8 Assessing Effects Through Laboratory Toxicity Testing

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8.1 INTRODUCTION

Toxicity testing in support of a risk assessment process for determining the potential impacts of chemicals to pollinator insects, and, more specifically, honey bees has typically involved both laboratory and field studies. Initially, tests are conducted that are intended to serve as a screen for whether a chemical represents a potential hazard. These tests are typically laboratory-based studies conducted on individual bees and are intended to provide conservative estimates of toxicity based on acute exposures of individual organisms under highly controlled environmental conditions. Based on the likelihood of exposure and the degree of sensitivity of

Au: There are two references with the same year in the reference list for the following references. They are:Alix et al., 2009 EPPO, 2010 OECD, 2007 Therefore to differentiate between them, the years have been changed by adding "a" and "b" along with year, for example, "Alix et al., 2009a and Alix et al., 2009b." Please specify their corresponding in-text citations.

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the test species in the initial laboratory tests, higher-tiered tests may be required to understand whether the effects observed in laboratory studies conducted on individual insects extend to the colony/population level under environmentally relevant exposure conditions.

For reasons discussed earlier, testing to determine the potential effects of chemicals on non-target organisms has typically relied on the use of surrogate test species. Selection of a surrogate species must consider the availability of the species and its ability to thrive under laboratory testing conditions. As such, the husbandry/environmental needs of the test species must be ~~well-known~~/documented so that tests can be readily conducted and reproduced/replicated. Ideally, the test species should be a relatively sensitive indicator of toxicity; however, it is generally recognized that the test species is unlikely to be the most sensitive of all species it is intended to represent. Although the European honey bee (*Apis mellifera*) has been used extensively in testing chemicals for potential effects, it is recognized that its biology is different from non-*Apis* bees (e.g., solitary bees) and other pollinating insects and that these differences may translate into significant differences in how the organism may be exposed/affected. The extent to which data from any surrogate test species are considered biased can only be elucidated through equally rigorous studies using other species. Currently, data for non-*Apis* bee species are limited; however, differences in the sensitivity of *Apis* and non-*Apis* bees may not be as pronounced as differences in potential exposure between honey bees and non-*Apis* bees. As an example, solitary ground-nesting bees of similar sensitivity to honey bees may be more vulnerable to exposure to soil treatments compared to honey bees.

The intent of toxicity tests is to provide measurement endpoints that can be used to assess the adverse effects from exposure to a particular stressor, for example, pesticides. Endpoints measured at the individual level are intended to provide insight on effects that are likely to impact entire populations/communities. In doing so, measurement endpoints drawn from laboratory-based tests should be readily linked to assessment endpoints (i.e., impaired survival, growth, or reproduction) that, in turn, are linked to protection goals. These assessment endpoints relate directly to maintenance of insect pollinators at the population/community level.

To ensure greater consistency in toxicity testing across chemicals, regulatory authorities have established guidelines that outline study design elements that should be considered as well as the nature of data to be collected. To conserve resources (i.e., focusing resources where they are most needed), and limit the number of animals required for testing, regulatory authorities have approached ecological risk assessment in a tiered manner. Laboratory-based studies (Tier 1), which can be conservative, relatively rapid and economical, are the first tier in evaluating chemicals for their potential (toxic) effects. Tier 1 tests provide an understanding of acute lethality and potential sublethal effects. This information should guide the decision of the assessor whether additional testing is needed. If, based on the outcome of Tier 1 laboratory-based studies, more refined studies are required, then their design should be informed by the Tier 1 study. A higher-tier study, such as a semi-field study, should be designed to answer questions identified in the lower-tier study(ies), which are limited. As such, a linkage should begin to be drawn between different tiers, that is, as moving from studies that look at the individual to studies that begin to look at the colony, and ultimately look at the colony in an environmentally realistic setting.

