Video Article

In Vitro Rearing of Solitary Bees: A Tool for Assessing Larval Risk Factors

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Abstract

Although solitary bees provide crucial pollination services for wild and managed crops, this species-rich group has been largely overlooked in pesticide regulation studies. The risk of exposure to fungicide residues is likely to be especially high if the spray occurs on, or near host plants while the bees are collecting pollen to provision their nests. For species of *Osmia* that consume pollen from a select group of plants (oligolecty), the inability to use pollen from non-host plants can increase their risk factor for fungicide-related toxicity. This manuscript describes protocols used to successfully rear oligolectic mason bees, *Osmia ribifloris* sensu lato, from egg to prepupal stage within cell culture plates under standardized laboratory conditions. The *in vitro*-reared bees are subsequently used to investigate the effects of fungicide exposure and pollen source on bee fitness. Based on a 2 × 2 fully crossed factorial design, the experiment examines the main and interactive effects of fungicide exposure and pollen source on larval fitness, quantified by prepupal biomass, larval developmental time, and survivorship. A major advantage of this technique is that using *in vitro*-reared bees reduces natural background variability and allows the simultaneous manipulation of multiple experimental parameters. The described protocol presents a versatile tool for hypotheses testing involving the suite of factors affecting bee health. For conservation efforts to be met with significant, lasting success, such insights into the complex interplay of physiological and environmental factors driving bee declines will prove to be critical.

Video Link

The video component of this article can be found at <https://www.jove.com/video/57876/>

Introduction

Given their role as the dominant group of insect pollinators¹, the global loss in bee populations poses a threat to food security and ecosystem stability^{2,3,4,5,6,7}. The declining trends in both managed and wild bee populations have been attributed to several shared risk factors including habitat fragmentation, emerging parasites and pathogens, loss of genetic diversity, and the introduction of invasive species^{3,4,7,8,9,10,11,12}. In particular, the dramatic increase in the use of pesticides, (e.g., neonicotinoids) has been directly linked to detrimental effects among bees^{13,14,15}. Several studies have shown that the synergism between neonicotinoids and ergosterol-biosynthesis-inhibiting (EBI) fungicides can lead to high mortality across multiple bee species^{16,17,18,19,20,21,22}. Nevertheless, fungicides, long considered to be 'bee-safe', continue to be sprayed on in-bloom crops without much scrutiny²³. Foraging bees have been documented to routinely bring back pollen loads contaminated with fungicide residues^{24,25,26}. The consumption of such fungicide-laden pollen can cause high mortality among larval bees^{27,28,29,30}, and a suite of sub-lethal effects among adult bees^{16,31,32,33,34}. A recent study suggests that fungicides may cause bee losses by altering the microbial community within hive-stored pollen, thereby disrupting the critical symbioses between bees and pollen-borne microbes³⁵.

Although solitary bees are vital for the pollination of several wild and agricultural plants^{36,37,38}, this diverse group of pollinators has received much less attention in pesticide monitoring studies. The nest of an adult solitary female contains 5-10 sealed brood chambers, each stocked with a finite mass of maternally-collected pollen and nectar, and a single egg³⁹. After hatching, the larvae rely on the allocated pollen provision, and the associated pollen-borne microbiota to obtain adequate nutrition^{40,41}. Because they lack the benefits of a social lifestyle, solitary bees may be more vulnerable to pesticide exposure⁴². For instance, while deficits in social bees following a spray may be compensated to some extent by workers and newly emerging brood, the death of a single adult solitary female ends all reproductive activity⁴³. Such differences in susceptibility highlight the need to incorporate diverse bee taxa in ecotoxicological studies to ensure adequate protection for managed and wild bees alike. However, aside from a handful of studies, investigations into the effects of fungicide exposure has primarily focused on social bees^{18,23,32,44,45,46,47,48,49}.

