Pollen Treated with a Combination of Agrochemicals Commonly Applied During Almond Bloom Reduces the Emergence Rate and Longevity of Honey Bee (Hymenoptera: Apidae) Queens

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Abstract

Honey bee (Apis mellifera L.) colonies that pollinate California’s almond orchards are often exposed to mixtures of agrochemicals. Although agrochemicals applied during almond bloom are typically considered bee-safe when applied alone, their combined effects to honey bees are largely untested. In recent years, beekeepers providing pollination services to California’s almond orchards have reported reductions in queen quality during and immediately after bloom, raising concerns that pesticide exposure may be involved. Previous research identified a synergistic effect between the insecticide active ingredient chlorantraniliprole and the fungicide active ingredient propiconazole to lab-reared worker brood, but their effects to developing queens are unknown. To test the individual and combined effects of these pesticides on the survival and emergence of developing queens, we fed worker honey bees in closed queen rearing boxes with pollen artificially contaminated with formulated pesticides containing these active ingredients as well as the spray adjuvant Dyne-Amic, which contains both organosilicone and alkyphenol ethoxylate. The translocation of pesticides from pesticide-treated pollen into the royal jelly secretions of nurse bees was also measured. Despite consistently low levels of all pesticide active ingredients in royal jelly, the survival of queens from pupation to 7 d post-emergence were reduced in queens reared by worker bees fed pollen containing a combination of formulated chlorantraniliprole (Altacor), propiconazole (Tilt), and Dyne-Amic, as well as the toxic standard, diflubenzuron (Dimilin 2L), applied in isolation. These results support recommendations to protect honey bee health by avoiding application of pesticide tank-mixes containing insecticides and adjuvants during almond bloom.

Key words: mixture toxicity, Royal Jelly, spray adjuvant, translocation
A variety of pesticides are often applied to almonds simultaneously in the form of tank mixtures (Mullin et al. 2015). Previous studies have found that mixtures of common agrochemicals can cause lethal and sublethal effects to developing queens. For example, DeGrandi-Hoffman et al. (2013) found that queens reared on diets containing the insecticide chlorpyrifos and the common fungicide Pristine (pyraclostrobin and bosalid) demonstrated increased viral titers as larvae and emerged from pupation at reduced rates. In colonies fed pollen treated with field-relevant levels of the fungicide active ingredients propiconazole (Pro) and chlorothalonil, rates of queen events and brood loss increased (Traynor et al. 2021). Studies on honey bee workers provide additional evidence that agrochemical mixtures pose high risk to developing queens. Wade et al. (2019) found that the combination of Pro and the insecticide chlorantraniliprole (Chl) had a pronounced synergistic effect on the mortality of lab-reared worker brood. This finding contrasts with the results of an earlier study on the toxicity of Chl in isolation on adult honey bees, which supported its labeling as toxicologically “inert” and undergo little testing for bee safety (but see USEPA 2021). This issue is especially relevant to California’s almond orchards, where the usage of organosilicone adjuvants in king boxes is among the most widely used (CDPR 2021).

In addition to pesticides, tank mixtures often contain spray adjuvants, which are used to improve aspects of pesticide performance (wetting, particle size, etc.) during application. Research on spray adjuvants containing organosilicone and ethoxylate compounds as principal functioning agents indicate that these compounds are toxic to honey bees when combined with pesticides (Mullin et al. 2015) and may be more toxic individually than certain pesticides (Mesnage and Antoniou 2017). Although the adjuvant Break-Thru was not found to affect queen survival during development (Johnson and Percel 2013), there are a wide variety of adjuvants used in almond fields for which the effects on bees is unknown. As with studies on agrochemical mixtures, most evidence of adjuvant toxicity in honey bees is derived from studies with workers. For example, a study on lab-reared workers found that larval exposure to 10 ppm of a commercial organosilicone synergized the pathogenicity of Black Queen Cell Virus (Fine et al. 2017). This has clear implications for queens because this virus infects and kills developing queens and was found to be prevalent in colonies contracted to pollinate almonds (Glenny et al. 2017). Despite growing evidence that some common adjuvants are toxic to honey bees, they are widely considered to be toxicologically “inert” and undergo little testing for bee safety (but see USEPA 2021). This issue is especially relevant to California’s almond orchards, where the usage of organosilicone adjuvants increased by more than 5-fold from 2001 to 2013 (Mullin et al. 2016). Of these, the adjuvant Dyn-Amic (Dyn), which contains both an organosilicone and an alkylphenol ethoxylate, is among the most widely used (CDPR 2021).

