Residues of neonicotinoid insecticides in pollen and nectar from model plants[1]

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Abstract

Systemic insecticides used for ornamental horticulture crops can protect all portions of a plant with long-lasting effects. However, they may be hazardous to pollinators foraging on contaminated nectar or pollen. Two model plant cultivars were chosen based upon their ability to produce large quantities of pollen or nectar, enabling examination of the level of nectar or pollen contamination (e.g., insecticide "residues") following insecticide treatments, rather than for the need or advisability to treat these plants in production nurseries or the landscape with systemic insecticides. These plants were sunflower (*Helianthus annuus* L. 'Taiyo') for pollen, and swamp milkweed (*Asclepius incarnata* L. 'Ice Ballet') for nectar. Plants were treated at labeled nursery rates with imidacloprid, dinotefuran, or thiamethoxam via foliar spray or soil drench at various times before bloom. Insecticide residues from pollen and nectar varied based upon application method, insecticide, and rate. Assuming that residues should be considered hazardous when they exceed 25 parts per billion (ppb) for nectar or 100 ppb for pollen, potentially bee-toxic concentrations of insecticide in sunflower pollen only followed high-rate drench treatments. Toxic concentrations of neonicotinoids were found in milkweed nectar when applied either as a drench or as a foliar spray up to six weeks before bloom. Label directions for nursery and greenhouse plants permit very high application rates relative to agronomic crops. These high rates can create hazardous conditions for pollinators, and should be avoided for ornamental crops that are highly attractive to bees.

Index words: sunflower (*Helianthus annuus* L.); swamp milkweed (*Asclepius incarnata* L.); dinotefuran; imidacloprid; thiamethoxam; pollinator; systemic insecticides.

Chemicals used in this study: dinotefuran (Safari 20 SG); imidacloprid (Xytect 2F); thiamethoxam (Flagship 25 WG).

Species used in this study: sunflower (*Helianthus annuus* L.); swamp milkweed (*Asclepius incarnata* L.).

Significance to the Horticultural Industry

Use of systemic pesticides in the production of ornamental horticulture crops presents a quandary: these materials can protect all portions of a plant with long-lasting effects and can be compatible with integrated pest management programs, because the residues are translocated throughout and are presented from inside the plant. However, based upon their systemic nature, they may also inherently pose risks to pollinators which avail themselves of contaminated nectar or pollen resources. Pesticide labels permit high rate applications in ornamental horticulture use of neonicotinoid insecticides, relative to agronomic uses. These rates can lead to elevated residues in pollen, and especially in nectar, which are predicted to be toxic to bees. Nurseries and ornamental greenhouse growers do need to recognize the potential for harm to pollinators from the use of nitroguanidine neonicotinoid systemic insecticides. Ways to mitigate the risk to pollinators include substituting foliar sprays over drenches, using the lowest effective application rates, switching to systemic insecticides that have lower intrinsic toxicity to bees when treating bee-attractive plants, or not using systemic insecticides on these plants. These mitigation approaches should be a high priority for research on establishing pollinator-safe practices in the ornamental horticulture industry.

Introduction

Recent public focus on the use of neonicotinoids in ornamental horticulture has led to demands from large retail stores that this class of insecticides not be used by growers in plant production, or that plants treated with these insecticides be labeled as such (McClellan 2014, Home Depot 2016). Significant data gaps related to the concentration of systemic insecticides ("residues") found in nectar or pollen of ornamental plants previously treated with these insecticides hampers efforts to assure the public and retail sellers of these plants that growers' practices result in plants that are safe to bees and other pollinators. Filling these data gaps requires assessing the risk to pollinators by measuring the concentration of neonicotinoids in nectar or pollen from treated plants. Risk assessment requires knowledge of (1) the concentration of insecticides or their toxic metabolites present in nectar and pollen, (2) the quantity of nectar or pollen from these flowers available to foraging bees, and (3) the inherent acute and chronic toxicity of these insecticides to non-target pollinators. The key limiting factor for evaluating concentrations of insecticides in nectar or pollen is the difficulty in obtaining sufficient quantities to conduct residue analyses.

Model plant systems facilitate measuring systemic pesticide residues. Few plants provide readily collected gram-quantities of nectar or pollen required for measuring the single-digit part per billion (ppb) concentrations of neonicotinoids needed for assessing hazard to pollinators.

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Table 1. Products used and application rates for treating container-grown 'Taiyo' sunflowers to investigate pollen residues of neonicotinoid insecticides.

Product	Active Ingredient	Drench[z]			Spray[y]		
		Low	Medium	High	Low	Medium	High
Xytect 2F	imidacloprid	2.75	14.0	71.5	7.9	15.9	31.7
Flagship 25WG	thiamethoxam	1.81	14.6	118	37.5	75	150
Safari 20SG	dinotefuran	11.5	37.2	121	30	60	120

[z]Application rates are given in milligrams of active ingredient per pot.

[y]Application rates are given in milligrams of active ingredient per liter of spray.

We have a conundrum: it is exactly those unusual plants that produce such copious amounts of nectar or pollen that would make them unwise to treat in a nursery or landscape setting with systemic pesticides. Such large quantities of nectar or pollen rewards make these plants highly attractive to pollinators, which, if their nectar or pollen are sufficiently contaminated, would put the pollinators at risk.

Neonicotinoid residues in nectar or pollen are anticipated to be governed by the species of plant, the physical-chemical properties of the active ingredient, how it is applied to the plant, and the duration of time between when it is applied and when the plant flowers. Our overarching goal was to provide a deeper understanding of the dynamics of movement of neonicotinoids into herbaceous plant nectar and pollen. Using model plants, we investigated the principles of systemic uptake into and contamination of nectar and pollen by three neonicotinoid insecticides registered for use in production of ornamental horticulture crops by manipulating active ingredient, application method, application rate, and time prior to bloom when making the insecticide application.

Materials and Methods

Part I. Sunflower pollen residues

Experimental design. A full factorial design was established to look at the main effects and the interactions between insecticide (3 levels: clothianidin, dinotefuran, and imidacloprid), application method (2 levels: foliar spray vs. drench), application rate (3 levels: low, medium, and high labeled rates), and time before bloom (5 levels: biweekly applications up until bloom), totaling 90 treatment combinations. With four single-plant replicates, there was a total of 360 individual plants. The factorial arrangement of treatments provides "hidden replication" which increases the statistical power of this experiment.

