Sublethal Effects of Pesticides on Queen-Rearing Success

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Abstract

The effects of sublethal pesticide exposure on queen emergence and immunity were measured. Queen-rearing colonies were fed pollen with chlorpyrifos (CPF) alone (pollen-1) and with CPF and the fungicide Pristine® (pollen-2). Fewer queens emerged when larvae were reared in colonies fed pollen-1 or pollen-2 than when larvae were reared in outside colonies without contaminated pollen. Larvae grafted from and reared in colonies fed pollen-2 had the lowest rate of queen emergence. Deformed wing virus (DWV) and black queen cell virus were found in nurse bees from colonies fed pollen-1 or pollen-2 and in outside colonies. The viruses also were detected in queen larvae. However, we did not detect virus in emerged queens grafted from and reared in outside colonies. In contrast, DWV was found in all emerged queens grafted from colonies fed pollen-1 or pollen-2 and reared either in outside hives or those fed pollen-1 or pollen-2. The results suggest that sublethal exposure of CPF alone but especially when Pristine® is added reduces queen emergence possibly due to compromised immunity in developing queens.

There are many reasons for colony losses: disease, Varroa, poor nutrition, and lethal exposure to pesticides. In recent years, one of the more common causes of colony losses is queen failure. Queens are being replaced at unprecedented rates in managed colonies. In some cases, when queens are lost, the colony is unable to rear a

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new queen to emergence. If the beekeeper does not introduce a new queen, the colony will die.

Though queenless colonies might not be able to replace their queen, a new queen can be purchased and introduced in the colony. However, queen breeders in California reported that they were unable to rear queens when they fed Queen-rearing colonies pollen collected from almond orchards. Sublethal levels of pesticides and fungicides that are often present in almond pollen were suspected of being a contributing cause.

To determine whether sublethal exposure to pesticides affected Queen-rearing, two of the most common contaminants in pollen were tested for their effects on queen emergence. The compounds were chlorpyrifos (CPF) often applied as Dursban® or Lorsban® and the fungicide Pristine® (PRS), that is, a combination of Boscalid and Pyraclostrobin (Mullin et al. 2010). CPF is applied before bloom in fruit and nut orchards to control scale insects. PRS can be applied during bloom to stone fruit (e.g., almonds, cherries, apricots, plums, and peaches) and pome fruit trees (apples and pears) to prevent brown rot, blossom blight, powdery mildew, scab, leaf spot, and shot hole, and to strawberries to prevent Botrytis gray mold, leaf spot, and powdery mildew (http://agproducts.basf.us/products/pristine-fungicide.html).

CPF is an organophosphate (OP) insecticide that affects the nervous system by inhibiting neurotransmitters (Pope 1999). However, there is increasing evidence that OPs have other biological effects (Duysen et al. 2001; Pettis et al. 2012, 2013). For example, OPs may disrupt metabolism (Adigun et al. 2010) and alter immune function by oxidative stress and subsequent tissue damage or stress-related immunosuppression (Li 2007).

Unlike insecticides that might target neural function, fungicides can affect basic cellular processes such as nucleic acid and protein synthesis, the structure and function of cell membranes, mitosis and cell division (Yang et al. 2011). The fungicide used in this study (PRS) affects the production of ATP, a molecule that supplies large amounts of energy for biochemical processes in cells. ATP is produced in specialized cellular structures called mitochondria. Fungicides that compromise ATP production in fungi can also reduce ATP levels in non-target organisms that synthesize this molecule in their mitochondria. Recently, PRS was reported to lower ATP levels in honey bees and reduce protein digestion (DeGrandi-Hoffman et al. 2015). There is increasing evidence that compounds that reduce ATP production also affect immune responses because these require the energy that ATP provides (Arnoult et al. 2009; Wu et al. 2012).

If sublethal exposure to pesticides affects immunity among immature and adult bees, colony losses attributed to viruses or other pathogens or parasites actually could be downstream effects of pesticides. High pathogen titers might also affect the ability of worker bees to rear new queens. In our study, we examined the effects that feeding colonies pollen contaminated with CPF alone and with added PRS might have on rearing queens to emergence. Virus titers in the colonies also were measured to determine whether consuming pollen with these pesticides was affecting immunity.

1 Experimental Procedures and Findings

To provide a greater understanding of how the effects of fungicides on queen development were determined, a brief description of the methods used in this study is provided. For a full description of all methods, see DeGrandi-Hoffman et al. (2013). Pollen was collected using pollen traps on hives placed in almond orchards for pollination. A pesticide analysis revealed the presence of CPF in the pollen.

