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Evaluating the effect of environmental chemicals on honey bee development from the individual to colony level

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TITLE:
Evaluating the effect of environmental chemicals on honey bee development from the individual to colony level

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KEYWORDS
Honey bee; *Apis mellifera* L.; pesticide; pyriproxyfen (PPN); basic larval diet (BLD); *in vivo* feeding

SHORT ABSTRACT
Herein we present a method to feed pesticide contaminated food to both an individual honey bee and a beehive colony. The procedure evaluates the pesticide effect on individual honey bees by *in vivo* feeding of basic larval diet and also on the natural condition of beehive colony.

LONG 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.

INTRODUCTION

The presence of pesticides in the environment is one of the most serious problems that impacts the life of a honey bee[1-3]. 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[9-11]. 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

1.1. Make 1 L of 50% sugar syrup. Dissolve 1 kg sucrose in 1 liter ddH_2O.

1.2. Prepare pyriproxyfen (PPN) solution in BLD. Make 1.1 L of 1000 ppm PPN stock solution and dilute 100 mL PPN solution in 1 L sterilized ddH_2O. Store at 4 °C.

1.3. 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.

1.4. 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.

1.5. **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).

1.5.1. 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.

1.6. 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].

2.1) Honey bee larvae selection and labeling.
2.1.1. 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.

2.1.2. 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 4th 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.

2.1.3. 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).

2.1.4. For each treatment, randomly select 50 one-day-old larvae (the 4th 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.

2.2) *In vivo* feeding.

2.2.1) 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.

2.2.2) Return the PPN-treated frames to the original colonies for further observations.

Note: Each treatment has four biological repeats in four colonies.

2.3) Observation of treated larvae at day 7.
2.3.1) 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.

2.4) Observation of treated pupae and eclosion rates at day 13.
2.4.1) Remove the bee wax of capped brood cells.

2.4.2) Put the soft tipped tweezers into brood cell and clamp the pupae very slightly then take pupae out gently.

2.4.3) 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.

2.4.4) Keep the 24-well tissue culture plates in an incubator at 34 °C and 70% relative humidity until emergence (c.a. 8 days).

2.4.5) Observe and record the pupae and emerged honey bees.

2.5) Statistics
2.5.1) Calculate the recorded data and present as a mean ± S.D.

2.5.2) 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

3.1) Set up the honey bee groups.
3.1.1. 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.

3.1.2. 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.

3.1.3. Cover the frame with transparent slide and nail the transparent slide to the edge of the frame with thumbtacks.

3.1.4. 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.

3.1.5. Return the labeled frame to part A for 3 days and then transfer the queen to part B to lay eggs.

3.1.6. 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.

3.1.7. Return the labeled frame to part B for 3 days and then transfer the queen to part A to lay eggs.

3.1.8. 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.

3.2) Treat honey bee colony with PPN sugar syrup on day 13 (Figure 2).

3.2.1) Add 1 L 50% PPN sugar syrup containing 10 or 100 ppm in a plastic bee feeder box (W×L×H= 20 cm × 30 cm × 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.

3.2.2) Feed the control group with 1 L 50% sugar syrup (no PPN) as described in step 2.2.

3.3) 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.

3.4) 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.

3.5) Observe pupae maturation and record the eclosion rate of 100 labeled brood cells for each group at day 17 (Figure 2).

3.5.1) 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.

3.5.2) Keep the 24-well tissue culture plates in an incubator at 34 °C and 70% RH until emergence.

3.5.3) 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.

3.6) Statistics
3.6.1) Calculate the recorded data and present as the mean ± SD.

3.6.2) 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, 3B, 3C and 3D. All parameters differed significantly at the higher dose (100 ppm) starting with group 3, except the hatching rate (Figure 3A, 3B, 3C and 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.

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].

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].

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[24-26]. 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, 25-28]. 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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Figure 1

