Journal of Visualized Experiments

Evaluating the Effect of Pesticides on the Larvae of the Solitary Bees

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE62946R2
Full Title:	Evaluating the Effect of Pesticides on the Larvae of the Solitary Bees
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TITLE:
Evaluating the Effect of Pesticides on the Larvae of the Solitary Bees

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SUMMARY:
The present protocol explains a method to feed pesticide-contaminated provisions to the larvae of the solitary bees, *Osmia excavata*. The procedure examines the ecotoxicity of the pesticide to the larvae of the solitary bees.

ABSTRACT:
Current ecological risk assessments of pesticides on pollinators have primarily considered only laboratory conditions. For the larvae of solitary bees, ingestion of provisions contaminated with pesticides may increase the mortality rate of the larvae, decrease the collection rate and the population of adult solitary bees in the next year from a demographic perspective. But there are limited studies on the effects of pesticides on the larvae of solitary bees. Therefore, understanding how pesticides influence the larvae of solitary bees should be considered an integral part of pesticide ecological risk assessment. This study presents a method to expose the larvae of solitary bee, *Osmia excavata,* to lethal or sublethal doses of pesticide, tracking larval weight gain, developmental duration, eclosion ability, and food consumption efficiency conversion of ingested food. To demonstrate the effectiveness of this method, the larvae of *O. excavata* were fed with provisions containing acute lethal and sublethal doses of chlorpyrifos. Then, the above indexes of the treated larvae were investigated. This technique helps to predict and mitigate the risk of pesticides to pollinators.

INTRODUCTION:
Pollinators play a critical role in the ecosystem services of modern global agriculture. While honey bees (*Apis mellifera*; Hymenoptera: Apidae) have traditionally been considered as the

essential economic pollinators of crops, recent research suggests that *Osmia* (Hymenoptera: Megachilidae) is also very important in improving pollination for certain crops, increasing fruit size and number of seeds, and reducing the proportion of asymmetric fruit in commercial orchards in different parts of the world[1]. *Osmia excavata* has been considered an ideal species for apple pollination, mainly in Asia, like in north and northwest China and Japan[2-4]. It can provide pollination services for certain crops with similar or sometimes with greater efficiency. In this respect, they have been shown to replace or work in synergy with the honey bees[4–6].

The biological characteristics of *O. excavata* are unique compared with social bees. Its univoltine, solitary, and nesting activity occurs mainly in spring and early summer. The nests of *O. excavata* are usually found in preexisting holes, typically in deadwood, hollow plants, straw tubes, and bamboo stem in the natural condition[3]. The adult *O. excavata* emerges from its cocoon to mate, gather pollen, and build a nest to lay eggs, which begin to hatch a week later. The fertilized eggs develop into females, while the unfertilized eggs develop into males[3]. Females are distributed in the bottom of the bee tube, and the corresponding provisions are more significant. In contrast, males were in the proximity of tube exit with minor provisions[7], so the males come out first, and the females come out later. The female mixes pollen with a small amount of nectar into a moist blob, the only food source for each larva in the cell[8].

Several studies have reported a decrease in the population of pollinating insects[9,10]. The extensive use of pesticides has been identified as one of the main factors for reducing pollinator abundance and diversity and may also endanger pollination services[11,12]. To reduce and mitigate the adverse effects of pesticides, it is necessary to conduct a pesticide risk assessment for pollinators. Some countries have established regulatory frameworks to ensure safety to bees from the pesticides used[13,14]. Recent studies have shown that *Osmia* was more susceptible to pesticides than honey bees[1,15].

Interestingly, most risk assessments were focused on adult honey bees[11,12]; little research has been conducted on *O. excavata*, especially the larvae. Furthermore, the mortality of *Osmia* directly caused by pesticides is most commonly considered[16]. Still, the chronic toxicities such as larval weight gain, developmental duration, feeding patterns, eclosion ability, subsequent adult behavior, and fecundity may have the same harm as the acute lethal toxicities and are often ignored because of a lack of an effective experimental method for the solitary bees[17].

Up to now, two methods are used to evaluate the effects of pesticides on the larvae of solitary bees: (1) an appropriate amount of pesticide was applied in the localized spot of provisions without removing the egg of solitary bees[1,18–20]; (2) replacing provisions with artificial pollen-nectar mixtures containing a specific amount of pesticide[21]. However, there are some limitations to the above two methods. The former can only measure acute toxicity, but not chronic toxicity because the larvae ingested the entire dose in a short period of time; the latter would lead to a high mortality rate because of human manipulation[1]. Here, the immersion method was described to study the ecotoxicity of pesticides to *O. excavata* under highly controlled research conditions by simulating the behavior of larval feeding on residual pesticide in the provisions in the real environment. The method of this study solves the

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disadvantages of the above two methods and is suitable for measuring the effects of a hazardous substance on acute and chronic toxicity.

PROTOCOL:

1. Preparation of the feeding tube

1.1. Punch a hole (~0.3 mm diameter) into the lid of a 2 mL centrifuge tube using an electric winding iron (see **Table of Materials**). Use such a centrifuge tube to maintain an *O. excavata* larva and its provision mass.

2. Preparation of pesticide

2.1. Dissolve the technical-grade pesticide (see **Table of Materials**) in acetone to acquire stock solutions of 1×10^4 µg a.i. mL^{-1}. Then, perform gradient dilutions of the solution to more than five concentrations.

NOTE: Chlorpyrifos at 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 µg a.i. mL^{-1} were used in this study.

3. Preparation of the provisions

3.1. Acquire plastic bee tubes containing provisions (see **Table of Materials**) and newly hatched larvae of *O. excavata* from a mass-rearing program.

NOTE: No pesticides were used from 20 days before flowering to the entire flowering period; chemical analysis results showed that commonly used pesticide contents in randomly selected fifty provisions were both below the minimum test levels.

3.2. Separate provisions and larvae gently using a soft brush. Select female larvae based on provision size and cell position within the nest[9]. Then, place uniform-sized provisions and selected female larvae in Petri dishes (60 mm diameter) and set them aside for use.

NOTE: Fifty provisions were randomly selected to analyze the contents of commonly used pesticides: chlorpyrifos, imidacloprid, fendifenuron, phoxim, avermectin. The soft brush parameters are (a) diameter of the brush: 0.3 mm, (b) length of the brush: 2 cm, (c) length of the pen: 18 cm.

4. Provision treatment with pesticide

4.1. Soak the selected evenly sized provisions (from step 3.2) in diluted pesticide (from step 2.1; chlorpyrifos at 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 µg a.i. mL^{-1}) for 10 s using a cage. Soak the control check (CK) in 0.2% solvent (acetone in this study).

NOTE: There are three replicates per concentration treatment, and each replicate consisted of 60 provisions. The difference of dosage of each provision can be reduced by selecting evenly sized provisions.

4.2. Measure the volume of pesticide solution before and after treating the provisions with the pesticide. Then, calculate the immersed volume of insecticide in each treatment, including 60 mass provisions (**Supplementary Table 1**). Place the provisions in separate centrifugal tubes with holes (from step 1.1) after air-drying on a sterile worktable.

NOTE: Before the experiment, put the cages containing the provisions into the pesticide solution, and then measure the volume of pesticide solution before and after soaking to eliminate the error.

4.3. Transfer female larvae individually to the surface of naturally dried provisions using a soft brush.

NOTE: One larva in one tube.

5. Growth conditions

5.1. Rear the larvae of O. *excavata* in a growth chamber in the dark, 65%–75% relative humidity, and 25 ± 2 °C[16].

6. Examination of the results

6.1. The acute lethal toxicity test

6.1.1. Measure the mortality of the larvae after placing them onto the treated and the control (CK) provisions for 48 h.

NOTE: The death criteria: when the larvae did not respond to mild touch using a soft brush under black-light lamps[22]. Black-light lamps were used to simulate the dark growth conditions of larvae and avoid the influence of light on the larvae when checking growth indicators. For eliminating human error, the mortalities with and without removal of larvae from the provisions after 48 h in control groups were also measured.

6.1.2. Weigh 60 provisions before and after 48 h of insect rearing trials to determine the amount of provision consumed by each larva.

6.1.3. Calculate the dose of pesticide at each concentration consumed by each larva according to the percentage of provision eaten and the pesticide content in each provision.

NOTE: The equation for dose calculation is[23]:

$$D = (W1\text{-}W2) \times (V1\text{-}V2) \times C/W1/60$$

where, D is the consumed dose of pesticide by each larva; W1 is the weight of 60 provisions before infusion of pesticide; W2 is the remaining weight of 60 provisions after 48 h; V1 is the volume of pesticide before immersion for 60 provisions; V2 is the volume of pesticide after immersion for 60 provisions; C is the concentration of the pesticide.

6.2. The sublethal toxicity test

6.2.1. Weigh the larvae before rearing trials and after 14 days of treatments to determine the larval weight gain.

6.2.2. Observe *O. excavata* daily during cocooning under black-light lamps to measure the larval development duration.

6.2.3. Weigh the remaining portions of provisions after 14 days of feeding on treated and CK provisions to calculate the consumption and the efficiency of conversion of ingested food (ECI)[24].

6.2.4. Examine the number of eclosions by sniping the cocoons using a small scissor when the control bees emerge into adults.

REPRESENTATIVE RESULTS:

The contents of commonly used pesticides, chlorpyrifos, imidacloprid, fendifenuron, phoxim, avermectin in provisions were less than the limit of quantification (0.01–0.02 mg kg^{-1}) in the control group; these results excluded the influence of pesticide residues on each treatment. The mortality with and without removing larvae from provisions after 48 h in control groups was evaluated; the results showed no significant differences (**Table 1**), indicating a minor human error.

In the acute lethal toxicity test (**Table 2**), provisions were soaked in seven diluted pesticide solutions (0.1, 0.2, 0.4, 0.8, 1.6, 3.2, and 6.4 µg a.i. mL^{-1} chlorpyrifos) and 0.2% acetone (as a control group). A log-probit regression analysis evaluated the median lethal dose (LD_{50} values) of pesticide to *O. excavata* according to ingested doses of pesticides (ranging from 0.0001–0.005 µg a.i. mL^{-1}) and corresponding mortality of larvae after 48 h of treatments. The results showed that the LD_{50} value of chlorpyrifos to the larvae of *O. excavata* was 0.001 (0.001–0.002) µg a.i. Bee^{-1}.

In the sublethal toxicity test, larval weight gain, developmental duration, eclosion rate, consumption, and ECI of *O. excavata* were evaluated under the soaking concentrations of 0.1, 0.2, 0.4, and 0.8 µg a.i. mL^{-1} of chlorpyrifos. An analysis of covariance (ANCOVA) was used to determine treatment-related changes in the development (except eclosion rate) and food utilization of *O. excavata*. In contrast, initial provision mass was used as a covariate. As the dose increased, the index values of larval weight gain, consumption, and ECI decreased for treatments, with the lowest values relative to the control observed in 0.013 µg a.i. bee^{-1} chlorpyrifos. Conversely, the most extended larval developmental duration was observed in 0.016 µg a.i. bee^{-1} chlorpyrifos compared to the control treatment (**Figure 1**).