The pollen collected in the traps was ground to a fine powder and spread on large aluminum trays. We applied PRS to half of the ground pollen at a rate of 3000 ppb of active ingredients (boscalid = 1966 ppb and pyraclostrobin = 998 ppb) using a hand sprayer (Fig. 1). The remaining half of the pollen was sprayed only with distilled water. By treating the pollen this way, we could feed the same source pollen to colonies and compare the effects of CPF alone (pollen-1) and with added PRS (pollen-2) on Queen-rearing success. We also measured the effects on immunity by measuring virus titers in the queens and the nurse bees that reared them.

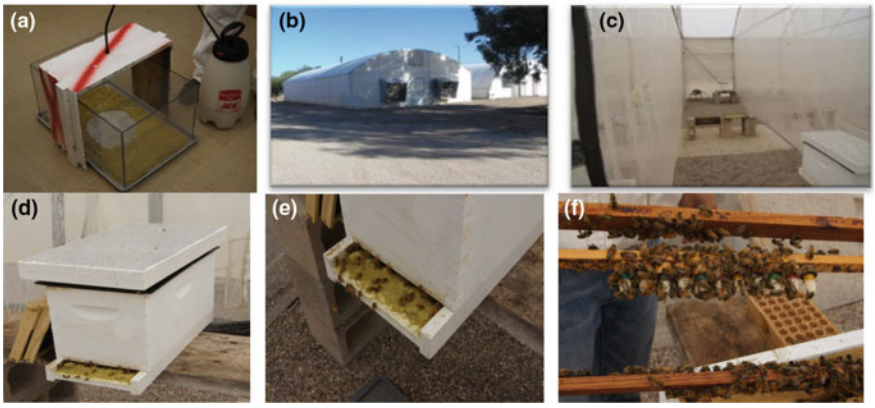

Fig. 1 **a** Applying either distilled water (pollen-1) or fungicide (pollen-2) to almond pollen contaminated with chlorpyrifos. **b** Colonies were placed in an enclosed flight area that has 10 separate sections that are 1.93 m wide, 8.25 m long, and 4.14 m high. **c** The sections are separated by cloth mesh and bees cannot fly between the sections. **d** The pollen-1 or pollen-2 was placed at the entrances of colonies in the enclosed flight area. **e** The bees collected the pollen, and **f** used it to rear queens we grafted with larvae from outside free foraging colonies or in colonies fed pollen-1 or pollen-2

2 Effects of Ingesting Contaminated Pollen on Queen-Rearing Success

All experiments were conducted at the USDA-ARS Carl Hayden Bee Research Center, Tucson, AZ. Colonies were comprised of Italian bees (*Apis mellifera ligustica*) and headed by commercially produced and mated European queens (Koehnen and Sons Inc., Glenn, CA, USA). The hives were established in an enclosed flight area in order to control their food source (Fig. 1). These hives were considered as 'enclosed colonies.' All colonies were comprised of about 3000 adult bees and a laying queen. The hives contained frames with foundation, and bees were fed sugar syrup to draw comb. When we saw larvae in the drawn comb, we began feeding the ground pollen to the colonies. There was no pollen in the colonies prior to our pollen feeding. Each colony was fed either pollen-1 or pollen-2. The ground pollen was placed at the hive entrance daily (Fig. 1), and the bees readily collected and stored it. Four colonies were provided with pollen-1 and five with pollen-2. After 4 weeks of feeding on the pollen, we began Queen-rearing experiments in these hives. This procedure insured that the queen cells evaluated in the study were tended by nurse bees reared entirely on the pollen we fed.

We established a second set of 5-frame nucleus hives in an apiary adjacent to the Bee Center. The colonies contained 3000–4000 bees with 2–3 frames of brood. The bees open foraged on native desert vegetation. These colonies are referred to as 'outside hives.'

Larvae (<36 h old) were grafted from worker cells into queen cups and reared into queens using the procedures described in Laidlaw (1979). Colonies used for Queen-rearing were made queenless for 24 h before queen cups containing larvae were introduced. Ten queen cups with larvae were placed in the center of each colony, and combs with bees and brood were placed on either side. The cells remained in the colony until queens were within 48 h of emergence. At that time, the cells were removed and placed in individual sterile vials in an environmental room with a temperature of 32–34°C (approximately 89–92°F) and 50% humidity. Each vial had a small piece of queen candy (a mixture of powdered sugar, honey, and water formed into a paste) for the emerged queen to feed on while in the vial. The queen cells were checked daily for emerged queens.

