Appendix 2: Elements of a Larval Study

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A2.1 PROPOSED ELEMENTS FOR A LARVAL STUDY

- Larvae at the L1 (first instar) stage are fed standardized amounts of a semi-artificial diet. Test items (pesticides or other products of interest) are incorporated into the food at different concentrations within an appropriate range in order to compute the following endpoints for larvae (L1 to L5), pupae (L5 to adult emergence), and adults (emergence to Day 22 post-emergence): LC50, LD50, and NOEC (the NOEC will be the principle target endpoint).
- The reference product is typically dimethoate.

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A2.2 LARVAE TERMINATION AND COLLECTION

- For one replicate, larvae are collected from a unique colony. Test colonies have to be healthy and must not show any visible clinical symptoms of pests, pathogens, and/or toxin stress. Tests should be conducted with summer larvae during a period from the middle of spring to the middle of autumn (the exact time of year varies by location). No varroa treatment with the exception brood removal should be applied within the 8 weeks preceding the beginning of experiments.

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- At Day 3 (prior grafting, Figure 4), the queen of the chosen colony is confined in its own colony onto a comb. This can be done using an excluder cage into which a comb (dark preferred) containing empty cells is placed or by using a smaller push-in cage (~10 × 10 cm) that can be used to confine a queen on a given section of comb containing empty cells. In both cases, the comb is placed close to other combs containing brood (Figure 1).
- At Day 2, with the verification that there are eggs, the queen is removed from the cage 22–26 hours after she was encaged. To ensure that larvae are available at Day 1 of the study it is recommended to cage the queens of 2–3 colonies in the event a queen is laying few or no eggs. Based on queen vigor, the queen's isolation time can be reduced in order to minimize variability in larval size (age).
- The comb containing the eggs is left caged to prohibit the queen from ovipositing further on the comb on the same position near the brood frames. The eggs develop until the larvae hatch at Day 1.
- At Day 1 (Figure A2.1), the comb containing first instar larvae is transferred from the hive to the laboratory for grafting. As L1 larvae are subject to desiccation, a wetted towel should be placed around the comb.

As per instruction, we have changed Figure 2 and Figure 3 cited in the appendices' text to Figure 10.2. Please specify how the citations of Figures 1 & 4 need to be changed.

Au: We have changed Figure 10.3 to Figure A2.1. Please check is it OK?

A2.3 PREPARATION OF REARING MATERIAL

A2.3.1 Rearing Cells

- Larvae (≤1 day old) are reared in polystyrene grafting cups (common among beekeeping equipment supply companies. Cells with rounded bottoms are best) having an internal diameter of approximately 9 mm. Before use, the cells are washed and sterilized in 0.4% MBC (methyl benzethonium chloride) water solution or ethanol and rinsed in sterile water then dried in a laminar-flow hood. Each larva is placed in a well of a 48-well tissue culture plate.
- Larvae plates with lids closed, are placed into a larval chamber such as a hermetic chamber (e.g., Plexiglas desiccator, a plastic container, etc.) into which a dish having a potassium sulfate (K_2SO_4) saturated solution is placed to maintain a water-saturated atmosphere (>90% relative humidity). The larval chamber is placed into an incubator at 34.5°C. It is important that this temperature is maintained within a small range since temperature can affect the toxicity of pesticides to immature bees (Medrzycki et al. 2010).

A2.3.2 Larval Food

- The food is composed of three diets for different days of the study with Diet A following the recipe of Vandenberg and Shimanuki (1987) and subsequent diets modified from this basic diet.
- Diet A (Day 1): 50% fresh royal jelly + 50% aqueous solution containing 2% yeast extract, 12% glucose, and 12% fructose. A recipe for 20 g diet contains 10 g royal jelly, 1.2 g glucose, 1.2 g fructose, and 0.2 g yeast extract mixed in 7 mL H_2O.
- Diet B (Day 3): 50% fresh royal jelly + 50% aqueous solution containing 3% yeast extract, 15% glucose, and 15% fructose. A recipe for 20 g diet contains 10 g royal jelly, 1.5 g glucose, 1.5 g fructose, and 0.3 g yeast extract mixed in 7 mL H_2O.
- Diet C (from Days 4 to 6): 50% fresh royal jelly + 50% aqueous solution containing 4% yeast extract, 18% glucose, and 18% fructose. A recipe for 21 g diet contains 10 g royal jelly, 1.8 g glucose, 1.8 g fructose, and 0.4 g yeast extract mixed in 7 mL H_2O.

