Video Article

Evaluating the Effect of Environmental Chemicals on Honey Bee Development from the Individual to Colony Level

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Abstract

The presence of pesticides in the beekeeping environment is one of the most serious problems that impacts the life of a honey bee. Pesticides can be brought back to the beehive after the bees have foraged on flowers that have been sprayed with pesticides. Pesticide contaminated food can be exchanged between workers which then feed larvae and therefore can potentially affect the development of honey bees. Thus, residual pesticides in the environment can become a chronic damaging factor to honey bee populations and gradually lead to colony collapse. In the presented protocol, honey bee feeding methods are described and applied to either an individual honey bee or to a colony. Here, the insect growth regulator (IGR) pyriproxyfen (PPN), which is widely used to control pest insects and is harmful to the development of honey bee larvae and pupae, is used as the pesticide. The presenting procedure can be applied to other potentially harmful chemicals or honeybee pathogens for further studies.

Video Link

The video component of this article can be found at https://www.jove.com/video/55296/

Introduction

The presence of pesticides in the environment is one of the most serious problems that impacts the life of a honey bee[1,2,3]. Several studies have demonstrated the common presence of pesticide residues in honey bee colonies and bee products. In Taiwan, the average application of pesticides was 11-12 kg/ha every year (from 2005 to 2013). The amount of pesticides used in Taiwan is higher than that of the EU countries, and the Latin American countries[4,5]. In other words, the apicultural environment is suffering serious pesticide stress, especially in Taiwan and possibly in other countries.

The honey bee *Apis mellifera* is one of the major pollinators in agricultural systems[6] and it also produces valuable products such as honey. However, honey bees are exposed to various pesticides and these pesticides can be brought back to beehives after foraging on flowers that have been sprayed with pesticides when collecting nectar and pollen[7,8]. They also can be exposed to pesticides by the beekeepers themselves aiming to control pest problems inside the hives[9,10,11]. Because honey bee larvae are fed by nurse bees for their development, larvae, drones and even the queen may be exposed to these pesticide-contaminated nectars and pollen[12]. The toxicity of various pesticides to honey bees needs to be addressed[13].

Many efforts have been made to evaluate the issues of environmental pesticide residues. Yang *et al.* tested the influence of the neurotoxic insecticide imidacloprid on the development of honey bee larvae in the beehive and reported that a sub-lethal dose of imidacloprid resulted in olfactory associative behavior of the adult bees[14]. Also, Urlacher *et al.* examined the sub-lethal effects of an organophosphate pesticide, chlorpyrifos, on a honey bee worker's learning performance under laboratory conditions[15]. In our previous study, we evaluated the impact of an insect growth regulator (IGR), pyriproxyfen (PPN), on larval honey bees[16].

In this paper, we present methods for evaluating the chemical impacts on the development of honey bees. Honey bee feeding methods were described and applied to either individual honey bees or to a colony. At first, we tested different concentrations of pesticide-contaminated basic larval diet (BLD) on larvae in the colonies to evaluate the impact of the pesticide on individual honey bees *in vivo*. We then proceeded to simulate the natural conditions of the pesticide by using pesticide-contaminated syrup within beehives. In this method, PPN, which is widely used against pest insects[17] and is harmful to the development of honey bee larvae and pupae[16,18,19], will be an indicator to represent the negative effect of the pesticide in the field.

Protocol

1. Preparations

1. Make 1 L of 50% sugar syrup. Dissolve 1 kg sucrose in 1 L ddH_2O.
2. Prepare pyriproxyfen (PPN) solution in BLD. Make 1.1 L of 10,000 ppm PPN stock solution and dilute 100 mL PPN solution in 1 L sterilized ddH_2O. Store at 4 °C.
3. Dilute the PPN stock solution to final concentrations of 0.1, 1, 10 and 100 mg/kg (ppm) in the BLD for the following experiment.
4. Make PPN-syrup (for the colony level). Dilute the PPN stock to final concentrations of 10 and 100 ppm in 50% sugar syrup for the following experiment.
5. Honey bee rearing.
   Note: Here, the experimental location is the National Ilan University (NIU) apiary, Yi-Lan City, Taiwan; (GPS coordinates: N24.747278, E121.746200).
   1. Check honey bee (*Apis mellifera* L.) colonies weekly for food quantity and feed with 1 liter 50% sugar syrup if necessary (the honey storage area is empty). Define a healthy colony as 9 frames of honey bee combs in each colony with a queen laying eggs normally.
6. Prepare 100 mL of basic larval diet (BLD). Dissolve 6% D-glucose, 6% fructose, and 1% yeast extract in sterilized distilled deionized water (ddH_2O) and supplement with 50% royal jelly. Store at 4 °C but not for more than 3 days.
   Note: Pre-warm to 35 °C before experiment and use within 3 days.