Plant species and culture. 'Taiyo' sunflower seeds (Seed Savers Exchange, Decorah, IA) were started in plug trays April 29, 2015, and transplanted to 12-L (#3) nursery pots on May 8 and May 15. This cultivar was chosen because it is a floriculture-type sunflower, is moderate in height (1.7 m [5.6 ft]), has a relatively early maturity (70 days), yet it is an heirloom cultivar and produces pollen. The potting mix consisted of a 12:3:8 mixture of composted hardwood chips, Sunshine #1 Mix (Sun Gro Horticulture, Agawam, MA), and sand. The high ratio of sand in this mix was designed to produce a very dense mix that would provide stability to the tall plants. Plants were placed in a gravel outdoor nursery area at the Valley Laboratory, Windsor, CT, and received daily overhead irrigation from impact sprinklers. Plants were provided liquid fertilizer containing 150 ppm of nitrogen on May 22 and 300 ppm on June 9 (Jack's Professional Water Soluble Fertilizer, J. R. Peters, Allentown, PA), and 30 g per pot with a surface application of controlled release fertilizer with micronutrients on May 26 (23-4-8 Nursery Mix, Everris N. A., Dublin, OH).

Plant treatment protocol – soil drench procedure. Insecticides used in these experiments belong to the nitroguanidine class of neonicotinoids, chosen because they are classified by the U.S. EPA as being highly acutely toxic to honey bees (U.S. EPA 2014), are commonly used in ornamental horticulture crops as both foliar sprays and drenches, and represent a wide range of solubility and mobility within plants. These insecticides were dinotefuran (Safari 20 SG, Valent Corp., Walnut Creek, CA), imidacloprid (Xytect 2F, Rainbow Treecare, Minnetonka, MN), and thiamethoxam (Flagship 25WG, Syngenta, Greensboro, NC). Labeled rates for ornamental horticulture use of these insecticides vary widely. The lowest labeled rates would result from broadcast applications that match the maximum EPA per acre rates for agronomic uses of 0.3, 0.44, and 0.3 kg active ingredient (a. i.) per hectare (0.266, 0.4, and 0.266 lb. a. i. per acre), for dinotefuran, imidacloprid, and thiamethoxam, respectively. These were the low rates in our experiments, applied in a drench to the pots. The labeled drench application rates for these three insecticides are 66, 36, and 65 times these amounts per acre, respectively, calculated for a 12-L (3 gal) pot with a diameter of 28 cm (11 in), and constituted the high drench rate. The medium rate for each insecticide was the geometric mean of the low and high application rates (this is the midpoint between the application rates, on a log scale; see Table 1). The imidacloprid nursery granular product label (Marathon 1% G, OHP Inc., Mailand, PA 19451) has a more extreme use rate, in which a 12-L pot could be treated with up to 250 mg a. i. per pot (89 times the broadcast application rate), but mixing with potting media may allow some irreversible binding prior to planting. Therefore, the use instructions for the drench procedure with Marathon II resulting in the lower pesticide loading within the pot were followed, as the application took place during plant growth.

Product treatment guidelines for drenching vary considerably among labels, with the volume suggested to be used for drenching a 12-L pot ranging from a low of 350 ml (12 fl. oz.) for the Safari 20SG label to 1.2 L (40 fl. oz.) with

Table 2. Protocols for QuEChERS extraction of sunflower pollen. Samples are vortexed, centrifuged, and the organic layer supernatant removed at the end of each step.

	Pollen sample quantity (mg)			
	100	200	500	1,000
Step 1				
Water (µl)	400	800	2,000	4,000
Acetonitrile (µl)	495	990	2,475	4,950
Carbamazepine[z] (µl)	5	10	25	50
Step 2				
Magnesium sulfate (mg)	200	400	1,000	2,000
Sodium acetate (mg)	50	100	250	500
Step 3				
Magnesium sulfate (mg)	25	50	125	250
PSA[y] (mg)	8	16	42	83
C18 (mg)	8	16	42	83
GCB (mg)	8	16	42	83
Acetonitrile (µl)	2,000	2,400	3,000	5,400

[z]Carbamazepine volume given is for a 1,000 ppm solution in acetonitrile

[y]Abbreviations: C18, 18-carbon length sorbent bound to silica; GCB, graphitized carbon black; PSA, primary secondary amine sorbent

the Flagship 25WDG label. The Marathon II label instructs the user to apply the specified quantity of insecticide in sufficient water to avoid leaching. For the purposes of this experiment, the amount of water required to reach saturation was determined from a sample of three untreated plant pots prior to each treatment. Those pots were drenched with 1-liter of water; water draining from the pot was captured and the volume measured. The volume applied in the drench was then 90% of the volume captured from the sample from which there was the least water retained in the pot. Insecticides were applied once to individual plants, with a two-week interval between treatment timing groups; application dates were June 4, June 18, July 2, July 16, or July 31.

Plant treatment protocol – foliar application procedure. Label directions for the insecticides being used in this experiment usually have a low and high rate. The rates tested were these labeled rates, plus one-half the lower labeled rate, as well. These rates were, for Flagship 25WG and Safari 20SG: 57, 114, and 228 g per 380 L (2, 4, and 8 oz. of formulated product per 100 gal.); and for Xytect 2F: 12.5, 25, and 50 ml per 380 L. Products were applied with a CO_2-pressurized research sprayer, with spray applied to wet foliage and stems without run-off. Plants were sprayed on the same days as the soil drench applications.

Sunflower pollen collection. Inverted paper bags tied near the bottom of the opening were used to enclose inflorescences to prevent visits by pollinators and to capture pollen shed by the flower. Flowers were enclosed at the start of anthesis. During flowering, the bags were partially removed, and the flower gently tapped to dislodge additional pollen into the bag, from which pollen was removed and frozen (-20 C [-4 F]) until processed for residue analysis. Once anthesis had proceeded to the center of the floral disk, the inflorescence was cut from the plant and pollen remaining on flowers was scraped off using a stainless steel laboratory spatula. Most pollen samples needed to be cleaned of contaminating anthers or other extraneous material, which was accomplished by passing the pollen through a 40-mesh soil sieve (openings of 420 µm). Pollen was stored in labeled 1.5 ml centrifuge tubes in a freezer until extracted.