Solitary bees belonging to genus *Osmia* (Figure 1) have been used worldwide as efficient pollinators of several important fruit and nut crops^{39,50,51,53,53}. As with other managed pollinator groups^{24,54,55,56,57,58}, adult *Osmia* bees are routinely exposed to fungicides sprayed on in-bloom crops⁴⁴. Adult females foraging on recently sprayed crops may collect and stock their brood chambers with fungicide-laden pollen,

which later forms the sole diet for the developing larvae. Consuming the contaminated pollen provisions can subsequently expose the larvae to fungicide residues⁴². The risk of exposure may be higher among oligolectic species that forage only on a few closely related host plants^{59,60,61}. Certain megachilid bees, for example, appear to preferentially forage for low-quality Asteraceae pollen, as a means of reducing parasitism⁶². However, the extent to which fungicides impact larval fitness among oligolectic solitary bees has not been empirically quantified. The goal of this study is to develop a protocol to test the main and interactive effects of fungicide exposure and pollen source on the fitness of *in vitro* reared solitary bees. To investigate, eggs of *O. ribifloris* sensu lato (s.l.) can be obtained commercially (Table of Materials). This population is ideal because of its importance as a native pollinator, and its strong predilection for the nectar-rich *Mahonia aquifolium* (Oregon grape) found within the region^{53,63,64} (Figure 2).

Figure 1. A high-resolution photo of an adult *Osmia ribifloris*. Photo credit Dr. Jim Cane, Research Entomologist, USDA-ARS [Please click here to view a larger version of this figure.](#)

Figure 2. Phragmite nesting reeds of *Osmia ribifloris* (s.l.) with a nesting female in the foreground. Chamber partitions and terminal plugs for the reeds are constructed from masticated leaves. Photo credit Mr. Kimball Clark, NativeBees.com [Please click here to view a larger version of this figure.](#)

The first objective of this study is to evaluate the effect of consuming fungicide-treated pollen on larval fitness (measured in terms of development time and prepupal biomass). While exposure to the commonly applied fungicide propiconazole has been linked to increased mortality among adult bees across several species^{23,24,32,44,45,54,55,56,57,58,65,66,67}, its impact on larval bees is less known. The second objective of this study is to evaluate the effects of consuming non-host pollen on larval fitness. Previous studies indicate that larvae of oligolectic bees fail to develop when forced to consume non-host pollen⁶⁸. Such results may be attributed to variations in bee physiology⁶⁹, pollen biochemistry⁷⁰, and the beneficial microbiome associated with natural pollen provisions⁷¹. The third objective of this study is to evaluate the interactive effects of fungicide treatment and dietary pollen on larval fitness.

Numerous biological traits including maternal body size, provisioning rate, foraging strategy, and pollen quantity^{72,73,74,75} are known to affect larval fitness among solitary bees. These factors can introduce significant variability between reeds, which poses a challenge in developing defensible experimental designs when assessing larval health. Moreover, given that larval development occurs inside sealed nesting reeds, the effects of such variability on the progeny are difficult to visualize and quantified without using non-lethal techniques (**Figure 3**). To overcome this challenge, all hypotheses within this study are tested using larvae reared outside of their nesting reeds. The experimental design represents

a fully crossed 2×2 factorial set-ups, with each factor consisting of 2 levels; Factor 1: Fungicide exposure (Fungicide; No fungicide); Factor 2: Pollen source (Host pollen, Non-host pollen). Bees are raised from the egg to the prepupal stage within sterile multiwell cell culture plates under controlled laboratory conditions. Each well is individually stocked with a standardized amount of pollen provision and a single egg. After hatching, the larva feeds on the allocated pollen within the well, completes larval development, and initiates pupation. Past studies have shown that unexplained mortality is lower among bees raised within this artificial rearing environment than that encountered in the wild^{49,76}. The use of *in vitro*-reared bees has several advantages over field-based studies: 1) it minimizes the confounding effects of natural variability and uncontrolled factors typically associated with field-based studies; 2) it allows multiple levels of manipulation for each factor(s) of interest to be tested simultaneously across treatment groups; 3) the number of replicates can be predetermined, and experimental factors for each replicate can be individually manipulated; 4) larval response variables can be easily visualized and recorded independently without disturbing adjacent larvae; 5) the protocol can be modified to accommodate more complex experimental designs involving multiple factors and response variables.

