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Research Article

The Effects of the Insect Growth Regulators Methoxyfenozide and Pyriproxyfen and the Acaricide Bifenazate on Honey Bee (Hymenoptera: Apidae) Forager Survival

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Abstract

The honey bee (*Apis mellifera* L. (Hymenoptera: Apidae)) contributes an essential role in the U.S. economy by pollinating major agricultural crops including almond, which depends entirely on honey bee pollination for successful nut set. Almond orchards are often treated with pesticides to control a variety of pests and pathogens, particularly during bloom. While the effects to honey bee health of some insecticides, particularly neonicotinoids, have received attention recently, the impact of other types of insecticides on honey bee health is less clear. In this study, we examined the effects to honey bee forager survival of three non-neonicotinoid pesticides widely used during the 2014 California almond bloom. We collected foragers from a local apiary and exposed them to three pesticides at the label dose, or at doses ranging from 0.5 to 3 times the label dose rate. The selected pesticides included the insect growth regulators methoxyfenozide and pyriproxyfen, and the acaricide bifenazate. We simulated field exposure of honey bees to these pesticides during aerial application in almond orchards by using a wind tunnel and atomizer set up with a wind speed of 2.9 m/s. Experimental groups consisting of 30–40 foragers each were exposed to either untreated controls or pesticide-laden treatments and were monitored every 24 hr over a 10-d period. Our results revealed a significant negative effect of all pesticides tested on forager survival. Therefore, we suggest increased caution in the application of these pesticides in almond orchards or any agricultural crop during bloom to avoid colony health problems.

Key words: almond, *Apis mellifera*, bifenazate, honey bee forager, insecticide, methoxyfenozide, pyriproxyfen

In the United States, honey bees (*Apis mellifera* L. (Hymenoptera: Apidae)) make the largest contribution to pollination services of agricultural crops, providing ~$17 billion to our economy annually (Calderone 2012). Almond (*Prunus dulcis*), which is among the major crop recipients of honey bee pollination services, depends completely on honey bees for successful nut set (Klein et al. 2012). The California almond industry produces ~80% of the world's almond supply (Klein et al. 2012), a feat accomplished through the use of about 60% (over 1.5 million) of all managed honey bee colonies in the country, which are transported to almond orchards during the crop's bloom in mid to late winter (Sumner and Boriss 2006). To avoid problems associated with pests and pathogens, almond orchards are frequently subjected to repeated chemical treatments during the bloom period (Bosch and Blas 1994).

While the use of insecticides has increased in parallel with greater agricultural production, only some pesticide classes (e.g., neonicotinoids) have been a focus of concern with respect to their impacts on bee health (Cresswell et al. 2011, Henry et al. 2012, Schneider et al. 2012, Goulson 2013), while others, including insect growth regulators (IGRs) and acaricides, have generally been overlooked. Despite this gap in knowledge, a few studies have been done on the effects of IGRs on honey bee health. For example, Chandel and Gupta (1992) found that exposure to the IGR diflubenzuron significantly increased mortality in early instar larvae and pupae, while exposure in the adult stage resulted in physical abnormalities. In a subsequent study, diflubenzuron exposure reduced worker weight and suppressed hypopharyngeal gland development (Gupta and Chandel 1995). Moreover, exposure to the IGR pyriproxyfen has been shown to negatively affect workers by inhibiting vitellogenin synthesis in the hemolymph

(Pinto et al. 2000). Similarly, topical exposure to pyriproxyfen was found to increase worker rejection by nestmates as well as morphological abnormalities, including misshapen wings (Fourrier et al. 2015).

Furthermore, although targeted toward non-insect arthropods, several acaricides are found frequently in hive products such as wax, honey, and pollen (Mullin et al. 2010), and thus, may constitute a significant threat to honey bee health. For instance, several acaricides including the pyrethroid fluvalinate and the organophosphate coumaphos have been shown to reduce sperm counts and sperm viability in honey bee queens (Rangel and Tarpy 2015, 2016). Likewise, the acaricides thymol, coumaphos, and formic acid negatively affect honey bee immune responses, including the expression of the c-Jun amino-terminal kinase pathway involved in detoxification (Boncristiani et al. 2012). Furthermore, coumaphos has been found to induce immunosuppression in workers by decreasing lysozyme expression (Garrido et al. 2016).