2. *In Vivo* Feeding Method

Note: *In vivo* feeding method has been modified from Hanley *et al.*[20]

1. **Honey bee larvae selection and labeling.**
   1. Insert a queen excluder to divide 9 frames of a healthy colony into 4 frames and 5 frames while limiting the queen to the 4 frame section. Leave at least one empty frame in the 4 frame section for laying eggs.
   2. After the queen laying eggs for 1 day, check the frames for the appearance of eggs and keep the eggs inside the beehives for 72 h (the 4^{th} day) until 1 day-old larvae hatch. Take one of the frames containing 1-day-old worker larvae (hatched within 24 h) out from the test hive by hand and remove the honey bee workers from the frame with a bee brush.
   3. Cover the frame with a transparent slide (Size = Length x Width x thick = 29.7 mm x 21 mm x 0.1 mm) and nail the transparent slide on the edge of frames with thumbtacks (**Figure 1A**).
   4. For each treatment, randomly select 50 one day-old larvae (the 4^{th} day) and mark each brood cell by using permanent marker pens on the transparent slide (**Figures 1A** and **1B**).
      Note: Write the information of each treatment on the frame and transparent slide as well by using permanent marker pens to avoid the confusion between different treatments. Remove the marked slides and keep for the *in vivo* feeding and observation.

2. ***In vivo* feeding.**
   1. Add different concentrations of PPN-BLD (0.1, 1, 10 and 100 ppm) to each labeled brood cell by pipetting at day 1, 2 and 3 with 10 µL, 10 µL and 20 µL, respectively, according to the intake quantity of the larvae age. Add the same amount of BLD (no PPN) to a control group. Thereby, the total dose of PPN-BLD in each labeled brood cell accumulates to 4, 40, 400, and 4,000 pg.
      NOTE: Recognize the labeled brood cells by the marked transparent slides and remove the marked transparent slides after feeding. Use fresh BLD for feeding to prevent the cleaning of the brood cells by the worker bees.
   2. Return the PPN-treated frames to the original colonies for further observations.
      NOTE: Each treatment has four biological repeats in four colonies.

3. **Observation of treated larvae at day 7.**
   1. To observe the PPN-treated larvae, return the labeled transparent slides to the frames to record the mortality and capping rates in the labeled brood cells.

4. **Observation of treated pupae and eclosion rates at day 13.**
   1. Remove the bee wax of capped brood cells.
   2. Put the soft tipped tweezers into brood cell and clamp the pupae very slightly then take pupae out gently.
   3. Transfer the pupae into 24-well tissue culture plates with a double-layer of laboratory tissues beneath each well. Record the damage and mortality during pupal transfer.
   4. Keep the 24-well tissue culture plates in an incubator at 34 °C and 70% relative humidity until emergence (*ca.* 8 days).
   5. Observe and record the pupae and emerged honey bees.

5. **Statistics**
   1. Calculate the recorded data and present as a mean ± S.D.
   2. Analyze the data using analysis of variance (ANOVA) by SAS and use the least significant difference (LSD) test to analyze the differences between two means of different treatments. Define statistically significant as *P*-value <0.05. Different letters in the same column of the table showed a significant effect by the statistical analysis.