Figure 3. Contents within a natural nesting reed of *Osmia ribifloris* (s.l.). Close up of (A) a dissected reed showing individual chambers, pollen provisions, and partitions, and (B) freshly harvested pollen provisions, and the associated eggs (indicated with a black circle). [Please click here to view a larger version of this figure.](#)

Protocol

1. Prepare Propiconazole Solutions for Fungicide Exposure Experiments

1. Prepare 0.1x fungicide solution by dissolving appropriate volumes of commercially purchased propiconazole 14.3% in sterile water the day of the experiment. Ensure that only freshly prepared fungicide solution is used for all treatments.
2. Add 23 μL of 0.1x fungicide solution per gram of pollen provision to obtain the maximum concentration of propiconazole previously reported from bee-collected pollen²⁴ (0.361 PPM or μg of active ingredient g^{-1} of pollen).

2. Harvest Eggs and Host Pollen Provisions from *Osmia* Reeds

1. Using a sterilized scalpel, dissect freshly plugged nesting reeds of *Osmia*, splitting it into two parts along the length of the reed to expose the individual chambers.
NOTE: Each nest may contain between 8 to 14 chambers and a single egg within a chamber.
2. Inspect the reeds visually to identify the chambers containing male eggs based on previously published guidelines⁷⁷. Use a sterilized bent needle to remove each pollen provision along with the associated egg from the nesting reed, and place in a clean weigh boat.
3. Gently separate the egg from the provision using a clean fine paint brush and record the fresh weight of the pollen provision and egg using a standard laboratory balance. Calculate the average weight of the male pollen provisions.
4. Perform the subsequent steps with minimum delay to reduce the chances of damage to the egg from exposure to excess temperature and dehydration.

3. Prepare Host Plant Pollen Provisions

1. Visually inspect the maternally collected host-plant pollen excavated from the nesting chambers to ensure that no parasites are present⁷⁸. In order to reduce any potential maternal bias, combine the pollen provisions into a single mass in a sterile petri dish and mix well using a sterilized needle.
2. Divide the combined mass into new pollen provisions, ensuring that the weight of each reconstituted provision is approximately equal to the average weight of a naturally allocated male provision (Mean $\pm$ SE, 0.35 ± 0.01 g, $N = 42$).
NOTE: Because *Osmia* sp. allocates smaller pollen provisions to the male offspring, this results in lower body weights of the male larvae compared to that of females⁷⁷. To avoid any such bias resulting from sex-specific differences, only use male eggs in the experiments.

4. Prepare Non-Host Plant Pollen Provision

1. Pulverize commercially purchased honey bee-collected pollen to a fine powder using a standard laboratory ball-mill.
2. Based on the moisture content of maternally-collected host pollen provisions (~20%), hydrate the pollen powder using appropriate volumes of 40% sterilized sugar solution⁷⁹ and mix thoroughly to form a dough-like consistency.
3. Divide into individual pollen masses, each weighing approximately the same as the average weight of a naturally allocated male provision.
NOTE: Moisture content of maternally collected host pollen provisions can be standardized in prior by comparing the fresh and dry weight of pollen provisions from 30 randomly selected male chambers⁸⁰. To obtain the dry weight, pollen provisions should be freeze-dried in a lyophilizer (1.5 Pa for 72 h).

5. Prepare Multiwell Cell Culture Plates

1. Line individual wells of sterile 48-well culture plate with autoclaved tin cups (5×9 cm). Using sterile forceps, gently flair out the top rim of the capsule so that it may accommodate the pollen provision.
2. Place a single mass of host or non-host pollen provision inside the tin cup using sterile tools based on the treatment group.
NOTE: To avoid cross-contamination, use separate plates for treatment and control groups.