A2.3.3 General Information Regarding Diet Preparation

Royal jelly can be stored frozen at −20°C in small aliquots to avoid multiple freezing which causes a change in the sugar crystals. It should be thawed by placing it at 4°C overnight, or at room temperature for 1–2 hours.

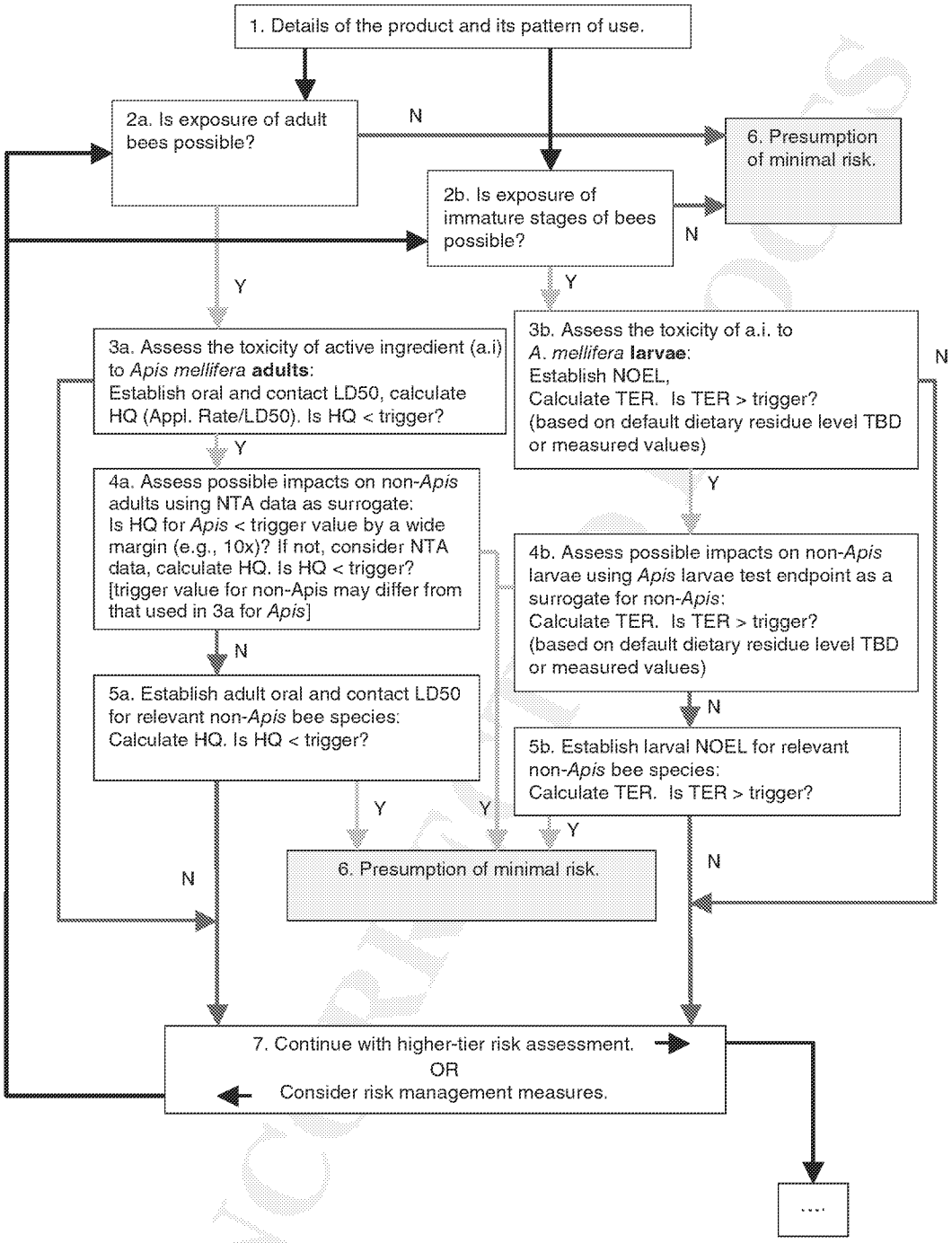

FIGURE A2.1 Insect pollinator screening-level risk assessment process for foliarly applied pesticides. (For a color version, see the color plate section.)

