Research article

Effects of common co-occurring pesticides (a neonicotinoid and fungicide) on honey bee colony health in a semi-field study

Sebastian Shepherd, Young-gyun Park, Christian H. Krupke [*]

Department of Entomology, Purdue University, West Lafayette, IN, USA

A R T I C L E   I N F O

A B S T R A C T

Multiple stressors are linked to declines of insects and important pollinators, such as bees. Recently, interactive effects of multiple agrochemicals on bees have been highlighted, including fungicides, which increase toxicity of neonicotinoid insecticides. Here, we use a semi-field study across two seasons in controlled foraging tunnels to test the effects of a field application of a commercial fungicide product with two active ingredients (pyraclostrobin and metconazole) applied at label rates. We also examine its interactive effects with the neonicotinoid insecticide clothianidin, at a conservative field-realistic dose of 2.23 ppb, on 48 honey bee colonies. We found combined effects of pesticide exposure, including additive 2.93-fold increases in mortality, and an additional effect of increased infestation levels of the ectoparasitic mite, *Varroa destructor*. Pesticide treatments also reduced colony activity, reduced colony weight, and increased sugar consumption of whole colonies. These findings indicate that typical sublethal exposure levels to common, co-occurring agrochemicals in the field significantly affect the health of whole honey bee colonies, highlighting an unintended consequence of increasing pesticide applications.

1. Introduction

Multiple interacting stressors, including climate change [1], land-use change [2], parasitism and viruses [3], along with pesticide use [4,5], are implicated in insect declines [6], including bee declines [7]. These stressors pose a threat to the important ecosystem services provided by insects globally [8]. Honey bees are exposed to a cocktail of pesticide compounds in the environment [9]. For example, a study by Long and Krupke [10] detected 32 different pesticides spanning nine chemical classes from just six hives. High-risk synergistic combinations of pesticides have been highlighted recently, including increased toxicity of neonicotinoid insecticides when co-exposed with fungicides [4,11–15]. For instance, clothianidin-induced mortality in honey bees more than triples when co-exposed with the ergosterol biosynthesis inhibitor (EBI) fungicide propiconazole [12]. Although fungicide-neonicotinoid interactions have been explored in bumble bee colonies with lab-exposure [16,17], these interactions have never been tested for honey bee colony health with a label-rate application of a commercial fungicide product in the field.

Maize is replacing grasslands in the central US, a region that supports over 40 % of the commercial US honey bee stock [2]. Consequently, foraging bees are invariably exposed to pesticides applied to maize. Headline AMP® (BASF, Research Triangle Park, NC, USA) is a fungicide containing two active ingredients: the EBI-fungicide metconazole (LD_{50} 87,000 ng/bee [18]) and the strobilurin pyraclostrobin (LD_{50} 73,000 ng/bee [18]). A restriction to prevent Headline AMP® applications from causing toxic exposure to bees

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* Corresponding author.
    E-mail address: ckrupke@purdue.edu (C.H. Krupke).

was removed from its label in 2015 (United States Environmental Protection Agency, Decision Number: 498344). Headline AMP® is commonly used on maize, with recommended application during the VT-R2 growth phases, which coincides with the period when maize pollen is available for consumption by obligate pollen-consuming pollinators, such as honey bees. In the Midwest/Central US, honey bees effectively utilize this highly available pollen resource, with maize comprising 28 % of honey bee pollen diet in summer months [19] and peaking at 71 % of honey bee pollen diet composition in the last week of July in our study region [19]. This means that that bees collecting maize pollen are likely to come into contact with residues of the fungicide Headline AMP® (and other agrochemicals applied to maize). Additionally, virtually all maize seed sold in the US is treated with neonicotinoid insecticides [20], so any bees using maize pollen as a food source will be exposed to sub-lethal levels of seed-applied neonicotinoids. Headline AMP® is typically applied aerially, and drift at above-canopy level in grasses/weeds in proximity to maize can be as high as 210–450 ng/cm^2 [21]. Drift/translocation of agrochemical residues to agricultural weeds is a common exposure route for bees [10,19,22,23]. According to the latest pesticide-use data from the United States Geological Survey [24], totals of 158,760–215,460 kg of metconazole, and 907,185–1,179,340 kg of pyraclostrobin were applied in the US in 2017, with applications predominantly in row crops in the Midwest/Central US.

Clothianidin is the most widely used neonicotinoid insecticide in the world [25] and acts as an insect neurotoxin by targeting nicotinic acetylcholine receptors [26]. It is highly toxic to bees (oral LD$_{50}$ 3.79 ng/bee [27]). Since initial licensing in the late 1990's, the use of neonicotinoid seed treatments in maize and soybean has been the principal driver [28] behind the 121-fold increase in bee toxic load in the Midwest US from 1997 to 2012 [20]. Bees encounter clothianidin, metconazole and pyraclostrobin through multiple exposure routes, including bee-collected pollen and wildflowers [29]. Clothianidin levels in pollen range from 2 to 23 ppb [10,29–32], metconazole has been detected at 19.0–94.4 ppb [15,29], and pyraclostrobin typically ranges from 9.8 to 265.0 ppb [9,10,29]. However, spike exposures have been recorded in proximity to crops, such as 27,000 ppb in bee-collected pollen [33] and 2170 ppb in beebread [34,35]. In nectar, clothianidin exposure typically ranges from 1 to 16 ppb [30–32,36]. As clothianidin levels in maize pollen specifically can be lower (e.g., 1.8–3.9 ppb [22,37]), we selected a 2.23 ppb clothianidin treatment in this study, which is field-realistic with respect to maize pollen and highly conservative with respect to clothianidin in other sources (other pollen types, nectar, water, etc.).

In this study, we investigated the combined effects of the fungicide Headline AMP® applied at 119.68 ml/ha (the lowest label-application rate) to wildflowers (20.06 g a.i./ha), and clothianidin delivered at 2.23 ppb via sugar feeders, on 48 nucleus honey bee colonies. Colonies were evenly split between four treatments: 12 × control, 12 × clothianidin, 12 × fungicide, and 12 × mix (2.23 ppb clothianidin + fungicide treated wildflowers). Treatments were conducted for two weeks in high tunnels, and changes in key colony health metrics that occurred over this period were measured.

2. Materials and methods

2.1. Bee colonies

Honey bee, *Apis mellifera* L., colonies were established from 1.36 kg packages (Bastin Honey Bee Farm, Knightstown, IN, USA) in new 24.45 cm deep nucleus hives (WW-203, Mann Lake, Hackensack, MN, USA) with five new 23.18 cm frames with wax foundation (WW-909, Mann Lake) in May of 2019 and 2020. Colonies were monitored for queen health and swarming, and five additional frames and nucleus boxes were added for colonies that required growth space. The colonies were kept in an organic wildflower meadow at Meigs Horticultural Farm (40.2856°, −86.8782°), part of the Throckmorton-Purdue Agricultural Center (TPAC, Lafayette, IN, USA, 40.2955°, −86.8944°), until experiments began.