6. Add Fungicides

1. Make a centrally placed depression within the pollen mass using a sterile wooden stick. Use a new stick for each pollen provision.
2. Add appropriate volumes of fungicide solution (for treatment), or sterile water (for controls) into the depression. Pinch the opening of the depression using sterile forceps to minimize surface contact between the fungicide/ sterile water and the egg.
3. Ensure that the factorial set-up of the experimental design aligns with that depicted in the schematic representation (Figure 4).

Figure 4. Schematic representation of the experimental setup. The experiment represents a fully-crossed 2×2 factorial setups. Factor 1 represents Fungicide exposure and consists of 2 levels: (i) No fungicide ($N = 10$), and (ii) Fungicide ($N = 10$). Factor 2 represents Pollen source and consists of 2 levels: (i) Host pollen ($N = 8$), and (ii) Non-host pollen ($N = 8$). [Please click here to view a larger version of this figure.](#)

7. Rear and Observe Larvae

1. Place a randomly selected male egg on the top surface of the pollen provision using a clean fine paint brush. Once eggs have been placed on all the provisions, replace the lid of the cell culture plate, securing it with labeling tape on the corners.
 2. Place the well plates on a clean tray and cover it with a dark cloth to obstruct contact with direct light. Place a 6 well plate containing 30 mL of sterile water within the tray to prevent desiccation. Leave incubation trays undisturbed inside an incubator at room temperature.
 3. Observe well plates daily under a dissecting microscope without removing the lid of the well plates. Ensure that the larvae are alive by checking for movement. If no movement is detected, discard the tin cup containing the dead larvae and the remaining pollen provision. Allow all surviving larvae to develop undisturbed within the well plates till they reach the prepupal stage.
 4. Remove the larva from the tin cup once it reaches the prepupal stage⁴¹. Use a brush to clean any defecate from the silk cocoon. Carefully cut through the silk cocoon using a dissecting microscope and extract the prepupa with rubber forceps.
 5. Handle the prepupa gently to ensure that the tools do not pierce the soft body. Record the fresh weight of the prepupa (prepupal biomass) and the developmental time from egg to the prepupal stage (larval developmental time).
- NOTE: Any dead larva should be discarded immediately to prevent undesired microbial growth on the cadaver and leftover pollen provision. This reduces the risk of infection to the remaining healthy larvae.

Representative Results

Larval fitness was quantified using three metrics (i) larval developmental time, (ii) prepupal biomass, and (iii) percent survivorship. A two-way ANOVA was conducted using Fungicide exposure (two levels: No fungicide, Fungicide) and Pollen source (two levels: Host pollen, Non-host pollen) as the independent variables, and larval developmental time as the dependent variable. The main effect for Fungicide exposure ($F_{1,28} = 1.24$, $P = 0.28$) was non-significant between the fungicide-treated (Mean $\pm$ SE) (28.14 ± 1.98 d, $N = 14$), and untreated (25.39 ± 1.65 d, $N = 18$) groups. The main effect for pollen source however, indicated a significant difference between developmental time for larvae raised on host pollen (20.00 ± 0.50 d, $N = 16$) and non-host pollen (33.19 ± 0.81 d, $N = 16$) ($F_{1,28} = 179.83$, $P < 0.001$). Bonferroni corrected Post-hoc comparisons indicated that larval developmental time did not vary significantly between fungicide-treated and untreated groups raised on host ($P = 0.57$) and non-host ($P = 0.32$) pollen. However, larval developmental time was significantly shorter for larvae raised on host pollen compared to non-host pollen for both fungicide-treated ($P < 0.001$) and untreated ($P < 0.001$) pollen. The interaction effect (Fungicide exposure $\times$ Pollen source) was not significant ($F_{1,28} = 0.09$, $P = 0.77$). The analysis was repeated using prepupal biomass as the dependent variable. The main effect for Fungicide exposure indicated a significant difference ($F_{1,28} = 4.66$, $P = 0.04$) between the fungicide-treated (0.123 ± 0.01 g, $N = 14$), and untreated (0.149 ± 0.01 g, $N = 18$) groups. The main effect for Pollen source ($F_{1,28} = 56.30$, $P < 0.001$) indicated a significant difference between the larvae raised on host pollen (0.170 ± 0.01 g, $N = 16$) and non-host pollen (0.105 ± 0.01 g, $N = 16$). Bonferroni corrected Post-hoc comparisons indicated that prepupal biomass did not vary significantly between fungicide-treated and untreated groups raised on host ($P = 0.22$) and non-host ($P = 0.08$) pollen. However, prepupal biomass was significantly higher among larvae raised on host pollen compared to non-host pollen for both fungicide-treated ($P < 0.001$) and untreated ($P < 0.001$) pollen. The interaction effect (Fungicide exposure $\times$ Pollen source) was not significant ($F_{1,28} = 0.132$, $P = 0.72$). **Figure 5** and **Figure 6** are graphical representations of results obtained from the aforementioned analysis. Independent samples t -test indicated a significant effect of pollen source on larval survivorship ($N = 18$, $t_9 = -2.45$, $P = 0.04$).

