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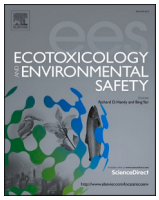

Field cross-fostering and *in vitro* rearing demonstrate negative effects of both larval and adult exposure to a widely used fungicide in honey bees (*Apis mellifera*)

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ABSTRACT

Pollinators and other insects are experiencing an ongoing worldwide decline. While various environmental stressors have been implicated, including pesticide exposure, the causes of these declines are complex and highly debated. Fungicides may constitute a particularly prevalent threat to pollinator health due to their application on many crops during bloom, and because pollinators such as bees may consume fungicide-tainted pollen or nectar. In a previous study, consumption of pollen containing the fungicide Pristine® at field-relevant concentrations by honey bee colonies increased pollen foraging, caused earlier foraging, lowered worker survival, and reduced colony population size. Because most pollen is consumed by young adults, we hypothesized that Pristine® (25.2% boscalid, 12.8% pyraclostrobin) in pollen exerts its negative effects on honey bee colonies primarily on the adult stage. To rigorously test this hypothesis, we used a cross-fostering experimental design, with bees reared in colonies provided Pristine® incorporated into pollen patties at a supra-field concentration (230 mg/kg), only in the larvae, only in the adult, or both stages. In contrast to our predictions, exposure to Pristine® in either the larval or adult stage reduced survival relative to control bees not exposed to Pristine®, and exposure to the fungicide at both larval and adult stages further reduced survival. Adult exposure caused precocious foraging, while larval exposure increased the tendency to forage for pollen. These results demonstrate that pollen containing Pristine® can induce significant negative effects on both larvae and adults in a hive, though the magnitude of such effects may be smaller at field-realistic doses. To further test the potential negative effects of direct consumption of Pristine® on larvae, we reared them *in vitro* on food containing Pristine® at a range of concentrations. Consumption of Pristine® reduced survival rates of larvae at all concentrations tested. Larval and adult weights were only reduced at a supra-field concentration. We conclude that consumption of pollen containing Pristine® by field honey bee colonies likely exerts impacts on colony population size and foraging behavior by affecting both larvae and adults.

1. Introduction

The pollination services of the honey bee (*Apis mellifera*) are of tremendous economic importance (Rucker et al., 2019; Calderone, 2012), contributing to the propagation of many human-used crops (Aizen et al., 2019). Though the number of honey bee colonies over the past decade have remained relatively stable (Ferrier et al., 2018), honey bees continue to endure health challenges, raising agricultural and environmental concerns (Spivak et al., 2011; Paudel et al., 2015). Several environmental stressors have been implicated as contributing factors to honey bee health difficulties (Naug, 2009; Smith et al., 2013; Sponsler et al., 2019), including pesticide exposure (Johnson et al., 2010). Fungicides are a class of synthetic chemicals that honey bees and other crop pollinators commonly encounter while foraging, because they are sprayed onto oilseeds (David et al., 2016) and the blooming flowers of nut, stone fruit and fruit crops (such as almonds, cherries, and strawberries) that are highly attractive to foraging bees (Mullin et al., 2010; Ostiguy et al., 2019). Fungicides often have sufficient lipid and

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water solubility to occur in both pollen and nectar, leading to their presence in both bee bread (pollen diluted with nectar and salivary secretions, (Kieliszek et al., 2018)) and comb wax (Mullin et al., 2010; Pettis et al., 2013; Ostiguy et al., 2019).

Recent meta-analyses suggest that effects of fungicides on bees have been relatively little-studied, especially in field conditions (Cullen et al., 2019; Noi et al., 2021). Exposure of honey bees to fungicides in the field has been correlated with a variety of sublethal disorders including poor brood rearing, queen replacement, colony weakening, reduced pollen consumption and digestion, and increased presence of disease (DeGrandi-Hoffman et al., 2013, 2015; Pettis et al., 2013; Simon-Delso et al., 2014; Zhu et al., 2014). In a survey of 330 colonies located in Belgian cropland, for example, fungicides were strongly correlated with hive disorders, while virus and pesticide loads were not (Simon-Delso et al., 2014). In contrast, Genersch et al. (2010) examined pesticide, including fungicide, contamination levels in pollen samples collected from nearly 120 apiaries, and found no correlation between pesticide contamination and overwintering survival (Genersch et al., 2010).

Based on acute laboratory studies, fungicides are relatively safe for pollinators, as they have low acute ingested or contact toxicity to bees. Ostiguy et al. (2019) compiled oral and contact short-term LD50s for 28 fungicides detected in U.S. apiaries, and compared these to levels measured in bee bread. No fungicide had an oral or contact LD50 lower than 250,000 μg/kg. In comparison, the highest mean concentration for any fungicide detected in pollen was 66 μg/kg, and the maximum concentration found in any pollen sample was 548 μg/kg (Ostiguy et al., 2019). These data suggested that exposure levels of fungicides are approximately three orders of magnitude lower than acute toxicities, with the associated conclusion that fungicides are safe for bees.