In addition to the pesticides and adjuvants identified above, almonds are regularly sprayed with insecticides during bloom, despite recommendations against this practice (Almond Board of California 2020). These insecticides are not acutely toxic to bees and include the previously mentioned chlorantraniliprole, which acts on ryanodine receptors in insect muscle, as well as insect growth regulators (IGRs), including diflubenzuron (Dif) and methoxyfenozide, that affect insect development. IGRs were shown to reduce the feeding ability of nurses as well as the emergence rate of queen-laid eggs (Fine 2020). Dif was previously found to reduce the survival of developing queens (Johnson and Percel 2013) and lab-reared worker brood (Wade et al. 2019).

In the present study, we investigated the individual and combined effects of the formulated products Altacor (35% Chl), Tilt (41.8% Pro), and Dyn on the survival of developing queens. Dimilin 2L (10% Dif) was included as a positive control. Queens were grafted into enclosed queen-rearing boxes (Spivak 1994, Johnson and Percel 2013) and were provided with nurse bees, syrup, and pollen. Pollen diets were either untreated (negative control) or treated with formulated agrochemicals. The translocation of each pesticide active ingredient from treated pollen into nurse bees and their royal jelly secretions was measured as well as queen survival throughout pupation, adult emergence, and to 7 d post-emergence.

**Methods**

**Queen-rearing Trials**

Experiments were conducted at Waterman Agricultural Research and Natural Resources Laboratory (WANRL) at the Ohio State University in Columbus, OH, from 2016-2018. Queen rearing trials were performed using a modified swarm box method (Johnson and Percel 2013, Spivak et al. 1994; Fig. 1). This approach limits the exposure of developing queens and their nurses to confounding variables associated with free-flying colonies (outside sources of pollen, weather events, etc.). Briefly, each swarm box was provisioned with 180 g of pollen and 2 liters of 50% (w/w) sucrose solution. Each box received thirty 24–48-h-old worker larvae, which were grafted into base mount JZ-BZ queen cups on a queen cell bar frame (Mann Lake Ltd., Hackensak, MN). Finally, each box received 3.15 liters of nurse bees (approximately 1.12 kg), which were shaken from multiple healthy colonies. Nurses did not receive any treatment prior to the start of each trial and were therefore only exposed to the treated pollen during the 96-h queen-rearing phase of each trial.

Experimental treatments were prepared by dissolving formulated products, alone or in combination, in distilled water to make a stock solution. The negative control contained only distilled water. Solutions were then blended with dried bee-collected pollen (Betterbee, Greenwich, NY) at a liquid:pollen ratio of 1:4 (w:w) using a food processor (Ninja Express Chop, SharkNinja Operating LLC, Chino, CA) to achieve target concentrations. The bulk pollen was thoroughly mixed prior to being portioned among trials. The target concentrations of the chemicals in pollen treatments were 40 ppm for Chl, 90 ppm for Pro, and 100 ppm for Dif. Diets with the adjuvant were treated to contain 0.8% Dyn by weight. Concentrations were chosen based on the maximum field application rates for each product in almonds (Supp Table 1 [online only]). These rates were chosen to simulate a high-exposure scenario immediately following a single pesticide application event. Five grams of treated pollen was sampled for pesticide analysis (described below) before the pollen was fed to each swarm box to determine the concentrations of each pesticide in treated pollen.