In the first experiment, larvae were grafted from the outside hives and placed in either enclosed colonies fed pollen-1 or pollen-2 or in different outside hives from those where they were grafted (Fig. 2). In Experiment 2, larvae were grafted into queen cups from the enclosed hives fed either pollen-1 or pollen-2. The queen cups were placed either back in the same enclosed colony from which they were grafted or in outside colonies (Fig. 3). The outside colonies were different from those used in the first experiment.

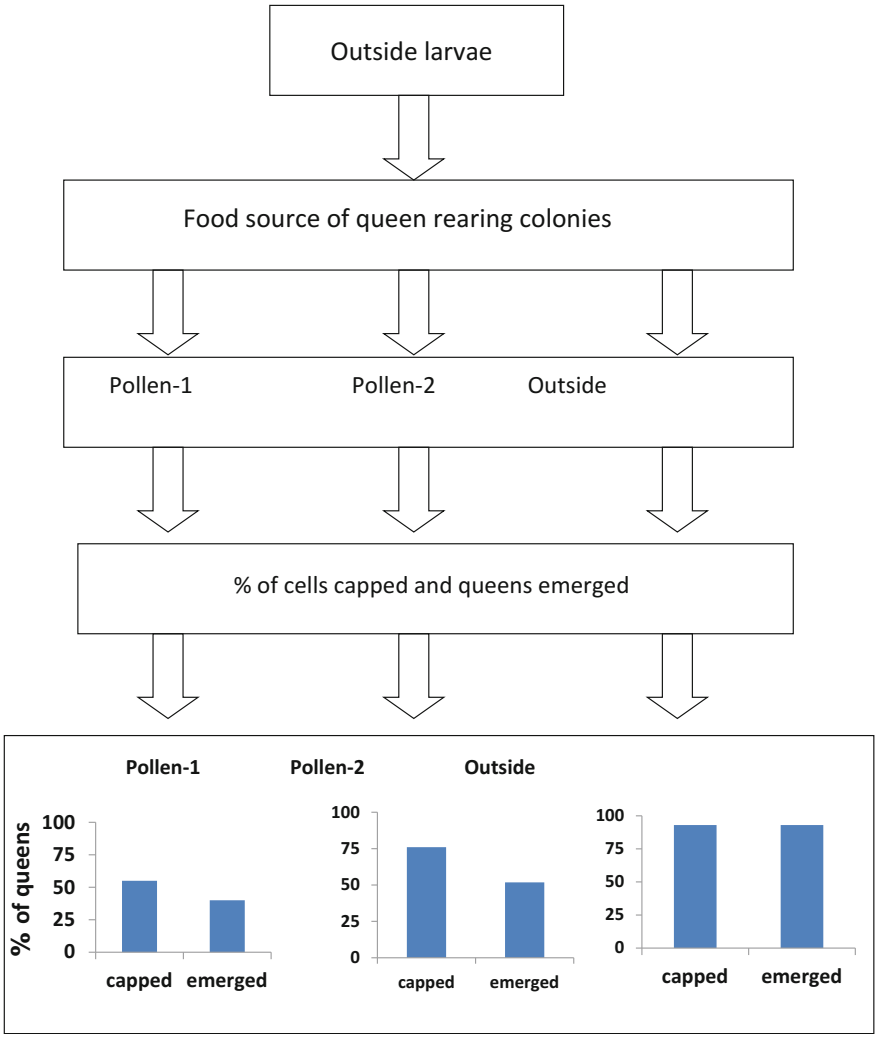

Fig. 2 Percentages of queen cells that were capped and queens that emerged when larvae from open foraging colonies outside an enclosed flight area were reared as queens in colonies fed pollen with chlorpyrifos alone (Pollen-1) or with added Pristine® fungicide (Pollen-2). Based on Chi-square (X^2) tests, significantly more queen cells were capped and had emerging queens when reared in colonies foraging on outside pollen compared with pollen-1 (55% survival to capped stage; $X^2 = 12.3$, $p < 0.0001$, and 40% emergence; $X^2 = 21.0$, $p = 0.0001$) or pollen-2 (76% survival to capped stage $X^2 = 4.03$, $p = 0.046$, and 51.8% emergence; $X^2 = 13.1$, $p < 0.0001$). More larvae survived to the capped brood stage in colonies fed pollen-2 than pollen-1, but the percentage of queens that emerged did not differ (pollen-1 = 40%, pollen-2 = 51.8%; $X^2 = 1.29$, $p = 0.255$)

