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Short-term persistence of foliar insecticides and fungicides in pumpkin plants and their pollinators

Short title: Foliar pesticide persistence in pumpkins and pollinators

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Abstract

To minimize the risk to bees and other beneficial insects, plant protection chemicals are typically applied to pollinator-dependent crop plants when flowers are absent or unopened. However, this approach does not entirely remove the risk of pollinator exposure. Much research has focused on negative effects of systemic insecticides (e.g., seed treatments) on pollinators, but less is known about the level of hazard posed by translocation of non-systemic foliar-applied pesticides to pollen and nectar that bees consume. In this study we assess the frequency and persistence of six foliar-applied pesticides in pumpkin (*Cucurbita pepo*) tissues and in their bee visitors. We analyzed residues of three insecticides (carbaryl, lambda-cyhalothrin, permethrin) and three fungicides (chlorothalonil, quinoxyfen, triflumizole) in pumpkin leaves, pollen, and nectar collected from five farms in the north-central USA, one day before a spray event, and one, three, and seven days after. Bees foraging on pumpkin flowers were collected one day before and one day after spray and screened for the same pesticides. Overall, insecticides were present in 56% of leaf samples. Compared to leaves, fewer pollen (insecticide detected in 16%, fungicide in 16%) and nectar samples (14%, 0%) contained pesticides. We detected one insecticide (carbaryl) in two out of 69 samples of foraging bees, and only in male squash bees (not in bumble or honey bees), which have life history traits that bring them into prolonged close contact with the sprayed crop plants. The persistence of some agrochemicals in leaves, pollen, and nectar up to a week following application merits consideration when managing pollinator-dependent crops. Even pesticides that are traditionally considered contact-based and applied when flowers are unopened can reach pollen and nectar and produce measurable risk to bees.

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Introduction

When applying chemical pesticides, growers must strike a balance between protecting crops and avoiding harm to non-target organisms like pollinating insects that contribute to crop yield [1]. As a result, much research has been done on the negative effects of agrochemicals on pollinators [2 – 4]. Recent research has focused on systemic insecticides applied to the soil or used as seed treatments, but less is known about the level and duration of hazard posed to pollinators by foliar-applied insecticides and fungicides [5 – 8]. In crops that are highly dependent on pollinators for fruit set, foliar sprays used in mid- to late-season to control diseases and insect pests are typically applied before the plants begin to bloom, or if flowers are present, late in the evening when bee activity is low and flower buds are closed [9]. That practice, while beneficial, does not entirely remove the risk of exposure for pollinators [10]. Pollinating insects may come into direct contact with recently sprayed foliage, soil, or contaminated dust particles, or consume contaminated pollen and nectar as adults or larvae [8, 11 – 14]. We do not fully understand the translocation of foliar-applied chemicals from leaves and unopened flowers to the nectar and pollen upon which bees feed, and their persistence in or on plant tissues under field conditions (but see [14]). The dearth of data reflects the expense of pesticide residue analysis, as well as the difficulty of obtaining sufficient pollen and nectar directly from flowers [13 – 14].

In this study, we assess the frequency and persistence of foliar-applied insecticides and fungicides in leaves, pollen, and nectar of pumpkin (*Cucurbita pepo* L.), as well as in bees visiting pumpkin flowers. Pumpkins, like other cucurbit crops, have separate male and female flowers, so they rely on pollinating insects, especially bees, to set fruit. In the USA, pumpkin flowers are most commonly pollinated by several widely distributed, dietary-generalist eusocial bee taxa (western honey bee, *Apis mellifera* (L.), and bumble bees, *Bombus* spp. (Latreille)) and

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by several solitary, *Cucurbita* pollen-specialist bees (*Xenoglossa* (*Peponapis*) *pruinosa* (Say) and other *Xenoglossa* spp., hereafter referred to collectively as "squash bees") [15 – 16]. *Cucurbita* specialists incur a higher risk from agrochemicals than do other bees. The females typically nest in soil directly beneath *Cucurbita* crop plants, and collect pollen exclusively from *Cucurbita* flowers, and the male bees spend much of their time sheltering in the wilted flowers [17 – 19]. For pollen-generalists like honey bees and bumble bees, *Cucurbita* nectar constitutes a smaller proportion of their diverse adult diets, and the larvae are also fed a mixture of pollen and nectar from many plant species. Although their diversified diets may dilute the agrochemical dose from any one source of contaminated pollen and nectar, eusocial bees in agricultural landscapes are subject to repeated long-term exposure from the food and other materials they collect and store in the nest [7, 20 – 21].

When *Cucurbita* flower availability coincides with a recent agrochemical spraying event, the risk to its pollinators can be high. In *Cucurbita,* both staminate and pistillate flowers offer a large nectar reward, incentivizing bees to visit these flowers intensely during the several hours after sunrise when they are open [17]. Meanwhile, foliar- and soil-applied pesticides readily translocate to bee-collected pollen and nectar in cultivated *Cucurbita* [10, 14, 22 – 24]. There are currently >40 insecticides and >40 fungicides approved for use with *Cucurbita* crops in the USA [25], and growers rotate their use of different chemical classes to minimize pest resistance. Therefore, if agrochemicals translocate to and persist in floral resources and in soil and other nesting materials, bees foraging in agricultural fields can be exposed to a variety of chemicals simultaneously [11, 21, 26 – 28]. Even in cases where insecticide exposures are not acutely lethal, there have been numerous sublethal effects reported in pollinators, including decreased foraging efficiency [29 - 32], increased susceptibility to disease or parasites [20, 33], reduced

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nest initiation [19, 34], and reduced offspring production [19, 35 – 37] or development [38]. Fungicides generally have low toxicity to adult foraging bees [39], but can be toxic to larvae [40] and may disrupt bee digestion and immunity by altering gut microbiomes or histology [41 – 43]. A further complication is that certain agrochemicals have additive, or even interactive, negative effects on bees [44]. For example, some pyrethroid and neonicotinoid insecticides can be made more toxic by the presence of azole fungicides [45].

To determine the degree to which pollinators are exposed to foliar-applied agrochemicals that were sprayed while flowers were closed, we analyzed residues of three insecticides and three fungicides in pumpkin leaves, pollen, and nectar collected from five farms in the north-central USA, one day before a spray event as well as one, three, and seven days after the spray event. Specifically, we asked the following questions:

(1) How frequently are foliar insecticide and fungicide residues found in pumpkin pollen, nectar, and leaves in the week after spray?