However, chronic consumption increases the toxicity of fungicides, and government agencies have recommended more extended testing than short-term LD50 trials and inclusion of toxicity tests on larvae (EFSA, 2013). The LD50 for adults chronically consuming the fungicide boscalid declined with age indicating that effects can accumulate, reaching values within the range of exposures calculated from field application rates (Simon-Delso et al., 2018). The fungicides captan and iprodione did not affect adult worker survival when administered in laboratory conditions as contact and oral treatments at levels well below the highest recommended field rate (Ladurner et al., 2005). However, captan, iprodione, and ziram, at concentrations in larval diet calculated from field dose application rates (1.9–10 mg/kg of formulated product), have been shown to prevent larval development to the adult stage *in vitro* (Mussen et al., 2004). Similarly, larval consumption of the fungicide chlorothalonil at the high end of measured concentrations in pollen (10 and 1.5 mg/kg, respectively) reduced successful development of larvae to adults by about 50%, and also induced morphological abnormalities, reduced adult body mass, reduced cell and nucleus sizes of adult hypopharyngeal glands, and induced expression of multiple detoxification enzymes (Tomé et al., 2020). In contrast, Wade et al. (2019) did not observe significant effects of the fungicides propiconazole, iprodione, boscalid and pyraclostrobin on larval survival though all fungicides tested reduced larval survival when combined with the insecticide diflubenzuron relative to the control (Wade et al., 2019). A number of fungicides have been shown to amplify the effects of other pesticides in the lab by interfering with physiological processes such as energy production and/or pesticide metabolism and excretion (Pilling et al., 1995; Iwasa et al., 2004; Tsvetkov et al., 2017; Tosi and Nieh, 2019). Wernecke et al. (2019) observed acute mortality in honey bee workers exposed to simulated tank mixtures of the neonicotinoid thiacloprid and the fungicides prochloraz, tebuconazole and a mixture of tebuconazole and triadimenol in laboratory, semi-field and field conditions (Wernecke et al., 2019).

There are a number of difficulties in linking the laboratory toxicity studies to understanding fungicide toxicity to honey bees in the field. First, risk depends on both toxicity and exposure, and estimating field exposures, especially over time, is challenging. Secondly, we do not know the concentrations of pesticides fed to larvae; these may be much lower than concentrations measured in pollen or wax as nurse bees may detoxify the compounds and they are diluted with glandular secretions (Lucchetti et al., 2018). Also, the responses of bees in a hive may differ from *in vitro* for many reasons, including social responses to stresses, different thermal, nutritional and microbiome conditions in hives compared to in the lab, and the very different behaviors exhibited by bees in the field (such as foraging). Thus toxicity tests performed under field conditions are necessary to validate findings from *in vitro* lab studies.

One very fundamental question concerning possible toxic effects of fungicides (and many pesticides) on pollinators is whether effects occur at specific life stages, or among particular classes of workers. Foragers may be sprayed directly or contact residues on flowers. However, the distribution of pollen and nectar (and pesticides therein) within the hive can be quite variable (Sponsler and Johnson, 2017). Adult workers consume a considerable amount of protein during the first days after eclosion, and thus will ingest fungicides in collected pollen, and adults of all ages consume nectar, potentially consuming water-soluble pesticides. Young adult bees use their consumed pollen and nectar to produce worker jelly from their hypopharyngeal glands that is fed to larvae (Naiem et al., 1999; Hu et al., 2019). If the nurses do not absorb pesticides, or if they metabolize them to less toxic compounds, this may partially or completely protect larvae from exposure to agrochemicals. The extent to which this occurs is unknown (Naiem et al., 1999) but may mirror larval protection conferred by the breakdown of plant toxins in pollen by nurses (Lucchetti et al., 2018). Larvae may also be affected indirectly due to pesticide effects on nurse bee physiology and larval care. Zaluski et al. (2020) observed that pyraclostrobin, one of the active ingredients of Pristine®, on its own and in combination with the systemic insecticide fipronil, reduced royal jelly protein expression in nurse bees, suggesting a decrease in capacities of nurse bees to feed the larvae. Such an effect on nurse bee productivity might reduce larval growth and/or survival though colonies may potentially counteract this outcome through elevated pollen foraging and consumption. Compromised royal jelly production may alter subsequent worker ontogeny by inducing foraging and death at earlier ages (Tomé et al., 2020). To date one study has examined the effect of larval exposure to fungicide on adult performance, observing significant increases in activity levels and mortality in bees that consumed pyraclostrobin and clothianidin-treated food (Tadei et al., 2019).

In this study, we assessed the effects of the commonly used fungicide, Pristine®, on honey bees, with a particular focus on determining whether toxic effects are mediated at the adult, larval or both stages. Pristine® is a commonly used fungicide on California almond orchards, which are pollinated by a large fraction of U.S. commercial bees (DeGrandi-Hoffman et al., 2019). It is labeled for use on grapes, berries, stone fruits, pome fruits, tree nuts, carrots, onions, and other bulb vegetables (Rucker et al., 2019). Pristine® contains 25.2% boscalid (2-chloro-N-[2-(4--chlorophenyl) phenyl] pyridine-3-carboxamide), which is a carboximide; and 12.8% pyraclostrobin (methyl *N*-[2-[[[1-(4-chlorophenyl)-1*H*-pyrazol-3-yl]oxy]methyl]phenyl]-*N*-methoxycarbamate), which is a strobilurin fungicide. By containing both carboximide and strobilurin components, Pristine® has potential effects at multiple stages of the respiratory chain, potentially increasing its mitochondrial toxicity. As an illustration of the magnitude of environmental pollution by such fungicides, in 2017, growers applied over 21,000 pounds of boscalid and 39,000 pounds of pyraclostrobin on California almonds (CDPR, 2019).

Consumption of Pristine® in pollen at field-realistic doses changes worker foraging behavior and reduces colony population size by lowering worker longevity (Fisher et al., 2021). However, the mechanisms by which these toxic effects occur remain unclear. A first key question is whether Pristine® has its effects on the larvae, adults or both. Most pollen is consumed by young adults, with the larvae eating only a few mg late in development (Crailsheim et al., 1992; Babendreier et al., 2004). Also, concentrations of boscalid and pyraclostrobin in nurse bees

were less than 5% of those in pollen, and concentrations of these fungicides in royal jelly produced by nurse bees fed Pristine® were undetectable, suggesting that the active ingredients in Pristine® are not passed to larvae in the brood food (DeGrandi-Hoffman et al., 2013). Finally, in our prior study, we did not observe effects of consumption of Pristine® on colony egg and brood levels (Fisher et al., 2021). Therefore, we hypothesized that Pristine® in pollen exerts its negative effects on honey bee colonies only by effects on the adult stage. To rigorously test this hypothesis, we used a cross-fostering experimental design, with bees reared in colonies provided Pristine® only in the larvae, only in the adult, or both stages, testing effects on survival and behavior of adult honey bee workers. We used a supra-field dose of Pristine® (about 10x above the highest average concentration of Pristine® in pollen collected by bees foraging in Pristine®-sprayed almond groves), as peak exposures are likely higher than average concentrations, and our goal was to exclude the possibility that larvae are negatively impacted by colonial Pristine® consumption in pollen. We further assessed the dose-dependent effects of Pristine® exposure *in vitro* by rearing honey bee larvae on Pristine®-tainted food. In contrast to our hypothesis, our results clearly demonstrate that when colonies consume Pristine® in pollen, there are negative effects on worker survival and behavior mediated by impacts on both larval and adult stages.