Foragers, the subset of a honey bee colony's worker force that is tasked with food collection (Huang and Robinson 1996, Abou-Shaara 2014), belong to the age cohort that is most susceptible to direct exposure to pesticides outside the hive (Pettis et al. 2013). Thus, foragers are particularly important in studies on the effects of pesticides used in agricultural crops on honey bee health. In this study, we exposed foragers to two IGRs and one acaricide widely applied during the almond bloom in California and assessed the effect of exposure to these pesticides on forager mortality over a 10-d period. Based on our results, we suggest that increased caution in the application of these pesticides in almond orchards or any agricultural crop during bloom should be encouraged in the industry's Best Management Practices efforts, as these products negatively affect honey bee forager longevity and thus, potentially impact overall colony health.

Materials and Methods

Pesticides Used

The pesticides selected for the study were the IGR methoxyfenozide (Intrepid: 22.6% methoxyfenozide; Dow AgroSciencies LLC, Indianapolis, IN), the IGR pyriproxyfen (Nyguard: 10% pyriproxyfen; McLoughlin Gormley King Company, Minneapolis, MN), and the acaricide bifenazate (Floramite: 22.6% bifenazate; MacDermid Agricultural Solutions, Waterbury, CT). These pesticides were chosen because they were widely applied during the almond bloom in California in 2014 (Summary of Pesticide Use Report Data; Table 1). All pesticides were applied individually at the manufacturer's recommended label dose or at different concentrations below and above the label dose rate (see below) following the experimental procedures outlined by Fisher et al. (2017). The label dose variants utilized ranged from half the label dose to three times the label dose rate. Dose variants above the label dose (i.e., 2×, 3×) represented scenarios where foragers could have been exposed repeatedly with the label dose (1×) application in different fields, or multiple times while performing the pollination services in the same field.

Experimental Treatment Groups

To assess the effects of the three pesticides on honey bee forager survival, five experimental groups were formulated for each pesticide tested, including a pesticide-free control group. The first set of trials included treatment with methoxyfenozide at differing concentrations derived from the recommended label dose compared with an untreated control group, which was tested in three separate trials conducted between March and May 2016 (Table 2). A second experimental trial set was conducted using pyriproxyfen at concentrations derived from the label dose compared with an untreated control group, which was used in three separate trials between January and March 2017 (Table 2). Finally, a third set of trials used the acaricide bifenazate at different concentrations derived from the recommended label dose compared with an untreated control group in three separate trials conducted between July and October 2016 (Table 2). For all trials, the control group consisted only of distilled water, which was the solvent used to dissolve the pesticides used in the treatment groups.

Forager Capture

Honey bee foragers were collected from a designated hive at the Janice and John G. Thomas Honey Bee Facility of Texas A&M University's RELLIS Campus in Bryan, TX. Foragers were captured by collecting bees from outer frames containing mostly food resources and no brood, since those frames are typically covered mostly by older adults, including foragers engaging in food collection and unloading tasks (Winston 1987). Bees were gently brushed off from the frames into bioassay cages composed of a circular cardboard frame of 15.2 cm in diameter, a single sheet of mesh fabric that was stretched over either side of the cage, and another thin cardboard holding ring slightly larger in diameter than the cage frame to secure the mesh in a taut position. Approximately 30–40 foragers were loaded into each bioassay cage and six bioassay cages were allocated to each experimental treatment group (Fig. 1a). Each bioassay cage was used only once and then discarded to avoid cross-contamination between trials.

Pesticide Exposure

The bioassay cages loaded with foragers were divided into experimental groups that were exposed to either pesticides at various concentrations or pesticide-free water, as described previously by Fisher et al. (2017). Foragers were exposed to the pesticides using a wind tunnel atomizer setup at the USDA-Agricultural Research Service Aerial Application Technology Laboratory in Bryan, TX. The wind tunnel-atomizer setup was designed and calibrated to accurately simulate the droplet size of aerial field applications for specific chemicals (Fritz et al. 2014). 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 speed of pesticides dispensed from an agricultural spraying aircraft. The bioassay cages were loaded one at a time onto a holding fork near the end of the wind tunnel chamber opposing