2.2. Experimental design

Colonies were exposed to one of four different treatments (control, clothianidin, fungicide, or mix) in two-week replicates. Exposure occurred in two (7.9 × 29.3 m) cold frame tunnels (102861, Clear Span, South Windsor, CT, USA) covered with an all-weather insect screen (allowing rain through; 100 % polyolefin 1 × 4 mm insect screen; Econet 100400, Hummert International, Earth City, MO, USA) to isolate exposed bees in the tunnels during the treatment period, at TPAC. The insect screen caused no increase in temperature and humidity above ambient levels in the tunnels and was purely used for the purpose of controlling bee foraging behaviour. Wildflowers were grown in the tunnels as a food resource and to deliver fungicide exposure (S. 1).

Four nucleus honey bee colonies were moved into each of the two tunnels on day one of a two-week replicate (eight total colonies per replicate between the two tunnels) ('colonies moved' in S. 2). Colonies were moved out of the tunnels on the morning of day 15 after the experimental times had elapsed.

On the morning that colonies were moved into the tunnel, while colony entrances were closed, in one tunnel (designated for fungicide and mix treatments), the fungicide product (Headline AMP®, BASF, Research Triangle Park, NC, USA) was applied at 119.68 ml/ha (the minimum label product use-rate per application for disease control on maize) (20.06 g a.i./ha) via a backpack sprayer to simulate non-target spray via aerial application onto wildflowers. In the other tunnel (designated for control and clothianidin treatments) wildflowers were untreated.

All eight colonies in each replicate were given a Boardman Entrance Feeder (FD-100, Mann Lake) for 50 % sucrose (w/v) solution supplementation during the treatment period, with custom built Styrofoam occlusion cases to prevent robbing. In each two-week replicate, out of the four colonies in each tunnel, two colonies were fed regular sugar solution, while the other two were fed sugar solution containing clothianidin at 2.23 ppb, prepared with analytical grade clothianidin (33589, PESTANAL, Millipore Sigma, St.

Louis, MO, USA). Thus, in each two-week eight-colony replicate, in one tunnel, there were two control colonies and two clothianidin colonies, and in the other tunnel there were two fungicide colonies and two mix colonies (S. 1C), where clothianidin was delivered independently to each colony orally in sugar solution, and fungicide was delivered through *ad libitum* contact/oral exposure on treated flowers. Bees were observed both foraging on wildflowers and feeding from sugar feeders. Sugar solution was replaced every two days ('sugar replaced' in S. 2) with freshly prepared solution, and entrance feeders were covered to prevent UV degradation of clothianidin.

Between August 6th and September 24th each year (2019 and 2020), two-week eight-colony replicates were repeated three times (total of 6), resulting in a total of 48 experimental colonies with 12 colonies in each treatment group (12 × control, 12 × clothianidin, 12 × fungicide, and 12 × mix). Colonies were matched for weight prior to allocation to treatments, and initial colony weight did not differ between treatment groups (RM One-Way ANOVA, $F_{3,33} = 0.09$, $P = 0.96$).

2.3. Wildflowers in tunnels

For floral resources in tunnels, a commercial mix of 23 species of annual wildflowers (AM016955, American Meadows, Shelburne, VT, USA) was supplemented with a sunflower/cosmos mix (AM01719, American Meadows) at a 10:1 ratio, resulting in a total of 24 species in the combined mix. The ground was cleared each year (S. 1A), and the seed mix was applied in the tunnels using a Gandy Orbit-Air Seed Applicator (Gandy, Owatonna, MN, USA) at a rate of 10.759 g/m^2 in late May. Tunnels were irrigated for 2.5 months so that experiments could begin with adult flowers in mid-August (S. 1B). To control for floral resources in each two-week replicate of the experiment, six × 1 m^2 quadrats were taken from randomized locations in both the fungicide tunnel and the control tunnel ('diversity quadrats' in S. 2).

2.4. Pesticide residues

As a control for residues of pesticides applied in treatments, residue analysis was conducted using previously described methods for analysing residues in pollen [10] with a modified QuEChERS method [38] and water [39], adapted here.

To confirm that fungicide residues did not accumulate in tunnels over successive treatments, the anthers of the most common flower available in the tunnels, *Cosmos sulphureus* (sulphur cosmos), were collected from the fungicide and control tunnels on days 1, 7 and 13 from each replicate (1–3 from 2019 and 4–6 from 2020) ('anthers collected' in S. 2). 1 g of *Cosmos sulphureus* anther tissue from each sample date was homogenized and separated into 15 ml centrifuge tubes. 10 ml of extraction solution (5 ml ddH_2O and 5 ml acetonitrile) was added to each centrifuge tube along with 10 μl of 10 ng/μl spiking solution containing deuterated internal standards of test active ingredients (metconazole and pyraclostrobin). Tubes were mixed thoroughly, and QuEChERs salts (2 g anhydrous magnesium sulphate [$MgSO_4$] and 0.5 g of sodium acetate [NaOAc]) were added to each tube. Samples were mixed on an agitator for 10 min and then centrifuged at 3500 RPM for 10 min. 1 ml of supernatant was removed and dispensed into 2 ml Agilent dispersive Solid Phase Extraction tubes containing 25 mg primary secondary amine, 7.5 mg graphitized carbon black, and 150 mg $MgSO_4$ (5982-5321, Agilent, Santa Clara, CA, USA). Tubes were vortexed and centrifuged at 13,000 RPM. The entire supernatant was transferred to 2 ml centrifuge tubes, ready for sample drying and storage for further analysis.

To ensure that clothianidin sugar solutions fed to bees had been prepared accurately, sugar solution samples from each two-week replicate treatment (1–3 from 2019 and 4–6 from 2020) were tested for clothianidin residues. These samples were prepared for analysis with high-performance liquid chromatography (HPLC) via solid-phase extraction. 1 ml of test treatment solution was spiked with 10 μl of 10 ng/μl spiking solution containing deuterated internal standards of test active ingredients (clothianidin) to form a test sample. Oasis HLB 1 cc filter cartridges (30 mg sorbent, 186008055, Waters, Milford, MA, USA) were attached to a Preppy 12-port vacuum manifold (Sigma-Aldrich, St. Louis, MO, USA). The test samples were passed through the cartridges and then eluted twice with 1.5 ml of HLPC-grade acetonitrile (Sigma-Aldrich). The eluted samples were collected in 5 ml centrifuge tubes, ready for sample drying and storage for further analysis.

Once in centrifuge tubes, water and anther test samples were dried in a Savant Automatic Environmental SpeedVac System (AES2010, Thermo Fisher Scientific, Waltham, MA, USA), and stored at −80 °C for further sample analysis. Samples were resuspended in 50 % acetonitrile/H_2O and transferred to 96-well plates for analysis by liquid chromatography (LC) and tandem mass spectrometry (MS) at the Bindley Bioscience Center at Purdue University using a 1200 Rapid Resolution LC system (Agilent) coupled to a 6460 series QQQ mass spectrometer (Agilent). Calibration curves were used to quantify levels of clothianidin in water samples and pyraclostrobin and metconazole in anther samples of *Cosmos sulphureus*.

2.5. Bee health metrics

Data on colony-level effects of pesticide treatment were collected during this assay. Some data were critical to observe periodically during replicates for repeated monitoring of colony health, while other data (with more invasive methods or for analytical reasons) were monitored before and after the two-week treatment period for the 'change' in the observed variable (S. 2).

