

Crop Pollination Exposes Honey Bees to Pesticides Which Alters Their Susceptibility to the Gut Pathogen *Nosema ceranae*

Informative, descriptive title in sentence form with a verb.
Please note that not all publications accept sentence titles.

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Overall, abstract is informative and concise

Use of "We" with active voice

Abstract

Recent declines in honey bee populations and increasing demand for insect-pollinated crops raise concerns about pollinator shortages. Pesticide exposure and pathogens may interact to have strong negative effects on managed honey bee colonies. Such findings are of great concern given the large numbers and high levels of pesticides found in honey bee pollen. Thus it is crucial to determine how field-relevant combinations and loads of pesticides affect bee health. We tested pollen from bee hives in seven major crops to determine [1] what types of pesticides bees are exposed to when for pollination of various crops and [2] how field-relevant pesticide blends affect bees' susceptibility to the gut pathogen *Nosema ceranae*. Our samples represent pollen collected by foragers for use by the colony, and do not necessarily reflect foragers' roles as pollinators. In blueberry, cranberry, cucumber, pumpkin and watermelon bees collected pollen exclusively from weeds and wildflowers during our sampling. Thus more attention must be paid to how honey bees are exposed to pesticides outside of the field in which they are placed. We detected 35 different pesticides in the sample pollen, and found high fungicide loads. The insecticides esfenvalerate and phosmet were at a concentration higher than their known lethal dose in at least one pollen sample. While fungicides are typically seen as fairly safe for honey bees, we increased probability of *Nosema* infection in bees that consumed pollen with a higher fungicide load. Our results need for research on sub-lethal effects of fungicides and other chemicals that bees placed in an agricultural setting are exposed to.

Principal conclusion places paper in appropriate context with other studies and highlights areas for future research

Principal objectives are clearly identified with numbers.

Introductory sentences provide background and context.

Results concisely summarized.

Citation: Pettis JS, Lichtenberg EM, Andree M, Stitzinger J, Rose R, et al. Crop Pollination Exposes Honey Bees to Pesticides Which Alters Their Susceptibility to the Gut Pathogen *Nosema ceranae*. PLoS ONE 8(7): e70182. doi:10.1371/journal.pone.0070182

Editor: Fabio S. Nascimento, Universidade de São Paulo, Faculdade de Filosofia Ciências e Letras de Ribeirão Preto, Brazil

Received March 25, 2013; **Accepted** June 16, 2013; **Published** July 24, 2013

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Funding: Funding for this study was provided by the National Honey Board (<http://www.honey.com/>) and the USDA-ARS Areawide Project on Bee Health (http://www.ars.usda.gov/research/projects/projects.htm?accn_no=412796). Neither the Honey Board nor USDA-ARS Program Staff had a role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: Dennis vanEngelsdorp is a PLOS ONE Editor. All other authors have declared that no competing interests exist. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

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As appropriate, introduction has a funnel shape.

Introduction begins broadly

and provides background

and context: in this case,

populations in many North American

the importance of

4] and increasing cultivation of honeybees to agriculture.

Habitat destruction, pesticide use, pathogens and climate change

are thought to have contributed to these losses [2,7,8]. Recent

research suggests that honey bee diets, parasites, diseases and

pesticides interact to have strong negative effects on managed

honey bee colonies [9,10].

exposure to sub-lethal doses of pesticides

may alter susceptibility to other

pathogens.

Introduction

Recent research is uncovering diverse sub-lethal effects of pesticides on bees. Insecticides and fungicides can alter insect and spider enzyme activity, development, offspring sex ratios, mobility, navigation, behavior, learning and immune function [9,13,14]. Reduced immune functioning is of particular interest because recent disease-related declines of bees including honeybees [15] and bumblebees [16] questions and foreshadows study's research. Pesticide and toxin exposure increases susceptibility to mortality from diseases including the gut parasite *Nosema* spp. [14,15]. These increases may be linked to insecticide-induced alterations to immune system pathways, which have been found for several insects, including honey bees [22,24–26].

Surveys of colony food reserves and building materials (i.e. wax) have found high levels and diversity of chemicals in managed honeybee colonies [18,27,28]. These mixtures have strong potential to affect individual and colony immune functioning. However, almost all research to-date on pesticides' effects on pathogen susceptibility has focused on a single chemical to test bees [16]. Because pesticides may have interactive effects on non-target organisms (e.g. [29]), it is crucial to determine how real world combinations and loads of pesticides affect bee health.

One pathogen of major concern to beekeepers is the endoparasitic fungal infections of the paragraph.

Introduction continues to narrow; here, the relevance of pathogens.

Funnel shape continues as introduction becomes more specific.

adversely affect honey bee colony health, and can result in complete colony collapse [30]. Infection with *Nosema* in the autumn leads to poor overwintering and performance the following spring [31], and queens can be superinfected soon after becoming infected with *Nosema* [32]. We chose *Nosema* as a model pathogen because earlier work [13,14] had demonstrated an interaction with pesticide exposure.

This study addresses two important questions. 1) What types of pesticides do bees be exposed to in major crops? While multiple studies characterized the pesticide profile of various materials in the nest [27,28], few have looked at the pollen blends back to the nest. 2) How do field-relevant pesticides blends affect bees' susceptibility to infection by the *Nosema* parasite?

Methods

Ethics Statement

Pollen was collected from honey bees with permission of the beekeepers and the land owners.

Hive Selection and Pollen Collection

We collected pollen carried by foraging honey bees returning to the hive for nine hives in seven crops: almond, apple, blueberry, cranberry, cucumber, pumpkin, and watermelon (Table 1). For each crop, we selected three fields that were separated by at least 1 km. These fields were deployed in these fields for pollination services to meet the needs. Within each selected field, we chose the hives with the strongest foraging forces by monitoring the bee yard for 5–10 min, and attached plastic pollen traps (Brushy Mountain Bee Farm, Moravian Falls, NC) to these hives. Pollen traps collect the pollen pellets bees carry on their hind tibiae in flattened regions called corbiculae. Bees use this pollen to make food for larvae. We checked traps after three days, and removed them if they contained less than 5 g of pollen. Traps with less than 5 g of pollen or for 10 days were replaced. We removed pollen from traps in 50 mL centrifuge tubes and stored the samples on ice until they could be transferred to a -29°C freezer in the lab.