Table 1. Top insecticides used during the California almond bloom in 2014*

Active ingredient	Main insecticide group	Mode of Action	Number of lbs applied	Number of agricultural applications	Number of acres treated
Methoxyfenozide	Insect growth regulator	Ecdysone receptor agonist	160,411.22	7,330	559,294.06
Pyriproxyfen	Insect growth regulator	Juvenile hormone mimic	4,529.84	2,285	240,012.20
Bifenazate	Acaricide	Electron transport inhibitor	48,673.22	1,071	73,446.86

*Application and treatment data obtained from the California Department of Pesticide Regulation "Summary of Pesticide Use Report Data 2014."

the large fans (Fig. 1b). Each pesticide was diluted in distilled water separately and sprayed at increasing concentrations corresponding to the label dose or other label dose variants (i.e., 1/2×, 1×, 2×, and 3×; Table 2). Approximately 10 ml of each pesticide solution was loaded into the twin fluid atomizer using a 10-ml syringe located at the end of the wind tunnel chamber where the large fans were located. A compressed air tank was connected to the atomizer and activated along with the wind tunnel fans propelling the pesticide solution through the atomizer and down the wind tunnel chamber. Each application lasted ~5 s to ensure the complete expulsion of the pesticide solution from the atomizer and propulsion down the chamber to the bioassay cage on the opposing end. Following exposure, the bioassay cages were removed from the holding fork and the atomizer was cleansed with distilled water between applications of each experimental treatment. This process was repeated for all bioassay cages allocated to each treatment group. All spray applications of a given pesticide were done starting with the lowest dose and ending with the highest dose tested. For the control group, bioassay cages were loaded into the wind tunnel and were sprayed only with distilled water not exposed to pesticides.

Monitoring Forager Survival

Following the application of each pesticide treatment, 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). To load bees, a wide brimmed funnel was placed over a containment unit and one of the holding rings on a bioassay cage was removed to facilitate transfer. 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, which was then gently shaken and topped with a lid to prevent foragers from escaping. This process was repeated until all foragers in each bioassay cage were transferred to the corresponding containment units. Two 1.5-ml Eppendorf tubes were inserted into pre-made holes in the lid of each containment unit to serve as a feeder and a water dispenser. Feeder tubes were loaded with ~1 ml of 1.5 M sucrose solution and water-dispensing tubes were loaded with ~1 ml of water. The containment units were kept in an incubator at 34.5°C and ~75% relative humidity. The units were checked every 24 hr for 10 consecutive days, recording the number of dead foragers at each 24-hr interval. A forager was considered dead if she exhibited a complete lack of movement, which often entailed the forager lying on her side with her proboscis permanently extruded.

Statistical Analysis

Due to unequal variances in the data, nonparametric Wilcoxon tests were performed for each trial to compare average forager mortality over a 10-d period between the untreated control group and each pesticide treatment group. A MANOVA test was also performed to assess interaction effects between trial and treatment for each pesticide. Kaplan–Meier survival analyses were performed to compare the survival rate between the untreated control groups and the treatment groups in each set of trials for the three pesticides tested. The level of statistical significance was set at $\alpha = 0.05$ for all tests. All descriptive statistics are reported as mean ± SEM. Statistical tests were performed with the software JMP 12.0 (SAS Inc., Cary, NC).

Results

The data obtained from the three separate trials conducted for each pesticide tested were pooled together to assess the overall average forager mortality and survival rate for each pesticide. We found an overall significant increase in average forager mortality due to methoxyfenozide exposure ($\chi^2 = 13.44$, $P = 0.009$), and obtained an interaction effect of trial and treatment ($F = 9.69$, $P < 0.0001$). Foragers were specifically affected by methoxyfenozide exposure at three concentration variants of the label dose (Table 2; Fig. 2a); namely,

Table 2. Experimental treatment groups used to test the effects of exposure to the IGRs methoxyfenozide and pyriproxyfen and the acaricide bifenazate at concentration variants of the formulated label dose on honey bee forager survival

Experimental treatment group	Application dose relative to the label dose rate	Methoxyfenozide concentration (ml/liter H_2O)	Pyriproxyfen concentration (ml/liter H_2O)	Bifenazate concentration (ml/liter H_2O)
Control	0 (no pesticide added)	0	0	0
1	1/2× label dose	0.37	0.53	0.32
2	1× label dose	0.75	1.06	0.64
3	2× label dose	1.5	2.12	1.28
4	3× label dose	2.25	3.18	1.92

