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ORIGINAL RESEARCH ARTICLE

Protocol for the *in vitro* rearing of honey bee (*Apis mellifera* L.) workers

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The *in vitro* rearing of worker honey bees (*Apis mellifera* L.) has become an increasingly important method in honey bee research in general, and in pesticide risk assessment specifically. Authorities from the European Organization for Economic Co-operation and Development and the United States Environmental Protection Agency are requesting data on pesticide impacts on immature bee survivorship prior to registering new crop protection products. Those using the current *in vitro* rearing protocols have had variable success with immature bee survival and protocol repeatability. Here, we present an improved method for the *in vitro* rearing of worker honey bees from larvae to adult emergence. We have achieved consistently high survival (>95%) in our control and solvent-control rearing trials. Changes in the proportion of diet components, royal jelly source, maintenance of the developing bee, and rearing environment are the main contributors for our high rearing success and are discussed herein. Our *in vitro* rearing protocol can be implemented as the standard protocol to determine the impact of pesticides on immature bees because of the protocol's high control survivability, ease in end point determination, and high overall repeatability.

Protocolo para la cría in vitro de obreras de *Apis mellifera*

La cría in vitro de las abejas obreras (*Apis mellifera* L.) se ha convertido en un método cada vez más importante en la investigación de la abeja de la miel en general, y específicamente, en la evaluación del riesgo de los plaguicidas. Las autoridades de la Organización Europea para la Cooperación y el Desarrollo, y la Agencia de Protección Ambiental de los Estados Unidos están solicitando datos sobre los efectos de los plaguicidas en la supervivencia de abejas inmaduras antes de registrar nuevos productos para la protección de los cultivos. Aquellos que utilizan actualmente los protocolos de la cría *in vitro* han tenido un éxito variable en la supervivencia de abejas inmaduras y la repetitividad del protocolo. Aquí presentamos un método mejorado para la cría *in vitro* de las abejas obreras a partir de larvas para la eclosión de los adultos. Hemos logrado consistentemente una alta supervivencia (>95%) en nuestro control y en los ensayos de cría con el disolvente del control. Los cambios en la proporción de los componentes de la dieta, la fuente de jalea real, el mantenimiento de las abejas en desarrollo, y el ambiente de cría son los principales constituyentes para nuestro alto éxito de cría y son discutidos en este trabajo. Nuestro protocolo de cría *in vitro* puede ser implementado como el protocolo estándar para determinar el impacto de los plaguicidas en las abejas inmaduras debido a la alta capacidad de supervivencia del control, la facilidad en la determinación del punto final, y la alta capacidad de repetitividad.

Keywords: honey bee; *Apis mellifera*; *in vitro* rearing protocol; artificial diet; pesticides

Introduction

The western honey bee (*Apis mellifera* L.) is an important pollinator species worldwide (Gallai, Salles, Settele, & Vaissière, 2009; Klein et al., 2007). Pesticides, nutrition, parasites, and/or diseases are thought to be major contributors to recent honey bee declines (Fairbrother, Purdy, Anderson, & Fell, 2014; National Agricultural Statistics Service, 2015; Neumann & Carreck, 2010; Staveley, Law, Fairbrother, & Menzie, 2014; Vanbergen et al., 2013). However, it is difficult to isolate the adverse impact of most of these stressors on honey bee health, particularly on immature honey bee health, because of the complexity associated with investigating multiple stressors simultaneously and the lack of quality, repeatable bioassays with which to do the work.

Substantial progress has been made with bioassays that can be used to rear immature honey bees in the laboratory over the past few decades (Aupinel et al., 2005; Crailsheim et al., 2013; Huang, 2009; Peng, Mussen, Fong, Montague, & Tyler, 1992; Rembold & Lackner, 1981; Vandenberg & Shimanuki, 1987). The *in vitro* methodology for workers was initially used to investigate queen/worker differentiation, nutrition, and pathogen impacts on immature bees (Rembold & Lackner, 1981; Vandenberg & Shimanuki, 1987). Rembold and Lackner (1981) were among the first to feed larvae a balanced diet of sugars, royal jelly, water, and yeast in order to rear larvae successfully within the laboratory. Vandenberg and Shimanuki (1987) further improved the *in vitro* methodology by optimizing diet composition and

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the amount of provisioned diet, both of which yielded higher rates of adult emergence and less queen intermediates. Peng et al. (1992) began utilizing the methodology for determining the toxicity of pesticides to developing honey bees. Other rearing strategies have been developed (Hendriksma, Härtel, & Steffan-Dewenter, 2011b; Huang, 2009). Most notably, Aupinel et al. (2005) further refined the methodology in a way that is easily adaptable to current regulatory requirements for accessing the toxicity of a target substance to developing honey bees. Their methodology was largely based on Vandenberg and Shimanuki (1987), but further refined the diet composition and amount being provisioned to the larvae, used plastic queen cells, and reduced the handling time by only feeding the larvae once daily.

The advancements in the *in vitro* rearing protocol have given government, industry, and academia a new tool for accessing the risks pesticides and other chemicals pose to immature honey bee development and survival (Aupinel et al., 2005, 2007, 2009; Crailsheim et al., 2013; Hendriksma, Härtel, & Steffan-Dewenter, 2011b; Huang, 2009; Peng et al., 1992; Zhu, Schmehl, Mullin, & Frazier, 2014). Honey bee *in vitro* rearing protocols have allowed researchers to standardize environmental conditions (e.g. temperature, humidity) in which the immature bees develop, provide a uniform diet to each individual, and reduce pathogen exposure.

There are some examples of successes using the Aupinel et al. (2005) methodology, particularly related to high survival throughout the larval stage of development. Hendriksma, Härtel, and Steffan-Dewenter (2011a) and Hendriksma, Härtel, Babendreier, von der Ohe, and Steffan-Dewenter (2012) demonstrated control bee survival of >90% up to the termination of the test at the prepupal stage of development. Krainer, Brodschneider, Vollmann, Crailsheim, and Riessberger-Gallé (2016) were able to achieve high survival through adult emergence, but this level of success has historically been atypical. Other *ad libitum* (Huang, 2009) and non-grafting (Hendriksma, Härtel, & Steffan-Dewenter, 2011b) rearing protocols yielded near 80% survival from the time of larva inclusion in the study to adult emergence but they have not been adopted for regulatory testing.

Despite advancements in the larval rearing protocol, there continues to be substantial difficulty in achieving consistent survival in untreated individuals and test reproducibility with existing protocols. Total bee survival from 48 h (2 days) must be 85% overall 7 days after grafting and 70% overall to adult emergence per thresholds set by regulatory agencies (OECD, 2014). Otherwise the data generated from those studies are not considered valid.

Generally speaking, consistently high control survival has been hard for investigators to achieve. In a 2005 and 2008 ring test, individuals from seven institutions, all with prior larval rearing experience, across five countries only achieved a rate of adult emergence greater than 80% in 43% of their control trials and greater than 90% in 17% of their control trials (Aupinel et al., 2009). Furthermore, several recently published studies based upon current protocols (Aupinel et al., 2005, 2007, 2009) noted very high control mortality. Zhu et al. (2014) reported larval control mortality six days after grafting to be over 15%. In our experience, this would translate to a poor rate of adult emergence given the already high rate of larval death which usually correlates with an equally high or higher rate of death in developing pupae. Other investigators experienced up to 60% mortality in controls prior to reaching adult emergence (Lüken et al., 2014). The reasons for low control survival and inconsistent results across different laboratories are unclear. Regardless, no *in vitro* rearing protocol developed to date yields consistent results, making them generally unfit for use as standard methodologies.