Periodic data included sugar consumption and mortality ('sugar' and 'mortality' in S. 2). Every two days that sugar solutions were replaced for colonies, the remaining sugar solution left in Boardman feeders was quantified to measure sugar consumption in terms of ml consumed per colony per day. For mortality, guidance was followed for standard methods for toxicology research in honey bees [40]. Collection trays (1548 cm^2) were placed at colony entrances as dead bee traps to collect cadavers removed by housekeeping bees. Dead bee traps were checked and emptied every two days to quantify relative mortality levels for each colony. The final mortality and sugar data were collected on the morning of day 15 as colonies were removed from the tunnels.

For change data, baseline measurements were made ('activity', 'weight', 'frame photos', and *Varroa* in S. 2), and changes from baselines were calculated. For colony activity, guidance was followed for standard methods of monitoring bee colony parameters [41]. Hand tally counters were used to record bee passes in and out of nucleus colonies for four × 2.5 min intervals between 11:00 a.m.–2:00 p.m. for all eight colonies in a replicate. The order by which four × 2.5 min intervals were sampled from colonies was randomized across the eight colonies during the above-mentioned sampling window, ensuring a representative 10 min total activity was monitored per colony per sample day. A 10 min representative time period is typical for studies monitoring honey bee activity in response to pesticide exposures [42], and for monitoring colony entrance activity, short repeat measurements are recommended for a representative sample [43]. A baseline recording of colony activity was taken on day 2 of the assay ('activity' in S. 2). This is because activity of colonies prior to placement in the tunnels is not representative of tunnel activity, as food resources external to the tunnels vary from internal resources and causes variability in colony behaviour. Additionally, baseline colony activity on day 1 in the tunnels is heightened immediately following the movement of colonies into a new location. Therefore, day 2 is the earliest representative day to collect baseline data. Colony activity was also sampled at the end of the replicate on day 14 ('activity' in S. 2), and changes in passes from baseline colony activity were calculated.

All colonies were weighed as they were placed in tunnels on day 1 and as they were removed from tunnels on day 15 ('weight' in S. 2). Weight changes from day 1 to day 15 were compared between treatments. 1–3 days prior to being placed in tunnels, and 1–3 days after being placed in tunnels, colonies were inspected following standard methods of monitoring honey bee colony parameters [41].

Photos were taken of both sides of all frames in each colony both before and after treatment in the tunnels. Photos were manually analyzed by visual examination of each cell in the frames, and the percentage of observed area of frames was determined [41] for multiple categories of colony health parameters, including pollen/bee bread, nectar, capped food (capped pollen or nectar), capped brood, larvae/eggs, drone comb, empty wax cells, and clear foundation. The presence of any queen cells in frame photos was also identified [41]. In total, 1420 photos were analyzed. The percentage change in colony health parameters from before to after treatment was calculated, as well as the presence of 'new' queen cells, i.e., queen cells on frames after treatment that were not present on frames before treatment. Colonies were sampled for loads of parasitic varroa, *Varroa destructor* (Anderson and Trueman), mites [44]. Using a *Varroa* EasyCheck monitoring tool (Véto-pharma, Palaiseau, France), an alcohol wash was conducted by collecting 300 bees from the frame in each colony that contained the highest level of brood. The number of *Varroa* mites/100 bees was calculated. Changes in *Varroa* load from parasite screens before and after treatments were used to compare treatment effects on parasite load.

2.6. Statistics

Data were analyzed using SPSS (v.26, IBM SPSS Inc., New York, NY, USA) and Graphpad Prism (v.8, Graph Pad Software Inc., San Diego, CA, USA). Where appropriate, homogeneity of variance and normality assumptions were tested. If required, equivalent non-parametric statistical tests were chosen.

Wildflower identification data (diversity, richness, and percentage cover) were analyzed with a two-way ANOVA with 'replicate' and 'tunnel' as main effects. Pesticide residue data were analyzed with a two-way RM ANOVA with 'treatment' and 'day' (1, 7, 13) as main effects and 'replicate' as a repeated measure. For both regular two-way ANOVAs and RM two-way ANOVAs, Bonferroni's correction for multiple comparisons was used for *post-hoc* analyses.

Mortality rates of bees collected in dead bee traps (dead bees/day), percentage changes in colony weight, sugar solution consumption rates, percentage changes in colony entrance activity, initial colony weights, and percentage changes in key colony structure parameters (brood, food resources, and empty cells), were analyzed using RM one-way ANOVAs, with treatment as the main effect, and replicate placement as a repeated factor. For RM ANOVAs if the assumption of sphericity was not met ($\varepsilon < 0.75$), then the Greenhouse-Geisser correction was applied (denoted by 'GG-Corrected' when reporting *F* statistic, adjusted degrees of freedom, and *P*-value). For non-parametric repeated measures analyses (the number of new queen cells in colonies and the change in *Varroa* loads in colonies), a Friedman test was used. In *post-hoc* analyses, Tukey's multiple comparison test was used for parametric RM One-Way ANOVAs, and Dunn's multiple comparison test was used for non-parametric Friedman's Tests. In both *post-hoc* analyses, *P*-values in multiple comparisons are adjusted for multiplicity of comparisons [45].

The number of colonies which 'contained new queen cells' or 'did not contain new queen cells' was analyzed with a chi-squared (χ^2) test. Pearson correlation coefficients were computed, and linear regressions fit with 95 % confidence intervals for relationships of initial colony weight *vs.* initial colony activity, end colony weight *vs.* end colony activity, change in colony weight *vs.* change in colony

Table 1

Simpson's index of diversity, species richness, and percentage cover (Mean ± SEM) for wildflowers in each tunnel and replication.

Year	Replication	Simpson's Index of diversity		Species Richness		Percentage Cover	
		Tunnel		Tunnel		Tunnel	
		Untreated	Treated	Untreated	Treated	Untreated	Treated
2019	1	0.83 ± 0.04	0.79 ± 0.03	10.17 ± 1.54	7.67 ± 1.33	94.67 ± 2.70	93.67 ± 2.11
	2	0.81 ± 0.04	0.83 ± 0.03	9.17 ± 1.56	9.33 ± 1.50	97.00 ± 1.61	95.67 ± 1.58
	3	0.84 ± 0.03	0.82 ± 0.02	9.83 ± 1.25	7.67 ± 0.76	96.33 ± 1.43	93.00 ± 2.02
2020	4	0.82 ± 0.02	0.87 ± 0.01	8.00 ± 1.24	10.67 ± 0.71	96.33 ± 1.33	96.83 ± 2.01
	5	0.78 ± 0.08	0.78 ± 0.04	8.33 ± 1.38	7.00 ± 1.48	97.67 ± 1.23	92.17 ± 2.41
	6	0.81 ± 0.03	0.84 ± 0.02	7.67 ± 1.02	9.00 ± 1.32	93.17 ± 2.68	92.33 ± 2.42

activity, percentage change in colony weight *vs.* change in mortality, and percentage change in colony activity *vs.* change in mortality. In both analyses of change in mortality data the $\log_{10}$-transformed mortality was used, as this provided a better fit in regression models.