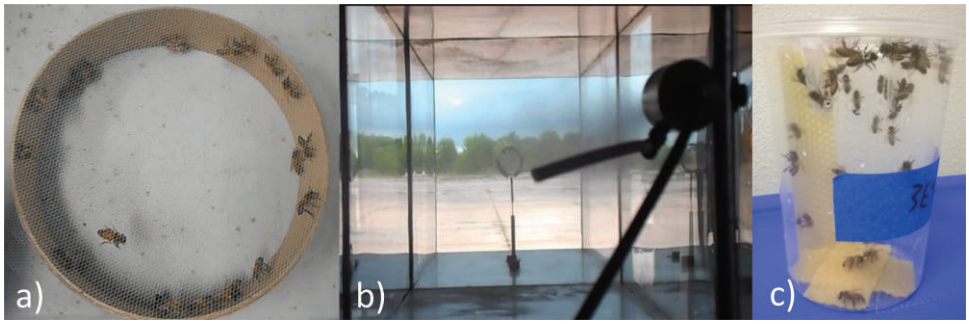

Fig. 1. Experimental set up used to test the effects of pesticide exposure on honey bee forager survival. First, (a) 30–40 bees were loaded into clean bioassay cages. Then, (b) the cages were placed in a wind tunnel and exposed to either a pesticide-free control or pesticide-laden atomized liquid treatment in increasing concentrations as shown in Tables 1 and 2. Once treated, the caged bees were transferred to (c) plastic holding units with feeders containing 50:50 sucrose solution and placed in an incubator held at 34°C to measure worker mortality every 24 hr for 10 d.

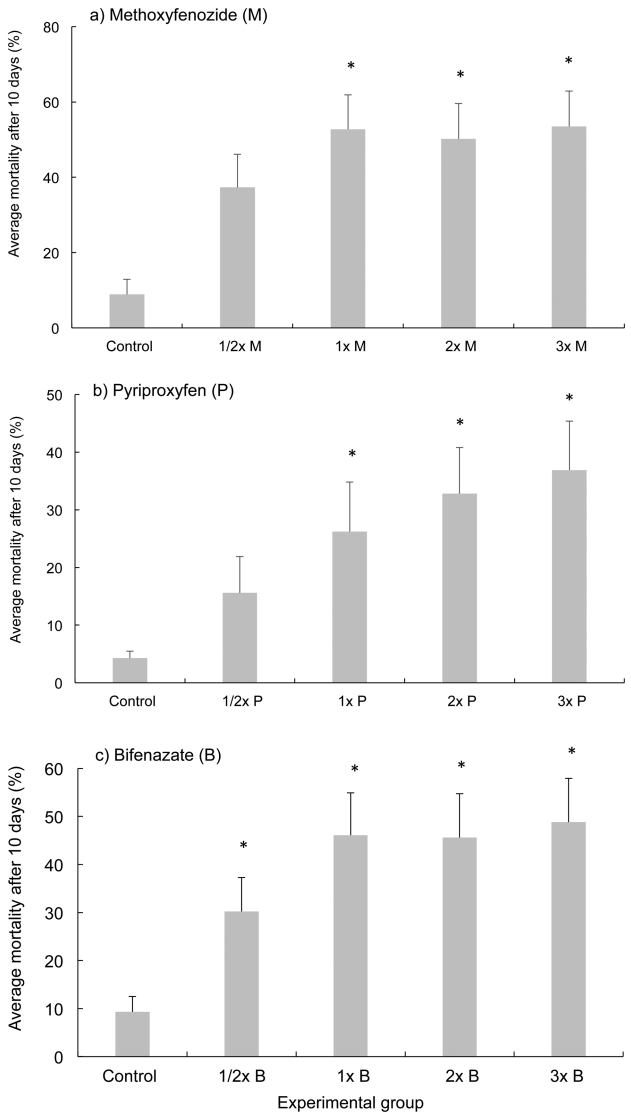

Fig. 2. Average honey bee forager mortality observed 10 d after bioassay cages containing 30–40 foragers were exposed in a wind tunnel to either (a) methoxyfenozide, (b) pyriproxyfen, (c) bifenazate or an untreated control group. The treatments included 1/2×, 1×, 2×, and 3× the label dose rate for each pesticide. *$P < 0.05$.

methoxyfenozide significantly increased average forager mortality at 1× the label dose ($Z = 2.86$, $P = 0.004$), at 2× the label dose ($Z = 3.36$, $P = 0.0008$), and at 3× the label dose ($Z = 2.61$, $P = 0.009$) compared with the control group.

