A standardized procedure for the *in vitro* Rearing of Honey Bee Larvae

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Testing Laboratories

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Non-Target Organism

1.0 INTRODUCTION

The Western honey bee (*Apis mellifera* L.) plays a vitally important role in United States (US) agriculture by providing pollination to the majority of fruits and vegetables in this country. The honey bee therefore remains one of the most important non-target organisms for registration of both pesticides and genetically modified organisms (GMO). Under current regulatory guidelines all biotechnology-derived products are tested against non-target organisms under a tiered testing regime (Romeis et al., 2008). For traits registered as insecticidal, Tier I tests include laboratory tests using larval and adult honey bees typically conducted at dose rates of at least 10X the expected environmental exposure. Under this testing regime if effects are observed in laboratory tests then more complex, *e.g.,* field tests may be warranted.

Laboratory tests play an important role for evaluating GMOs because field tests are usually more variable, more time consuming, more costly, and frequently do not improve the certainty of regulatory risk assessments. More importantly, in most cases we do not know if bee larvae actually have consumed the introduced material or if worker bees removed them. Laboratory tests therefore are critical for evaluating effects on immature stages of honey bees.

With support from the ABSTC, MSU Apicultural Laboratory has been improving and standardizing the commonly used honey bee larval *in vitro* rearing method, originally developed by Vandenberg and Shimanuki (1987). This protocol has resulted in an average survival rate of 80% from the first instar larvae to adults (N = 1,000). In addition, the majority (>98%) of the resultant adults are of the worker phenotype and very few became queens. The following is a brief description of the standardized procedure.

2.0 MATERIALS AND METHODS

2.1 Test System

2.1.1 Larval Honey Bee Diet Preparation

1. Heat 50 ml of distilled water until boiling. Let boil for 5 min. Let the water cool.
2. Weigh 6 grams of D-glucose (6%), 6 grams of D-fructose, and 1 gram of yeast extract. These can all be in the same beaker (50 ml).
3. Thaw royal jelly (frozen at -80°C, BeeNatura.com) either by placing it at 4°C overnight, or at room temperature for 1-2 hrs. Weigh 50 grams of royal jelly by slowing dripping the jelly into a 100 ml beaker on a digital balance. Remove any visible pieces of beeswax.
4. When the water cools to about 45-55°C (cool enough for hands to touch), measure 37 ml of water, add to the beaker containing 6 grams of D-glucose (6%), 6 grams of D-fructose, and 1 gram of yeast extract. Swirl the beaker until everything is dissolved. If the water is at the right temperature this step does not require a spatula to get everything into solution.

5. Decant the dissolved sugars and yeast extract into the 100 ml beaker containing royal jelly. Mix them thoroughly using a spatula.
6. The larval diet is now ready for use. Label the beaker with date. This should be used for 3 days only (stored at 4°C). Alternatively, diet for the whole exposure period may be prepared at study initiation and stored at -80°C.

   Bake all glassware coming into contact with larval diet (beakers, measuring cylinder, etc.) in a self-cleaning oven at the setting of "self clean". This usually bakes at high temperature (500°C) for 3.5 hours. Do not use a spatula to remove sugars/yeast to the weighing paper but lightly tap the containers of chemicals to get the correct weight. This prevents chemical contamination. If there is too much material, use a spatula to remove the excess and discard them.

2.1.2 Bee Experimental Groups

The study will initially comprise only a larval honey bee diet. Subsequent tests may include a test substance (protein), a control substance (buffer), an assay control substance (water), and a reference substance (potassium arsenate).

2.1.3 Species

The study will be conducted using second instar larval workers of the honey bee (*Apis mellifera* L.).

2.1.4 Justification of Test System

The western honey bee (*Apis mellifera* L.) plays a vitally important role in US agriculture by providing pollination to the majority of fruits and vegetables in this country. The honey bee therefore remains one of the most important non-target organisms used for testing for the registration of both pesticides and organisms (GMO).

