 1.2 ng/ml); we diluted untreated samples 1:30, which was the minimum dilution to achieve the necessary volume for the test. We performed the ELISA according to manufacturer's instructions and calculated neonicotinoid concentrations via a standard curve. We then multiplied test values by the dilution factor to determine concentration in ng/ml (ppb). Due to variation in cross-reactivity between compounds, the ELISA recognizes clothianidin and imidacloprid at 121% and 100%, respectively. We adjusted values obtained for clothianidin to account for this cross-reactivity. The experiment was repeated twice with a separate set of plants. Using honey spiked with 0.5 ppb imidacloprid, we measured the ELISA kit recovery as 98.8%.

Parasitoid Bioassay

To examine the effects of feeding on neonicotinoid-treated EFN, we conducted a bioassay using parasitoid females. We transferred 40 newly eclosed parasitoids to individual 473 ml inverted plastic cups (Global Supply Store, Inc., Pomona, CA) and randomly assigned each wasp to one of five treatments: untreated EFN, clothianidin-treated EFN, imidacloprid-treated EFN, honey, or water (n = 8 per treatment). We conducted the bioassays in a growth chamber (same conditions as above).

We provided wasps with water and a 4 µl droplet of EFN. For positive and negative control treatments, we provided wasps with droplets of honey solution or distilled water, respectively. To ensure wasps could feed ad libitum, we replenished droplets of EFN, honey, or water every 3 d. We collected EFN from plants as described above and stored it at –20°C until a sufficient volume had been collected for the experiment. We diluted EFN and honey approximately 1:2 with distilled water to achieve Brix values of 32–33% as measured using a refractometer. We diluted the EFN because the high concentration of sugars in the EFN meant that evaporation of the droplets would rapidly lead to crystallization of the sugars and impede wasp feeding (Lange et al. 2017). Although dilution of EFN and subsequent evaporation would affect neonicotinoid concentration, EFN sugar concentration varies temporally with evaporation and stomatal closure (Lange et al. 2017), indicating that insects would likely be exposed to this variation in the field. We recorded mortality twice daily until all wasps had died. As a proxy for wasp size, we also measured tibia lengths using a stereomicroscope (Olympus SZX10, Tokyo, Japan) with the measuring tool included in cellSens Standard 1.6 software.

To examine potential sublethal effects of neonicotinoid-treated EFN, we measured parasitism success for the EFN and honey treatments (n = 8 wasp per treatment). Because wasps from the water treatment died after just 72 h (Fig. 3), they could not be included in this experiment. On the sixth day of the experiment, we introduced

one male wasp into each container for 24 h to allow mating. We then removed males and added 20 s instar fall armyworm for 3.5 h to allow females the opportunity to parasitize. We transferred fall armyworm larvae to individual cups with artificial diet and monitored until parasitoid egression, caterpillar pupation (indicating unsuccessful parasitization), or death. We also recorded parasitization rate, development time, cocoon weight (to the nearest 0.001 mg) and sex of the parasitoid offspring.

Acute Toxicity Assays

To determine acute oral toxicity of clothianidin and imidacloprid to *C. marginiventris*, we conducted feeding assays with spiked honey for both male and female wasps. We dissolved technical grade clothianidin and imidacloprid (Chem Service Inc, West Chester PA) in acetone to achieve a concentration of 10^6 ppb (1 mg/ml) and serially diluted these stock solutions in acetone to concentrations of 10^5 ppb, 10^4 ppb, and 10^3 ppb. To make spiked honey, we combined 9 parts honey solution (33.3%) and 1 part neonicotinoid solution to produce 30% honey solutions with the appropriate neonicotinoid concentration. We tested the following concentrations of each neonicotinoid: 10^2 ppb, 10^3 ppb, 10^4 ppb, and 10^5 ppb. A 9:1 honey to acetone solution served as a control. We stored all solutions at –20°C in the dark and prepared spiked honey freshly before use.

