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Research article

The Synergistic Effects of Almond Protection Fungicides on Honey Bee (Hymenoptera: Apidae) Forager Survival

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Abstract

The honey bee (*Apis mellifera* L.) contributes ~$17 billion annually to the United States economy, primarily by pollinating major agricultural crops including almond, which is completely dependent on honey bee pollination for nut set. Almond growers face constant challenges to crop productivity owing to pests and pathogens, which are often controlled with a multitude of agrochemicals. For example, fungicides are often applied in combination with other products to control fungal pathogens during almond bloom. However, the effects of fungicides on honey bee health have been so far understudied. To assess the effects of some of the top fungicides used during the 2012 California almond bloom on honey bee forager mortality, we collected foragers from a local apiary and exposed them to fungicides (alone and in various combinations) at the label dose, or at doses ranging from 0.25 to 2 times the label dose rate. These fungicides were Iprodione 2SE Select, Pristine, and Quadris. We utilized a wind tunnel and atomizer set up with a wind speed of 2.9 m/s to simulate field-relevant exposure of honey bees to these agrochemicals during aerial application in almond fields. Groups of 40–50 foragers exposed to either untreated controls or fungicide-laden treatments were monitored daily over a 10-d period. Our results showed a significant decrease in forager survival resulting from exposure to simulated tank mixes of Iprodione 2SE Select, as well as synergistic detrimental effects of Iprodione 2SE Select in combination with Pristine and Quadris on forager survival.

Key words: Almond, *Apis mellifera*, fungicide, honey bee forager survival, Iprodione 2SE

Honey bees (*Apis mellifera* L.) contribute ~$17 billion annually to the United States economy, primarily through pollination of major agricultural crops (Calderone 2012, Zhu et al. 2015). Among the main crops pollinated by honey bees is almond (*Prunus dulcis*), which relies almost entirely on honey bee pollination for nut set (Klein et al. 2012). The almond industry in California produces about 80% of the almonds consumed worldwide (Klein et al. 2012), employing ~60% of all managed honey bee hives in the country to provide pollination services during the crop's bloom in mid- to late-winter (Sumner and Boriss 2006). In protecting almond orchards from various pests and pathogens, heavy chemical treatments are employed during bloom (Bosch and Blas 1994). However, despite their ubiquitous use, the effects on honey bee health of the various pesticides used repeatedly in almond orchards are not well understood.

In particular, little is known about the effects on honey bee health of fungicides used in almond orchards during bloom, although a few studies have shown that some fungicides affect colony health at various stages of bee development. For example, Mussen et al. (2004) and Mussen (2013) reported negative impacts on brood survival in vitro and in the field when the brood was fed pollen that was artificially contaminated with fungicides (Mussen et al. 2004, Mussen 2013). Similarly, Kubik et al. (1999) recorded high levels of the fungicides vinclozolin and iprodione in stored pollen and honey collected from colonies used for pollination in cherry orchards (Kubik at al. 1999). Furthermore, Vandamme and Belzunces (1998) observed negative sublethal effects of combinations of the fungicides prochloraz and difenoconazole, and the insecticide deltamethrin, on thermoregulation in adult workers (Vandamme and Belzunces 1998). Combinations of fungicides, acaricides, and insecticides have also been shown to cause synergistic detrimental effects on adult worker and queen mortality (Pilling and Jepson 1993, Johnson and Purcell 2013). Moreover, an examination of the combined application of some insecticides, a fungicide (tetraconazole), and a herbicide in crop systems such as cotton, rice, and corn, revealed significant negative synergistic impacts of these chemicals on adult worker survival in vitro (Zhu et al. 2015).

A recent review of studies on the toxicity to honey bees of fungicides and other pesticides found in pollen, wax, and honey, noted an alarming persistence of fungicide residues in most samples, including noticeable detection levels of the fungicides boscalid, captan, and

myclobutanil in pollen and adult bees, often at higher frequencies than other pesticide classes (Johnson et al. 2010). In a different study, the fungicide chlorothalonil was found at high frequency and concentration in stored pollen from commercial apiaries from across the United States (Mullin et al. 2010). Chlorothalonil contamination is associated with entombing behavior, whereby workers cap contaminated pollen cells with propolis, presumably to protect the colony from further exposure to the chemical (vanEngelsdorp et al. 2009). However, the precise health effects of high levels of chlorothalonil in pollen are not well understood.

Honey bee foragers, which begin their food-seeking tasks 21 d postemergence (Huang and Robinson 1996, Abou-Shaara 2014), comprise the age group that is most susceptible to direct exposure to agrochemicals when visiting flowers for pollen and nectar collection (Pettis et al. 2013). Foragers are thus an attractive age cohort for studying the effects of field-relevant concentrations of fungicide tank mixes used in agricultural crops on colony health. In this study, we exposed foragers with three fungicides widely applied during the almond bloom in California to assess the potential synergistic effects of these agrochemicals in simulated tank mixes on forager mortality. In light of our findings, we propose a more careful consideration of fungicide application in almond orchards or any agricultural crop during bloom, because it might negatively affect honey bee colony health in ways that are still poorly understood.