Here, we outline an improved protocol for the *in vitro* rearing bioassay that has allowed us to reach over 95% survival consistently in both our control and solvent treatments. We provide an updated protocol with a revised method in a step-by-step format to outline how to rear honey bee workers artificially. The Crailsheim et al. (2013) COLOSS *BEEBOOK* publication for the artificial rearing of honey bees provides guidance for understanding the historical context of the methodology and the different rearing approaches that are utilized by various researchers. The "standardized methods" are, however, difficult to follow and interpret for a researcher who is not familiar with the methodology. Additionally, we highlight the crucial points of the rearing process that are necessary to achieve a high percentage of adult emergence with the goal of increasing rearing success worldwide and providing a higher level of resolution between data produced from rearing bees on test diets (pesticides, nutritional supplements, etc.) and control ones.

Protocol

(1) Prepping tools/supplies- A list of all equipment and supplies is provided in Table 1. They must be acquired and prepared as discussed below before initiating the *in vitro* rearing protocol.
  (1.1) Chinese grafting tool
    (1.1.1) Some Chinese grafting tools (Figure 1(A), Table 1, item V) should be modified when used for transferring honey bee larvae from combs to larval sterile tissue culture plates (STCPs, Table 1, item W). In these instances, the flexible filament tip of the tools should be trimmed to approximately 2.5 mm in width (Figure 1(B)) to allow for

Table 1. Tools and supplies needed for the *in vitro* rearing protocol.

	Item letter corresponds to the first mention of the item in the text	Section letter corresponds to section of text in which the item is discussed	Item and Description	Quantity (minimum)
Equipment	A	1.4.1	Heratherm incubator (Thermo Scientific, #IMH750-S), or equivalent. The incubator must maintain temperature within ±0.5 °C.	1 unit
	B	2.2	Freezer (−20 °C)	1 unit
	C	2.3.6	Refrigerator (+4 °C)	1 unit
	D	1.4.5	Clean hood, positive pressure (air flow set at 0.5 inches of water)	1 unit
	E	3.2.1	Microwave, 1000 watt	1 unit
	F	1.4.5	Space heater, 1500 watt ceramic (Comfort Zone, #C2442WN)	1 unit
	G	1.4.1	30.5 cm^3 desiccators (Thermo Scientific, #08-642-21)	2 units
	H	1.4.2	Data loggers (Onset, HOBO #UX100-011)	2 units
Queen caging and transportation	I	3.1	Honey bee colonies (Langstroth hive)	3 units
	J	3.1	Zinc queen excluder push-in cage (10 cm × 10 cm × 3 cm, L × W × H)	4 cages
	K	3.2.1	ThermiPaq heat packs, clay based, 15 cm × 30 cm (Medical Supply 123, #201)	2 packs
	L	3.2.2	Five-frame honey bee hive box with two telescoping outer covers	1 box with two lids
Larval diet preparation	M	2.3.3	D-fructose (Fisher, #L95-500), store at room temperature	1 container
	N	2.3.3	D-glucose (Fisher, #D16-500), store at room temperature	1 container
	O	2.3.4	Bacto yeast extract (Bacto, #288620), store at room temperature	1 container
	P	2.3.5	Royal jelly (Stakich). The source of royal jelly is very important and it should be vetted appropriately (see Discussion-"Larval Diet Composition" for more details) prior to use. Stakich brand royal jelly has been used successfully and reliably for rearing larvae. All royal jelly should be shipped via overnight delivery. It should be stored at −20 °C upon arrival.	1 container
	Q	2.3.1	ddH_2O or autoclaved water	1 gallon
	R	2.3.1	0.22 μm sterile Durapore PVDF membrane syringe filters (Fisher, #SLGV033RS)	1 pack
	S	1.3.3	Stainless steel laboratory spatula (Fisherbrand, #14-373)	3 tools
	T	1.3.3	Stainless steel laboratory scoopula (Fisherbrand, #14357Q)	3 tools
	U	1.3.3	100 mL glass beakers (Corning Life Sciences, #07-250-054), or equivalent	3 beakers
Honey bee grafting and maintenance	V	1.1.1	Chinese grafting tools (GloryBee, #14513) or similar grafting tool. The grafting tools should be made of plastic or metal so that they can be sterilized easily. Wooden grafting tools are not acceptable.	10 tools
	W	1.1.1	48-well tissue culture plates, sterile (Falcon, #353230) or equivalent. Plates will be prepared in two different ways for larval rearing. The larvae will be reared in larval sterile tissue culture plates (STCP), while the pupae will be transferred to and maintained in pupal STCP.	1 case

(*Continued*)

Table 1. (*Continued*).

	Item letter corresponds to the first mention of the item in the text	Section letter corresponds to section of text in which the item is discussed	Item and Description	Quantity (minimum)
	X	1.2.1.1	Brown plastic cell cups (Mann Lake LTD, #QC-110). The cell cups need to fit within the STCP wells; therefore, other cell cup designs may not work for rearing protocol.	1 bag
	Y	4.2.6	Pipette, variable volume 10–100 µL (Sigma-Aldrich, #Z683809)	1 unit
	Z	4.2.6	Pipette tips, sterile filtered 1–200 µL (Sigma-Aldrich, #CL54823)	1 box
	AA	1.2.2.1	Kimwipes (Kimberly Clark, #06-666A)	1 box
	BB	1.4.3	Potassium Sulfate (K_2SO_4, Sigma Aldrich, #223492-2.5Kg)	1 container
	CC	1.4.3	Sodium Chloride (NaCl, Sigma Aldrich, #S7653-1Kg)	1 container
	DD	1.4.5	Fiber optic light source, or equivalent	1 unit
Sterilization	EE	1.3.2	Face mask (Global Industries, #T9F954219)	2 masks
	FF	1.3.3	UV light	1 light
	GG	1.3.2	Nitrile gloves (Fisher Scientific, #19-167-051)	1 box
	HH	1.3.3	Aluminum foil	1 box
	II	1.4.1	Bleach (10% v/v)	1 container

the larvae to be picked up from the base of the cell unhindered. Be sure to confirm that the plunger of the grafting tool completely contacts the filament when the plunger is depressed.

(1.1.2) Other Chinese grafting tools should be modified differently when used to transfer prepupae from larval STCPs to pupal STCPs (Figure 1(C)). The tools should be modified by removing their springs and plungers (Figure 1(D)). The plunger and spring are easily removable by pulling firmly on the top of the plunger, and sliding off the plunger and spring from the tool. The tool can be reassembled without the spring and plunger after these components are removed.

(1.2) 48-well sterile tissue culture plates (STCP)

(1.2.1) Larval STCP (Figure 2(A))

(1.2.1.1) Place a single cell cup (Table 1, item X) into each well of the STCP.

(1.2.2) Pupal STCP (Fig. 2(B))

(1.2.2.1) Cut Kimwipes (Table 1, item AA) into 2.0 × 1.0 cm pieces.

(1.2.2.2) Place a piece of Kimwipe at the bottom of each well in a new STCP. A properly placed Kimwipe will cover the bottom of a well and fold equally up the sides of the well.

(1.3) Other tools, materials, and supplies.

(1.3.1) All tools, materials, and supplies must be sterilized, whenever possible, prior to their use to prevent contamination or the introduction of pathogens to the developing bees.

(1.3.2) Always wear clean nitrile disposable gloves (Table 1, item GG) and a face mask (Table 1, item EE) during material preparation.