Two separate experiments were conducted. The first experiment included treatments of Altacor (Chl), Tilt (Pro), and a combination of Altacor + Tilt (Chl+Pro). The second experiment also included treatments of Dyn, Altacor + Dyn (Chl+Dyn), and Altacor + Tilt + Dyn (Chl+Pro+Dyn). An additional treatment with the insecticide Dimilin 2L (Dif) was included in the first experiment as a positive control. Each experiment was performed in three replicated trials. A detailed protocol for setting up the swarm boxes and conducting the rest of the experiment is provided (Supp File 2 [online only]).

Prepared swarm boxes, with grafted larvae, were stored in a dark room at 20–28°C for 96 h. At this time, 5 g of nurse bees (found clustering on the queen cell frame) and 5–7 capped queen cells were
removed from each swarm box for pesticide residue analyses (Fig. 1). The number of queen cells that were sampled varied between treatments as different numbers were needed to yield at least 1 g of royal jelly for chemical analysis. In trials receiving the Dif treatment, queen cells were not sampled for chemical analysis if survival was already low by day 4. This ensured that Dif trials could still serve as a positive control for all timepoints during survival analysis. Royal jelly from the sampled queen cells was manually extracted using a microspatula and stored in airtight microcentrifuge tubes at −20°C. The remaining queen cells were moved to a strong colony where they were incubated until adult queens emerged. On the eighth day of the trial, all capped queen cells were counted and individually caged to protect the cells and confine the adult queens once they emerged. The individually caged cells were checked every 2–3 d to record the number of queens that had emerged. Queen survival following emergence was recorded until 7 d after the first queen emergence was noted.

**Pesticide Residue Analysis**

Pollen, nurse bees, and royal jelly samples were stored at −20°C prior to being sent to The University of Guelph’s Agricultural and Food Laboratory for analysis by LC/MS/MS. Concentrations of each pesticide active ingredient (Chl, Dif, and Pro) were determined for each sample. Five grams of the untreated commercial pollen was used. The concentrations detected and the limits of detection for Chl, Dif, and Pro from experimental samples were 26, 88.5, and 66 ppm, respectively (Fig. 2, Supp Table 2 [online only]). The median concentrations of Chl, Pro, and Dif in treated pollen were 26, 88.5, and 66 ppm, respectively (Fig. 2, Supp Table 2 [online only]). The R code for all analyses and the associated datasheets can be found at https://doi.org/10.6084/m9.figshare.14541918.v2.

**Results**

**Pesticide Residue Analysis**

The median concentrations of Chl, Pro, and Dif in treated pollen were 26, 88.5, and 66 ppm, respectively (Fig. 2, Supp Table 2 [online only]). The concentrations of each active ingredient were 1–2 orders of magnitude lower between successive hive components (pollen > bees > jelly, Fig. 2). Residues of pesticides that were not applied as experimental treatments (contaminants) were either not detected or only detected at a fraction of the concentration of chemicals that were applied as treatments. The concentrations detected and the limits of detection for Chl, Dif, and Pro from experimental samples are provided in Supp Tables 3 and 4. None of the pesticide active ingredients used for this study (Chl, Pro, Dif) were detected in the untreated commercial pollen that was used.

A Shapiro–Wilk test found that the translocation rates of Chl ($n = 27$, $w = 0.869$), Dif ($n = 7$, $w = 0.738$), and Pro ($n = 20$, $w = 0.869$) were not normally distributed ($P < 0.05$) for both pollen and nurse bees. For each chemical, a nonparametric Kruskal–Wallis rank sums test was performed across mixtures. Differences between the total translocation of each active ingredient from pollen into royal jelly were also tested for significance with a Kruskal–Wallis rank sums test, followed by a post-hoc Dunn's test with a Bonferroni correction, using the R package dunn test (Dinno 2017). For all tests, adjusted $P$ values $< 0.05$ were considered statistically significant.