To assess toxicity of the neonicotinoid-spiked honey solution, we set up containers similar to those used for the bioassays described above with a water wick and a 5 µl droplet of spiked or control honey solution. We replenished droplets after 48 h. We transferred five newly eclosed male or female wasps into each container and recorded the time. At each concentration, we set up between 5 and 7 containers for males and females, giving a total of between 20 and 30 individuals per control dose, and 30–35 for most other doses. Based on the initial 30 wasps we tested, female wasps had a considerably higher tolerance for imidacloprid than males. Thus, for the two highest doses of 10^4 ppb and 10^5 ppb, we tested an additional 25 wasps, giving a total of 55 individuals for each of these doses.

We monitored wasps at 2, 4, 8, 12, 24, 48, 72, and 96 h to determine time of death. To confirm death, we gently prodded wasps with forceps and carefully observed them for movement of limbs or antennae.

Statistical Analyses

We analyzed EFN quantity as total soluble sugars per plant using a two-way ANOVA with seed treatment and damage as factors and experimental run as the blocking variable. We used SAS 9.4 (SAS Institute, Cary NC) to perform these analyses.

To analyze metabolomics data, we removed two compounds that were likely contaminants prior to further analysis: triethanolamine and phthalic acid. For the remaining compounds, we first normalized the data to reduce the effect of instrument sensitivity drift caused by machine maintenance, aging, and tuning parameters. The total average peak height (APH) sum of all identified (genuine) metabolites (APH_{total}) across treatments was used for normalization. To normalize each compound, we used the following formula for each metabolite i of sample j:

$$metabolite_{ij,\ normalized} = \left(\frac{metabolite_{ij,\ raw}}{APH_j}\right) * APH_{total}$$

We then scaled the normalized data using the Pareto method (van den Berg et al. 2006, Yang et al. 2015), and analyzed global multivariate differences in metabolite composition by seed treatment using ANOVA-simultaneous component analysis (ASCA) with 1000 permutations (Smilde et al. 2005) in R 3.4.3 (R Core Team 2017). We also conducted one-way ANOVA to test for differences in abundance of each individual metabolite between seed treatments using MetaboAnalyst 4.0 with Bonferroni corrections for multiple comparisons (Chong et al. 2018).

To determine differences in parasitization success between treatments, we used a one-way ANOVA after logit transformation to fulfill ANOVA assumptions, including normality and homogeneity of variance. We conducted this analysis in R 3.4.3 (R Core Team 2017).

To examine differences in longevity of female wasps feeding on different food sources, we used Kaplan–Meier analysis. We further analyzed the EFN and honey treatments using a Cox-Proportional Hazards model, including hind tibia length as a proxy for parasitoid size and quality (Visser 1994, Sagarra et al. 2001) as a covariate. We conducted survival analyses in R. 3.4.3 (R Core Team 2017) using the 'survival' and 'survminer' packages.

We calculated LC_{50} values for *C. marginiventris* wasps by probit analysis using the LC_probit function of the 'ecotox' package in R 3.4.3 (R Core Team 2017).

Results

Mechanically damaged cotton plants produced significantly more EFN than undamaged plants (F = 161.34; df = 2, 56; P < 0.0001). There was no effect of seed treatment (F = 0.65; df = 2, 56; P = 0.525) or its interaction with damage (F = 0.19; df = 2, 56; P = 0.831) on the amount of EFN produced by cotton plants (Fig. 1).

Untargeted GC-TOF-MS analysis of the EFN metabolome of cotton detected 196 compounds, of which 49 could be identified. Identified analytes included sugars, sugar alcohols, fatty acids, organic acids, and esters (Supp Material [online only]).