Extraction and analysis of insecticide residues. Pesticide analyses were conducted using a slightly modified version of the standard procedures in our laboratory for a multi-pesticide residue screen that had been used for both pesticide residues in fruits and vegetables (Krol et al. 2014) and pollen and nectar from cucurbits (Stoner and Eitzer 2012). A modified version of the QuEChERS (for Quick, Easy, Cheap, Effective, Rugged and Safe) protocol (Anastassiades et al. 2003) was used (Table 2). Unless otherwise noted, reagents used were pesticide analysis grade obtained from Sigma-Aldrich (St. Louis, MO). Samples (100 to 1,000 mg of pollen) were spiked to contain 50 ppb of carbamazepine as an internal standard (David et al. 2016), combined with water and acetonitrile (Pesticide Grade, Thermo Fisher Scientific, Waltham, MA), then vortexed. A mixture of magnesium sulfate and sodium acetate salts were added and mixed to cause the solvent layers to separate. The upper (acetonitrile) layer was removed following centrifugation, and further magnesium sulfate, primary and secondary amine exchange material (PSA Bonded Silica, Sigma Aldrich), 18-carbon length silica-bound sorbent (Discovery DSC-18, Sigma Aldrich) and graphitized carbon black (ENVI-Carb SPE bulk packing, Sigma-Aldrich) were added to remove interfering materials and improve quantification of the insecticide residues. Following vortexing and centrifugation, samples were split: one half of the supernatant volume was removed and concentrated under a stream of nitrogen to 1 ml for instrumental analysis; the other half of the sample was used for ELISA (enzyme-linked immunosorbent assay) determinations.

Extracts analyzed with high performance liquid chromatography/mass spectrometry used a Dionex Ultimate 3000 liquid chromatograph (Thermo Fisher Scientific): 2 µl of the extract were injected onto a Zorbax SB C-18, 2.0 by 150 mm, 1.8 µm column (Agilent, Santa Clara, CA). The column was gradient eluted at 0.2 ml per minute. The elution program was 5% acetonitrile/water for 1 minute, increased to 50% acetonitrile/water at 12 minutes, and 95% acetonitrile/water at 15 minutes before returning to the initial condition at 18.5 minutes to equilibrate for the next injection. Both solvents had 0.1% formic acid added. The effluent from the liquid chromatograph was coupled via positive ion electrospray with the Thermo Velos Pro Linear Ion Trap (Thermo Fisher Scientific). The Velos Pro mass spectrometer was set up with a unique scan function for each compound. This mode allows the parent ion for each pesticide to be uniquely isolated and fragmented with detection of the fragments. The pesticides were quantified using solvent-based standards containing the pesticides and the carbamazepine internal standard. These analysis conditions average 95 ± 18% recovery with detection limits ranging from 0.5 to 2 ppb depending on matrix, compound, and the amount of sample available.

For use in ELISA analyses, the acetonitrile was evaporated under nitrogen in a hood and the dried residues

suspended in 1.000 ml of water. The internal standard method does not assist quantification of residues with the ELISA method. To properly calculate the concentrations in the original sample, the volumes of solvent used for each step in the QuEChERS protocol (Table 2) and the volumes recovered for each step of the process for every sample were recorded. This allowed the percent recovery to be established, based on loss of solvent relative to the total amounts of solvent used, and so the milligram equivalents of pollen represented at the end of the process could be calculated.

ELISA plates (96-wells per plate) were obtained from Envirologix, Portland, ME (imidacloprid); Beacon Analytical, Portland, ME (thiamethoxam), and Horiba Instruments, Irvine, CA (dinotefuran). The sensitivity for each of these plates differed, requiring different degrees of dilution of samples prior to analysis. The strategy for dilution was informed by results from representative samples for different treatment combinations which had been analyzed via HPLC-MS/MS. Samples were diluted in distilled water, and the resulting solution was placed directly into a well of the ELISA plate with buffer solution and conjugate. Following an incubation period, the contents of the wells were removed and thoroughly washed with tap water from the plates. A substrate solution was added to the wells, and after incubating, a stop solution was added and the absorbance of light at 450 nm recorded with a microplate reader. The concentration of the insecticide was then determined from the standard curve obtained from triplicate sets of standards run on the same ELISA plate, and the original sample concentration in the microplate well determined by multiplying the dilution factor by the ELISA-determined well concentration. If the concentration was above the highest value from the standard curve, then the sample was diluted further and retested. The ELISA-determined concentrations was then adjusted for the number of milligram equivalents of pollen to establish the concentration of insecticide in the original pollen sample.

Insecticide residue data required logarithmic transformation [log (x)] prior to statistical analysis to establish homogeneity of variance. Following transformation, data were subjected to factorial analysis of variance with Statistix 9 (Analytical Software 2008), treating the experiment as a completely randomized design.

Part II. Milkweed nectar residues

Plant species and culture. 'Ice Ballet' milkweed plant in 7.5-L (#2) pots were purchased May 5, 2015, from a local nursery, and were grown in the same gravel nursery areas and under the same growing conditions as the sunflowers. These plants were used in a factorial design experiment, using the same insecticides and treatments as in the sunflower experiment, but only using the high application rate spray and drench applications. Insecticides were applied once to individual plants, with a two-week interval between treatment timing groups; application dates were June 10, 24, and July 10. The pot diameter was 21.6 cm, and so the potting mix drench application rates were adjusted to be proportional to pot surface area, giving quantities of active ingredient applied per pot of 72.1, 42.7, and 70.3 mg for dinotefuran, imidacloprid, and thiamethoxam, respectively. The concentrations used in the foliar spray were the same as for the high rate foliar spray for the three insecticides used in the sunflower experiment (Table 1).

Milkweed nectar collection. Nectar samples were collected July 2 to 29. Plants with open flowers were brought into a greenhouse and a clear plastic bag used to protect the flower from pollinator visits. When nectar could be seen filling nectaries, the bags were carefully removed, the portion of the inflorescence containing nectar was snipped from the plant, and dental floss used to hold the flowers upside-down within a 50 ml plastic centrifuge tube. The ends of the dental floss projected beyond the threads of the closed cap to firmly hold the flowers near the top of the tube. The tube with flowers was then centrifuged at 5,000 rpm in a Sorvall Superspeed RC-2B centrifuge (DuPont USA, Wilmington, DE) for 5 minutes to remove the nectar from the flowers. The volume of nectar was then measured and stored in 1.5 ml centrifuge tubes until processed further. Because nectar collected from bagged flowers may be more dilute than from flowers that are openly exposed (Wyatt et al. 1992), a 20 µL sample was used from the aggregated nectar from each plant to measure the degrees Brix (Rhino Brix30, Reichert Technologies, Depew, NY). This allowed later estimation of nectar contamination from non-bagged conditions, by assuming that nectar would measure 50 °Brix (Wyatt et al. 1992).

Sample processing and analysis. A subset of nectar samples representing the different treatment combinations was split to compare analyses via HPLC/MS-MS and ELISA. Unlike the pollen samples, only the aliquot being analyzed by HPLC was processed through a modified QuEChERS protocol. The clean-up procedures were the same as in Table 2, except that use of graphitized carbon black in Step 3 was omitted. Just as with the pollen samples, results from the HPLC subset were used to calculate optimal dilution of samples for ELISA analyses. Insecticide residue data required logarithmic transformation [log (x)] prior to statistical analysis to establish homogeneity of variance. Following transformation, data were subjected to factorial analysis of variance, treating the experiment as a completely randomized design.