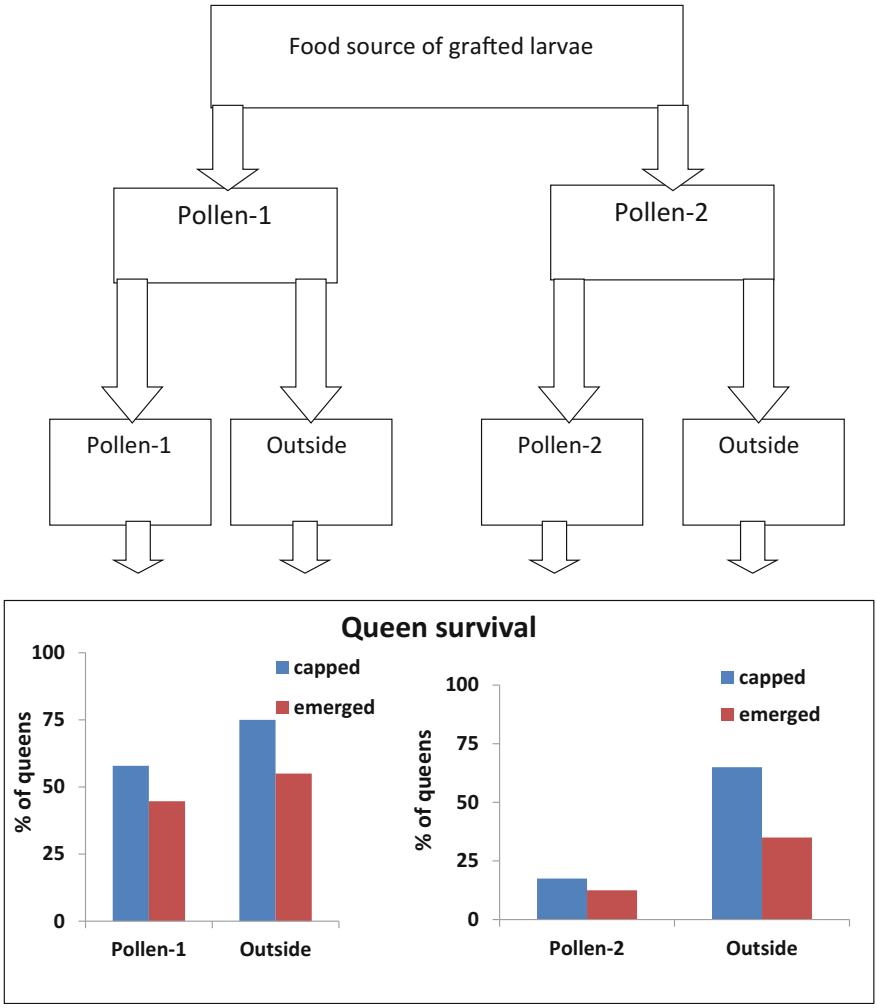

Fig. 3 Percentage of queen cells that were capped and had emerged queens when larvae were grafted from colonies in an enclosed flight area (EFA) that were fed pollen with chlorpyrifos alone (Pollen-1), added Pristine® fungicide (Pollen-2), or were in open foraging colonies outside the EFA (outside). Based on Chi-square (X^2) tests, percentages of larvae grafted from colonies fed pollen-1 that survived to the capped stage did not differ between those reared in colonies fed pollen-1 (57.9%) or in outside colonies (75%) ($X^2 = 1.65$, $p = 0.198$). The percent that emerged also did not differ ($X^2 = 0.55$, $p = 0.457$). Significantly, fewer larvae from colonies fed pollen-2 and reared in those colonies survived to the capped stage ($X^2 = 13.54$, $p < 0.0001$) and emerged as queens compared with those reared in outside colonies ($X^2 = 4.21$, $p = 0.04$). The percentage of larvae grafted from and reared in colonies fed pollen-1 that survived to the capped stage or emerged as queens was significantly higher than when larvae were grafted from and reared in colonies fed pollen-2 (capped stage: $X^2 = 13.6$, $p < 0.0001$, emerged queens: $X^2 = 10.0$, $p = 0.002$)

When larvae were grafted from and reared in outside colonies (Experiment 1), 93% emerged as queens (Fig. 2). This was a significantly higher emergence success than when the larvae were reared in colonies fed either pollen-1 or pollen-2. Only about 40% of the larvae emerged as queens in colonies fed pollen-1, and about 50% emerged in colonies fed pollen-2. In Experiment 2, the fewest larvae emerged as queens when they were grafted from and reared in colonies fed pollen-2 (Fig. 3). Less than 25% of the queen cells survived to the capped stage and even fewer emerged. Larvae grafted from colonies fed pollen-2 and reared in outside colonies also had relatively low queen emergence.