Multivariate ASCA analysis detected no differences in overall metabolite composition between EFN from different seed treatments (P = 0.158). These data were visualized using a principal components analysis (Fig. 2), although the first two principal components explained just 22.3 and 20.0% of the variation. We also found no

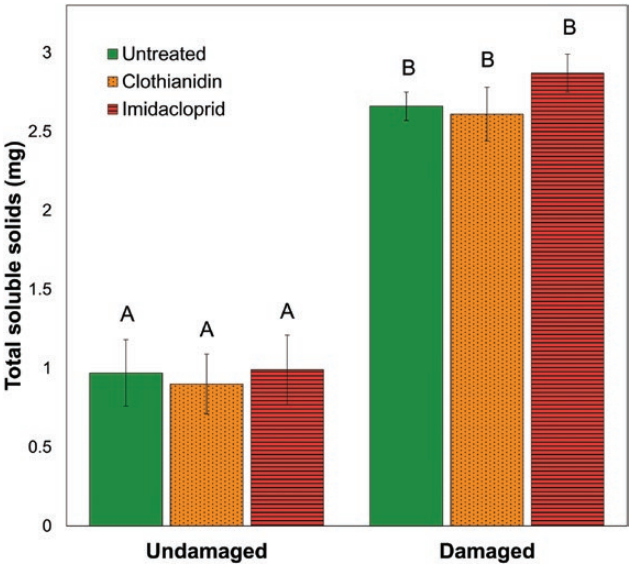

Fig. 1. Total extrafloral nectar produced by cotton plants grown from seeds that were untreated, treated with clothianidin, or treated with imidacloprid. Plants were left undamaged, or damaged mechanically. We quantified extrafloral nectar 48 h after damage. Bars represent means with standard errors. Letters above the bars denote significant differences at P < 0.05.

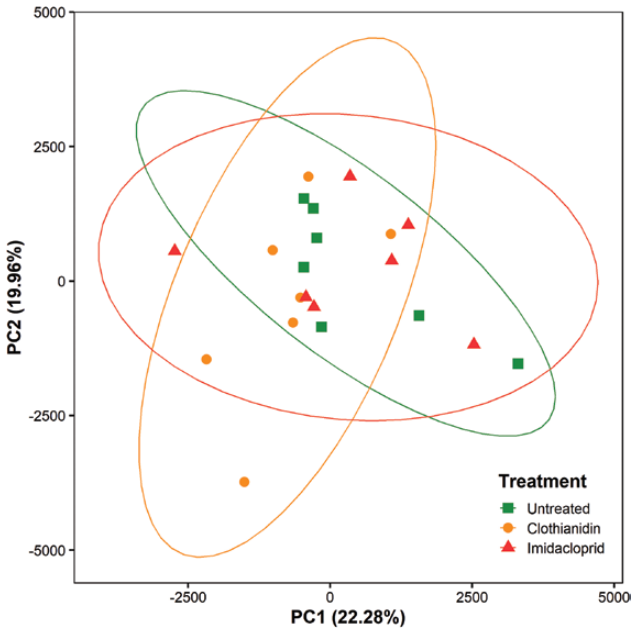

Fig. 2. Principal components analysis of metabolites detected from extrafloral nectar of cotton plants grown from seeds that were untreated, treated with clothianidin, or treated with imidacloprid. Multivariate analysis via ANOVA-simultaneous component analysis (ASCA) with 1,000 permutations indicated that there was no significant difference in metabolite composition between treatments ($P = 0.158$).