Because our first round of pollen trapping in cranberry fields yielded little pollen, we collected pollen from each hive in cranberry fields twice: early in the flowering season and late in the season. We separate these samples in data analyses, referring to them as "Cranberry early" and "Cranberry late."

We measured the wet weight of each pollen sample, and compared the quantity of pollen collected by hives in different

Justification for research model

Crop Pollens Affect Bee Health

Words like "then" help readers understand the sequence of experimental procedures.

crops via a Kruskal-Wallis test followed by a parametric Tukey-type test (using the R pack [33]). We then divided each sample into three subsamples. Each subsample was sorted by color and then each group of colored pollen pellets were identified (see Fig 1). One subsample was sent to the USDA's Agricultural Marketing Service Laboratory in Gastonia, NC for pesticide analysis; and a 10 g subsample was sent to the USDA-ARS Bee Research Laboratory (Beltsville, MD) for the *Nosema* assay. Because almond pollen was collected after all other crops, we were unable to include it in the pesticide analysis. In cases where the total amount of pollen collected from a single colony was less than 6 g, all the pollen was used for pesticide analysis.

Past tense used, as is appropriate in a methods section.

Research questions clearly identified with numbers.

Many journals require an Ethics Statement.

Use of "we" with active voice makes methods section easier to read.

Explanation of why methods were appropriate

Pollen Identification

Each 5 g pollen subsample was dehydrated in a drying oven at 40°C. We considered a sample to be dry when its weight did not change between two consecutive time points (measured every 4–6 h). Typically pollen dried in 12–18 h. To identify pollen types collected by the bees, we sorted the pollen in each color, quantified each color by comparing to Standard color palettes, re-weighed after color separation.

Addresses method limitations

color from each subsample on a separate slide. We prepared each slide by grinding 2 pollen pellets in 2 mL water and letting them dissolve to form a slurry. We placed a small amount of slurry on a slide with a drop of silicon oil, and covered slides and sealed with clear nail polish after letting air bubbles escape for 48 h. We visually identified each pollen type under 400x magnification by comparing with published reference collections [34–36]. Visual identification of pollen grains through comparison with voucher or reference specimens is standard in pollination biology.

Genus and species names provided.

Similarities between closely related pollens, however, prevent identification to genus or species with this method [39].

Because of this limitation, we assumed that all pollen collected in apple (*Malus domestica*) orchards that was identified as *Malus* sp. was from apple trees, and that all pollen in the Cucurbitaceae family

collected in cucumber (Cucurbitaceae, *Cucumis sativus*) fields was from cucumber flowers.

Table title is informative; table is understandable without reference to the text.

For each subsample, we estimated pollen diversity as the number of different pollen colors collected from that bee hive. We also calculated the proportion, by weight, of the pollen that was identified as belonging to the target crop's genus. Many samples

could only be identified to genus, so assessing target genus rather than target crop permitted a more inclusive estimate.

Table 1. Quantity and diversity of pollen collected in pollen traps on individual honey bee hives.

| Crop | Location | Mean grams of pollen collected (se) | Mean number of pollen types (se) |
|--------------------------|-----------------------------------|-------------------------------------|----------------------------------|
| Almond | Rosedale, CA; Kern County | 42.0 (9.1) ^{a,b} | 1.7 (0.2) ^{a,b} |
| Apple | York Springs, PA; Adams County | 26.7 (2.6) ^a | 4.9 (0.5) ^c |
| Blueberry | Deblois, ME; Washington County | 4.1 (1.5) ^b | 6.0 (1.0) ^c |
| Cranberry (early season) | Hammonton, NJ; Atlantic County | 13.0 (2.5) ^{a,b} | 4.0 (1.0) ^{b,c} |
| Cranberry (late season) | Hammonton, NJ; Atlantic County | 13.9 (3.8) ^{a,b} | 4.1 (0.6) ^{b,c} |
| Cucumber | Cedarville, NJ; Cumberland County | 8.1 (2.7) ^b | 5.5 (1.3) ^{b,c} |
| Watermelon | Seaford, DE; Sussex County | 27.1 (11.2) ^{a,b} | 7.1 (1.2) ^c |
| Pumpkin | Kutztown, PA; Berks County | 98.6 (29.0) ^{a,b} | 3.7 (0.6) ^{b,c} |

Letters indicate statistically different groups.

doi:10.1371/journal.pone.0070182.t001

Kruskal-Wallis tests to determine whether either of these measures differed with the crop in which sampled bee hives were placed.

Pesticide Analysis

We determined the identity and abundance of pollen types present in pollen samples collected from

For each field sampled ($n=19$), we pooled pollen from the three hives for analysis. One early-season cranberry field and one cucumber field did not yield sufficient pollen in traps for pesticide analysis. Methods follow the LC/MS-MS and GC/MS methods for pollen analysis described in Mullin et al. [27]. We used these data to determine the total number of pesticides detected in each sample, each sample's total pesticide load, and the diversity and

in each of 10 categories: insecticides, fungicides, several insecticide types (carbamates, cyclodienes, nematicides, organophosphates, oxadiazines and permit comparison between categories with different numbers of elements, we calculated diversity as the proportion of pesticides from a category found in a given sample, and load as the total load divided by the number of chemicals in that category. We only calculated diversity for categories with at least three chemicals.