Considerable testing has been conducted with honey bees under relatively standardized conditions ~~which has resulted~~ in a sizeable database on the acute contact toxicity of a wide range of chemicals. This toxicity data generated through relatively standardized testing enables risk assessors to compare the relative toxicity of chemicals to bees across chemical classes with highly divergent modes of action. Workshop participants believed that since Tier 1 laboratory studies often serve as the basis on which further testing is or is not required, these studies are relied upon to be accurate, informative, and efficient. Further, studies must be designed and harmonized to provide the highest quality data with the least amount of variability. This chapter provides an overview of existing toxicity tests and their strengths/weaknesses and discusses proposed modifications to existing studies, or additional studies that could address limitations in the current battery of studies.

8.2 OVERVIEW OF LABORATORY TESTING REQUIREMENTS AMONG SEVERAL COUNTRIES

8.2.1 Overview of Honey Bee Laboratory Testing in the European Union

To assess the potential hazard of pesticides to honey bees, regulatory agencies in different world regions have developed varied approaches and requirements for hazard testing in support of ecological risk assessment. The requirements for regulatory testing on honey bees in the European Union (EU) can be found in Annex II and III of EU Directive 91/414 (EU Directive 91/414)~~. Additional~~ regulatory guidance ~~is being~~ provided ~~by the EU Terrestrial Guidance Document, SANCO/10329/rev 2 final, 2002, and recently revised EPPO documents~~ (~~SANCO/10329/rev 2 final, 2002~~; OECD, 2007; EPPO, 2010, 2011). A new EU Regulation (EC ~~No~~ 1107/2009), intended to replace EU Directive 91/414, was published in October 2009, but the data requirements and risk assessment criteria to support this new directive have not been established ~~(EC No 1107/2009).~~

European testing has always followed a sequential testing scheme, that is, starting with laboratory-based testing and then moving on to higher-tier studies if warranted. Where there is only one route of exposure (e.g., oral exposure in case of soil application of systemic products), the acute testing can be restricted to that route (i.e., contact or oral). ~~For~~ systemic products applied as a seed dressing, the acute oral toxicity of the active substances has to be determined ~~as oral exposure is a relevant route of exposure.~~ However, in recent years, information and incidents have indicated that contaminated dust associated with planting pesticide treated seed is an exposure route that should be considered. (Alix et al., 2009; Forster, 2009; Pistorius et al., 2009). In such a case, potential routes of exposure would include oral contact and, therefore, effects testing would be required to account for both routes of exposure. Acute tests with the formulated product, that is, active ingredients (a.i.) plus inerts, are required if the product contains more than one active substance, or if the toxicity of a new formulation cannot be reliably predicted to be either the same or lower than a formulation ~~tested~~ (EU ~~Directive~~ 91/414~~, point 10.4.1~~).

In the EU, regulatory authorities may require a bee brood feeding test to assess potential hazard of a pesticide on honey bee larvae. Currently, this testing must be carried out when the active substance may act as an insect growth regulator, or when available data indicate that there are effects on development at immature stages. Larval testing may be carried out according to the method described by Oomen et al. (1992) in which colonies are fed pesticide concentrations in sugar syrup. Dose levels used in this test should reflect maximum levels (of active substance) expected in the applied product.

If results of either the adult or larval tests indicate that a presumption of minimal risk cannot be made, then further testing such as a semi-field or field testing is triggered in order to determine whether any toxicity is observed under realistic exposure conditions. OECD guidance document No 75 (OECD, 2007) and EPPO 170 (EPPO, ~~2010; PP1/170~~) provide recommendations on testing honey bee brood under semi-field and field conditions.

8.2.2 Overview of Honey Bee Laboratory Testing for Regulatory Purposes in North America

Similar to the EU, North America (United States Environmental Protection Agency (~~US~~EPA), and Canada's Pest Management Regulatory Agency (PMRA)) employs laboratory-based tests as a first step for evaluating the potential toxicity of chemicals to insect pollinators. The ~~US~~EPA's data requirements for insect pollinator testing are defined in the US Code of Federal Regulations 40 (CFR 40~~; Protection of the Environment)~~ Part ~~158 (Data Requirements for Pesticides) Subpart G (Ecological Effects) §158.630 (40 CFR Part 158, 2012).~~ Similar to the European process, the North American process also follows a tiered approach.