Materials and Methods

Fungicides Used

The fungicides selected for our study were Iprodione 2SE Select (Prime Source, LLC, Evansville, IN) (active ingredient: 23.8% iprodione), Pristine BASF Corporation, Research Triangle Park, NC (active ingredients: 25.2% boscalid, 12.8% pyraclostrobin), and Quadris Syngenta Crop Protection, Inc. Greensboro, NC (active ingredient: 22.9% azoxytrobin), which were among the top 50 pesticides most widely used during the almond bloom in California in 2012 (Pesticide Action Network Pesticides Database 2012; Table 1). All fungicides were purchased from a commercial source (Amazon Inc.) and applied individually or in combination with other fungicides at either the manufacturer's recommended label dose, or at different concentrations (see section 2 below).

Experimental Treatment Groups

To assess the effects on forager mortality of each selected fungicide, alone and in various combinations with other fungicides, seven experimental groups were formulated including a fungicide-free control group. The fungicide treatment groups included Iprodione 2SE Select (iprodione) at differing concentrations derived from the recommended label dose (Table 2). Other treatment groups included in the study were combinations of iprodione and Pristine or iprodione and Quadris at the exact recommended label dose. The control group consisted only of the solvent, distilled water, which was used to dissolve all fungicides used in the treatment groups. Three separate trials were conducted between September and November 2015 (Table 2), and increased rainfall and corresponding decreases in available forage were observed over this period.

A fourth experimental trial was conducted in December 2015 by applying each fungicide at twice the recommended label dose rate, reflecting a potential worst-case scenario in the application of these chemicals to honey bee foragers (Table 3).

Forager Capture

Honey bee foragers were collected from a designated hive located at the Janice and John G. Thomas Honey Bee Facility of Texas A&M University's Riverside Campus in Bryan, TX. Honey bee adults covering frames that contained little to no brood but contained ample food resources were selected for the exposure experiments. Such frames were targeted because they likely had a higher number of older adult bees on them, including foragers, which take on food collection rather than brood maintenance, the task of younger workers (Winston 1987). Foragers were gently brushed off the frames into bioassay cages composed of a circular cardboard frame, holding rings, and mesh side panels. The bioassay cage frames had a diameter of ~15.2 cm. To enclose the cages, a single sheet of mesh fabric was stretched over either side of the cage, then a thin cardboard holding ring with a slightly larger diameter than the cage frame was forced around the frame securing the mesh in a taut position. Approximately 40–50 foragers were loaded into each bioassay cage, and a total of six bioassay cages were allocated to each experimental treatment group (Fig. 1a). The bioassay cages were disposed of after every use.

Fungicide Exposure

Bioassay cages loaded with 40–50 foragers were divided into experimental groups that were either exposed to fungicides at various concentrations, or exposed to fungicide-free water, the diluent used in every treatment (see Tables 2 and 3 for details). Contact exposure was conducted utilizing a wind tunnel atomizer setup at the USDA-Agricultural Research Service Aerial Application Technology Laboratory at the Riverside Campus of Texas A&M University, located in Bryan, TX. Large fans at one end of the wind tunnel setup propelled air at a speed of 2.9 m/s down the wind tunnel chamber, simulating the wind speed of pesticides dispensed from agricultural aircraft. Labeled bioassay cages were loaded, one at a time, onto a holding fork near the end of the wind tunnel chamber opposing the large fans (Fig. 1b). The fungicides were diluted in water and sprayed at concentrations corresponding to the label dose or preselected label dose variants. Approximately 10 ml of each fungicide solution was loaded into the twin fluid atomizer located at the end of the wind tunnel chamber corresponding to the large fans. A 10-ml syringe was used to transfer fungicide solution through a plastic tube attached directly to the atomizer. A compressed air tank was connected to the atomizer and activated along with the wind tunnel fans propelling fungicide solution through the atomizer and down the

Table 1. Top four fungicides used in California during the almond bloom in 2012[a]

Fungicide	Chemical class	Gross number of pounds applied	Application rate (lbs/acre)	No. of acres planted	No. of acres treated	Percentage of acres treated
Azoxystrobin	Strobin	44,481	0.19	223,847	231,044	103
Boscalid	Anilide	62,143	0.21	337,528	327,122	97
Pyraclostrobin	Strobin	34,589	0.11	337,242	327,122	97
Iprodione	Dicarboximide	151,968	0.48	310,766	315,097	101

[a] Data obtained from the Pesticide Action Network Pesticides Database "*Pesticide Use on Almonds in 2012*."

wind tunnel chamber. Each application lasted for ~5 s to ensure the complete expulsion of fungicide solution from the atomizer and propulsion down the chamber to the bioassay cage on the opposing end. Following exposure, bioassay cages were removed from the holding fork and the atomizer was cleansed with acetone between the application of each experimental treatment. This process was repeated for all bioassay cages allocated to each treatment group. The control group bioassay cages were loaded into the wind tunnel but were spared fungicide exposure; instead, they were sprayed with water propagated through the atomizer.