(1.3.3) Place all grafting tools, laboratory tools (Table 1, items S and T), STCPs, glass beakers (Table 1, item U), and aluminum foil (Table 1, item HH) under a UV light (Table 1, item FF) for 15 min and then turn them over for an additional 15 min to sterilize the other side. This adequately sterilizes all materials for use. The UV output of different light manufacturers will vary and may require a different time duration for adequate sterilization of tools and materials.

(1.3.4) Once sterilization is complete, use gloved hands to place plate covers

Figure 1. Plastic Chinese grafting tool (A), modified grafting tool (B), pupal transfer tool (C) and removed components from pupal transfer tool (D).

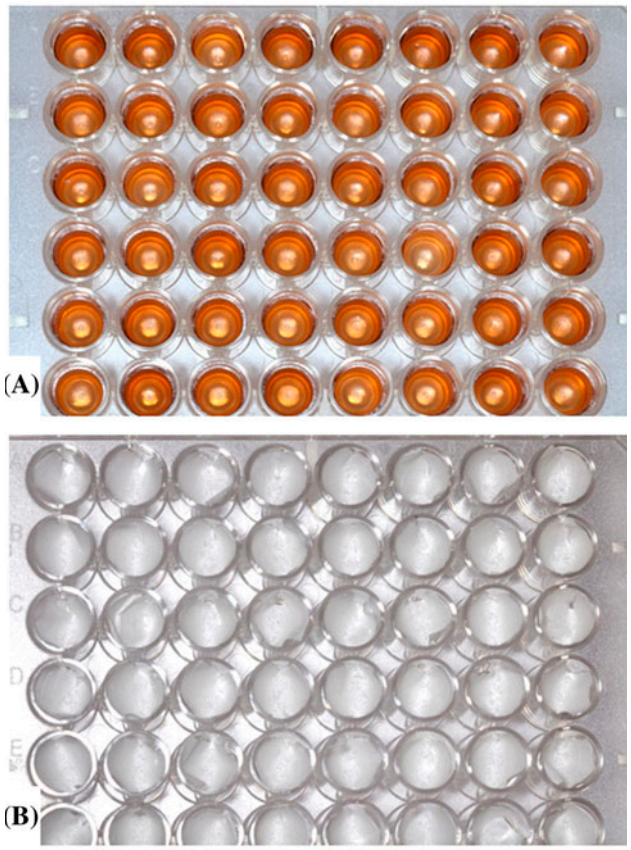

Figure 2. Larval sterile tissue culture plate with a plastic cell cup placed within each well (A), and pupal sterile tissue culture plate with a 2 × 1 cm piece of Kimwipe placed within each well (B).

onto the STCPs, cover the glass beakers with the UV-exposed surface of the aluminum foil facing down on the beakers, and wrap the tools in aluminum foil (sterile surface contacting the tools) until use.

(1.4) Prepare the desiccators and incubator (Figure 3(A)).

(1.4.1) Clean all interior surfaces of the desiccators (Table 1, item G) and incubator (Table 1, item A) with a 10% v/v bleach/H_2O solution (Table 1, item II) before each new round of larval grafting. Be sure to let the desiccators and incubator dry completely before placing any bees into the rearing environment.

(1.4.2) Place the desiccators in an incubator that has a temperature set-point at 35 °C. The temperature within the incubators should not vary more than ±0.5 °C from the set-point. Data loggers (Table 1, item H) should be used to confirm the temperature within the incubators and temperature/humidity within the desiccators.

(1.4.3) Prepare supersaturated salt solutions of K_2SO_4 (Table 1, item BB, for the larval stage, ~160 g K_2SO_4 to 1 l H_2O) and NaCl (Table 1, item CC, for the pupal stage, ~400 g NaCl to 1 l H_2O) by mixing the salts in 45 °C tap water.

(1.4.4) The referenced desiccators contain a leak-proof tray that can hold the salt solutions. The trays should be filled with about 400 ml of salt solution. Different sized desiccators may require different amounts of saturated salt solutions to achieve the correct humidity. To fill the trays, remove all shelves from the desiccator, pour the salt solution into the tray, and replace the shelves. The K_2SO_4 solution is used in desiccators where larval STCPs are held and the solution produces

Figure 3. Incubator containing desiccators used to house larval and pupal sterile tissue culture plates (A), and positive flow hood used for *in vitro* larval grafting, feeding, and monitoring for mortality (B).

a R.H. of ~94% at the incubator temperature of 35 °C. The NaCl solution is used in desiccators where pupal STCPs are held, producing a R.H. of ~75% at the incubator temperature of 35 °C. We recommend that at least two desiccators be used, one for housing larval STCPs and a second for housing pupal STCPs. Monitor the amount of salt solution in the tray daily and be sure to refill when the solution is getting low due to evaporation. Replace the salt solutions weekly to prevent mold growth in the salt solutions.

(1.4.5) All manipulation with the immature bees, including larval grafting and feeding, should be done in a positive flow clean hood (Figure 3(B), Table 1, item D). The hood should be sterilized with a 10% v/v bleach/H_2O solution before and after the larvae are fed or inspected. Alternatively, the hood can be equipped with a UV light that can be turned on for 15 min (or according to UV light manufacturer's directions) prior to any manipulation with immature bees. The hood should be located in close proximity to the incubator in which the larvae/pupae are kept. This will minimize any disturbance to the immature bees during grafting and feeding. The hood should be equipped with a light source (Table 1, item DD) for proper illumination of the immature bees and a space heater (Table 1, item F) to keep the hood space warm (~31 °C) during larval transfers, feedings, and mortality inspections.

(2) Preparing the larval diet

(2.1) Honey bee larvae are fed a diet of sterile water, sugars, yeast, and royal jelly over a period of six days post grafting.

(2.2) Royal jelly should be stored at −20 °C until use. Freeze the royal jelly in aliquots of 40–50 ml to prevent repeated freezing and thawing. Any unused royal jelly from the larval diet preparation can be placed in a refrigerator at 4 °C for up to one month.

(2.3) The diet should be created using sterile laboratory tools and beakers. Allow all diet components to warm to room temperature prior to mixing the diet. Mix the diet components using a spatula or vortex mixer. Do not shake the components when mixing the diet which introduces unwanted air into the diet and reduces the density of

Table 2. Amount and percentage of diet components in the larval diet necessary to feed approximately 400 larvae.

	Amount of diet components (g)			Percentage of diet components in total diet		
Diet component	Diet A	Diet B	Diet C	Diet A	Diet B	Diet C
Royal jelly	4.43	4.30	25.00	44.25	42.95	50.00
Glucose	0.53	0.64	4.50	5.30	6.40	9.00
Fructose	0.53	0.64	4.50	5.30	6.40	9.00
Yeast extract	0.09	0.13	1.00	0.90	1.30	2.00
Water	4.43	4.30	15.00	44.25	42.95	30.00
Total	10	10	50	100	100	100

the diet. One ml of diet will weight approximately 1.10–1.15 g. Mix the diet in the following order:

(2.3.1) Filter the water (Table 1, item Q) in excess of the amount needed using a 0.22 µm membrane syringe (Table 1, item R)

(2.3.2) Measure the amount of filtered H_2O needed.

(2.3.3) Add the two sugars, D-fructose (Table 1, item M), and D-glucose (Table 1, item N), to the water and mix until the sugars dissolve completely.

(2.3.4) Add the yeast (Table 1, item O) and mix until completely dissolved.

(2.3.5) Add the royal jelly (Table 1, item P) and mix until it is homogenous. There are three different diets (Diet A, B, and C) fed to the honey bees during larval development. The three diets differ in the amount and proportion of ingredients they contain (Table 2). The sample amounts of diets given in Table 2 (10 g of diet A, 10 g of diet B, and 50 g of diet C) will feed a minimum of 400 larvae throughout their entire larval development.