Tier 1 consists of an acute contact toxicity test with young adult honey bees~~, USEPA Guideline 850.3020,~~ (USEPA, 2012a). Until recently, ~~US~~EPA has typically required just the acute contact toxicity test; however, in efforts to better harmonize with its counterparts in Canada and Europe~~,~~ and in recognition that exposure occurs through ingestion of pesticide residues as well as through contact, the United States has begun to require oral toxicity tests consistent with OECD ~~Guideline 214~~ (OECD, 1998~~a~~). Higher tier studies may be required if the results of the acute toxicity tests indicate that the LD50 <11 μg a.i./bee toxicity, and/or if other lines of information, such as data in the open literature and incident data indicate that additional information is needed.

Currently, higher tier tests include laboratory-based toxicity of residues on foliage~~ test, that is, USEPA Guideline 850.3030~~ (USEPA, 2012b) and field-based pollinator ~~study, USEPA Guideline 850.3040~~ (USEPA, 2012c). The toxicity of residues on foliage test is based on the work of Johansen et al. (1977) and Lagier et al. (1974) and is intended to provide data on the residual toxicity of a compound to honey bees. In this study, the test substance is applied to a sample of crop material (alfalfa is preferred) at the typical label rate and placed ~~in~~ with caged test bees which ~~are allowed to~~ forage on the treated plant material. Mortality and adverse effects are recorded after 2, 8, and 24 hours of exposure to the treated foliage. If the mortality of bees exposed to 24-hour-old residues is greater than 25%, sampling is continued at 24-hour intervals until mortality of bees exposed to treated foliage is not significantly greater than the controls.

Beyond the toxicity test of residues on foliage, if any of the following conditions are met, EPA may require a pollinator field study (~~OPPTS Guideline 850.3040;~~ USEPA, 1996):

- Data from other sources (e.g., open literature, beekill incidents) indicate potential adverse effects on colonies, especially effects other than acute mortality (reproductive, behavioral, etc.).
- Data from toxicity of residue on foliage studies indicate extended residual toxicity.
- Data derived from studies with terrestrial arthropods other than bees indicate potential chronic, reproductive, or behavioral effects.

Field pollinator testing is intended to examine the potential effects of a chemical on the whole honey bee colony, and the nature of these studies is discussed in Chapter 9. ~~US~~EPA testing requirements stipulate ~~that~~ acute contact toxicity tests ~~be conducted using technical grade active ingredient (purity~~ >95%~~)~~, while higher-tier tests are typically conducted using the formulated product.

8.3 UNCERTAINTIES IN CURRENT TESTING PARADIGMS

Laboratory-based acute toxicity testing of honey bees in the United States has not formally included studies examining the potential effects of pesticides on honey bee larvae (brood). In addition, while test guidelines stipulate that sublethal effects must be reported in acute tests, the typical endpoint reported from these tests is the median lethal dose (LD50) and rarely is a median effect concentration (EC50) based on sublethal effects reported. Given that the current US test guidelines are designed to yield regression-based endpoints, that is, LD_x values, endpoints such as no-observed-adverse-effect concentrations (NOAEC) and lowest-observed-effect concentrations (LOAEC) which require hypothesis testing are not likely attainable since treatments are not sufficiently replicated.

Also, as noted earlier, under the US testing process, the honey bee is used as a surrogate for other pollinator insects and for terrestrial invertebrates. In the EU, however, specific test guidelines are available for examining the effects of pesticides on non-target arthropods and beneficial insects based on the ESCORT 2 guidance (Candolfi et al., 2000) independent of the studies examining toxicity to honey bees. Uncertainties regarding the use of honey bees as surrogates for other non-*Apis* bees were identified at the ~~Pellston~~ workshop. These uncertainties are centered on the fact that the life history and social biology of honey bees are significantly

different from those of other bees and arthropods. At this time, there are insufficient data to determine whether or not honey bees serve as reasonable surrogates for other non-*Apis* bees or insect pollinators in general (i.e., whether laboratory studies conducted with *A. mellifera* provide endpoints sufficiently protective of the range non-*Apis* bees or other insect pollinator insects and/or terrestrial invertebrates). However, it was noted by ~~Pellston~~ participants that since laboratory studies are intended to examine the intrinsic toxicity of a chemical to a particular test organism, differences in the biology of the test organism relative to those species for which it is intended to serve as a surrogate may not be critical. Table 8.1 provides a comparison of the acute laboratory toxicity tests (OECD ~~213~~, OECD ~~214~~, and ~~OPPTS 850.3020~~) currently required by regulatory authorities in the EU and United States.