Table 2. Fungicide treatment groups devised to test the effects of Iprodione 2SE Select at various concentrations and iprodione in combination with Quadris and Pristine to test the effects of fungicides on honey bee forager mortality

Experimental treatments	Fungicides used (percentage of label dose rate)	Fungicide concentration (ml or g/liter H_2O)
Control	N/A	N/A
1	1/4× Iprodione 2SE Select	5.5 ml
2	1/2× Iprodione 2SE Select	11 ml
3	1× Iprodione 2SE Select	22 ml
4	2× Iprodione 2SE Select	44 ml
5	1× Iprodione 2SE Select+ 1× Quadris	22 mL + 125 ml
6	1× Iprodione 2SE Select + 1× Pristine	22 mL + 21.8 g

Table 3. Fungicide treatment groups devised to test the effects of Iprodione 2SE Select, Quadris, and Pristine, at twice the recommended label dose rate, on honey bee forager mortality

Experimental treatment	Fungicides used (label dose rate)	Fungicide concentration (mL or g/liter H_2O)
Control	N/A	N/A
1	2x Quadris	250 ml
2	2x Pristine	43.6 g
3	2x Iprodione 2SE Select	44 ml

Monitoring Forager Survival

Following the application of fungicide treatments, foragers in each bioassay cage were transferred to a labeled plastic containment unit (~1 quart in volume), containing strips of wax foundation attached to the side and bottom of the unit (Fig. 1c). A wide brimmed funnel was placed over a containment unit, and then one of the holding rings on a bioassay cage was removed to facilitate the transfer of foragers. The bioassay cage was secured over the funnel and one of the mesh side panels was removed, allowing foragers in the bioassay cage to migrate into the containment unit. The containment unit was gently shaken and a lid was swiftly placed over it to secure the foragers within. This process was repeated until all foragers in each bioassay cage were transferred to corresponding containment units. A pair of 1.5-ml Eppendorf tubes was inserted into premade holes in the lid of each unit. The Eppendorf tubes served as feeders and water dispensers. Feeder tubes were loaded with ~1 ml of sugar syrup composed of a 50:50 mixture of water and sucrose, while water dispensing tubes were loaded with ~1 ml of water. The containment units were kept in an incubator set at 34.5 °C and ~75% relative humidity. The units were checked every 24 h for 10 consecutive days, noting the number of dead workers to determine the total number of workers that died every day within the 10-d period. A forager was considered dead if it exhibited a complete lack of movement, which was often accompanied by the forager lying on its side with its proboscis permanently extruded.

Statistical Analysis

To compare the average forager mortality over a 10-d period between the untreated control group and each individual fungicide treatment, we performed Student's t-tests (JMP 12.0, SAS Inc., Cary, NC). To compare the survival rate between control and treatment groups, we performed Kaplan–Meier survival analysis (JMP 12.0, SAS Inc., Cary, NC). For all tests, the level of statistical significance was set at $\alpha = 0.05$. All descriptive statistics are reported as mean ± standard error of the mean (SEM).

Results

Following exposure to Iprodione 2SE Select alone at various concentrations, and Iprodione 2SE Select in combination with other fungicides (Table 2), foragers tended to experience significantly high

Fig. 1. Experimental set up used to test the effects of fungicides on honey bee forager mortality. First, (a) about 40–50 bees were loaded into a clean bioassay cage. Then, (b) cages were consecutively placed in a wind tunnel and exposed to either a fungicide-free control or fungicide-laden treatment in increasing concentrations as shown in Tables 2 and 3. Once treated, the caged bees were transferred into (c) plastic holding units with feeders containing 50:50 sucrose solution ad libitum and placed in an incubator held at 34 °C to measure worker mortality every 24-h for 10 d.