When larvae were grafted from colonies fed pollen-1, the percentages that survived to the capped stage were similar between those reared in colonies fed pollen-1 (57.9%) and in outside colonies (75%). The percentages of larvae grafted from and reared in colonies fed pollen-1 that survived to the capped brood stage or emerged as queens were significantly higher than when larvae were grafted from and reared in colonies fed pollen-2. When queen cells that did not emerge in both Experiments 1 and 2 were opened, we found that either the larvae did not successfully pupate and were a black viscous mass in the cell or were fully formed dark black pupa but were dead in the cells (Fig. 4).

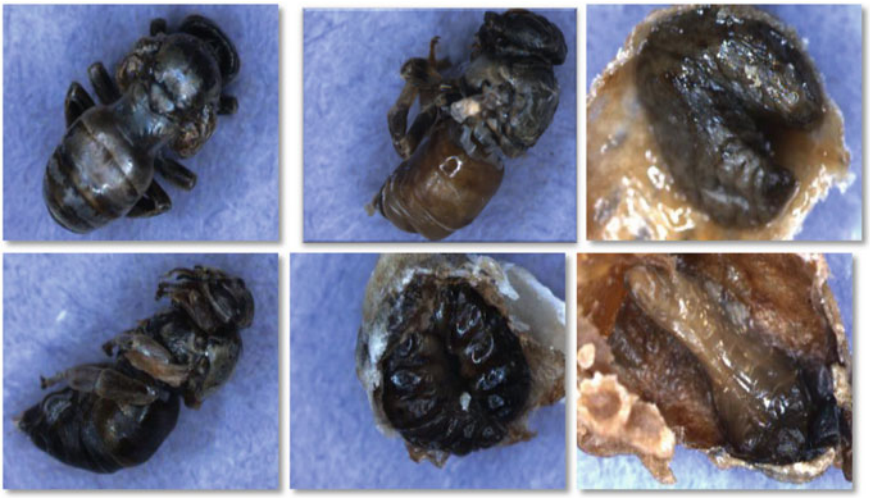

Fig. 4 Examples of queens that failed to emerge when reared in colonies fed almond pollen with either chlorpyrifos alone or with chlorpyrifos and the fungicide Pristine®. Queens that died prior to pupation or were fully formed and pigmented but did not emerge occurred in colonies fed either type of pollen

3 The Effects of Ingesting Contaminated Pollen on Virus Levels

To determine whether ingesting contaminated pollen affects immunity in developing queens, virus titers were measured in: (1) nurse bees tending queen cells, (2) queen larvae developing in the cells, and (3) emerged queens. The samples for virus analyses were collected from colonies in Experiments 1 and 2.

RNA extraction techniques (Chen et al. 2005) were used to test for the presence and relative quantity of 7 common bee viruses including *acute bee paralysis virus* (ABPV), *black queen cell virus* (BQCV), *chronic bee paralysis virus* (CBPV), *deformed wing virus* (DWV), *Kashmir bee virus* (KBV), *Israeli acute paralysis virus* (IAPV), and *Sacbrood virus* (SBV). We consistently found only *deformed wing virus* (DWV) and *Black Queen Cell Virus* (BQCV) in the samples. Detection of the virus does not indicate that the adult bee or larvae showed symptoms of viral disease.

In Experiment 1, DWV was detected in all nurse bees tending the queen cells in both outside colonies and in the enclosed colonies fed pollen-1 or pollen-2 (Fig. 5). About 70% of the queen larvae reared in outside colonies and all of those reared in enclosed colonies had DWV. We did not detect DWV in emerged queens reared in outside colonies. However, about 30% of the emerged queens grafted from larvae in colonies fed pollen-1 and 75% of those from colonies fed pollen-2 had DWV.