2.1.5 Preparation for executing the protocol

The critical step in this test system is familiarity with the art of larval transfer from the hive to plates, and the transfer of mature larvae to pupation plates. These procedures require training and practice before commencing this protocol. The procedure will be done on at least 100 larvae or until 70% (ideally 80%) survival to adult emergence is obtained by the person transferring larvae. Before a technician can be considered qualified they must repeatedly demonstrate a high level of proficiency transferring larvae. For example, the technician must demonstrate 80% survival to adult emergence with control larvae 3 times before being considered qualified.

2.1.6 Origin and Acclimation

Larvae will be obtained under the respective testing laboratory's standard operating procedure (SOP). If large numbers of larvae are being tested (>200 per day), it is advisable to confine a queen inside a cage with a frame so that it becomes easier to obtain large number of larvae with similar ages, about 4-5 days later. The cage can be constructed using queen excluder material to allow workers to pass but restrict the queen to be inside. The cage is readily available in China but no US suppliers carry it.

2.1.7 Handling

A soft larval transferring tool consisting of a thin flexible plastic scoop with a spring-loaded bamboo plunger designed to push the larvae off once it has been harvested (queen grafting tool, China, **HD-390**, mannlakeltd.com) is used for the transfer. Larvae that are damaged, flipped, or dropped during transfer will not be used. Larvae will be transferred and oriented to allow their spiracles access to air. To reduce cross contamination by pathogenic microorganisms the larval transferring tool is sanitized with 70% ethyl alcohol and blotted dry with a Kimwipe at regular intervals of approximately 10 transfers.

2.2 Physical System

2.2.1 Containers

The containers used in the study will be 24-well cell culture plates (3.4 mL/well, Corning). Containers will be cleaned/siphoned daily during the study to remove old diet prior to adding fresh diet. Care will be taken not to adversely disturb the larvae. Each test container will be labeled with the treatment, replicate and study number.

2.2.2 Replication and Control of Bias

Each treatment group will have four to six replicates from 4 to 6 hives each containing approximately 24 honey bee larvae at initiation (approximately 96 to 144 honey bee larvae per test group). Larvae are assigned to each treatment well in a non-systematic manner. Each participating laboratory will use its SOP for labeling and tracking of both honey bees and test material. Larvae that are injured by handling during the course of the study will be censored from the analysis.

2.2.3 Required Equipment and Supplies

- Desiccators (the desiccator is used with the sulfuric acid and glycerol to maintain high humidity and limit exposure).
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- Laminar flow hood
- Oven for sterilizing glass
- Fiber optic light (used to aid with transfer of larvae from the frames)
- Growth chamber
- Cell culture growth trays
- A vacuum source
- Tubing for vacuum
- Fine tip glass fitting
- Grafting tool
- Kimwipes
- Ethyl alcohol
- 100 mL beaker
- Cage of queen exclusion material (refer to section 2.1.6)
- D fructose (F3510, sigmaaldrich.com)
- D glucose (G7021, sigmaaldrich.com)
- Royal Jelly
- Yeast Extract (212750, Bacto Yeast Extract, Becton, Dickinson and Company)
- Distilled water

2.3 Study Conditions

2.3.1 Temperature

Cell culture plates will be maintained at 34 ± 2ºC. Temperature will be continuously monitored using a minimum/maximum thermometer placed in the incubator. Readings of temperature extremes will be recorded daily.

2.3.2 Relative humidity

The test system will be placed in an incubator designed to maintain the relative humidity at 90 ± 5% for larvae and 75% ± 5% for pupae.

2.3.3 Lighting

Larvae will be incubated in darkness to simulate hive conditions. However, larval honey bees will be exposed to laboratory lighting for approximately 30 minutes each day during the daily replacement of diet.