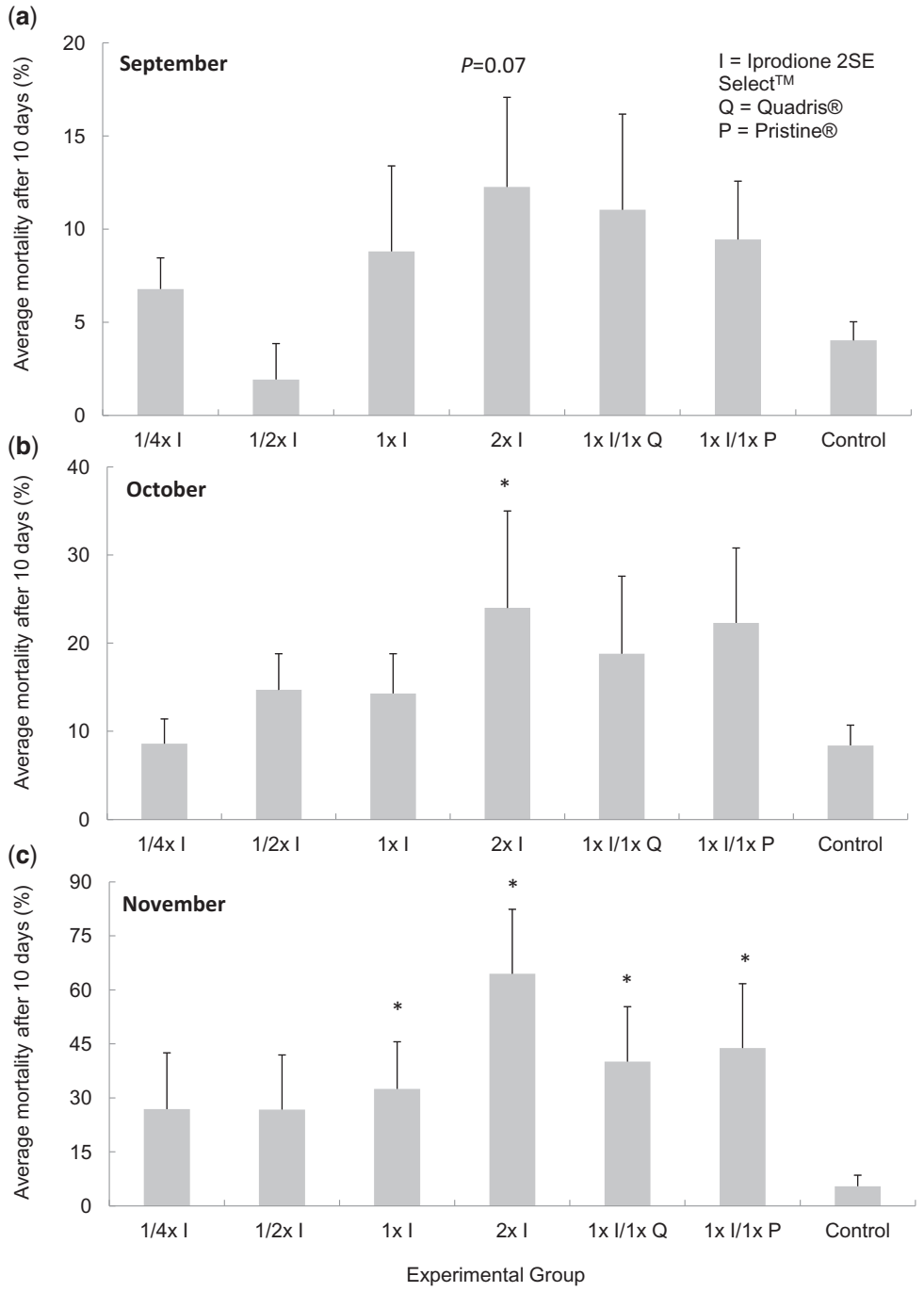

Fig. 2. Average honey bee forager mortality observed after 10 d during three trials in which bioassay cages ($n = 6$) containing 40–50 foragers each were exposed in a wind tunnel to six fungicide treatment groups and an untreated control group. The trials were conducted in (**a**) September 2015, (**b**) October 2015, and (**c**) November 2015. The treatments included Iprodione 2SE Select at 1/4×, 1/2×, 1×, and 2× the label dose rate, as well as combinations of Iprodione 2SE Select and Quadris, and iprodione and Pristine, all at the label dose rate. The "*" symbols represent values of $P < 0.05$.

mortality that increased in severity over the course of the first three trials (Fig. 2). For instance, during the September 2015 trial, no statistically significant difference was observed when the treatment groups were compared with the untreated control (Fig. 2a). But in the October 2015 trial, this difference was statistically significant ($t = 1.06$, $P = 0.04$), with foragers exposed to Iprodione 2SE Select at twice the label dose having an average 10-d-mortality of 24% ± 11% compared with 8.4% ± 2.3% for the untreated control group (Fig. 2b). Differences in worker mortality were more striking during the November 2015 trial (Fig. 2c), with a significantly higher average mortality observed in the Iprodione 2SE Select treatment at the label dose (32.5% ± 13.1%; $t = 1.99$, $P = 0.04$), the Iprodione 2SE Select at twice the label dose (64.5% ± 17.9%; $t = 3.23$, $P = 0.01$), the combinations of Iprodione 2SE Select and Quadris at the label dose (40.1% ± 15.3%; $t = 2.21$, $P = 0.04$), and the combinations of Iprodione 2SE Select and Pristine at the label dose (43.9% ± 17.8%), compared with the untreated control group (5.5% ± 3.1%; $t = 2.11$, $P = 0.04$).

Table 4. Fungicide treatment groups used in experiment 1 that resulted in a significant decrease in honey bee forager survival relative to the control group

Trial	Month	Pairwise treatment comparison	χ^2 value	*P* value
1	Sept. 2015	2× Iprodione vs. Control	11.05	<0.001
		1× Iprodione 2SE Select + 1× Quadris vs. Control	11.21	<0.001
		1× Iprodione 2SE Select + 1× Pristine vs. Control	6.14	0.01
2	Oct. 2015	1/2× Iprodione 2SE Select vs. Control	6.26	0.01
		1× Iprodione 2SE Select vs. Control	4.19	0.04
		2× Iprodione 2SE Select vs. Control	18.11	<0.0001
		1× Iprodione 2SE Select + 1× Quadris vs. Control	9.15	0.003
		1× Iprodione 2SE Select + 1× Pristine vs. Control	15.9	<0.0001
3	Nov. 2015	1/4× Iprodione 2SE Select vs. Control	72.5	<0.0001
		1/2× Iprodione 2SE Select vs. Control	43.6	<0.0001
		1× Iprodione 2SE Select vs. Control	65.9	<0.0001
		2× Iprodione 2SE Select vs. Control	266.4	<0.0001
		1× Iprodione 2SE Select + 1× Quadris vs. Control	126.2	<0.0001
		1× Iprodione 2SE Select + 1× Pristine vs. Control	172.3	<0.0001