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Chlorpyrifos's impacts on eclosion rate were evaluated using one-way analysis of variance (ANOVA) and Tukey's least significant difference (LSD) test. Pearson's correlation was also conducted to analyze the relationship between ingested dosages of chlorpyrifos and the eclosion rate of *O. excavata.* Here, the results of this analysis showed that a significant negative linear relationship exists for the treatments ($R^2 = 0.82$, $P = 0.03$). The eclosion rate was considerably lower when the ingested dosages exceeded 0.002 μg a.i. bee^{-1} than those in the control treatment (**Figure 2**).

FIGURE AND TABLE LEGENDS:

Figure 1: Effect of chlorpyrifos on the growth, development, and feeding of *O. excavate.* (**A**), (**C**), (**D**): after 14 days of treatment; (**B**): before cocooning of *O. excavate*. Different lowercase letters indicate significant differences between treatments at $P < 0.05$. The numbers for each data point average with SD.

Figure 2: Relationship between ingested dosages of chlorpyrifos and eclosion rate of *O. excavate*. Different lowercase letters indicate significant differences between the treatments at $P < 0.05$; The numbers for each data point average with SD.

Table 1: The mortality with and without removing larvae from provisions after 48 h in control groups. The same lowercase letters indicate no significant differences between treatments at $P < 0.05$.

Table 2: Toxicity of Chlorpyrifos to *Osmia excavata* after 48 h of treatments. SE – standard error; Df – degree of freedom; χ^2 – values of Chi-square; CI – confidential interval.

Supplementary Table 1: The immersed volume of insecticide in each treatment, including 60 mass provisions.

DISCUSSION:

For adult pollinators, there are two main methods for measuring the ecotoxicity of pesticides. One is the contact method, in which the pesticide is applied to the prothorax of the adult insects; the other is the gastric toxicity method, in which the adult pollinators are fed with honey water containing pesticide[25,26]. In recent years, it has been found that the pollination effect and eclosion rate of *O. excavata* are relatively low[27]. It is speculated that the influence of pesticide application on the growth and development of larvae is one of the main reasons. However, there are few reports on toxicity assessment methods of pesticides to the larvae of *O. excavata*. In this study, an effective method for evaluating the impact of pesticides on the mortality, growth, and development, and feeding of the larvae of *O. excavata* is proposed by contaminating the provision masses with pesticides.

Many studies used sucrose solution containing lethal medium concentrations to evaluate the toxicity of pesticides to honey bees[28–30]. The main routes of pesticide exposure to solitary bees were larval or adult ingestion, contact, and transovarial transmission[31]. The method in this study simulated the response of solitary adult bees on direct contacting and feeding on food containing pesticides in the field. For solitary bee larvae, its response was feeding on the residual pesticide in the provision masses according to the biological characteristic. Additionally, the pesticide exposed to provisions in the field would incur degradation, volatilization, conduction to other tissues before being eaten by the larvae of *O. excavata.* Therefore, it is better to evaluate the ecotoxicity of pesticides to *O. excavata* by analyzing the pesticide dose ingested by larvae than using the pesticide concentration for immersion.

The provisions vary significantly in size, which can substantially affect the mass of larvae and adults. Provisions and female larvae were selected to minimize the error based on provision size and the cell position within the nest. Additionally, after screening by the above method, provisions with similar sizes were further selected. Although this part of the workload is relatively large, it is essential for the statistics of food consumption per larva and the volume of pesticides at each concentration in the present study. Accordingly, the intake amount of the pesticide could be accurately calculated. Follow-up work determining the pesticide residue in provisions at different times after field application will help to guide the releasing time of the adult *O. excavata* and reduce the adverse effect of pesticides on the larvae of *O. excavata.*

Chlorpyrifos has a high lethality rate to the larvae of *O. excavata*, which was similar to results reported on adult pollinators (*Apis mellifera* and *Apis cerana*)[32,33]. It can be seen that the method in this study can predict the toxicity of pesticides to the larvae of *O. excavata.* However, previous studies have found that low mortality is not a uniform stress response and does not indicate any adverse effects on pollinators. For example, neonicotinoids are insufficient to cause the acute death of bees[34] but can impair the ability of olfactory learning and memory and nesting and gathering activities[35–40]. Thus, it is essential to evaluate the chronic toxicity of pesticides on the larvae of *O. excavata* for a comprehensive understanding of the ecotoxicity of pesticides to pollinators from a demographic perspective. But this method assessed larval weight gain, developmental duration, feeding patterns, and eclosion ability of the larvae of *O. excavata*. The ability to flight and fecundity after emergence into adults was not evaluated.

The present study still has some limitations. It was assumed that the provisions absorbed 100% of the immersed solution. Still, this assumption should be verified analytically since the volumes and humidity of each provision may result in different concentrations, indicating that both ingested food and the verification of the nominal test concentrations used in food are required when reporting toxicity endpoints on a dose basis. Thus, it is still needed to verify the pesticide concentrations in the provisions using an analytical method in the future.

In summary, the method presented will help researchers to improve the ecological risk of pesticides to the larvae of solitary bees by assessing endpoints related to mortality, larval weight gain, developmental duration, eclosion ability, and feeding patterns. The technique can potentially enhance the safety of pesticide use by generating quantitative data relating to the larvae of solitary bees that would be difficult to acquire using semi-field and field experiments. The adverse effects of pesticides on solitary bees can be better predicted and mitigated utilizing this technique.

DISCLOSURES:

The authors have no conflicts of interest to declare.

ACKNOWLEDGEMENTS:

This study was supported by the National Key R&D Program of China (2017YFD0200400), Major Scientific and Technological Innovation Project (2017CXGC0214), Bee Industry Innovation Team of Shandong Province, Agricultural Science and Technology Innovation Project of Shandong Academy of Agricultural Sciences (CXGC2019G01), and Agricultural Science and Technology Innovation Project of Shandong Academy of Agricultural Sciences (CXGC2021B13).

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Figure 1

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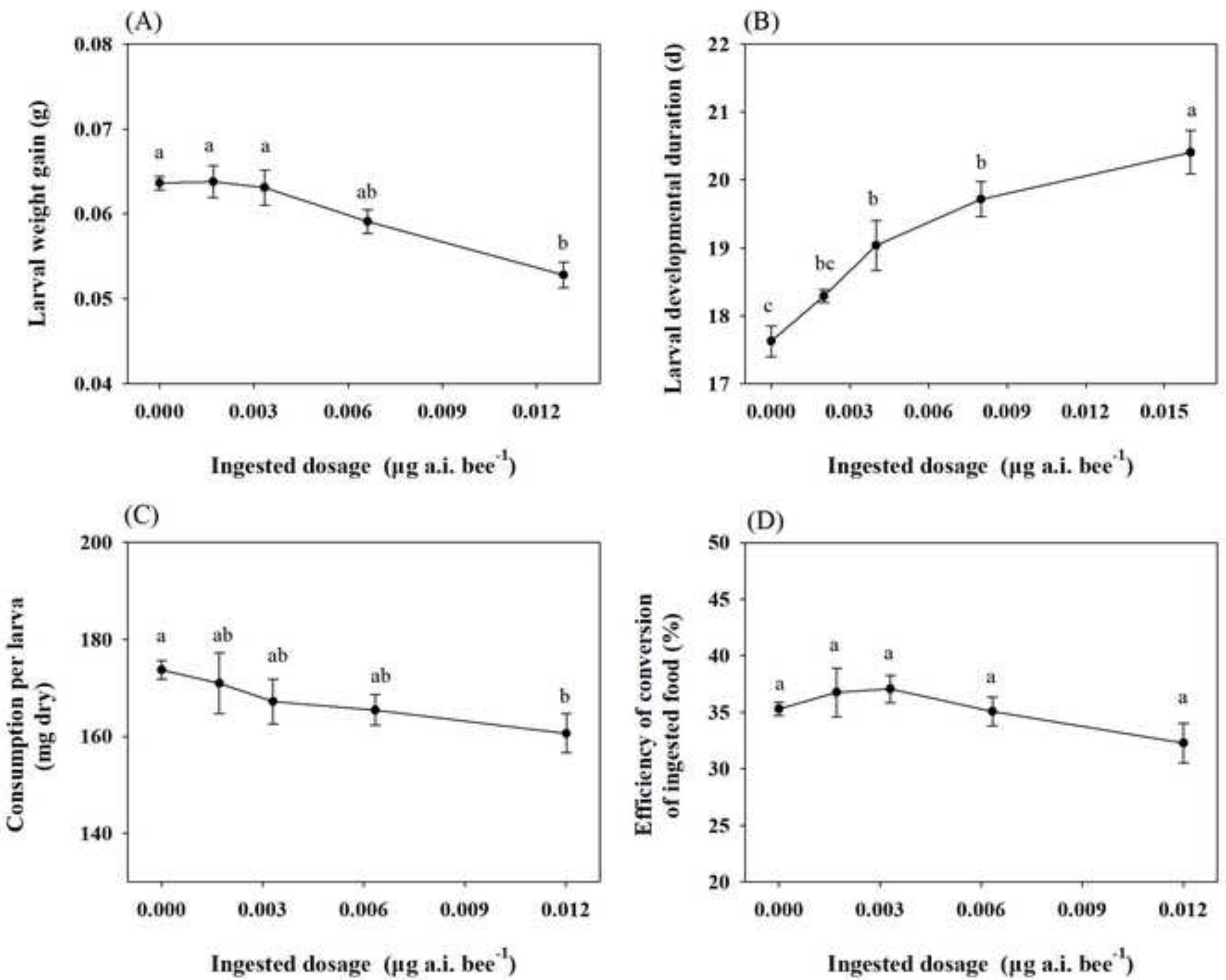
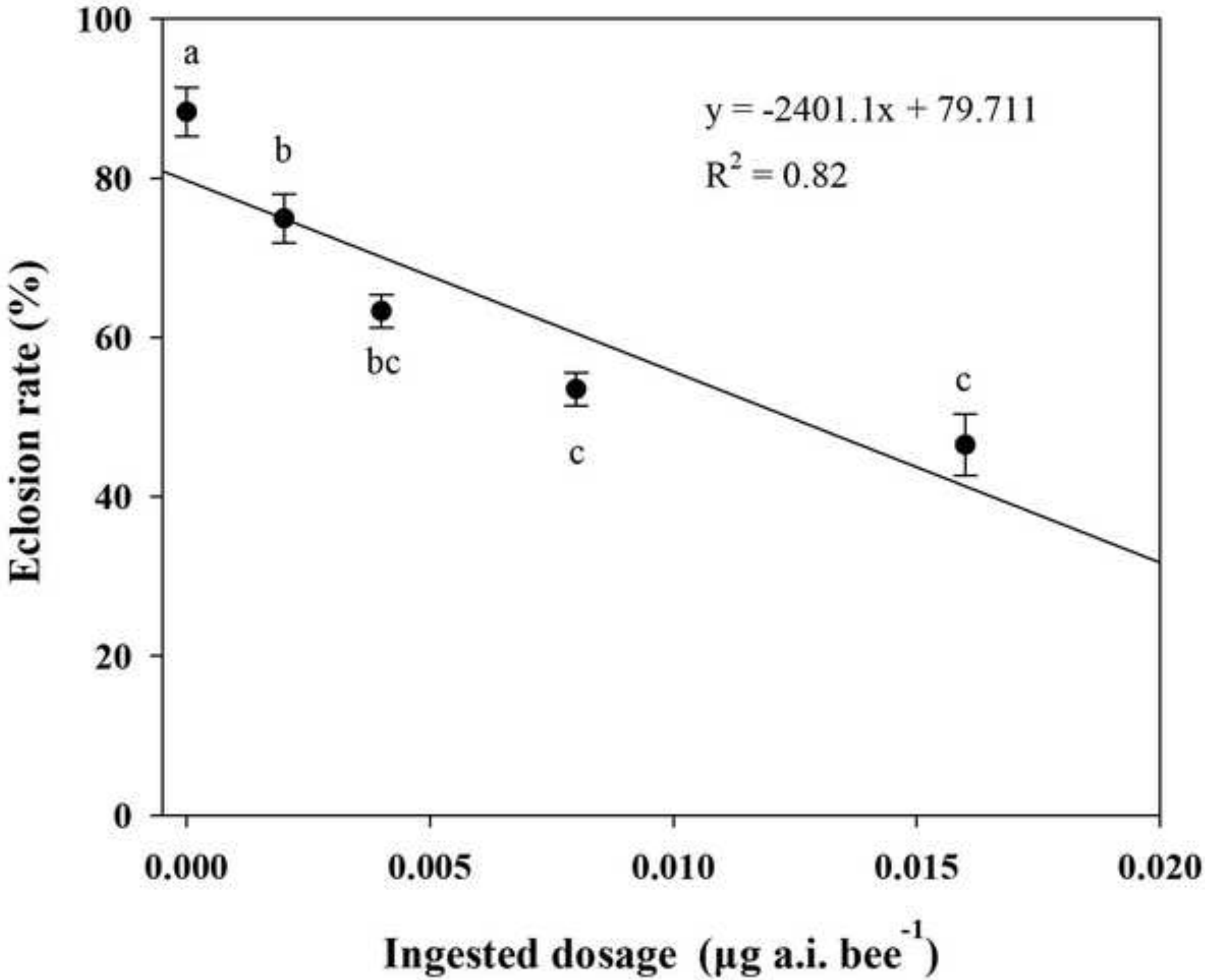