(2) Does detection of residues in pumpkin tissues attenuate with time since spray?

(3) Are foliar insecticides and fungicides detected in bees visiting pumpkin flowers, and what level of hazard do they pose to pumpkin pollinators?

Methods

Study System

Our study took place on five pumpkin farms in central Ohio, USA, in 2019 (Supporting Information, S1 Table**)**. The farms were all conventional, commercial-scale working farms, with pesticide application decisions reflecting typical management regimes for our study region (ranging from prophylactic applications to integrated pest management). All growers used seeds

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126 treated with FarMore® FI400, which contains three fungicides (mefenoxam, fludioxonil,
127 azoxystrobin) and the neonicotinoid insecticide thiamethoxam. Foliar sprays were applied mid-
128 season during flowering to manage insect pests and fungal diseases.

129 Pumpkin flowers in our system are visited by free-living bumble bees and squash bees
130 [23]; nevertheless, four of the five farms also contracted or kept honey bees to supplement
131 pollination. As in other cucurbit crops, pumpkin flowers are short-lived, with each flower
132 opening around sunrise and lasting only several hours before wilting. The ephemeral nature of
133 these flowers limits the routes through which foliar-applied chemicals may enter pollen and
134 nectar; the flowers we surveyed for residue analysis (see below) opened for the first time on the
135 day we collected the pollen and nectar.

136 # Data Collection

137 To determine the level of hazard to bees foraging in pumpkin fields after mid-season application
138 of foliar sprays, we analyzed residues of six agricultural chemicals (three insecticides and three
139 fungicides) in pumpkin leaves, pollen and nectar from flowers, and bee visitors to flowers.
140 Growers decided independently what chemicals to apply and when. At each farm, we
141 opportunistically assayed pumpkin tissues one day before, as well as one day, three days, and
142 seven days after foliar spray events that involved one of our target agrochemicals (but not the
143 day of the spray event), based on planned application schedules communicated by our
144 collaborating growers. During each survey, we collected 20 pumpkin leaves as well as pollen and
145 nectar from about 40 male flowers, from individual plants haphazardly selected across the entire
146 field. We also collected up to 10 individuals of each of the following types of bees foraging on
147 pumpkin flowers one day before and one day after spray events: bumble bee workers, honey bee

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148 workers, and male and female *X. pruinosa* squash bees. We prioritized bee collections on the day

149 before and after spray when exposure was most likely and did not collect bees on day 3 or 7.

Table 1. Summary of agrochemical sprays analyzed in pumpkin leaf, pollen, and nectar, and in bee visitors.

Chemical Residue	Category	Class	Contact Acute LD_{50} Honey Bee	Oral Acute LD_{50} Honey Bee	$logK_{ow}$	pK_a	Molecular Weight
Carbaryl	Insecticide	Carbamate	0.14	>0.21	2.36	10.4	201.2
Cyhalothrin, total	Insecticide	Pyrethroid	0.038	0.027	6.8	9	449.9
Permethrin	Insecticide	Pyrethroid	0.024	0.13	6.1	NA	391.3
Chlorothalonil	Fungicide	Organochlorine	> 101	>63	2.94	neutral	265.9
Triflumizole	Fungicide	Imidazole	20	14	4.77	3.7	345.8
Quinoxyfen	Fungicide	Quinoline	>100	>100	5.1	neutral	308.1

LD_{50} values (the amount of a chemical that is lethal to 50% of a test population) are in µg / bee for contact and oral acute LD_{50} in honey bees, *Apis mellifera*. Octanol-water partition coefficient ($logK_{ow}$) is the relative concentration of a chemical in n-octanol versus water at pH 7, 20°C. Higher values of $logK_{ow}$ indicate greater lipophilicity (and a lower affinity for water) and values above 4.5 are considered likely to adsorb and accumulate in lipid-rich tissues such as cuticular waxes or pollen. A chemical's ionizability is given as pK_a, the pH at which a chemical is 50% ionized, or in equilibrium between its undissociated and ionized state (calculated as the negative base-10 logarithm of the acid dissociation constant at 25°C). Chemicals with $pK_a < 7$ are most likely to reach vascular tissue and mobilize systemically throughout the plant. A 'neutral' pK_a indicates the chemical does not ionize under relevant plant conditions. Chemicals with molecular weights below 800 g mol^{-1} are able to penetrate plant cuticles. Pesticide properties were obtained from the University of Hertfordshire Pesticide Properties Database [46].

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We collected the pumpkin leaves into resealable plastic bags. Bees were collected from flowers into individual microcentrifuge vials. We collected pollen and nectar by harvesting entire male flowers: anthers were collected into a 50-ml centrifuge tube, and nectar was extracted from the unopened nectar well (i.e., not depleted or contaminated by foraging bees) using a clean 50-µl micropipette tip glued onto the needle adapter of a 5-ml syringe. All samples were kept on ice and transferred to a -80°C freezer for storage.

Pesticide residue analysis was conducted on 1 g samples of leaf, pollen (half pollen and half anther tissue, hereafter referred to as 'pollen'), nectar, and bee tissue. We cut thin latitudinal strips from stacked pumpkin leaves at the widest part of the leaves near the center. These strips were chopped into small pieces using a sterile razor blade and from these pieces, a 1-g sample of tissue was weighed and placed in a 1.5-mL microcentrifuge tube. Pollen samples were scraped from the anthers with a sterile spatula to obtain a 0.5-g sample to which we added 0.5 g of tissue removed from the outer layer of 2 - 3 anthers due to the difficulty of obtaining 1 g of pure pollen. A 1-mL sample of nectar was measured into a clean 1.5-mL microcentrifuge tube. For the bee samples, it was necessary to combine 1 - 5 bees of the same species and sex collected from the same farm on the same day in order to make a 1-g sample (mean = 2.6 bees, SD = 1.4). All tissue samples were kept at -80°C until shipped overnight on ice to SGS North America (Brookings, SD) for analysis.

Tissues were homogenized and residues extracted using the QuEChERS method (EN15662) [47]. Chemical residues were analyzed using protocols outlined in the Association of Official Agricultural Chemists method [48]. Carbaryl and triflumizole residues were analyzed using liquid chromatography and tandem mass spectrometry (LC-MS/MS). Chlorothalonil, cyhalothrin (total), permethrin (cis and trans), and 5,7-dichloro-4-(p-fluorophenoxy) quinoline

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(i.e., quinoxyfen) were analyzed using gas chromatography mass spectrometry (GC-MS/MS) and concentrated from 2 mL to 1 mL. The concentrations of each residue were determined by comparing to standard curves including triphenyl phosphate (TPP) as the internal standard. Chemical concentrations were recorded in µg / kg (parts per billion, hereafter 'PPB'). The lower limit of quantification for most chemicals was 10 µg / kg, but was 20 µg / kg for cyhalothrin and triflumizole.