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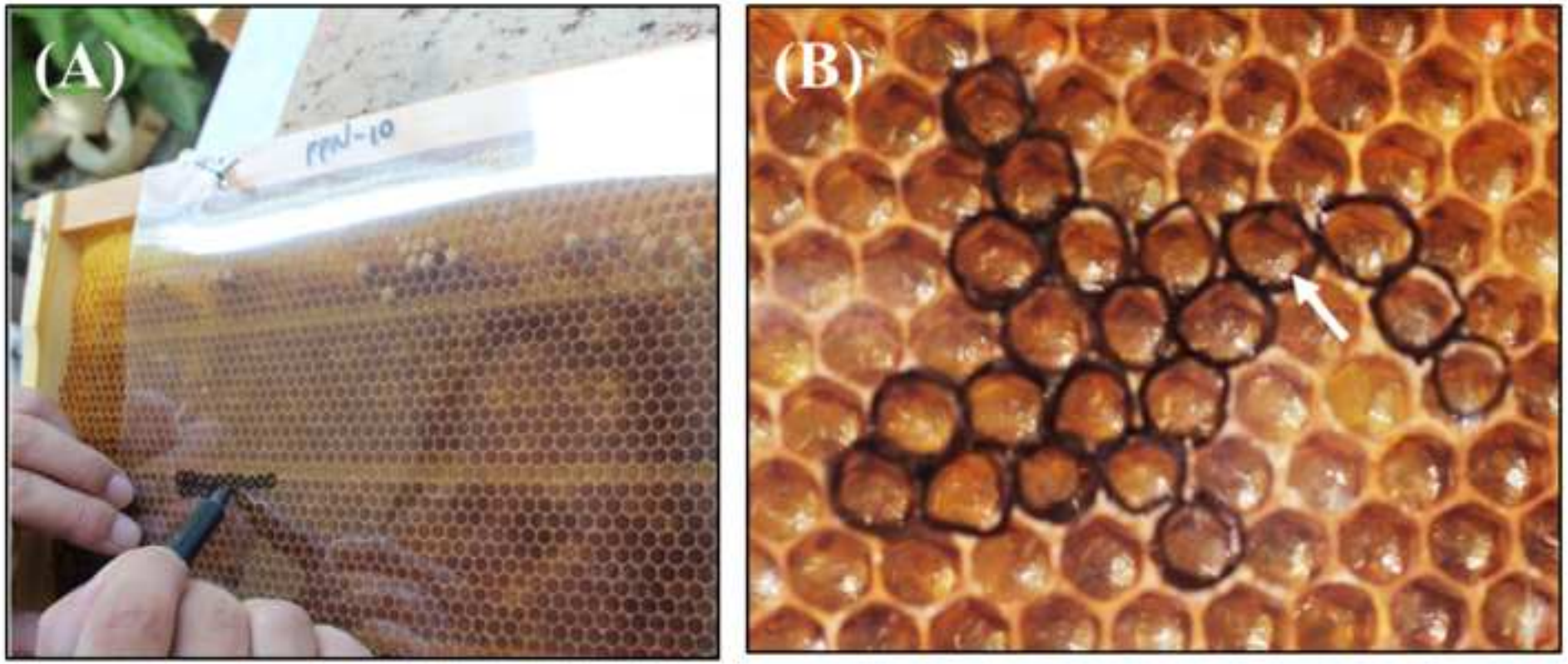

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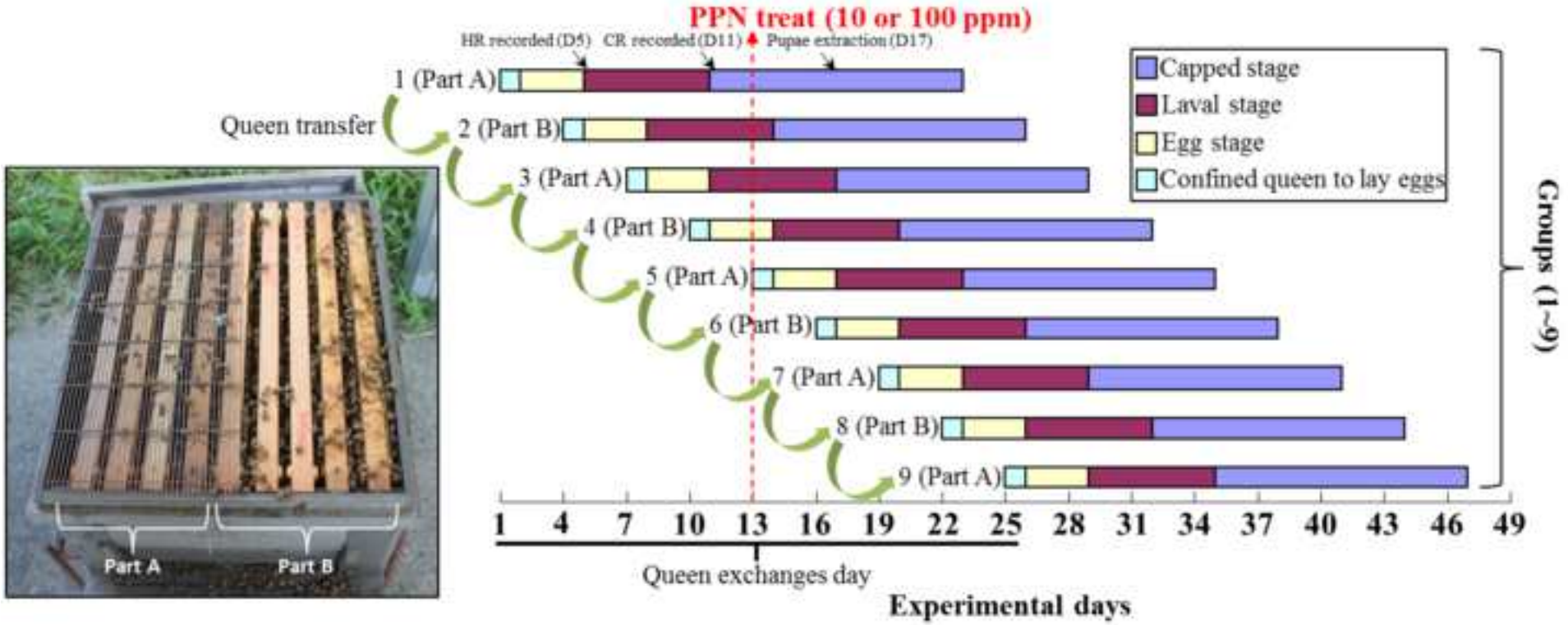