Results and Discussion

Sunflower pollen was collected between July 13 and August 24. The total pollen collected from individual flowers varied considerably. The yield from some flowers colonized by insect pests was minimal, whereas the maximum yield (from two flowers) was just over one gram each (Fig. 1). Overall, the yield of pollen per flower was 376 ± 223 mg (mean ± sd).

The ELISA-based residue data included the full set of samples, whereas the HPLC-based data essentially duplicated a small subset of the ELISA results. Therefore, the ELISA-based residue data were used in the analysis of variance and to generate graphs. Residues of insecticides

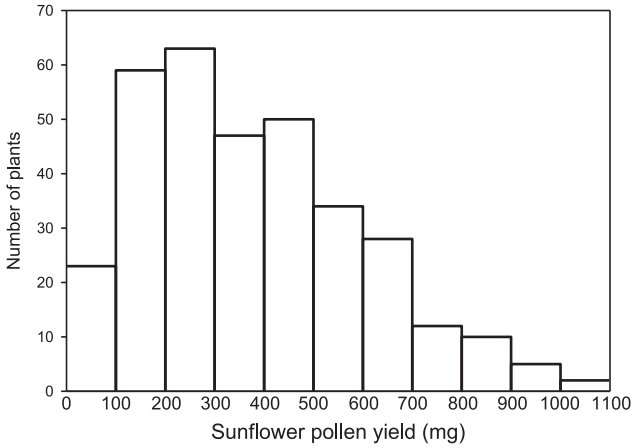

Fig. 1. Frequency histogram for the combined pollen weights from each sunflower plant.

found in the sunflower pollen varied with insecticide, rate, application method, and time elapsed between application and bloom (Fig. 2); with all main effects, two-way, and three-way interactions (except for the insecticide by rate by week and method by rate by week) interactions being highly significant (Table 3). Of these influences, method of application gave the greatest differences in residues, with foliar spray application resulting in lower concentrations than a soil drench, with the exception of the low dose applications of imidacloprid. We assume that a value of 100 ppb for presence in pollen may be a threshold for toxicity to honey bees, based upon the 25 ppb NOEL (no observable effect level) threshold for colony level effects from chronic exposure to nectar contaminated with imidacloprid, and the observation that considerably more nectar is consumed by worker bees than pollen (U. S. EPA 2014, U. S. EPA 2016). The four-fold increase in pollen vs. nectar thresholds for toxicity may be conservative, as the BeeREX model (U. S. EPA 2014) estimates maximum consumption of pollen to be about 3% that of the maximum consumption of nectar. The assumptions of similar toxicity among neonicotinoids is based upon their similar acute oral toxicities to individual bees; interpretations may need to be revised as more precise measures of toxicity from chronic exposure are published. The 100 ppb threshold was exceeded for all drench applications applied at the high labeled rates, for most of the medium rate drench applications, and for dinotefuran drenched at even the lowest application rate within six weeks of bloom. The high concentrations of dinotefuran found in pollen may result from its greater water solubility and higher mobility in plant tissues than the other two active ingredients (Richard S. Cowles, unpublished data). Imidacloprid is known to have the lowest mobility of these insecticides within plant tissues, therefore it is expected to take a longer time from application to reach potentially toxic concentrations in pollen. The low rate imidacloprid drench was the only application rate, method, and insecticide found to result in increasing concentrations as the time since application increased; the drench 10 weeks prior to bloom was the only timing for the low rate imidacloprid drench that resulted in approximately 100 ppb concentrations in pollen.

The concentrations found in sunflower pollen following foliar spray applications were, for some insecticides and application rates, greatly less than those resulting from drench applications. The greatest differences were for dinotefuran, in which the middle application rate caused an

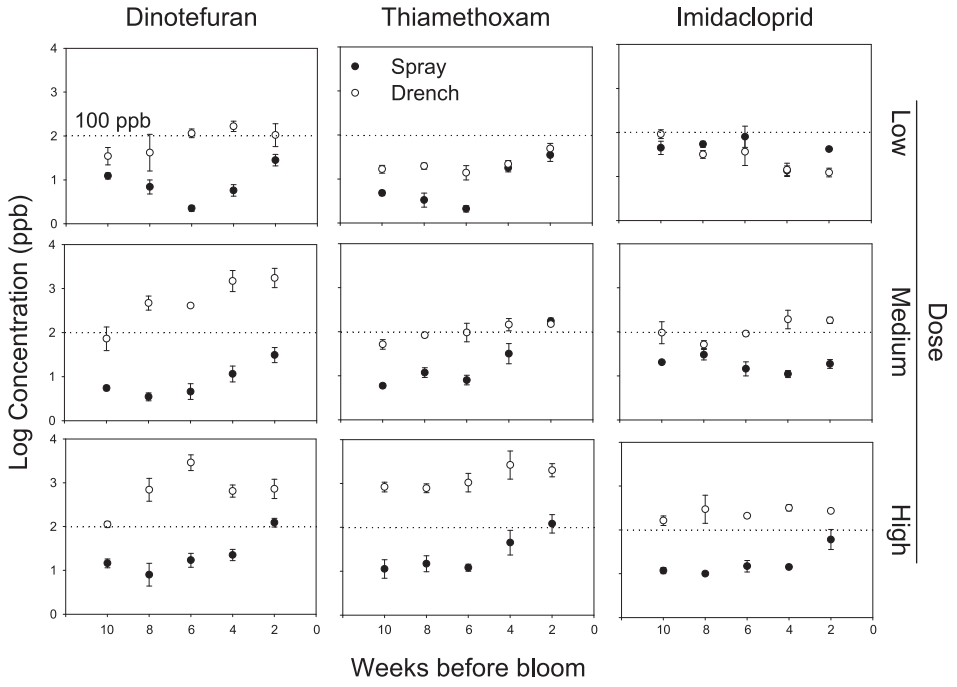

Fig. 2. The relationship between application rate, timing, and method with the residues of three neonicotinoid insecticides applied to *Helianthus annuus* 'Taiyo', as determined from pollen samples by ELISA following sample clean-up using a modified QuEChERS protocol. Data shown are mean ± se; $n = 3$ replicates. The dashed line at 100 ppb represents the threshold above which these insecticides may be expected to cause harm to honey bees.

