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 ddH2O.”

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 4th 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.

C:\Users\user\Desktop\Figure 3.tif

**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 Elsevier16

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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