Reverse osmosis water or distilled water should be used, boiled for 10 minutes, and cooled to 45–55°C (cool enough for hands to touch) before using it for mixing. Water, sugars, and yeast should be mixed thoroughly (all solid materials should be broken up with a sterile spatula) in lab ware (preferably glass lab ware such as a beaker) that has been autoclaved. The mixture should be vortexed for 30 seconds. Once the bubbles have settled, the total volume should be adjusted to 10 mL with the prepared water. Finally when the mixture has room temperature, 10 g of royal jelly should be added to the mixture and the mixture vortexed for 30 seconds. The diets prepared for a test should be stored in a refrigerator at ~5–10°C during the test.

A2.3.4 Pupation and Emergence

- At Day 7 (prepupal stage), the plates with open lids are transferred into a pupal chamber (i.e., a hermetic Plexiglas desiccator, a plastic container, etc.). The chamber should be maintained with a saturated atmosphere (~75% relative humidity), this can be achieved by placing a dish with an NaCl saturated solution into the chamber.
- The container is then placed into an incubator at 34.5°C.
- At Day 15, each plate is transferred into an emergence box (~11 × 15 × 12 cm) with a cover that is aerated with wire gauze. The emergence chamber should contain a piece of comb (~3 × 5 cm) that attracts the emerging bees. Emerging bees are fed *ad libitum* with a sucrose syrup solution (50% sucrose/distilled water by volume) that is supplied in a 2 mL eppendorf tube with a hole below. The emergence box is returned to the pupal chamber.

A2.3.5 Grafting and Feeding of Larvae

- The rearing cells in the well plate are prepared by pipetting 20 μL of Diet A into each cell. The comb is placed angular on a clean table and a cold light or LED light is used for illumination to prevent larvae from drying.
- The grafting of the L1 larvae is performed by quick transfer from the comb to each plastic cell cup and placed on the surface of the diet using a grafting instrument of choice (a grafting spoon, paint brush size 00, Chinese grafting tool, etc.).
- If grafting is performed from several combs or a comb is not used for a moment it should be covered by a wetted towel. The grafting should be performed randomly to maintain treatment heterogeneity.
- When a plate is completed with 48 larvae, it is placed into the larval chamber and then into the incubator immediately.
- The larvae are fed once a day (except at Day 2) at the same time of day (± 1 hour) three different diets in different amounts using a stepwise pipette with sterile tips. Prior to administration to the larvae, the diet is warmed to 34.5°C by placing in the incubator 1 hour before feeding. The diet should be pipetted on the inner side wall of the cell to slide slowly down in order to avoid the larvae from drowning. It must be avoided that the diet is placed on the larvae to prevent blocking of the spiracles.

A2.3.6 Experimental Groups

- The experimental unit is a single larva in a cell and a treatment group consists of minimum 24 larvae (half of a 48 tissue culture plate). For each test, the following treatment groups should be used:
  - one control diet without solvent (24 larvae)
  - one control diet with solvent (24 larvae)

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- five test item concentrations (24 larvae each)
- one reference treatment with dimethoate (24 larvae)

Each test (all eight groups of test larvae) should be replicated across three independent colonies (unrelated queens).

A2.4 PREPARATION OF THE PESTICIDE SOLUTIONS

- The test pesticide is dissolved in water (the preferred solvent) or acetone if the pesticide is not water soluble. If a solvent other than water is used, a second solvent control group must be used consisting of control larvae fed with diet containing the solvent at the same concentration as the treated samples.
- Dilutions of the stock solutions are made with non-chlorinated, sterile drinking water using disposable pipette tips equipped with a filter. The amount of test solution administered must not exceed 10% of the final volume. In all cases, one must include the same final volume of water or solvent in all treatments and controls.