Figure 2


100
80
60
40
20
0
a
b
bc
c
c
y = -2401.1x + 79.711
R² = 0.82
Eclosion rate (%)
0.000
0.005
0.010
0.015
0.020
Ingested dosage (μg a.i. bee⁻¹)

Treatments	Mortality	
	Repetition	Mean
With removal of larvae	11.91%	
	7.63%	9.67% [a]
	9.46%	
Without removal of larvae	6.88%	
	7.37%	8.28% [a]
	10.59%	

Table 2

Insecticide	**Slope ± SE**	**Df**	**χ^2 (*P*)**	**LD_{50} (95% CI) (μg a.i. bee^{-1})**	**LD_{90} (95% CI) (μg a.i. bee^{-1})**
Chlorpyrifos	y=3.23+0.30x	5	5.38 (0.37)	0.001 (0.001-0.002)	0.02 (0.012-0.038)

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Table of Materials
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Dear editor:

On behalf of my co-authors, we thank **JoVE** for giving us an opportunity to revise our manuscript, we appreciate you and the reviewers very much for the constructive comments and suggestions on our manuscript entitled “**Evaluating the effect of pesticides on the larvae of solitary bees”. (Article ID: JoVE62946R1)**

We have studied reviewer's and editorial comments carefully and have made revisions which marked in red in the paper. We have tried our best to revise our manuscript according to the comments. Please find the revised version which we would like to submit for your kind consideration.

We would like to express our great appreciation to you and the reviewer for the helpful comments in our paper. We are looking forward to hearing from you.

Thank you and best regards.

Yours sincerely,
Yingying Song

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E-mail: menxy2000@hotmail.com

List of Responses

Responses to editor:

Comment: Please include the table entitled "The immersed volume of insecticide in each treatment including 60 mass provisions" as a Supplementary table and also include the description of the same in the manuscript text. A legend for the same must be included in the Figure and table legend section.

Response: Thanks for your helpful comments. We have added the table and the same legend as a supplementary table, and we also added the description, that is "Then calculate the immersed volume of insecticide in each treatment including 60 mass provisions (**Table S1**)", in the manuscript text in lines 141-142.

Table S1 The immersed volume of insecticides in each treatment including 60 mass provisions.

Concentration (μg a.i. mL^{-1})		0.1	0.2	0.4	0.8	1.6	3.2	6.4
Immersed volume per treatment (mL)	1	1.23	1.18	1.19	1.18	1.24	1.19	1.24
	2	1.18	1.24	1.20	1.23	1.17	1.22	1.20
	3	1.22	1.21	1.23	1.20	1.21	1.19	1.17
	Mean±SD	1.21±002	1.21±0.02	1.21±0.01	1.20±0.01	1.21±0.02	1.20±0.01	1.20±0.02

Comment: Please address the comments marked in the attached manuscript.

Response: Thanks very much for your helpful comments. We have addressed the comments marked in the attached manuscript. The responds are listed as follows.

Comment: lines 1-2: Please proofread the manuscript. The title has been made concise by removing *Osmia excavate*. Please approve

Response: Thanks very much. We have proofread the manuscript, and approved the amendment.

Comment: lines 23-24, 28-29, 35-37, 58-65, 69-72, 87-89, 92-94, 122-123, 175-176, 208-210, 224-225, 275-277, 290-292. Reworded for clarity, please check.

Response: Thanks very much. We have checked and approved the amendment.

Comment: lines 77-81: Please include citations

Response: Thanks very much. We have added related reference, "Lin, Z., Meng, F., Zheng, H., Zhou, T., Hu, F. Effects of neonicotinoid insecticides on honeybee health. *Acta Entomologica Sinica*. **57** (5), 607–615 (2014)".

Comment: line 108: What is this?

Response: Thanks. It is the short for available ingredient. We have added the relevant explanation.

Comment: lines 108-109: Please specify the gradient dilutions used.

Response: Thanks for your suggestion. We have specified the gradient dilutions used: chlorpyrifos at 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 μg a.i. mL^{-1} in this study.

Comment: line 112: Please include the statement that the provisions were allowed to air dry in an appropriate place

Response: Thanks. We have added the statement: after being air-dried on a sterile worktable in line 143.

Comment: lines 132-133: Please mention the step number from which this is extracted

Response: Thanks very much. We have added the step number: ...selected provisions (from step 3.2) in diluted pesticide (from step 2.1)...

Comment: line 132: Please clarify what is meant by selected provisions?

Response: Thanks. We have added "evenly sized" in line 132, and added "The difference of dosage of each provision can be reduced by selecting evenly sized provisions" in lines 137-138.

Comment: line 132: Please specify the concentration

Response: Thanks very much. We have specified the concentration, that is, "chlorpyrifos at 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 μg a.i. mL^{-1}" in line 133.

Comment: lines 133-134: Please clarify

Response: Thanks very much. In order to make this part clearer, we have changed "As a control check (CK), soak other selected provisions in..." to "Soak the control check (CK) in 0.2% solvent"

Comment: line 134: What is the solvent used here?

Response: Thanks. Acetone was used in this study, we have added related information.

Comment: lines 140-141: Phrase changed, please check

Response: Thanks very much. We have checked and approved the amendment.

Comment: line 143: Step number added, please check

Response: Thanks very much. We have checked and approved the amendment.

Comment: line 145: Phrase added, please check

Response: Thanks very much. We have checked and approved the amendment.

Comment: lines 149-150: One larvae in one cage/tube? Or how this is done?

Response: Thanks. Yes, one larvae in one tube, in order to make this part clearer, we have added related information in line 152.

Comment: line 156: Are the larvae reared along with the provisions ? How long they are kept in this condition? 48h?

Response: Thanks. Yes, the larvae reared along with the provisions. They are kept in this condition until 48h for the acute lethal toxicity test or 14 d for the sublethal toxicity test or until eclosion for eclosion rate test, we can find related information in the "6. Examine the results" section.

Comment: line 178: Please include citation for the equation

Response: Thanks. We have added the citation for the equation.

Comment: line 195: How is this done?
Response: Thanks. *O. excavata* would not emerge from their cocoons until the next spring, but they have already emerged into adults in August of the first year in China. Only by artificial dissection of the cocoons can we see the eclosion of *O. excavata* in each treatment. Thus, we sniped the cocoons using a small scissor to examine the number of eclosions.

Comment: line 196: Why suddenly month is mentioned here? Is it important? In that case, please mention all the time periods, like when the rearing starts and all
Response: Thanks very much. *O. Excavata* would emerged into adults in August of the first year in China. We have changed "in late August" to "when the control bees emerged into adults", considering that the eclosion time of *O. Excavata* bees may be different in each country.

Comment: line 206: Please add a Figure/table as a result of this test
Response: Thanks. We have added a table as a result of this test.

Comment: line 251: Please include a paragraph on the limitations of the method in this section
Response: Thanks very much for your helpful suggestions. We have added limitations in lines 299-305, that is, "The present study still existed some limitations of the method. We assumed that 100% of the immersed solution was absorbed by the provisions, but this assumption should be verified analytically since the volumes and humidity of each provision may result in different concentrations, indicating that both ingested food and the verification of the nominal test concentrations used in food are required when reporting toxicity endpoints in dose basis. Thus, it is still needed to verify the pesticide concentrations in the provisions using an analytical method in the future".

List of Responses

Dear Reviewer:

Thank you very much for your processing and the comments. Those comments are all valuable and very helpful for revising and improving our manuscript. As suggested by you, we have revised our manuscript carefully and have made corrections which we hope meet with your approval. Revised portion are marked in red in the paper. The main corrections in the paper and the responds are listed as follows.

Responses to reviewer 2:

Comment: L125-126, L199-201: analytical verification of commonly used pesticide contents in provisions from the origin of the mass provisions are now referred but no details are provided about which pesticides are those and the levels of residues found on them. Not clear the meaning of "contents in provisions were below minimum test levels" in the added text.