(2.3.6) The mixed diets can be stored for a maximum of three days at 4 °C (Table 1, item C) after which, they should not be used.

(3) Collection of larvae

(3.1) A honey bee queen in a suitable queenright colony (Table 1, item I, see Discussion section for details on colony selection criteria) is confined on a frame of wax comb using a queen excluder push-in cage (Figure 4(A), Table 1, item J), or equivalent. Worker bees can travel through the cage; thus they are able to tend the developing brood and queen. Frames with plastic foundation, rather than ones with wax foundation, work best when using push-in cages

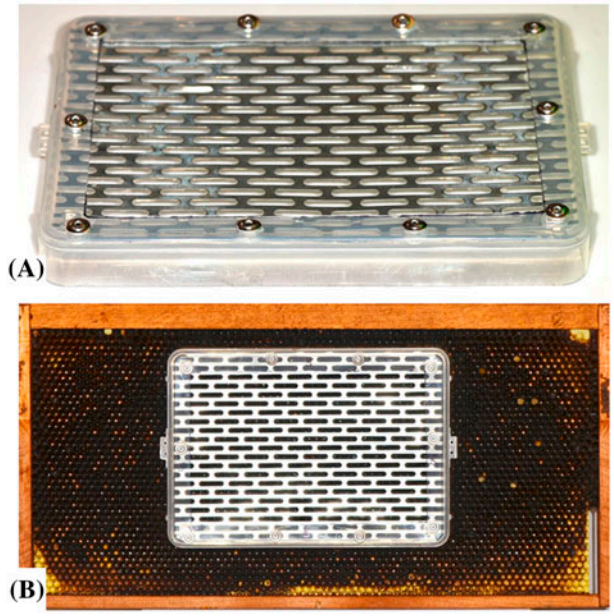

Figure 4. Push-in zinc queen excluder cage (A) and position of queen excluder cage on a honey bee hive frame (B).

because the plastic foundation provides a solid stopping point for the cage. Such a cage can be used to obtain at least 400 larvae. Cage design can vary though we have found it is useful to use smaller cages rather than larger ones because the age and size of the larvae are standardized in a concentrated area on the frame.

(3.1.1) Place the queen inside the queen excluder cage by putting the queen on the comb and securing the cage around her (Figure 4(B)). Firmly press the edges of the cage into the wax comb to prevent the queen from escaping. Place the frame with the excluder cage into the middle of the brood nest to ensure the proper incubation of the eggs prior to hatching.

(3.1.2) Release the queen from the cage after a period of 24 h (Table 3). Confirm that the queen has laid

Table 3. *In vitro* rearing time reference points.

Age of bee d (h) from $t = 0$. All times are ±0.5 d or 12 h.	Time d (h) since initiating *in vitro* rearing protocol	Time d (h) recognizing grafting as $t = 0$	Task performed	Sample daily time schedule
−0.5 (−12)	0 (0)	−4 (−99)	Cage queen	10:00
0.5 (12)	1 (24)	−3 (−75)	Release queen	10:00
1.5 (36)	2 (48)	−2 (−51)	N/A	
2.5 (60)	3 (72)	−1 (−27)	N/A	
3.625 (87)	4 (99)	0	Graft/Feeding	13:00
4.625 (111)	5 (123)	1 (24)	Inspection	13:00
5.625 (135)	6 (147)	2 (48)	Feeding/ Inspection	13:00
6.625 (159)	7 (171)	3 (72)	Feeding/ Inspection	13:00
7.625 (183)	8 (195)	4 (96)	Feeding/ Inspection	13:00
8.625 (207)	9 (219)	5 (120)	Feeding/ Inspection	13:00
9.625 (231)	10 (243)	6 (144)	Pupal transfer/ Inspection	13:00
10.625 (255)	11 (267)	7 (168)	Pupal transfer/ Inspection	13:00
11.625 (279)	12 (291)	8 (192)	Pupal transfer/ Inspection	13:00
12.625 (303)	13 (315)	9 (216)	Inspection	13:00
13.625 (327)	14 (339)	10 (240)	Inspection	13:00
14.625 (351)	15 (363)	11 (264)	Inspection	13:00
15.625 (375)	16 (387)	12 (288)	Inspection	13:00
16.625 (399)	17 (411)	13 (312)	Inspection	13:00
17.625 (423)	18 (435)	14 (336)	Inspection	13:00
18.625 (447)	19 (459)	15 (360)	Inspection	13:00
19.625 (471)	20 (483)	16 (384)	Inspection	13:00
20.625 (495)	21 (507)	17 (408)	Inspection	13:00
21.625 (519)	22 (531)	18 (432)	Inspection/Adult emergence	13:00
22.625 (543)	23 (555)	19 (456)	Inspection/Adult emergence	13:00
23.625 (567)	24 (579)	20 (480)	Inspection/Adult emergence	13:00
24.625 (591)	25 (603)	21 (504)	Inspection/Adult emergence	13:00
25.625 (615)	26 (627)	22 (528)	Inspection/Adult emergence	13:00

Notes: For the "age of bee from $t = 0$," 0 is the midpoint of the time the queen was caged. Once the queen is released, the eggs she laid are 12 ± 12 h old if she was caged for 24 h. We discuss the tasks performed at each time point in the "Task performed" column. We also provide a sample time schedule that aligns with the mentioned tasks and puts all tasks associated with the rearing protocol at reasonable times of the day.

eggs in the cells. The age of the eggs when the queen is released is estimated to be 12 ± 12 h when the queen has been caged for 24 h, i.e. the midpoint of caging is $t = 0$ h. The ±12 h can be reduced if necessary by limiting the amount of time the queen remains caged.

(3.1.3) Replace the push-in cage on the comb in the same position it was in when it contained the queen. This will prohibit the queen from laying additional eggs in the target brood section.

(3.2) Larvae are transported at $t = 87 \pm 12$ h (75 h after the queen is released, Table 3) to a sterile lab environment for grafting.

(3.2.1) Pre-warm two clay-based heat packs (Table 1, item K) in a microwave (Table 1, item E) for 90 s.

(3.2.2) Place the heating packs on the inside base of a box (Table 1, item L) made to accommodate the number of frames being taken from the apiary. The heat pads will provide the larvae with adequate warmth for about 30 min, while they are being transported from the field to the laboratory.

(3.2.3) At $t = 87 \pm 12$ h (Table 3), remove the frame from the colony, gently brush off the bees, and transport the frame to the laboratory in the heated hive box. Be sure to con-