2.3.4 Study Initiation

Culture plates will have 100 µL of warmed (approximately 34ºC) larval diet added to each well of a 24-well cell culture plate. Second instar larvae will be transferred in a laminar flow hood. Second instar larvae (hatched within 24 to 48 hrs) will be individually chosen in a non-systematic manner and transferred gently into each cell of the 24-well cell culture plates.

3.0 LARVAE GRAFTING AND REARING

To obtain 1st instar larvae of worker bees, a queen should be confined using a queen excluder cage (workers can pass through the cage grid while the queen can not) for 36 hours on a worker comb. The worker bees could move in and out of excluder box freely. The cage is placed into the center of the hive, so that the queen and the larvae can be well-tended by nurse bees. After 36 hours, check for the presence of eggs in the confined comb and released the queen. On the fourth day, remove the frame from the hive and bring it to the laboratory.

Add 100 µL of diet to each well of a 24-well cell culture plate (3.4 ml/well, Corning), pre-warmed (34 °C, 5 minutes) before grafting larvae. The larval transfer is carried out in a laminar flow hood. First instar larvae (hatched within 24 hrs) are transferred gently into cells of the 24-well cell culture plates at a density of one larva per well. It is helpful to use a fiber optic light to help visualize the larvae in cells. A soft larvae transferring tool (queen grafting tool, China, Fig. 1) is used for the transfer. To reduce cross contamination by pathogenic microorganisms the larval transferring tool is sanitized with 70% ethyl alcohol periodically (about every 10 larvae).

The larval rearing plates are transferred into a desiccator where the relative humidity is maintained at 95% (10% sulfuric acid solution in water, v/v). The desiccator is kept in a dark incubator maintained at 34 °C. Each day, removed excess diet using a vacuum (utilizing the lab supplied vacuum with a glass flask to trap the liquid, a glass pipette (Pasteur) is attached at the end for aspiration). Next, pre-warm fresh diet to incubator temperature and add to the larval rearing plate. The amount of food to add each day is shown in Table 1. Larvae will defecate when they are ready to pupate. Defecation is characterized by the presence of uric acid crystals and stringy material in the well (Fig. 2). When a larva defecates, it is ready to be transferred to a pupation plate.

Table 1. Amount of diet to add to the larval rearing plates per day.

Days	1	2	3	4	5	6	Total
Amount (µl)	100	100	100	200	200	200	900

Record mortality each day. Dead larvae are recognized by the following symptoms: absence of movement when observed under a dissecting microscope, lack of turgidity, flattened body, tissue that is starting to disintegrate.

4.0 TRANSFER OF LARVAE TO PUPATION PLATES

Pupating plates are prepared with the same type of 24-well plates except that each well is matted with 2 layers of dust-free Kimwipes. The Kimwipes should be previously rinsed with 70% ethyl alcohol and dried before being cut into pieces of 3 x 1.4 cm. The mature larvae are dried on 2 layers of pre-cleaned Kimwipes before being transferred to the pupation plates at the density of one larva per well. Transfer larvae to pupation plates using a modified Chinese grafting tool (with the plunger removed, Fig. 3). To avoid contamination, the operations are carried out in a laminar hood, and the larval transferring tool is sanitized with 70% ethyl alcohol prior to each use. The pupation plates are kept in an incubator at 34 °C and 75% relative humidity (maintained by saturated NaCl solution, or a RH regulated incubator), until adult bees emerge (this happens around 10-11 days after larval transfer) (Fig. 4). Do not disturb the bees to check for survival for approximately 3-4 days after the transfer to pupation plates as the pre-pupal stage is the most sensitive stage to movement and manipulations.

5.0 TYPICAL RESULTS

				Survival rates (%)		
	# of larvae transferred	# of pre-pupae	# of emerged adults	Larvae to pre-pupae	Pupae to adults	Larval to adults
N	168	156	133	92.9%	85.3%	79.2

The critical step is the familiarity with the art of larval transfer from the hive to plates, and the transfer of mature larvae to pupation plates. Because there is no daily transfer of larvae, MSU has obtained over 80% average total survival (from larvae to adults) over the last three years. This is considerably better than the reported 50% survival in controls in the recent publications.