Figure 5. Bar graph showing metrics for larval fitness based on larval developmental time and prepupal biomass. Larval fitness metrics are clustered based on (A) and (B) Pollen source; and (C) and (D) Fungicide exposure. (Mean $\pm$ 1 SE). *** $P < 0.001$ [Please click here to view a larger version of this figure.](#)

Figure 6. Interaction plot for larval fitness metrics. Interactive effects of Fungicide exposure and Pollen source on (A) larval developmental time, and (B) prepupal biomass. (Mean $\pm$ 1 SE). [Please click here to view a larger version of this figure.](#)

Pearsons correlation was used to explore the relationship between larval developmental time and prepupal biomass (**Figure 7**). A significant negative correlation was noted across all treatment groups ($r = -0.83$, $P < 0.001$, $N = 32$), and across fungicide treatments (No fungicide: $r = -0.76$, $P < 0.001$, $N = 18$; Fungicide: $r = -0.92$, $P < 0.001$, $N = 14$). While there was a significant negative correlation for larvae raised on non-host pollen ($r = -0.64$, $P < 0.01$, $N = 16$), no such relationship was observed for larvae raised on host-pollen ($r = -0.01$, $P = 0.98$, $N = 16$).

Figure 7. Relationship between larval developmental time and prepupal biomass. Pearson correlation between developmental time and prepupal biomass across **(A)** all treatment groups ($P < 0.001$) **(B)** Pollen source (Host pollen: $P = 0.98$, Non-host pollen: $P < 0.01$); **(C)** Fungicide exposure (No fungicide: $P < 0.001$, Fungicide: $P < 0.001$). For panels **(B)** and **(C)**, trendlines are color-matched with symbols in the figure legend. [Please click here to view a larger version of this figure.](#)

Animated Figure 1. Fifth stage larval instar of *O. ribifloris* within a single well of a multiwell plate. The larva is noted to have started spinning a silken cocoon in preparation for pupation. [Please click here to view this video.](#) (Right-click to download.)

Discussion

Rearing bees outside their natural nesting reeds, under laboratory conditions, allows the testing of multiple hypotheses pertaining to larval fitness. To the extent that unidentified factors continue to cause bee mortality, risk assessment studies using *in vitro* experiments can help identify potential threats and inform management practices for this species-rich group of wild pollinators^{12,38,49,76,81,82}.