The total number of pesticides present and total load did not meet parametric assumptions. We thus analyzed how these variables differ between crops using non-parametric Kruskal-Wallis tests. When separated by category and log-transformed, pesticide loads did meet parametric assumptions. We thus determined whether load varied by pesticide category using a general linear mixed model with sample as a random effect, to control for the fact that our regression included one data point per category from each sample. Insufficient degrees of freedom prevented us from expanding this model to include crop. We thus asked whether the pesticide load and diversity varied with crop for each category using one Kruskal-Wallis test per category and applying a sequential Bonferroni correction [40] across pesticide categories to control for multiple comparisons.

Nosema Infection

**Includes material
information such as
company and
tion**

The *Nosema* infection experiment is similar to published methods [10] disease-free honey bees from each of three commercial bee breeders at the Bee Research Laboratory. Each bee was placed in a cage with two other bees in a group upon emergence, with the ten bees in each cage from the same colony housed together in a single cage (12×12×12 cm). Each group of bees was fed 1 g of pollen mixed with 0.5 mL of syrup (1:1 sucrose to water by weight), which they fully consumed in 2–4 days. These pollen cakes were placed in small petri dishes with the laboratory cages. Pollen from either one of the crop fields or one of two control diets were used. The pollen control group (“BRL”) was fed a mixed pollen diet prepared by the USDA-ARS Bee Research Laboratory. This pollen was collected in the desert Southwest (Arizona Bee Products, Tucson, AZ) and tested as pesticide-free by the USDA Agricultural Marketing Service prior to use. A protein control group was fed an artificial honey bee pollen substitute, MegaBee®. The *Nosema* inoculum was freshly prepared by mixing *Nosema* spores isolated from an infected colony (details provided in [26]) with 50% sucrose solution to obtain a concentration of ca. 2 million spores per 5 mL. We fed 5 mL of the *Nosema* inoculum to each cage during the first two days of adult life, then provided bees with *ad libitum* access to clean 50% (w/v) sucrose solution. We collected bees 12 days after infection and examined them for the presence or absence of *N. ceranae* spores by homogenizing individual abdomens in 1 mL distilled water. Here we focus only

Subheadings guide readers through the paper.

Citation of another paper for methods description

on infection prevalence, the number of individuals and spores.

To look for potential effects of individual susceptibility to *Nosema* infection, we calculate and its 95% confidence interval for bees becoming infected after consuming pollen with a specific pesticide. Relating the chance of developing a disease after a particular exposure [14], here each pesticide. A relative risk value of one indicates that the probability of infection is equal between exposed and non-exposed groups.

the reader and explain why methods were appropriate.

We further tested effects of pesticides in pollen on measured *Nosema* prevalence using a generalized linear mixed model with a bee's *Nosema* status as the response variable, the source hive and pesticide variables as fixed effects, and the pollen sample fed to the bee as a random effect. Collinearity prevented developing a full model to investigate in detail how pesticides and pollen source affect bees' susceptibility to *Nosema* infection. We thus selected for analysis two measures that vary with crop and are not nested: total pesticide diversity and fungal load. To graph logistic regression results in a meaningful manner, we followed recommendations [42,43] and a modification of the `logistf` function in the R `poppbio` package [44] that shows our model's fit in parentheses.

Words like "further tested" help orient the reader and explain why methods were appropriate.

Results

Pollen Collection

Bee colonies collected different amounts of pollen in the different crops (Table 1; Kruskal-Wallis $p = 0.0001$). Pollen diversity, estimated by number of differently colored pollen pellets caught in traps, varied by crop (Table 1; Kruskal-Wallis $p = 0.0014$). The proportion of pollen that bees brought to the target crop, except for almond and watermelon (mean \pm se = 0.33 ± 0.05 ; Table S1), like pollen weights, this proportion dramatically differed between crops (Fig. 1; $H_7 = 44.86$, $p < 0.0001$). Notably, none of the pollen trapped from hives in blueberry, cranberry (early and late), pumpkin or watermelon fields was from the target crop.

Statistical values
are in parentheses,
making results
section easier to
read.

Pesticide Analysis

All pollen collected in this study contained pesticides (Table 2; mean \pm se = 9.1 ± 1.2 different chemicals, range 3–21). Pesticide loads ranged from 23.6 to 51,310.0 ppb ($11,760.0 \pm 3,734.2$ ppb). The maximum pesticide concentration in any single pollen sample exceeded the median lethal dose (LD_{50} , the dose required to kill half a population within 24 or 48 h) for esfenvalerate and phosmet (Table 2). The number of pesticides detected in trapped pollen varied by the crop in which the bee hives were located (Kruskal-Wallis test: $H_6 = 12.96$, $p = 0.04$), but the total not ($H_6 = 11.21$, $p = 0.08$) (Fig. 2).

Sentences are short and concise, allowing results to be clearly understood

We found insecticides and fungicides in all 1 in 23.6% of, pollen samples. Insecticides collected by the bees came from seven categories: oxadiazines in 10.5%, neonicotinoids in 15.8%, 31.6%, cyclodienes in 52.6%, formamidines in 52.6%, organophosphates in 63.2%, and pyrethroids in 100% of pollen samples. Both neonicotinoids and oxadiazines were present only in pollen collected by bees in apple orchards (Figs. 3, S1). Within a sample, pollen fungicide loads were significantly higher than loads of herbicides or any of the insecticide categories (Fig. 4; GLMM, likelihood ratio test: $\chi^2 = 121.9$, $df = 8$, $p < 0.0001$). short and concise, allowing results to be clearly understood.