Data Analysis

As growers decided independently what and when to spray (Supporting Information, S1 Table), the number of samples tested and detected was tallied separately for each chemical (and for each of two categories – insecticide and fungicide) using only the data from farms where each was used. We present the percent detection of each chemical in each tissue type over time (from 1 d before through 7 d after spray). All analyses were conducted using the R programming language, version 4.3.2 [49].

To assess risk to bees from contact with pumpkin leaves or consumption of pollen and nectar after foliar spray, three risk indices were calculated – Hazard Quotient (*HQ*), a variation of Hazard Quotient (HQ_{PPB}), and US EPA BeeREX Risk Quotient (*RQ*). First, Hazard Quotient (*HQ*) was calculated for each tissue type as the concentration of a chemical residue in plant or bee tissue divided by acute LD_{50} (oral for pollen and nectar, contact for leaf, average of contact and oral for bee) [50]. Honey bee LD_{50} was used as LD_{50} was not consistently available for bumble and squash bees (**Table 1**). Second, we created a variation of *HQ* (hereafter referred to as HQ_{PPB}) that first converts toxicity into the same units as our chemical concentrations (PPB) to allow for direct comparisons between pesticide load and toxicity. In calculating *HQ*, the LD_{50} is expressed as the standard µg / bee. However, for HQ_{PPB}, LD_{50} values are first converted from µg

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214 / bee to µg / kg of bee tissue by dividing LD_{50} values by 0.00012 kg (the average weight of a
215 honey bee worker [51]). HQ_{PPB} is a more conservative way to assess acute risk to bees from
216 agrochemicals than *HQ*. For example, the maximum concentration of carbaryl we detected in
217 nectar 1 d after spray was 44.1 µg / kg. The oral LD_{50} of carbaryl is 0.21 µg / bee (**Table 1**), or
218 1750 µg carbaryl per kg of nectar after conversion to PPB. Therefore, HQ_{PPB} = 0.03, which
219 indicates that concentration would not pose a high risk to bees because it represents only a small
220 proportion of LD_{50}. In terms of risk to individual bees, if an adult honey bee foraging for pollen
221 consumed 0.0000435 kg of pumpkin nectar the day after spray [52] at a concentration of 44.1 µg
222 carbaryl / kg nectar, the bee would have ingested 0.002 µg of carbaryl, which is 1% of the LD_{50}
223 of 0.21 µg / bee. By comparison, for the same carbaryl concentration *HQ* = 210, which would be
224 interpreted as a potential risk to bees using a common level of concern (LOC) of HQ > 50 [53 –
225 54].

226 In addition to *HQ* and HQ_{PPB}, we calculated contact and dietary *Risk Quotient* ($RQ_{contact}$,
227 RQ_{pollen}, RQ_{nectar}) using the U.S. Environmental Protection Agency's BeeREX tool, which is
228 intended for foliar sprays applied to crops in bloom [52]. Risk quotient has the advantage over
229 *HQ* of taking into account the amount of the contaminated substance consumed or encountered
230 by a typical honey bee forager [53]. The BeeREX tool calculates dietary *RQ* separately for adult
231 honey bee pollen and nectar foragers and compares empirical values of residue concentrations in
232 pollen and nectar, multiplied by the typical amount of pollen and nectar consumed by a honey
233 bee nectar or pollen forager, to oral acute LD_{50}. Contact *RQ* is calculated by comparing the
234 chemical application rate, multiplied by a constant that represents the typical amount of chemical
235 encountered by a honey bee forager if it flies through a cloud of spray, to the contact acute LD_{50}.
236 Ideally, to assess bee risk of pesticide contamination from leaf contact, this index should be

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further modified to also take into account the proportion of a bee's body that comes into contact with the plant when it is resting on foliage, but that data was not available. For further details on how the BeeREX tool calculates risk quotient, see the US EPA document Guidance for Assessing Pesticide Risks to Bees, Appendix 3 [55].

To interpret risk to bees associated with each tissue type and chemical, risk index values were compared to a pre-determined level of concern (LOC) for each index and interpreted as 'low risk' if below the LOC or 'low risk cannot be concluded' if exceeding LOC [53]. *HQ* values above 50 [53 – 54] and *RQ* values above 0.4 [55] are considered a potential risk to pollinators. A threshold LOC has not yet been established for HQ_{PPB}, but a simple and direct way to interpret it would be that values greater than 1 indicate a significant likelihood of acute bee toxicity (because if $HQ_{PPB} > 1$, the quantity of pesticide in a pollen or nectar sample that has the same mass as an average honey bee worker exceeds honey bee LD_{50}).

Results

Foliar insecticide and fungicide spray residues were detected more frequently and in greater concentrations in pumpkin leaves than in pollen, nectar, or foraging bees (**Fig 1**, **Fig 2**). Overall, more than half of leaf samples (56%) tested positive for at least one insecticide and 12% for at least one fungicide (**Table 2**). Based on risk assessment quotients (**Table 3,** Supporting Information **S2 Table**), insecticide concentrations in leaves often exceeded levels of concern (**Fig 1**). However, those indices assume that a foraging bee would actually come into contact with all the chemical present on or in the leaf sample. Compared to leaves, fewer pollen (insecticide detected in 16%, fungicide in 16%) and nectar samples (14%, 0%) contained

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pesticide residues and those that did typically had lower concentrations (**Table 2**). However, pollen contained relatively high levels of the insecticides carbaryl and permethrin (**Table 3**). Of the six chemicals, only one insecticide (carbaryl) was detected in nectar. Similarly, we had low detection of insecticides (3%) and no fungicides in adult foraging bees (**Table 2**). Carbaryl was present in two bee samples at levels below acute LD_{50}; both of which were male *X. pruinosa* that were collected one day after a spraying event.