3. Results

3.1. Wildflowers in tunnels

There was no effect of 'time' or any difference between 'tunnels' for all measured variables including Simpson's Index of Diversity (Two-Way ANOVA: Time, $F_{5,60} = 0.70$, $P = 0.62$; Tunnel, $F_{1,60} = 0.06$, $P = 0.81$), species richness (Two-Way ANOVA: Time, $F_{5,60} = 0.47$, $P = 0.80$; Tunnel, $F_{1,60} = 0.16$, $P = 0.68$), or percentage cover (Two-Way ANOVA: Time, $F_{5,60} = 0.99$, $P = 0.43$; Tunnel, $F_{1,60} = 2.69$, $P = 0.11$) (Table 1). This indicates that there was no difference between the diversity, richness, or cover of wildflowers between tunnels or between replicates. Seventeen out of 24 species in the wildflower seed mix were identified in the tunnel quadrats. Additionally, over the course of two years, 25 flowering weed species were identified in tunnel quadrats, resulting in a total observed species richness of 42 for wildflowers in the experiment (S. 3), with a high total Simpson's Index of Diversity of 0.93813. The overall percentage cover of wildflowers in the tunnels was 94.9 %, with the most common species being *Cosmos sulphureus* (sulphur cosmos), *Cosmos bipinnatus* (wild cosmos), *Setaria faberi* (giant foxtail), *Cyperus esculentus* (yellow nutsedge), *Coreopsis tinctoria* (Plains coreopsis), and *Abutilon theophrasti* (velvetleaf).
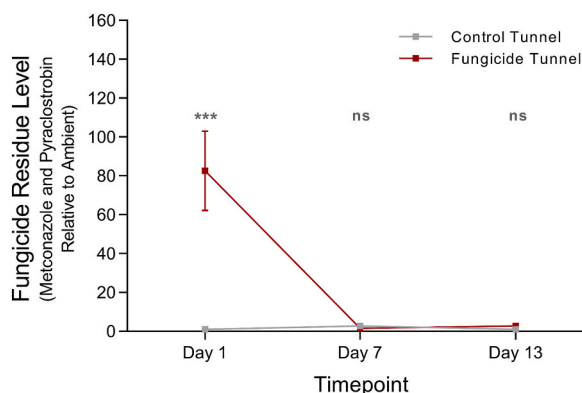
3.2. Pesticide residues

Following the analysis of fungicide active ingredient residues, there was a significant interaction effect between the 'time' during the replicate and 'treatment tunnel' on the combined relative level of residues of metconazole and pyraclostrobin (RM Two-Way ANOVA, $F_{2,10} = 15.87$, $P = 0.0008$) (Fig. 1). Across all six replicates on day 1 (immediately after fungicide application), the combined residue levels of metconazole (5.726 ± 0.462 ppm) and pyraclostrobin (4.841 ± 0.290 ppm) were significantly higher in the treated tunnel than in the control tunnel (Bonferroni: $P = 0.0001$). On both day 7 (Bonferroni: $P > 0.9999$) and day 13 (Bonferroni: $P > 0.9999$), there was no difference between the residues of pyraclostrobin and metconazole found in anther samples of *Cosmos sulphureus* in either of the tunnels, showing that residues returned to ambient levels at least seven days after the fungicide product was applied, and that fungicide active ingredient residues did not accumulate between successive replicates with this experimental design (Fig. 1).

In the analysis of clothianidin residues in sugar solutions fed to bees, the mean clothianidin levels were 2.23 ± 0.03 ppb. This confirms accurate mixing of clothianidin in treatments, which through the rest of this paper are referred to as 2.23 ppb.
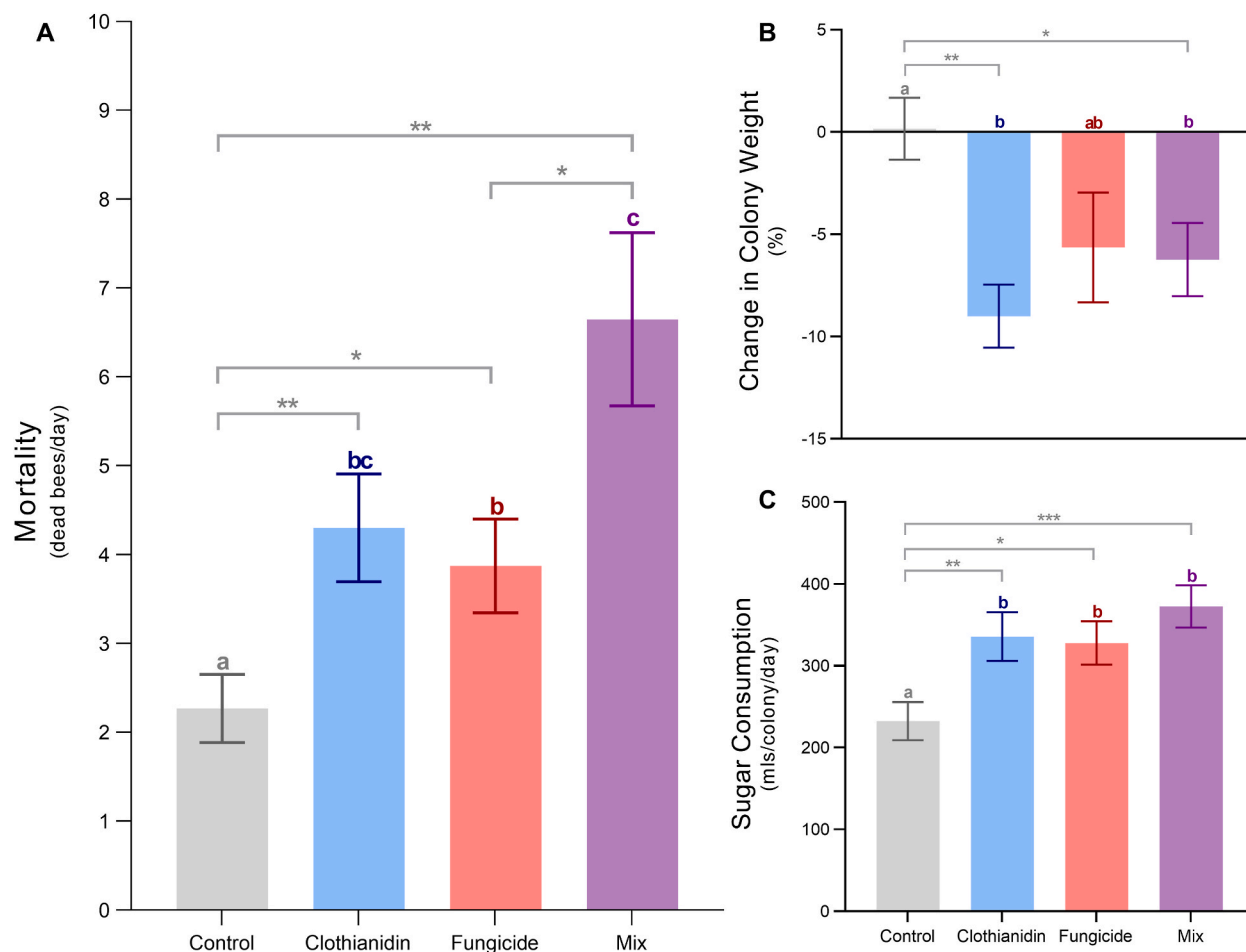
3.3. Mortality

After exposure of 12 colonies per treatment to control or pesticide treatments, in control colonies, the mortality observed in dead bee traps was 2.27 ± 0.38 dead bees/day, whereas clothianidin mortality was 4.30 ± 0.61, fungicide mortality was 3.88 ± 0.53, and mix mortality was 6.65 ± 0.98 dead bees/day (Fig. 2A).