3. Toxicity of PPN at Colony Level in Beehive

1. Set up the honey bee groups.
    1. Insert a queen excluder vertically to divide 9 frames of a healthy colony into 4 frames (Part A) and 5 frames (Part B) while limiting the queen to the 4 frame section . Leave at least one empty frame in the 4-frame section for laying eggs.
    Note: Put another queen excluder the on top of the queen part to prevent the queen from moving between the parts.
    2. After the queen laying eggs for 1 day, check the frames for the appearance of eggs. Take the appropriate frames containing eggs out from part A by hand and remove the honey bee workers from the frame with a bee brush.
    3. Cover the frame with transparent slide and nail the transparent slide to the edge of the frame with thumbtacks.
    4. Randomly select 100 brood cells containing eggs and mark each brood cell by using permanent marker on the transparent slide. Assign these 100 brood cells as Group 1. Write the information of each treatment on the frame and transparent slide using permanent marker to avoid the confusion between different treatments.
    5. Return the labeled frame to part A for 3 days and then transfer the queen to part B to lay eggs.
    6. After 1 day, check the frames of part B, choose one appropriate frame and label 100 brood cells containing eggs as described in step 3.1.4. Assign these 100 brood cells as Group 2.
    7. Return the labeled frame to part B for 3 days and then transfer the queen to part A to lay eggs.
    8. Repeat the queen exchange between Part A and Part B 6 more times and assign groups numerically, respectively (**Figure 2**). There should be total of 9 groups.

2. **T**reat honey bee colony with PPN sugar syrup on day 13 (**Figure 2**).
    1. Add 1 L 50% PPN sugar syrup containing 10 or 100 ppm in a plastic bee feeder box (W x L x H = 20 cm x 30 cm x 3.5 cm) and put the box on top of the frames in the experimental colonies.
    Note: Group 1 does not receive the PPN at 13 days as the brood cells have been sealed.
    2. Feed the control group with 1 L 50% sugar syrup (no PPN) as described in step 2.2.

3. Count 1 day-old larvae and record as the egg hatching rate of 100 labeled brood cells for each group at day 5 (**Figure 2**). Honey bee eggs usually take 3 days to hatch into 0 day larvae; therefore, check and label 100 eggs-containing brood cells at day 1 and count the number of 1 day-old larvae for each group at day 5 to obtain the percentage of hatching rate.
4. Count the capped brood cells and record as the larval capping rate of 100 labeled brood cells for each group at day 11 (**Figure 2**). 6 to 7 day-old larval brood cells will be capped with bee wax by honey bee workers for larvae pupating.
5. Observe pupae maturation and record the eclosion rate of 100 labeled brood cells for each group at day 17 (**Figure 2**).
    1. Remove the bee wax of capped brood cells and take pupae out with soft tipped tweezers gently. Transfer the pupae into a 24-well tissue culture plates with a double-layer of laboratory tissues beneath each well.
    2. Keep the 24-well tissue culture plates in an incubator at 34 °C and 70% RH until emergence.
    3. Observe and record the pupae and emerged honey bees for each group until Group 9 (49 experimental days).
    Note: Each treatment has four biological repeats.

6. Statistics
    1. Calculate the recorded data and present as the mean ± SD.
    2. Analyze the significant differences between pairs of treatments (*e.g.* 0 ppm/10 ppm, 10 ppm/100 ppm and 0 ppm/100 ppm) in each group by using the Student's two-tailed *t*-test. Define as statistically significant if *P*-value < 0.05.

Representative Results

For the honey bee field test, a queen was limited to the 4-frame section for laying eggs. This step could increase the brood density in one frame and facilitate subsequent observations. Each treatment was labelled, and the honey bees' development was clearly observed through a transparent slide. *In vivo* feeding of PPN-BLD to honey bee larvae in the beehive was performed to precisely evaluate the influence of PPN on the development of honey bees in the colony. Using the *in vivo* feeding method facilitated the observation of the impacts of the chemical treatments on the beehive.

For each dose of PPN-BLD, a total of fifty brood cells were marked and treated. After adding PPN-BLD, the treated frames were returned to the original colonies for observation under natural conditions. The negative effects of PPN-BLD on larval-stage honey bees were easily observed. A dosage-dependent effect was observed. **Table 1** present the number of honey bees that died at the larval stage at the two higher doses of 10 and 100 ppm. As shown in **Table 1**, at the two doses (0.1 and 1 ppm), the capping rates, days to emergence and eclosion rates were not significantly different from 0 (BLD food added) or the unfed control, with only a significantly higher percentage of bees with deformed wings at 1 ppm. Melanization of pupae was observed at low concentrations of PPN. Furthermore, the proportion of adult honey bees appeared with deformed wings increased with higher doses of PPN (**Table 1**).