Fig 1. Insecticide detection in pumpkin tissues and bee visitors within one week after foliar spray application. The percent of samples in which a residue was detected is shown for each tissue type and time since spray. Percent detection of each chemical was calculated separately using only the data from farms where each was applied. Chemicals are color coded as follows: carbaryl (black), permethrin (orange), lambda cyhalothrin (yellow). Point size corresponds to the following six categories of maximum chemical concentration: < 100 PPB, 100 – 500, 501 – 1000, 1001 – 5000, 5001 – 10000, >10000. A diamond shape indicates that at the maximum concentration detected, HQ_{PPB} exceeded the level of concern for bee risk assessment ($HQ_{PPB} > 1$). A circle indicates $HQ_{PPB} < 1$.

Fig 2. Fungicide detection in pumpkin tissues and bee visitors within one week after foliar spray application. The percent of samples in which a residue was detected is shown for each tissue type and time (days since spraying event). Percent detection of each chemical was calculated separately using only the data from farms where each was applied. Chemicals are color coded as follows: triflumizole (green), quinoxyfen (blue), chlorothalonil (gray). Point size and shape are as in Fig 1.

Table 2. Detection and concentration of six agrochemical residues in pumpkin tissues and in bee visitors to flowers within one week after foliar spray application.

	Leaf				Pollen				Nectar				Bee			
Chemical	**N**	**$N_{Pos.}$**	**Min**	**Max**	**N**	**$N_{Pos.}$**	**Min**	**Max**	**N**	**$N_{Pos.}$**	**Min**	**Max**	**N**	**$N_{Pos.}$**	**Min**	**Max**
Carbaryl	28	15	**12.4**	**<u>27980.0</u>**	27	3	**26.7**	**583.1**	24	4	**11.0**	**44.1**	69	2	**22.8**	**89.1**
Permethrin	8	3	**<u>2071.7</u>**	**<u>4755.5</u>**	7	1	**57.9**	**57.9**	7	0	-	-	44	0	-	-
L. Cyhalothrin	4	2	**<u>677.6</u>**	**<u>1362.9</u>**	4	0	-	-	4	0	-	-	0	-	-	-
Insecticide	32	18	-	-	32	5	-	-	28	4	-	-	69	2	-	-
Triflumizole	8	3	305.4	**9023.1**	8	2	23.3	24.7	6	0	-	-	0	-	-	-
Quinoxyfen	0	-	-	-	17	4	26.8	37.1	13	0	-	-	15	0	-	-
Chlorothalonil	16	0	-	-	16	0	-	-	15	0	-	-	25	0	-	-
Fungicide	24	3	-	-	25	4	-	-	21	0	-	-	25	0	-	-

Chemicals are ordered from most to least frequently detected within the two categories – insecticide and fungicide. The number of samples tested (*N*) and the number in which a chemical residue was detected (number positive, $N_{Pos.}$) is given for each tissue. The number tested and detected was tallied separately for each chemical (and for each category) using only the data from farms where that chemical was applied. Minimum and maximum chemical concentrations are given in µg / kg (PPB). Bold font indicates that a concentration exceeded level of concern (LOC) for at least one of our three risk assessment indices ($HQ > 50$, $HQ_{PPB} > 1$, $RQ > 0.4$); bold underlined font indicates it exceeded LOC for all three. The number of farms where each chemical was applied, the rate of application, and the total number of spray events is provided in Supporting Information S1 Table.

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Table 3. Risk assessment for bees visiting pumpkin flowers within one week after foliar spray application, based on the maximum chemical concentrations found in each tissue.

Tissue	Chemical	*HQ*	HQ_{PPB}	*RQ*
Leaf	Carbaryl	**199857.14**	**23.98**	**96.06**
	Permethrin	**198145.83**	**23.78**	**67.19**
	Cyhalothrin	**35865.79**	**4.30**	**14.14**
	Triflumizole	**451.16**	0.05	0.05
	Chlorothalonil	ND	ND	0.07
Pollen	Carbaryl	**2776.67**	0.33	0.01
	Permethrin	**445.38**	0.05	<0.01
	Cyhalothrin	ND	ND	0.00
	Triflumizole	1.76	0.00	<0.01
	Quinoxyfen	0.37	<0.01	<0.01
	Chlorothalonil	ND	ND	0.00
Nectar	Carbaryl	**210.00**	0.03	0.06
	Permethrin	ND	ND	<0.01
	Cyhalothrin	ND	ND	0.00
	Triflumizole	ND	ND	<0.01
	Quinoxyfen	ND	ND	<0.01
	Chlorothalonil	ND	ND	0.00
Bee	Carbaryl	**509.14**	0.06	NA
	Permethrin	ND	ND	NA
	Quinoxyfen	ND	ND	NA
	Chlorothalonil	ND	ND	NA

Three risk quotients were calculated for each tissue: *HQ*, HQ_{PPB}, and *RQ*. RQ_{pollen} and RQ_{nectar} estimate risk for a honey bee foraging on pollen or nectar, respectively, based on our empirical values of chemical concentration in pollen and nectar. RQ_{leaf} (or contact *RQ*) was based on application rate of the pesticide and not on empirical concentration in the samples. Therefore, a value for chlorothalonil leaf *RQ* is given even though it was not detected in our leaf samples. Bold font indicates the value exceeded LOC ($HQ > 50$, $HQ_{PPB} > 1$, $RQ > 0.4$) and, therefore, low risk cannot be concluded [53]. Only pesticides for which data were available were included in this table. ND indicates that a tissue was tested for the chemical, but not detected. For risk assessment based on minimum chemical concentrations, see Supporting Information S2 Table.

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316 Among the six agrochemicals tested, only carbaryl, the most commonly used insecticide,
317 was detected in all four tissue types – leaves, pollen, nectar, and bees (**Table 2**). No additional
318 chemicals were found in nectar or bee samples. The insecticides lambda cyhalothrin and
319 permethrin were both detected in leaves, but only the latter was present in pollen as well. Two of
320 three fungicides (triflumizole and quinoxyfen) were detected in pumpkin tissues – quinoxyfen in
321 pollen only, and triflumizole in both leaves and pollen. The fungicide chlorothalonil was not
322 detected in any pumpkin tissues (similar to the results of Bloom et al. [23]), even though it was
323 used by three out of five of our growers (Supporting Information, S1 Table). The maximum
324 concentration varied strongly among chemicals (**Table 2**).
325 Typically, either the proportion of contaminated samples or the maximum concentration
326 of insecticides in pumpkin tissues decreased over the week following foliar application (**Fig 1**).
327 Overall, insecticide was found in 75% of leaves the day after spray, and 50% on Days 3 and 7
328 (**Table 4**). The likelihood of detecting insecticides in pollen the day after spray was 29%, and
329 decreased to 13% by Day 7. The day after spray 43% of nectar samples tested positive for the
330 insecticide carbaryl, which decreased to 0% by Day 7. Although detection decreased over time,
331 our data suggest that some insecticides persisted in leaves and pollen beyond one week. For
332 example, carbaryl was found in 4 out of 7 leaves sampled the day before spray (**Table 5**) across
333 three farms, including one positive sample from a farm where that chemical was not applied
334 during our study period. At one farm that applied carbaryl, then applied it again nine days later at
335 a lower rate, carbaryl was detected in leaves during both sampling rounds except for Day 7 after
336 the second application. It was not common among growers in this study to apply multiple
337 insecticides at a time, but at one farm that did use two in the same spray event, 50% of leaves the

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next day (but not pollen, nectar, or bees) contained both chemicals (Supporting Information, **S3 Table**).