After adjusting for multiple comparisons, pesticide loads did not vary by crop for any pesticide category (Fig. S1). We calculated

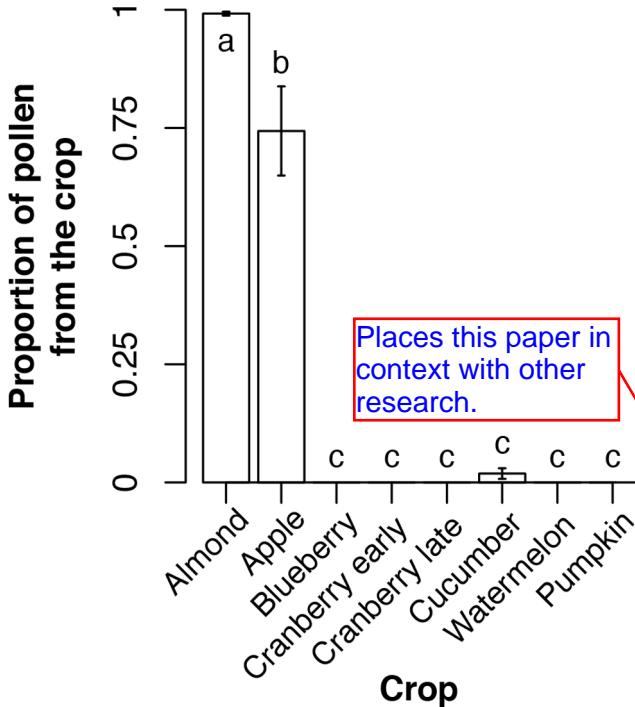


Figure 1. Pollen collection from the crop where a hive was placed for most crops. Bars show mean \pm se. Letters indicate significant differences ($p < 0.05$). doi:10.1371/journal.pone.0070182.g001

Important, representative results are clearly identified; repetitive data are shown in tables.

Nosema Infection

147 of the 630 bees (23.3%) fed *Nosema* spores became infected. 22 of the 35 pesticides (62.9%) found in our pollen samples were relative risk values significantly different from zero. Pesticides (22.9%) were associated with increased prevalence, while the remaining 14 were associated with decreased prevalence.

Results are provided but not discussed; discussion is saved for the next section.

Two of the three detected pesticides applied by control hive mites (marked with a * in Table 2) had a relative risk value greater than two, indicating *Nosema* prevalence in bees feeding those chemicals (DMPF and flualinate) was double the *Nosema* prevalence in bees that did not feed those chemicals. Of the seven pesticides found in pollen from over half, or at least four, of the crops, the majority were associated with higher *Nosema* prevalence in bees that consumed them. Both control diets had relative risk values not significantly different from one.

A pollen sample's fungicide load significantly affected *Nosema* prevalence among bees fed that pollen (Fig. 5; GLMM, likelihood ratio test: $\chi^2 = 5.8$, df = 1, $p = 0.02$), but pesticide diversity did not ($\chi^2 = 1.7$, df = 1, $p = 0.19$). A bee's source colony, included as a blocking variable, also did not affect *Nosema* prevalence ($\chi^2 = 2.0$, df = 2, $p = 0.36$). Replacing fungicide load with chlorothalonil load obtained the same result (chlorothalonil load: $\chi^2 = 5.3$, df = 1, $p = 0.02$; pesticide diversity: $\chi^2 = 1.5$, df = 1, $p = 0.23$; source colony: $\chi^2 = 2.0$, df = 2, $p = 0.36$; fungicide load model AIC = 612.71, chlorothalonil load model AIC = 613.15). Chlorothalonil was also the most abundant fungicide in our samples, and comprised $50.0 \pm 10.2\%$ (mean \pm se) of the per sample total fungicide load.

Unexpected results are identified and discussed.

Discussion begins with study results, then broadens in scope.

Discussion

The results from this study highlight several patterns that merit further attention. First, despite being rented to pollinate specific crops, honey bees did not always return to the nest with corbiculate pollen from those crops. These findings support other research with honey bees and native bees indicating that native bees may be more efficient pollinators. Fungicides were present at high levels in both pollen collected by bees. Third, two fungicides (pyraclostrobin, and two miticides used by bees against varroa infestation (amitraz and flualinate) had an effect on bees' ability to withstand parasite infections. Pesticides' effects on bee health has focused almost entirely on insecticides (e.g. fipronil [15] and the neonicotinoids imidacloprid [13,14] and thiacloprid [15]). Finally, several individual pollen samples contained loads higher than the median lethal dose for a specific pesticide. While multiple studies have shown negative effects of specific pesticides on honey bee individual and colony health [14,15,22,26] and high pesticide exposure [27,28], ours is the first to demonstrate how real world pollen-pesticide blends affect honey bee health.

Our results show that beekeepers need to consider not only pesticide regimens of the fields in which they are placing their bees, but also spray programs near those fields that may contribute to pesticide drift onto weeds. The bees in our study collected pollen from diverse sources, often failing to collect pollen from the target crop (Fig. 1). All of the non-target plants able to identify to genus or species was from the genus *Malus* (S1), suggesting the honey bees were collecting small amounts of pollen from weeds surrounding our focal fields. The two exceptions to this were hives placed in almond and apple orchards. Almond flowers early in the year, and almond orchards are large, thus providing honey bees with little access to other flowers. Honey bees rarely collect pollen from blueberry or cranberry flowers, which only release large quantities of pollen after being vibrated by visiting bees (buzz pollination) [46,47]. Honey bees are capable of buzz pollination and thus are able to collect large amounts of pollen from these plants to help offset nectar availability. Bumble bees, which can buzz pollinate, collect mainly pollen when placed in blueberry fields [48]. Interestingly, the two crops that saw high levels of pollen collection by honey bees are Old World crops that evolved with honey bees as natural pollinators. Crops native to the New World, where honey bees have been introduced, yielded little or no pollen in our samples.

It is possible that bees were exposed to pollen containing no pollen from our focal crops, even though they were placed in fields with no pollen from those crops. Because pollen intended for consumption by bees is corbiculate pollen intended for consumption by humans, data indicate only flowers from which bees are actively collecting pollen and not all flowers they visited. Several studies have detected pesticides in floral nectar and pollen [49,50], sometimes in concentrations with sublethal effects on honey and bumble bees [51,52]. Honey bees may collect nectar from blueberry and cranberry flowers via legitimate visits or "robbbing" through slits cut at the base of flower corollas [53]. However, exposure to pesticides via nectar may be unlikely in cucumber, pumpkin and watermelon. Beekeepers often report poor honey production when their hives are placed in these crops (pers. obs.).