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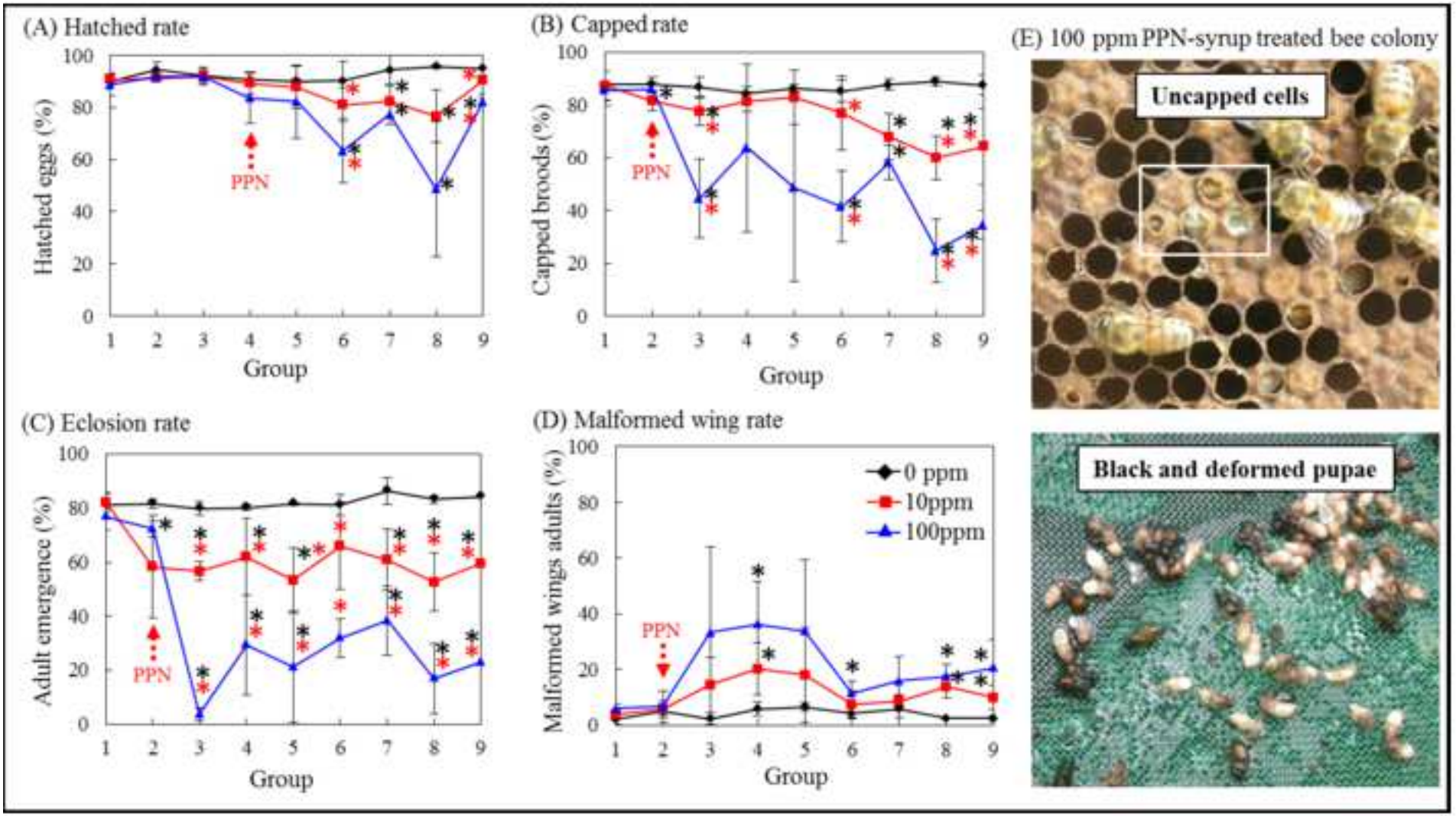

