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Colony field test reveals dramatically higher toxicity of a widely-used mito-toxic fungicide on honey bees (*Apis mellifera*)☆

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ABSTRACT

Honey bees (*Apis mellifera*) and other pollinator populations are declining worldwide, and the reasons remain controversial. Based on laboratory testing, fungicides have traditionally been considered bee-safe. However, there have been no experimental tests of the effects of fungicides on colony health under field conditions, and limited correlational data suggests there may be negative impacts on bees at levels experienced in the field. We tested the effects of one of the most commonly used fungicides on colony health by feeding honey bee colonies pollen containing Pristine® (active ingredients: 25.2% boscalid, 12.8% pyraclostrobin) at four levels that bracketed concentrations we measured for pollen collected by bees in almond orchards. We also developed a method for calculating per-bee and per-larva dose. Pristine® consumption significantly and dose-dependently reduced worker lifespan and colony population size, with negative health effects observed even at the lowest doses. The lowest concentration we tested caused a 15% reduction in the worker population at an estimated dosage that was three orders of magnitude below the estimated LD_{15} values for previous acute laboratory studies. The enhanced toxicity under field conditions is at least partially due to activation of colonial nutritional responses missed by lab tests. Pristine® causes colonies to respond to perceived protein malnutrition by increasing colony pollen collection. Additionally, Pristine induces much earlier transitioning to foraging in individual workers, which could be the cause of shortened lifespans. These findings demonstrate that Pristine® can negatively impact honey bee individual and colony health at concentrations relevant to what they experience from pollination behavior under current agricultural conditions.

1. Introduction

Honey bees are declining in North America, threatening over $12 billion in agriculture that depends on pollination services (Calderone, 2012; Zhu et al., 2015). The alarming decline in this important agricultural pollinator reflects a much broader and now well-documented reduction in pollinators, with damaging ecological impact (Naug, 2009; Johnson et al., 2010). Ongoing pollinator decline is attributed to a variety of factors, including habitat loss, monocultural floral resources, pathogens, parasites, and pesticides (Naug 2009; Johnson et al., 2010; Williams et al., 2010; Smith et al., 2013; Sponsler et al., 2019). The relative importance of any of these factors remains unclear, however, in part because detailed well-controlled studies are needed to evaluate their individual effects (Naug 2009; Johnson et al., 2010; Williams et al., 2010; Smith et al., 2013; Sponsler et al., 2019). Fungicides are the agrochemicals most frequently encountered by foraging honey bees, as well as other bees on agricultural crops such as stone and pome fruits and berries. This is due to the application of fungicides during periods of bloom, when bee pollen collection is at its peak (Pettis et al., 2013). Fungicides are also among the most common agrochemical contaminants found in the wax comb and pollen reserves of honey bee hives, indicating that colony exposure likely extends beyond the bloom period (Mullin et al., 2010). Indeed, Stoner and Eitzer (2013) calculated pollen hazard quotients based on application rates and LD_{50} values suggesting that the dangers of some fungicides are orders of magnitude greater than many insecticides including neonictinoids (Stoner and Eitzer, 2013). As yet, we lack an

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understanding of the colony-level effects of any fungicide on any social insect pollinator.

There is emerging correlative and epidemiological evidence that at least some fungicides can be harmful to bee health (Pilling and Jepson, 1993; Pettis et al., 2013; McArt et al., 2017). However, these claims lack causal demonstration for any fungicide that exposure to fungicides at field-appropriate levels has detrimental effects on colonies of pollinators. Oral and contact LD_{50}s for fungicides measured on individual bees in the lab are usually found to be at least four orders of magnitude greater than concentrations found in honey bee food stores (Ostiguy et al., 2019), producing the expectation that field dosages are "safe". However, some fungicides have been shown to have sublethal negative effects on bee behavior and health and to cause lethality in longer-term studies, although it is unclear whether the doses used in the lab are well-matched to field exposures (Bernauer et al., 2015; Campbell et al., 2016; Fisher et al., 2017; Mao et al., 2017; Simon-Delso et al., 2018). More commonly, in laboratory studies, fungicides applied individually have often been shown to be nonlethal, with some able to synergize the toxicity of other pesticides (Pilling et al., 1995; Iwasa et al., 2004; Johnson et al., 2013; DeGrandi-Hoffman et al., 2015; Tsvetkov et al., 2017; Zaluski et al., 2017; Tosi and Nieh, 2019).

Extrapolating toxicity of pesticides on individual bees in the lab to responses of field colonies can be problematic. As complex societies, social insect colonies may have protective responses not obvious from laboratory tests on individuals. For example, nurse bees could conceiveably prevent fungicide transfer to larvae by metabolizing fungicides or by selectively using food that is not contaminated, and/or colonies might be able to rear more brood in response to worker deaths, minimizing potential negative effects of fungicides on colonies. Conversely, pesticides could produce stronger effects on colonies than measured in individuals by interfering with social communication signals (for example by inducing absconding from the nest). The first study to document fitness effects of a fungicide on bee colonies in the field showed that spraying plants with the fungicide chlorothalonil at prescribed doses caused bumblebee colonies caged with those plants to produce fewer workers and have lighter queens, but the mechanisms for these effects are unclear (McArt et al., 2017).

Here we focus on honey bees (*Apis mellifera*) and their exposure to the fungicide Pristine®, which is applied during bloom for many crops, including almonds (Janousek and Gubler, 2010). Like many fungicide treatments, Pristine® is applied during crop bloom, a critical period where pollinators can collect and consume both nectar and pollen contaminated with the fungicide (Janousek and Gubler, 2010). Pristine® has two active ingredients, the anilide fungicide boscalid, and the strobilurin fungicide, pyraclostrobin, both of which inhibit mitochondrial respiration in fungal targets (Avenot and Michailides, 2007). Pyraclostrobin has recently been shown to damage the midgut epithelia of honey bees when fed to bees in the lab (Costa Domingues et al., 2020; Tadei et al., 2020). To assess the concentration of Pristine® that honey bees are exposed to when pollinating almond orchards, we measured the concentrations of boscalid and pyraclostrobin in pollen collected by honey bee colonies in California almond orchards. To confirm that observed effects are due to the fungicide, we conducted a dose-response experiment with colonies fed pollen containing Pristine® at four doses bracketing the boscalid and pyraclostrobin concentrations detected in the pollen collected by bees across multiple months, as may occur if bees are used to pollinate multiple crops sprayed with this fungicide. We show, for the first time using a longitudinal study in the field, that Pristine® has dose-dependent negative effects, potentially causing protein malnutrition that induces earlier foraging, shorter worker lifespan, and smaller colony sizes at doses three orders of magnitude lower than laboratory studies. These findings indicate that testing standards (OECD 1998; OECD 2017) for assessing pesticide hazard for honey bees are inadequate in scope by not accounting for in-hive factors and environmental interactions.