**Survival Analysis**

Counts of living and dead queens at 4, 8, 12 (emergence), and 19 d post-grafting (7 d post-emergence) were used to calculate the probability of queens surviving to each timepoint for each trial. Trials were omitted from the analysis according to two criteria: (1) trials with (negative) control mortality greater than 50% on day 12, or (2) trials with positive control (Dif) survival on day 12 greater than the corresponding survival of queens in the negative control group. A comparison of the overall survival between treatment groups was performed with a pairwise log-ratio test with a Bonferroni correction using the pairwise_survdiff function in the R package survival (Tehrano 2021). This test is suitable for analyses in which some number of subjects are censored from the study prior to the conclusion of the study. Censored queens in our study included those that were removed on day 4 in order to sample the royal jelly in their cells. On day 12, another subset of queens were removed for a companion study on the reproductive effects of the agrochemicals used in the present study. Finally, the survival of a subset of queens were measured up to day 19, the rest of which were censored from the study on day 12 (Supp Table 4 [online only]). The R code for all analyses and the associated datasheets can be found at https://doi.org/10.6084/m9.figshare.14541918.v2.
Survival Analysis

For each treatment group, 89–180 queens from 3–6 queen boxes were included in the survival analysis (Table 1). Raw survival data is presented in Supp Table 6 [online only]. By day 12, the mean survival rates of all experimental groups were less than that of the control group, except for the Pro group (Table 1, Supp Fig. 2 [online only]). Differences with the control group became more pronounced on day 19. A pairwise log-rank test found significant differences in the overall survival curves of the control group and the Chl+Pro+Dyn (P = 0.006) and Dif (P < 0.001) groups (Fig. 4). In addition, the survival of the positive control group, which was treated with Dif, was significantly different from all other groups (P < 0.05). Differences in survival for all other pairwise comparisons were non-significant (P > 0.05, Supp Table 7 [online only]).

Discussion

In agreement with previous studies (DeGrandi-Hoffman et al. 2013, Johnson and Percel 2013, Dively et al. 2015, Böhme et al. 2018, 2019, Milone et al. 2021), we found that the translocation rates of chemicals into royal jelly were quite low and never exceeded 1% of the concentrations in treated pollen (Fig. 3). Despite the low levels of chemicals detected in royal jelly, we found that the average probability of emergence was reduced by about 75% in groups reared on pollen containing the positive control Dimilin 2L (Dif) and by nearly 30% in groups reared on pollen containing a combination of Altacor (Chl), Tilt (Pro), and Dyne-Amic (Dyn), relative to the negative control group (Table 1, Supp Fig. 2 [online only]). Concentrations of pesticide active ingredients were 2–3 orders of magnitude greater in treated pollen relative to the royal jelly secretions of nurse bees, supporting a filtering role of nurses against the exposure of brood to food-borne toxicants. Notably, chemical concentrations were 1–2 orders of magnitude greater in samples of nurses relative to the royal jelly we collected from queen cells.

Our results indicate that nurses can effectively mitigate queen exposure to pesticides, but their protective function can be

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### Table 1. The number of trials, number of queens (omitting queens that were removed for chemical analysis), and mean probabilities of survival (± 1 standard deviation) for each treatment group at each timepoint