The combination of high pesticide loads and increased *Nosema* infection rates in bees that consumed greater quantities of the fungicides chlorothalonil and pyraclostrobin suggest that some fungicides have stronger impacts on bee health than previously

Results are discussed meaningfully and not simply repeated; in this case, patterns are analyzed.

Clearly identifies practical applications

Addresses study limitations

Transition words provide flow between sentences.

Information reads from left to right, making information easier to follow.

Table 2. Pesticides found in pollen trapped off honey bees returning to the nest.

| Pesticide | Insecticide family | LD ₅₀ (ppm) ^a | Crops in which detected ^c | Detections | Quantity detected, mean \pm se (max) (ppb) | Relative risk (95% CI) |
|--------------------------------------|---|-------------------------------------|--------------------------------------|------------|--|------------------------|
| Fungicides | | | | | | |
| Azoxystrobin | | >1,562.5 [64] | Cr, Cu, Wa | 10 | 60.3 \pm 25.6 (332) | 0.75 (0.56, 1.02) |
| Captan | | >78.13 [65] | Ap, Cr, Cu, Wa | 9 | 976.9 \pm 734.4 (13,800) | 0.59 (0.42, 0.81)† |
| Chlorothalonil | | >1,414.06 [66] | Ap, Bl, Cr, Cu, Pu, Wa | 17 | 4,491.2 \pm 2,130.7 (29,000) | 2.31 (1.35, 3.94)† |
| Cyprodinil | | >6,125 [67] | Ap | 3 | 996.9 \pm 707.5 (12,700) | 0.31 (0.15, 0.65)† |
| Difenoconazole | | >781.25 [68] | Ap | 3 | 171.4 \pm 119.4 (2,110) | |
| Fenbuconazole | | >2,282.65 [69] | Ap, Cr, Cu | 10 | 227.3 \pm 89.2 (1,420) | |
| Pyraclostrobin | | 573.44 [70] | Cr, Pu | 4 | 2,787.1 \pm 1,890.1 (27,) | |
| Quintozene (PCNB) | | >0.78 [71] | Cr | 2 | 0.3 \pm 0.3 (4.7) | |
| THPI | Captan metabolite | | Cr, Cu | 3 | 832.1 \pm 531.8 (9,470) | |
| Herbicides | | | | | | |
| Carfentrazone ethyl | | >217.97 [72] | Cr | 1 | 0.1 \pm 0.08 (1.6) | 1.05 (0.54, 2.05) |
| Pendimethalin | | >388.28 [73] | Ap, Cr, Pu | 5 | 5.1 \pm 3.7 (69.5) | 1.47 (1.08, 1.99)† |
| Insecticides | | | | | | |
| 2,4 Dimethylphenyl formamide (DMPF)* | Amitraz (formamidine) metabolite | | Bl, Cu, Pu, Wa | 10 | 171.5 \pm 117.0 (2,060) | 2.13 (1.56, 2.92)† |
| Acetamiprid | Neonicotinoid | 55.47 [60] | Ap | 3 | 59.1 \pm 32.2 (401) | 0.31 (0.15, 0.65)† |
| Bifenthrin | Pyrethroid | 0.11 [74] | Pu, Wa | 3 | 6.6 \pm 3.8 (53.1) | 2.08 (1.53, 2.83)† |
| Carbaryl | Carbamate | 8.59 [75] | Ap, Cu, Wa | 6 | 57.8 \pm 30.0 (403) | 0.42 (0.27, 0.66)† |
| Chlorpyrifos | Organophosphate | 0.86 [16] | Ap, Cr, Cu, Pu | 7 | 3.1 \pm 1.1 (15.5) | 0.89 (0.64, 1.23) |
| Coumaphos* | Organophosphate | 35.94 [16] | Bl, Cr, Cu | 6 | 2.2 \pm 1.0 (17.5) | 0.62 (0.43, 0.91)† |
| Cyfluthrin | Pyrethroid | <0.31 [76] | Cr, Wa | 2 | 0.6 \pm 0.4 (5.4) | 1.31 (0.85, 2.02) |
| Cyhalothrin | Pyrethroid | 0.30 [77] | Ap, Pu, Wa | 7 | 14.6 \pm 7.9 (131) | 0.94 (0.69, 1.29) |
| Cypermethrin | Pyrethroid | 0.18–4.38 [78] | Cr | 1 | 0.4 \pm 0.4 (6.9) | 1.05 (0.54, 2.05) |
| Deltamethrin | Pyrethroid | 0.39 [79] | Cr | 1 | 4.5 \pm 4.5 (85.3) | 1.05 (0.54, 2.04) |
| Diazinon | Organophosphate | 1.72 [80] | Ap, Cr | 3 | 1.4 \pm 1.0 (19.8) | 0.56 (0.32, 0.97)† |
| Endosulfan I | Cyclodiene | 54.69 [16] | Ap, Cr, Cu, Pu, Wa | 8 | 1.5 \pm 0.7 (12.9) | 1.60 (1.20, 2.14)† |
| Endosulfan II | Cyclodiene | 54.69 [16] | Ap, Cr, Cu, Pu | 6 | 0.8 \pm 0.3 (5.3) | 1.41 (1.04, 1.91)† |
| Endosulfan sulfate | Endosulfan metabolite | | Cr, Cu | 4 | 0.3 \pm 0.2 (2.1) | 0.79 (0.52, 1.19) |
| Esfenvalerate | Pyrethroid | 0.13 [81] | Ap, Cr, Cu | 7 | 16.9 \pm 12.0 (216) | 0.51 (0.35, 0.75)† |
| Fluvalinate* | Pyrethroid | 1.56 [82] | Bl, Cr, Cu, Pu, Wa | 16 | 42.4 \pm 29.7 (570) | 2.43 (1.49, 3.96)† |
| Heptachlor epoxide | Heptachlor ^b (cyclodiene) metabolite | | Cr | 1 | 0.6 \pm 0.6 (12) | 1.05 (0.54, 2.04) |
| Imidacloprid | Neonicotinoid | 0.23 [83] | Ap | 3 | 2.8 \pm 2.0 (36.5) | 0.31 (0.15, 0.65)† |
| Indoxacarb | Oxadiazine | 1.41 [84] | Ap | 2 | 0.5 \pm 0.5 (9) | 0.28 (0.11, 0.73)† |
| Methidathion | Organophosphate | 1.85 [85] | Cr | 1 | 1.6 \pm 1.6 (31) | 1.05 (0.54, 2.04) |
| Methomyl | Carbamate | <3.91 [86] | Wa | 1 | 13.6 \pm 13.6 (259) | 1.54 (0.91, 2.61) |
| Phosmet | Organophosphate | 8.83 [85] | Ap, Cr, Cu | 5 | 798.7 \pm 772.4 (14,700) | 0.36 (0.21, 0.61)† |
| Pyrethrins | Pyrethroid | 0.16 [16] | Cr | 1 | 5.1 \pm 5.1 (97.4) | 1.05 (0.54, 2.05) |
| Thiacloroprid | Neonicotinoid | 114.06 [60] | Ap | 2 | 1.1 \pm 0.8 (12.4) | 0.35 (0.15, 0.82)† |
| Control diets | | | | | | |
| BRL | NA | NA | NA | NA | NA | 0.58 (0.23, 1.48) |
| MegaBee | NA | NA | NA | NA | NA | 0.74 (0.33, 1.67) |