Table 1

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

Excel Spreadsheet- Table of Materials/Equipment

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Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
Honey bee box	SAN-YI Honey Factory	W1266	Honeybees rearing
Queen excluder (between frames)	SAN-YI Honey Factory	I1575	Queen limitation
Queen excluder (on top)	SAN-YI Honey Factory	I1566	Queen limitation on top
Bee brush	SAN-YI Honey Factory, Taiwan	W1414	clean the bees on frame gently
Bee feeder	SAN-YI Honey Factory, Taiwan	P0219	feed sugar syrup to colony
Transparent slide	Wan-Shih-Chei, Taiwan (http://www.mbsc.com.tw/a01goods.asp?s_id=40)	1139	Mark the larval area on the frames (Material: Polyethylene Terephthalate, PET) (Size= Length*Width*thick= 29.7mm*21mm*0.1mm)
24 well tissu culture plate	Guangzhou Jet Bio-Filtration Co., Ltd	TCP011024	Rearing pupae from extraction
Autoclave	Tomin medical equipmenco., LTD.	TM-321	Make sterilized distilled deionized water (ddH2O)
P20 pipetman	Gilson	F123600	Add PPN into bee larval food pool
Incubator	Yihder Co., Ltd.	LE-550RD	Rearing pupae from extraction
Kimwipes	COW LUNG INSTRUMENT CO., LTD	KCS34155	Rearing pupae from extraction
Royal jelly	National Ilan University (NIU)	NIU	Make basic larval diet (BLD)
D-(+)-Glucose	Sigma	G8270	Make basic larval diet (BLD)
D-(-)-Fructose	Sigma	F0127	Make basic larval diet (BLD)
Yeast extract	CONDA, pronadisa	1702	Make basic larval diet (BLD)
Sucrose	Taiwan sugar coporation	E01071010	Make sugar syrup for bee food
Pyriproxyfen (11%)	LIH-NUNG CHEMICAL CO.. LTD.	Registration No. 1937	Insect growth regulator (IGR) used in the experiment

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Author(s): Chong-Yu Ko, Yue-Wen Chen and Yu-Shin Nai

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Article Title: procedures for evaluating the effect of environmental chemicals on honeybee's development from individual to a colony level.

Signature: Yu-Shin Nai Date: 2016 / 7 / 29

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Dear Dr. Nai,

Your manuscript JoVE55296R2 "Procedures for evaluating the effect of environmental chemicals on honey bees' development from individual to a colony level" has been editorially reviewed and the following comments need to be addressed.

Please employ professional copy-editing services if this manuscript is to be accepted. There continues to be significant language and grammatical issues in the manuscript that severely compromises the clarity and science of the manuscript. The Discussion section is especially difficult to understand. Furthermore, there is a lack of consistency between terms in the manuscript Eclosion rate in the manuscript but emergence rate in the Figure.

Please revise the manuscript to address the reviewer comments. Please incorporate the rebuttal comments into the manuscript text to strengthen the manuscript.

→ Thank you for your suggestions. We have downloaded the file “55296_R2_RE” for editing and this revision were addressed the editorial comments. For the discussion section, we also have revised it again and made it easy to understand. Moreover, we have also incorporated the rebuttal comments into the manuscript text and corrected “Emergence rate” to “Eclosion rate” in the Figure and table.

This manuscript has sent to AJE for English editing to improve the language and grammatical issues and the editorial certificate is also submitted for your reference.

Comments of last revision:

Editorial comments:

The manuscript has been modified by the Science Editor to comply with the JoVE formatting standard. Please maintain the current formatting throughout the manuscript. The updated manuscript (55296_R1_081616.docx) is located in your Editorial Manager account. In the revised PDF submission, there is a hyperlink for downloading the .docx file. Please download the .docx file and use this updated version for any future revisions.

1. Grammar:

-Line 83: “Honey bees rearing” Honey bee

→ Yes, we have corrected to “Honey bee”

-Line 37: “In the presenting protocol” presented

→ Yes, we have corrected to “presented”

-Line 23 and throughout “Basic Larvae Diet” should be larval?

→ Yes, we have corrected all “Basic Larvae Diet” to “Basic Larval Diet”

-Line 124 “circled each brood cell by using permanent markers”

→ We have corrected to “marked each brood cell by using permanent marker pens”. The marker pens, which we used in this experiment, are available in the following website: https://www.9x9.tw/mod/product/index.php?REQUEST_ID=55a65ce32a0f9d7015e5755b7e11e3b506e2734aeafc88747d578b6eb13f1b75

-Line 171 “select 100 brood cells containing eggs and circled each brood cell”

→ We have corrected to “select 100 brood cells containing eggs and mark each brood cell”

-Line 273. “Modified figure and legend reproduced with permission of the ELISIVER” Elsevier.

→ Yes, we have checked it.

2. Additional detail is required: Line 309: “that workers removed little larvae from the added food to brood cells” I’m not clear on what this means. Please reword or explain further.

→ Yes, we have revised it as below: “…and it was found that workers removed little larvae from the brood cells, which contained added or contaminated food.”

Editor’s Note: We do not require in depth or novel results for publication in JoVE, only representative results that demonstrate the efficacy of the protocol. However, please ensure that all claims made throughout the manuscript are supported by either results or references to published works.

→ Thank you for your kindly remind, we have checked and applied the copyright from previous

published work (Elsevier license terms and conditions" in Jul 18, 2016) for *JoVE* manuscript and in our previous work, the results demonstrate the efficacy of the current protocol.

Reviewer #1:

Manuscript Summary:

This manuscript attempts to describe a method for assessing the effects of the insect growth regulator, pyriproxyfen, on honey bee larvae and pupae. The authors presented data for exposures through a dietary route and evaluated the impacts of the pesticide exposure on individual bees and the colony.

Major Concerns:

The manuscript is very poorly written and was a challenge to follow. The flow is very choppy and disjointed. It appears as though much of the research presented in the manuscript was previously published (ref #16) as most of the figures were replicates from this previously published paper. The queen limited laying method and queen exchanged methods are not adequately described. No rationale is provided concerning the dosing levels used. Are these levels environmentally-relevant? The linkage between larval, pupal and adult exposures and effects is very poor. Overall, the structure of the manuscript would need to be completely redone to clarify its meaning. However, it merely appears to be an add on to the earlier published paper and therefore not publishable without additional research.