2. Materials and methods

2.1. Measurement of boscalid and pyraclostrobin in pollen collected from Pristine®-sprayed orchards

In 2010 and 2011, hives in two California almond orchards were outfitted with pollen traps on the hive entrance, and accumulated pollen in the traps was pooled together to obtain four samples. Pooled samples were analyzed for pesticide residues at the USDA-AMS National Science Laboratory (Gastonia, NC). The concentrations of boscalid and pyraclostrobin in corbicular pollen, (reported in Table 1), were used as the basis for the dose formulation for the experimental colonies.

2.2. Colony initiation

Forty experimental colonies of the Italian honey bee (*Apis mellifera ligustica*) were established from 3-lb (1.36 kg) bee packages (~10,000 bees) in April 2018. All packages were obtained from Pendell Apiaries, Inc. in Stonyford, CA (39.376956, −122.558801). The forty experimental colonies were placed into Apimaye insulated hives (Kaftan LLC Tempe, AZ). To control for location effects, colonies were placed into eight clusters of five hives, with one hive from each treatment group in a cluster. Each cluster was arranged in a circular pattern around a different tree (for shade) in the apiary at the Arizona State University Polytechnic Campus honey bee lab in Mesa, AZ (33.293173, −111.684520), clusters were about 6 m away from each other. To ensure that colonies were not exposed to comb with previous agrochemical content, each hive was initially stocked with five wooden frames outfitted with a plastic worker cell template foundation, so that workers constructed new comb. We also collected bee bread samples from each hive during the experiment, in June 2018, and had these analyzed by the USDA-AMS National Science Laboratory. Bee bread samples were pooled by treatment group resulting in five samples, each incorporating bee bread from eight hives. Pesticide residue analyses found no other agrochemicals present above detection levels other than a few herbicides: diuron, flumeturon, and hexazinone. The highest detection level for any herbicide was 12 ppb. A metabolite (DMPF) of the miticide amitraz was detected at higher levels (max 147 ppb). Amitraz was the active ingredient of the Varroa mite treatments we applied in the Fall of 2018 and Spring of 2019.

All hives were supplied with 30% sugar syrup for the first 3 weeks after their establishment to assist comb building. Hives were also outfitted with internal pollen traps to reduce access to pollen collected in the surrounding environment. Hives were maintained with 50 g pollen patties consisting of a 1:1:1 ratio of dry pollen, sucrose and fondant sugar (8% inverted); approximately 8% of each pollen patty consisted of deionized H_2O which was added after the dry ingredients were thoroughly mixed. Pollen patties on 60×15 mm petri dishes were placed into each hive. Experimental hives were maintained in this manner from their initial setup in April 2018 until May 1, 2018 when colony growth assessments commenced. A week prior to the initial colony growth assessment, the experimental hives were equalized so that they had approximately the same number of drawn combs (five) and adult population.

Table 1
Boscalid and pyraclostrobin concentrations detected in corbicular pollen.

Year	Pyraclostrobin (ppm)[a]	Boscalid (ppm)[a]	Approx. Pristine® range level (ppm)
2010	1.73	6.06	13.51–24
2011	0.4	1.49	3.13–5.9
2011	0.69	2.25	5.39–8.94
2011	0.5	1.76	3.91–7

[a] Obtained from pesticide residue analyses conducted by USDA-Agricultural Marketing Service National Science Laboratory (Gastonia, NC, USA) right.

2.3. Fungicide treatment

Beginning on May 1, 2018 we modified the pollen patty supplied to designated experimental hives by incorporating Pristine® (BASF Corporation, Research Triangle Park, NC). Four doses of Pristine® (25.2% boscalid, 12.8% pyraclostrobin) were used: 0.23; 2.3; 23; and 230 ppm, while the control group continued to receive pollen without Pristine®. Each hive within each five-hive cluster was randomly allocated to one of the five experimental treatment groups such that a single representative of each treatment group was present in each cluster to equalize environmental effects. We fed each hive fungicide-treated pollen patties *ad libitum*, with a new patty supplied as soon as the previous patty was entirely consumed. If the pollen patty was not completely consumed within one week, it was replaced to maintain freshness. Each colony was monitored every other day to verify pollen patty availability and remaining patties in each dish were weighed at least once a week to record weekly consumption. Weekly pollen patty preparation for each treatment group included mixing equal parts ground pollen, sucrose and dry fondant sugar (147 g each) with deionized water (39 g) forming a 480 g (0.480 kg) pollen mixture which accounted for a 50 g ration for each of eight colonies (400 g total) plus an extra 20 percent (80 g). Pristine was added into the water allowing it to dissolve and mix evenly through the resulting pollen mixture. The amount of Pristine® added was calculated by solving for mg of solute (Pristine®) per kg of the complete pollen patty (pollen, sugar, water mixture). As an example, for the 230 ppm dose group, 230/1000000 * 0.480 kg of pollen mixture = 110.4 mg of Pristine® added to the pollen mixture to form the treatment for the 230 ppm dose group. For the 0.23, 2.3 and 23 ppm dose groups, 0.11, 1.10, 11.0 mg of Pristine® were added, respectively, to each of their corresponding 0.480 kg pollen mixtures.

2.4. Colony demography and growth assessments

For each colony, we assessed the number and proportion of frames occupied by eggs, larvae, pupae, honey, pollen and adult workers every two weeks, with 20 hives assessed per week. Hive assessments were divided between two groups of two observers such that each group assessed two clusters (10 hives) a week. Each cluster had a single representative from each treatment group and the control such that all treatment groups were assessed at the same time. Assessments continued from May to Nov 2018. We utilized Canon® EOS Rebel T5 cameras to take two sets of photographs of both sides of each frame in each hive, one with and one without adult workers present. To measure adult populations, observers estimated the number of bees on a frame (±5 bees) after being trained with 25 reference pictures with worker populations ranging from 10 to approximately 1000 bees. For all colonies, pictures of the adults on each frame were taken before other hive measures. To minimize disruption, frames were handled slowly and carefully. Capped brood, honey and pollen were estimated from a second set of images of each frame taken after the workers had been gently shaken and brushed off the frame. An 877.2 cm^2 grid was overlaid on the frame, and the number of cm^2 occupied by pupae, honey or pollen was counted. For incompletely filled grid cells, a cell fraction was calculated based on the number of cells filled by the parameter of interest divided by 23 (the number of cells per 6.45 cm^2). Our estimates on the amount of eggs and larvae were conducted visually in the field with the 877.2 sq. cm grid overlaid on the frame, because eggs and young larvae were difficult to discern in photographs.