In general, shorter developmental times and higher prepupal biomass is associated with higher larval fitness. Across all treatments, duration of larval development was negatively correlated with prepupal biomass. However, there were significant differences between the duration of larval development and prepupal biomass across the four groups. Larvae that did not receive any fungicides and were allowed to consume host pollen had the shortest larval developmental time, and highest prepupal biomass. In contrast, those larvae that consumed fungicide-treated non-host pollen, took the longest to complete larval development, and had the lowest prepupal biomass. However, the trends for larval survivorship were less clear, with mortality being noted only for the fungicide-treated group raised on host pollen. Deconstructing the main and interaction effects of Fungicide exposure and Pollen source revealed that: (i) The main effect of consuming fungicide-treated pollen had a significant negative impact on prepupal biomass, but not larval developmental duration. While a previous study has demonstrated acute oral toxicity in adult *Osmia* at higher concentrations, these results suggest that oral exposure to propiconazole at far lower concentration can affect fitness by reducing prepupal biomass²³. (ii) The main effect of consuming non-host pollen has an adverse effect on larval fitness. These results are consistent with previously published studies that suggest pollen quality (i.e., presence of toxins, protective compounds, lack of essential nutrients), and differences in bee physiology can restrict bees from utilizing non-host pollen⁶⁸. It is also likely that the absence of beneficial microbiota typically acquired from host-pollen and/or bee crop may exacerbate this effect. (iii) There was no significant interaction between Fungicide exposure and Pollen source on larval fitness. Across both fungicide-treated and untreated groups, larvae consuming non-host pollen had significantly lower prepupal biomass and longer larval developmental time compared to larvae raised on host pollen. Regardless of pollen source, larvae consuming fungicide-treated provisions had significantly lower prepupal biomass compared to larvae raised on untreated pollen. Aside from the significant main effects, the interactive effect of both factors conformed to the additive model, i.e., the negative effects of fungicide exposure and pollen type on larval fitness were not synergistic. Uncertainties arising from the interplay of various determinants of bee health (as detailed here), constrain the effectiveness of pollinator management strategies. By helping predict the interactive effects of multiple risk factors, results from similar laboratory-based experiments can help circumvent this long-standing challenge in bee conservation efforts.

There are several critical steps within this protocol that can influence the outcome of the experiment. Whenever possible, it is advisable to use organic pollen that is free of pesticide residues. Using pollen from unknown sources increases the risk of contamination with various agrochemicals, which can confound experimental findings. It is important to obtain freshly plugged *Osmia* nesting reeds so that only unhatched eggs or very young larvae are used in the study. This ensures that the larvae are raised almost entirely on the intended pollen treatment type. The nesting reed should be dissected using a shallow incision, failing which the pollen provisions and eggs may be damaged during harvesting. Once eggs have been removed they must be handled gently to prevent damage and retained in the weigh boats in a cool dark hygienic environment (e.g., biosafety cabinet) till they are transferred. This holding time should be kept to a minimum (< 30 min) to ensure that the quality of the egg is not compromised. All procedures should be performed in a biosafety cabinet to ensure a clean working environment and reduce chances of contamination. To ensure their efficacy, only use freshly prepared fungicide solutions for treatments. Given that pollen is hydrophobic, the fungicide solution/ sterile water should be introduced into the depression made within pollen provisions. This maximizes the volume of liquid permeating through the provision. However, it is important that the depression does not pierce the entire depth of the provision, as it would

result in loss of volume from the solution adhering to the capsule floor. The individual treatments and controls should be conducted in separate well plates to reduce chances of cross-contamination from volatile compounds and/or pollen-borne microbiota. Pieces of folded tape should be attached to the edges of the plate to allow sufficient air gap once the lids are in place. During daily observations, the plates should be handled gently to minimize disturbance to the larvae. Observations should be made under the microscope with minimum light intensity, and the lids should not be removed unless to discard dead larvae. In case of unexpected widespread mortality, both larvae and their pollen provisions must be visually inspected to check for signs of infection and infestation. The well plates containing the compromised replicates should be immediately discarded, the workspace disinfected, and tools sterilized to prevent the spread of infection.