8.4 LIMITATIONS AND SUGGESTED IMPROVEMENTS FOR TIER 1 TESTING

8.4.1 Adult *A. Mellifera* Worker Acute Toxicity

Exposure of honey bees can be from direct overspray while the bees are foraging, by contact with contaminated surfaces of the plant, or by intake of contaminated pollen and nectar. The hazard posed by short-term exposures can be assessed using acute toxicity tests. As discussed in the preceding section, acute honey bee testing under laboratory conditions has been conducted for some time according to several different test guidelines and published methods~~, for example, EPPO 170 (1992, and updated in 2010), SETAC (1995), Stute (1991), and EPA OPPTS 850.3020 (USEPA, 2012a)~~. Workshop participants considered the OECD test guidelines (OECD, 1998a, 1998b) to be the most detailed of those available for assessing the acute toxicity of pesticides to honey bees for the reasons presented below.

Acute honey bee tests performed according to OECD ~~213 (acute oral toxicity; OECD, 1998b), and OECD 214 (acute contact toxicity; OECD, 1998a)~~, can be designed as limit tests or as dose–response studies (with a minimum of five doses and a minimum of 3 replicates of 10 bees at each dose). The bees are held under controlled temperature and humidity conditions ~~and~~ mortality and behavior is monitored for a minimum of 48 hours (this is extended if effects are prolonged). The reported data include the LD50 (with 95% confidence limits), at 24 hours, 48 hours and, if relevant 72 hours and 96 hours time points (in µg test substance per bee), the slope of dose–response curves, and any other observed abnormal bee responses. Both tests include ~~both~~ a control (treated with the same concentration of solvent as in the treated doses) and a toxic standard (e.g., dimethoate) with defined acceptance criteria.

The ~~OECD 214~~ acute contact test (OECD, 1998a) involves direct application of the test substance (active ingredient or formulation), usually as a 1 µL drop, diluted in an organic solvent or water as required, applied directly to the dorsal thorax of the bee. Among the advantages of the ~~OECD 214~~ acute contact test guidelines are:

- replication (at least three replicates);
- no in-hive treatments for 4 weeks prior to use in a study are permitted;
- higher number of test organisms is specified (30 bees);
- prescriptive environmental conditions;
- stringent control mortality is specified (10%);
- a toxic standard is required and validity criteria are stated; and,
- test duration is prolonged in case of delayed effects.

~~The~~ only internationally accepted oral acute toxicity test guideline ~~is OECD 213~~ (OECD, 1998~~b~~). The test is similar in ~~design to the OECD 214 (OECD, 1998a),~~ acute contact toxicity test, but consists of group feeding ~~of~~ a known volume of treated sucrose solution over a maximum period of 6 hours ~~to the replicate bees within~~

TABLE 8.1

Comparison of Acute Contact Test Guidelines (OECD ~~214~~ and ~~EPA OPPTS 850.3020~~) and Acute Oral Test Guideline (OECD ~~213~~)