Table 4. Detection of insecticide and fungicide residues in pumpkin tissues and bees by days since foliar spray application.

Tissue	Time	Any Insecticide(s)	Any Fungicide(s)
Leaf	-1	50	0
	1	75	17
	3	50	17
	7	50	17
	total	56	13
Pollen	-1	0	14
	1	29	20
	3	14	17
	7	13	17
	total	16	16
Nectar	-1	0	0
	1	43	0
	3	17	0
	7	0	0
	total	14	0
Bee	-1	0	0
	1	8	0
	total	3	0

The percent of samples (rounded to the nearest integer) in which any one or more insecticides or fungicides were detected is given by tissue type and time since spray (-1 = one day before, 1 = one day after, 3 = three days after, 7 = seven days after). Percent detection was calculated separately for insecticides and fungicides using only data from farms where sprays from each category were used.

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Table 5. Detection of carbaryl insecticide in pumpkin and bee tissues before a spray event through one week after.

Tissue	Time	N	$N_{Pos.}$	Min	Max	*HQ*	HQ_{PPB}	*RQ*
Leaf	-1	7	4	19.0	225.2	**1608.5**	0.19	NA
	1	7	6	13.8	27980.0	**199857.1**	**23.98**	NA
	3	7	3	12.4	19020.0	**135857.1**	**16.30**	NA
	7	7	2	145.3	244.9	**1749.3**	0.21	NA
Pollen	-1	7	0	-	-	0.00	0.00	0.00
	1	7	2	26.7	108.8	**518.1**	0.06	0.01
	3	6	1	583.1	583.1	**2776.6**	0.33	<0.01
	7	7	0	-	-	0.00	0.00	0.00
Nectar	-1	7	0	-	-	0.00	0.00	0.00
	1	7	3	14.4	44.1	**210.1**	0.03	0.06
	3	5	1	11.0	11.0	**52.4**	0.01	0.02
	7	5	0	-	-	0.00	0.00	0.00
Bee	-1	43	0	-	-	0.00	0.00	NA
	1	26	2	22.8	89.1	**509.2**	0.06	NA

Time since spray is indicated as -1 = one day before, 1 = one day after, 3 = three days after, 7 = seven days after. The number of samples tested (*N*) and the number in which carbaryl was detected ($N_{Pos.}$) is given for each tissue and time. Minimum and maximum concentrations are in units of µg / kg (PPB). Risk quotients *HQ*, HQ_{PPB}, and RQ_{pollen} *and* RQ_{nectar} are based on the maximum residue concentration. RQ_{leaf} is not available by day because it was estimated from initial application rate, not leaf concentration. Bold font indicates concentration exceeded a threshold level of concern of $HQ > 50$, $HQ_{PPB} > 1$, or $RQ > 0.40$. For detection of the other chemicals by day and tissue type, see Supporting Information S4 – S7 Tables.

The percent of pumpkin tissue samples that contained fungicides remained relatively constant in the week following application (**Table 4**), though the maximum concentrations in leaves decreased with time (**Fig 2**). When growers applied more than one fungicide over the course of the study period, multiple were taken up by pollen, but not by other tissues (Supporting Information, **S3 Table**). For example, at one farm that applied quinoxyfen and triflumizole fungicides a week apart, both were detected in 2 out of 6 pollen samples after the second spray event.

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Discussion

Much research has already reported acute and sublethal effects of systemic insecticides on beneficial insects like pollinators in agricultural landscapes [5, 7 – 8]. There have been fewer empirical studies on translocation of non-systemic pesticides sprayed on crop foliage to non-target plant regions (but see [56]), but models predict these chemicals can move into floral tissues under certain conditions based on their chemical properties [57]. Here we add to the emerging knowledge of frequency and persistence of non-systemic foliar-applied pesticides in crop leaves, pollen, nectar, and pollinating bees. As expected, we detected insecticide residues with high frequency and concentration in pumpkin leaves through the week following application in concentrations that may pose a risk to bees if they have extended contact with leaves (e.g., while females groom pollen onto their scopa or rest under foliage during inclement weather). On day 1 after spray, insecticides and fungicides were present in floral tissue (especially pollen), which raises questions about whether spray droplets leak into unopened flowers at the time of application or translocate later through foliage or petals and become incorporated into nectar and pollen. We detected insecticide residues in few bee samples the day after spray, and the bees that tested positive (male squash bees) have life history traits that bring them into prolonged contact with sprayed crop plants.

A key strength of our study is that it was a ‘natural’ experiment conducted in fields managed by local growers using chemicals and rates of application that they typically use. Tradeoffs of this approach are an unbalanced design and low sample sizes for some agrochemicals and time points, which limited our ability to draw broader conclusions about their mobility in plants and the hazard they pose to pollinators.

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Detection of foliar-applied pesticides in pumpkin tissues and pollinators in the week after spray

There are two likely explanations for how non-systemic pesticides sprayed on foliage still reached pumpkin pollen, nectar, and foraging male squash bees, even though flowers were unopened during application. First, chemical-laden droplets may have leaked into flowers during spray and directly contaminated pollen and nectaries if the buds were not tightly sealed. Similarly, spray droplets may have also accumulated in wilted flowers where male squash bees were resting for the night, although only one chemical out of six (carbaryl) was detected in squash bees. Second, chemicals on the leaf surface or in stomal apertures may have crossed lipid boundaries, mobilized in phloem, and become incorporated into developing flowers. Environmental conditions, the addition of adjuvants (*e.g.*, surfactants), and the chemical properties of pesticides such as water solubility, lipophilicity, ionizability, and molecular weight all contribute to their ability to infiltrate a plant's epidermis and translocate to non-target regions [58 – 62]. These xenobiotic chemicals move through plants along both lipophilic and aqueous pathways, and undergo complex and transformative interactions [61]. Pesticides applied to crop plants are typically slightly lipophilic so that they can permeate the cuticle, but not be completely adsorbed in intra-cuticular non-crystalline wax [60]. They are also often weak electrolytes that can dissolve in water and be transported to different plant compartments [58 – 59]. Overall, pesticides that are somewhat lipophilic weak acids are most mobile; they are bioactive under certain conditions yet do not ionize at the pH levels of aqueous reservoirs inside plant cells and become 'ion trapped' before reaching phloem to disperse [58].