2.5. Pollen foraging and consumption assessments

Environmental pollen collection rates were calculated weekly by dividing the mass of pollen accumulated in the pollen traps by the number of days since the tray was emptied. Foraging behavior was assessed weekly through the Summer and early Fall by monitoring returning foragers at the hive entrance. We observed each hive entrance for 5 min a day in the morning and counted the number of pollen and non-pollen foragers returning to each hive. All forty colonies were assessed on the same morning, from 6:30–7:30 a.m., ensuring that all treatment groups experienced the same weather and pollen availability. Consumption of the pollen patties was calculated by weighing the pollen patties before and after replacement, and dividing by the number of days the pollen patty was within the colony.

2.6. Assessment of age of first foraging and adult worker longevity

In February 2019, three hives were randomly selected from each treatment group to assess the effects of Pristine® consumption on age of first foraging and adult longevity. A minimum of one frame stocked with capped brood was placed in an incubator (34 °C, 70% relative humidity) overnight from each hive. Newly emerged bees were collected the next day or over a span of the next few days until a minimum of 300 individuals were collected from each hive. Each newly emerged adult was marked with paint on the mesonotum of the thorax with differing paint colors applied according to treatment group, date and hive of origination. Marked bees were reintroduced to their hive of origin within 90 min after all newly emerged bees were collected and completely marked.

The age of first foraging for marked individuals was recorded by observing the hive entrance for 10-min sessions each day. Due to cool morning early spring temperatures in occurring when these experiments were run, observations started in the late morning (10 a.m.), when external honey bee activity was observed to start, and extended into the afternoon (12:30 p.m.) such that all fifteen colonies were observed for 10 min each.

The number of surviving marked bees was assessed weekly, each Wednesday afternoon following re-introduction, by opening each hive and recording the number of marked individuals observed on each frame. Hand clickers were used to record marked bees observed frame by frame. Once assessed, frames were placed aside to prevent recounting the same individuals.

2.7. Pollen trap efficiency

In order to assess pollen consumption per bee (and effective

Pristine® concentration in the consumed pollen), we needed to assess what fraction of hive pollen consumption came from the pollen patties vs. pollen collected by foragers that penetrated the pollen trap. To assess the efficiency of the internal pollen traps (Goodwin and Perry, 1992), twenty hives were each observed across five morning hours from 6 to 11 a.m. In two 10 min sessions each hour, observers watched the hive entrance and counted the number of incoming pollen foragers. Foragers with two fully loaded corbiculae were distinguished from foragers with a single loaded corbicula or reduced loads on both corbiculae. Following each of the ten observations the bottom board was removed and accumulated pollen loads screened by the pollen trap were counted. Expected numbers of pollen loads were calculated by multiplying the total number of fully loaded foragers by two and adding to the number of half and reduced loaded foragers. Efficiency was calculated by dividing the number of pollen loads collected by the pollen trap by the number of pollen loads expected based on counts of returning pollen foragers.

2.8. Statistical analysis

For data that visually suggested dose-dependence of Pristine® consumption (adult population levels, adult worker longevity, pollen foraging, collection and storage), data were analyzed using general linear regression tests (dependent variable vs. Pristine® concentration and sampling bout as parametric factors) with colony as a random effect. For assessment of averages (average worker population, average longevity, age of first foraging, average pollen foraging, collection, storage and consumption) we also tested for significant effects using general linear regression tests, and all post hoc analyses were conducted using Tukey HSD. The average worker population levels (Fig. 1b), the averages for pollen foraging, collection and storage (Fig. 3b, d, f) and average adult longevity (Fig. 2c) compared the average number of marked bees observed within each replicate (colony) for each treatment across the duration of the experiment, with colony as a random effect. The average age of first foraging (Fig. 2d) was the comparison of average day post-emergence that marked individuals were first observed foraging for each replicate for each treatment. Overwintering survival (Fig. 1c) data were assessed using contingency analysis pooling all Pristine®-treated groups as there was no obvious dose-dependence of mortality and we lacked the power to test all individual treatment groups against the control group. All data analyses were performed using JMP Pro 14 (SAS Inc., Cary, NC). Statistical significance was set at $\alpha = 0.05$ for all tests performed.

3. Results

Concentrations of boscalid and pyraclostrobin in corbicular pollen collected from honey bees at almond orchards.

We measured boscalid and pyraclostrobin levels in pollen collected from pollen traps for commercial honey bee colonies pollinating almond orchards that had been sprayed with Pristine. Boscalid levels ranged from 1.49 to 6.06 ppm and pyraclostrobin levels ranged from 0.4 to 1.73 ppm (Table 1). Given the percent representation of boscalid (25.2%) and pyraclostrobin (12.8%) in Pristine®, these measurements translated to Pristine® levels of 3–24 ppm (Table 1).

3.1. Effect of Pristine® exposure on adult population levels and winter survival

We monitored worker populations and brood levels across six months (May–Oct), and subsequently measured overwinter survival (Oct–Feb). Over the entire experiment, there was a significant linear effect of Pristine® dose on worker populations, with an average decrease of 21% relative to control colonies (Fig. 1A, Supplementary Fig. 1). There was no significant dose-dependent effect on overwintering mortality, but when Pristine®-treated colonies were pooled across doses, overwinter survival was significantly (30%) lower than controls (Fig. 1B) [$P = 0.02$]. Colonies consuming the lowest dose, less than 10% of the lowest concentration of Pristine® measured in almond pollen, also showed a significant decline in worker population (Fig. 1A, Supplementary Fig. 1) [$P < 0.0001$]. Declining worker populations could be due to reduced worker production or increased mortality or both. We found no signficant dose-dependent effect of Pristine® on brood levels (eggs, larvae, sealed brood, Supplementary Figs. 2-4), suggesting that Pristine® did not affect brood production rate but rather primarily influences worker mortality.