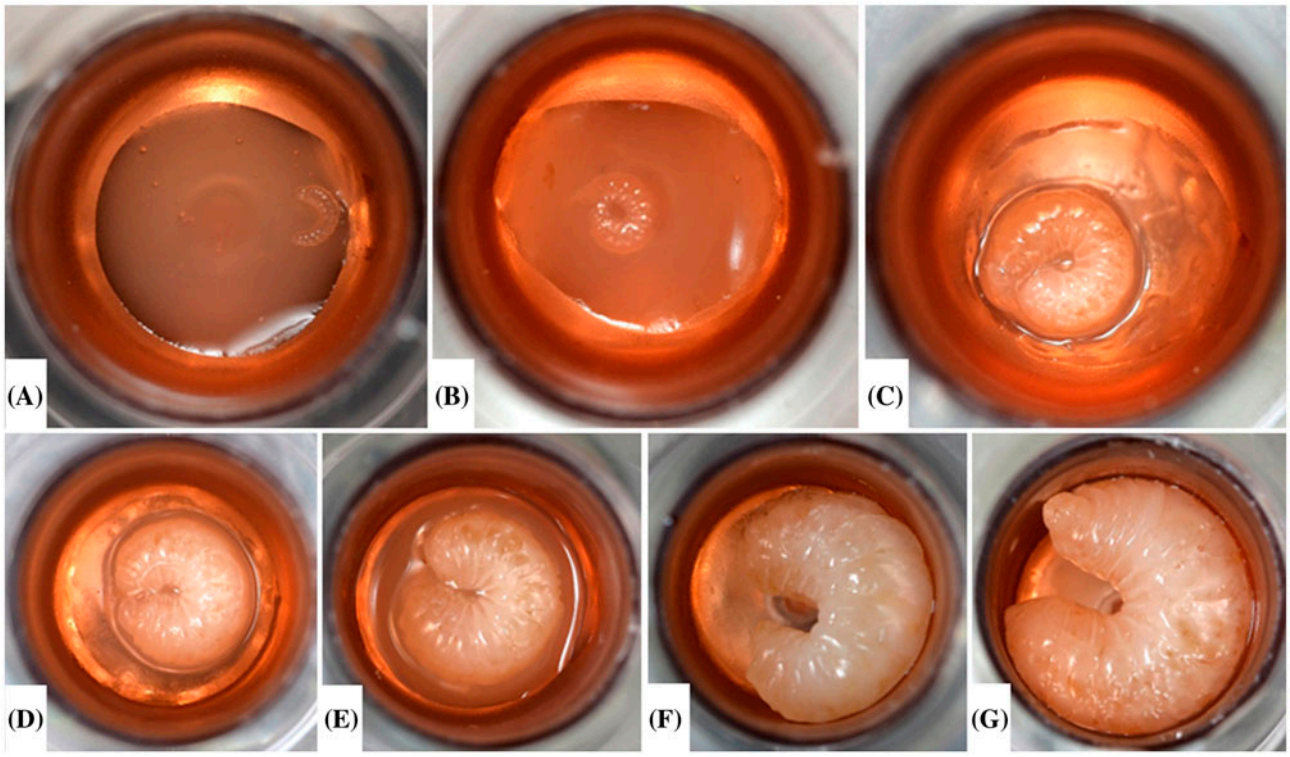

Figure 5. Larval development from the day of grafting (A, t = 3.625 ± 0.5 d), one day (B, t = 4.625 ± 0.5 d), two days (C, t = 5.625 ± 0.5 d), three days (D, t = 6.625 ± 0.5 d), four days post graft (E, t = 7.625 ± 0.5 d), five days (F, t = 8.625 ± 0.5 d), and six days (G, t = 9.625 ± 0.5 d) post grafting. The prepupa in G has fully consumed its diet and is ready to be transferred into the pupal plate. All pictures were taken prior to any scheduled feedings.

Table 4. Schedule of larval feeding during the *in vitro* protocol.

Time after grafting	Diet	Amount of diet (µl)
t = 0	A	20
t = 24 h (1 day)	n/a	0
t = 48 h (2 days)	B	20
t = 72 h (3 days)	C	30
t = 96 h (4 days)	C	40
t = 120 h (5 days)	C	50

firm the presence of larvae and never shake the frame of larvae while manipulating the frame or during its transit.

(3.2.4) At the laboratory, place the collected frame in an incubator maintained at 35 °C and constant dark until the time of grafting (no longer than 3 h).

(4) Larval grafting and maintenance (Figure 5)

(4.1) All three diets (A, B, and C) are fed to the larvae during their development. The amount of the diet fed to the larvae varies throughout larval development according to the schedule in Table 4. There are a total of five feedings over a six day period, with no feeding occurring on the second day of rearing.

(4.2) Young larvae are transferred from the comb (Fig. 6(A)) to the cell cups (Fig. 6(C)) in the prepared and sterilized larval STCP.

(4.2.1) Pre-warm diet A in the incubator until it reaches approximately 35 °C. Prewarming the diet takes approximately one hour prior to grafting and can be placed into the incubator prior to transporting larvae from the apiary to the laboratory.

(4.2.2) Place the sterilized larval STCP and grafting tools in the clean hood and put on nitrile gloves and a face mask.

(4.2.3) Turn on the space heater to a low setting (~31.0 °C) and locate the heater about 15 cm from the edge of where the frame will be in the hood. A light source should be used to facilitate seeing the larvae in the cells.

(4.2.4) Place the comb with the larvae in the hood. The comb should be

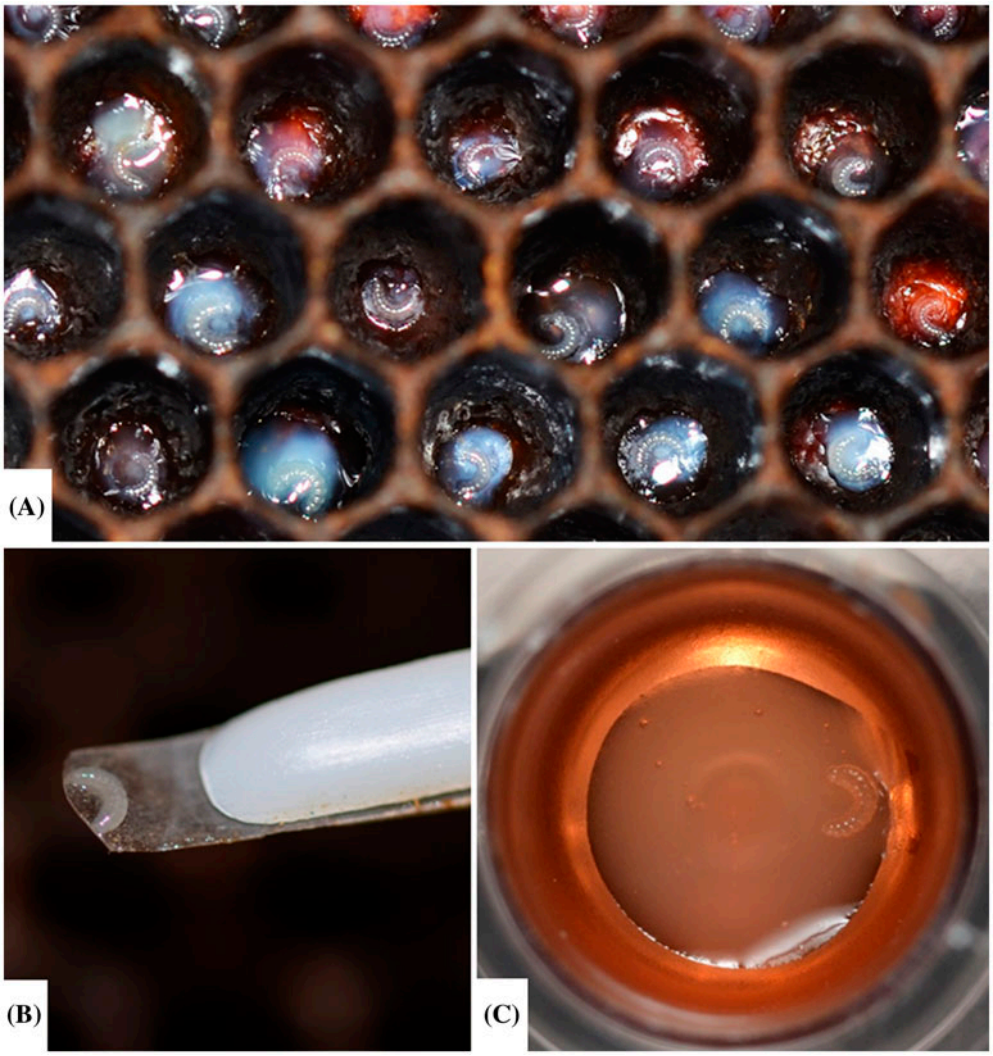

Figure 6. Young larvae in frame prior to grafting (A), larva on the filament tip of a Chinese grafting tool while grafting from the frame to the cell cup (B) and larva within cell cup after grafting (C).

slightly tilted up, approximately 5–10° from the flat position and toward the grafter, to allow the grafter to see into the back of the cells.