Table 3. Analysis of variance for neonicotinoid insecticide residues found in sunflower pollen following foliar spray or soil drench applications

Source	DF	SS	MS	F	P
Insecticide	2	0.6852	0.3426	3.68	0.0267
Method	1	68.6508	68.6508	737.48	0.0000
Rate	2	23.4802	11.7401	126.12	0.0000
Week	4	9.1291	2.2823	24.52	0.0000
Insecticide × Method	2	8.2093	4.1047	44.09	0.0000
Insecticide × Rate	4	6.1060	1.5265	16.40	0.0000
Insecticide × Week	8	5.3861	0.6733	7.23	0.0000
Method × Rate	2	11.6561	5.8280	62.61	0.0000
Method × Week	4	3.5259	0.8815	9.47	0.0000
Rate × Week	8	2.3706	0.2963	3.18	0.0019
Insecticide × Method × Rate	4	4.6540	1.1635	12.50	0.0000
Insecticide × Method × Week	8	3.7630	0.4704	5.05	0.0000
Insecticide × Rate × Week	16	2.3905	0.1494	1.61	0.0686
Method × Rate × Week	8	0.6741	0.0843	0.91	0.5129
Error	227	21.1310	0.0931		
Total	300				

approximate 100-fold reduction of residues (two log units, Fig. 2) found in pollen following a spray application, compared to a drench applied at the same time. The smallest differences were observed for the longer-residual active ingredients (imidacloprid and thiamethoxam) applied at the low and medium application rates. Overall, foliar applications of neonicotinoids did not result in concentrations exceeding 100 ppb in sunflower pollen, except for any of the three insecticides applied at the highest rate within 2 weeks of bloom, or the medium rate of thiamethoxam applied within two weeks of bloom.

Milkweed nectar was collected daily from July 2 to 24, 2015. Aggregated samples contained 10 to 2,150 µL (653 ± 586 µL, mean ± sd) (Fig. 3). These samples ranged from 8.5 to 43 °Brix (20.3 ± 4.9, mean ± sd). The ELISA determinations included the full set of samples and so were used for statistical analyses and for generating graphs. Concentrations of insecticides found in the nectar were consistently greater than the U. S. EPA established threshold of concern for imidacloprid of 25 ppb (U.S. EPA 2016) (Fig. 4), with significant differences between insecticide active ingredients, method of application, and

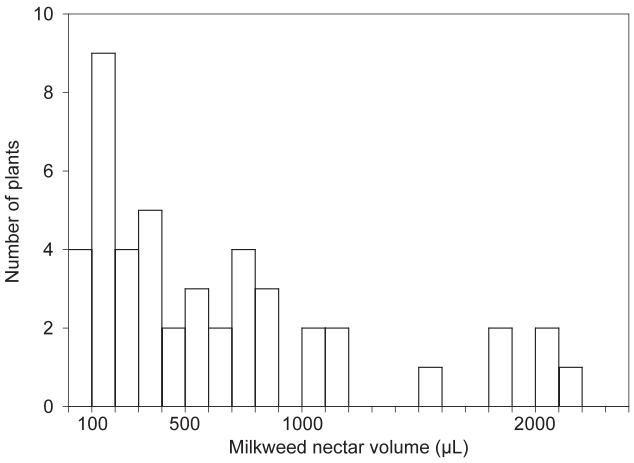

Fig. 3. Frequency histogram for the combined nectar volumes from each milkweed plant.

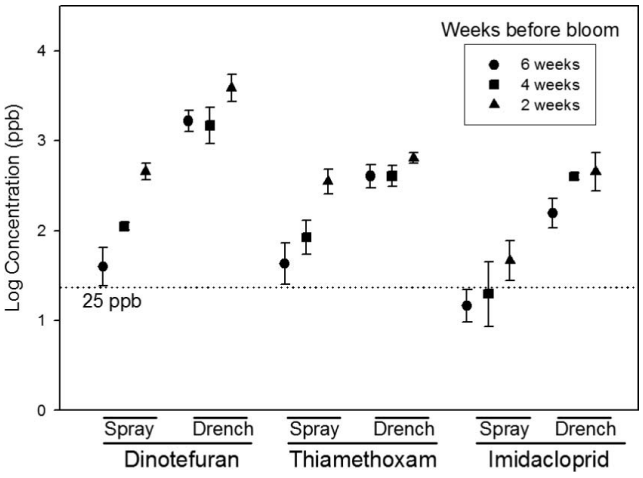

Fig. 4. The relationship between application timing and method with the residues of three neonicotinoid insecticides applied to *Asclepias incarnata* 'Ice Ballet', as determined from nectar samples by ELISA. Data shown are mean ± se; $n = 3$ replicates. Data have been adjusted for method bias as determined from HPLC/MS-MS analysis of split samples. The dashed line at 25 ppb represents the threshold above which these insecticides may be expected to cause harm to honey bees.

time between application and collection of nectar (Table 4). The general patterns were consistent with the results observed with residues found in pollen from sunflowers. For example, the greatest differences between residues resulting from drench vs. spray applications were observed with dinotefuran (10, 10 and 40-fold differences, for 2, 4, and 8 weeks since treatment, respectively). For thiamethoxam, these amounts were 2, 6, and 10-fold differences, and for imidacloprid were 2, 27, and 10-fold differences, approximately, for 2, 4, and 8 weeks since treatment, respectively. There was a steep decline in concentration found in nectar as time increased, with the reductions in residues being greatest with dinotefuran. The thiamethoxam residues found in nectar 2 weeks after application were equivalent with either foliar spray or drench, indicating the great efficiency of this insecticide to be absorbed into plants and transported systemically. For the other two active ingredients, there were significant differences in residue concentrations between foliar and drench applications: these differences among insecticides in response probably explain the significant insecticide by application method term ($F = 4.06$; df = 2, 41; $P = 0.0245$) in the analysis of variance (Table 4). Residues in sunflower pollen for the medium and low rate applications of thiamethoxam spray or drench at 2 weeks after treatment were also equivalent.

The results of pollen residue analyses via ELISA were compared with those from HPLC/MS-MS by log-transforming data, graphing and conducting linear regression, followed by the homogeneity of slopes test (Analytical Software 2008). A more sensitive test to investigate possible method bias was conducted by analyzing a paired t-test of log-transformed data. If both methods arrived at exactly the same residue measurement, then all the data would fall on a diagonal line with a slope of 1.00. Overall,

Table 4. Analysis of variance for neonicotinoid insecticide residues found in milkweed nectar following foliar spray or soil drench applications