Here, there was a significant effect of treatment on mortality (RM One-Way ANOVA, GG-Corrected $F_{2.019,22.21} = 13.41$, $P = 0.0001$) as all pesticides increased mortality (Tukey: clothianidin, $P = 0.0029$; fungicide, $P = 0.0265$; mix, $P = 0.0012$). The effect of pesticides on mortality was additive (mix induced higher mortality than fungicide; Tukey: $P = 0.0418$) as fungicide exposure increased mortality 1.72-fold relative to the control, clothianidin did so at 1.89-fold, the expected mix mortality increase was 2.61-fold, and the observed mix treatment increased mortality by 2.93-fold.

Fig. 1. Total fungicide resides of metconazole and pyraclostrobin relative to ambient levels in *Cosmos sulphureus* pollen (Mean and SEM are plotted). (*** significance indicates $P < 0.001$. 'ns' indicates no significant difference. Fungicide levels do not aggregate over time.

Fig. 2. Impacts of pesticide treatment on mortality, weight, and resource consumption of honey bee colonies. (A) Impacts of treatment (control, clothianidin, fungicide, or mix) on mortality, measured by the number of dead bees found per day in dead bee traps at colony entrances. (B) Impacts of treatment on the percentage change in colony weight. (C) Impacts of treatment on sugar consumption (ml of treatment solution consumed per colony per day). All bars plot mean values for treatments, and error bars are SEM. Significance stars (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) and letters identify treatments that are significantly different from multiplicity adjusted P – values in *post hoc* analysis.

3.4. Weight change and sugar consumption

Following a two-week exposure, colony weight decreased (Fig. 2B) after all pesticide treatments (clothianidin -9.00 ± 1.52 %, fungicide -5.64 ± 2.69 %, mix -6.24 ± 1.79 %), whereas weight did not change for controls (colonies increased $+0.15$ % ± 1.52 %). Treatment affected colony weight (RM One-Way ANOVA, GG-Corrected $F_{2.159,23.75} = 3.625$, $P = 0.0393$), as clothianidin (Tukey: $P = 0.0033$) and mix (Tukey: $P = 0.0249$) treatments reduced colony weight. Furthermore, colonies exposed to pesticides all consumed more sugar solution (Fig. 2C) than the 232.2 ± 23.4 ml/day consumed by control colonies (RM One-Way ANOVA, $F_{3,33} = 7.775$, $P = 0.0005$), (Tukey: clothianidin, 335.8 ± 29.8 ml/day, $P = 0.0089$; fungicide, 327.8 ± 26.5 ml/day, $P = 0.0174$; mix, 372.7 ± 25.9 ml/ day, $P = 0.0003$). This indicates that despite increased consumption, weight was reduced in pesticide-treated colonies.