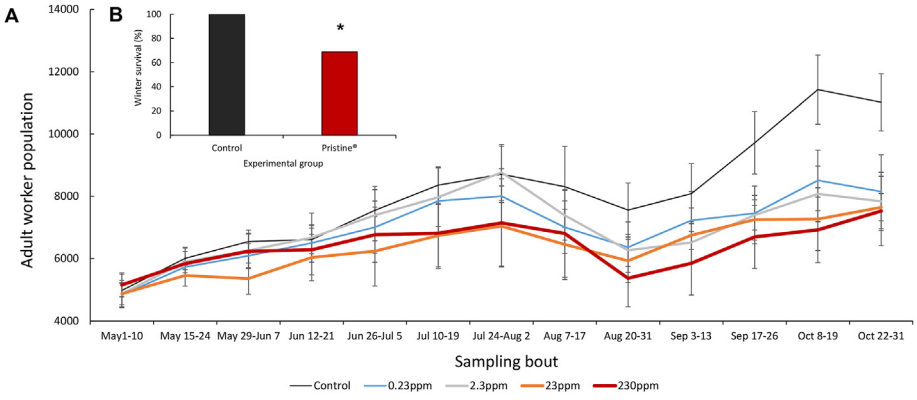

Fig. 1. The effects of Pristine® consumption on adult worker population and winter survival. *(A)* Adult worker population through time for colonies given pollen with increasing Pristine® concentrations. Pristine® consumption decreased worker populations in a dose dependent manner (General Linear Regression, $R^2 = 0.41$, $P < 0.0001$), post hoc analyses were conducted using Tukey HSD. To account for repeated measures for each treatment group, colony was included as a random effect in the regression model. The control group had a significantly higher population than all of the Pristine®-treated groups by the August 20–31 sampling date and thereafter through Oct. 31. For this graph, means and standard errors are shown. N = 8 colonies for each treatment group. *(B)* Winter survival for colonies consuming pollen with or without Pristine® fungicide. Pristine® treatment significantly affected winter survival (Contingency Analysis, $X^2 = 5.24$, $P = 0.02$). The control group did not undergo any colony-level mortality. N = 8 for control colonies, N = 32 for Pristine®-treated colonies. Dose effects on colony over-winter mortality were not linear so Pristine®-treated colonies were pooled for this analysis.

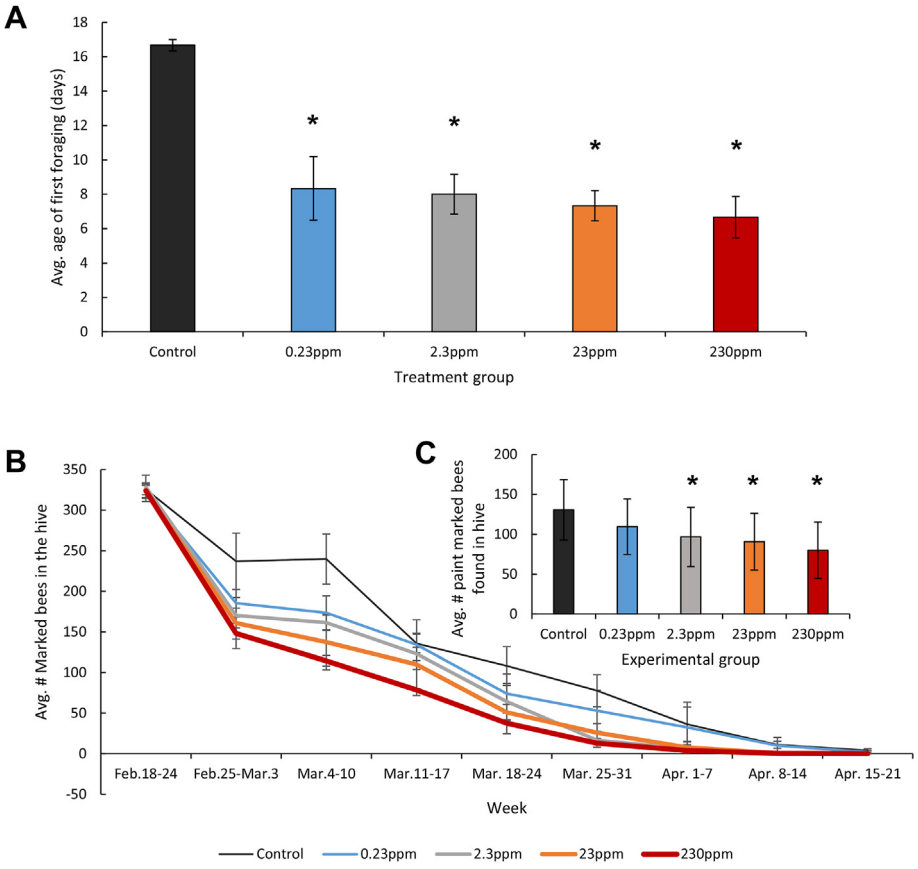

Fig. 2. Pristine®-treated bees foraged earlier and had reduce longevity. *(A)* Marked workers from Pristine®-treated hives began foraging significantly earlier (General Linear Regression, $R^2 = 0.83$, $P = 0.0008$). For this and all subsequent graphs, asterisks indicate treatment groups that differ significantly from the control, n = 3 colonies for each treatment group. *(B)* Paint marked adult workers observed in the hive over time for colonies consuming pollen with zero or four doses of Pristine® fungicide. There was a significant linear and negative effect of Pristine® dose on the number of bees surviving over time (General Linear Regression, $R^2 = 0.33$, $P < 0.0001$). To account for repeated measures for each treatment group, colony was included as a random effect in the regression model *(C)* Average number of marked workers observed over the entire experimental duration. Significantly fewer marked workers were observed in Pristine®-treated hives (General Linear Regression, $R^2 = 0.98$, $P < 0.0001$).

3.2. Pristine® induced differences in worker longevity and foraging ontogeny

To assess Pristine® effects on individual worker longevity, we paint-marked and reintroduced day-old workers from three colonies for each experimental group. When marked bees were first observed foraging, we then made daily 10-min counts of returning marked foragers. Pristine® consumption was associated with earlier foraging and with reduced worker longevity. Bees originating from fungicide-treated hives initiated foraging activity approximately a week earlier than control hives, though this effect was not dose-dependent (Fig. 2A). Correspondingly, the number of surviving marked workers in Pristine®-exposed colonies was significantly lower than in control colonies by the second week of observation. Worker survival over time continued to decline with dose-dependent effects of Pristine® concentration (Fig. 2B and C).