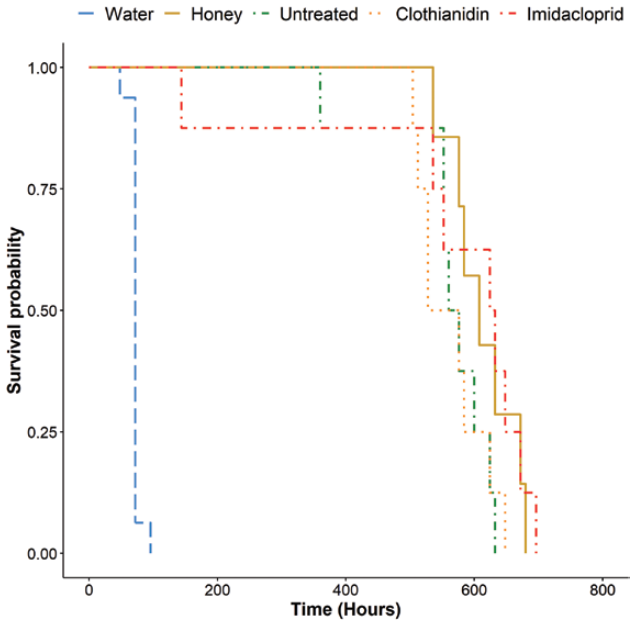

Fig. 3. Kaplan–Meier survival curve depicting longevity of *Cotesia marginiventris* female wasps provided with different food sources: water, honey, or extrafloral nectar collected from cotton plants grown from seeds that were untreated, treated with clothianidin, or treated with imidacloprid. Overall there was a significant difference in survival between treatment groups ($P < 0.001$); pairwise comparisions via log-rank test indicate that only the water treatment was different from other treatments ($P < 0.05$).

differences in the amount of any individual metabolite between seed treatments ($P > 0.05$; data not shown).

ELISA assays indicated that neonicotinoids were present in EFN of treated plants. In clothianidin- and imidacloprid-treated plants, the active ingredients were detected at concentrations of 77.3 ± 17.3 and 122.6 ± 11.5 ppb, respectively. In untreated plants, neonicotinoid concentrations were below the limits of detection for the test.

When analyzed using the Kaplan–Meier method, the longevity bioassay with *C. marginiventris* female wasps indicated that there was an overall significant difference in survival times between treatment groups that received different food types ($P < 0.001$; Fig. 3). Pairwise comparisons using a log-rank test revealed that only the water treatment was significantly different from the honey and EFN treatments ($P < 0.05$). Further analysis of the honey and EFN treatments using Cox-Proportional Hazards model, including hind tibia length as a covariate, found no difference in survival times between treatments ($P = 0.162$).

We found no difference in parasitization success between females fed honey or different EFN treatments (Table 1; $F = 0.99$; df = 3, 26; $P = 0.411$). The offspring of these females exhibited no difference in development time (Table 1; $F = 1.78$; df = 3, 26; $P = 0.179$) or cocoon weight (Table 1; $F = 1.75$; df = 3, 26; $P = 0.184$) between treatments. Across all treatments, only male offspring were produced.

For clothianidin, 48-h LC_{50} values for males and females were similar (8,267 and 7,827 ppb, respectively; Table 2; Fig. 4). For imidacloprid, the 48-h LC_{50} for males was 7,291 ppb, substantially lower than the 48-h LC_{50} of 49,800 ppb calculated for females. Non-significant ($P > 0.05$) χ^2 values for all models indicate that the data fitted well to the probit analysis model (Table 2). At 72 and 96 h, the LC_{50} decreased for both neonicotinoids in male and female wasps (Tables 3 and 4). However, significant χ^2 values for probit analysis of females receiving imidacloprid and males receiving clothianidin at 96 h indicate a poor fit of the data and confidence intervals were unable to be calculated for these two LC_{50} values (Table 4). Mortality in the control treatments was never above 4%.

Table 1. Performance of offspring from *Cotesia marginiventris* females fed on honey or extrafloral nectar (EFN) from untreated, clothianidin-treated, or imidacloprid-treated cotton plants

Treatment	% Larvae parasitized	Development time (d)	Cocoon weight (mg)
Honey	64.1 ± 13.3	12.42 ± 0.09	2.10 ± 0.02
Untreated EFN	81 ± 6.8	12.32 ± 0.08	2.14 ± 0.01
Clothianidin EFN	68.8 ± 7.3	12.55 ± 0.08	2.15 ± 0.01
Imidacloprid EFN	54.9 ± 15.3	12.30 ± 0.09	2.13 ± 0.02

Wasps developed in *Spodoptera frugiperda* larvae. Development time indicates the number of days from egg to adult eclosion.