(4.2.5) Visually inspect and omit use of any larvae that have noticeable defects or are not synchronized in growth (i.e. only use larvae that are about the same size).

(4.2.6) Place 20 µl of diet A into each of the 48 cell cups of the larval STCP using a calibrated variable volume pipette (Table 1, item Y) or equivalent equipped with a disposable sterile filtered pipette tip (Table 1, item Z).

(4.2.7) Insert the flexible tip of a Chinese grafting tool (the tool modified for grafting larvae) along the side of a cell containing a larva (Figure 6(A)) and gently slide the tip underneath the larva.

(4.2.8) Gently lift the grafting tool straight out of the cell with the larva firmly on the end of the tip (Figure 6(B)). Only insert the tool inside the cell once to graft the larva. Avoid grafting any larvae that were not secured on the first attempt (i.e. a single larva should be manipulated only once and then removed from the cell/disposed if she was not secured on the first attempt or if her health was believed to be compromised in any way).

(4.2.9) Place the tip of the grafting tool containing the larva into a sterile cell cup until the tip of the tool is bent slightly against the base of the cup.

(4.2.10) Depress the plunger slowly to force the larva off of the tip of the grafting tool and onto the top of diet A at the base of the cup (Figure 6(C)).

(4.2.11) Slowly and gently remove the grafting tool from the cell cup, being careful not to disturb the larva. The larva should be lying with the same side facing up as they were in the wax cells. Any larvae believed to be damaged in any way during the grafting process should be removed (cup included) and replaced with a new sterile cup and diet.

(4.2.12) Wash the grafting tool after every eighth grafted larva. The tool should be washed in 75% ethanol and then rinsed with ddH_20. Allow the tool to dry by placing it on a Kimwipe before grafting additional larvae. It is better to use multiple tools during the grafting process so that some are available for use while others are clean and drying.

(4.2.13) Confirm that each grafted larva is sitting on top of the diet (Figure 6(C)). If any larvae are grafted improperly, remove the entire larva and cell cup from the experiment and replace with a new cell cup into which diet A and a larva are placed.

(4.2.14) The target time for grafting 48 larvae into one STCP should be less than 20 min.

(4.2.15) Once a larval STCP is filled, place the STCP horizontally (cup openings facing upwards) in the larval desiccator maintained at 94% R.H. using the K_2SO_4 salt solution. Do not move the desiccator suddenly as this can disturb the developing larvae and spill the salt solution.

(4.3) Larval survival is monitored daily by removing the larval STCP, placing the STCP within the heated positive flow hood, and visually inspecting the larvae. Monitor larval mortality prior to adding new diet.

(4.3.1) Remove and dispose of cell cups containing dead larva (do not reuse the cell cups). Dead larvae can be identified by appearing deflated/flaccid (Figure 7) or by the presence of black spots.

(4.3.2) Do not touch the larvae while assessing mortality.

Figure 7. The appearance of a dead larva has a characteristic sunken in appearance and excess diet within the well.

(4.3.3) Once finished assessing mortality, return the larval STCPs to their appropriate desiccator.

(4.4) Subsequent larval feedings. The larval diet is fed to the larvae as outlined in Table 4. The feedings always should be performed in the positive flow hood with the space heater set about 31 °C.

(4.4.1) Remove the larval STCP from the incubator and place the STCP within the heated positive flow hood.

(4.4.2) Any leftover diet visible at the next scheduled feeding is left for the developing larvae. The new scheduled diet gets added to any leftover diet. Do not remove any leftover diet as this increases the risk of damaging the larva.

(4.4.3) Using a pipette, place the new diet along the interior surface of the cell cup. Be careful not to submerge the larvae during application. The diet should be released slowly from the pipette. Pipette tips should be changed between STCPs or treatment groups.

(4.4.4) Do not touch the larvae with the pipette tips at any time during larval development.

(5) Pupae transfer and maintenance (Figure 8)

(5.1) Larvae will begin to be transferred from the larval STCP to the prepared pupal STCP at $t = 9.625 \pm 0.5$ d (Table 3, six days after grafting) to allow the larvae to pupate into

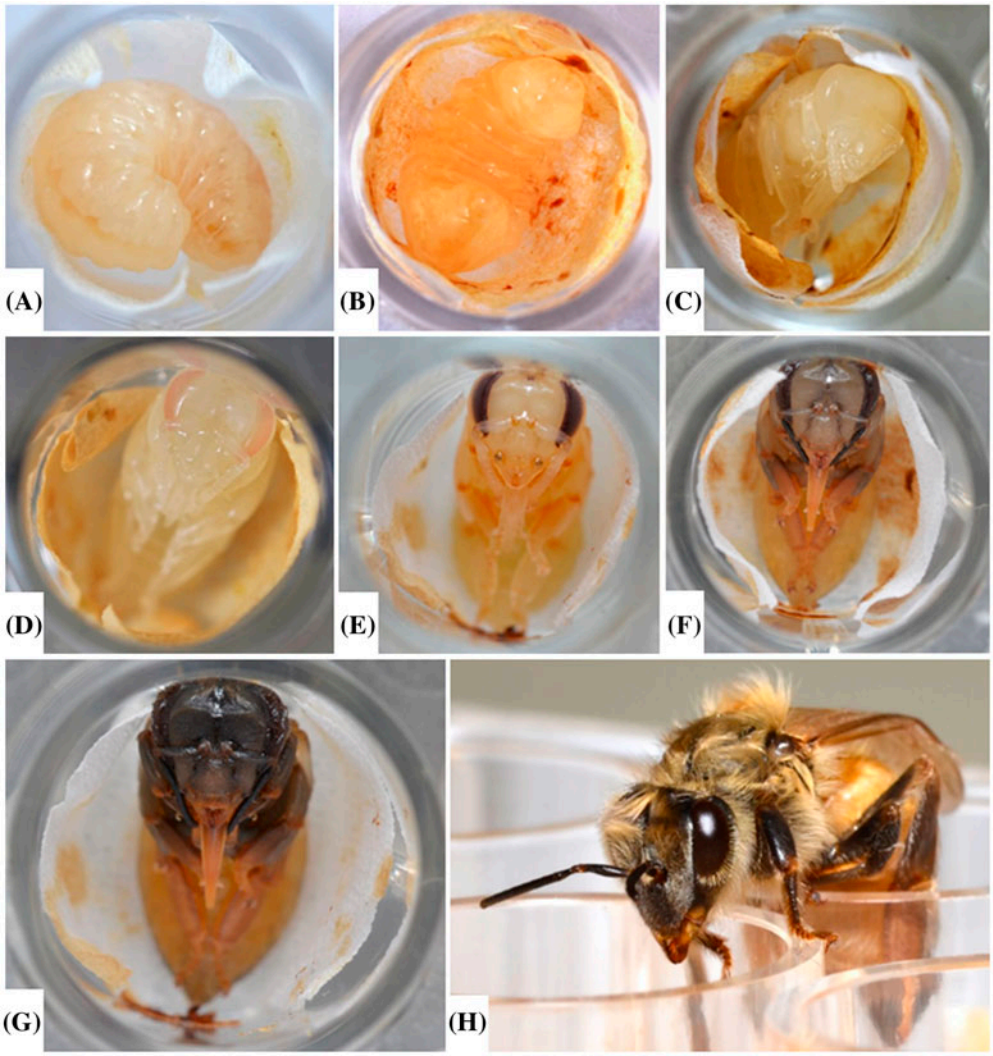

Figure 8. Pupal development from immediately after the pupal transfer (A) through adult emergence (H). Some days during the pupal development stage are not pictured. Note the pigmented color of the eyes and the darkening cuticle as the pupating bee continues to develop within the pupal STCP. A piece of Kimwipe lines the bottom of each well.

adults. The larvae are considered ready to be transferred to the pupal STCP only once they have consumed all of the available diet. Larval readiness for transfer can occur over a period of a few days within a single larval STCP, depending on specific experimental treatments (i.e. pesticides causing delayed development). Only larvae that have consumed their diet are moved. All other larvae are left in the larval STCP and not provided new food.