Source	DF	SS	MS	F	P
Insecticide	2	11.1075	5.5537	48.46	0.0000
Method	1	13.6354	13.6354	118.97	0.0000
Week	2	3.0184	1.5092	13.17	0.0000
Insecticide × Method	2	0.9317	0.4659	4.06	0.0245
Insecticide × Week	4	0.1492	0.0373	0.33	0.8593
Method × Week	2	0.5041	0.2520	2.20	0.1238
Insecticide × Method × Week	4	0.3508	0.0877	0.77	0.5541
Error	41	4.6991	0.1146		
Total	58				

the measurement of residues with HPLC/MS-MS did not significantly differ from the measurements obtained with the ELISA method (Fig. 5) for dinotefuran data (linear regression $R^2 = 0.85$, paired t-test $P = 0.62$). For imidacloprid samples ($R^2 = 0.86$, paired t-test $P = 0.054$), the ELISA method at concentrations <25 ppb yielded higher values than did HPLC/MS-MS, which resulted in the imidacloprid regression having a slope with a 95% confidence interval ($1.118 \leq \beta \leq 1.626$) that did not include 1, which was significantly greater than that observed for the thiamethoxam residues ($0.80 \leq \beta \leq 1.015$), though not different from dinotefuran ($0.766 \leq \beta \leq 1.319$). The ELISA analyses for thiamethoxam ($R^2 = 0.92$) were significantly higher than from HPLC determinations (paired t-test, $P = 0.024$), by a factor of 1.32. Reexamination of averages for log-transformed residue values generated from split samples revealed that, similar to the thiamethoxam results, there is generally a higher estimation of residues from ELISA determinations than from HPLC analyses. For imidacloprid and dinotefuran, these were 1.54- and 1.42-fold differences, respectively. As the paired t-test comparisons were not significant for two of the three analytes (an analyte is a chemical for which residue levels were quantified), data presented in Fig. 2 have not been corrected for method bias.

The results of nectar residue analyses via ELISA vs HPLC/MS-MS were compared in the same manner as the pollen samples. Residues determined by the ELISA method were systematically greater than those found through HPLC ($P < 0.0001$, paired t-test). The slopes for the regression lines were very slightly > 1.0 for dinotefuran ($1.01 \leq \beta \leq 1.18$), and did not significantly differ from 1.0 for the other two analytes: $0.92 \leq \beta \leq 1.50$ and $0.82 \leq \beta \leq 1.19$ for imidacloprid and thiamethoxam, respectively. These split sample analyses served to calibrate the ELISA method: we re-scaled the ELISA values to values predicted to be observed from HPLC/MS-MS analysis by dividing the ELISA-determined values by 1.4-, 2.2-, and 4.3-fold for imidacloprid, dinotefuran, and thiamethoxam, respectively (Fig. 6). The nectar residues presented in Fig. 4 have been corrected for both sample Brix and method bias. This adjustment was large enough to shift the level of contamination resulting from the imidacloprid spray treatment at six weeks before bloom to be below the level of concern.

There was systematic bias when comparing ELISA to HPLC/MS-MS residue determinations, with greater levels

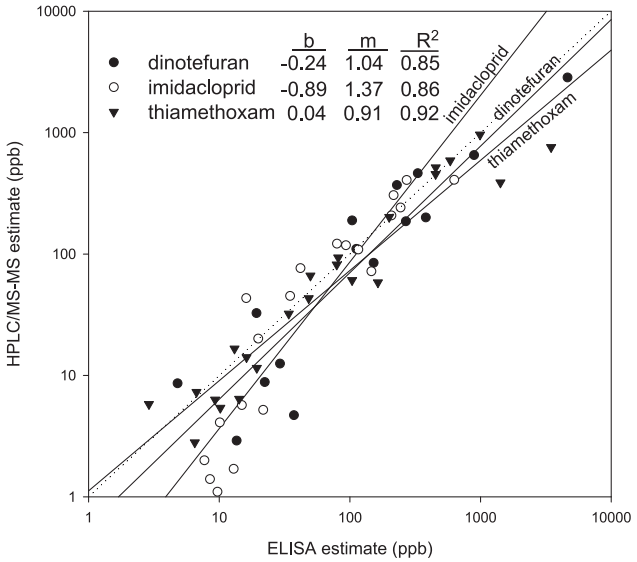

Fig. 5. The regression of residues from sunflower pollen estimated by ELISA vs HPLC/MS-MS methods; m and b are the slope and intercept estimates, respectively, for the regression of the log-transformed data. The dotted line along the diagonal of the graph depicts perfect agreement between the two methods. The significantly steeper slope for imidacloprid results from higher residue estimates with ELISA for low-concentration samples.

of residues determined, on average, with the ELISA method. These could have arisen from (1) matrix effects and non-specific binding, resulting in falsely elevated readings with the ELISA tests, or (2) possibly poorer sensitivity of HPLC due to overreliance on our internal standard. HPLC/MS-MS can underestimate true residue values if co-eluting substances suppress the signal for detecting the insecticide, relative to the signal being measured for the internal standard. Deuterated spiked internal standards correct for this possibility by co-eluting with the analyte, but use of such a standard was not possible in our study because samples were being split for analysis via both ELISA and HPLC/MS-MS. Matrix effect interference was likely to have influenced ELISA determinations for dinotefuran and thiamethoxam in nectar, as those were much greater than the results obtained through HPLC/MS-MS. The better agreement between ELISA and HPLC determinations with pollen samples, which used sample clean-up for both methods, suggests that sample clean-up of nectar samples might also be necessary to improve ELISA determinations of neonicotinoid residues in nectar.

There had previously been little known about the concentrations of neonicotinoids in nectar and pollen of ornamental crops. Extreme concentrations in whole flowers have been measured from ornamental plants following high-application rate, nursery-labeled use instructions (Krishik et al. 2015), but the actual degree of exposure through pollen and nectar cannot be equated from analysis of whole flowers (Johnson 2012). However, there has been a recent review on the subject of environmental risks posed by neonicotinoids, including to bees (Goulson 2013), and the literature on acute and chronic toxicity to bees has also

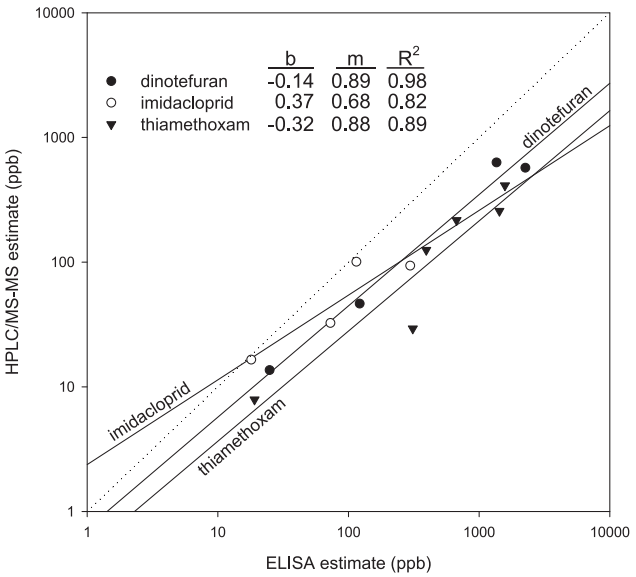

Fig. 6. The regression of residues from milkweed nectar estimated by ELISA vs HPLC/MS-MS methods; m and b are the slope and intercept estimates, respectively, for the regression of the log-transformed data. The dotted line along the diagonal of the graph depicts perfect agreement between the two methods. The significant displacement of residue estimates from this line indicates that the ELISA method consistently overestimated residues compared with HPLC/MS-MS. Samples analyzed by HPLC were cleaned with a modified QuEChERS procedure; the ELISA samples were not.