BQCV was found more frequently in nurse bees from colonies fed pollen-2 than in nurses from outside colonies or those fed pollen-1. The virus was not detected in queen larvae reared in colonies fed pollen-1 or pollen-2 or in emerged queens. These results might have occurred because those with BQCV died shortly after being grafted and were removed by the bees before we sampled them.

In Experiment 2, we detected DWV in all nurse bees, queen larvae, and virgin queens in both outside and enclosed colonies. BQCV was detected in 83% of the nurse bees in outside colonies and in all of those in the enclosed colonies. More than half of the queen larvae reared in outside colonies and all of those reared in the enclosed colonies had BQCV. All virgin queens grafted from enclosed colonies fed pollen-1 and half of those grafted from colonies fed pollen-2 and reared in outside colonies had BQCV. The virus also was detected in 67% of the virgin queens grafted from and reared in enclosed colonies fed pollen-1 and 33% of those fed pollen-2.

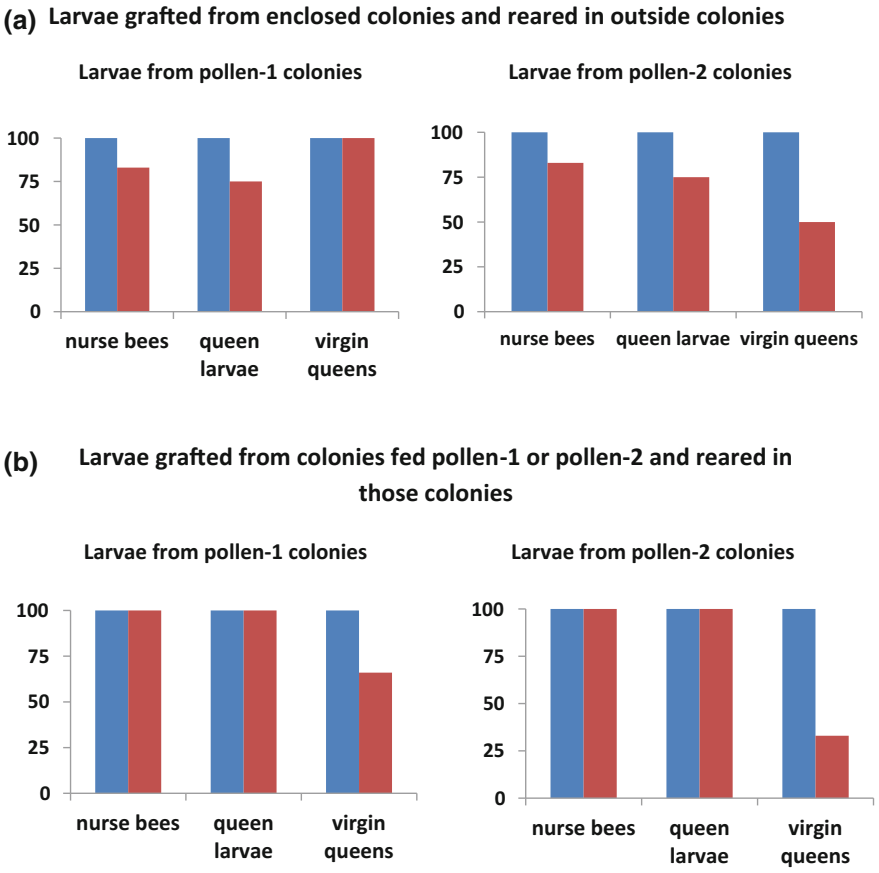

Fig. 5 Percentage of samples testing positive for deformed wing virus ( ) and black queen cell virus ( ) when larvae were grafted from outside colonies and reared into queens in hives fed almond pollen with chlorpyrifos (pollen 1) or with chlorpyrifos + Pristine® fungicide (pollen 2) (**a**), or were grafted from and reared in colonies fed either pollen-1 or pollen-2 (**b**)

4 Conclusions

Larvae either grafted from or reared as queens in colonies fed pollen contaminated with chlorpyrifos alone or with the fungicide Pristine® were less likely to emerge and more likely to test positive for both DWV and BQCV than those grafted from and reared in colonies without contaminated pollen. Though chlorpyrifos alone appears to reduce queen emergence, the reductions were greater when the fungicide also was present.