Likewise, we observed an overall significant increase in average forager mortality from exposure to the IGR pyriproxyfen ($\chi^2 = 15.13$, $P = 0.004$) with two particular label dose variants significantly impacting average mortality (Fig. 2b); specifically, pyriproxyfen at 2× ($Z = 3.39$, $P = 0.0007$) and 3× the label dose ($Z = 3.08$, $P = 0.002$) significantly affected forager mortality compared with the untreated control group.

We also found an overall significant increase in average mortality of foragers exposed to the acaricide bifenazate ($\chi^2 = 18.27$, $P = 0.001$) and obtained an interaction effect of trial and treatment ($F = 2.82$, $P = 0.009$). Bifenazate significantly increased average forager mortality at 1/2× ($Z = 2.94$, $P = 0.003$), at 1× ($Z = 3.33$, $P = 0.0009$), at 2× ($Z = 3.22$, $P = 0.001$), and at 3× the label dose ($Z = 3.50$, $P = 0.0005$) compared with the untreated control group (Fig. 2c).

Similar to average mortality, foragers exposed to the concentration variants of each pesticide experienced a significantly lower survival rate compared with the untreated control groups. We found a significant overall decrease in forager survival due to methoxyfenozide exposure ($\chi^2 = 429.50$, $P < 0.0001$; Fig. 3a). When analyzed individually, each label dose variant of methoxyfenozide caused lower survival compared with the control group including 1/2× ($\chi^2 = 148.31$, $P < 0.0001$), 1× ($\chi^2 = 343.98$, $P < 0.0001$), 2× ($\chi^2 = 329.84$, $P < 0.0001$), and 3× the label dose ($\chi^2 = 398.48$, $P < 0.0001$). A similar outcome resulted from pyriproxyfen exposure, which induced a significant overall decrease in forager survival compared with the control group ($\chi^2 = 213.10$, $P < 0.0001$; Fig. 3b). When analyzed individually, a significant decrease in forager survival was found for pyriproxyfen at 1/2× ($\chi^2 = 31.62$, $P < 0.0001$), 1× ($\chi^2 = 93.03$, $P < 0.0001$), 2× ($\chi^2 = 173.30$, $P < 0.0001$), and 3× the label dose ($\chi^2 = 180.04$, $P < 0.0001$) compared to the control group. Finally, exposure to bifenazate had a similar negative overall effect of lowering forager mortality ($\chi^2 = 273.13$, $P < 0.0001$; Fig. 3c). Specifically, we found significantly lower forager survival from exposure to bifenazate at 1/2× ($\chi^2 = 69.54$, $P < 0.0001$), 1× ($\chi^2 = 174.17$, $P < 0.0001$), 2× ($\chi^2 = 192.28$, $P < 0.0001$), and 3× the label dose ($\chi^2 = 246.41$, $P < 0.0001$) compared with the control group.

Discussion

Our study shows that the IGRs methoxyfenozide and pyriproxyfen and the acaricide bifenazate, which were widely used during the 2014 almond bloom in California, cause significant negative effects to honey bee forager survival post exposure. All three pesticides caused significant increases in average forager mortality after 10 d, and Kaplan–Meier survival analysis revealed a significant decrease in forager survival over the 10-d period when foragers were exposed to various pesticide concentrations compared with untreated controls, with only slight variations in the magnitude of the effect between the pesticides used. Exposure to methoxyfenozide at all label dose variants (except for 1/2× the label dose) and to bifenazate at all label dose variants caused significantly higher average forager mortality compared with untreated controls. Conversely, only the dose variants of pyriproxyfen above the label dose affected average forager mortality.