Discussion

EFN is an important plant resource for predators, parasitoids, and other beneficial arthropods. Yet the widespread use of NSTs can alter this resource and potentially affect natural enemies. Exposure to neonicotinoids can have both lethal and sublethal effects on non-target insects (Müller 2018). The majority of published studies on risks of neonicotinoids have focused on bees (Decourtye and Devillers 2010), with impacts on natural enemies less well understood (Wood and Goulson 2017). In this study, we sought to address possible impacts of NSTs on natural enemies via EFN.

EFN can contribute to biological control of plants by attracting and sustaining natural enemies (Jones et al. 2017). Plants with reduced production of EFN are often associated with fewer natural enemies, leading to declines in plant fitness and fruit production (Mathews et al. 2007). Contrary to our hypothesis, we did not find

Table 2. Oral toxicity of clothianidin and imidacloprid (mg/liter) at 48 h to *Cotesia marginiventris* males and females calculated using probit analysis

		n	Slope + SE	48-h LC_{50} (95% CI)	χ^2	*P*-value
Clothianidin	Male	128	1.90 + 0.31	8.27 (5.25–12.88)	1.737	0.420
	Female	120	2.20 + 0.42	7.83 (5.00–12.20)	0.404	0.817
Imidacloprid	Male	131	1.98 + 0.33	7.29 (4.76–11.24)	0.718	0.698
	Female	160	3.00 + 0.45	49.8 (36.39–65.00)	< 0.001	1.000

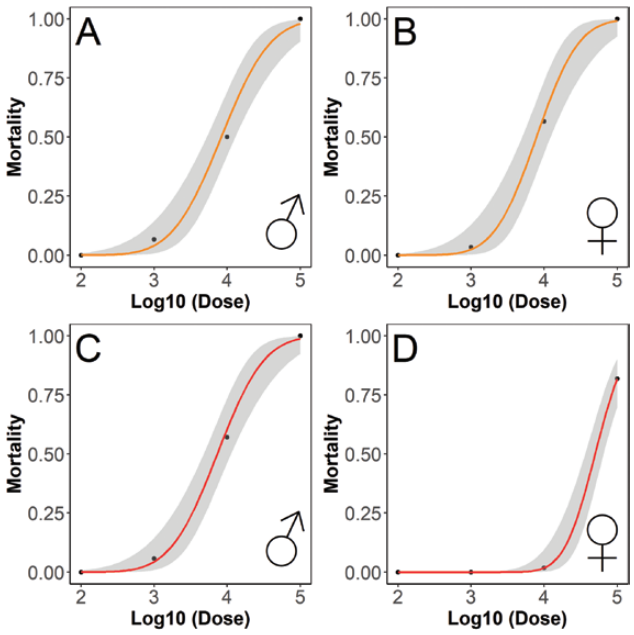

Fig. 4. Survivorship curves of *Cotesia marginiventris* wasps receiving neonicotinoids via oral feeding assays at 48 h: (A) males receiving clothianidin, (B) females receiving clothianidin, (C) males receiving imidacloprid, and (D) females receiving imidacloprid. For clothianidin, the LD_{50} was similar between males and females at 8.3 mg/liter (8300 ppb) and 7.8 mg/liter, respectively. For imidacloprid the LD_{50} values between males and females were disparate at 7.3 mg/liter (7300 ppb) and 49.8 mg/liter (49800 ppb) for males and females, respectively. Shaded areas on plots represent the 95% CI.

evidence that cotton plants treated with imidacloprid or clothianidin produced different amounts of EFN, either constitutively or in response to damage. Available evidence suggests that neonicotinoids can upregulate the SA pathway in several plant species (Ford et al. 2010, Szczepaniec et al. 2013), which can be antagonistic to JA-mediated defenses such as EFN (Heil et al. 2001, Schmitt et al. 2018). We did not measure SA and JA of cotton plants in this study, so it is unclear if the NSTs affected plant defense pathways. Although NSTs did not appear to suppress EFN production in cotton plants, we found that plants translocated these insecticides to the EFN, and thus treated plants produce similar amounts of a potentially toxic resource.