recently been reviewed (EFSA 2012, APVMA 2014). One of the best-studied crops with respect to floral concentrations has been sunflower, because it has been an agronomic crop for which neonicotinoids have been applied through seed treatments. As has been observed with other seed-treated crops, the concentrations of imidacloprid found in seed-treated sunflower crops have ranged from being undetectable (Schmuck 2001, with a limit of detection [LOD] of 1.5 ppb) to an average of 3.3 ppb and a maximum of 11 ppb (Bonmatin et al. 2003). The mechanisms of floral resource contamination with systemic insecticides are best understood from studies that have observed and compared concentrations of neonicotinoids found in foliage, whole flowers, pollen, and nectar. Johnson (2012) studied the potential impact to honey bees of imidacloprid applications to red maples (*Acer rubrum* L.) growing in New York City for the Asian longhorned beetle (*Anaplophora glabripennis* Motschulsky)] quarantine. In that work, the concentrations of imidacloprid found in various sources were approximately (values in parentheses are concentrations in ppb): foliage (10,000), whole flowers (200), pollen (5), and nectar (not detectable). This trend is one that should be expected since foliage is a sink for xylem sap because leaves conduct photosynthesis and utilize large volumes of xylem sap during evapotranspiration over a long period of time, during which neonicotinoids present in sap may accumulate. Whole flowers do have stomates, but are not usually photosynthetic or long lasting. Therefore, they are unlikely to accumulate systemic insecticides to the same degree as leaves. Pollen is even less of a sink for xylem-mobile products; we might expect that the concentrations could be similar to that found in xylem sap at the time of formation of the pollen grains. Nectar is essentially sugar-water with some amino acids. This would suggest that nectar would be supplied by phloem tissue. However, even within the Asteraceae, nectaries can be provisioned from phloem, both phloem and xylem, or neither (Wist and Davis, 2006). Therefore, the concentration of neonicotinoids in nectar may be idiosyncratically related to the species being investigated and its nectar production physiology.

The decline of residues in leaf tissues is fairly well known for foliar applications on herbaceous plants, in which there can be expected to be translaminar absorption (the degree of which is specific to the neonicotinoid) followed by degradation through metabolism and exposure to light. Generally, the half-life for foliar residues are approximately 3 to 5 days for imidacloprid (Mukherjee and Gopal 2000). Decline of residues following foliar treatment of woody plants may be more complex, due to bark absorption of residues with subsequent lateral transport to xylem and eventual upward movement to leaves and flowers (Coppel and Norris 1966; McCullough et al. 2007). For woody ornamental plants, storage of systemic insecticide in phloem tissue may occur, which can result in multiple-year detection of residues in new growth. The storage phenomenon is extreme in conifers, in which a single soil application can result in multiple years of pest suppression (Cowles et al. 2006; Benton et al. 2016).

Soil applications provide a challenge for modeling the movement into plants and contamination of floral resources. Once neonicotinoids are incorporated into soil, those that are relatively stable in this environment can provide long-residual protection of plants against various pests. Depending on the neonicotinoid, insecticide binding to soil organic matter competes with availability to plants of the active ingredient in soil solution, and so besides soil residue half-life considerations, nearly irreversible binding to organic matter in soil leads to diminishing availability of insecticide to be absorbed and transported to above-ground tissues. Because it is likely that the concentrations in nectar and pollen will be governed by the flux of insecticide moving in the sap at the time that these tissues develop, the time-course for concentrations expected in nectar or pollen following a soil application should peak soon after application and gradually decline, but the shape of this relationship should be highly influenced by insecticide active ingredient, potting mix composition, and concentration of insecticide initially applied. Neonicotinoid product labels permit extremely high application rates on ornamental nursery crops and for treatment of individual trees and shrubs, relative to the 0.3 to 4 kg per ha (0.266 to 0.4 lb active ingredient per acre) maximum use rate found in agricultural crops. For imidacloprid, potting mix incorporation of nursery granular products can reach 3.8 g per m^2, which would translate when pots are closely packed, of up to 38.2 kg active ingredient applied per ha, or about 87 times the highest agronomic use rate.

These high application rates, on a per unit area basis, may not translate to high nectar or pollen contamination in trees (Johnson 2012), because the insecticides are diluted

within a large plant's biomass. As the size of the treated plant decreases, we can expect that these high rates will result in increasing residues (Cowles 2010). Therefore, ornamental horticulture use of these systemic insecticides on herbaceous plants and shrubs should be expected to present the greatest risk for residues in floral resources to reach potentially toxic levels for pollinators.

Obtaining nectar or pollen samples of sufficient size to conduct residue analyses can be very difficult. Larson et al. (2015) centrifuged over 10,000 individual clover inflorescences (0.4 to 1.4 mg per inflorescence) to obtain 36 nectar samples for chemical residue analyses. There are two basic approaches for aggregating enough nectar or pollen to conduct residue analyses. One method is to use bumble bees caged with multiple plants of one type, all treated similarly, and to "rob" these bees of their pollen loads and honey pots. For a fully replicated experiment with multiple insecticides, application rates, and time points for residue decline estimation, (as presented in this study) such an approach quickly becomes impractical. Each treatment combination and replicate would require its own cage and bumble bee colony, and have large numbers of plants from each treatment combination and replicate to support collection of their pollen or nectar by bees.

The second method is to use a model plant system, in which the plants are chosen because they produce large quantities of nectar and/or pollen. Flowers from these plants are notably favored by pollinators, and yields of nectar or pollen per acre of these plants are sometimes known (Crane et al. 1984). Plants in the mint family, *Sesamum* spp., some borages (e.g. *Echium* spp.), *Phacelia tanacetifolia*, *Telephium* spp. (*Sedums*), *Nicotiana* spp., and *Asclepias* spp. produce so much nectar that quantities suitable for conducting residue analyses might be obtained. Commercial floriculture cultivars of sunflowers (*Helianthus annuus* L.) are mostly male-sterile, to avoid the messiness of shed pollen. However, there are still heirloom floriculture cultivars, such as the 'Taiyo' used in this study and those grown for their seeds that are not male sterile. We found that a single sunflower inflorescence was sufficient to provide enough pollen to conduct residue analyses. One challenge to communicating results from the current study will be to emphasize that the plants were specifically chosen for the high yields of nectar or pollen, and that nitroguanidine neonicotinoid application using the high nursery use rates is inappropriate for such plants that are highly attractive to bees. Treating *Asclepias* spp. with systemic insecticides would be especially unwise, because customers are likely to purchase these plants expressly for their value in supporting development of monarch butterfly larvae.