3.3. Differential pollen collection and storage resulting from exposure to Pristine®

Pristine® exposure increased the fraction of foragers collecting pollen (Fig. 3A, Supplementary Fig. 5), increased the amount of pollen collected in pollen traps (Fig. 3B, Supplementary Fig. 6), and reduced levels of pollen stores (Fig. 3C, Supplementary Fig. 7), all in an approximately dose-dependent manner. We broadly estimated per capita consumption of the pollen patties by dividing the rate of pollen patty consumption by the number of adult bees in the hive. Pristine® exposure increased per capita pollen patty consumption (Fig. 3D) relative to the control though the effect of dose was nonlinear. These findings are consistent with the hypothesis that bees under Pristine® exposure consume more pollen, but perhaps are unable to completely digest or absorb pollen nutrients. Honey reserves did not seem to be affected by Pristine® treatment (Supplementary Fig. 8), supporting the hypothesis that Pristine® specifically affects pollen processing. Thus multiple lines of indirect evidence, ranging from individual consumption through colony foraging effort, suggest that Pristine® may interfere with the ability of honey bees to process pollen, potentially explaining their earlier foraging and demise.

3.4. Estimation of dose received per bee

Prior toxicological studies have not been able to estimate doses (either as rates of intake or mass) for field colony experiments. Here we develop a method for calculating dose per bee based on our measurements of the rate at which hives consumed the Pristine-containing pollen patty, the number of larvae and pupae in each hive, and estimates from the literature on the relative consumption of pollen by adults and larvae. This estimate assumes that larvae only receive boscalid and pyraclostrobin in pollen; it is certainly plausible that some of these active ingredients are passed to the larvae in the hypopharyngeal gland secretions that are fed to larvae

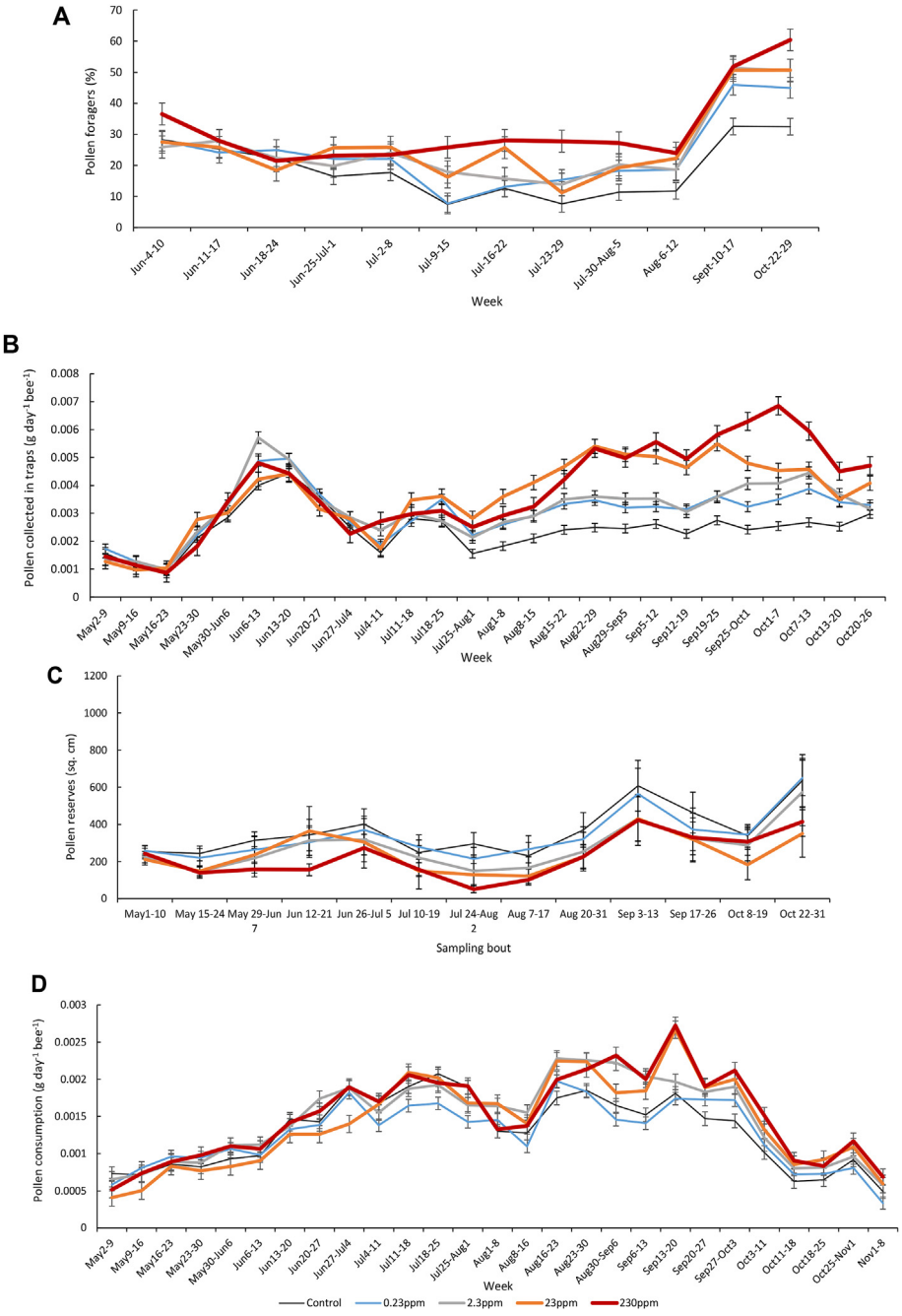

Fig. 3. Pristine® consumption increased pollen foraging and consumption. *(A)* Pristine® had a significant linear positive effect on the number of pollen foragers observed (General Linear Regression, $R^2 = 0.07$, $P < 0.0001$). Colony was included as a random effect in the regression model for this and all subsequent regression analyses. N = 8 colonies for each treatment group. *(B)* Pristine® had a significant linear positive effect on weekly pollen collection per capita (General Linear Regression, $R^2 = 0.72$, $P < 0.0001$). N = 8 colonies for each treatment group. *(C)* Pristine® had a significant linear negative effect on the quantity of pollen stores (General Linear Regression, $R^2 = 0.12$, $P < 0.0001$). N = 8 colonies for each treatment group. N = 8 colonies for each treatment group. *(D)* Pristine® treatment significantly affected the per capita rate at which pollen patties were consumed (General Linear Regression, $R^2 = 0.89$, $P < 0.0001$). N = 8 colonies for each treatment group.

(which would increase larval dose above what we estimate here). We estimated the dose of Pristine, boscalid and pyraclostrobin received by the bees in the hive during the time period of June 27-July 25, as adult populations and levels of brood were relatively constant over this time period (Fig. 1, S1-S3), as were consumption rates of the pollen patties (Fig. 3D), facilitating a steady-state analysis.