Response: Thanks for your helpful comments. We have added commonly used pesticides: chlorpyrifos, imidacloprid, fendifenuron, fendifenuron, phoxim, avermectin in line 126, and changed "Commonly used pesticide contents in provisions were below minimum test levels in the control group" to "The contents of commonly used pesticides chlorpyrifos, imidacloprid, fendifenuron, fendifenuron, phoxim, avermectin in provisions were less than the limit of quantification (0.01-0.02 mg kg^{-1}) in the control group" to clear the meaning of "contents in provisions were below minimum test levels" in lines 199-201.

Comment: L130-143, L175-176, L 267-268: the authors assume that 100% of the immersed solution is absorbed by the provisions but this assumption should be verified analytically since the volumes and humidity of each provision may result in different concentrations. The authors claim that it is better to evaluate the ecotoxicity of pesticides to *O. excavata* (or other bee species) by using only the pesticide dose ingested by larvae because consider unreliable the analytical measurements. The reviewer respectfully disagrees with the authors. To calculate accurately any ingested dose both the amount of provision ingested (this is measured in this study) as well as the analytical measurement of the pesticide concentrations in the provisions (not measured in this study) are needed. In regulatory chronic bee studies with honeybees both measurements are required when reporting toxicity endpoints in dose basis, i.e. both ingested food and the verification of the nominal test concentrations used in food (e.g. royal jelly in the case of honeybees). The usual practice in those regulatory bee studies is to develop an analytical method covering the concentration range of the dose preparations that are used in the studies. These analytical methods must be also validated. According to the OECD test guidelines for bee testing, if evidence is available to demonstrate that the concentration of the test substance has been satisfactorily maintained within 20 percent of the nominal or measured initial concentration throughout the test, then the results can be based on nominal or measured initial values.
The reviewer recommmends these points should be at least reported by the authors as limitations in the manuscript.

Response: Thanks very much for your helpful suggestions. We have added these points as limitations in the manuscript in lines 299-305, that is "The present study still existed some limitations of the method. We assumed that 100% of the immersed solution was absorbed by the provisions, but this assumption should be verified analytically since the volumes and humidity of each provision may result in different concentrations, indicating that both ingested food and the verification of the nominal test concentrations used in food are required when reporting toxicity endpoints in dose basis. Thus, it is still needed to verify the pesticide concentrations in the provisions using an analytical method in the future".

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We have studied reviewer's comments carefully and have made revisions which marked in red in the paper. We have tried our best to revise our manuscript according to the comments. Please find the revised version which we would like to submit for your kind consideration.

We would like to express our great appreciation to you and the reviewer for the helpful comments in our paper. We are looking forward to hearing from you.

Thank you and best regards.

Yours sincerely,
Yingying Song

Corresponding author:
Name: Xingyuan Men
E-mail: menxy2000@hotmail.com

List of Responses

Dear Editor and Reviewers:

Thank you very much for your processing and the comments. Those comments are all valuable and very helpful for revising and improving our manuscript. As suggested by you and the reviewer, we have revised our manuscript carefully and have made corrections which we hope meet with your approval. Revised portion are marked in red in the paper. The main corrections in the paper and the responds are listed as follows.

Responses to editor:

Comment: Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Response: Thanks for your helpful comments. In order to improve the quality of the manuscript, our manuscript has been reviewed by a English-speaking scientist. Besides, we also used the 'spell-check' and 'grammar-check' functions of the word processor to avoid unnecessary errors.

Comment: Please provide an email address for each author.

Response: Thanks for your suggestion. We have added an email address for each author in lines 13-15.

Comment: Before the abstract, please provide a Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

Response: Thanks very much. We have added a Summary to clearly describe the protocol and its applications in lines 17-20.

Comment: Please revise the following lines to avoid overlap with previously published work: 54-56, 112-113, 187-190.

Response: Thanks for your suggestion. We have reedited the language to avoid overlap with previously published work.

Comment: Please ensure that the Abstract is 150-300 words long.

Response: Thanks. We have revised the Abstract according to your suggestion.

Comment: Please revise the Introduction to include all of the following:

a) A clear statement of the overall goal of this method

b) The rationale behind the development and/or use of this technique

c) The advantages over alternative techniques with applicable references to previous studies

d) A description of the context of the technique in the wider body of literature

e) Information to help readers to determine whether the method is appropriate for their application

Response: Thanks for your helpful comments.

a) The overall goal of this method has been described "Here a immersion method was described to study the ecotoxicity of pesticides to *O. excavata* under highly controlled research conditions...." in lines 81-83.

b) The rationale behind the development and/or use of this technique has been described "...by simulating the method of larval feeding on residual pesticide in the provisions in real environment" in lines 82-83.

c) We have added the advantages over previous studies "Up to now, two methods are used to evaluate the effects of pesticides on the larvae of solitary bees: a) a appropriate amount of pesticide was applied in the localised spot of provisions without removing the egg of solitary bees (Sgolastra et al., 2015; Gradish et al., 2012; Hodgson et al., 2011; Konrad et al., 2008); b) replacing provisions with man-made pollen-nectar mixtures containing a certain amount pesticide (Abbott et al., 2008). However, there are some limitations for above two methods. The former one can only measure acute toxicity, but not chronic toxicity, because the larvae ingested the entire dose in a short period of time; the latter one would lead to high mortality rate because of human manipulation (Sgolastra et al., 2015)....The method of this study solves the disadvantages of the above two methods" in lines 74-81, 83-84.

d) We have added some related literatures, i.e. Sgolastra et al., 2015; Gradish et al., 2012; Hodgson et al., 2011; Konrad et al., 2008.

e) We have added the related description, "...suitable for measurement the effects of hazardous substance on the acute and chronic toxicity" in lines 84-85.

Reference:

Sgolastra, F., Tosi, S., Medrzycki, P., Porrini, C., Burgio, G. Toxicity of spirotetramat on solitary bee larvae, *Osmia cornuta* (hymenoptera: megachilidae), in laboratory conditions. *Journal of Apicultural Science*. **59** (2), 73–83 (2015).

Gradish, A.E., Scott-Dupree, C.D., Cutler, G.C. Susceptibility of *Megachile rotundata* to insecticides used in wild blueberry production in Atlantic Canada. *Journal of Pest Science.* **85**, 133–140 (2012).

Hodgson, E.W., Pitts-Singer, T.L., Barbour, J.D. Effects of the insect growth regulator, novaluron on immature alfalfa leafcutting bees, Megachile rotundata. *Journal of Insect Science*. **11**, 43 (2011). DOI: 10.1673/031.011.0143

Konrad, R., Ferry, N., Gatehouse, A.M.R., Babendreier, D. Potential effects of oilseed rape expressing oryzacystatin-1 (OC-1) and of purified insecticidal proteins on larvae of the solitary bee *Osmia bicornis*. *PLoS ONE.* **3**, 7 e2664 (2008). DOI: 10.1371/journal.pone.0002664

Abbott, V.A., Nadeau, J.L., Higo, H.A., Winston, M.L. Lethal and sublethal effects of imidacloprid on Osmia lignaria and clothianidin on Megachile rotundata (Hymenoptera: megachilidae). *Journal of Economic Entomology.* **101**, 784–796 (2008).

Comment: Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Response: Thanks for your helpful suggestion. We have avoided using of personal pronouns, especially in the protocol.

Comment: Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be

written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.
Response: Thanks very much. We have revised the protocol section, ensured all text in the imperative tense in complete sentences, avoided usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol, and added notes when some texts cannot be written in the imperative tense.

Comment: Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
Response: Thanks for your helpful suggestion. We have added detailed operation steps and added related references.

Comment: Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT UP TO 3 of protocol text for inclusion in the protocol section of the video.
Response: Thanks for your helpful suggestion. We have revised the format of the manuscript, such as paragraph Indentation, line spacings, include a single line space and so on.

Comment: Please remove the embedded figure(s) from the manuscript. All figures should be uploaded separately to your Editorial Manager account. The legends should appear only in the Figure and Table Legends section after the Representative Results.
Response: Thanks. We have removed the embedded figures from the manuscript. And the legends have appeared in the Figure and Table Legends section after the Representative Results.

Comment: Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”
Response: Thanks very much. We did not acquire explicit copyright permission. We deleted the results about abamectin, and replaced with the results about chlorpyrifos, which have not been published.

Comment: After the protocol (before discussion), please discuss all figures in the Representative Results section. However, for figures showing the experimental setup, please reference them in the Protocol. Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the

figures. Data from both successful and sub-optimal experiments can be included.
Response: Thanks for your suggestion. We have discussed all figures in the Representative Results section, included at least one paragraph of text to explain the Representative Results in the context of the technique, referred to all of the figures, and included data from both successful and sub-optimal experiments.

Comment: As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
Response: Thanks for your helpful suggestion.
a) Critical step within the protocol has been discussed in line 211-217, "For solitary bee larvae, its response was by feeding on the residual pesticide in the provision masses according to the biological characteristic. Additionally, the pesticide exposed to provisions in the field would incur degradation, volatilization, conduction to other tissues before being eaten by the larvae of *O. excavata.* Therefore, it is better to evaluate the ecotoxicity of pesticides to *O. excavata* by calculating the pesticide dose ingested by larvae than by using the pesticide concentration for immersion".
b) Modifications and troubleshooting of the technique have been discussed in line 219-225, "The provisions vary greatly in size, and this can have a significant effect on the mass of larvae and adults. In order to minimize error, provisions and female larvae were selected based on provision size and cell position within the nest. Additionally, after screening by the above method, provisions with the similar size were further selected. Although this part of the workload is relatively large, it is very important for the statistics of food consumption per larva and the volume of pesticides at each concentration in the present study, accordingly, the intake amount of pesticide could be accurately calculated".
c) Limitations of the technique have been discussed in line 239-241, "But this method just assessed larval weight gain, developmental duration, feeding patterns and eclosion ability of the larvae of *O. excavata*, the ability to flight and fecundity after emergence into adults was not evaluated".
d) The significance with respect to existing methods been discussed in line 245-249, "The technique has the potential to enhance the safety of pesticide use by generating quantitative data relating to the larvae of solitary bees that would be difficult to acquire using semi-field and field experiments. The adverse effects of pesticides on solitary bees can be better predicted and mitigated using this technique".
e) Future applications of the technique have been discussed in line 243-245, "the method presented will help researchers to better evaluate the ecological risk of pesticides to the larvae of solitary bees by assessing endpoints related to the mortality, larval weight gain, developmental duration, eclosion ability and feeding patterns".

Comment: Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. The table legend or caption (title and description) should appear in the Figure and Table Legends section after the

Representative Results in the manuscript text.
Response: Thanks for your helpful suggestion. We have removed embedded Table from the manuscript.

Comment: Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ITALICS). Volume (BOLD) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names. Make sure all references have page numbers or if early online publication, include doi.
Response: Thanks very much. We have revised references according to your advises.

Comment: Please sort the Materials Table alphabetically by the name of the material.
Response: Thanks for your helpful suggestion. We have sorted the Materials Table alphabetically by the name of the material.