→ This manuscript is focusing on methodology and the *JoVE* journal does not require in novel results for publication, only representative results, which can demonstrate the efficacy of the protocol. In our case, we already applied for the permission from the "Elsevier license terms and conditions" on Jul 18th, 2016, to reproduce our previous data in this manuscript. Moreover, all figures were modified to meet the context of this manuscript. The descriptions of queen-limited method were listed in Line111-122 and queen-exchange method Line 173-203; using queen-exchange method in a colony could help us to select the same egg age groups easier. One of the rationales for these dosages (100, 10, 1, 0.1 ppm) was that we have surveyed the pesticide residues, which used in the agricultural environment from fresh pollen samples, in these data (unpublished), PPN also be found in the pollen sample with 0.4 ppm; it means some of the honey bee colonies suffered this dosage of PPN. Therefore, we tried to test the impacts of higher dosage on honey bee's development in colony level in order to another rationale that provide the information to the government for reference. For honey bees, environmental pesticides can be brought back to the beehives after foraging on crops or flowers. These contaminated foods can be exchanged between workers which then feed larvae. Therefore, to better understand the chemicals impacts on the development of honey bees colonies, we set up this experiment. There are different developmental stages (eggs, larvae, pupae and adult bees) of honey bees in a colony, we tried to simulate the natural condition and to know the chemical impacts on the development stages of honey bees under dynamic environment. In our experiment, for example, after PPN treating (13 days), we could observe the uninfluenced capping rate in group 1, influenced capping rate in the other eight groups, influenced larval stages in group 2 and 3 etc.. In such condition, we

could know the real situations within the colony and how the chemical disperses to the whole colony.

Reviewer #2:

Manuscript Summary:

The draft "Procedures for evaluating the effect of environmental chemicals on honey bees' development from individual to a colony level" by Ko et al. presents two techniques to assess the impact of ecotoxins on honey bee larvae survival. In one experiment, they add basic larval diet to brood cells in vivo und successively record larval mortality. This has before been established by Hanley et al., 2003. In the second experiment, colonies are fed sugar syrup spiked with the testing compound, and larval mortality is observed in age cohorts obtained by caging and exchanging the queen from one compartment of the hive to another one.

Major Concerns:

In particular the second experiment raises some questions on experimental design, that a method describing paper should answer. For example, it is not absolutely clear to the reader, how many colonies should be used to test for different concentrations and you do not at all present any statistics on your results of this experiment. Further, the problem that I see is the not so clear separation of non-exposure and exposure groups. You also do not present, how much syrup was consumed, because it could be that for some days larvae are fed sugars from (uncontaminated) reserves from the bees honey stomach.

→ For this experiment, each PPN concentration (10 or 100 ppm) was performed in one colony to prevent the PPN contaminations and four colonies were used for biological repeats. Every PPN concentration including nine groups (Group1~9 for different time durations) and each group contained 100 honey bee individuals. In group 1, it had no any PPN because of PPN treated at 13 days and it was in the capping stage in Group 1 supposed there was no any feeding motion. It was difficult to control the feeding quantity of each larva in whole colony level; however, in this experiment, we set up a PPN (or other chemicals)-contamination model to test how the chemical influences whole colony. Indeed, more individuals could get supported data, but based on our previous data (Chen et al., 2016), 100 individuals could present the significant impacts between low (10ppm) and high (100ppm) PPN treatments.

Minor Concerns:

The introduction is clear and addresses all the relevant literature.

Protocol:

Line 88: What are the conditions to judge if feeding sugar syrup is necessary?

→ The frames should be checked and the colony should be feed when the honey storage area in the frame is empty. We have added the condition for feeding process as below: “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)"

L 91 and elsewhere: I suggest writing basic larval diet instead of basic larvae diet.

→ Yes, we have corrected it to "basic larval diet".

L 97: Dissolving 1 kg of sucrose in 2 liter water does not give 2 L of 50% sucrose solution.

→ We have checked and corrected to "Make 1 L of 50% sugar syrup. Dissolve 1 kg sucrose in 1 liter ddH_2O."

L 109: In this method...?

→ We have deleted it.

L 114 and elswhere: reword "spawning"

→ We have corrected to "laying eggs"

L124-125: Is that day 1 of your experiment?

→ No, we have added "(the 4^{th} day)".

L 133-135 and Discussion: Be more precise on why bees did not take out the added BLD.

→ In fact, it is, according to our observation, if the added BLD is not fresh, the worker bees will clean the whole brood cell including larvae. Therefore, we have to note this removal action during the experiment. We added the note at Line138: Use fresh BLD for feeding to prevent the worker bees' brood cells clean action. In the discussion section, we have revised it as below: "…and it was found that workers removed little larvae from the brood cells, which contained added or contaminated food."

L 143-144: Discuss the role of touching pupae with tweezers.