A2.4.1 Treatments

- In acute toxicity tests, larvae are treated at Day 4 with Diet C containing the test item solutions at their respective test concentrations.
- For chronic toxicity tests, larvae are treated daily (except Day 2) with the diets containing the test item solutions at test concentrations. In order to assess the adequate endpoints (NOEC and LC50), run a preliminary experiment with appropriate test material, varying geometrically through 5–10 different concentrations, which can be determined.

A2.4.2 Toxic Reference

- The toxic reference is typically the organophosphate dimethoate:
  - in acute toxicity tests: 3 μg/larva is mixed with Diet C and provided at Day 4,
  - in chronic toxicity tests: it is mixed with the three diets at test concentrations of 20 μg/kg diet.

A2.4.3 Definition of Mortality

- Larva: An immobile larva (not breathing or moving when viewed under a dissecting scope) is recorded as dead. If a larva's mortality is in doubt, examine the larva the following day.
- Pupa: A non-emerged individual at Day 22 is considered as dead during the pupal stage.
- Adult: An immobile adult that does not react to a tactile stimulation is recorded as dead.

A2.4.4 Mortality Assessments

- Larva: Daily (except Day 2) when larvae are fed, all dead larvae are removed for sanitary reasons. Specific mortality checks are made according to the type of test (acute or chronic). In the acute test, where exposure is at Day 4, a first mortality check is made at Day 4 in order to replace the dead larvae before they have started consuming the diet containing the insecticide. Mortality must also be recorded at Days 5, 6, and 7. In the test with chronic exposure mortality is noted at Day 7.
- Pupa: Non-emerged bees are counted at Day 22.

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- Adult: Short-term survival: dead adults and living (emerged) adult bees that left their cell and show a normal development are counted at Day 22.
- Long-term survival: living adult bees and dead adults are assessed daily through 10 days post-emergence. Typically, control mortality increases from Days 12 to 14.

A2.4.5 Validity Range of Data

- For the test to be considered valid, bees fed the control diet must adhere to the following:
  - Larvae: ≤10% mortality (number of dead larvae/24)
  - Pupae: ≤20% mortality (number of dead pupae at Day 22/24)
  - Adult: ≤10% mortality (number of dead adults at Day 10 post-emergence/total number of emerged adults)

If the mortality in the control groups is higher than that outlined here, the test is invalidated.
The mortality rate within the dimethoate control should be:

- Acute test: ≥50% mortality at Day 6 for larvae exposed to 3 μg dimethoate/larva at Day 4.
- Chronic test: ≥50% cumulative mortality at Day 7 after exposure to 20 mg dimethoate/kg diet.

The calculated LC50 must be in each case between the concentrations tested; the LC50 must not be extrapolated outside of the tested concentration.

A2.4.6 LD50 and LC50 Calculation

- Mortalities are expressed in percentage of the reference populations after an adjustment according to the Abbott formula (1925):

$$\text{Raw mortalities}: M = \frac{(P - T)}{S} \times 100$$

$$\text{Percent mortalities}: M = \frac{(\%P - \%T)}{100 - \%T} \times 100$$

- M is the adjusted mortality expressed in percentage of the initial population, initial number of larvae (24) for a larval mortality, number of living prepupae at Day 7 for pupal mortality, number of emerged (adult) bees at Day 22 for adult mortality
- P, mortality due to the treatment
- T, control mortality
- S, surviving number in control
- $\%P$, mortality percentage due to the treatment
- $\%T$, control mortality percentage

The results will be analyzed using regression and/or probit modeling. All raw and adjusted data must appear in the study report. The lethality graphs and their equations must be reported. The results should include LC50 values for 24 and 48 hours expressed in terms of μg per individual (for the acute test), and for an LC50 in μg per liter of solution (ppb) for the chronic test. These calculated variables should include their respective 95% confidence intervals.

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A2.4.7 Determination of the NOEC

The NOEC is the highest concentration which does not induce mortality significantly higher than that observed in controls. This analysis is typically performed using a chi-square test (one-tail test, at $\alpha = 0.05$).

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