^aWe divided LD₅₀ values given as µg/bee (g) by 0.128 (equivalent to multiplying by 7.8) to obtain ppm when necessary [85]. If multiple values have been published, we include only the smallest.

^bHeptachlor has been banned for use on cranberries since 1978 [87], but can persist in the soil for extended periods of time.

^cAp = apple, Bl = blueberry, Cr = cranberry, Cu = cucumber, Pu = pumpkin, Wa = watermelon.

*Used by beekeepers within the hive for parasitic mite control.

[†]Relative risk different from 1 at the 95% confidence level.

NA indicates information that is not relevant to control diets.

doi:10.1371/journal.pone.0070182.t002

Legend allows readers to understand abbreviations used in the table.

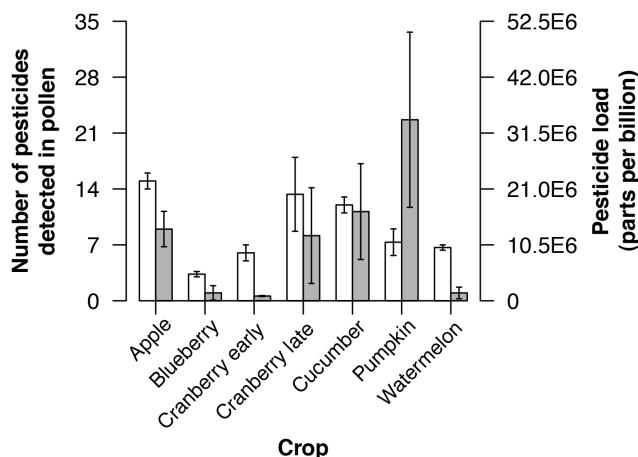
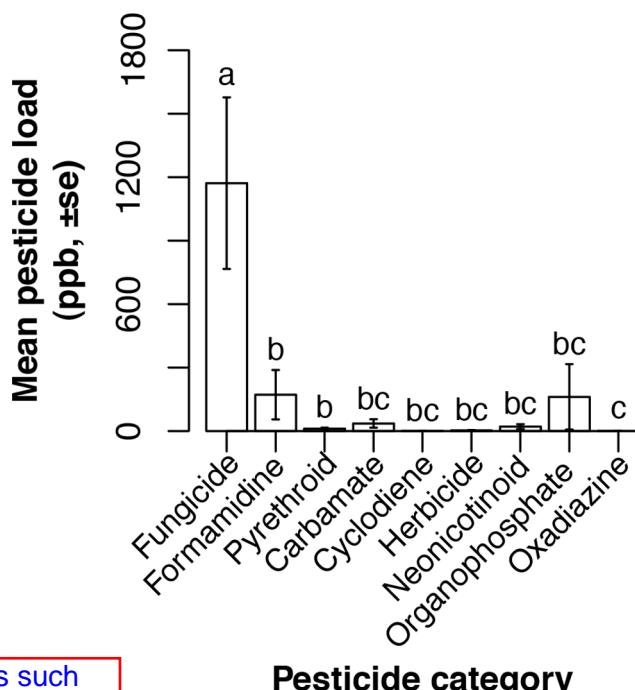


Figure 2. Pesticide diversity found in pollen samples, but not pesticide load, varied by crop. White bars show pesticide diversity, gray bars show pesticide load (mean \pm se). Post-hoc testing found the following groups, where letters indicate statistically significant differences: apple a, b; blueberry c; cranberry_early d; cranberry_late b, d, e, f; cucumber e; pumpkin c, d, f; and watermelon d.

doi:10.1371/journal.pone.0070182.g002

thought. *Nosema* infection was more than twice the risk (>2) in bees that consumed these fungicides [54]. In our study, neonicotinoids entered the nest only via apple pollen. However, we found fungicides at high loads in our sampled crops. While fungicides are typically less lethal to bees than insecticides (see LD₅₀ values in Table 2), these chemicals still have potential for lethal [55] and sub-lethal effects. Indeed, the fungicides chlorothalonil (found at high concentrations in our pollen samples) and myclobutanil increases gut cell mortality to the same degree as imidacloprid [56], an insecticide with numerous sub-lethal effects (e.g. [21,57]). Exposure to fungicides can also

Transition words such as "However," "While," and "Indeed" increase flow between sentences.