The rate (g day^{-1}) at which Pristine ($Pr_{in\text{-}hive}$), boscalid ($B_{in\text{-}hive}$) and pyraclostrobin ($Py_{in\text{-}hive}$) was consumed per hive can be calculated from the rate at which the pollen patty is consumed (PPC_{hive}, g day^{-1}) multiplied by the decimal fraction of each in the pollen patty (Pr_c, B_c, Py_c, g g^{-1}), as in equation (1):

$$Pr_{in-hive} = PPC_{hive} * Pr_c \quad (1)$$

We calculated pollen patty consumption for each hive from the weekly measures of pollen patty consumption over this time period, and then calculated the mean across all treatments since during this period the treatments did not differ in rates of pollen

patty consumption.

Pollen is consumed by both young adult bees and larvae. Crailsheim et al. (1992) demonstrated that the number of pollen grains in a young bee's gastrointestinal tract increased up to 6–10 days of age in two different colonies, so we estimated that adult bees consumed pollen up to 8 days of age. The duration of the pupal period is twelve days in honey bees, as pupal numbers were relatively constant over this period, we estimated the average worker production per day as the number of pupal cells divided by 12. Fungicide treatment did not affect the number of pupal cells (Fig. S4), so we used the average across treatments. During the target time period, the average colony had 1347 square cm of pupae. As there were 3.57 cells cm^{-2}, this corresponded to 4810 pupae. Dividing by 12, yields an average daily eclosion rate for adult bees of 401 bees day-1. As there is little mortality during the pre-foraging stage in a healthy hive (Rueppell et al., 2007), the number of adult bees 1–8 days old equals 8 * 401 = 3208. Again, because the number of pupae did not vary among treatment groups over this period we used this same number of adult bees for all treatment groups when calculating per larva and per adult dose.

Pollen is consumed by the larvae during the latter half of development, so we estimated the number of larvae consuming pollen by dividing the number of larval cells by two. As for the pupae, there was no significant effect of treatment on the number of larvae in the colonies throughout the experiment (Fig. S3), so we used the average larval abundance (438 cm^2 * 3.57 cells cm^{-2} * ½) to calculate that, on average, there were 782 larvae consuming pollen in each hive during our target time period.

The workers consume considerably more pollen than larvae, who receive most of their protein in the form of hypopharyngeal gland secretions from the young adults (Babendrier et al., 2004). Crailsheim et al. (1992) estimated that the average adult worker consumes 60 mg of pollen during development, while Babendrier et al. (2004) estimated that the average larva consumed 2 mg of pollen during their development. Therefore to simplify the math for dose estimation, we assumed that adults consumed 30× more pollen than larvae.

According to mass balance, the rate at which Pristine® in the pollen patty is consumed must equal the rate of Pristine® consumption of young adult workers and larvae summed:

$$\mathrm{Pr}_{in-hive} = (3,208 adults * 30x) + (782 larvae * x) \quad (2)$$

where x is the rate of Pristine consumption of each larva (grams day^{-1}). Rearranging and solving for x:

$$\mathrm{Pr}_{larva} = \mathrm{Pr}_{in-hive}/97,022 \quad (3)$$

$$\mathrm{Pr}_{adult} = 30{*}\mathrm{Pr}_{larva} \quad (4)$$

Where $\mathrm{Pr}_{\mathrm{larva}}$ and $\mathrm{Pr}_{\mathrm{adult}}$ indicate the rates of Pristine® consumption by each larva and adult, respectively, in grams day^{-1}. The rate of boscalid consumption is 25% of Pristine consumption rate, whereas pyraclostrobin is consumed at 12.8% of the Pristine consumption rate.

These rates of intake can be converted to total mass doses for each larva ($\mathrm{Pr}_{\mathrm{m\text{-}larva}}$, grams) and each adult ($\mathrm{Pr}_{\mathrm{m\text{-}adult}}$) by multiplying the rate of intake times the number of days over which pollen consumption occurs:

$$\mathrm{Pr}_{m-larva} = \mathrm{Pr}_{larva}{*}3 days \quad (5)$$

$$\mathrm{Pr}_{m-adult} = \mathrm{Pr}_{adult}{*}8 days \quad (6)$$

On the lowest Pristine® concentration (0.23 ppm in the pollen patty), the estimated Pristine® dose was 0.086 ng for each larva and about 6.9 ng per each adult, increasing up to 1000× on the highest dose (Table 2). Boscalid dose was calculated by multiplying Pristine® dose by 0.252, and the pyraclostrobin dose by multiplying the Pristine® dose by 0.128, reflecting the mass % of the active ingredients in this fungicide (Table 2).

3.5. Estimation of pollen consumption per larva and per adult

To estimate the mass of the pollen patty that was consumed by each larva and adult ($\mathrm{PPC}_{\mathrm{m\text{-}larvae}}$, $\mathrm{PPC}_{\mathrm{m\text{-}adult}}$, grams), we followed the logic of equations (2)–(4) (assuming that adults consume 30× more pollen than larvae), converting from a rate to an amount by multiplying the rate of pollen consumption from the Pristine®-pollen patty (grams day^{-1}) times the number of days that the larvae (3) and adults (8) consume pollen:

$$PPC_{m-larva} = 3{*}(PPC_{hive} / \ / 97,022) \quad (7)$$

$$PPC_{m-adult} = PPC_{m-larva}{*}30{*}8 \quad (8)$$

For the 0.23 ppm treatment group, estimated pollen intake from the pollen patty was 0.4 mg for a single larva, and 31 mg for a single adult; calculated per-individual pollen patty consumption for all treatment groups were within 15% of these values.

Direct measures of pollen in the digestive tracts of larvae and adults indicate that they consume about 2 and 60 mg of pollen, respectively (Babendreier et al., 2004; Crailsheim et al., 1992, Table 3), more than the 0.4 and 31 mg of pollen patty intake we estimated per larva and adult, respectively. However, our pollen traps did not successfully screen out most environmental pollen collected by foragers (Fig. S10), indicating that a substantial amount of each colonies' pollen resources were non-treated. Pollen trap efficiency was approximately 32% (Fig. S10). For the 0.23 ppm treatment group, the average amount of pollen collected from pollen traps was 5 g day^{-1}. Correcting for pollen trap efficiency, this indicates that (0.68/0.32 * 5) = 10.6 g day^{-1} of Pristine®-free outside pollen entered the hive. The 0.23 ppm treatment group hives consumed, on average, 12.1 g day^{-1} of pollen patty. Therefore we calculated total pollen intake for per larva ($\mathrm{PolTotal}_{\mathrm{larva}}$, grams) and per adult ($\mathrm{Poltot}_{\mathrm{adult}}$, grams) from:

$$Poltot_{larva} = ((12 + 10.6) / 12){*}PPC_{m-larva} \quad (9)$$

Table 2
Fungicide treatment concentrations in pollen patties and estimated per larva and per adult doses of Pristine®, boscalid and pyraclostrobin, as calculated with equations (5) and (6), respectively, using measured pollen patty consumption (12 g day^{-1} $hive^{-1}$), the estimated number of 0–8 day old adults (3,200) and 4-6 day-old larvae (780) for the June–July period when these were relatively stable and did not differ significantly among treatment groups (Fig. S3 and S4). For the 0.23 ppm treatment group, mass-consumption of the Pristine®-pollen patty was estimated as 0.4 ng per lava and 31 mg per young adult; the other treatment groups were within 15% of this value.