To simulate honey bee colonies suffering from environmental PPN residue, feeding of PPN syrup to the entire honey bee colony was performed. Before treatment, each colony was divided into 9 different temporal groups at 3 day intervals by two queen excluders (**Figure 2**). Therefore, the effects of PPN on the rates of hatching, capping and eclosion were observed simultaneously under conditions similar to those in the natural environment.

The colonies were fed syrup with 10 or 100 ppm PPN at day 13 (indicated by the red dotted in **Figure 2**). Theoretically, group 1 was a PPN-free control because the PPN-syrup was fed at day 13 and the honey bees in group 1 were in the pupal stage and capped; the honeybees in the larval stage began to be affected in groups 2 and 3, and the eggs will be affected in groups 4-9 due to the longer exposure time in the PPN-contaminated beehives (**Figure 2**).

In this trial, we fed honey bee colonies with PPN syrup and observed development when the adult honeybees consumed the syrup and presumably fed the queen and larvae. This assumption was confirmed by the hatching, capping and eclosion rates, and deformed winged bees were observed after the PPN treatments, as shown in **Figures 3A**-**3D**. All parameters differed significantly at the higher dose (100 ppm) starting with group 3, except the hatching rate (**Figure 3A**-**3D**). Moreover, after the PPN-syrup treatments, many pupae died with black cuticles or by failing to emerge. In the 100 ppm PPN treatment, the capped cells were destroyed, and the injured pupae were removed from the colony (**Figure 3E**).

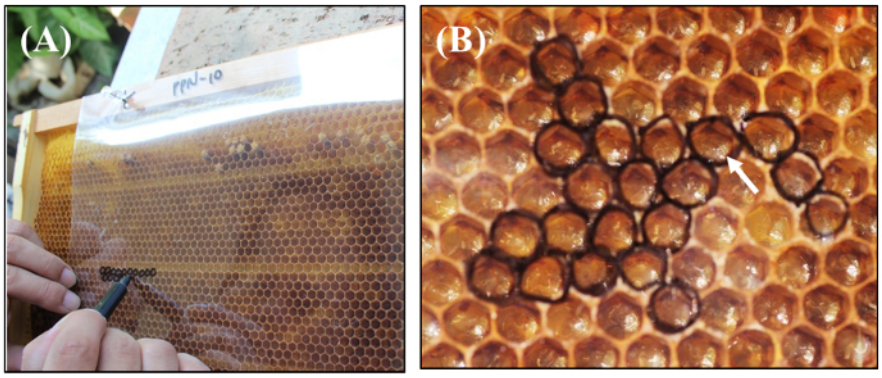

Figure 1: Schematics of brood-cells labeling. (**A**) 1 day-old worker larvae covered by transparent slide papers and were labeled by permanent marker. (**B**) Labeled 1 day-old worker larval brood cells; the white arrow indicates a 1 day-old worker larva inside. Please click here to view a larger version of this figure.

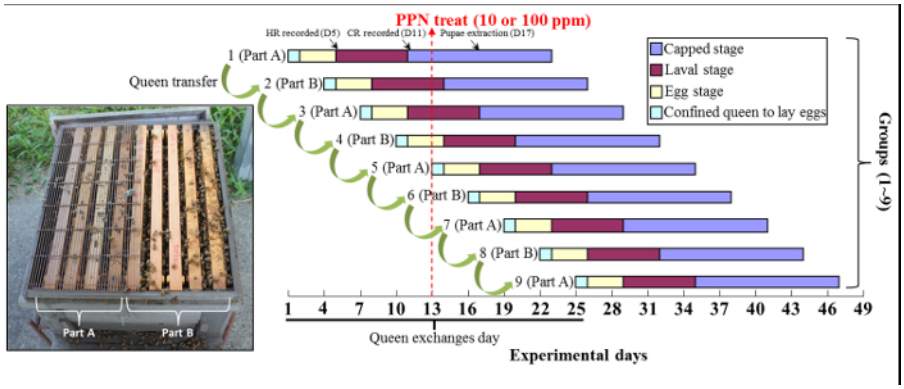

Figure 2: Timeline for when PPN is started relative to the 9 experimental groups. Different concentrations of PPN were fed at day 13 (red dotted line). The treated colony was divided into Part A and Part B. The queen exchanges from part A to part B and *vice versa* for laying eggs are shown. This has been reproduced with permission from Elsevier[16]. Please click here to view a larger version of this figure.