The lipophilicity and other properties of our target agrochemicals can partially explain their biological fate in pumpkin tissues in our dataset [58 – 61]. For example, three of the four

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426 pesticides we detected in pollen – permethrin, quinoxyfen, and triflumizole – are lipophilic
427 ($logK_{OW} > 4.5$, **Table 1**), making them likely to adsorb onto lipid-rich tissues like the pollen coat,
428 if they are not first trapped in leaf cuticle waxes. Another insecticide we detected in pollen –
429 carbaryl – has only moderate lipophilicity ($logK_{OW} = 2.4$), giving it more potential to mobilize
430 vascularly and be incorporated into developing floral tissue. Consistent with this reasoning, we
431 recorded a five-fold increase in carbaryl concentrations in pollen from the first to the third day
432 after spray (**Table 5**). However, our low sample sizes make us unable to resolve whether those
433 values are within the typical range of variation for pollen or are evidence of systemic uptake.
434 Another consideration for carbaryl is that it also has a low molecular weight and is a very weak
435 acid (dissociates more readily at low pH levels), making it likely to cross membranes and to
436 ionize and bind with compounds in relatively acidic compartments inside cells like vacuoles (pH
437 ~ 5) before it reaches phloem (pH ~ 8) [58]. These properties contribute to its persistence in
438 leaves, instead of translocation to pollen and nectar that bees eat. This example of carbaryl
439 demonstrates the complexity of interactions pesticides undergo within crop plants; no one factor
440 can fully explain their presence.

441 In addition to the properties of the agrochemicals, differences in the composition of plant
442 tissues and the timing of their availability also influence their uptake of pesticides. For example,
443 pollen has a higher lipid and protein content [63] and takes more time to develop than nectar,
444 which may contribute to its greater pesticide load in ours and other studies (reviewed in [14]).
445 We detected four out of six agrochemicals (two insecticides and two fungicides) in pollen after
446 foliar application. Fungicide was detected almost as frequently in pollen as in leaves (**Table 4**).
447 In contrast, only one insecticide and no fungicides were found in nectar. Nectar is secreted after

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448 flower anthesis [64], and so would most likely not have been present in unopened flowers during
449 spray.

450 Based on our detection of pesticides in pollen, it is likely that bee larvae fed on pumpkin
451 pollen from these fields were exposed to multiple agrochemicals simultaneously, with the
452 potential for additive, or even interactive toxicity [40, 44]. However, in this field study, wild bee
453 nest locations were unknown and we were not able to test larvae from the nests of the adult
454 foragers we collected. Laboratory trials with contaminated pollen fed to larvae of honey bees or
455 other agriculturally important pollinators could illuminate the effects of consuming pesticide
456 residues on larval development and survival [38, 40, 65 – 66]. Population-level studies that
457 investigate multiple exposure routes and outcomes for larvae and adult bees such as that by
458 Willis-Chan and Raine [19] are best able to assess total risk.

459 Consumption of contaminated pollen and nectar is arguably a greater concern for bees
460 than leaf contact exposure because it can impact larvae as well as adults and lead to population-
461 level effects [13 – 14]. However, the persistence of the insecticide carbaryl in leaves between
462 spraying events also raises concern from the pollinator perspective because it prolongs their risk
463 of exposure. In addition, the high concentrations of pesticides in leaves during the week after
464 foliar spray led to the highest bee risk quotient values in our dataset (**Table 3**). However, risk
465 assessments that aim to quantify bee toxicity from leaf contact may overestimate the true risk to
466 bees since they assume that a bee actually encountered the entire dose of the chemical present in
467 the leaf sample. Instead, only the bee's lower legs and tip of the abdomen contact a leaf on which
468 it is resting (*authors' personal observations*).

469 Bee contamination was infrequent in our dataset. We detected one insecticide (carbaryl,
470 the most widely used by growers in this study) in two out of 69 bee samples and at

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concentrations that were not acutely lethal. Both samples consisted of bees that we expected to have the highest risk of exposure – males of *X. pruinosa*, a *Cucurbita* pollen specialist bee species, which spend much of their adult lives resting in the wilted flowers. Since so few bees tested positive the day after spray application (when they would have come into contact with the greatest concentrations of pesticides), it seems unlikely that additional sampling on Days 3 and 7 would have revealed higher contamination rates. However, our data may be subject to survivorship bias; our sampling method only captured living bees that were still able to forage the day after a spray event, and not those that had encountered or accumulated a lethal dose. As no other foraging bee species (bumble bees or honey bees) tested positive, it seems likely the squash bees came into direct contact with the agrochemical, rather than consuming it through contaminated nectar. We note that wild bees foraging or nesting in pumpkin fields are also at risk of exposure to agrochemicals through other non-dietary media (*e.g.,* contaminated soils or water in or near crop fields), which can contribute to multiple additive exposures, but these routes are beyond the scope of our study [12, 67].

Conclusions

Our results strengthen the evidence that agricultural chemicals applied to foliage are present in crop plant pollen and nectar, as well as leaves after spray, at least in the short term. Therefore, applying pesticides to crops when flowers are present, but unopened, fails to completely protect pollinators from exposure. The six chemicals we tested differed in their patterns of detection in pumpkin tissues, likely due to their physicochemical properties. Pesticides were infrequently found in adult bees foraging on pumpkin flowers in our dataset, and only in squash bees and not in social taxa, likely because of bee natural history traits. The persistence of some pesticides in leaves and pollen up to a week following application, often at concentrations that exceeded

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494 levels of concern for bees, should be a consideration when managing pollinator-dependent crops.
495 If standardized translocation rates for crop plant pollen and nectar were included in pesticide
496 properties databases, it would allow for better integrated pest management decisions in
497 pollinator-dependent crops. More research is also needed on the effects of consumption of
498 contaminated pollen by bee larvae and how that translates to population-level effects.
499

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Supporting Information Captions

S1 Table. Summary of pesticides applied during the study period. To preserve the anonymity of growers, farms are listed by identifiers A – E. The approximate location of each grower's farm, rounded to the nearest 1/10th decimal degree for anonymity, is as follows: A – 40.1N, -82.8W; B – 40.0, -82.4; C – 40.1, -82.7; D – 40.0, -82.6; E – 39.9, -82.8. Spray events during the study period are numbered sequentially within farm. For example, at farm B spray event 1, the grower applied both carbaryl and permethrin on the same day. Pesticide residue names are given as their active ingredients (for commercial names see main text Table 1). The rate of application is given in fluid ounces per hectare, either as a range or a single concentration, as reported by the growers. A † after the chemical name indicates that we were not able to test for it.