2. Materials and methods

2.1. Field-rearing experiment

2.1.1. Honey bee colonies and experimental administration of fungicide

A full description of the methods used here is presented in Fisher et al. (2021). Briefly, 40 experimental colonies of the Italian honey bee (*Apis mellifera ligustica*), headed by sister queens, were established from packages. Newly established packages were observed to be free of clinical symptoms of infection with viruses, *Nosema* sp. and *Varroa* mites. To ensure that control 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 and had these analyzed by the USDA-AMS National Science Laboratory. Pesticide residue analyses found no other agrochemicals present above detection levels other than a few herbicides: diuron, flumeturon, and hexazinone, none of which were higher than 12 μg/kg. We previously treated hives for *Varroa* mites approximately five months before the start of the cross-fostering experiment, a metabolite (DMPF) of the miticide amitraz was detected at higher levels (max 147 μg/kg) than other pesticides in the bee bread.

Hives were outfitted with internal pollen traps to restrict 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) mixed with deionized H_2O containing Pristine® (BASF Corporation, Research Triangle Park, NC). Pollen used for this study was obtained from Bulk Foods (https://bulkfoods.com/health-foods/bee-pollen-granules.html) which is appraised as pesticide free. We fed each hive fungicide-treated pollen patties (230 mg/kg) *ad libitum*, with a new patty supplied as soon as the previous patty was entirely consumed or each week. Control hives were also maintained on pollen patties but were spared fungicide exposure. Each colony was monitored every other day to verify pollen patty availability.

2.1.2. Cross fostering assay and adult longevity assessments

To assess the relative influence of Pristine® exposure on honey bees when exposure occurs during the larval or adult stage, we conducted a cross-fostering experiment (Silva et al., 2013) and tested the effect on longevity and behavior. Newly-emerged bees were collected from three control (non-fungicide treated) colonies and three colonies treated with 230 mg/kg Pristine® fungicide formulation mixed into pollen as described above. At least one brood frame from each control and Pristine®-treated source colony was temporarily removed and placed in wire mesh frame cages (L x W x H: 53.3 × 5.1 × 27.9 cm) to contain newly emerged bees. Frames were kept in an incubator (34 °C, 90% relative humidity) until a sufficient number of individuals emerged (~540 per frame). Newly emerged bees from the three source control colonies were pooled together after marking them on the mesonotum with a distinct paint color corresponding to treatment, date of emergence, and hive of origin. Pooled control bees were then divided into four equal subsets (~410 bees per subset, composed of ~135 bees per source colony) with two subsets placed in two of the source control colonies and the remaining two subsets placed in two of the source Pristine®-treated colonies. Similarly, newly emerged bees from Pristine®-treated colonies were marked, pooled, and distributed to the same source Pristine® and control colonies as the control subsets. This process resulted in the formation of two cross-fostered groups (Larval-0/Adult-P, Larval-P/Adult-0) which had undergone larval development in either control (0) or Pristine®-treated (P) conditions and adulthood in the opposite condition (Table 1). Two homogenous groups (Larval-0/Adult-0, Larval-P/Adult-P) were also formed, having undergone larval development and adulthood in the same conditions. The use of three source colonies for control and Pristine®-treated bees further resulted in three strains within each treatment group.

To assess longevity, marked bees were counted weekly following reintroduction. Each hive was opened and the number of marked individuals observed on each frame was recorded. Hand clickers were used to differentially record the number of marked bees for either treatment group present in a hive. Once assessed, frames were placed in an empty brood chamber away from the hive to prevent recounting the same individuals.

2.1.3. Foraging behavior assessments

To assess the age of first foraging, previously marked individuals were differentially recorded according to strain and forager type (pollen or non-pollen) based on the presence or lack of pollen loads on the corbiculae for all four experimental hives. This allowed for comparisons between treatment groups but also within a treatment group by accounting for the onset of foraging among the different strains within a treatment group. Foragers were recorded by observing the hive entrance for 30-min sessions each morning (9 a.m.) starting three days after newly emerged bees were marked and placed in hives. Outgoing and incoming marked individuals were considered foragers if they were observed to bear pollen on their corbiculae or were not clearly engaged in orientation flights.

Once marked individuals were observed to start foraging, pollen foraging rates among experimental groups were assessed for the first week immediately following the first observation of foraging behavior. Each hive was monitored for one hour in the morning (9–11 a.m.) each day by observing the hive entrance and counting the total and pollen-carrying incoming marked individuals.

2.2. In vitro rearing experiment

2.2.1. Larval preparation

To obtain a sufficient number of bees of the same age, a non-experimental queen-right colony was selected and the queen was confined over empty cells on a drawn empty comb frame from the

Table 1
Treatment group rearing conditions.

Treatment group	Treatment condition	Larval condition	Adult condition
Larval-0/Adult-0	Homogenous	Control	Control
Larval-P/Adult-0	Cross-fostered	Pristine	Control
Larval-P/Adult-P	Homogenous	Pristine	Pristine
Larval-0/Adult-P	Cross-fostered	Control	Pristine

queen's hive for 24 h using an excluder cage. This confinement encouraged the queen to lay eggs in the empty cells ensuring that resulting worker bees would be approximately the same age. The frame was removed from the hive and taken into the laboratory four days after initial confinement of the queen to graft newly eclosed larvae (Kaftanoglu et al., 2011).