Average forager mortality data was also analyzed over the course of 10 d using Kaplan–Meier survival analysis. In addition to the assessment of the general effect of the collective treatment groups on forager mortality for all treatment groups, the survival rate of foragers exposed to each individual treatment was compared with the untreated control group. The treatment groups at twice the label dose of Iprodione 2SE Select, Iprodione 2SE Select + Quadris, and Iprodione 2SE Select + Pristine were observed to significantly decrease forager survival in all three trials of the first experiment (Table 4). Similarly to our average mortality results, fungicide-treated foragers experienced a significant and progressive decrease in their survival rate, which occurred earlier in time over the course of the first three trials, compared with the untreated control group (Fig. 3). In the first trial, which was done in September 2015 (Fig. 3a), a significant decrease in forager mortality was observed as a general effect of fungicide exposure ($\chi^2 = 25.04$, $P < 0.001$). Iprodione 2SE Select at twice the label dose, the combination of Iprodione 2SE Select and Quadris at the label dose, and Iprodione 2SE Select and Pristine at the label dose significantly decreased forager survival compared with the untreated control group. In the second trial, which was done in October 2015 (Fig. 3b), the overall effect of fungicide exposure was more pronounced ($X^2 = 30.30$, $P < 0.0001$). The treatment groups Iprodione 2SE Select at the label dose and Iprodione 2SE Select at half the label dose significantly decreased forager survival. Also, as with the September 2015 trial, the treatments of Iprodione 2SE Select at twice the label dose, Iprodione 2SE Select in combination with Quadris, and Iprodione 2SE Select in combination with Pristine all demonstrated a significant decrease in forager survival compared with the untreated control group. Interestingly, forager survival rate was most highly impacted in the November 2015 trial (Fig. 3c), whereby all treatment groups experienced a significant decline in survival relative to the control group ($\chi^2 = 328.7$, $P < 0.0001$). The treatment groups included Iprodione 2SE Select at a quarter of the label dose, at half the label dose, at the label dose, and at twice the label dose, Iprodione 2SE Select combined with Quadris, and Iprodione 2SE Select combined with Pristine (Table 4).

In a second experiment (with only one trial) conducted in December 2015, foragers were exposed to individual applications of Iprodione 2SE Select, Pristine, and Quadris, each at twice the label dose rate (Table 5). During this trial, foragers experienced significantly higher average mortality when exposed to iprodione at twice the label dose rate compared with the untreated control group ($t = 2.22$, $P = 0.04$; Fig. 4). Mortality data using Kaplan–Meier survival analysis revealed an overall significant effect of all three of the fungicide treatment groups in significantly decreasing forager survival rate over the 10-d experimental period ($\chi^2 = 31.5$, $P < 0.0001$; Fig. 5). When the survival rate of each individual fungicide treatment group was compared with the control group, each pairwise treatment comparison revealed a significant decrease in forager survival rate (Table 5).

Discussion

Our examination of the most frequently used fungicides during almond bloom in California during the 2012 season revealed a significant negative effect of Iprodione 2SE Select, alone and in combination with Quadris or Pristine, on honey bee forager survival. Overall, we observed significant drops in forager survival (as measured by Kaplan–Meier survival analyses) when foragers were exposed to the various fungicides treatment groups compared with untreated control groups. These effects were consistent in three separate trials conducted in Fall 2015, with the overall effect of the fungicide treatments progressively intensifying in trials conducted later in the year, thus suggesting a severe seasonal effect of fungicide exposure on forager mortality.

The causes of decreased forager survival due to fungicide exposure are likely due to disruption of key physiological processes within exposed foragers. However, the mode of action of fungicides on honey bee physiology has been poorly examined. It is possible that exposed workers may have inadvertently increased the concentration of fungicides that end up inside their bodies through allo- and self-grooming (Scheiner et al. 2013), thus licking fungicides on their cuticle while confined in the containment units after being treated with the fungicides. This idea remains to be tested, however. In addition, the transition to winter physiology in honey bees appears to coincide with reduced immunological processes (Steinmann et al. 2015), specifically in the reduction of the expression of genes associated with microbial resistance. This process of reducing immune strength apparently coincided with our continuous application of fungicides, perhaps clarifying why the same treatment groups had an enhanced negative effect on forager survival over time.