S2 Table. Risk assessment for bees visiting pumpkin within one week after foliar spray, based on the minimum chemical concentrations detected in each tissue type. Three risk quotients were calculated for each tissue: *HQ* (Hazard Quotient), HQ_{PPB} (Hazard Quotient in which LD50 was first converted to bee-relevant PPB), and *RQ* (Risk Quotient from the US EPA BeeREX tool). See Methods for details of index calculations. Bold font indicates the index value exceeded the threshold level of concern ($HQ > 50$, $HQ_{PPB} > 1$, $RQ > 0.40$), and, therefore, low risk cannot be concluded. Only pesticides for which data were available were included in this table. For risk assessment based on the maximum chemical concentrations found in each tissue, see main text Table 3.

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S3 Table. Detection of one versus multiple insecticide and fungicide residues in pumpkin and bee tissues at farms that applied multiple chemicals. The percent of samples (rounded to the near integer) in which any one or two different chemicals were detected was calculated separately for insecticides and fungicides using only data from farms where multiple sprays from each category were used. Four out of five farms used a mix of both insecticides and fungicides during the study period, but no farms sprayed all three of our focal insecticides or all three fungicides during the study. Time since foliar application is indicated as -1 = one day before, 1 = one day after, 3 = three days after, 7 = seven days after.

S4 Table. Detection of permethrin insecticide in pumpkin and bee tissues before a spray event through one week after. Time since spray is indicated as -1 = one day before, 1 = one day after, 3 = three days after, 7 = seven days after. The number of samples tested (*N*) and the number in which permethrin was detected ($N_{Pos.}$) is given for each time. Minimum and maximum concentrations are in units of µg / kg (PPB). Risk quotients *HQ*, HQ_{PPB}, and RQ_{pollen} *and* RQ_{nectar} are based on the maximum residue concentration. RQ_{leaf} is not available by day because it was estimated from initial application rate, not leaf concentration. Bold font indicates concentration exceeded a threshold level of concern of $HQ > 50$, $HQ_{PPB} > 1$, or $RQ > 0.40$.

S5 Table. Detection of lambda cyhalothrin insecticide in pumpkin and bee tissues before a spray event through one week after. Time since spray is indicated as -1 = one day before, 1 = one day after, 3 = three days after, 7 = seven days after. The number of samples tested (*N*) and the number in which lambda cyhalothrin was detected ($N_{Pos.}$) is given for each time. Minimum and maximum concentrations are in units of µg / kg (PPB). Risk quotients *HQ*, HQ_{PPB}, and RQ_{pollen} *and* RQ_{nectar} are based on the maximum residue concentration. RQ_{leaf} is not available by day because it was estimated from initial application rate, not leaf concentration. Bold font indicates concentration exceeded a threshold level of concern of $HQ > 50$, $HQ_{PPB} > 1$, or $RQ > 0.40$.

S6 Table. Detection of triflumizole fungicide in pumpkin and bee tissues before a spray event through one week after. Time since spray is indicated as -1 = one day before, 1 = one day after, 3 = three days after, 7 = seven days after. The number of samples tested (*N*) and the number in which triflumizole was detected ($N_{Pos.}$) is given for each time. Minimum and maximum concentrations are in units of µg / kg (PPB). Risk quotients *HQ*, HQ_{PPB}, and RQ_{pollen} *and* RQ_{nectar} are based on the maximum residue concentration. RQ_{leaf} is not available by day because it was estimated from initial application rate, not leaf concentration. Bold font indicates concentration exceeded a threshold level of concern of $HQ > 50$, $HQ_{PPB} > 1$, or $RQ > 0.40$.

S7 Table. Detection of quinoxyfen fungicide in pumpkin and bee tissues before a spray event through one week after. Time since spray is indicated as -1 = one day before, 1 = one day after, 3 = three days after, 7 = seven days after. The number of samples tested (*N*) and the number in which quinoxyfen was detected ($N_{Pos.}$) is given for each time. Minimum and maximum concentrations are in units of µg / kg (PPB). Risk quotients *HQ*, HQ_{PPB}, and RQ_{pollen} *and* RQ_{nectar} are based on the maximum residue concentration. RQ_{leaf} is not available by day because it was estimated from initial application rate, not leaf concentration. Bold font indicates concentration exceeded a threshold level of concern of $HQ > 50$, $HQ_{PPB} > 1$, or $RQ > 0.40$.