2.2.2. Larval diet preparation

To assess the effects of Pristine ® on honey bee larval survival and mass, five artificial larval diets were prepared consisting of royal jelly thoroughly mixed with glucose, fructose, yeast extract and distilled water based on diet 3 in Kaftanoglu et al. (2011). Kaftanoglu et al. (2011) contained several procedural overlaps with OECD 239 (OECD, 2013) with one major distinction being the administration of all larval food at the start of development, rather than the daily feeding specified in OECD 239. We chose the Kaftanoglu et al. method as it reduces disturbance to the developing larvae, and we obtained high larval survival with this method. Four different nominal concentrations of Pristine® fungicide formulation (Table S2) were each incorporated into separate larval diet sets. These concentrations were chosen to bracket the concentrations measured in pollen for honey bees foraging at a California almond orchard recently sprayed with Pristine® (Fisher et al., 2021). In that study, Pristine® in pollen ranged from 3 to 24 mg/kg. Here we used two concentrations in that range (2.3 and 23) and a concentration an order of magnitude lower and higher. A control diet was also prepared including the same ingredients with the exception of the fungicide. Larval diet preparation began by mixing 12 g of glucose, 12 g of fructose and 2 g of yeast extract into 74 g of sterilized distilled water to form a 100 g stock solution. This stock solution was then divided evenly into five autoclaved 118.3 mL glass containers such that each container held 20 g of the water, sugar and yeast mixture. Differing Pristine® concentrations were added to four of the containers which were mixed, dissolving Pristine® in solution. Afterward, 20 g of royal jelly (Cockett Honey Inc., Tempe, AZ, USA) was then added to each container and thoroughly mixed to complete the preparation of the larval food. Each container was refrigerated at 4 °C for 24 h to allow for the escape of air bubbles. Analytical balances and pipettors (Mettler-Toledo Rainin, LLC Oakland, CA, USA) used to weigh larval food components during preparation and dispense the completed larval food mixture had a readability of 0.1 mg and an error range of ± 2.5%, respectively.

2.2.3. Grafting and maintenance

Brown queen cell cups (Mann-Lake, Hackensack, MN, USA) were placed into petri dishes (60 × 15 mm) designated to different experimental groups (~20 cups per dish). About 170 mg (± 4.7 mg) of larval food was placed in each cup, larvae were supplied only with this single 170 mg allotment intended to last for the duration of larval development. A total of one thousand 1st instar larvae were grafted from a single frame pulled from a non-experimental hive. Each treatment group had two hundred larvae distributed in ~10 petri dishes per treatment group). To minimize stress, larvae were grafted for 30 min, followed by a 30-min rest interval where the source frame was placed back in its hive of origin. This grafting process was repeated until one thousand larvae were grafted into the prepared cell cups over the course of 5 h. Once fully stocked with larvae, the petri dishes were randomly distributed and maintained in an incubator set at 34 °C and 90% relative humidity.

2.2.4. Larval survival and weight

Larval survival was assessed daily for the 5 days required for larval development. Dead larvae and their cell cups were removed daily to prevent infection. On the sixth day after grafting, larvae were placed in a petri dish (100 × 15 mm) lined with tissue paper to promote defecation overnight. The next day, following defecation, the larvae were transferred directly from the filter paper-lined petri dishes to 24-well plates (well diameter: 2 cm) with one larva per well. Twenty of the surviving larvae from each experimental group were randomly selected and weighed. The well plates were then maintained in an incubator (34 °C, 70% rh) for the entirety of pupation.

2.2.5. Adult assessment

Adults were collected as they eclosed, which was approximately 20 days after grafting, or 14 days after larvae were transferred to the well plates. Once eclosion began, we checked the incubator and collected all eclosed adults hourly until all surviving bees had eclosed (approximately 24 h). Surviving adult bees randomly selected from each experimental group were weighed individually to assess the effects of the larval diet on adult weight.

2.3. Statistical analysis

For cross-fostering experiment comparisons, the age of first foraging and pollen foraging tendency (Fig. 1 and 2) were analyzed using ANOVA with hive as a random effect. Strain (hive of origin within a treatment group) was tested and not found to have a significant effect, and so was excluded from further models. Post hoc analyses were performed using Tukey-HSD. The comparison of survival rates between homogenous and cross-fostered bees (Fig. 3) and *post hoc* analyses (Table S3) was performed using a Kaplan-Meier survival analysis.

To compare the survival rates among control and Pristine-treated *in vitro* reared larval groups (Fig. 4), we performed Kaplan–Meier survival and *post hoc* analyses (Table S4). Average larval and adult weights were compared using ANOVA (Fig. 5), *post hoc* analyses were conducted using Tukey-HSD. For all tests, the level of statistical significance was set at $\alpha = 0.05$. JMP 14.0 Pro (SAS Inc., Cary, NC) software was used to perform all tests, all descriptive statistics are reported as mean and standard error of the mean (SEM).

3. Results

3.1. Cross-fostering experiment

Exposure to Pristine® during adulthood induced early foraging ($F_{3,23} = 50.0$, $P = 0.02$; Fig. 1); however, larval exposure to Pristine® did not affect the age of first foraging. In contrast, larval exposure to Pristine® determined pollen foraging tendency. Larval Pristine® exposure increased the percentage of bees that foraged for pollen whether adults were in Pristine®-treated colonies ($F_{1,19} = 34.87$, $P < 0.0001$; Fig. 2A), or control colonies (Larval-0/Adult-0) ($F_{1,19} = 9.81$, $P = 0.006$; Fig. 2B).