Another important factor is the source of royal jelly. MSU is still trying to determine if there are large variations in the quality among different suppliers. The current supplier, BeeNatura.com, has consistently provided quality product based on MSU results of the last three summers.

6.0 REFERENCES CITED

Romeis J, et al. (2008) Assessment of risk of insect-resistant transgenic crops to nontarget arthropods. Nature Biotechnology 26: 203-208

Vandenberg, J.D. and Shimanuki, H., 1988. Technique for rearing worker honeybees in the laboratory. Journal of Apicultural Research 26: 90–97

Figure illustration

Fig. 1. Grafting a first day larva using the Chinese larval grafting tool (the faint hexagons in the background are brood cells).

Fig. 2. Uric acid crystals and fiber-ish material from larval defecation

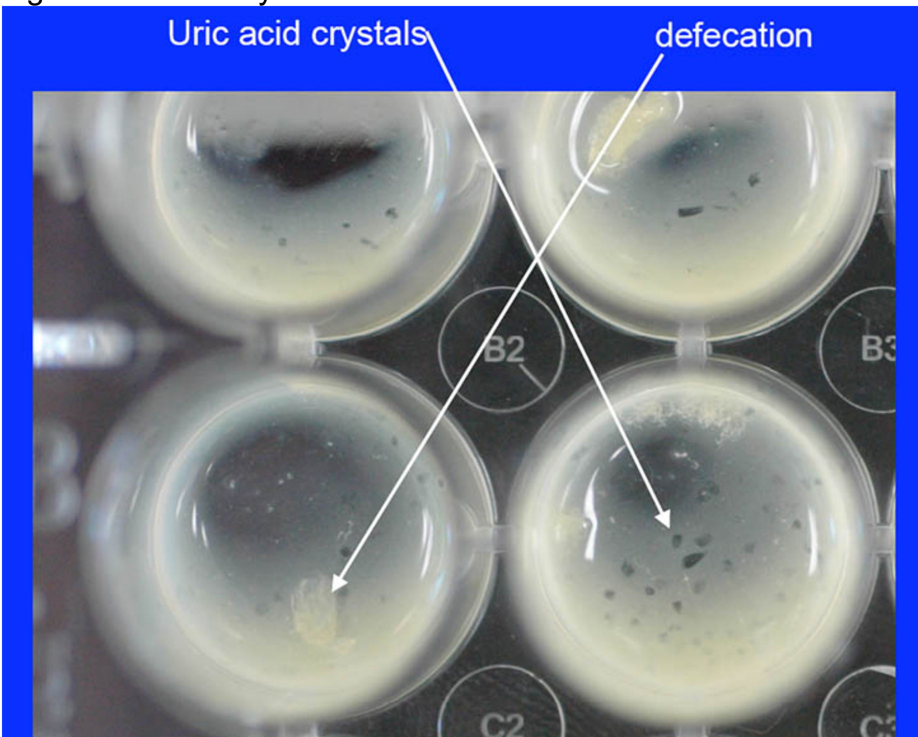

Fig. 3. Transfer of mature larva for pupation, using a modified grafting tool.

Fig. 4. A newly enclosed adult worker in a cell from pupation plate.

Sources of chemicals/supplies

1. D(+) glucose, tissue culture tested, G7021, Sigma
2. D(-) fructose, tissue culture tested, F3510, Sigma
3. Bacto yeast extract, Becton and Dickinson, catalog # 212750
4. Water: regular distilled water, after 5 min boiling
5. 24 cell tissue culture plates, Costar 3526, Corning, NY 14831
6. Royal Jelly
   (MSU supplier: Bee Natura (www.BeeNatura.com))
7. Grafting tool, standard soft Chinese grafting tool
   (http://www.beeworks.com/catalog/index.php?main_page=product_info&products_id=2)
8. Kimwipes, Kimtech Science