	Pristine	Boscalid	Pyraclostrobin
Pollen patty, ppm	0.23	0.06	0.03
Per larva dose, ng	0.086	0.022	0.011
Per adult dose, ng	6.9	1.73	0.88
Pollen patty, ppm	2.3	0.6	0.3
Per larva dose, ng	1.0	0.25	0.13
Per adult dose, ng	79.7	20.1	10.2
Pollen patty, ppm	23	6	3
Per larva dose, ng	8.3	2.09	1.06
Per adult dose, ng	663	167.1	84.9
Pollen patty, ppm	230	60	30
Per larva dose, ng	89.9	22.7	11.5
Per adult dose, ng	7194	1813	921

Table 3
Estimated total pollen intak per larva and per adult based on our measures of pollen consumption, brood cell number, and pollen trap efficiency, for our 0.23 ppm treatment group, compared to estimated pollen intake by two more direct methods.

Stage	Pollen intake, mg	Estimation Method	Reference
1. Larva	2	Pollen grain counts	Babendreier et al. (2004)
2. Adult	60	Mass of pollen in gut	Crailsheim et al. (1992)
3. Larva + Adult	62	Row 1 + 2	
4. Larva	0.8	Equation 9	This study
5. Adult	58	Equation 10	This study
6. Larva + adult	58.8	Row 4 + 5	This study

$$Poltot_{adult} = ((12 + 10.6) / 12) * PPC_{m-adult} \quad (10)$$

For larvae, our estimated total pollen intake was 0.8 mg per larva and 58 mg per adult (Table 3). The summed larva plus adult total pollen intake is within 10% of the estimated larva plus adult total pollen intake measured by direct weighing in other studies (Table 3), providing a strong validation of our estimated per individual total pollen consumption and per-individual mass dose of pesticide.

4. Discussion

Our observations that Pristine® reduced worker population levels, reduced worker longevity and caused overwintering failure demonstrate for the first time that a fungicide can directly impair colony health of honey bees. The effects occurred at concentrations previously determined to be safe for bees based on standard laboratory tests on caged individual workers. Determining the importance of Pristine® and other fungicides in the ongoing decline of pollinators will require careful study of the extent and duration of exposure of pollinators to fungicides (Sponsler et al., 2019). However, the fact that we observed significant negative effects on worker populations of a dose less than one tenth that of the lowest concentration of Pristine® found in pollen strongly suggests that direct effects of fungicides may have been a contributing factor to those effects.

Our data also suggest a possible mechanism by which Pristine® may cause these negative effects on colony demography. Previous research on the effects of Pristine® consumption showed increased protein content in fecal material, suggesting that protein digestion or absorption is inhibited (DeGrandi-Hoffman et al., 2015). Further, the damage that pyraclostrobin causes to midgut epithelia of honey bees (Costa Domingues et al., 2020; Tadei et al., 2020) may impair digestion and/or absorption of nutrients. Poor pollen quality reduces worker lifespan (Crailsheim, 1990), and low levels of hemolymph proteins induce earlier foraging (Nelson et al., 2007). These prior findings led us to hypothesize that the accelerated behavioral development and reduced lifespans of Pristine®-exposed workers could be due to poor pollen utilization, possibly induced by the inhibition of intestinal mitochondria by Pristine®. The earlier foraging and increased pollen foraging observed in Pristine®-treated colonies may suggest that Pristine® causes colonies to respond as though they have a deficiency in pollen quantity and/or quality or experience developmental alterations that shorten worker lifespan. Earlier foraging is generally associated with a shorter lifespan in honey bees, due to increased physiological aging and risk accumulation (Neukirch, 1982; Rueppell et al., 2007). The link between the reduction in lifespan and early foraging is supported by our finding of reduced longevity and earlier onset of foraging among Pristine®-treated colonies (Fig. 2). Such colony-level effects cannot be measured in lab tests with caged workers, emphasizing the need for colony-level studies of pesticides in social insects.

Our experiment found that continuous, six-month exposure to Pristine®-contaminated pollen caused progressive, accumating effects on adult populations. As noted above, this duration of continuous exposure is unlikely in the field, but it seems plausible that colonies kept near many large bee-pollinated crops may experience exposure to Pristine® and other fungicides for multiple months. U.S. migratory colonies are often left for three weeks in almond orchards in California, after which hives may be transported to other crops including citrus, cherries, apples, pears, blueberries, strawberries and curcubits, most of which are approved crops for Pristine® use (Rucker et al., 2019). Further, pollen and nectar resources collected from Pristine® -sprayed crops will remain in the colony for weeks to months afterward, extending exposure, albeit at lower levels. While prolonged exposure leads to cumulative differences in worker populations between control and Pristine® -exposed colonies (Fig. 1), it is not clear whether this matters to the behavioral and mortality effects observed. The negative health effects of Pristine® may be simply due to the concentration ingested in pollen, or Pristine® may have also accumulated in wax over time, enhancing the toxic effect. Our findings of accumulating differences between control and pesticide-treated colonies supports observations that high colony mortality of bees is associated with management strategies in which colonies pollinate sequences of monoculture crops, causing pollinators to be exposed to pesticides and restricted diet diversity for longer durations (Aizen et al., 2019).

The health effects on colonies of Pristine® that we observed were similar to what has been described as Colony Collapse Disorder (CCD), including reduced worker longevity and high winter mortality of colonies (vanEngelsdorp et al., 2009; Williams et al., 2010). CCD is thought to have multiple causes (vanEngelsdorp et al., 2009; Williams et al., 2010), and likely only a subset of affected colonies experience fungicide exposure; however, our data do show a similar impact of fungicide exposure on colony health, with overexposure producing fragile colonies that enter winter with lower population numbers and suffer consequent reduced survival. Arizona winters are relatively mild, so overwintering losses might be even higher in colder climates (Medrzycki et al., 2010).