	OECD ~~214~~ (Acute Contact)	EPA OPPTS 850.3020 (Acute Contact)	OECD 213 (Acute Oral)
Status and background	Adopted September 21, 1998 Based on EPPO GL 170 (1992) and improvements considered made by ICPBR (1993) Other GLs considered: SETAC (1995), Stute (BBA) (1991), EPA OPPTS 850.3020 (2012a)	Public draft April, 1996 Based on OPP 141-1 (1982)	Adopted September 21, 1998 Based on EPPO GL 170 (1992) and improvements considered made by ICPBR (1993) Other GLs considered: SETAC (1995), Stute (BBA) (1991), EPA OPPTS 850.3020 (1995)
Test species and test organisms	Young, healthy, adult worker bees (*Apis mellifera*), same race, similar age and feeding stage, from queen-right colony, known history Bees collected from frames without brood are suitable Bees should not have been treated chemically for at least 4 weeks	Young test bees, 1–7 days old (*A. mellifera*), may be obtained directly from hives or from frames kept in an incubator, from same source	Young, healthy, adult worker bees (*A. mellifera*), same race, similar age and feeding stage, from queen-right colony, known history Bees collected from frames without brood are suitable Bees should not have been treated chemically for at least 4 weeks
Test cages	Clean and well-ventilated, made of any appropriate material, for example, stainless steel, wire mesh, plastic, disposable wooden cages Groups of 10 bees	Test chambers may be constructed of metal, plastic, wire mesh, or cardboard, or a combination of these materials Groups of at least 25 bees	Clean and well-ventilated, made of any appropriate material, for example, stainless steel, wire mesh, plastic, disposable wooden cages Groups of 10 bees
Handling, feeding, preparation	Food–*ad libitum*—as sucrose solution (50% w/v),for example, via glass feeders Bees may be anaesthetized with carbon dioxide (CO_2) or nitrogen (N_2) for application. Amount should be minimal Moribund bees should be rejected before testing	A 50% sugar/water solution should be provided *ad libitum* (purified or distilled water should be used) Bees may be anaesthetized with carbon dioxide (CO_2) or nitrogen (N_2) for application	Food–*ad libitum*—as sucrose solution (50% w/v), for example, via glass feeders Feeding system should allow recording of food intake (e.g., glass tubes 50 mm long, 10 mm wide, and narrow end) Bees may be starved for up to 2 hours before test initiation Moribund bees should be rejected before testing

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TABLE 8.1
(Continued)

	OECD 214 (Acute Contact)	EPA OPPTS 850.3020 (Acute Contact)	OECD 213 (Acute Oral)
Solvents	Test substance applied as solution in a carrier, that is, organic solvent—acetone preferred—or a water solution with a (commercial) wetting agent Two separate control groups, that is, water and solvent /dispersant	A solvent is generally used to administer the test substance. The solvent of choice is acetone (or other volatile organic solvents) Two concurrent control groups, that is, water and solvent (or carrier) control	Test substance applied as 50% sucrose solution in a carrier, that is, organic solvent (e.g., acetone), emulsifiers or dispersants at low concentration up to max 1% should not be exceeded Two separate control groups, that is, water and solvent/dispersant
Test and control groups	Normally five doses in geometric series with a factor ≤ 2.2 covering the range of LD50 for definitive test (ranger-finder proposed) Minimum of three replicates with 10 bees for each dose rate and control (Minimum of 30 bees for each dose) Max. ≤ 10% control mortality at test end	A minimum of five dosage levels spaced geometrically. Recommended spacing for each dosage level to be at least 60% of the next higher level. Three or more dosages should result between 0 to 100% mortality Minimum of 25 bees for each dosage Max. ≤ 20% control mortality during the test	Normally five doses in geometric series with a factor ≤2.2 covering the range of LD50 for definitive test (ranger-finder proposed) Minimum of 3 replicates with 10 bees for each dose rate and control (Minimum of 30 bees for each dose) Max. ≤ 10% control mortality at test end
Limit test	100 μg a.i./bee in order to demonstrate that the LD50 is greater than this value	25 μg a.i./bee in order to demonstrate that the LD50 is greater than this value	100 μg a.i./bee in order to demonstrate that the LD50 is greater than this value
Toxic standard	At least 3 dose rates with 3 × 10 bees to demonstrate, for example, the toxic standard, dimethoate, is within the reported contact LD50 of 0.10–0.30 μg a.i./bee (Gough et al., 1994). Other toxic standards are acceptable	A concurrent positive control is not required A lab standard is recommended; also when there is a significant change in source of bees	At least three dose rates with 3 × 10 bees to demonstrate, for example, the toxic standard, dimethoate, is within the reported contact LD50 of 0.10–0.35 μg a.i./bee (Gough et al., 1994). Other toxic standards are acceptable