We demonstrated that a relatively inexpensive analysis using ELISA methods can provide data of similar quality to the standard method using HPLC/MS-MS, when the samples have first been cleaned with the QuEChERS procedure. HPLC/MS-MS costs $200 to 250 per sample to analyze, whereas ELISA determinations cost $4.76 to 14.88 per well – the total cost per sample for ELISA analysis depends on the number of wells required (1 to 6 wells per sample, depending on whether duplicate tests are run and the number of dilutions required, described below). Due to the higher cost for HPLC determinations, we limited the number of samples analyzed by HPLC/MS-MS to 120 samples. A subset of samples were analyzed by both HPLC/MS-MS and ELISA, permitting more efficient use of ELISA plates and effective quality control evaluation of the ELISA results. Using both HPLC/MS-MS and ELISA methods for a subset of our samples allowed us to convert the semi-quantitative results from ELISA into quantitative values. The chief problem with ELISA is that both an insecticide (the parent compound) and its metabolites may interact with the antibodies used for the colorimetric analysis. For example, for the imidacloprid ELISA kit, imidacloprid, imidacloprid olefin, and the hydroxy-imidacloprid compounds (three of them) all may contribute to the "imidacloprid" signal (Envirologix 2015). The result is that the ELISA method can overestimate the amount of parent compound present. When a sample is analyzed by both ELISA and HPLC/MS-MS, the relative contribution of the parent compound and its metabolites can be determined, and so all samples obtained from plants under the same treatment conditions can be assumed to have a similar relative parent/metabolite profile. For our samples, the imidacloprid olefin was present at about 10% of the parent compound. For comparing the ELISA and HPLC/MS-MS results, the level for these two analytes (as determined by HPLC/MS-MS) were added together, and found to closely match the value found with ELISA.

Using HPLC/MS-MS together with ELISA permitted more efficient use of ELISA plates. Often, several ELISA wells are used to analyze a single sample, because the range quantifiable within the standardization curve may only consist of a 10- to 30-fold range of concentrations (e.g., 0.2 to 6 ppb for imidacloprid is a 30-fold range). When samples contain higher concentrations than the standard curve can quantify (e.g., anything greater than 6 ppb, such as 100 or 1000 ppb), then sequential dilution and retesting may be necessary to bring the sample concentration within the standard curve. Since the HPLC/MS-MS analysis provided residue concentrations for a subset of samples, these determinations provided the information required to optimize the dilution of samples so that results for all similar samples would fall within the standard curve for ELISA analyses.

Nitroguanidine neonicotinoid contamination of nectar is of greater concern for honey bee health than equally contaminated pollen, because they consume more nectar to satisfy their caloric needs. Worker honey bees consume the equivalent of 4 mg of carbohydrates daily (Brodschneider and Crailsheim 2010), originating from the equivalent of 8 to 20 mg of nectar (assuming 20 to 50 Brix for nectar), and about 4 mg of pollen per day on average (Crailsheim et al. 1992). The consumption of pollen by worker bees is greatly affected by their needs for protein, with nurse bees consuming nearly 12 mg per day between ages of 4 to 9 days, and 2 mg or less after 16 days as an adult (Crailsheim et al. 1992). Honey bees feed newly hatched larvae glandular secretions (jelly) rather than directly feeding them unprocessed nectar or pollen; after three days and until they pupate, larvae are fed jelly along with a total of

about 1 to 6 mg of unprocessed pollen, representing only about 5% of the protein in their diet (Babendreier et al. 2004, Brodschneider and Crailsheim 2010). The processing of nectar and pollen by nurse bees to feed larvae jelly permits the adult workers to metabolize pesticides (Suchail et al. 2004, Creswell et al. 2014) found in both foodstuffs, which may protect larvae from pesticide exposure. For other bees, contamination of pollen with insecticides may be of greater concern than for honey bees; we can expect that pollen could be hazardous to other species of bees at similar concentrations as for nectar, because they feed pollen mixed with nectar directly to developing young. Recent studies (Creswell et al. 2014, Rundlöf et al. 2015) suggest that bumble bees and solitary bees are more sensitive to nitroguanidine neonicotinoids than honey bees, and solitary bees may be more sensitive than bumble bees. These studies suggest a number of mechanisms for greater sensitivity of bumble bees and solitary bees, including poorer detoxification and more pronounced effects on behavior of adult bees.

Our results with sunflower suggest that certain use practices for insecticides would not present concentrations in pollen hazardous to honey bees. Because we did not assess nectar from these same plants, we cannot state with certainty that these practices would be "bee-safe", however.

Two recent studies using honey bees to sample pollen from landscapes in the Northeast and Northwest U. S. found levels of contamination of about 2 ppb for imidacloprid, and approximately 6 ppb for imidacloprid equivalents, when combining the exposure risk from all neonicotinoid insecticides into imidacloprid toxicity equivalents (Lu et al. 2015; Lawrence et al. 2016). These empirical data indicate that current use practices for neonicotinoids do not generally result in hazardous exposure for honey bees. Our data suggest that it is possible to treat plants at the labeled rates on pesticide labels in a manner that would result in much higher level exposures from landscape ornamental plants than would be indicated by these surveys of exposure from honey bee-collected pollen. It is very likely that ornamental plants being treated with these insecticides in the landscape (1) are treated in a manner that results in much lower levels of contamination than found in these experiments or (2) that treated ornamental plants provide only a small portion of the nectar or pollen being collected by honey bees, effectively diluting the residues from floral resources treated with these insecticides.

Nurseries do need to recognize the potential for harm to pollinators from the use of nitroguanidine neonicotinoid systemic insecticides resulting in contamination of nectar and pollen on highly pollinator-attractive flowers. Systemic insecticides vary widely in their acute and chronic toxicity to bees. For example, the non-nitroguanidine neonicotinoid, acetamiprid, is about 1,000 times less acutely toxic to bees than the insecticides we studied (Iwasa et al. 2004). Ways to mitigate the risk to pollinators include (1) switching to systemic insecticides that have lower intrinsic toxicity to bees (e.g., using acetamiprid vs. dinotefuran, imidacloprid, or thiamethoxam), and (2) to reserve use of systemic insecticides that are highly toxic to pollinators for plants that do not produce significant quantities of nectar or pollen.

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