A significant difference in overall survival rates was observed among all experimental groups ($X^2 = 141.78$, $P < 0.0001$; Fig. 3). Bees reared

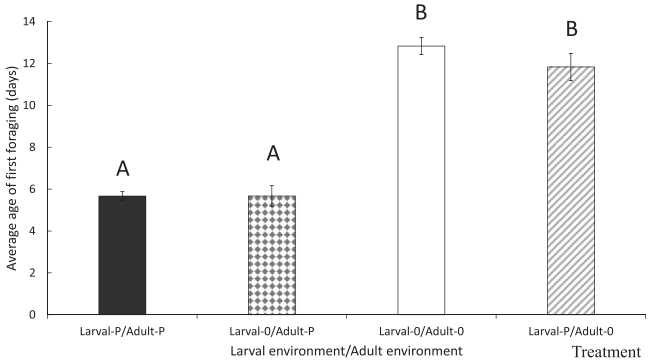

Fig. 1. Treatment affected the age of first foraging (ANOVA, $F_{3,23} = 50.0$, $P = 0.02$). The age of first foraging for experimental groups was reduced by adult but not larval Pristine® exposure. For this and subsequent bar graphs, means and standard errors are shown. Here means are averages of the first day foraging was observed for bees of the six different source-colonies. No color print.

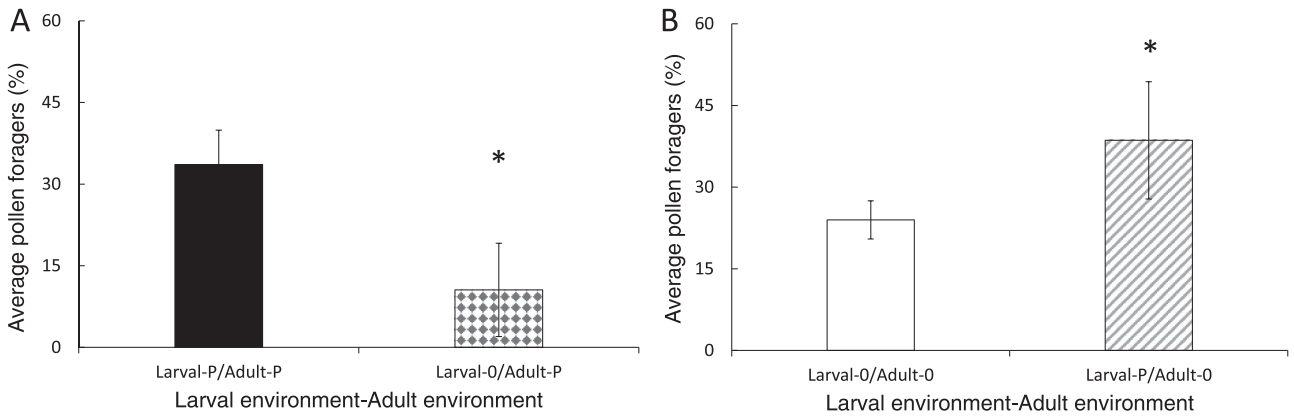

Fig. 2. (A) When adults were reared in colonies with Pristine®-treatment, bees that were exposed to Pristine as larvae (Larval-P/Adult-P) foraged more for pollen as adults than bees that were not exposed to Pristine as larvae (Larval-0/Adult-P, ANOVA, $F_{3228} = 34.87$, $P < 0.0001$).). (B) When adults were reared in control colonies without Pristine exposure, if the bee had been exposed as larvae to Pristine (Larval-=P/Adult-0, they foraged for pollen more than bees that underwent their entire lifecycle (Larval-0/Adult-0) in control colonies (ANOVA, $F_{3429} = 9.81$, $P = 0.006$). N = 2 colonies for each experimental group. Differing letters indicate a significant difference between groups. No color print.

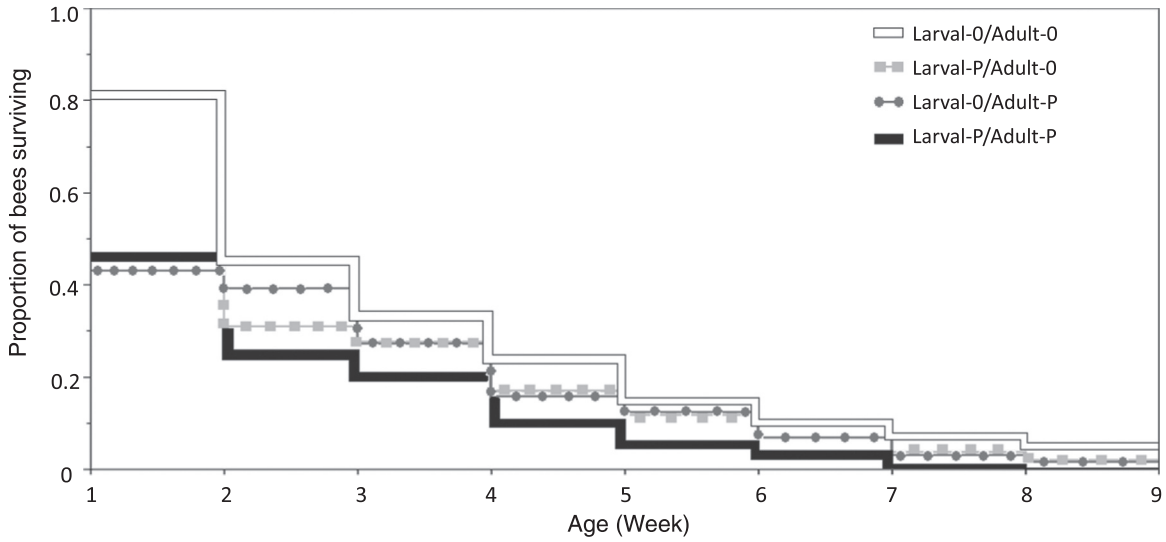

Fig. 3. Survival over time for the treatment groups. The Larval-0/Adult-0 group had a significantly higher survival rate than all other groups (Kaplan-Meier survival analysis, $X^2 = 15.41$, $P = 0.004$). The two cross-fostered groups, Larval-P/Adult-0 (Kaplan-Meier survival analysis, $X2 = 81.98$, $P < 0.0001$) and Larval-0/Adult-P (Kaplan-Meier survival analysis, $X^2 = 16.92$, $P < 0.0001$) also had significantly higher survival rates than the Larval-P/Adult-P treatment group. For each experimental group, approximately 825 newly emerged adults were marked and evenly distributed between two colonies. No color print.

entirely in control colonies (Larval-0/Adult-0) had a significantly higher survival rate than all other groups (Table S3). The cross-fostered groups (Larval-0/Adult-P, Larval-P/Adult-0) had significantly higher survival rates than the bees that were reared entirely in Pristine®-treated colonies (Larval-P/Adult-P) (Table S3).