Interestingly, when the fungicide prochloraz was topically applied along with pyrethroid insecticides to workers, it enhanced the toxicity of both compounds by inhibiting the activity of detoxifying cytochrome P450 monooxygenases (Pilling et al. 1995). In a more recent study, topical application of prochloraz was observed to interact with the acaricides tau-fluvalinate, coumaphos, and fenpyroximate, increasing their toxicity to treated workers (Johnson et al. 2013). Four other fungicides, including chlorothalonil, boscalid, pyraclostrobin, and a combination of boscalid and

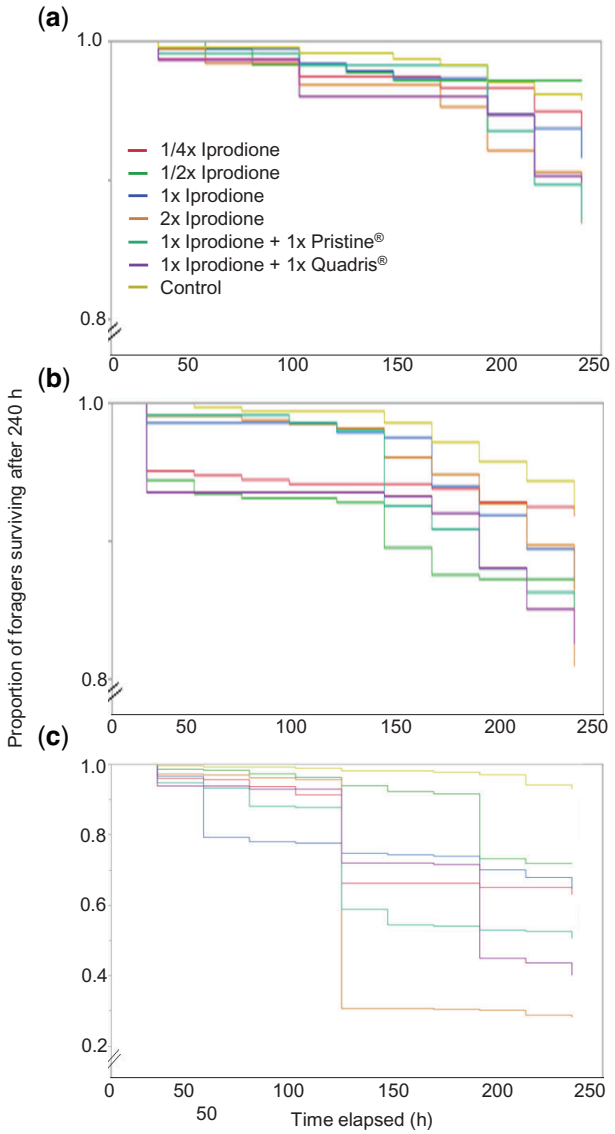

Fig. 3. Proportion of honey bee foragers contained in groups of 40–50 individuals that survived in an incubator held at a constant temperature of 34 °C, 24-h after exposure in a wind tunnel to either one of six fungicide treatment groups or an untreated control group. Trials were conducted in (**a**) September 2015, (**b**) October 2015, and (**c**) November 2015. See "*Materials and Methods*" for more details.

Table 5. Fungicide treatment groups tested in December 2015 that resulted in a significant decrease in honey bee forager survival relative to the control group

Pairwise treatment comparison	χ^2 value	*P* value
2x Iprodione 2SE Select vs. Control	21.64	<0.001
2x Pristine vs. Control	27.31	<0.001
2x Quadris vs. Control	32.12	<0.001

pyraclostrobin (the active ingredients in Pristine), similarly increased the toxicity of tau-fluvalinate (Johnson et al. 2013) via topical applications to workers. In that study, inhibition of detoxifying P450 monooxygenases was proposed as the mechanism by which the fungicides enhanced the toxicity of the acaricides used (Johnson et al.

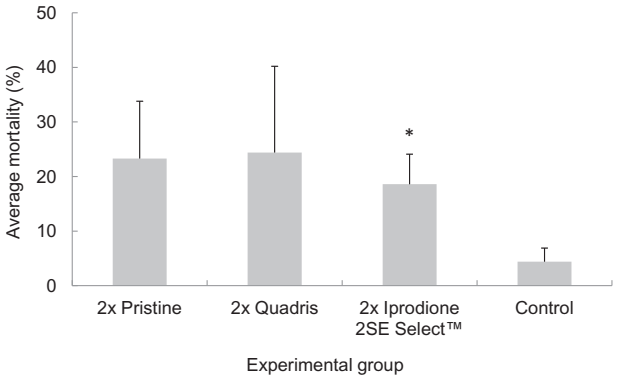

Fig. 4. Average honey bee forager mortality observed after 10 d during a trial conducted in December 2015, in which bioassay cages containing 40–50 foragers were exposed in a wind tunnel to three fungicide treatment groups and an untreated control group. The fungicide treatments included Pristine, Quadris, and Iprodione 2SE Select at twice the label dose rate. The "*" symbol represents a value of $P < 0.05$.