Passes of bees in and out of colony entrances were recorded at the start/end of the experiment, and the percentage change in activity compared. Activity reduced slightly in control colonies between the start and end of the experiment (-6.11 ± 2.65 %), but was reduced by greater amounts in clothianidin (-18.02 ± 4.33 %), fungicide (-17.20 ± 4.72 %), and mix (-21.56 ± 3.42 %) treated colonies (Fig. 3A). Treatments reduced activity (RM One-Way ANOVA, GG-Corrected $F_{2.231,24.54} = 3.298$, $P = 0.0492$) for both clothianidin (Tukey: $P = 0.0324$) and mix (Tukey: $P = 0.0073$) exposures. There was a strong relationship between colony weight and activity both at the start (Fig. 3B, Pearson $R^2 = 0.92$, $P < 0.0001$) and end (Fig. 3C, Pearson $R^2 = 0.79$, $P < 0.0001$) of the experiment, as heavier colonies had higher activity. There was also a significant relationship between changes in these factors. Colonies that exhibited greater weight reductions also showed greater reductions in activity (Fig. 3D, Pearson $R^2 = 0.23$, $P = 0.0006$). Moreover, there were higher rates of mortality in colonies that exhibited larger reductions in weight (Fig. 4A, Pearson $R^2 = 0.17$, $P = 0.0032$) and in colonies that exhibited greater reductions in colony entrance activity (Fig. 4B, Pearson $R^2 = 0.15$, $P = 0.0074$).
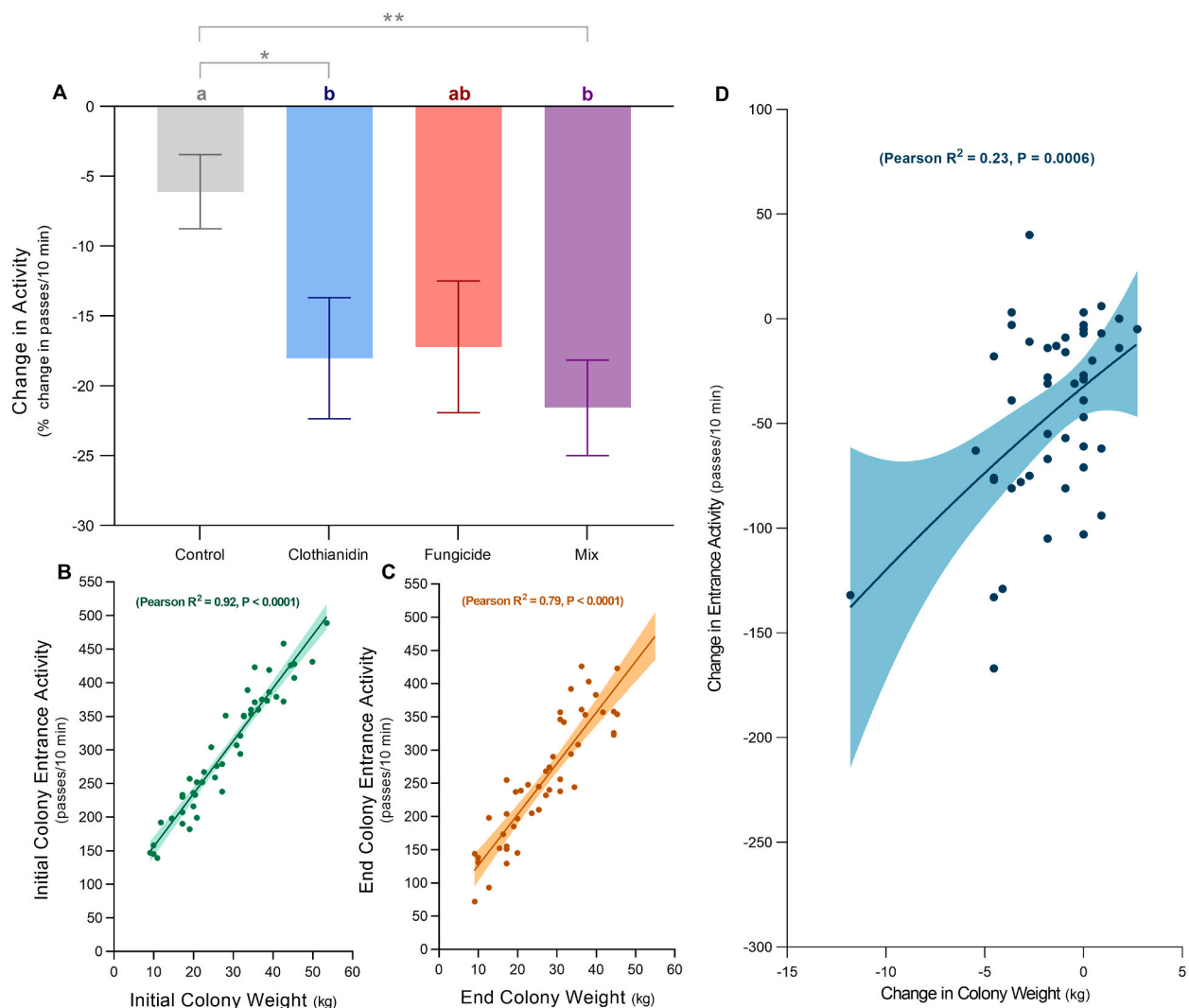
Fig. 3. Relationships between pesticide treatment, colony activity and colony weight. (A) Impacts of treatment (control, clothianidin, fungicide, or mix) on the percentage change in colony activity, measured by the percentage change in the passes of bees from the entrance of colonies. All bars plot mean values for treatments, and error bars are SEM. Significance stars (*$P < 0.05$, **$P < 0.01$) and letters identify treatments that are significantly different from multiplicity adjusted P – values in *post hoc* analysis. (B) The relationship between initial colony weight (kg) and initial entrance activity (passes of bees per 10 min at colony entrances. (C) The relationship between end colony weight (kg) and end entrance activity (passes of bees per 10 min at colony entrances). (D) The relationship between the change in colony weight (kg) and the change in entrance activity (passes/10 min). For all correlations (B–D), a linear regression is fitted with 95 % confidence intervals. Pearson correlation coefficients (R^2) and P – values for relationships are displayed.
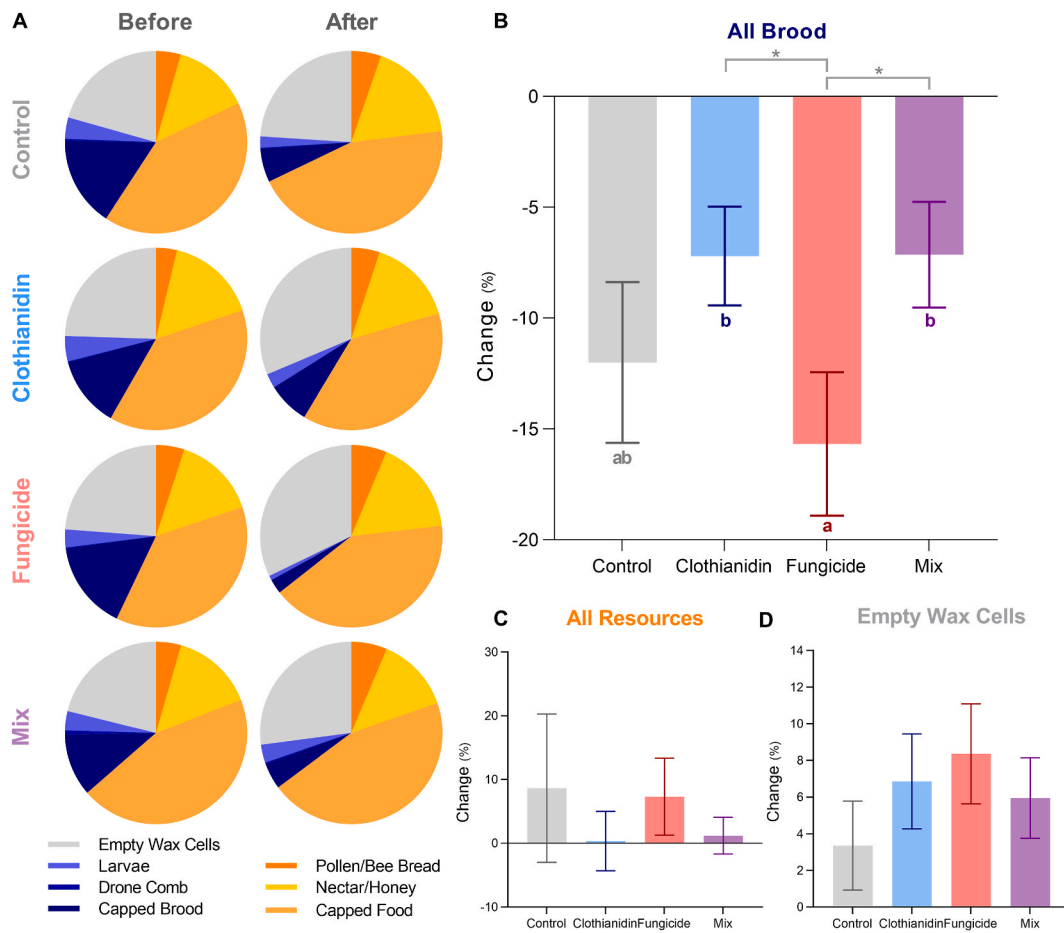
3.5. Colony structure

Photographic analysis of all the frames from experimental colonies before/after treatment determined key aspects of colony structure (Fig. 5A), including changes in the amount of brood (larvae, drone comb, and capped brood) (Fig. 5B), food resources (nectar, pollen/bee bread, and capped food) (Fig. 5C), and empty cells (Fig. 5D). Treatments generally did not affect colony structure. There was a slight effect of treatment on brood (RM One-Way ANOVA, $F_{3,33} = 4.070$, P = 0.0145) as fungicide colonies exhibited greater brood reductions (-15.68 ± 3.24 %) than clothianidin (-7.20 ± 2.22 %, Tukey: $P = 0.0299$) and mix (-7.14 ± 2.38 %, Tukey: $P = 0.0285$) colonies. Control colonies lost more brood (-12.00 ± 3.62 %) than clothianidin and mix colonies, but not as much as fungicide colonies. However, these changes were statistically significant. There was no effect on the food resources in the colonies (RM One-Way ANOVA, $F_{11,33} = 1.46$, $P = 0.24$) or empty cells (RM One-Way ANOVA, $F_{11,33} = 0.87$, $P = 0.44$).