Notwithstanding its broad-ranging application, there are certain limitations to this method. For instance, while it is best practice to use organic pollen whenever available, raising plants within a greenhouse setting to yield sufficient amounts of pure uncontaminated pollen is logistically prohibitive. In such cases, wild-collected pollen may be used, provided that it is screened for the presence of pesticide residues. Another strategy to reduce the risk of contamination when using wild-collected pollen is to obtain pollen from a source that is less likely to have been sprayed (e.g., pristine undisturbed areas located far from agricultural farms). The host pollen used in this study was obtained from nesting reeds that were placed in natural woodlands and grasslands surrounding the foothills of the Wasatch Range near Kaysville, Utah. Given that this region is dominated by wild, unmanaged natural forests far from any commercial agricultural areas, and that these bees do not fly as far as honey bees when foraging^{83,84,85}, it is extremely unlikely that the pollen they collected would have been sprayed. Thus, pesticide residues within the pollen collected here are likely to be trivial. Foraging in such wild landscapes, the adult female is less likely to encounter contaminated pollen, reducing the risk of exposure among larvae. The commercially purchased honeybee pollen used in this study is collected from natural forested areas in northern Wisconsin and Michigan. Marketed for human consumption, publicly available information and personal communication with the supplier indicates that the hives are not chemically treated, and the pollen is sold in its natural, raw form without any modifications⁸⁶. Therefore, it is reasonable to assume that the contaminant load in the commercially purchased honeybee pollen would be minimal. For studies that do not obtain pollen from unmanaged areas with wild natural vegetation, it is advisable to have direct empirical evidence from pollen chemistry analysis to ensure that pollen used in risk assays is contaminant free. Another limitation involves the contrivance introduced by the artificial rearing environment. Despite best efforts, it is not logistically feasible to replicate the exact microenvironment within a natural nesting reed (e.g., moisture, oxygen concentration, the three-dimensional structure of individual chambers), which may impact larval fitness to unknown degrees. To defensibly simulate the characteristics of the natural diet, preliminary data from nesting reeds must be obtained prior to *in vitro* diet manipulation studies. Although the non-host pollen used in this study is obtained from areas where the Oregon grape is absent or rare⁸⁷, there may be traces of host pollen mixed within the commercially purchased pollen, potentially affecting the results. Another drawback of this technique is that handling stress during the experimental period may cause adverse effects on the bees. Finally, while it is common to encounter unhatched eggs in nature⁶³, under laboratory conditions it is difficult to ascertain whether the failure to hatch was due to handling stress, experimental treatment, or a result of natural causes. Since these factors may introduce unknown degrees of bias into the study, one must use caution while interpreting the results obtained.

By controlling for factors that can demonstrably bias experimental outcome (e.g., maternal foraging efficiency⁷⁵, sex-specific variations⁷⁷, and dietary pollen^{68,72}), the described protocol provides substantial improvements over previously published techniques⁷⁶, and offers a more rigorous framework for hypotheses testing. For instance, using *in vitro*-reared bees allows investigations into sex-specific responses to xenobiotic treatment, which would otherwise be challenging to study among wild populations of cavity-nesting bees^{49,88}. The potential for manipulating and testing multiple interacting factors (e.g., diet quality and quantity, diet-associated microbiota, exposure to synergistic pesticides), can provide valuable insights into the key determinants of bee fitness. The flexibility of the protocol readily allows modifications (e.g., using different sized well plates to accommodate larvae of varying sizes, changing the type and amount of diet), making it amenable for use with several other species of solitary bees and wasps⁷⁶. While this study evaluated fitness based on the development and survivorship at the feeding stage, insects can be incubated until emergence to obtain additional data on emergence rate, food-to-body conversion, and post-emergence longevity⁴⁹. This information can help assess the sub-lethal effects of treatments in toxicity assays. With increasing interest in the function of the pollen-microbiome, laboratory-based studies can identify sensitive versus resistant bee species based on their pollen microbiota⁷¹. Differences in fungicide susceptibility across bee groups may be explored by sequencing the microbiota within hive-stored pollen. This can help ascertain the role of the pollen microbiome in conferring varying degrees of resistance to xenobiotic stressors. Future studies may also help identify differences between the natural microbiota of host and non-host pollen, which may serve as an underlying factor driving oligolectic behavior within select bee species.

The *in vitro* rearing of larval solitary bees may help control for the natural variability experienced in the wild, thereby delineating the role of individual and interacting factors in affecting bee fitness. This accessible and inexpensive technique expands the entomologists' toolkit by allowing for the manipulation of multiple parameters, which can be easily modified to address specific research objectives. If evidence from *in vitro* experiments can help identify at-risk populations, the impact on bee conservation strategies will be substantial.

Disclosures

The authors have nothing to disclose.

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