Responses to reviewer#1:

Comment: It is not possible to know a priori the level of pesticide exposure in the provisions because it depends on the quantity of solution absorbed by provision. This, in turn, can depend on the level of humidity in the provisions, on their pollen composition and on their size.
Response: Thanks for your helpful comments. In order to minimize error, we selected provisions based on provision size and cell position within the nest, which have been described in lines 101-102 (Select female larvae according to provision size and cell position within the nest. Then place pollen provisions of uniform size and selected female larvae respectively in petri dishes (60 mm diameter) and set aside for use), additionally, the volumes of insecticide were measured for each treatment including sixty pollen provisions with uniform size, instead of each provision being treated individually. The immersed volume of insecticide in each concentration including 60 pollen provisions has shown in below table. There were no significant difference between each treatment, which may also indicate little difference in provision size.

Table The immersed volume of insecticides in each treatment including 60 mass provisions.

Concentration (μg a.i. mL^{-1})		0.1	0.2	0.4	0.8	1.6	3.2	6.4
Immersed volume per treatment (mL)	1	1.23	1.18	1.19	1.18	1.24	1.19	1.24
	2	1.18	1.24	1.20	1.23	1.17	1.22	1.20
	3	1.22	1.21	1.23	1.20	1.21	1.19	1.17
	Mean±SD	1.21±002	1.21±0.02	1.21±0.01	1.20±0.01	1.21±0.02	1.20±0.01	1.20±0.02

Comment: It is not sure that the pesticide is absorbed by provision in homogenous way. This can affect the effects on larvae.
Response: Thanks very much. The immersed volume of insecticide in each concentration including 60 pollen provisions can be found in above table.

Comment: Why did the authors calculate pesticide exposure (e.g. consumed dose) after 48h? Because the authors measured several endpoints after the complete larval development (e.g. larval duration, eclosion rate) I would expect the calculation of the consumed dose considering all larval period, which lasts several weeks.
Response: Thanks for your comments. The consumed doses after 48h (see lines 130-139) and 14 d (see lines 145-146) of treatments were both calculated, the former was used to determine the acute lethal toxicity, the latter was used to measure the sublethal toxicity (e.g. larval duration, eclosion rate, consumption).

Comment: In ecotoxicological study it is very important to verify the nominal dose with the actual dose. I would have expected that the actual pesticide concentration in the provisions were verified with chemical analysis
Response: Thanks very much. The main purpose of pesticide ecotoxicity test is to guide the safe dose range for pesticide use in the field and prevent the adverse effects on non-target organisms. The method in this study can determine the actual dose of pesticides by calculating the feeding amount of *O. excavata* (see detailed formula in the manuscript). We do not believe that the chemical analysis method is more accurate, because some samples can be lost during grinding and solvent dissolving operations, resulting in errors. The method in this study can also be used to guide the application of pesticides in the field. For example, in many pesticide toxicity tests, insects are treated by formulating and calculating gradient concentrations of pesticides, rather than using chemical analysis methods (Ceuppens et al., 2015; Jiang et al., 2019; Hedayati et al., 2019).

References:

Ceuppens, B., Eeraerts, M., Vleugels, T., Cnops, G., Roldan-Ruiz, I., Smagghe, G., 2015. Effects of dietary lambda-cyhalothrin exposure on bumblebee survival, reproduction, and foraging behavior in laboratory and greenhouse. J Pest Sci. 88 (4), 777–783.

Jiang, J., Liu, X., Huang, X., Yu, X., Zhang, W., Zhang, X., Mu, W., 2019. Comparative ecotoxicity of neonicotinoid insecticides to three species of *Trichogramma* parasitoid wasps (Hymenoptera: Trichogrammatidae). Ecotox Environ Safe. 183, 109587.

Hedayati, M., Sadeghi, A., Maroufpoor, M., A Güncan. (2019). Transgenerational sublethal effects of abamectin and pyridaben on demographic traits of *Phytonemus pallidus* (Banks) (Acari: Tarsonemidae). Ecotoxicology. 28, 467–477.

Responses to reviewer#2:

Comment: The reviewer recommends to cite previous similar studies like Sgolastra et al (2015), Ladurner et al. (1999) and Abbot et al. (2008). Discuss what improvements/novelties (if any) their test method introduces compared to those precedent published methods.
Response: Thanks for your helpful suggestion. We have added the advantages over previous studies "Up to now, two methods are used to evaluate the effects of pesticides on the larvae of solitary bees: a) a appropriate amount of pesticide was applied in the localised spot of provisions without removing the egg of solitary bees (Sgolastra et al., 2015; Gradish et al., 2012; Hodgson et al., 2011; Konrad et al., 2008); b) replacing provisions with man-made pollen-nectar mixtures containing a

certain amount pesticide (Abbott et al., 2008). However, there are some limitations for above two methods. The former one can only measure acute toxicity, but not chronic toxicity, because the larvae ingested the entire dose in a short period of time; the latter one would lead to high mortality rate because of human manipulation (Sgolastra et al., 2015)....The method of this study solves the disadvantages of the above two methods" in lines 74-81, 83-84.

Reference:

Sgolastra, F., Tosi, S., Medrzycki, P., Porrini, C., Burgio, G. Toxicity of spirotetramat on solitary bee larvae, *Osmia cornuta* (hymenoptera: megachilidae), in laboratory conditions. *Journal of Apicultural Science*. **59** (2), 73–83 (2015).

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Abbott, V.A., Nadeau, J.L., Higo, H.A., Winston, M.L. Lethal and sublethal effects of imidacloprid on Osmia lignaria and clothianidin on Megachile rotundata (Hymenoptera: megachilidae). *Journal of Economic Entomology.* **101**, 784–796 (2008).

Comment: For the reviewer there is no sufficient information to be able to assess the efficacy of the test method because of lack of relevant information: no data on variability in control mortality (non-treated groups), lack of analytical verification of possible contaminants from the origin of the mass provisions, lack of verification of applied concentrations using appropriate analytical methods, lack of information in the test conditions, etc.

Response: Thanks for your helpful comments.

a) We assessed the effects of the manipulation (i.e. with and without removal of larvae from provisions) without pesticide treatments. The result showed that there were no significant differences of mortality with and without removal of larvae from provisions. The mortality for pesticide treatment has also measured, and has been used to calculate the LD^{50} value. In order to make this part clear, we have added mortality (non-treated groups) in manuscript (line 127-129, 153-155).

Table 1 The mortality with and without removal of larvae from provisions after 48 h in control groups.

Treatments	Mortality	
	Repetition	Mean
With removal of larvae	11.91%	9.67% a
	7.63%	
	9.46%	
Without removal of	6.88%	8.28% a

larvae	7.37%	
	10.59%	

The same lowercase letters indicate no significant differences between treatments at $P < 0.05$

b) Fifty provisions were randomly selected for chemical analysis before the start of the study, results showed that commonly used pesticide contents in provisions were below minimum test levels. We have added related information in the manuscript (line 104-105, 151-152).

c) The method in this study can determine the actual dose of pesticides by calculating the feeding amount of *O. excavata* (see detailed formula in the manuscript). We do not believe that the chemical analysis method is more accurate, because some samples can be lost during grinding and solvent dissolving operations, resulting in errors. The method in this study can also be used to guide the application of pesticides in the field. For example, in many pesticide toxicity tests, insects are treated by formulating and calculating gradient concentrations of pesticides, rather than using chemical analysis methods to accurately calculate the concentration of each treatment (Ceuppens et al., 2015; Jiang et al., 2019; Hedayati et al., 2019).

References:

Ceuppens, B., Eeraerts, M., Vleugels, T., Cnops, G., Roldan-Ruiz, I., Smagghe, G., 2015. Effects of dietary lambda-cyhalothrin exposure on bumblebee survival, reproduction, and foraging behavior in laboratory and greenhouse. J Pest Sci. 88 (4), 777–783.

Jiang, J., Liu, X., Huang, X., Yu, X., Zhang, W., Zhang, X., Mu, W., 2019. Comparative ecotoxicity of neonicotinoid insecticides to three species of *Trichogramma* parasitoid wasps (Hymenoptera: Trichogrammatidae). Ecotox Environ Safe. 183, 109587.

Hedayati, M., Sadeghi, A., Maroufpoor, M., A Güncan. (2019). Transgenerational sublethal effects of abamectin and pyridaben on demographic traits of *Phytonemus pallidus* (Banks) (Acari: Tarsonemidae). Ecotoxicology. 28, 467–477.

Comment: Also, the authors claim that this test method could be used in pesticide risk assessment. Regulatory agencies will consider the protocols efficients (valid) for risk assessment when the test methods are sufficiently reliable, repeatable and reproducible by different laboratories. This can not be assessed based on this single study.

Response: Thanks very much. We have revised the statement, that is, changed "..... could be used in pesticide risk assessment..." to "..... has the potential to enhance the safety of pesticide use".

Comment: Finally, I also have a several suggestions to the authors for major revisions because several statements and references, especially in the introduction, are considered incorrect or inappropiated:

Response: Thanks very much. We have revised according to your suggestions, the detail changes can be found in the following parts.

Comment: L22: The statement "current ecological risk assessment for pollinators rely heavily on adult bees in laboratory conditions" should be revisited. Several regulatory risk assessment schemes currently in force around the world (EU, North America, Brazil, Japan, etc) require studies with immature stages of bees (larvae) in laboratory conditions. i.e. they are part of the data requirements to authorize pesticides. Two official protocols for testing honeybee larvae in laboratory are available at OECD level: test guideline 237 and OED guidance document 239 (see https://www.oecd.org/chemicalsafety/pesticides-biocides/work-related-beespollinators.htm).

Response: Thanks for your helpful suggestion. We have changed "Current ecological risk assessment for pollinators rely heavily on adult bees in laboratory conditions" to "Current ecological risk assessments of pesticides on pollinators have been largely concerned in laboratory conditions".

Comment: L43-44: *Osmia excavata* has been considered an ideal species for apple pollination mainly in Asia, like in north and northwest China and Japan. It would be recommendable to add references about the geographical distribution of this species, e.g. Shu et al. 2002, etc.

Response: Thanks for your helpful suggestion. We have added references about the geographical distribution of this species.

References:

Shu, G.W., Ren, W., Smirle, M.J., Huan, L.X. Release of *Osmia excavata* and *Osmia jacoti* (hymenoptera: megachilidae) for apple pollination. *Canadian Entomologist*. **134** (3), 369–380 (2002).

Men XY, et al. Biological characteristics and pollination service of Mason bee. *Chinese Journal of Applied Entomology.* **55** (6), 973–983 (2018).

Comment: L48: The biological characteristics of *O. excavata* does not seem to differ to other cavity-nesting species in the genus Osmia or even in the family Megachilidae, so it is recommended to revisit the statement "has special biological characteristics"

Response: Thanks very much. We have revised the statement, that is, added "compared with social bees".