Metabolomics analysis of cotton EFN revealed that this resource predominantly comprised sugars, namely fructose, glucose, sucrose, 1-kestose and raffinose. Cotton EFN also had small amounts of fatty acids, sugar alcohols, and esters. Interestingly, our analysis detected only one amino acid, glycine. A previous study identified 24 amino acids from cotton EFN (Hanny and Elmore 1974). The high number of unidentified compounds in our study may reflect that untargeted metabolomics was insufficient to accurately identify all amino acids present in cotton EFN. Nevertheless, we did not find differences in overall metabolite composition or individual compounds between EFN collected from untreated and treated plants. This result mirrors our finding that NSTs did not affect the quantity of EFN produced by cotton nectaries and suggests that these seed treatments did not appreciably alter metabolic pathways regulating EFN production in cotton.

We detected neonicotinoids in EFN of cotton plants grown from seeds treated with two commonly applied neonicotinoids, clothianidin, and imidacloprid, at concentrations of 77 and 123 ppb, respectively. Neonicotinoids have been detected in floral nectar of plants applied with NSTs at concentrations ranging from <1 to 16 ppb (EFSA 2012, Rundlof et al. 2015). Our finding that neonicotinoids are translocated from seed treatments to EFN corroborates results from a recent study that found thiamethoxam occurred at concentrations of 1–5 ppb in EFN of greenhouse-grown sunflowers (Bredeson and Lundgren 2018). Neonicotinoids are highly water-soluble, and the discrepancy between studies could be due to a number of factors, including application rate, plant species, plant age (Alford and Krupke 2017), watering regime, and soil type. Future studies should examine neonicotinoid concentrations in EFN from field-grown cotton plants.

EFN is a food resource for many natural enemies, including ants, parasitoid wasps, lacewings, beetles, and spiders (Hespenheide 1985, Taylor and Foster 1996, Limburg and Rosenheim 2001, Taylor and Pfannenstiel 2008, Klein et al. 2016). Honey bees (*Apis mellifera*) have also been observed foraging at extrafloral nectaries (Koptur 1992, Cuautle and Rico-Gray 2003). Honey bees are often used as an indicator species for insecticide toxicity (Medrzycki et al. 2013), and have oral LD_{50} values of 0.0079 and 0.0037 µg/bee for clothianidin and imidacloprid, respectively (DiBartolomeis et al. 2019). On the basis of these values, bees would need to consume approximately 0.103 ml and 0.03 ml of imidacloprid- and clothianidin-containing EFN to experience 50% mortality. Honey bee foragers consume up to 321 mg (approximately 0.321 ml) of nectar per day (Rortais et al. 2005). These calculations suggest that bees using EFN as a significant food resource could ingest quantities of neonicotinoids that approach or exceed their oral LD_{50}. In contrast with floral nectar that is only produced by mature, flowering plants, cotton EFN is produced by all leaves including the cotyledons, and is available as early as 1 to 2 wk after planting (personal observation). Thus, in certain environments EFN from neonicotinoid-treated plants may represent a consistent and ongoing route of insecticide exposure to beneficial insects.