(5.1.1) Monitor the larvae daily and move them when the diet is consumed fully. Delaying the time of transfer past the time when the diet is consumed will result in increased mortality to the developing bees which may be due to developmental changes in the bee or the presence of fecal material in the cups in the STCP. We found significant differences in the overall survivorship when between larvae moved prior to defecation and those moved after defecation (Figure 9).

(5.1.2) Transferring the mature larvae to the pupal STCP.

(5.1.2.1) Remove the cell cup from the larval STCP.

(5.1.2.2) Gently invert the cell cup at a 45° angle over a well in the pupal STCP.

(5.1.2.3) Guide the larva into the well of the pupal STCP using a Chinese grafting tool modified as described in 1.1.2.

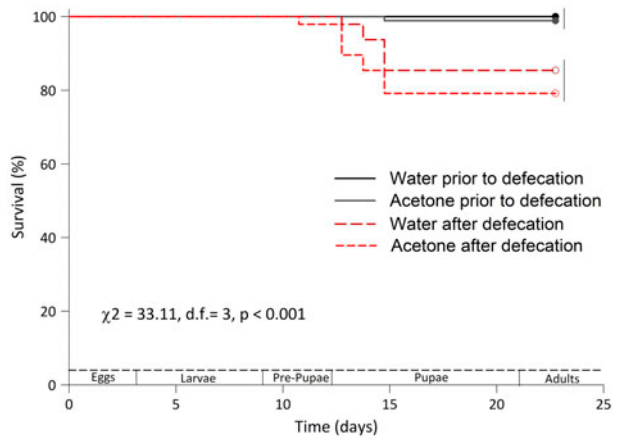

Figure 9. Survival plots of honey bee workers reared *in vitro* and fed just the diet or the diet containing solvents (water and acetone) used to dissolve pesticides. The survival of larvae moved prior to and after defecation was evaluated. Differences in the survival between bees moved prior to and after defecation were detected using a LOG RANK test. Survival curves grouped by the same vertical bar on the right are not significantly different per Holm-Sidak's test ($p > 0.05$).

(5.2) After all larvae ready that day have been transferred, place the pupal STCP horizontally in a desiccator maintained at 75% R.H. using the NaCl salt solution and replace any untransferred larvae back in the larval 94% RH desiccator.

(5.2.1) The developing bees are not fed as pupae.

(5.2.2) Do not move the desiccator suddenly as this can disturb the developing pupae and spill the salt solution.

(5.3) Pupal survival is monitored daily by removing the pupal STCP from the desiccator, placing it in the positive flow hood, and visually inspecting the pupae.

(5.3.1) Remove and dispose of dead or dying pupae and their cups using a sterile pair of forceps.

(5.3.2) Do not keep the pupal STCP out of the incubator during the monitoring process for more than 5 min.

(5.3.3) Do not touch the pupae while assessing mortality.

(6) Adult emergence

(6.1) Adult bees will begin to emerge after a period of approximately $t = 21.625 \pm 0.5$ d (18 days after grafting, Table 3). Check for emerged adults at least once daily as some adult emergence may be delayed for a number of reasons (environment, diet, treatment influences, etc.). Emerging adults can be maintained by feeding them pollen and a 50% sugar water solution (w/v) in an artificial bioassay cage (Aupinel et al., 2005; Williams et al., 2013).

(7) Measurable endpoints for risk assessment

(7.1) Mortality is measured daily throughout the rearing process and can be used to calculate the larval, pupal, and total immature bee survival rate (or percent survival). The equation is: percent survival at given life stage (larva, pupa, etc.) = (total number individuals completing the life stage/total number of individuals entering the life stage) × 100.

(7.2) Fresh weight at adult emergence can be measured by removing and weighing adult bees when they emerge within the pupal STCP.

Discussion

With our methods, we have optimized the success of honey bee *in vitro* larval rearing to adult emergence. From our experiences, we detail below five critical areas for achieving low mortality rates and high reproducibility: (1) larval diet source and composition; (2) grafting environment; (3) plate conditions; (4) rearing environment; and (5) prepupal transfer. We did not measure the individual contribution of each step, but the collective sum translated into excellent survival and adult emergence rates of >95%, neither of which has been achieved consistently before, the latter being indicated in published reports where the various *in vitro* rearing protocols were used.

Larval diet source and composition

The most important considerations with the larval diet are quality components, the percentage of each ingredient in the diet, and a properly vetted royal jelly source and batch. One of the main differences between our larval diet recipe and those listed in previous protocols (Aupinel et al., 2005; Crailsheim et al., 2013) are the increase in the water content and a reduction in the royal jelly content of diets A and B. We found that the diets noted in the literature (Aupinel et al., 2005; Crailsheim et al., 2013) were prone to drying out in the experimental conditions under which we reared larvae. Consequently, we increased the amount of water added to our diets over that recommended by Aupinel et al. (2005) and Crailsheim et al. (2013). Larvae fed the new, modified diet never appeared to develop into queen-worker intermediate castes and the diet never appeared to dry, however we did not conduct any detailed caste analyses. Geometric morphometrics, which extracts spatial information from morphological structures (De

Souza et al., 2015) could be used in future studies to confirm that the *in vitro* workers were indeed workers and not intercastes.

One of the difficulties in including royal jelly in a standardized larval rearing protocol is that royal jelly composition varies from one royal jelly source and batch to the next (Sabatini, Marcazzan, Caboni, Bogdanov, & Almeida-Muradian, 2009; Zheng, Hu, & Dietemann, 2010). We discovered that the source of royal jelly is critical in larval rearing success. In pilot experiments (data not shown), royal jelly sourced from different companies yielded different rates of immature bee survival to adult emergence. Survivorship of the developing bees in our study using the same protocol but otherwise different batches of royal jelly varied from 25 to 100%. In our case, we achieved high levels of reproducibility and adult emergence using Stakich brand royal jelly which is sourced and sold in Michigan, USA. It is crucial to compare the survival rates from immature honey bees to adult emergence using different batches of royal jelly sourced from different companies. We set aside a small amount of royal jelly from a Stakich batch that yielded high immature bee survivability rates. We, then, compared survival rates resulting from larvae feeding on all royal jelly we acquired for future studies to the batch we knew produced high rates of survival.

Be sure the royal jelly is maintained properly during transit and storage. All royal jelly should be shipped via overnight delivery, placed in 30–50 ml aliquots upon arrival, and stored at −20 °C. This may have to be negotiated with the supplier as most royal jelly is sold for human consumption that, otherwise, does not have the same shipping requirements. After receiving and opening the royal jelly, we recommend splitting the batch among several small (~30–50 ml) vials. This will limit the freeze/thaw cycle associated with using royal jelly from a single batch when needed. This way, only the number of vials needed can be thawed for use. The vial of working royal jelly stock, i.e. the royal jelly made ready for immediate use, can be maintained within the fridge at 4 °C for up to a month.