Comment: L54: Is the reference #6 appropriate for this description? Other cites related to the biology and ecology of O. excavata would be more appropriate.

Response: Apologize for our mistake. We have deleted the reference #6, and cites related to the biology and ecology of *O. excavata*, that is , Men et al., (2018).

Comment: L58: It is recommended to use others or additional references to the one used (#7), in this paper this species is not described.

Response: Thanks. We have updated the reference, the new reference was Liu et al., (2018).

Reference:

Liu, L., et al. Population investigation and restriction factors analyses of *Osmia excavata* Alfken in Jiaodong. *Apiculture of China*. **69** (9), 68–71 (2018).

Comment: L63-65: It is recommended to revisit the statement "it is necessary and urgent to fully conduct risk assessment for pollinators". Although this statement may be applicable for specific countries without regulatory frameworks for pesticides and pollinators, a comprehensive body of legislation and specific bee risk assessments have been established in multiple countries to evaluate the safety of pesticides uses to pollinator bees (see for example Bireley et al., 2019).

Response: Thanks for your helpful suggestion. We have revised the statement, the new sentence is "In order to reduce and mitigate the adverse effects of pesticides, it is necessary to conduct pesticide risk assessment for pollinators and some countries have established regulatory frameworks to ensure the safety of pesticides uses to bees"。

Reference:

Bireley, R., et al. Preface: Workshop on pesticide exposure assessment paradigm for non-Apis bees. *Environmental Entomology.* **48** (1), 1-3 (2019).

Comment: L66: The study referred for this statement (#13) is not correct for such statement because O. excavata was not studied. Another nesting solitary bee of the genus Osmia, Osmia bicornis, was studied.

Response: Apologize for our mistake. We have changed "Recent studies have shown that *O. excavata* was more susceptible to pesticides than honey bees" to "Recent studies have shown that *Osmia* was more susceptible to pesticides than honey bees", and added another reference "Sgolastra, F., Tosi, S., Medrzycki, P., Porrini, C., Burgio, G. Toxicity of spirotetramat on solitary bee larvae, *Osmia cornuta* (hymenoptera: megachilidae), in laboratory conditions. *Journal of Apicultural Science*. **59** (2), 73–83 (2015)."

Comment: L101-106: Given that each nest is usually provisioned by a single female, did the distribution between treatments accounted for genetic and age similarities of female larvae?

Response: Thanks for your helpful suggestion. These solitary bees were acquired from a mass-rearing program, and gradually bred and cultivated from a small number of similar populations, and have been bred continuously for more than 14 generations in the same environment, such as similar feeding and flying range for adults, similar storage conditions for bee tubes. The standard deviation from figure 1-2 also showed relatively small difference within each treatments. Thus, we speculated that these bees have genetic and age similarities, which have little influence on each treatment.

Comment: L101-102: Was the sex confirmed after emergence of the bees from the cocoons?

Response: Yes.The sex was confirmed after emergence of the bees from the cocoons, because the female was larger than the male.

Comment: L101&103: The larvae were removed using a soft brush from the provision before the application and transferred back again after the application. Were the effects of this manipulation assessed? A comparison of mortality with and without removal of larvae from provisions in control groups could help to elucidate these potential effects related to the methodology.

Response: Thanks very much. We just assessed the effects of this manipulation without pesticide treatments. The result showed that there were no significant differences of mortality with and without removal of larvae from provisions. In order to make this part clear, we added these results in the manuscript.

Table 1 The mortality with and without removal of larvae from provisions after

48 h in control groups.

Treatments	Mortality	
	Repetition	Mean
With removal of larvae	11.91% 7.63% 9.46%	9.67% a
Without removal of larvae	6.88% 7.37% 10.59%	8.28% a

The same lowercase letters indicate no significant differences between treatments at $P < 0.05$

Comment: L119: Can the authors justify the selection of the relative humidity and temperature based on the known biology of *O. excavata*? A reference would be recommendable.
Response: Thanks very much. We have investigated a lot of beekeepers in China, the rearing condition was set according to them. Furthermore, this growth condition is favorable for *O. excavata* to emerge. We have added related reference.

Reference:
Yuan, R., et al. Toxicity and hazard assessment of six neonicotinoid insecticides on *Osmia excavata* (hymenoptera:megachilidae). *Acta Entomologica Sinica*. 61 (8), 950–956 (2018).

Comment: L119: What was light regime used?
Response: Thanks for your comments. The larvae of *O. excavata* grow in the relatively dark environment in the bee tube during the whole growth period. In this study, black-light lamps were used to simulate the dark growth conditions of larvae and avoid the influence of light on the larvae when checking growth indicators, such as the development duration. In order to make this part clear, we have added related information in the manuscript.

Comment: L139: In regulatory bee studies each applied dosage is confirmed through chemical analysis. Were the tested nominal concentrations of pesticides verified/confirmed analytically? Given that the provisions were acquired from a mass-rearing program in the field, the provisions should be also analyzed for chemicals that could contaminated such provision before the start of the study. Such verification is critical to avoid confounding results.
Response: Thanks very much. The colonies of *O. excavata* with mass provisions were acquired from a continuous mass-rearing program in a 6-ha apple orchard in Qixia City, Shandong Province, China. There were no other flowering crops nearby during the bee nesting period and no pesticides were applied 20 days before flowering and throughout the flowering period of apple trees. Fifty provisions were randomly selected for chemical analysis before the start of the study, results showed that commonly used pesticide contents were below minimum test levels.
The main purpose of pesticide ecotoxicity test in this study is to guide the safe dose range for pesticide use in the field and prevent the adverse effects on non-target organisms. Insects are treated by formulating and calculating gradient concentrations of pesticides, the dose of pesticide at each

concentration consumed by each larva was calculated according to the percentage of edible mass provision and the content of pesticide in each mass provision, rather than using chemical analysis methods. We do not believe that the chemical analysis method is more accurate, because some samples can be lost during grinding and solvent dissolving operations, resulting in errors, and these method were similar to other studies (Ceuppens et al., 2015; Jiang et al., 2019; Hedayati et al., 2019).

References:

Ceuppens, B., Eeraerts, M., Vleugels, T., Cnops, G., Roldan-Ruiz, I., Smagghe, G., 2015. Effects of dietary lambda-cyhalothrin exposure on bumblebee survival, reproduction, and foraging behavior in laboratory and greenhouse. J Pest Sci. 88 (4), 777–783.

Jiang, J., Liu, X., Huang, X., Yu, X., Zhang, W., Zhang, X., Mu, W., 2019. Comparative ecotoxicity of neonicotinoid insecticides to three species of *Trichogramma* parasitoid wasps (Hymenoptera: Trichogrammatidae). Ecotox Environ Safe. 183, 109587.

Hedayati, M., Sadeghi, A., Maroufpoor, M., A Güncan. (2019). Transgenerational sublethal effects of abamectin and pyridaben on demographic traits of *Phytonemus pallidus* (Banks) (Acari: Tarsonemidae). Ecotoxicology. 28, 467–477.

Comment: L182: To assess the sensitivity and reliability of the proposed protocol and these results, more information is needed on the control group as well as the possible presence of other chemicals in the provisions before the start of the study. This verification was not done despite it is a normal practice in regulatory bee study.

Response: Thanks for your comments. The colonies of *O. excavata* with mass provisions were acquired from a continuous mass-rearing program in a 6-ha apple orchard in Qixia City, Shandong Province, China. There were no other flowering crops nearby during the bee nesting period and no pesticides were applied 20 days before flowering and throughout the flowering period of apple trees. Fifty provisions were randomly selected for chemical analysis before the start of the study, results showed that commonly used pesticide contents were below minimum test levels. In order to make this part clear, we have added related information in lines 98-100, 151-152.

Comment: L202-203: (…) there are few reports on toxicity assessment of pesticides to pollinator larvae. This statement is incorrect. As explained before, there are official reports and official validated test methods for honeybee larvae regularly used in pollinator risk assessments for pesticides.

Response: Apologize for our mistake. We have revised this statement, the new sentence is "...there are few reports on toxicity assessment methods of pesticides to the larvae of *O. excavata*"

Comment: L207-208: The author disagrees that this method can be used to simulate the response of adult pollinators. In Osmia adults, the exposure is mainly via ingestion of nectar, not pollen.

Response: Thanks very much. Because the adults mainly feed on nectar, not pollen, many studies used sucrose solution containing lethal medium concentrations of pesticide to evaluate the toxicity of pesticides to adult pollinators (such as Meikle et al., 2021). Larvae of *O. excavata* response was mainly via ingestion of pollen-nectar provision containing pesticide. In order to make this part clear, we have added "sucrose solution containing" in L207.

Reference:

Meikle, W.G., Adamczyk, J.J., Weiss, M., Ross, J., Beren, E. Sublethal concentrations of clothianidin affect honey bee colony growth and hive CO_2 concentration. Scientific Reports. 11(1), 4364 (2021). DOI: 10.1038/s41598-021-83958-8

Comment: L215-217: The reviewer disagrees that this test method can accurately determine toxicity to pesticides to larvae of O. excavata only weighting the bee provisions. To accurately quantity ingested dosages of pesticides both weight of diet (in this case provisions) and measurement of nominal tested concentrations using appropriate analytical methods are needed. These two measurements are normal practices in regulatory bee larvae studies (see OECD 237 and 239) and several bee adult studies (see OECD 245, 246 and 247).

Response: Thanks for your helpful comments. The main purpose of pesticide ecotoxicity test in this study is to guide the safe dose range for pesticide use in the field and prevent the adverse effects on non-target organisms. We believe that chemical analysis method is necessary to determine the pesticide content in provisions before the test, but it is not necessary to determine the concentration of each treatment during the test, because some samples can be lost during grinding and solvent dissolving operations, resulting in errors. A large number of similar studies also did not use chemical analysis methods to determine concentrations (Ceuppens et al., 2015; Jiang et al., 2019; Hedayati et al., 2019).

References:

Ceuppens, B., Eeraerts, M., Vleugels, T., Cnops, G., Roldan-Ruiz, I., Smagghe, G., 2015. Effects of dietary lambda-cyhalothrin exposure on bumblebee survival, reproduction, and foraging behavior in laboratory and greenhouse. J Pest Sci. 88 (4), 777–783.

Jiang, J., Liu, X., Huang, X., Yu, X., Zhang, W., Zhang, X., Mu, W., 2019. Comparative ecotoxicity of neonicotinoid insecticides to three species of *Trichogramma* parasitoid wasps (Hymenoptera: Trichogrammatidae). Ecotox Environ Safe. 183, 109587.

Hedayati, M., Sadeghi, A., Maroufpoor, M., A Güncan. (2019). Transgenerational sublethal effects of abamectin and pyridaben on demographic traits of *Phytonemus pallidus* (Banks) (Acari: Tarsonemidae). Ecotoxicology. 28, 467–477.