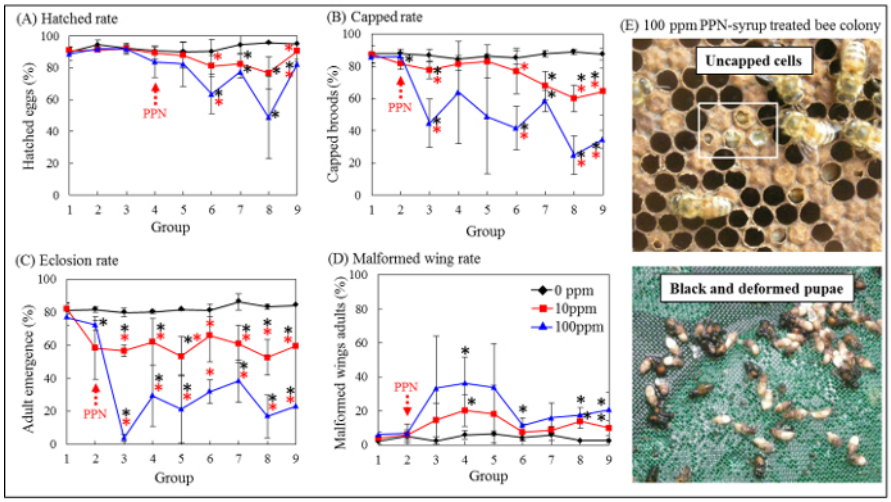

Figure 3: Development of honey bee larvae before and after feeding 1 kg PPN syrup into tested bee colonies. A total of nine groups were surveyed in this experiment: (**A**) hatching rate; (**B**) capping rate; (**C**) eclosion rate; and (**D**) malformed wing rate. Mean ± SD are presented; Red arrows indicate the time during which PPN may start acting on bees. Black asterisks show statistical significance compared to the control (0 ppm). Red asterisks show statistical significance between 10 and 100 ppm; (**E**) 100 ppm PPN syrup treated bee colony showed uncapped cells, presumably deformed pupae and black and deformed pupae. This has been reproduced with permission from Elsevier[16]. Please click here to view a larger version of this figure.

PPN-BLD (ppm)	Larvae No.	Larval development		
		Capped rate (%)	Eclosion rate (%)	Malformed wings (%)
100	140	0b	0b	-
10	139	22.2 ± 33.2b	0b	-
1	140	72.3 ± 17.9a	67.7 ± 17.6a	7.7 ± 5.7a
0.1	136	78.3 ± 17.5a	75.4 ± 22.8a	1.4 ± 2.8b
0	138	78.9 ± 5.4a	78.9 ± 5.4a	0b
Non-feeding	122	87.8 ± 9.1a	86.1 ± 7.2a	0.8 ± 1.5b

Table 1: Effects of continued 3 days feeding of PPN on 1 day-old larvae. To each larval cell, 10, 10 and 20 µL of BLD were added from days 1 to 3, respectively. Each assay contained 25-38 larvae in a colony and 4 colonies were tested. Mean ± SD are presented. Different letters in the same column are significantly different by the least square difference test ($P < 0.05$) after ANOVA showed a significant effect. This has been reproduced with permission from Elsevier[16].

Discussion

The queen-limited egg-laying method and queen-exchange method are critical steps for setting up honey bee groups for field testing within this protocol. The queen-limited egg-laying method permits synchronization of the life cycle of honey bees. Consequently, researchers can select 1 day-old larvae of the same age for treatment with different doses of pesticide. For the queen-exchange method, the queen was exchanged between part A (4 frames) and B (5 frames) to obtain different developmental stages of honey bee for field testing to evaluate the impacts of pesticide and pesticide residues. Moreover, a large number of selected brood cells were recorded in the field test using transparent slides for labelling. However, egg over-laying occasionally results in insufficient brood cells for the queen laying eggs. Therefore, the preparation of empty frames is required for the queen-exchange method. Alternatively, the queen-limited egg-laying method could be also used to prepare different honey bee groups for testing in the beehive. The separation of the frames into 2 frames in part A and 7 frames in part B and the placement of the queen in part A (2 frames) may limit the eggs laid by the queen within the 2 frames.