When analysing frame photos, the presence of new queen cells in colonies was recorded as an indicator of stress/queen loss (Fig. 6A). More new queen cells were produced (Fig. 6B) in treated colonies (Control = 1; Clothianidin = 17, Fungicide = 11, Mix = 6), and more treated colonies exhibited increases (Fig. 6C) in queen cells (Control = 1; Clothianidin = 3, Fungicide = 2, Mix = 4).
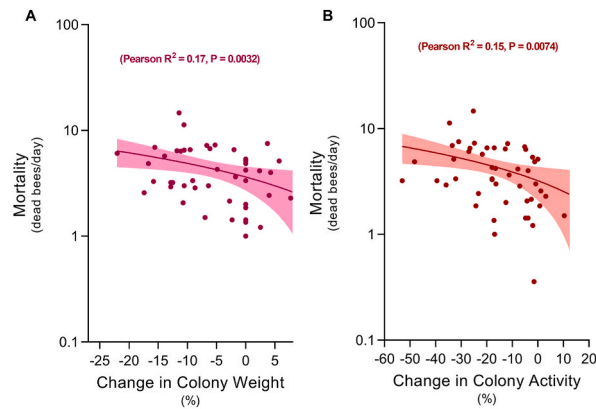
Fig. 4. Relationships between mortality and the percentage change in colony weight and activity. (A) The relationship between the percentage change in colony weight and ($\log_{10}$-transformed) mortality (dead bees/day in dead bee traps). (B) The relationship between the percentage change in colony activity and mortality. In both panels mortality is plotted on a $\log_{10}$ scale and a linear regression is fitted with 95 % confidence intervals. Pearson correlation coefficients (R^2) and P – values for relationships are displayed.

Fig. 5. Effects of pesticide treatment on honey bee colony structure. (A) The percentages of key colony structure parameters (pollen/bee bread, nectar/honey, capped food, capped brood, drone comb, larvae, and empty wax cells) before and after treatments occurred in tunnels. (B–D) the changes in the percentages of key colony structure parameters including (B) 'all brood' (larvae, drone comb and capped brood), (C) 'all resources' (pollen/bee bread, nectar/honey, and capped food), and (D) 'empty wax cells'. All bars for B-D plot mean values for treatments, and error bars are SEM. Significance stars (*$P < 0.05$) and letters identify treatments that are significantly different from multiplicity adjusted P – values in *post hoc* analysis.
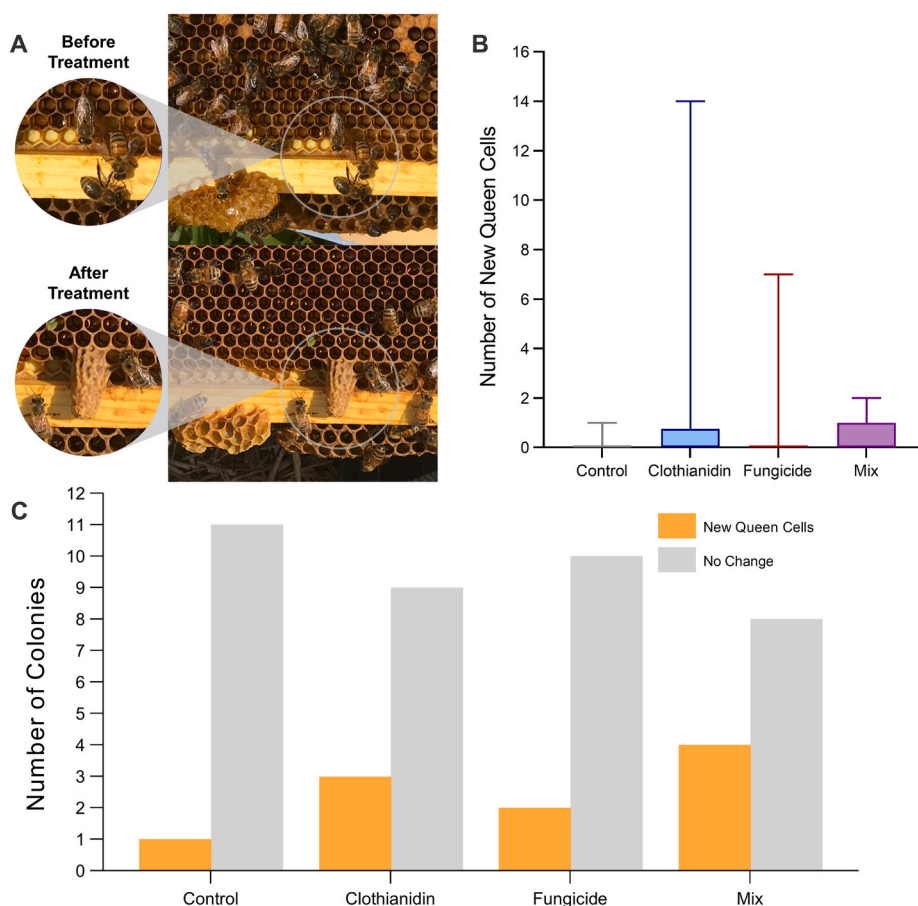
Fig. 6. Effects of pesticide treatment on the development of new queen cells in experimental colonies. (A) Photographic example of queen cell development. Before treatment (*top inset*) shows the same frame from an experimental colony as after treatment (*bottom inset*), and the development of a new queen cell in the treated colony (the levels of which were quantified in the analysis of 1420 photos of frames). (B) The effect of treatment on the numbers of new queen cells in treated colonies (mid-line represents medians, the box represents the interquartile range, and whiskers represent the total range for each data-set). (C) The number of colonies within each treatment group that exhibited 'new queen cells' or exhibited 'no change'.

However, there were few colonies (10/48, only 21 %) with new queen cells for statistical analysis, and there was no significant treatment effect on the amount of new queen cells (Fig. 6B; Friedman Test Statistic $= 1.94$, *d.f.* $= 3$, $P = 0.58$) or the number of colonies with new queen cells (Fig. 6C; $\chi^2 = 2.526$, *d.f.* $= 3$, $P = 0.47$).

3.6. Parasitic mite load

Screens of colonies for the parasitic mite *V. destructor* were taken before and after treatment, and the change in mite loads compared between treatments (Fig. 7). Treatment affected *Varroa* infestation in honey bee colonies (Friedman Test Statistic $= 11.16$, *d.f.* $= 3$, $P = 0.0109$). Median *Varroa* levels remained stable in control (Interquartile Range [IQR] 0.00–0.33 mites/100 bees) and fungicide colonies (IQR 0.00–0.58 mites/100 bees) and increased slightly by 0.33 mites/100 bees (IQR 0.08–0.67) in clothianidin treated colonies. Only with mix exposure did the change in *Varroa* levels increase significantly from control (Dunn's, $P = 0.0431$), with a median increase in *Varroa* by 0.83 mites/100 bees (IQR 0.42–1.00). *Varroa* loads were generally low, which is typical for newly established nucleus colonies. Only 1 out of 48 colonies (a mix treated colony with 4.33 mites/100 bees) exceeded the 3–4% infestation threshold that would merit autumnal *Varroa* treatment.