We conducted a bioassay with the parasitoid wasp *C. marginiventris* to explore effects to natural enemies from feeding on EFN from neonicotinoid-treated cotton plants. We did not find significant differences in mortality between females feeding on untreated and treated plants. Similarly, the lady beetle *Coleomegilla maculata* did not experience differences in mortality rates from feeding on artificial nectar spiked with thiamethoxam and clothianidin at concentrations up to 100 ppb (Bredeson and Lundgren 2018). Although *Microplitis croceipes* parasitoids displayed reduced longevity and

Table 3. Oral toxicity of clothianidin and imidacloprid (mg/liter) at 72 h to *Cotesia marginiventris* males and females calculated using probit analysis

		n	Slope + SE	72-h LC_{50} (95% CI)	χ^2	*P*-value
Clothianidin	Male	128	2.08 + 0.36	5.69 (3.67–8.70)	0.258	0.879
	Female	120	2.24 + 0.43	7.31 (4.68–11.33)	0.268	0.875
Imidacloprid	Male	131	2.44 + 0.41	4.48 (3.02–6.56)	0.018	0.991
	Female	160	3.15 + 0.45	46.2 (33.70–60.20)	< 0.001	1.000

Table 4. Oral toxicity of clothianidin and imidacloprid (mg/liter) at 96 h to *Cotesia marginiventris* males and females calculated using probit analysis

		n	Slope + SE	96-h LC_{50} (95% CI)	χ^2	*P*-value
Clothianidin	Male	128	1.79 + 0.29	3.53 (NaN[a])	12.67	0.002
	Female	120	2.41 + 0.47	5.95 (3.83–9.03)	0.059	0.971
Imidacloprid	Male	131	2.54 + 0.42	4.21 (2.85–6.11)	0.008	0.996
	Female	160	2.19 + 0.29	26.6 (NaN[a])	37.02	<0.001

[a]Confidence intervals were unable to be calculated due to a poor fit of the data to the probit model.

foraging ability after feeding on EFN from cotton plants sprayed with imidacloprid (Stapel et al. 2000), insecticide concentrations in the nectar were not measured, making it difficult to compare directly with our results. Honeydew, another sugary food resource, contaminated with thiamethoxam at concentrations of approximately 18 ppb, was highly toxic to the hoverfly pollinator *Sphaerophoria rueppellii* and moderately toxic to the parasitoid wasp *Anagyrus pseudococci* (Calvo-Agudo et al. 2019). Honeydew with imidacloprid concentrations of 15–68 ppb was moderately toxic to *S. rueppellii* but did not alter mortality of *A. psuedococci* (Calvo-Agudo et al. 2019). These findings suggest that the concentrations of neonicotinoids we found in cotton EFN could be toxic to some beneficial insect species.

To examine acute oral toxicity of clothianidin and imidacloprid to *C. marginiventris*, we conducted feeding assays with spiked honey. We determined 48 h LC_{50} concentrations for clothianidin as 8.3 mg/liter (8300 ppb) and 7.8 mg/liter (7,800 ppb) for males and females, respectively, and for imidacloprid as 7.3 mg/liter (7,300 ppb) and 49.8 mg/liter (49,800 ppb) for males and females, respectively. It is unclear why the LC_{50} value for females exposed to imidacloprid was considerably higher than for males, although sex-specific responses to neonicotinoids have been reported (Nielsen et al. 2008, Mobley and Gegear 2018). For clothianidin, the LC_{50} for males and females is approximately 100 times greater than the concentration we detected in EFN. For imidacloprid the LC_{50} is approximately 60 and 400 times greater than the concentration we measured in EFN for males and females, respectively. This suggests that *C. marginiventris* wasps are unlikely to experience direct mortality from feeding on neonicotinoid-treated cotton EFN and corroborates results from our bioassay that found no difference in mortality between treatments.