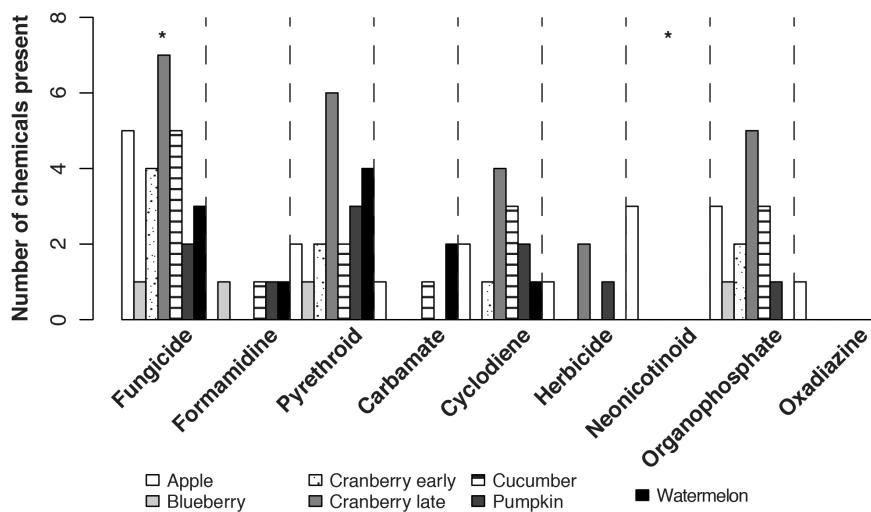


Pesticide category

load varied by pesticide category. Letters indicate significant differences. The total load for each category is the number of chemicals in that category, to facilitate comparison across categories.

doi:10.1371/journal.pone.0070182.g004

make bees more sensitive to acaricides, reducing medial lethal doses [58]. In our study, consuming pollen with higher fungicide loads increased bees' susceptibility to *Nosema* infection. This result is likely driven by chlorothalonil loads. The pesticide with the highest relative risk was the fungicide pyraclostrobin. Bees that consumed pollen containing pyraclostrobin were almost three times as likely (relative risk = 2.85, 95% CI 2.16–3.75; Table 2)



Study conclusions are discussed in relation to previous research.

Figure 3. Fungicide and neonicotinoid diversities varied by crop. Bars show the total number of pesticides in each category found in each crop. Kruskal-Wallis test statistics comparing pesticide diversity between crops are: fungicides, $H_6=16.1$, $p=0.01$; cyclodienes, $H_6=6.9$, $p=0.33$; neonicotinoids, $H_6=17.9$, $p=0.007$; organophosphates, $H_6=14.3$, $p=0.03$; pyrethrins, $H_6=7.8$, $p=0.26$. We only compared pesticide diversities for categories containing at least three chemicals. Sequential Bonferroni adjusted critical values are: 0.01, 0.0125, 0.0167, 0.025, 0.05. A * indicates that the total number of pesticides varied between crops within that pesticide category.

doi:10.1371/journal.pone.0070182.g003

Use of "First," "Second," and "Third" guide readers through the paper.

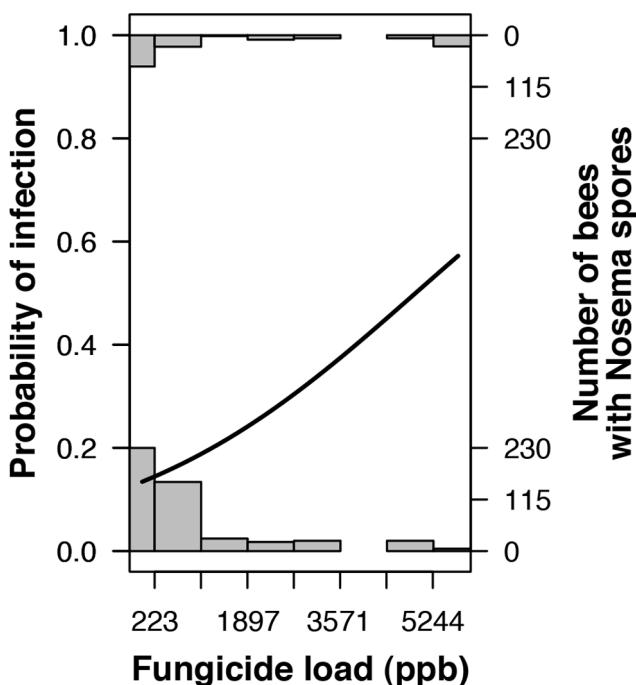


Figure 5. Probability of *Nosema* infection increased with fungicide load in consumed pollen. Histograms show the number of bees with (top) and without (bottom) *Nosema* spores as a function of the fungicide load in the pollen they were fed. The curve shows the predicted probability of *Nosema* infection.
doi:10.1371/journal.pone.0070182.g005

than bees consuming pollen without this chemical to become infected after *Nosema* exposure. Our results show the necessity of testing for sub-lethal effects of pesticides on bees, and advocate for testing more broadly than the insecticides that are the targets of most current research.

A similarly large increased risk of *Nosema* infection was associated with consumption of DMPF and flualinate, miticides applied by beekeepers to help control the highly-destructive *Varroa* mite [3]. The path from in-hive application of these miticides to

Significance of results in relation to broader research questions; however, the word "suggests" avoids overstating the implications.

returning to the hive is unclear. An increasingly common method of removing mites from hives is to rotate combs out of hives to remove mites, is expected to reduce miticide levels in hives, and may also reduce spread of these chemicals to the potential extra-nest sources, however, would slow miticide accumulation and slow the development of these chemicals.