Aside from pyrethroids, honey bees are generally considered to be highly susceptible to insecticides (Johnson et al. 2006). While cytochrome P450 enzymatic activity has been implicated in the detoxification of some insecticides, particularly neonicotinoids such as thiacloprid and acetamiprid (Iwasa et al. 2004), immunological pathways such as oxidative stress and antimicrobial peptide production appear to be the main targets of many insecticides, including neonicotinoids and IGRs (James and Xu 2012). Although the modes of action of pyriproxyfen, as well as methoxyfenozide and bifenazate on honey bee foragers are poorly understood, studies examining the effects of these pesticides in other arthropods may offer insight into how these chemicals may affect honey bee foragers. For instance, pyriproxyfen is a juvenile hormone analog that functions primarily in inhibiting adult emergence from developmental life stages (Ohba et al. 2013). It has been observed to induce sterility in adult females of the mosquito *Aedes albopictus* (Ohba et al. 2013). Additionally, contact exposure of pyriproxyfen by adults of the flea *Ctenocephalides felis* resulted in increased mortality, reduced fat bodies, and swollen midguts (Meola et al. 1993). In contrast, *C. felis* was not observed to experience significant mortality resulting from feeding bioassays containing pyriproxyfen (Meola et al. 2000). With regards to honey bees, only one study to date has shown that

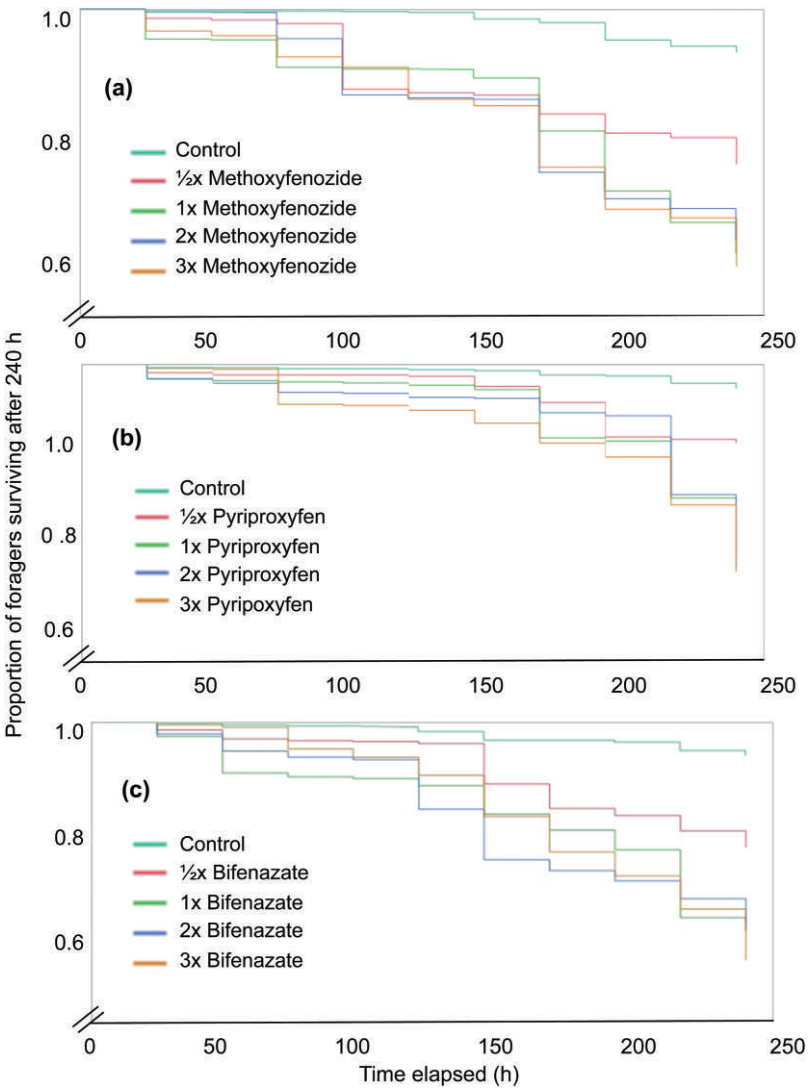

Fig. 3. Proportion of honey bee foragers contained in groups of 30–40 individuals that survived over a 240-h period in an incubator held at a constant temperature of 34°C, 24 hr after exposure in a wind tunnel to either (a) methoxyfenozide, (b) pyriproxyfen, (c) bifenazate or an untreated control group. See "Materials and Methods" for more details.

exposure to pyriproxyfen affects young honey bees by inhibiting vitellogenin synthesis (Pinto et al. 2000), but its effects on older foragers is yet unknown.