Other studies investigating acute toxicity from neonicotinoids in natural enemies have found highly variable results depending on the species and chemical tested. LC_{50} values for the parasitoid *Trichogramma confusum* after topical application of acetamiprid, imidacloprid, thiacloprid, and thiamethoxam were 93.21 mg/liter (93,210 ppb), 754.2 mg/liter (75,4200 ppb), 176.5 mg/liter (176,500 ppb), and 0.24 mg/liter (240 ppb), respectively (Wang et al. 2013). Likewise, LC_{50} values from contact exposure to acetamiprid for the four parasitoid wasps *Aphytis melinus*, *Gonatocerus ashmeadi*, *Eretmocerus eremicus*, and *Encarsia formosa* were 0.005 mg/liter (5 ppb), 0.134 mg/liter (134 ppb), 12.02 mg/liter (12,020 ppb), and 108.27 mg/liter (108,270 ppb), respectively (Prabhaker et al. 2007). However, because the chemicals in these studies were administered topically, they are not directly comparable to the oral feeding assays we presented in this study. Neonicotinoids are usually more toxic when ingested compared with contact exposure, likely due to low penetration of the cuticle (Decourtye and Devillers 2010).

Although lethal thresholds are informative, they do not give a complete picture of the impact of an insecticide on an insect species (Müller 2018). Insecticides can have a wide range of sublethal effects on insect behavior and physiology, although it can be difficult to assess the concentration at which these effects occur (Müller 2018). We examined whether exposure to field-realistic rates of neonicotinoid insecticides via EFN can influence female parasitization rate and development rate. Although we did not find significant differences between treatments, it is unknown whether higher concentrations would have altered parasitization and development rates. If neonicotinoid application rates on cotton seeds continue to increase as they have on maize seeds (Tooker et al. 2017), it is likely that field-realistic concentrations of neonicotinoids in EFN will rise, potentially having a greater chance of influencing parasitoid fitness. Parasitoids that ingested honey containing the LC_{10} (10 mg/liter; 10,000 ppb) and LC_{20} (20 mg/liter; 20,000 ppb) of imidacloprid experienced reduced parasitization rates (Liu et al. 2010), although these values are also about 100–200 times higher than neonicotinoid concentrations we detected in EFN.

We did not explore parasitoid preference for EFN from treated and untreated plants, but given that there was no difference in *C. marginiventris* mortality between EFN treatments, it seems unlikely that wasps would avoid neonicotinoid-containing nectar in a no-choice scenario. A previous study found that gustatory neurons of bumble bees and honey bees did not respond to stimulation by imidacloprid, thiamethoxam, and clothianidin, indicating that bees cannot taste neonicotinoids (Kessler et al. 2015). Moreover, bees did not avoid sugar resources containing these neonicotinoids, despite the negative effects of thiamethoxam and clothianidin on bee survival (Kessler et al. 2015).

Crops grown from neonicotinoid-treated seeds are ubiquitous throughout the United States, yet there is still much to learn about how these insecticides affect plants and beneficial insects in agricultural systems. We found that cotton plants translocated clothianidin and imidacloprid to EFN, indicating that insects consuming this

resource would be exposed to neonicotinoids. Although we did not detect differences in mortality in *C. marginiventris* females feeding on treated EFN in the laboratory, we recognize the limitation of testing just one parasitoid species from one lab colony. Toxicity of insecticides can vary between related species (Prabhaker et al. 2007, Wang et al. 2013), and between different populations of the same species (Huseth et al. 2016).

Furthermore, lab-based studies may not fully reflect how insects respond to neonicotinoids in the field. The effect of insecticides on insects is a complex interaction between susceptibility and exposure, with the latter dictated by the insect's behavior in an agroecosystem (Stark et al. 1995). Moreover, because insects are exposed to pathogens, other pesticides, and other stresses (e.g., limited food, temperature extremes) in the field, interactions between these stressors and insecticide exposure could affect survival and fitness (Cresswell 2011, Doublet et al. 2015, Poquet et al. 2016, Grassl et al. 2018). Further investigation of how NSTs affect EFN of field-grown crops is warranted, including examination of lethal and sublethal impacts on arthropods using this food source.

Supplementary Data

Supplementary data are available at *Environmental Entomology* online.

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