For the *in vivo* feeding method, PPN-BLD was added to each brood cell. The honey bees exhibited higher acceptance of BLD than syrup[16,20]. Honey bee larvae can survive and grow on an artificial diet composed of royal jelly, sugars, yeast extract, and distilled water[22,23]. The glucose and fructose composition of the BLD did not affect the survival rates of the larvae *in vitro*[21], and therefore, BLD would be more stable for honey bee growth in the beehive. Notably, the use of fresh BLD for feeding could also prevent the worker bees' larval exclusion during the larval feeding experiment. Moreover, during the feeding process, gentle feeding is initially required to avoid the death of 1 day-old larvae.

During field tests, chemically contaminated BLD in the beehive occasionally caused larval exclusion due to the high olfactory sensitivity of nursing bees. The sugar composition significantly affects the average larval survival, pre-pupal larval weights, adult weights, and ovariole numbers[21]. When syrup was used to deliver transgenic pollen or a positive control pesticide diazinon to honey bee larvae, the workers removed few larvae from the brood cells containing added or contaminated food[21]. Thus, the sugar composition should be noted. Increasing the number of 1 day-old larvae or dispersing the tested individual brood cell sites may also improve the problems. Indeed, based on the observation of the PPN-BLD treatment processes and the dramatic dose-dependent impacts of PPN-BLD on the development of honey bees, we assumed that the nurses did not remove the artificially added BLD to the brood cells. Low concentrations of PPN cause melanization of pupae, possibly due

to increased phenoloxidase activity, which regulates melanization and pupation[24,25,26]. Based on the use of an artificial feeding method for each brood cell, the dramatic impacts of PPN-BLD on the development of honey bees might be due to direct contact with PPN from the first day of hatching.

The chemical dosages used in the feeding experiments could be designed based on survey data for pesticide residues in fresh pollen samples collected from honey bee hives. There are a variety of routes of pesticide contamination of colonies in the natural environment. Chemical contaminants could be brought back to the honey bee colonies and ingested by larvae due to the feeding motion of worker honey bees, eventually influencing the development of larvae. To evaluate the impact of PPN at the colony level in the beehive, syrup was used instead of pollen for feeding in this study as the fastest and most direct way to ensure that the honey bees consumed the chemical. Furthermore, the condition of the syrup can be defined, whereas the content of pollen is difficult to control (*e.g.*, pathogens or pesticide contamination).

Under field conditions, different developmental stages (eggs, larvae, pupae and adult bees) of honey bees in a honey bee colony suffer the same environment factor. In our experimental setup, natural conditions were simulated to evaluate the impacts of the chemical on the development stages of honey bees in a dynamic environment. Therefore, after PPN treatment (13 days), we could observe the uninfluenced capping rate in group 1, the influenced capping rate in the other eight groups, and the influenced larval stages in groups 2 and 3, *etc.* Under these conditions, the true effects within the colony and the route of dispersal of the chemical to the whole colony are clear.

The honey bee colonies treated with PPN syrup exhibited distortion of capped cells and removal of pupae from the colony. Furthermore, extensive melanization of pupae was observed in the 100 ppm PPN treatment. Thus, the PPN-syrup feeding method allowed the dynamic impact of chemicals on the life cycle of honey bees in the beehive to be observed. In this trial, the syrup taken up by the worker and nursing bees was presumably fed to the queen and larvae. For confirmation, our future studies will use chemical markers (such as an edible dye) in the chemical-containing syrup to further facilitate the study of the dynamics of chemically contaminated beehives.

The food source of nurse bees should come from foragers or in beehive and larvae are fed mandibular and hypopharyngeal gland secretions produced by nurse bees[21,25,26,27,28]. When larvae are fed by nurse bees, secretions produced by the hypopharyngeal gland in the mandibles may dilute the PPN-syrup. This biological dilution effect more closely resembles the natural conditions but explains the differing results to the *in vivo* feeding method. Several materials including other pesticides, heavy metal and honey bee pathogens could be applied to this feeding method for evaluating and addressing the roles in honey bee populations.

Disclosures

The authors declare that they have no competing financial interests.

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