Our study is one of the first long-term longitudinal studies of the effect of an environmental contaminant on honey bee colony health. We clearly show that Pristine® can have direct, negative effects on honey bee colonies at concentrations similar to those ingested in pollen when foraging on fungicide-sprayed fields. The results highlight the need for further assessment of the role of fungicides in pollinator decline using the variation in field conditions and timescales relevant to pollinator diversity and associated colony health.

Long-term exposures of most honey bee hives to Pristine® and its active ingredients is likely lower than what we studied here. In a recent measure of boscalid and pyraclostrobin levels in bee bread within colonies without extensive exposure to monoculture crops (Ostiguy et al., 2019), levels of boscalid (0.003 ppm) and

pyraclostrobin (0.006 ppm) were approximately an order of magnitude lower than the lowest concentrations we tested (0.06 and 0.03 ppm in pollen patties (Table 2). The lowest levels of boscalid (mean 0.32 ppm) and pyraclostrobin (mean 0.15 ppm) measured in corbicular pollen of bees foraging in almond groves (Table 1) fit within in the range of samples of hive pollen obtained from Florida and California beekeepers, though these fungicides were only detected in a small proportion of hives (Mullin et al., 2010). Boscalid was found in 32% of the pollen samples collected from forty Belgian apiaries, at levels ranging from 0.007 to 0.512 ppm (Simon-Delso et al., 2017). Together with our measures for bees known to be foraging on Pristine®-sprayed orchards (3–24 ppm Pristine® in corbicular pollen), these data suggest that some honey bee colonies experience very little exposure to these fungicides, and those that do likely experience a pulse of fungicide exposure while foraging on a sprayed crop, continuing as the collected pollen is consumed, followed by a washout of the fungicide as new pollen stores are collected. Field tests of the toxicity of such pulsed exposures and the lower concentrations found in many stationary apiaries will be needed to assess the broader role of Pristine® and other fungicides in honey bee health.

To our knowledge, no previous study has estimated the per-individual dose of pesticide in a whole-hive experiment. To estimate the number of adults and larvae consuming the fungicide-laden pollen patties, we used our measurements of pupal and larval cells in the colonies, larval and adult development time, and literature measurements of the relative pollen consumption of larvae and adults. The daily rate of fungicide and active dose by each larva or adult was then calculated, and total dose received by multiplying by the number of days of pollen consumption (Table 2). This method should be broadly useful for estimating per-indidual pesticide doses for experimental field hives.

The increased mortality of honey bees in the 0.23 ppm treatment group occurred at doses much lower than reported in prior acute laboratory studies. For the 0.23 ppm treatment group, the summed boscalid + pyraclostrobin treatment dose for both the larval and adult stage was 2.6 ng (Table 2). Following standard acute oral toxicity testing procedures (OECD 1998), the LD_{50} value of a mixture of boscalid and pyraclostrobin, the active ingredients of Pristine®, is reported as 115 μg/bee (EPA 2013). The hives fed 0.23 ppm pollen had approximately 15% fewer workers, so we estimate a 15% mortality on average. Although a probit slope was not reported for the acute lab toxicity study, assuming a Hill slope of 1, the LD_{15} would be 17 μg/bee, more than three orders of magnitude higher than the dose received by bees in our 0.23 ppm treatment group. Ideally there would be a chronic laboratory study of Pristine® effects for comparison, but to our knowledge, this does not exist. Simon-Delso et al. (2018) used chronic exposure of foragers, who fed on boscalid in sugar water daily (as compared to our bees who consumed pesticide in pollen, and so likely did not receive pesticide after about 8 days of age). They reported a lethal cumulative dose that killed 50% of 0.76 mg (Simon-Delso et al., 2018). Assuming a Hill slope of 1, this would predict a lethal cumulative dose to kill 15% of the bees as 0.23 mg (230,000 ng) boscalid bee^{-1}, whereas the summed larval + adult boscalid intake of the bees in our 0.23 ppm treatment group was 1.75 ng of boscalid (Table 2), about five orders of magnitude lower. Of course, Pristine® also contains pyraclostrobin, which likely increases its toxicity.

Given the much higher toxicity observed in the field than lab, it is important to ask, are our per bee dose calculations reasonable? We addressed this question by testing whether our estimates of per-individual pollen consumption were reasonable. Our estimates of summed larval and adult per capita pollen consumption were within 10% of literature data (Table 3), strongly supporting the validity of our estimated doses of fungicide per bee. Calculating per bee pollen consumption required us to estimate how much pollen leaked through our pollen traps, which turned out to be only 32% efficient at removing pollen from foragers (Fig. S10). Since approximately half of the pollen consumed by the bees in the Pristine®-treated hives was “clean”, the effective concentrations of Pristine® in pollen consumed by bees were approximately half those that we supplied in the pollen patties.

It is not clear why toxicity is so much greater for colonies under field conditions than in acute laboratory studies. Toxicity of the “inert” adjuvant ingredients of Pristine® may be important (Mullin et al., 2015). The field environment may be more stressful than living in an incubated cage with *ad lib* sugar water. Additionally, behavioral interactions that occur in eusocial insect colonies seem likely to be very important in this case. Honey bee foragers collect food for the colony, and foraging is regulated by complex colonial factors including brood production, colony nutrient stores, and forage availability (Seeley, 1985). For social insects such as honey bees, colony-level tests are required to properly evaluate pesticide risk, as the social interactions, activities, and environmental conditions may alter the effect from that observed for an individual bee in a warm lab incubator fed *ad lib* sugar syrup.

5. Conclusions

In conclusion, in addition to synergizing effects of other pesticides (Pilling et al., 1995; Iwasa et al., 2004; Johnson et al., 2013; DeGrandi-Hoffman et al., 2015; Tsvetkov et al., 2017; Zaluski et al., 2017; Tosi and Nieh, 2019), fungicides are likely to have important direct negative effects on pollinator health. Because negative effects of fungicides have also been shown for bumblebees and solitary bees (Artz and Pitts-Singer, 2015; McArt et al., 2017), these negative effects may also contribute more broadly to pollinator decline. Ultimately, the effect of pulsed exposure to pesticides that cause significant but not total mortality on colony fitness will depend on conditions throughout the rest of the year, including exposure to other pesticides and pathogens, as well as climate and access to good forage, emphasizing the importance of management strategies that minimize pollinator exposure to pestides and maximize access to diverse high-quality forage.

Author statement

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Declaration of competing interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.115964.

Data statement

All datasets generated for this study will be uploaded to Mendeley Data.

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