Comment: L219-222: Although the selection of provisions and females' larvae were made based on provision size and cell provision, these had to differ in size unavoidably. Such size difference increase background variability among tested individuals. One way to overcome the size difference in provisions may be mixing all individual provisions to form a common mass provision. In this way, both provision composition and levels of test solution could be homogenized.

Response: Thanks very much. We have tried to mix all individual provisions to form a common mass provision before this study, which may increased human error and lead to a higher mortality rate (about 70%) in the control treatment. Thus, we chosed the method in this study.

Responses to reviewer#3:

Comment: I have many concerns about the lack of details in this protocol and that there is no validation of the pesticide consistency and distribution in the provision masses. There is more work to be done for this method (and others already published) before any can satisfy the needs of regulatory agencies and researchers. I have attached an annotated PDF with comments to highlight these concerns.

Response: Thanks for your helpful comments. We have added details according to your suggestions. In the chronic toxicity test, provision of each treatment was almost completely eaten, then we believed that whether the solution is absolutely evenly distributed has little influence on the results. We randomly cut some provisions in the middle after immersion and found that the provisions in the middle were also moist. In addition, the larvae feed from the top down on the pollen, so we think the method of this study is feasible.

Comment: Other authors have already published on this topic. There is no validation (e.g. chemical analysis) of the delivery of pesticides throughout the provision masses, no true uniformity in provision sizes and quality (they are natural provision whose size and nutritional quality can vary annually or by crop due to resource availability), and no evidence that several classes of pesticides (as single a.i. or formulation) and required water, solvents, or other additives used with them will have the same kind of uptake as the one example given.

Response: Thanks very much.

a) In order to minimize error, we selected provisions based on provision size and cell position within the nest, which have been described in lines 101-103 (Select female larvae according to provision size and cell position within the nest. Then place pollen provisions of uniform size and selected female larvae respectively in petri dishes (60 mm diameter) and set aside for use), additionally, the volumes of insecticide were measured for each treatment including sixty pollen provisions with uniform size, instead of each provision being treated individually. The immersed volume of insecticide in each concentration including 60 pollen provisions has shown in below table. There were no significant difference between each treatment, which may also indicate little difference in provision size.

Table The immersed volume of insecticides in each treatment including 60 mass provisions.

Concentration (μg a.i. mL^{-1})		0.1	0.2	0.4	0.8	1.6	3.2	6.4
Immersed volume per treatment (mL)	1	1.23	1.18	1.19	1.18	1.24	1.19	1.24
	2	1.18	1.24	1.20	1.23	1.17	1.22	1.20
	3	1.22	1.21	1.23	1.20	1.21	1.19	1.17
	Mean±SD	1.21±002	1.21±0.02	1.21±0.01	1.20±0.01	1.21±0.02	1.20±0.01	1.20±0.02

b) The method in this study can determine the actual dose of pesticides by calculating the feeding amount of *O. excavata* (see detailed formula in the manuscript). We do not believe that the chemical analysis method is more accurate, because some samples can be lost during grinding and solvent dissolving operations, resulting in errors. The method in this study can also be used to guide the application of pesticides in the field. For example, in many pesticide toxicity tests, insects are treated by formulating and calculating gradient concentrations of pesticides, rather than using chemical analysis methods (Ceuppens et al., 2015; Jiang et al., 2019; Hedayati et al., 2019).

References:

Ceuppens, B., Eeraerts, M., Vleugels, T., Cnops, G., Roldan-Ruiz, I., Smagghe, G., 2015. Effects of dietary lambda-cyhalothrin exposure on bumblebee survival, reproduction, and foraging behavior in laboratory and greenhouse. J Pest Sci. 88 (4), 777–783.

Jiang, J., Liu, X., Huang, X., Yu, X., Zhang, W., Zhang, X., Mu, W., 2019. Comparative ecotoxicity of neonicotinoid insecticides to three species of *Trichogramma* parasitoid wasps (Hymenoptera: Trichogrammatidae). Ecotox Environ Safe. 183, 109587.

Hedayati, M., Sadeghi, A., Maroufpoor, M., A Güncan. (2019). Transgenerational sublethal effects of abamectin and pyridaben on demographic traits of *Phytonemus pallidus* (Banks) (Acari: Tarsonemidae). Ecotoxicology. 28, 467–477.

Comment: There are some problems with the writing and clarity. Details are missing for the figures. One reference is repeated.

Response: We apologize for the mistake. We have revised according to your suggestions. The detail changes can be found in the following parts.

Comment: L24 This makes no sense unless it refers to pesticides found in the provision mass that a bee collects for each larva. but this is not stated here.

Response: Thanks for your helpful comments. We have revised this statement, that is, changed "which is closely related to the collection rate of solitary bees, even the pollination efficiency of the adult from a demographic perspective" to "For the larvae of solitary bees, ingestion of provisions contaminated with pesticides may increase the mortality rate of the larvae, decrease collection rate and the population of adult solitary bees in the next year from a demographic perspective. But limited research has been found on the effects of pesticides on the larvae of solitary bees".

Comment: L26 I don't understand the meaning here. Does it mean the age of the adult pollinator? If so, I don't see how it relates to larval pesticide exposure and pollination efficiency.

Response: Thanks very much. Yes, it mean the age of the adult pollinator. In order to make this part clear, we have revised this statement, that is, changed "which is closely related to the collection rate of solitary bees, even the pollination efficiency of the adult from a demographic perspective" to "For the larvae of solitary bees, ingestion of provisions contaminated with pesticides may increase the mortality rate of the larvae, decrease collection rate and the population of adult solitary bees in the next year from a demographic perspective. But limited research has been found on the effects of pesticides on the larvae of solitary bees".

Comment: L29 This is nonsensical. One does not expose "toxicity of the pesticide." One can expose a larva to a toxic dose of pesticide that can be lethal or sublethal.

Response: We apologize for the mistake. We have changed "... present a method to expose the acute lethal toxicity of pesticide to the larvae of *Osmia excavata*" to "... present a method to expose the larvae of *Osmia excavata* to lethal or sublethal dose of pesticide".

Comment: L31 Wouldn't these parameters be co-variates of weight gain and development?
Response: Thanks very much. Even so, we believed that these indicators (food consumption and the efficiency of conversion of ingested food) can reflect whether pesticides have adverse effects on insect feeding and nutrient utilization.

Comment: L33 Solitary bee provisions are made of pollen and nectar. They are more correctly referred to as "pollen-nectar mass provisions" or "mass provisions" after the composition is defined.
Response: We apologize for the mistake. We have changed "pollen provisions" to "provisions" in the full manuscript.

Comment: L39 I this equating Osmia to honey bee pollination is only possible in some cases.
Response: Thanks for your helpful comments. We have revised this sentence according to your suggestions.

Comment: L43 In what country or region is it important. It is not found globally.
Response: Thanks very much. *Osmia excavata* has been considered an ideal species for apple pollination mainly in Asia, like in north and northwest China and Japan[2, 3, 4]. We have added related references about the geographical distribution of this species.

References:

Shu, G.W., Ren, W., Smirle, M.J., Huan, L.X. Release of *Osmia excavata* and *Osmia jacoti* (hymenoptera: megachilidae) for apple pollination. Canadian Entomologist. **134** (3), 369–380 (2002).

Men XY, et al. Biological characteristics and pollination service of Mason bee. *Chinese Journal of Applied Entomology.* **55** (6), 973–983 (2018).

Comment: L50 A naive reader would not understand this. Also, it seems to be the only artificial nest tunnel mentioned and is out of place among the natural ones (unless bamboo is also provided commercially).
Response: Thanks for your comments. The nests of O. excavata are usually found in preexisting holes, typically in dead wood, hollow plants, straw tubes, and bamboo stems in the natural condition rather than artificial nest tunnel. In order to make this part clear, we have revised this sentence and added a related reference.

References:

Men XY, et al. Biological characteristics and pollination service of Mason bee. *Chinese Journal of Applied Entomology.* **55** (6), 973–983 (2018).

Comment: L51 I am surprised that cell walls have leaf or mud. Unless the bees are patching up a hole or crevice, I don't know of other Osmia spp that line the walls. I referred to Rust 1976 (Biology of O. ribifloris in J Kansas Ent Soc) who cites Maeta 1978 about nest construction. There is no mention of the cell wall being covered.

Response: Thanks very much. We have deleted "Cell wall, nest plug and partitions are composed of leaf materials or mud"

Comment: L56 This is written above in a different manner.
Response: Thanks very much. We have deleted "After mating, the females collect pollen, build nests and lay eggs".

Comment: L62 To what does "it" refer? The decline in pollinators or the use of pesticides?
Response: Thanks. In this part, "it" refers to the use of pesticides. In order to make this part clear, we have deleted "it".

Comment: L64 Authors should also refer to EFSA Guidance Document on the risk assessment of plant protection products on bees (Apis mellifera, Bombus spp. and solitary bees) 2013
Response: Thanks very much. We have added this reference "European Food Safety Authority. EFSA Guidance Document on the risk assessment of plant protection products on bees (*Apis mellifera*, *Bombus* spp. and solitary bees). *EFSA Journal 2013*. **11** (7), 3295 (2013). DOI:10.2903/j.efsa.2013.3295".

Comment: L81 This seems out of place. It also needs a citation.
Response: Thanks very much. We have deleted "The mortality of *Osmia* directly caused by pesticides is most commonly considered, but the chronic toxicity such as larval weight gain, developmental duration, feeding patterns, eclosion ability, behavior and fecundity may be more harm from a demographic perspective and is often ignored..." to line 68-72, and added a citation.

References:
Yuan, R., et al. Toxicity and hazard assessment of six neonicotinoid insecticides on *Osmia excavata* (hymenoptera:megachilidae). *Acta Entomologica Sinica*. **61** (8), 950–956 (2018).

Comment: L71 More harm than what? ("More" implies that a comparison is being made.)
Response: Thanks for your comments. We have changed "" to "...have the same harm as the acute lethal toxicities...".

Comment: L71 Again the meaning is unlear.
Response: Thanks very much. We have deleted "from a demographic perspective".

Comment: L89 A hole (~.3 mm dia.) was punched into the
Response: Thanks very much. The sentence has been changed to "A hole (about 0.3 mm diameter) was punched into the lid of 2 mL centrifuge tube using a electric winding iron...".

Comment: L89 Such a centrifuge tube could then be used to maintain an O. excavata larva and its provision mass.
Response: Thanks for your helpful suggestion. The sentence has been changed according to your advice.

Comment: L93 Would this be different for a different pesticide? Do you already know how much ai is potentially toxic to the bee larvae?

Response: Thanks for your helpful comments. This would be same for a different pesticide. Because 1×10^4 μg a.i. mL^{-1} is a stock solution, the test concentration treatments for a different pesticide were much smaller than this concentration. The test concentrations were gradiently diluted on the basis of stock solution.