3.2. *In vitro rearing experiment*

We observed a significant impact of all concentrations of Pristine® tested on larval survival ($X^2 = 15.41$, $P = 0.004$; Fig. 4). Individual comparisons between the control group and each Pristine® concentration group further supported the significant impact of all concentrations tested on larval survival (Table S4). Pristine® treatment significantly affected larval weights ($F_{4,99} = 10.61$, $P < 0.0001$, Fig. 5) and newly emerged adult weights ($F_{4,84} = 25.69$, $P < 0.0001$; Fig. 6), but only the highest concentration group, 230 mg/kg, differed significantly from the control.

4. Discussion

In contrast to our hypothesis that consumption of Pristine® in pollen by honey bee colonies would only affect adult honey bees, we found clear evidence of effects on both the larval and adult stages. Our conclusion that larvae are negatively affected by Pristine® was supported by our *in vitro* larval rearing experiments, in which Pristine® consumption at a range of concentrations reduced survival. Together these results indicate that Pristine® can have widespread effects on honey bee colonies, and that short-duration exposures even on one life-stage may negatively impact the colony. However, the mechanisms of the effects on larvae and adults still require clarification.

While our results allow us to reject the hypothesis that consumption of Pristine®-tainted pollen only affects adult bees, an important caveat is that it is conceivable that a different conclusion might be reached at lower concentrations. The concentration of Pristine® used here was in excess of most but not all previously reported levels in pollen. Simon-Delso et al. (2018) calculated field exposure of honey bees in boscalid sprayed fields as 26.2 mg/kg, corresponding to approximately

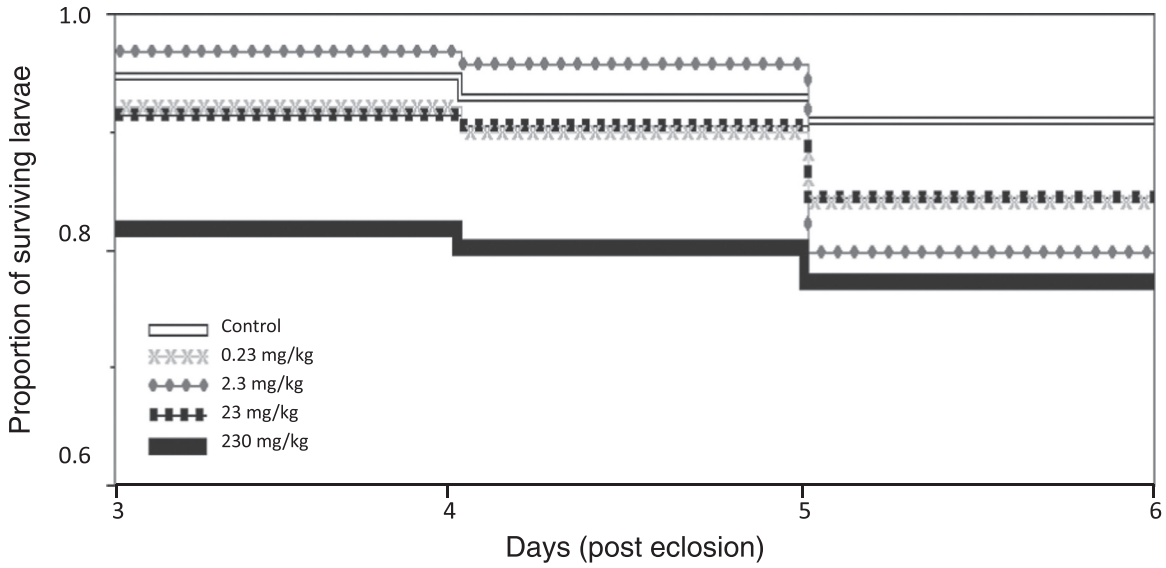

Fig. 4. Larval survival over time among honey bee larvae reared *in vitro* on different concentrations of Pristine®. Larval mortality was not observed for any group until day 3 post-eclosion, All experimental groups experienced significantly lower survival rates than control larvae (Kaplan-Meier survival analysis, all $P < 0.05$). N = 200 larvae for each experimental group. No color print.

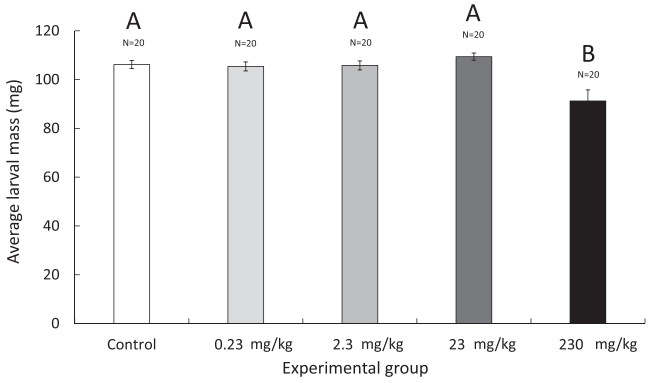

Fig. 5. Average larval weight for each experimental group. Fifth instar larvae were weighed 6 days post eclosion. Larval weight was significantly affected by treatment group ((ANOVA, $F_{4,99} = 10.62$, $P < 0.0001$), but only the 230 mg/kg treatment group was significantly lighter than controls (Tukey HSD, $q = 2.78$, $P < 0.0001$). N = 20 larvae for each experimental group. No color print.