→ To prevent the disruption of honey bee pupae, soft tip tweezers should be used. The researcher should remove the wax cap at first and put the tweezer into brood cell and then clamp the body part of pupae very slightly. Besides, more details actions will present in the video.

L 154-156: Statistics need to be explained in more detail

→We have added more detail of statistics at Line 161-168 as below:

2.5) Statistics

2.5.1) Calculate the recorded data and present as a mean ± SD.

2.5.2) 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.

L 171: circle(d)

→ We have corrected to “mark” each brood cell by using permanent marker pens on the transparent slide.

L 172: Asign instead of design?

→ We have corrected to “Assign”.

Results:

The days to emergence (shown in Table 1) are not presented in protocol, nor in Results. How was the data obtained?

→ We have deleted the data in this table.

L 245-247: I think this is one of the most crucial points in your experimental setup: You state that the observations are made at the same time, but this is not true. The time differences is a few days only, but still there could be differences in environmental conditions (see Schmickl and Crailsheim, 2001 and 2002).

→Thank you for your concern. It occurred within the same colony that different developmental stages (eggs, larvae, pupae and adult bees) of honey bees suffer the same environment factor. Although there were still differences in environmental conditions, we tried to simulate the natural condition and to know the chemical impacts on the development stages of honey bees under dynamic environment. In our experiment, for example, after PPN treating (13 days), we could observe the uninfluenced capping rate in Group 1, influenced capping rate in the other eight groups, influenced larval stages in group 2 and 3 etc.. In such condition, we could know the real situations within the colony and how the chemical disperses to the whole colony.

In Line 249-252 you point out, that the non-exposure vs. exposure period is not strictly separated, and you do not come up with a clear solution on how to handle this!

→In Group 1, it was PPN-free control because of PPN treated at 13 days and group 1 was in growing into capping stage; we supposed there was no any feeding motion in capping stage. We have added one sentence in this section: ”Theoretically, group 1 was a PPN-free control, because of the PPN-syrup fed at 13 days and the honey bees in group 1 were under pupal stage and capped;…”

Line 254-263: Statistics missing.

→We have added the “Statistics” at Line242-247 and revised figure 3 as below:

3.6) Statistics

3.6.1) Calculate the recorded data and present as Means ± SD.

3.6.2) Analyze the significant differences between pairs of treatments (e.g. 0ppm/10ppm, 10ppm/100ppm and 0ppm/100ppm) in each group by using Student's two-tailed *t*-test. Define statistically significant as *P*-value <0.05.

In figure 3, we have added the asterisks for indicating significant differences between two treatments; the black one means significant difference to control (0ppm) and red one means 10ppm/100ppm was significant difference.

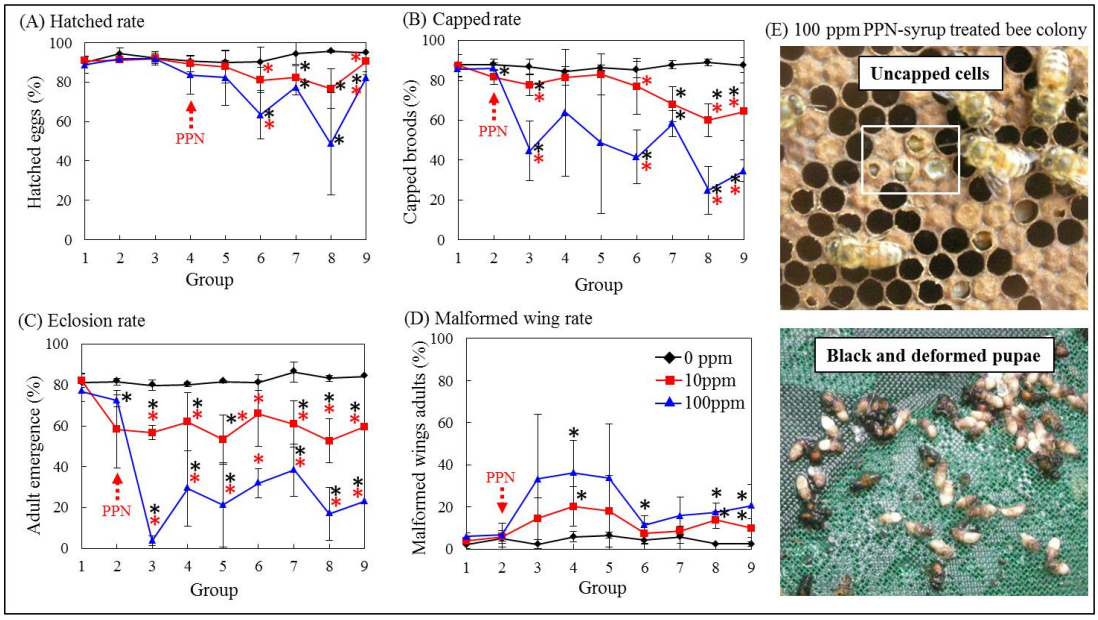

Figure 3: Development of honey bee larvae before and after feeding 1 kg PPN syrup into tested bee colonies. A total of nine groups were surveyed in this experiment (A) Hatching rate; (B) Capping rate; (C) Eclosion rate; (D) % of bees with deformed wing rate; Means ± SD are presented; Arrows indicate the time during which PPN may start acting on bees; Black asterisks= Significant to control (0ppm); Red asterisks= Significant between 10 and 100ppm; (E) 100 ppm PPN syrup treated bee colony showed uncapped cells, presumably deformed pupae and black and deformed pupae. Modified figure and legend reproduced with permission of the Elsevier[16]

Line 273: Elsevier?