The differences in queen emergence rates between larvae reared in outside colonies and enclosed colonies fed contaminated pollen indicate that both the larvae and the Queen-rearing environment were affected by the pesticides. The highest queen emergence occurred when larvae were grafted from and reared in the outside colonies. We did not detect virus in these queens. However, when larvae from the outside colonies were reared in hives fed either pollen-1 or pollen-2, only about 50% of them emerged and DWV was detected in all of them. The difference in queen emergence and virus titers between rearing environments might be partially attributed to greater stress experienced by enclosed colonies compared with those outside where bees were open foraging. Though bees foraged in the enclosed area, the confinement might have produced stress that was not experienced by the outside colonies. Still, the pesticides in the pollen seemed to have an effect on Queen-rearing that was greater than differences in the location of the Queen-rearing colonies. For instance, when larvae were grafted from enclosed colonies and reared in the outside colonies, they had lower emergence rates and tested positive for virus with greater frequency than when larvae were grafted from and reared in outside colonies. Though the larvae selected for grafting were less than 36 h old, the effects of the pesticides were already present.

The findings from this study suggest that there could be severe reductions in queen cell capping and emergence if colonies contain pollen contaminated with pesticides. Colonies that have lost their queen or that are established from queenless splits should be checked frequently to be sure that a new queen is reared to emergence. If beekeepers see cells that were started and then torn down or are sealed but queens do not emerge, the pollen might be too contaminated for the bees to successfully rear queens. Similarly, queen producers should not use pollen in their Queen-rearing colonies that might be contaminated with pesticides and fungicides even if the worker bees seem to be unaffected. Beekeepers that rear queens in colonies with contaminated pollen should be aware that the queens that do emerge might have DWV and thus extend the impact of pollen contamination into the colonies headed by those queens. Though the effects of DWV on the queens themselves seem to be minimal, there is an association between DWV infection in the ovaries and the degeneration of individual follicles (Gauthier et al. 2011). More importantly, DWV is transmitted vertically from queens to their offspring (Chen et al. 2005; Yue et al. 2006; DeMiranda and Fries 2008) resulting in a persistent latent infection circulating in the colony population. Under appropriate environmental or biological stressors such as Varroa mites, the viruses become activated and cause various pathologies in the hosts. These include behavioral deficiencies (Iqbal and Mueller 2007), wing deformity, and significantly reduced life expectancy (DeMiranda and Genersch 2010). There also is a strong association between DWV in worker bees and colony mortality over winter (Gauthier et al. 2011; Highland et al. 2009; Berthoud et al. 2010; Genersch et al. 2010; DiPrisco et al. 2011). Thus, the sublethal effects of pesticides might be contributing to the pervasive presence of DWV in managed colonies and to colony losses when the viruses are activated by stress factors such as Varroa and the miticide treatments used to control this parasite (Locke et al. 2012).

Though CPF alone affected queen emergence rates and virus titers, the impact was greater when combined with PRS. The combination of a neurotoxin (CPF) and an inhibitor of mitochondrial function (PRS) might have caused the care of the queen cells by nurse bees to be compromised so that the queen larvae were nutritionally stressed. This alone could have reduced the number of capped cells and emerged queens. In addition, CPF and PRS also could have been affecting innate immunity in the nurse bees so that they were transmitting higher virus titers to the larvae. Indeed, there was higher mortality prior to the capped cell stage when larvae were grafted from and reared in colonies containing pollen contaminated with CPF and PRS. Thus, the effects of CPF that is a common contaminant of pollen might be amplified if it is present when PRS is applied to crops in bloom.

Large pollen stores collected from orchards are tempting to use for Queen-rearing colonies or for queenless splits. However, the pollen might be contaminated with pesticides particularly fungicides applied during bloom. To be sure that queens can be reared using the pollen source, beekeepers should graft just a few bars of queen cells to determine whether the queens emerge. If they do, the pollen probably is fine to use for Queen-rearing. However, if very few queen cells are sealed or emerge, the pollen might contain levels of pesticides that are affecting queen development. Determining the effects of the pollen source on queen emergence on a small scale by grafting a few bars could save time in the end if few queens emerge.