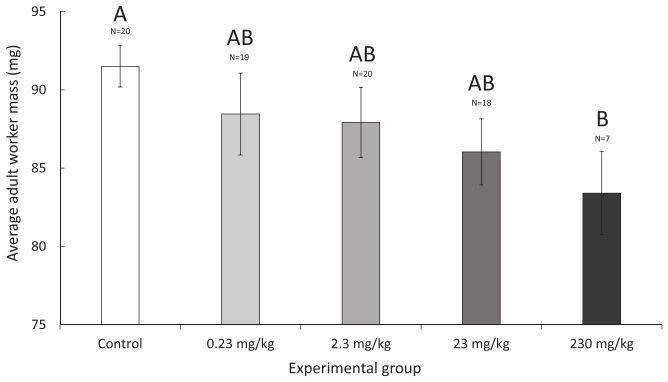

Fig. 6. Average adult weight for each experimental group. Weights of adult bees were significantly affected by treatment group (ANOVA, $F4,84 = 3.25$, $P = 0.02$), only the 230 mg/kg treatment group were significantly lighter than control bees (Tukey HSD, $q = 2.80$, $P = 0.04$). Sample sizes varied between adult groups due to differences in larval and pupal survival. No color print.

105 mg/kg Pristine®. However, Simon-Delso's study of Belgian apiaries (Simon-Delso et al., 2014) found boscalid and pyraclostrobin concentrations in bee bread of 0.1 mg/kg each, leading to a predicted concentration of Pristine® in pollen of 0.7–1.4 mg/kg. Efforts to monitor risks faced by honey bees, including pesticide exposure, is an organized process in several European nations where fungicides have been reported to contribute to bee poisoning incidents (Barnett et al., 2007; Porrini et al., 2016; Pistorius, 2017). Mullin's 2010 study of U.S. commercial apiaries found similar levels of boscalid (0.11 mg/kg) and pyraclostrobin (0.084 mg/kg) in bee bread (Mullin et al., 2010), which would correspond to 0.44–0.65 mg/kg Pristine® in pollen. Ostiguy et al. (2019) study of apiaries associated with U.S. universities found lower levels, with 0.003 mg/kg boscalid and 0.006 mg/kg pyraclostrobin in bee bread, corresponding to Pristine® concentrations in pollen of 0.02–0.08 mg/kg. Of course, such samples of bee bread concentrations will reflect whatever pollen was collected recently, not necessarily after exposure to Pristine®-sprayed fields. Assessments of corbicular (bee collected) pollen have also generally found lower levels of boscalid and pyraclostrobin (Stoner and Eitzer, 2013; Friedle et al., 2021). Stoner and Eitzer (2013) examined bee collected pollen from five apiaries located in areas with varying levels of urbanization over a five year period and reported boscalid residue levels at an average of 0.04 mg/kg but max detections of 0.85 mg/kg (Stoner and Eitzer, 2013). Friedle et al. (2021) reported even lower levels of boscalid and pyraclostrobin (max detections of 201 and 49 ng/g, respectively) measured in corbicular pollen (Friedle et al., 2021). However, the Friedle paper measured pesticides in a single hive surrounded by diverse fruit crops that likely were sprayed at different times, likely causing maximal pesticide levels to be diluted relative to the situation in large US almond groves in which hives are likely foraging exclusively on pollen from almonds (Friedle et al., 2021).

We recently measured the concentration of boscalid and pyraclostrobin in corbicular pollen collected over multiple weeks from honey bees foraging at Pristine®-sprayed California almond orchards, and boscalid levels ranged from 1.5 to 6 mg/kg, and pyraclostrobin levels ranged from 0.4 to 1.7 mg/kg, corresponding to concentrations of Pristine® in pollen of 3–24 mg/kg (Fisher et al., 2021). Thus the 230 mg/kg concentration of Pristine® used in our pollen is an order of magnitude higher than the highest average concentration measured, so it is not yet clear whether significant survival effects on adults due to exposure at single developmental stages will occur at field concentrations. However, the fact that we observed significant decreases in

survival when bees consumed 0.23 mg/kg pollen during months-long colony exposure (Fisher et al., 2021) suggests that even short-term exposure during only the larval or adult stage is likely to affect the survival of workers foraging in Pristine®-treated orchards. Discrepancies in residue levels between almond pollen (Fisher et al., 2021) and other floral sources (Stoner and Eitzer, 2013; Friedle et al., 2021) may be indicative of elevated use of chemical treatments in almond orchards. Limited sample sizes (Friedle et al., 2021) and potential uncertainty of exposure (Stoner and Eitzer, 2013; Friedle et al., 2021) may also account for the reported differences.

How Pristine® exposure to larvae causes reduced life-spans in adults is unclear. As noted above, larvae consume only a few mg of pollen, resulting in an estimated dose of 1–8 ng of Pristine® for colonies consuming field-relevant doses (Fisher et al., 2021). The lowest dose we tested here *in vitro* (0.23 mg/kg in the food consumed throughout the larval period) represents a dose of 39 ng of Pristine®, if the larvae consumed all of the 170 mg of food provided (Table S5). Thus, it is plausible that there is a direct effect of Pristine® consumption on larval development despite the small amount of pollen that larvae consume, but further experiments will be necessary to confirm. Another possibility is that Pristine affects larvae indirectly. Perhaps consumption of Pristine®-tainted pollen by young nurses causes them to produce brood food of lower quality or quantity.