| Treatment   | Trials (n) | Queens (n) | Mean ± SD | Queens (n) | Mean ± SD | Queens (n) | Mean ± SD | Queens (n) | Mean ± SD |
|-------------|------------|------------|-----------|------------|-----------|------------|-----------|------------|-----------|
| Chl         | 6          | 180        | 0.85 ± 0.10 | 143        | 0.81 ± 0.12 | 143        | 0.75 ± 0.13 | 105        | 0.58 ± 0.19 |
| ChlDyn      | 3          | 90         | 0.86 ± 0.11 | 73         | 0.81 ± 0.15 | 73         | 0.70 ± 0.20 | 64         | 0.70 ± 0.20 |
| ChlPro      | 3          | 89         | 0.80 ± 0.15 | 73         | 0.80 ± 0.16 | 73         | 0.75 ± 0.18 | 54         | 0.56 ± 0.26 |
| ChlProDyn   | 3          | 90         | 0.67 ± 0.23 | 74         | 0.67 ± 0.23 | 74         | 0.53 ± 0.30 | 59         | 0.42 ± 0.40 |
| Control     | 3          | 90         | 0.88 ± 0.13 | 71         | 0.86 ± 0.14 | 71         | 0.76 ± 0.16 | 45         | 0.65 ± 0.21 |
| Dif         | 6          | 179        | 0.81 ± 0.12 | 143        | 0.45 ± 0.05 | 143        | 0.20 ± 0.06 | 106        | 0.03 ± 0.06 |
| Dyn         | 3          | 89         | 0.68 ± 0.16 | 70         | 0.68 ± 0.16 | 70         | 0.59 ± 0.29 | 53         | 0.46 ± 0.44 |
| Pro         | 6          | 180        | 0.91 ± 0.05 | 145        | 0.91 ± 0.05 | 145        | 0.90 ± 0.06 | 96         | 0.52 ± 0.25 |
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via royal jelly. Walsh et al. (2020) found that queens reared on wax and pollen treated with a combination of pesticides at field-relevant levels had reduced sperm viability in their spermathecae. This was observed despite negligible levels of direct oral exposure to pesticides can cause developmental effects to their hypopharyngeal glands that can ultimately impair their ability to tend to brood (Heylen et al. 2011, Hatjina et al. 2013, Zaluski et al. 2017). In queen-rearing experiments, this has been directly linked to reductions in both the quantity and metabolomic profile of royal jelly (Degrandi-Hoffman et al. 2015, Milone et al. 2021). We did not measure the effects of our treatments on nurses, which may include effects to their hypopharyngeal glands as well as their nursing behavior. This remains an interesting avenue for future studies.

In a previous study, Chl and Pro were found to have a synergistic effect on larval mortality (Wade et al. 2019). The present study extends this work to developing queens. Like other sterol biosynthesis inhibiting (SBI) fungicides, Pro is designed to inhibit enzymes that are closely related to key detoxification enzymes, the cytochrome P450 monoxygenases, in honey bees (Johnson 2015). Several studies have found synergistic toxicity between SBI fungicides such as Pro and insecticides in the pyrethroid and neonicotinoid classes (Johnson et al. 2013, Robinson et al. 2017, Carnesecchi et al. 2019), as well as quercetin, a naturally-occurring phytochemical common in pollen (Mao et al. 2017). In a large-scale survey of commercial bee colonies across the United States, SBI residues in beeswax were a significant predictor of both colony collapse and queen mortality (Traynor et al. 2016).

Although we did not find that the combination of Altacor (Chl) and Tilt (Pro) reduced queen survival relative to treatments receiving just Altacor, Tilt, or the negative control, these differences may have become evident if queen health had been tracked over a longer timeframe, or if additional measures of queen fitness were included. For example, Milone and Tarpy (2021) found that queens reared on wax and pollen treated with a combination of pesticides at field-relevant levels had reduced sperm viability in their spermathecae. This was observed despite negligible levels of direct oral exposure via royal jelly. Walsh et al. (2020) found that queens reared on wax treated with common pesticides, including common miticides used in beekeeping, produced fewer eggs as adults, had smaller worker retinues, and produced profiles of mandibular pheromones that were less attractive to worker bees in behavioral assays. Importantly, the effects of agrochemical mixtures on queens will likely be exacerbated by their effects on other members of the colony. For example, the viability of drone sperm was found to be reduced in drones reared on wax contaminated with pesticides, which may have long-term effects to the productivity of mated queens (Fisher and Rangel 2018). Finally, there are many other agrochemicals applied in almonds whose combined effects may have been more or less severe than those included in the present study. Fisher et al. (2017) found that combinations of the dicarboximide fungicide Iprodione 2SE Select reduced the survival of foragers following spray exposure when combined with certain strobilurin-containing fungicides (Pristine or Quadris). This is notable given that these mixtures lacked any insecticides. Subsequently, Fisher et al. (2021) found that the growth of field colonies was reduced when fed pollen containing just Pristine at field-relevant concentrations.