Grafting environment

It is important to maintain the right grafting environment to ensure high survivorship using the protocol. First instar larvae are incredibly sensitive to temperature fluctuations and other disturbances when they are transported into the laboratory for grafting. We recommend ensuring that the area where grafting will occur is heated sufficiently (~31 °C). We speculate that the introduction of pathogens is limited by grafting in a positive-pressure clean hood. The grafter always should be mindful of sterility and wear a dust mask and gloves while grafting.

Plate conditions

Plate conditions also are an important part of obtaining high survivorship using the protocol. The plate conditions dictate the environment encountered by the bees during their development. Always keep the cover on the larval and pupal STCPs except when the STCPs are in the clean hood. Keeping the STCPs closed helps prevent contamination and maintain temperature and humidity. Furthermore, the introduction of pathogens into the rearing environment is reduced by keeping the lid on the larval STCP while moving the plate between the hood and the incubator. It is not necessary to apply glycerol solution to the cotton placed in the plates, below the cell cups, though others have suggested that it is necessary to maintain humidity within the larval STCP (Aupinel et al., 2005, 2009; Crailsheim et al., 2013; OECD, 2014).

Rearing environment

A controlled rearing environment prevents the developing bees from being adversely affected by shock throughout their development into an adult bee. An incubator with less than 1.0 °C fluctuation over a 24-h period will maintain a consistent rearing temperature and is especially necessary if one is using the rearing protocol to measure the impacts of pesticides on bees. Medrzycki et al. (2010) demonstrated that small fluctuations in temperature can impact a pesticide's toxicity to developing bees. Temperature and relative humidity are controlled further by placing the STCP within desiccator chambers. Removing the desiccator chambers from the incubator while a test is being performed is an unnecessary disturbance and may reduce bee survival substantially.

Prepupal transfer

The fifth critical consideration for achieving low mortality rates and high reproducibility with the *in vitro* rearing protocol is the timing of prepupal transfer. The larvae are transferred to a new pupal STCP once the larval diet is consumed completely (approximately $t = 9.625 \pm 0.5$ d, or 6 days after grafting). Our results indicate that leaving the pupae in the same STCP in which they develop may decrease the rate of adult emergence and stunt bee growth. We believe that it is important for bee survival to transfer the developing bee to the pupal STCP after the bee fully consumes the diet but before she defecates. After transferring the larva to the pupal STCP, the bee defecates while it rests on the Kimwipe. The Kimwipe quickly absorbs the fecal waste and reduces contact between the bee and its waste. Increased mortality in the pupal stage of development noted using previous protocols (Aupinel et al., 2005; Crailsheim et al., 2013) may be associated with the contact between the developing bee and its feces.

Furthermore, bees reared using our protocol did not display any adverse morphological deformations such as stunted growth or shortened abdomens, as has been reported by others (Brodschneider, Riessberger-Gallé, & Crailsheim, 2009; Riessberger-Gallé, Vollmann, Brodschneider, Aupinel, & Crailsheim, 2008). We saw no indication of a reduction in bee survivorship when manually transferring the bees to a pupal STCP at the end of their larval development.

Additional considerations

The selection of suitable colonies in the apiary can improve the success of the rearing methods. We have preliminary data that suggest survivorship in the *in vitro* rearing protocol may correlate partially with the colony from which the larvae were grafted. Only colonies that have functional laying queens that are producing solid brood patterns (<10% empty cells in the pattern), are free of symptomatic diseases or pests, and are not undergoing any treatments for pests/pathogens at protocol initiation should be used for acquiring young larvae.

Queens occasionally escape or do not lay eggs while caged. The type of push-in cage used in our proposed method works best with plastic foundation to reduce the possibility of an escaped queen. Regardless, for time-sensitive trials, it is advisable to cage multiple queens in the event that one fails to lay eggs or otherwise escapes.

Our proposed method produced a consistent rate of survival over 95% in both our control and solvent groups. The validity criteria for the proposed OECD repeated-exposure (chronic) larval test guideline in Europe is 85% survival from 72 h (3 d) after grafting until 168 h (7 d) after grafting and 70% survival to adult emergence (OECD, 2014). These high levels of mortality are problematic when resolving potential toxic effects of a test compound on bee larvae. Furthermore, the OECD guidelines permit the tester to replace any dead larvae with living ones at 48 h (2 d) after grafting, immediately before administering diet B (OECD, 2014). We did not have to do this as the survivorship in our larvae was high throughout the study. Thus, our protocol permits the user to begin administering pesticides to the very first diet provided to the grafted larvae rather than 2–3 d after grafting the larvae as required via other protocols. We believe this makes our protocol useful for testing the chronic impact of pesticides on developing bees.

The use of alternative *in vitro* rearing methods has yielded variable rates of success (Hendriksma, Härtel, & Steffan-Dewenter, 2011b; Huang, 2009). Non-grafting methods (queen eggs laid directly into artificial comb within the hive that are used throughout the rearing protocol) have resulted in mortality rates between 2 and 8% in larvae and adult emergence rates near 80% (Hendriksma, Härtel, & Steffan-Dewenter, 2011b). While limiting direct human interference with developing bees may yield a higher rate of survival than Aupinel et al. (2007) experienced using their protocols, our results indicate that human interference does not cause a reduction in bee survival directly given that we transferred our post-food consumption larvae to new plates. Huang (2009) suggests a*d libitum* feeding during the larval development stage. They experienced an adult emergence rate of approximately 80% from the time of grafting. However, *ad libitum* feeding makes it impossible to determine the level of bee exposure to a test substance provided in the larval diet. Our current protocol allows for the dose of a test solution to be calculated for bees consuming the total diet provided, i.e. 160 µl of diet. The dose cannot be determined for bees that fail to survive until the end of the larval stage since the diet is not consumed completely by the larvae prior to death.

High mortality rates and difficulty reproducing quality results within and between laboratories may not be related entirely to methods, but be attributed to experimental grafting error, temporal and spatial effects, genetic variation in the bee source, pathogens, and larval size at grafting. Furthermore, there are several unknowns in key parts of any *in vitro* rearing protocol including royal jelly quality and the functionality of *in vitro* reared workers. Royal jelly has some remarkable biological activities (Viuda-Martos, Ruiz-Navajas, Fernández-López, & Pérez-Álvarez, 2008), but the percentage of certain royal jelly components may vary across different geographic regions (Wei et al., 2013). It is necessary to import royal jelly in some countries and customs may require the irradiation of royal jelly which may alter any potential beneficial microbe community within the diet or otherwise impact diet quality.

Few have studied the functionality of *in vitro* reared worker bees (Brodschneider et al., 2009). Some protocols (Huang, 2009) are prone to producing worker-queen intercastes which may not be functional in the hive. Brodschneider et al. (2009) observed similar flight performances between artificially reared and colony-reared honey bees, but in the future, investigators using our improved protocol can explore the functionality of *in vitro* reared workers by comparing anatomical, behavioral, and physiological parameters of *in vitro*- and hive-reared worker honey bees.

We have discussed an improved protocol for the *in vitro* rearing of larval worker honey bees to adult emergence. Our rearing protocol produces high survivorship in control individuals and can be used to assess the potential risks of crop protection products to, the contribution of nutrition to, and the effects of brood diseases on bee health. In conclusion, our *in vitro* rearing protocol can be implemented as the standard protocol used to determine the impact of stressors on immature bees because of the protocol's high control survivability, ease in end point determination, and high overall repeatability.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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