Interestingly, age of first foraging was affected by Pristine® exposure during adulthood, while the tendency to forage for pollen was affected by larval Pristine® exposure. One possible mechanism for the effect of adult exposure to Pristine® on age of first foraging is that this is a consequence of Pristine®'s apparent interference with the digestion and absorption of pollen (DeGrandi-Hoffman et al., 2015), perhaps due to inhibition of mitochondria in the gut epithelia, or to disruptions of the microbiome. Pollen foraging for honey bee colonies is indicative of nutritional needs and colonies will direct more effort into collecting pollen based on colony demographics (Ghosh et al., 2020). Thus the potential interference of Pristine® on protein processing could have driven elevated pollen foraging due to perceived protein deficits relative to colony demographics. Exposure to colony starvation, pollen deprivation or disease commonly induces precocious foraging (Janmaat and Winston, 2000; Schulz et al., 1998; Toth and Robinson, 2005; Woyciechowski and Morón, 2009), which can be advantageous in the short term by increasing nutrient intake but problematic in the face of a chronic stressor (Perry et al., 2015). Nutritional stresses that reduce pollen availability or quality also induce pollen foraging and earlier foraging (Camazine, 1993; Mattila and Otis, 2006; Silva et al., 2013). Plausibly, protein deprivation may induce decreases in the egg yolk synthesis protein, vitellogenin, which functions as a regulator of age-related task transitions in worker honey bees (Guidugli et al., 2005; Amdam et al., 2006; Page et al., 2006). The mechanisms by which larval exposure to Pristine® cause an increased tendency to forage for pollen are also unclear. This could be due to colonial perception of low protein-nutritional status of the larvae, or direct physiological effects of juvenile stress on adult physiology and behavior. Larval starvation causes changes in juvenile hormone levels, energy stores, metabolic rates, ovary size and sugar sensitivity (Wang et al., 2016a, 2016b); conceivably Pristine® may cause similar physiological alterations, causing effects on the tendency to forage for pollen.

Differential survival among the cross-fostered treatment groups suggests that fungicide consumption, even when it occurs exclusively during the larval or adult stage, negatively affects worker lifespan. Reduced lifespan is associated with costs in colony productivity (Rueppell et al., 2008) with lifespan reductions at least partly determined by the age at which foraging starts (Rueppell et al., 2008). Protein processing deficiencies conferred by Pristine® consumption (DeGrandi-Hoffman et al., 2015) may represent a cost to colony productivity, perhaps through inadequate larval provisioning. Thus, adult bees in Pristine®-treated colonies, through direct and indirect effects of exposure, and through larval signals (Pankiw et al., 1998), may forage early to compensate, resulting in early death. Also, cross-fostered groups had significantly higher survival rates than the homogenous Pristine®--treated group pointing to a dosage effect on survival (Fig. 3), as also indicated by the larval experiments (Fig. 4) and dose-response experiments for whole field colonies (Fisher et al., 2021). Adult exposure alone resulted in a reduction in survival relative to the control but not to the extent that exposure at all life stages induced. This may suggest that larval signals induced early foraging in homogenous and cross-fostered groups influencing resulting survival, but exposure duration differentially impacted individual physiology determining the difference in survival. The lack of early foraging, yet reduced survival, among cross-fostered bees in control colonies suggest that larval Pristine® consumption caused changes in bee physiology that reduced long-term health independent of the age at which foraging commenced.

Factors that may temper our findings include the detection of an amitraz metabolite (DMPF) in bee bread samples analyzed in experimental hives. Exposure to amitraz contaminants in wax reduces honey bee drone fertility (Fisher and Rangel, 2018) while consumption of amitraz reduces larval survival and developmental rate in laboratory conditions (Dai et al., 2018). The detection of amitraz in experimental hives may have indicated the potential for synergistic effects though a previous study examining the effects of topical application found no evidence of synergism of amitraz combined with boscalid, pyraclostrobin or the combination of boscalid and pyraclostrobin (Johnson et al., 2013). Amitraz retention in beeswax appears to be low, Martel (2007) reported a lack of amitraz detection in wax following treatment of bee hives with Apivar®, the active ingredient of which is amitraz (Martel, 2007). Additionally, Wu et al. (2011) examined the effects of pesticide residues in wax, including DMPF a metabolite of amitraz, on brood longevity and development finding that the negative impact of exposure was significantly reduced within two weeks (Wu et al., 2011). These findings suggest that our application of amitraz five months prior to the start of our cross-fostering experiment may have had a negligible impact on resulting outcomes. Another caveat to our results is that in assessing the effects of Pristine® on larvae reared *in vitro*, we used only one source hive which may not have reflected the full range of possible responses to Pristine® exposure.

5. Conclusions

In contrast to our expectations, feeding colonies Pristine®-tainted pollen affected worker survival and behavior regardless of whether the bees were exposed at the larval or adult stage. While Pristine® exposure at both larval and adult life stages elicited the strongest negative effects on survival, limited exposure during a single life stage negatively impacted honey bee health and behavior, albeit at concentrations exceeding field exposure levels. This suggests that limited exposure over short durations may elicit significant negative impacts on overall colony health and population over time, though further tests with field-realistic doses and exposure durations are needed. Results from the *in vitro* reared larval studies also support the hypothesis that larvae are sensitive to Pristine, though again, further tests are required to determine the specific mechanisms and dose-responses across a wider range of lower exposures. These findings support the need to address the general lack of sublethal studies of fungicides (Cullen et al., 2019; Noi et al., 2021) and the need for stringent evaluation of the safety of fungicides to pollinators in field conditions at field realistic levels.

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CRediT authorship contribution statement

Adrian Fisher: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Gloria DeGrandi-Hoffman**: Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Writing - review & editing. **Brian Smith**: Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Writing - review & editing. **Cahit Ozturk**: Investigation, Methodology, Data curation. **Osman Kaftanoglu**: Conceptualization, Funding acquisition, Methodology. **Jennifer H. Fewell**: Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Writing - review & editing. **Jon F. Harrison**: Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.112251.

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