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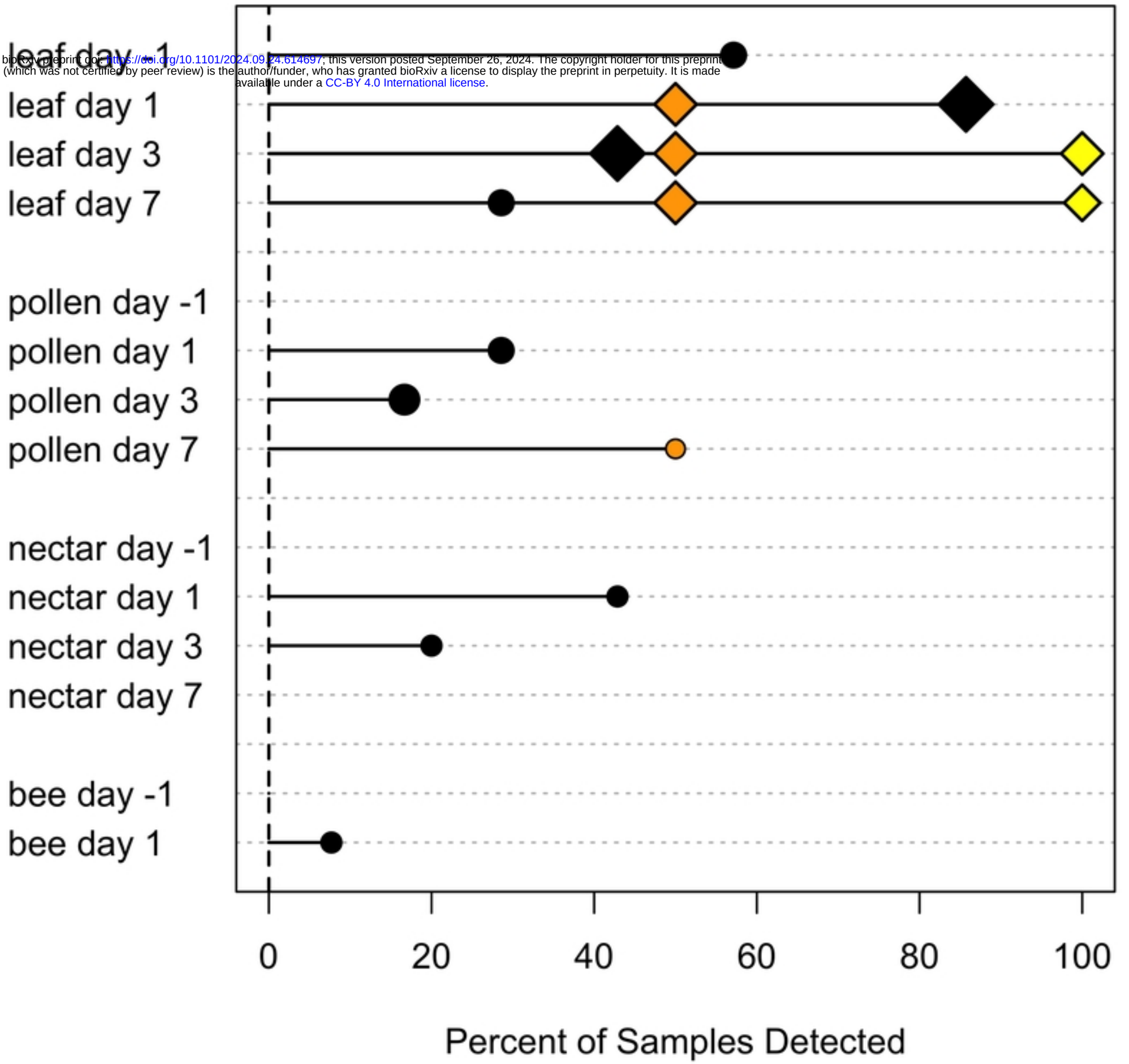

Figure 1

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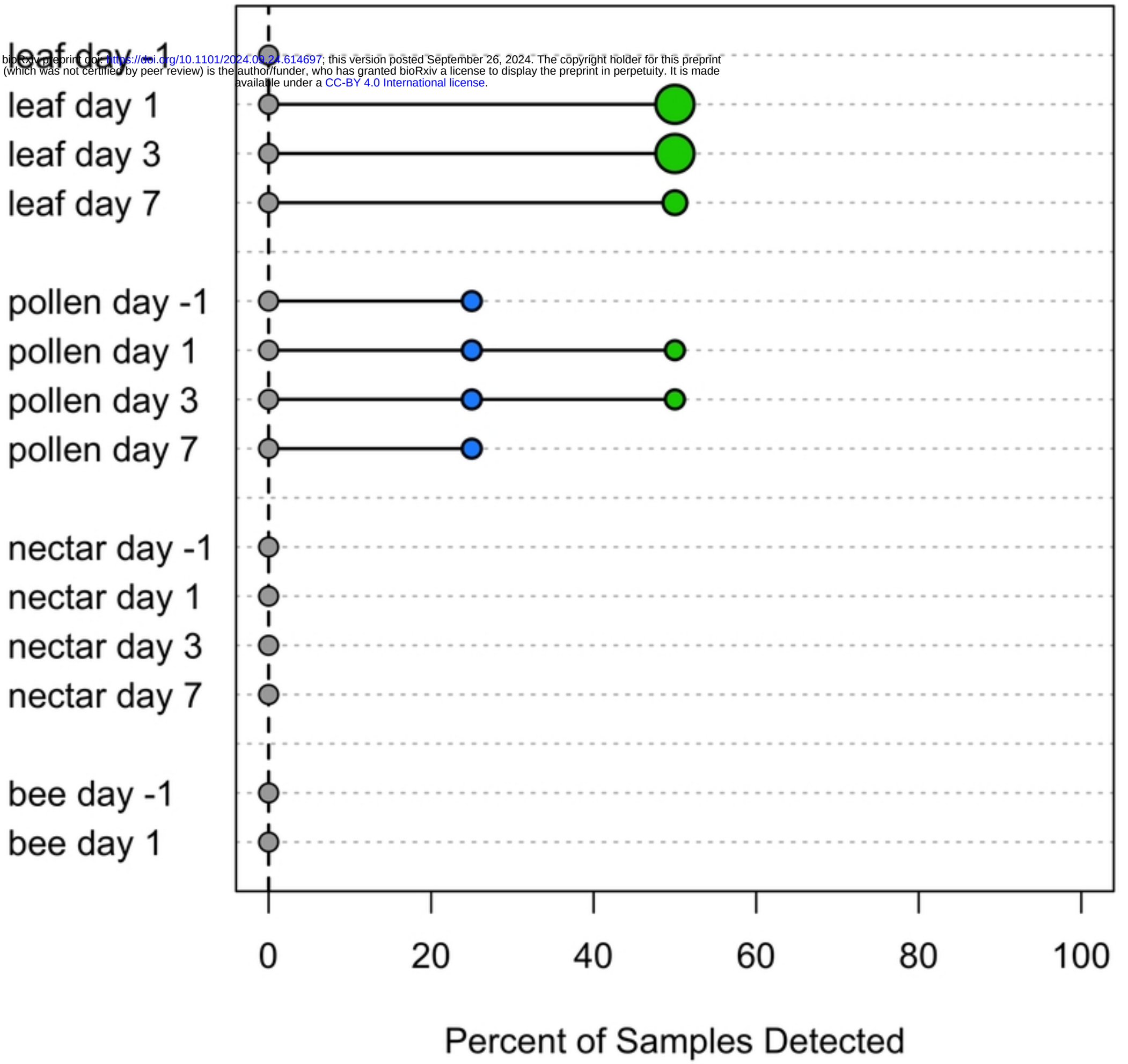

Figure 2