→ We have corrected it.

Line 343: forgers?

→ We have corrected it to "foragers".

Reviewer #3:

Manuscript Summary:

The manuscript "Procedures for evaluating the effect of environmental chemicals on honey bees'

development from individual to a colony level" written by Ko C.Y., et al. has described a research protocol to study the effect of environmental pesticides on the honey bee development at an individual level and colony level. I found this protocol the authors presented was used in another paper "The impact of pyriproxyfen on the development of honey bee (*Apis mellifera* L.) colony in field" which was published by the same group in Journal of Asia-Pacific Entomology in 2016. However, I do have some major comments on the protocol.

Major Concerns:

1. The authors used the exact same tables, figures and pictures which were published in the previous paper. I don't see any modifications which were claimed to be made by the group. This is not usually allowed by the both journals which published your previous paper and which they was submitted to. Please extract the core information of your previous figures and remake new figures.

→ This manuscript is focusing on methodology and the *JoVE* journal does not require in novel results for publication, only representative results, which can demonstrate the efficacy of the protocol. In our case, we already applied for the permission from the "Elsevier license terms and conditions" on Jul 18th, 2016, to reproduce our previous data in this manuscript. Moreover, all figures were modified to meet the context of this manuscript.

2. On line 144, were the mortality and damage caused or increased by removing the pupae from the cells to culture plates in the lab? Have you considered that and mentioned it in the manuscript?

→ Yes, this is an important step and it needs to operate very carefully by soft tip tweezers. All the mortality and damage were recorded (Table 1) and the data were subjected to statistical analysis. We have added a note: Record the damage and mortality during pupal transfer at Line 154.

3. On line 177, why did the authors wait for 3 days after they identified and labeled the experimental frame? Why not directly move the queen to part B after the labeling procedures? It would be more intuitive to move the queen earlier because the queen would lay eggs on other frames during those three days and it would affect availability of the frame and the future labeling.

→Thank you for your suggestion. In this case, we have to considerate the experimental interval in order to match the feeding of PPN.

4. The figure 2 is not clear for me. Did that procedure produce different ages of bees? If it is true, did the figure 3 indicate 1-9 as ages when the larvae were treated by the chemicals?

→Because of the queen could lay eggs freely at part A or B, thus, there were many different ages of larvae, in this experimental design, we can just select 100 eggs with same age and set up as a group, then repeat this procedure for the other eight times to set up 9 different time groups for testing. Yes, in figure 3, the group 1-9 were as different ages.

5. It is not clear to me how long did the authors perform the chemical feeding to the colonies. If the authors fed them bees all the way to the end of the experiment, does it mean the bees received the chemicals at the different ages and also for the different length of the time?

→We fed honey bee colony with 1 liter of PPN-syrup only once at 13 days (see the red dot line in figure 2). According to our observation, the PPN-syrup was consumed within one day. Yes, the bees in different groups received the chemicals at the different ages and also for the different length of the time, therefore, according to the recorded data, we could see the short term and long term impacts on the bee colony.

6. In the right figure of figure 2, if we consider each horizontal bar is a frame, does it mean the first one did not receive the chemical at all because the chemical was provided on the 13th day when the cells have been sealed? And the larvae on the second frame were treated at the end of pupal stage… This should be stated clearly in the protocol since it is essential for interpreting the results.

→Yes, the first one group did not receive the chemicals. We have added this in the protocol (Line 211) and result section (Line 272-276).

7. Can authors make it clear that what is the goal to use this protocol especially for the part of figure 2? Why did the author use this complicated design to investigate the chemical effects? What is the advantage to use this design to transfer queens between two parts of the colony?

→ For honey bees, environmental pesticides can be brought back to the beehives after foraging on crops or flowers. These contaminated foods can be exchanged between workers which then feed larvae. Therefore, to better understand the chemicals impacts on the development of honey bees colonies, we set up this experiment. There are different developmental stages (eggs, larvae, pupae and adult bees) of honey bees in a colony, we tried to simulate the natural condition and to know the chemical impacts on the development stages of honey bees under dynamic environment. In our experiment, for example, after PPN treating (13 days), we could observe the uninfluenced capping rate in group 1, influenced capping rate in the other eight groups, influenced larval stages in group 2 and 3 etc.. In such condition, we could know the true situations within the colony and how the chemical disperses to the whole colony. The advantage of queens-exchange method in a colony is that it could help us select the same egg age groups easier.