Relative risk values showed an interesting pattern: all relative risk values significantly different than one were almost all in the same direction. The formamidine (DMPF) and two of the three pyrethroids (bifenthrin and flualinate, but not esfenvalerate) were associated with an increased risk of *Nosema* infection. The carbamate (carbaryl), all neonicotinoids (acetamiprid, imidacloprid and thiacloprid), organophosphates (coumaphos, diazinon and phosmet) and the oxadiazine (indoxacarb) were associated with reduced risk of *Nosema* infection. Esfenvalerate and coumaphos have previously been found to be associated with colonies without Colony Collapse Disorder [59]. These patterns suggest that insecticides' modes of action have differential effects on honey bee immune functioning. Because of the relatively small number of pesticides we found in each insecticide family,

however, additional sampling is necessary to determine how robust this pattern is.

The large numbers of pesticides found per sample and the high concentrations of some pesticides are concerning. First, two pollen samples contained one pesticide each at a concentration higher than the median lethal dose. Esfenvalerate ($LD_{50} = 0.13$ ppm) was measured at 0.216 ppm in pollen collected by bees in a cucumber field, and phosmet ($LD_{50} = 8.83$ ppm) at 14.7 ppm in one apple orchard. While the mean loads for these pesticides are well below their respective median lethal doses (0.0169 ppm for esfenvalerate, 0.7987 ppm for phosmet), our data indicate some bee colonies are being exposed to incredibly high levels of these chemicals. Second, research suggests that simultaneous exposure to multiple pesticides decreases lethal doses [58,60] or increases supersedure (queen replacement) rate [61]. Our pollen samples contained an average of nine different pesticides, ranging as high as 21 pesticides in one cranberry field. Thus published LD_{50} values may not accurately indicate pesticide toxicity inside a hive containing large numbers of pesticides. Research looking at additive and synergistic effects between multiple pesticides is clearly needed. Third, pesticides can have sub-lethal effects on development, reproduction, learning and memory, and foraging behavior. The mean and maximum imidacloprid loads in our samples (0.0028 and 0.0365 ppm, respectively) are higher than some published imidacloprid concentrations with sub-lethal effects on honey and bumble bees (0.001–0.0098 ppm [21,54,62]).

It is not surprising that total pollen collection varied by crop. Bee foraging activity levels vary with weather [63], thus outcomes of short-term measurements may be sensitive to temperature, cloud cover or humidity during data collection. Because we collected pollen samples from different parts of the country and on different days, weather conditions undoubtedly differed between crops. Crop flowering timing and landscape-level floral availability can also affect bee activity levels. We focused our analyses on variables less affected by these factors, such as the diversity of pollen types found in samples and the proportion of a sample that was from the target crop.

Our results are consistent with previous analyses of pollen collected from bee nests [16,18,27]. The more diverse sampling of Mullin et al. [16] found nearly triple the number of pesticides we found, but the average number of pesticides per sample (7.1) is slightly lower than our 9.1. In our study and those listed above, pesticides applied to control hive pests were present in a large proportion of samples, often in quantities higher than most pesticides applied to crops.

Our results combined with several recent studies on specific pesticides' effects on *Nosema* infection dynamics [13–15] indicate that a detrimental interaction occurs when honey bees are exposed to both pesticides and *Nosema*. Specific results vary, and may depend on the pesticide or dose used. For example, bees exposed to imidacloprid and *Nosema* can have lower spore counts than bees only infected with the pathogen but also exhibit hindered immune functioning [13]. Our study improves on previous methodologies by feeding pollen with real-world pesticide blends and levels that truly represents the types of exposure expected with pollination of agricultural crops. The significant increase in *Nosema* infection following exposure to the fungicides in pollen we found therefore indicates a pressing need for further research on the sub-lethal effects of fungicides on bees. Given the exposure to pesticides we show, and increasing evidence that pesticide blends harm bees [16,18,58], there is a pressing need for

Conclusions are clearly stated and significance of paper discussed.

Citations are placed within the sentence (and not at the end) when appropriate.

Significance of work in relation to other research

Some journals allow supplements.

further research on the mechanisms underlying pesticide-pesticide and pesticide-disease synergistic effects on honey bee health.

Supporting Information

Figure S1 Pesticide loads did not differ by crop for any pesticide category. Kruskal-Wallis test statistics comparing pesticide loads between crops are: fungicides, $H_6 = 10.6, p = 0.10$; herbicides, $H_6 = 8.3, p = 0.22$; carbamates, $H_6 = 13.4, p = 0.04$; cyclodienes, $H_6 = 6.7, p = 0.35$; formamidines, $H_6 = 13.6, p = 0.03$; neonicotinoids, $H_6 = 17.8, p = 0.007$; organophosphates, $H_6 = 14.5, p = 0.02$; oxadiazines, $H_6 = 11.3, p = 0.08$; pyrethroids, $H_6 = 9.6, p = 0.14$. Sequential Bonferroni adjusted critical values are: 0.0055, 0.0063, 0.0071, 0.0083, 0.01, 0.0125, 0.0167, 0.025, 0.06.

(DOCX)

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Crop
Acknowledgments are courteous and specific.

Table S1 Plant sources of pollens collected by bees placed in seven crops.
(DOCX)

Acknowledgments

We thank David Hackenberg and David Mendes for letting us work with their bee hives, John Baker and Rob Snyder for field assistance, and Simonds for pesticide identification, and Vic Levi and assistance with *Nosema* assays.

The views expressed in this article are those of the authors and may not necessarily represent the policies or positions of the US Department of Agriculture (USDA).

Some journals require author contributions.

Author Contributions

Conceived and designed the experiments: JSP RR DV. Performed the experiments: JSP MA JS DV. Analyzed the data: EML DV. Wrote the paper: JP EML DV.

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