In order to determine the appropriate concentration of each pesticide, we did a pilot experiment before the formal experiment. Firstly, we determined the LD_{50} values of insecticides to other insects according to the references; second, we set a series of concentration gradients to conduct this study according to above LD_{50} values, and fond out the concentrations which resulted in mortality rates of *Osmia excavata* between 20% and 80%; thirdly, we set a series of concentration gradients (more than 5 concentration treatments) again between the above concentration range (resulting in 20-80% mortality rates), because the accuracy of LD_{50}/LC_{50} is the highest in this concentration range, then we began the formal experiment for acute lethal and sublethal tests.

Comment: L93 in a solvent? What was the solvent?

Response: Thanks very much. We have added the solvent, "in acetone".

Comment: L97 in which nests were made in paper tubes. (I assume this is true because you mention straw tubes earlier.) Perhaps the nests were in something else, and if so, you show state how you were able to get the provisions and eggs or larvae.)

Response: Thanks very much. Nests were made in plastic tubes. Plastic bee tubes containing provisions and newly hatched larvae of *O. excavata* were acquired from a mass-rearing program. In order to make this part clear, we have added related information.

Comment: L101 Where were larvae kept once separated from the provision?

Response: Thanks. Pollen provisions and selected larvae were respectively placed in Petri dishes (60 mm diameter), theses have described in line 103.

Comment: L101 An artist paint brush? A soft brush can be large.

Response: Thanks very much. The soft brush is bot large. Diameter of the brush: 0.3 mm, length of the brush: 2 cm, length of the pen: 18 cm. In order to make this part clear, we have added above information in line 105-106.

Comment: L103 Did you weigh the provisions to know the uniformity? Were the provisions also from among those made for female larvae?

Response: Thanks for your helpful comments. Yes, the provisions were weighed. We randomly selected 100 provisions for weighing, per provision weight was 0.2 ± 0.001 g. These provisions were also from those made for female larvae.

Comment: L103 Was there a limit on how long the provisions were left outside a container so that they would not dry out? Were they covered during this time? Where were larvae

Response: Thanks very much. It takes about 2 hours. They were covered the lid of the Petri dish during this time to avoid dry out. Larvae were placed in another Petri dishes, the young larvae eat very little and have little effect on them during this time.

Comment: L108 As a control check (CK), other selected provisions were soaked in 0.2% solvent.
Response: Thanks very much. We have advised according to your advise.

Comment: L112 Volume of what? The pesticide solution? This item needs a better description.
Response: Thanks for your helpful comments. We have changed to "the volumes of pesticide solution".

Comment: L116 Does this mean that the provisions were allowed to air dry prior to putting larvae onto them?
Response: Yes, the provisions were allowed to air dry to restore to the pre-infusion state.

Comment: L119 A "darkroom" refers to a room where light-sensitive film is developed. I think here you mean that the larvae were reared in darkness. Were they in an incubator or growth chamber, rather than a room?
Response: We apologize for the mistake. The larvae were reared in a growth chamber with darkness. We have advised related information.

Comment: L130 Define what is meant here: 48 hrs after larvae were placed onto treated and CK provision masses.
Response: Thanks very much. We have advised related information.

Comment: L132 This is an interesting way of determining the dose. I worry however, that immersion of the provision in the pesticide does not equally distribute the pesticide in the whole provision mass. Has there been a chemical analysis of the provisions to see if the pesticide has penetrated the provisions? Eeraerts et al. 2020 Apidologie use homogenized pollen from honey bees to feed to larvae. They can then mix the pesticide into the food and also make the provision uniform in size.
Response: Thanks for your comments. We have tried to mix all individual provisions to form a common mass provision before this study, which may increased human error and lead to a higher mortality rate (about 70%) in the control treatment. Sgolastra et al., (2015) also reported that provisions with man-made pollen-nectar mixtures would lead to high mortality rate because of human manipulation. Although we did not perform a chemical analysis, we randomly cut some provisions in the middle after immersion and found that the provisions in the middle were also moist. In addition, the larvae feed from the top down on the pollen, so we think the method of this study is feasible. For the chronic toxicity test, provision of each treatment was almost completely eaten, then we believed that whether the solution is absolutely evenly distributed has little influence on the results.

Comment: L132 How do you know that soaking the provision in pesticide solution for 10 s allowed the pesticide to be evenly distributed throughout the provision mass?
Response: Thanks for your comments. We randomly cut some provisions in the middle after

immersion and found that the provisions in the middle were also moist. In addition, the larvae feed from the top down on the pollen, so we think the method of this study is feasible. For the chronic toxicity test, provision of each treatment was almost completely eaten, then we believed that whether the solution is absolutely evenly distributed has little influence on the results.

Comment: L141 Did you know if the larvae were in the 2nd, 3rd, 4th or 5th instar? If not, how could you compare developmental rates to cocooning?
Response: Yes. Before the beginning of this study, we have observed and recorded the whole process of larval development in different instar, pupation and eclosion, as well as their duration.

Comment: L141 Were all larvae cocooned after 14 days? Did the weight of the cocoon matter?
Response: No, all larvae were not cocooned after 14 days. By this time the larvae have eaten all the food, and they're still in the larval stage. We did not weight of the cocoon matter.

Comment: L148 How did you see the eclosion to adult in the cocoon. Or by eclosion, do you mean emergence in the spring? Were the cocooned adults artificially wintered under controlled conditions and then warmed to emerge? If they did not emerge, was their life stage recorded (prepupa, pupa, adult) after opening the cocoon?
Response: Thanks very much. *O. excavata* would emerge into adults in late August every year and don't emerge from cocoon until the following spring. In this study, small scissors were used to snip the cocoons in late August and then the eclosion of *O. excavata* in each treatment was investigated. In order to make this part clear, we have added related information.

Comment: L157 These were the dilutions for the immersions, but not the doses per amount of provision, right. Wouldn't a treatment be what the larvae were actually fed?
Response: Thanks very much. These were the dilutions for the immersions. The actually fed doses have been used to calculate LD_{50} and determine relationship between ingested dosages and growth, development, feeding and eclosion rate of *O. excavata*, and these actually fed doses can be found in Figure 1-2.

Comment: L170 What is this?
Response: Thanks very much. We have changed "above index value" to "the index values of larval weight gain, consumption, ECI and eclosion rate".

Comment: L171 Above it was stated that there were treatments. In figure 1, I only see 5 values for the results, with the first one being the CK (0.00 dose). The legend for figure 1 does not explain that the numbers for each data point are averages with SD or SE. Does n = 60 at each point, or was there a lot of mortality with fewer larvae representing the data for the highest pesticide does?
Response: Thanks for your helpful comments.
In the acute lethal toxicity test, provisions were soaked in 7 diluted pesticide (0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 μg a.i. mL-1 chlorpyrifos) and 0.2% acetone (as a control group). These were used to calculate LD_{50}. In the sublethal toxicity test, larval weight gain, developmental duration, eclosion rate, consumption and ECI of *O. excavata* were evaluated under the soaking concentrations of 0.1,

0.2, 0.4 and 0.8 μg a.i. mL-1 chlorpyrifos. Thus, only 5 values for the results, with the first one being the CK (0 dose) in Figure 1-2.
The numbers for each data point are averages with SD.
We used 60 larvae × 3 replications = 180 larvae per concentration per insecticide . Although each replicate included 60 bees, the results were shown for each bee (n = 1 at each point). And we can see that from the equation in the manuscript.
In the acute lethal toxicity test, the mortality was about 80% under the highest soaking concentrations of 6.4 μg a.i. mL^{-1} abamectin. In the sublethal toxicity test, the mortality was less than 50% under the highest soaking concentrations of 0.8 μg a.i. mL^{-1} abamectin.
In order to make this part clear, we have added related information.

Comment: L181 Same missing information as in Fig 1.
Response: Thanks very much. We have added related information.

Comment: L200 This could only be understood by someone familiar with the management of solitary bees for commercial pollination.
Response: Thanks. In order to make this part clear, we have changed "cocoon recovery rate" to "eclosion rate".

Comment: L200 This is only one example for *O. excavata*. Is this topic of this sentence meant for only one species or for many solitary species?
Response: Thanks very much. The topic of this sentence meant for only *O. excavata*. In order to make this part clear, we have changed "solitary bees" to "*O. excavata*".

Comment: L201 Proper application is also likely to be harmful is no risk assessment has prohibited application where bees are flown.
Response: Thanks for your helpful comments. We have deleted "improper".

Comment: L207 This paragraph needs a lot more info for the naive reader. For a thorough review of this problem about pesticides for solitary bees, see: Kopit, A. M., and T. L. Pitts-Singer. 2018. Routes of pesticide exposure in solitary, cavity-nesting bees. Environ. Entomol. 47: 499–510.
Response: Thanks for your helpful suggestion. We have added more information about pesticides for solitary bees, that is, "The main routes of pesticide exposure to solitary bees were larval or adult ingestion, contact, and transovarial transmission"in line 208-209 and cited this reference.

Comment: L215 Are you sure the parts eaten by the larvae were equally dose, and were they able to avoid portions with the highest concentration? I don't understand the juxtaposition here.
Response: Thanks for your helpful comments. The implication of this statement is that it is better to evaluate the ecotoxicity of pesticides to *O. excavata* by calculating the pesticide dose ingested by larvae than by using the pesticide concentration for immersion. In order to make this part clear, we have revised this statement.

Comment: L221 Already stated in introduction.
Response: Thanks very much. We have deleted repeated statement.

Comment: L227 Indeed, for *Osmia* I know, one provision mass represents one day of collecting nectar and pollen to make one provision mass. Therefore, time since the spray is important be cause newly opened flowers will not be contaminated unless the pesticide is systemic.
Response: Thanks very much. Many commonly used insecticides are systemic, such as organophosphorus, organic nitrogen, carbamates. Therefore, determining the effect of pesticide on *O. excavata* is very important in enhancing the pollination value of *O. excavata*.

Comment: L232 This assumes that the pesticide is equally distributed in the provision masses and has equal uptake. None of the data for uptake of pesticide after immersion was shown in this demonstration.
Response: Thanks for your helpful comments. We have changed "... accurately determine the acute lethal toxicity of pesticides to ..." to "... predict the toxicity of pesticides to ...".

Comment: L259 This reference is the same as #11.
Response: We apologize for the mistake. We have revised the reference.

Concentration (μg a.i. mL^{-1})		0.1	0.2	0.4	0.8	1.6	3.2	6.4
Immersed volume per treatment (mL)	1	1.23	1.18	1.19	1.18	1.24	1.19	1.24
	2	1.18	1.24	1.20	1.23	1.17	1.22	1.20
	3	1.22	1.21	1.23	1.20	1.21	1.19	1.17
	Mean±SD	1.21±0.02	1.21±0.02	1.21±0.01	1.20±0.01	1.21±0.02	1.20±0.01	1.20±0.02