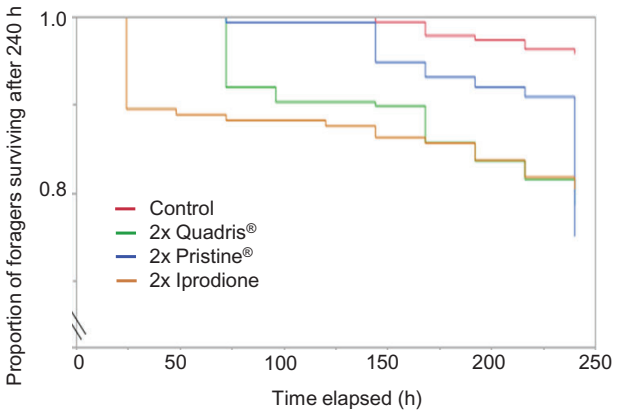

Fig. 5. Proportion of honey bee foragers contained in groups of 40–50 individuals that survived in an incubator held at a constant temperature of 34.5 °C, 24-h after exposure in a wind tunnel to either Quadris, Pristine, or Iprodione 2SE Select at twice the label dose rate, or to an untreated control group.

2013). In fungal targets, boscalid and pyraclostrobin function as a succinate dehydrongenase inhibitor and a quinone outside inhibitor, respectively (Fernández-Ortuño et al. 2012). Thus Pristine disrupts mitochondrial metabolism and ATP synthesis within cells.

The active ingredient of Quadris, azoxystrobin, functions in a similar manner in that it disrupts the electron transport chain, thereby inhibiting ATP synthesis (Bartlett et al. 2002). Azoxystrobin also induces oxidative stress through electron liberation from the process of respiration (Kim et al. 2007). Finally, iprodione, though its mode of action is not fully understood, appears to inhibit glutathione synthesis (Dierickx 2004), which is essential for detoxification processes in the cell's mitochondria (Ribas et al. 2014).

The previously demonstrated lethal effects of field-relevant doses of iprodione on honey bee brood, although applied in vitro (Mussen et al. 2004), suggest the potential for contaminated foragers to inadvertently cause rapid population declines in exposed colonies. Intensive application of iprodione in almond orchards may lead to its prevalence in food stores, particularly pollen, as has been noted in other agricultural systems such as cherry orchards (Kubik et al. 1999). The resilience in wax of fungicide residues with respect to other pesticide classes (Johnson et al. 2010) may make them an insidious threat to colony

health, especially when considering the general lack of attention to this class of agrochemicals. Interestingly, boscalid, one of the active ingredients of Pristine, was among the most highly detected fungicides found in wax by a review of studies examining in-hive pesticide toxicity (Johnson et al. 2010). This suggests that along with Iprodione 2SE Select, Pristine may also impact colony health through persistent contamination of pollen reserves. Mussen et al. (2004) may provide an accurate basis of comparison for the sustenance of brood on contaminated pollen in field conditions, an indication of further colony effects beyond short-term forager mortality.

Given their effects on fungal targets and insight on the effects of a few fungicides on honey bees (Pilling et al. 1995, Johnson et al. 2013), fungicides may overall exert similar effects to those caused by acaricides and other chemicals in inhibiting detoxifying components and processes. However, despite reports of the persistence of fungicides in a hive environment (Kubik et al. 1999, Johnson et al. 2010), the focus to honey bee health has been on the effect of fungicides in augmenting the toxicity of other pesticides classes (Pilling and Jepson 1993, Pilling et al. 1995, Vandamme and Belzunces 1998, Johnson and Purcell 2013, Johnson et al. 2013), therefore leaving a distinct vacancy in our understanding of the precise effects of fungicides on their own on honey bee health. By examining fungicides as individual applications and in combination with other fungicides, we will create a stronger basis for understanding the potential threat that fungicides alone may pose to honey bee health.

In conclusion, despite the importance of honey bee foragers in supplying food resources to their colony, the precise mechanisms of action of fungicides used during floral bloom have not been characterized in honey bees. When considering the use of fungicides during the almond bloom, cautious fungicide application in almond orchards is recommended to prevent unplanned forager exposure to these chemicals. Perhaps avoidance of such applications during bloom or applying fungicides during times of low honey bee forager activity, such as late evenings, would help mitigate the direct and potential secondary effects of fungicides to honey bee colony health.

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References Cited

Abou-Shaara, H. F. 2014. The foraging behaviour of honey bees, *Apis mellifera*: A review. Vet. Med. 1–10.

Bartlett, D. W., J. M. Clough, J. R. Godwin, A. A. Hall, M. Hamer, and B. Parr-Dobrzanski. 2002. The strobilurin fungicides. Pest Manag. Sci 58: 649–662.

Bosch, J., and M. Blas. 1994. Foraging behaviour and pollination efficiency of *Osmia cornuta* and *Apis mellifera* on almond (Hymenoptera, Megachilidae and Apidae). Appl. Entomol. Zool. 1–9.

Calderone, N. W. 2012. Insect pollinated crops, insect pollinators and US agriculture: Trend analysis of aggregate data for the period 1992–2009. PLoS ONE 7: e37235.

Dierickx, P. J. 2004. Cytotoxicity of the dicarboximide fungicides, vinclozolin and iprodione, in rat hepatoma-derived Fa32 cells. Altern. Lab. Anim. 369–373.

Fernández-Ortuño, D., F. Chen, and G. Schnabel. 2012. Resistance to pyraclostrobin and boscalid in *Botrytis cinerea* isolates from strawberry fields in the Carolinas. Plant Dis. 1198–1203.

Huang, Z. Y., and G. E. Robinson. 1996. Regulation of honey bee division of labor by colony age demography. Behav. Ecol. Sociobiol. 147–158.