4. Discussion

We have demonstrated that the combined effects of commonly encountered neonicotinoid insecticides and fungicides observed on individual honey bee mortality in the lab [4,11–14] also occur when entire colonies are exposed to in-field, sub-lethal rates of these compounds. Both fungicide active ingredients, metconazole as an EBI-fungicide [46], and pyraclostrobin, which targets mitochondrial respiration [47], may contribute to increased clothianidin toxicity. EBI-fungicides are known to increase neonicotinoid toxicity [11–14], and boscalid (which targets mitochondrial respiration) more than doubles clothianidin toxicity in honey bees [4]. These
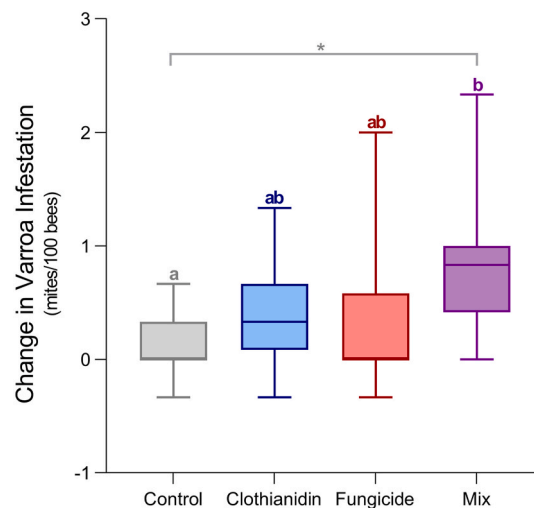
Fig. 7. Effects of pesticide treatment on the change in the levels of the parasitic mite *Varroa destructor*. Treatments include control, clothianidin, fungicide, and mix. *Varroa* infestation changes are measured in the mites/100 bees for each colony over the two-week treatment period. Mid-lines represent medians, the box represents the interquartile range, and whiskers represent the total range for each data-set. Significance stars (*$P < 0.05$) and letters identify treatments that are significantly different from multiplicity adjusted P – values in *post hoc* analysis.

effects may be brought about through physiological mechanisms, such as modulating the activities of enzymes like gluta-thione-*S*-transferase (GST), acetylcholinesterase [14], and cytochrome P450's [13], which are involved in stress/insecticide detoxi-fication pathways.

Some of the effects we observed have clear implications for honey bee colony health: the key observation was that application of pesticides led to increased mortality in treated colonies and a decline in the overall populations of bees within these colonies. Furthermore, we found a synergistic effect of clothianidin and fungicide on honey bee mortality, consistent with previous studies [4, 11–14]. Additionally, we observed diminished activity levels and weight reductions in the treated colonies. Our analysis uncovered a significant correlation between honey bee activity and both mortality and weight, implying a decline in the overall fitness of the honey bee colony due to increased mortality.

Moreover, pesticide exposure caused increased sugar consumption. Similar effects have been observed before with dosed solutions in the lab, where combined exposure to the neonicotinoid imidacloprid, the EBI-fungicide difenoconazole, and the herbicide glyph-osate, increased sugar consumption in honey bees [14]. There are multiple hypotheses for this effect [14], and while preferences for solutions containing neonicotinoids have been shown [48], more recent studies have shown that bumblebee preferences for neonicotinoid-treated sugar water might be acquired with experience [49], and other bumblebees (e.g. *Bombus impatiens*) show no evidence of preferences for neonicotinoid-treated sugar water compared to untreated control solutions [50]. The time in season when we performed the experiments may be a key variable here; future work could explore whether documented differences across the season in honey bee nectar vs. pollen feeding preferences affect consumption of pesticide formulations introduced with sugar solutions. Another hypothesis is that pesticide-induced energetic stress elicits greater feeding responses in bees [14], which may explain the effects we observed.

In addition to direct bee mortality, the population dynamics of honey bee colonies may be affected by combined pesticide exposure, which is arguably a more important parameter in evaluating pesticide effects on social insects. This has been shown to be important to social insect colony dynamics (and survivorship) through a range of empirical [51,52] and modelling approaches [53]. For example, with combined clothianidin and fungicide exposure, we found increased *Varroa* mite infestations. The global spread of *Varroa* mites has driven an epidemic of deformed wing virus (DWV) in honey bees [3]. Clothianidin exposure has been shown [54] to reduce immune response (against both the feeding activity of *Varroa* mites and the DWV they carry), which increases *Varroa* infestations and DWV infections in honey bees. In our colonies, fungicides might have exacerbated the impact of clothianidin on *Varroa* infestations by interfering with pesticide detoxification pathways [13,14]. Additionally, clothianidin [55] and pyraclostrobin [56] affect development of hypopharyngeal glands (which secrete royal jelly) of nurse bees, which can have detrimental effects on colony main-tenance/nutrition, and brood and queen care (potentially leading to population-level colony effects). Interestingly, while differences were not statistically significant here, the average reduction in brood levels in controls (which is normal this time of year in the attrition of honey bee colonies [57,58]) was greater than the brood reduction in treatments containing clothianidin. Clothianidin exposure has been shown to increase brood initiation rate (as a means of compensating for mortality) [55]. Thus, as *Varroa* mites reproduce in sealed brood cells, higher brood levels dramatically increase the population growth of *Varroa* mites [57]. Therefore, pesticide-induced changes in honey bee population dynamics may indirectly increase ectoparasite load, via increased breeding reservoirs.

This study demonstrates that field applications at the lowest label rate of a commonly-used commercial fungicide have com-pounding stress effects on whole honey bee colonies when combined with sub-lethal, field relevant levels of the neonicotinoid

insecticide clothianidin. Due to logistical and budgetary limitations, our experiments were conducted within field cages and using nucleus colonies, and these factors should be considered when extrapolating results to honey bees foraging from unenclosed colonies. Conversely, a benefit of our approach is uncovering effects that are subtle, unlikely to be attributed to pesticides, and unlikely to be detected by beekeepers. Effects such as increased mortality [59] and heightened levels of *Varroa* [60], can weaken and eventually cause the failure of honey bee colonies, often experienced as overwintering losses. Reduced colony population size also directly reduces the profits beekeepers gain from rentals for pollination ecosystem service delivery [61]. Pesticide combinations are likely to be encountered by many wild pollinators and other pollen and nectar foragers as well, and fungicide/neonicotinoid interactions have been highlighted in bumblebees [12,16,17] and solitary bees [12,62]. A recent study highlighted the interactions between exposure to fungicide and butenolide (flupyradifurone) insecticides in a key pollinator of cucurbits, the solitary, ground-nesting squash bees [63].

The prevalence of agrochemical interactions is unavoidable due to widespread neonicotinoid applications to row crop seeds, which is done prophylactically [20,25,64], and has led to steep increases in environmental toxicity encountered by pollinators in the Midwest US [20,28]. This practice continues, despite a lack of evidence that these seed-treatments provide demonstrable yield benefits to row crop farmers [23,65,66]. Given the diverse cocktail of agrochemical stressors, including fungicides, which pollinators are exposed to alongside neonicotinoids across much of the US [9,10,19], our findings re-emphasize [66] the urgent need to reconsider the current prophylactic mindset guiding pesticide applications in the most widely planted commodities.

Data availability statement

The data supporting the findings of this study are available in supporting information.

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CRediT authorship contribution statement

Sebastian Shepherd: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Young-gyun Park:** Data curation, Formal analysis, Writing – review & editing. **Christian H. Krupke:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Christian H. Krupke reports financial support was provided by National Institute of Food and Agriculture.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e29886.

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