Methoxyfenozide is an ecdysone receptor agonist that induces molting abnormalities including peculiar cuticular deposition and early apolysis, fatally preventing larval transition to later life stages (Carlson et al. 2001). Similar to pyriproxyfen, methoxyfenozide has been observed to induce sublethal effects on adult insects, particularly in lepidopteran species, by lowering fertility and fecundity (Sun et al. 2000, Pineda et al. 2007, Ohba et al. 2013). While reproduction is not a task of honey bee workers, fat bodies are important for a variety of insect physiological functions including energy storage and metabolism (Arrese and Soulages 2010). Thus pyriproxyfen, and possibly methoxyfenozide, may specifically affect foragers by negatively impacting fat body reserves inducing energetic costs.

Of the three pesticides examined, bifenazate is least understood with regard to its mode of action. Van Nieuwenhuyse et al. (2012) reported inhibition of the mitochondrial complex III in the spider mite *Tetranychus urticae* after bifenazate exposure. Expectation of a similar effect on honey bees exposed to bifenazate may be tempered, however, by the apparent difference in insect and arachnid metabolism of bifenazate (Van Nieuwenhuyse et al. 2012). Thus, the detrimental effect of exposure we observed in honey bee foragers may potentially be attributable to a different impact on mitochondria functionality or other cellular processes.

Although pesticides are often applied at levels below acute mortality for honey bees, our study suggests that honey bee forager survival is significantly reduced even at levels below the manufacturer's recommended application dose rates. Fisher et al. (2017) recently conducted a similar study looking at the effects to forager survival of fungicides commonly used in California for the protection of almond orchards. In their study, the authors tested the effects of dose variants of iprodione ranging from 25% of the label dose rate to two times the label dose rate, as well as iprodione in combination with a formulation of boscalid and pyraclostrobin (Pristine) or azoxystrobin (Quadris) at the label dose, on honey bee forager mortality over a 10-d period. They found that iprodione at all concentration variants and in combination with other fungicides negatively affected forager survival, particularly during trials conducted in fall and winter (Fisher et al. 2017). As observed in our examination of

methoxyfenozide and bifenazate, the effect of iprodione and iprodione combinations found in that study were increasingly pronounced over time, verifying a potential increase in seasonal susceptibility of foragers to pesticide exposure.

We also found an interaction effect between trial and treatment for methoxyfenozide and bifenazate. The intensity of the impact of exposure increased progressively over time across trials for both of these pesticides, suggesting a potential seasonal effect on forager survival that should be considered when exposing honey bees to these chemicals. The trials for methoxyfenozide were conducted from March to May, while those for bifenazate were conducted from July to October. Thus, combined, the trials encompassed a period of time that spanned from spring to summer, or from summer to fall. The progressively more pronounced negative effects of bifenazate exposure on forager survival over time may have occurred because honey bee physiology changes between seasons, particularly in the transition to winter physiology, which entails reduced expression of genes involved in pathogenic resistance (Steinmann et al. 2015).

Although we found that pyrirpxifen, methoxyfenozide, and bifenazate negatively impact forager survival at field-relevant concentrations, the did not cause the entire forager population in each confinement unit to die. This may suggest intracolonial differences in susceptibility to these chemicals, which is a potential route for future studies. In addition, a study of the potential routes of transmission of pesticides to larvae, pupae, and adults may provide further insight into how each route of pesticide exposure might affect colony health. As more studies are published on the sub-lethal effects of pesticides on honey bee health (Colin et al. 2004, Henry et al. 2012, Schneider et al. 2012, Fourrier et al. 2015, Garrido et al. 2016), a more careful consideration of the importance of integrated pollinator and pest management needs to be addressed. For instance, measures reducing pesticide exposure to honey bees during almond bloom are being more thoroughly described by the Almond Board of California's Best Management Practices (http://www.almonds.com/pollination#BeeBMPs), including recommendations for pesticide application during alternative hours coinciding with low honey bee forager activity (i.e., between dusk and dawn) to aid in reducing the impact of pesticides on honey bee health. Avoidance strategies may present the best option for defending crops while minimizing pollinator loss, given that our study indicates that a lack of immediate mortality may not indicate a lack of long-term health risks for honey bees.

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