Our trials with Dimilin 2L, which served as a positive control for survival analysis, reinforce past studies indicating that it poses unacceptable risk to honey bee brood. Assuming that queens consume 380 µg of jelly over their development (Dietz and Lambremont 1970), queen larvae in our study consumed up to ~ 0.152 µg of Dif. This is an order of magnitude lower than the acute oral dose used in a previous study (2.28 µg) (Wade et al. 2019). It is also well below acute contact LD50s observed in worker larvae exposed between the third and sixth instar (2.42–6.02 µg/larva) (Gupta and Chandel 1995). Although we found Dif residues in jelly were below these acutely toxic levels, queens reared with Dif-treated pollen had significantly lower survival relative to all other treatments (Fig. 4, Supp Fig. 2 [online only]). These results corroborate the findings of Thompson et al. (2005), who observed half as many new eggs per day and a 6-fold increase in the rate of brood replacement in colonies treated with sucrose solution containing Dimilin Flo at a concentration mimicking its maximum field application rate in the United Kingdom.

We found a significant difference in the translocation rates of Pro and Chl (Fig. 3), but the mechanisms underlying the relative translocation of these and other food-borne contaminants of honey
P values are presented in Supp Table 5 [online only]. Items in the legend are ordered by their mean rate of survival on day 19. (P < 0.05). Exact dead queens were taken on days 4 (capping), 8, 12 (emergence), and up to 7 d post-emergence (day 19). Letters in the legend indicate significant differences in Fig. 4.

Adjuvant synergized the pathogenicity of common honey bee viruses. Fine et al. (2017) found that the exposure of larvae reared in vitro to an organosilicone adjuvant synergized the pathogenicity of common honey bee viruses, pyraclostrobin (DeGrandi-Hoffman et al. 2015). Fine et al. (2017) also found that the exposure of larvae reared in vitro to an organosilicone adjuvant synergized the pathogenicity of common honey bee viruses.

Fig. 4. Kaplan–Meier survival curves for queens reared with each pollen treatment. Data for each chemical were pooled across all trials. Counts of living and dead queens were taken on days 4 (capping), 8, 12 (emergence), and up to 7 d post-emergence (day 19). Letters in the legend indicate significant differences (P < 0.05). Exact P values are presented in Supp Table 5 [online only]. Items in the legend are ordered by their mean rate of survival on day 19.

The effects of agrochemicals on brood can interact with other stressors associated with the long-distance movement of colonies between crop blooms, such as increased rates of viral transmission (Cavigli et al. 2016). This is important, in part, because the combination of stressors faced by migratory colonies may undercuts the profitability of almond pollination for beekeepers (DeGrandi-Hoffman et al. 2019). DeGrandi-Hoffman et al. (2013) found increased virus titers in queen larvae exposed to the insecticide chlorpyrifos and the fungicide Pristine, which has been commonly used in almonds outside the blooming period. A similar result was found in adult workers exposed to pollen treated with the fungicides boscalid and pyraclostrobin (DeGrandi-Hoffman et al. 2015). Fine et al. (2017) found that the exposure of larvae reared in vitro to an organosilicone adjuvant synergized the pathogenicity of common honey bee viruses.

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Author Contributions
R.M.J. and C.H.L. conceptualized and designed the study. C.H.L. developed the methodology and conducted the experiments. D.F.R. analyzed data and wrote the initial draft of the manuscript. All authors contributed equally to editing and reviewing of the manuscript.

Supplementary Data
Supplementary data are available at Journal of Insect Science online.

Conclusion
Agrochemical mixtures remain a plausible cause of queen health issues occurring around almond bloom, particularly in combination with the other stressors involved in the annual migration of honey bees for pollination (vanEngelsdorp et al. 2013). Given the low levels of pesticide active ingredients detected in royal jelly, the effects of agrochemical mixtures on developing queens likely resulted from indirect effects on nurses in addition to direct toxicity to queens. These findings support current best management practices recommending that neither insecticides nor adjuvants be combined with fungicides applied to almonds during bloom when honey bees are present for pollination (Almond Board of California 2020).
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