Reviewer #4:

Manuscript Summary:

This manuscript presents a useful and interesting method for testing the effects of chemical contamination on honey bee development, particularly pyriproxyfen. This is done through in vivo feeding of developing larvae with contaminated artificial diet and through whole-hive feeding with contaminated syrup. Overall, this is good protocol. In particular, I think the hive-level approach with the stepped groups of bees is particularly useful, as it shows a nice trend over time using the same

queen and hive environment (i.e., the data shown in Fig. 3)

Minor Concerns:

First, I think the manuscript should be edited again for correct English grammar and punctuation. Overall, it is understandable, but there are many errors in the manuscript that are somewhat distracting. I do not think this is a critical problem, but should be addressed before publication. I also have a few comments about the protocol where I think additional details will help the protocol's clarity, and I also feel some information could be added to the discussion.

Line 91-93: section 1.2 - roughly how long can the BLD diet be stored at 4C?

→ We have added "(Do not exceed 3days)" in this section.

Line 121 - Section 2.1.3 - what is the 'slide"? What is it made of? How thick is it? It is important because the authors nail thumbtacks into it as part of the protocol and not all materials would work for this

→ Thank you for this suggestion, we have added the information in the materials table. The material of transparent slide is polyethylene terephthalate, PET; the size of transparent slide= Length*Width*thick= 29.7mm*21mm*0.1mm.

Line 1310 Section 2.2.1 - Where are the frames of focal brood cells kept? Are they returned to the colony daily after feeding? Are they kept in an incubator? Is the slide removed for feeding, then replaced? Or is it left off for this duration? None of this is clear.

→ The focal frames were returned to the origin colonies daily (see section 2.2.2); the slides were removed from the frames after marking the focal brood cells. The marked slides were kept for *in vivo* feeding and observation. We have added sentences in the Line127: "Remove the marked slides and keep for the *In vivo* feeding and observation." and Line 138: "Note: Recognize the labeled brood cells by the marked transparent slides and remove the marked transparent slides after feeding."

Line 137 - Section 2.2.2 - do the authors remove the slide before returning the frame to the colony? If not, does this affect worker thermoregulation?

→We have added sentences in the Line127: "Remove the marked slides and keep for the *In vivo* feeding and observation." and Line 138: "Note: Recognize the labeled brood cells by the marked transparent slides and remove the marked transparent slides after feeding."

Line 149 - Section 2.4.3 - approximately how long does it take for emergence of pupae from this point?

→ We have added "(c.a. 8 days)."

Line 160-162 - Section 3.1.1 - I am confused at the setup of the divided colony. The authros say the put a queen excluder on, and the figure shows an excluder on top. But is another excluder placed vertically in the colony to separate Part A and Part B? This must be something special to prevent the queen from moving between the parts. I think the excluder system needs to be described in more detail and/or the photo of the setup needs improvement.

→ Yes, there was a vertical queen excluder to divide part A and B. We have corrected the sentence to "Insert a queen excluder "vertically" to divide 9 frames….; and we also added a note at line 168: Note: Put another queen excluder on the top of the queen part to prevent the queen from moving between two parts.

Line 219 - Section 3.5.4 - Is this observation repeated daily, every other day, etc, through the experimental groups (i.e., as somewhat shown in Figure 2?). At this point, the protocol just says to record data on Day 17, but the figure shows through Day 48. Context suggests this be repeated with the groups but I think it is valuable to be specific here.

→ The observation depend on each group, for example, in group1, the observation day were indicated on the time bar (HR recorded, D5, CR recorded, D11 and Pupae extraction, D17) and so on. Once the experiment start, the parameters of each group will be observed and recorded at each time point (D5, D11 and D17) until Group 9. We have added one sentence to explain it: Line 226: Observe and record the pupae and emerged honey bees "for each group until Group 9 (49 experimental days)"

Discussion:

In the discussion, I think the authors need to discuss how field relevant the approach for hive-level exposure is. For example, the in vivo approach is very controlled but less realistic, but provides very clear data - the authors describe this. However, they then argue the whole-hive application is more realistic. This is true. On the other hand, they deliver the chemical via syrup feeders - is this the realistic route of entry for PPN? Is there evidence that contamination comes in via nectar and not via pollen or adherence to the cuticle? I know for many insecticides pollen contamination is more realistic and there has been criticism of studies that only use high doses of insecticides dissolved in syrup, as it is unlikely bees are being exposed in this way. I do no think this totally precludes the usefulness of the approach, but I do think the authors should discuss it. In summary, I think they need to say whether syrup/nectar delivery is realistic and if it is not, why it is still useful.

→ We appreciate your useful suggestions! It is true that the natural environment has variety routes which the pesticides could be brought into colonies. The chemical would contaminate honey bee colonies. Indeed, pollen is one of the pesticide residues sources, based on our surveyed, the pesticide used in the agricultural environment could be detected in fresh pollen samples in Taiwan. From our data (unpublished), PPN was also found in the pollen sample with the concentration of 0.4 ppm; however, these chemical contaminations could be brought back to the colonies and would be ingested by larvae and influenced their development eventually by feeding motion of worker honey bees. In

our experiment, instead of pollen, we used syrup for feeding, it is the fastest and most direct way to ensure that honey bees already intake the chemical- syrup; From this experimental design, we could easily get clean and pure sugar, while pollen is difficult to control its content (e.g., pathogens or pesticide contaminations). We have added the discussion at Line 355-363.

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English editing certificate