References

Adigun AA, Seidler FJ, Slotkin TA (2010) Disparate developmental neurotoxicants converge on the cyclic AMP signaling cascade revealed by transcriptional profiles *in vitro* and *in vivo*. Brain Res 1316:1–16

Arnoult D, Carneiro L, Tattoli I, Girardin SE (2009) The role of mitochondria in cellular defense against microbial infection. Semin Immunol 21:223–232

Berthoud H, Imdorf A, Haueter M, Radloff S, Neumann P (2010) Virus infections and winter losses of honey bee colonies (*Apis mellifera*). J Apic Res 49:60–65

Chen YP, Pettis JS, Feldlaufer MF (2005) Detection of multiple viruses in queens of the honey bee, *Apis mellifera* L. J Invert Pathol 90:118–121

De Miranda JR, Fries I (2008) Venereal and vertical transmission of deformed wing virus in honeybees (*Apis mellifera* L.). J Invertebr Pathol 98:184–189

De Miranda JR, Genersch E (2010) Deformed wing virus. J Invertebr Pathol 103:48–61

DeGrandi-Hoffman G, Chen Y, Simonds R (2013) The effects of pesticides on queen rearing and virus titers in honey bees (*Apis mellifera* L.). Insects 4:71–89, doi:10.3390/insects4010071

DeGrandi-Hoffman G, Chen Y, Watkins deJong E et al (2015) Effects of oral exposure to fungicides on honey bee nutrition and virus levels. J Econ Entomol 108:2518–2528, doi:10.1093/jee/tov251

DiPrisco G, Zhang X, Pennacchio F et al (2011) Viral dynamics of persistent and acute virus infections in honey bee. Viruses 3:2425–2441

Duysen EG, Li B, Xie W, Schopfer LM, Anderson RS, Broomfield CA, Lockridge O (2001) Evidence for nonacetylcholinesterase targets of organophosphorus nerve agent: Supersensitivity of acetylcholinesterase knockout mouse to VX lethality. J Pharmacol Exp Ther 299: 528–535

Gauthier L, Ravallec M, Tournaire M, Cousserans F, Bergoin M, Dainat B, deMiranda JR (2011) Viruses associated with ovarian degeneration in *Apis mellifera* L. queens. PLoS ONE 6:e16217

Genersch E, von der Ohe W, Kaatz H et al (2010) The German bee monitoring project: A long term study to understand periodically high winter losses of honey bee colonies. Apidologie 41:332–352

Highland AC, El Nagar A, Mackinder LCM et al (2009) Deformed wing virus implicated in overwintering honeybee colony losses. Appl Environ Microbiol 75:7212–7220

Iqbal J, Mueller U (2007) Virus infection causes specific learning deficits in honeybee foragers. Proc Roy Soc Lond B Biol Sci 274:1517–1521

Laidlaw HH (1979) Contemporary queen rearing. Dadant & Sons, Hamilton, IL, USA

Li Q (2007) New mechanism of organophosphorous pesticide-induced immunotoxicity. J Nippon Med Sch 74:92–105

Locke B, Forsgren E, Fries I, deMiranda JR (2012) Acaricide treatment affects viral dynamics in *Varroa destructor*-infested honey bee colonies via both host physiology and mite control. Appl Environ Microbiol 78:227–235

Mullin CA, Frazier M, Frazier JL, Ashcraft S, Simonds R, vanEngelsdorp D, Pettis JS (2010) High levels of miticides and agrochemicals in North American apiaries: Implications for honey bee health. PLoS ONE 5:e975

Pettis JS, vanEngelsdorp D, Johnson J, Dively G (2012) Pesticide exposure in honey bees results in increased levels of the gut pathogen Nosema. Naturwissenschaften 99:153–158

Pettis JS, Lichtenberg EM, Andree M, Stitzinger J, Rose R, vanEngelsdorp D (2013) Crop pollination exposes honey bees to pesticides which alters their susceptibility to the gut pathogen *Nosema ceranae*. PLoS ONE 8(7):e70182. doi:10.1371/journal.pone.0070182

Pope CN (1999) Organophosphorus pesticides: they all have the same mechanism of toxicity? J Toxicol Environ Health Part B Crit Rev 2:161–181

Wu JY, Smart MD, Anelli CM, Sheppard WS (2012) Honey bees (*Apis mellifera*) reared in brood combs containing high levels of pesticide residues exhibit increased susceptibility to Nosema (Microsporidia) infection. J Invertebr Pathol 109:326–329

Yang C, Hamel C, Vujanovic V, Gan Y (2011) Fungicide: modes of action and possible impact on nontarget microorganisms. ISRN Ecol. doi:10.5402/2011/130289

Yue C, Schroder M, Bienefeld K, Genersch E (2006) Detection of viral sequences in semen of honeybees (*Apis mellifera*): evidence for vertical transmission of viruses through drones. J Invertebr Pathol 92:105–108

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