Johnson, R. M., and E. G. Purcell. 2013. Effect of 'Bee-Safe' insecticides and fungicides on honey bee queen development and survival. Poster presented at 2nd International Conference on Pollinator Biology, Health and Policy, Aug. 14–17, 2013. Pennsylvania State University.

Johnson, R. M., M. D. Ellis, C. A. Mullin, and M. Frazier. 2010. Pesticides and honey bee toxicity – USA. Apidologie 312–331.

Johnson, R. M., L. Dahlgren, B. D. Sigfried, and M. D. Ellis. 2013. Acaricide, fungicide and drug interactions in honey bees (*Apis mellifera*). PLoS ONE 8: e54092. doi:10.1371/journal.pone.0054092.

Kim, J. H., B. C. Campbell, N. Mahoney, K. L. Chan, R. J. Molyneux, and G. S. May. 2007. Enhanced activity of strobilurin and fludioxonil by using berberine and phenolic compounds to target fungal antioxidative stress response. Lett. Appl. Microbiol. 134–141.

Klein, A. M., C. Brittain, S. D. Hendrix, R. Thorp, N. Williams, and C. Kremen. 2012. Wild pollination services to California almond rely on semi-natural habitat. J. Appl. Ecol. 723–732.

Kubik, M., J. Nowacki, A. Pidek, Z. Warakomska, L. Michalczuk, and W. Goszczynski. 1999. Pesticide residues in bee products collected from cherry trees protected during blooming period with contact and systemic fungicides. Apidologie 521–532.

Mullin, C. A., M. Frazier, J. L. Frazier, S. Ashcraft, R. Simonds, D. vanEngelsdorp, and J. Pettis. 2010. High levels of miticides and agrochemicals in North American apiaries: Implications for honey bee health. PLoS ONE 5: e9754.

Mussen, E. C. 2013. Problems with almond bloom sprays. University of California Cooperative Extension.

Mussen, E. C., J. E. Lopez, and C.Y.S. Peng. 2004. Effects of selected fungicides on growth and development of larval honey bees, *Apis mellifera* L. (Hymenoptera: Apidae). Environ. Entomol. 1151–1154.

Pesticide Action Network Pesticides Database 2012. Pesticide Use on Almonds in 2012. (http://www.pesticideinfo.org/DS.jsp?sk=3001) (Accessed 3 December 2015).

Pettis, J. S., E. M. Lichtenberg, M. Andree, J. Stitzinger, R. Rose, and D. vanEngelsdorp. 2013. Crop pollination exposes honey bees to pesticides which alters their susceptibility to the gut pathogen *Nosema ceranae*. PLoS ONE 8: e70182. doi: 10.1371/journal.pone.0070182

Pilling, E. D., and P. C. Jepson. 1993. Synergism between EBI fungicides and a pyrethroid insecticide in the honeybee (*Apis mellifera*). Pesticide Sci. 293–297.

Pilling, E. D., A. C. Bromley-Challenor, C. H. Walker, and P. C. Jepson. 1995. Mechanism of synergism between the pyrethroid insecticide–cyhalothrin and the imidazole fungicide prochloraz, in the honey bee (*Apis mellifera* L.). Pesticide Biochem. Physiol. 1–11.

Ribas, V., C. García-Ruiz, and J. C. Fernández-Checa. 2014. Glutathione and mitochondria. Front. Pharmacol. 1–19.

Scheiner, R., C. I. Abramson, R. Brodschneider, K. Crailsheim, W. M. Farina, S. Fuchs, B. Grünewald, S. Hahshold, M. Karrer, G. Koeniger, et al. 2013. Standard methods for behavioural studies of *Apis mellifera*. J. Apic. Res. 52: 52.4.04.

Steinmann, N., M. Corona, P. Neumann, and B. Dainat. 2015. Overwintering is associated with reduced expression of immune genes and higher susceptibility to virus infection in honey bees. PLoS ONE 10: e0129956. http://doi.org/10.1371/journal.pone.0129956

Sumner, D. A., and H. Boriss. 2006. Bee-conomics and the leap in pollination fees. Found. Agric. Econ. 9–11.

Vandamme, R., and L. P. Belzunces. 1998. Joint actions of deltamethrin and azole fungicides on honey bee thermoregulation. Neurosci. Lett. 57–60.

vanEngelsdorp, D., J. D. Evans, L. Donovall, C. Mullin, M. Frazier, J. Frazier, D. R. Tarpy, J. Hayes, Jr., and J. S. Pettis. 2009. "Entombed Pollen": A new condition in honey bee colonies associated with increased risk of colony mortality. J. Invert. Pathol. 147–149.

Winston, M. L. 1987. The biology of the honey bee. Harvard University Press, Cambridge, MA.

Zhu, Y. C., J. Adamcyzk, T. Rinderer, J. Yao, R. Danka, R. Luttrell, and J. Gore. 2015. Spray toxicity and risk potential of 42 commonly used formulations of row crop pesticides to adult honey bees (Hymenoptera: Apidae). J. Econ. Entomol. 108(6): 2640–2647