(Continued)

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TABLE 8.1
(Continued)

	OECD 214 (Acute Contact)	EPA OPPTS 850.3020 (Acute Contact)	OECD 213 (Acute Oral)
Exposure	1 μL per bee applied on dorsal side of thorax (higher volumes, if justified) via micro-applicator Temperature: 25 ± 2°C Relative humidity: 50–70% Test duration: 48 hours (If mortality increases by >10% between 24 hours and 48 hours, the duration is prolonged to maximally 96 hours provided that the control does not exceed 10%)	5 μL per bee should not exceeded Temperature: 25–35°C Relative humidity: 50–80% Test duration: 48 hours	100–200 μL per 10 bees of 50% sucrose solution in water (or higher) provided for 3–4 (max. 6) hours Amount consumed is measured Temperature: 25 ± 2°C Relative humidity: 50–70% Test duration: 48 hours (If mortality increases by >10% between 24 hours and 48 hours, the duration is prolonged to maximally 96 hours provided that the control does not exceed 10%)
Observations	Mortality at 4 hours, 24 hours, 48 hours, and potentially at 72 hours and 96 hours Abnormal behavioral effects during the test period should be recorded	Mortality at 4 hours, 24 hours, 48 hours All signs of intoxication and other abnormal behavior (e.g., ataxia, lethargy, hypersensitivity) during the test period should be recorded	Mortality at 4 hours, 24 hours, 48 hours, and potentially at 72 hours and 96 hours Amount of diet consumed per group should be measured to determine palatability of diet Abnormal behavioral effects during the test period should be recorded
Data reporting	Range-finding data LD50 plus 95% confidence limits, that is, at 24 hours, 48 hours and, if relevant 72 hours and 96 hours (in μg test substance per bee) and slope of curves Mortality statistics (e.g., probit analysis, moving-average, binominal probability) Other biological effects and any abnormal bee responses Deviations from test guideline	Range-finding data LD50 plus 95% confidence limits, that is, at 24 hours, 48 hours, and slope of curves, goodness-of-fit test results Mortality statistics (e.g., probit analysis, moving-average, binominal probability) Signs of intoxication and other abnormal behavior Deviations from test guideline	Range-finding data LD50 plus 95% confidence limits, that is, at 24 hours, 48 hours, and if relevant 72 hours and 96 hours (in μg test substance per bee) and slope of curves Mortality statistics (e.g., probit analysis, moving-average, binominal probability) Other biological effects and any abnormal bee responses Deviations from test guideline

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~~a cage~~ and then untreated sucrose is supplied *ad libitum*. Group feeding can be used to administer the dose of test substance because honey bees exhibit trophallaxis, i.e., the transfer of food among colony members; the applicability and repeatability of this is demonstrated by the toxic reference chemical (e.g., dimethoate), which is stable within a testing facility. ~~The test requires monitoring of the actual intake of the treatment to determine the intake of the test substance per bee as some~~ pesticides, such as pyrethroids, are repellent and the total dose may not be consumed.

Participants of the Workshop discussed the limited number of cases which would compel specific deviations from the OECD acute test guidelines, such as when working with the Africanized bee (Nocelli, personal communication). However, changes in study design can affect outcomes and reliability of the resulting data. Before data generated from modified study designs can be used reliably in risk assessment, the methodology and the resulting data should undergo a separate validation exercise (e.g., determination of appropriate toxic reference and control data).

8.5 ADULT ORAL CHRONIC TOXICITY—*APIS BEES*

Undertaking an adult oral chronic toxicity study is a refinement step in the proposed risk assessment scheme. Currently, there is no standardized guideline for chronic toxicity testing with bees, but method proposals and study design elements from acute toxicity tests which may be applicable to longer-term studies can be found in a number of publications~~, for example,~~ Schmuck ~~(~~2004~~)~~, Suchail et al. ~~(~~2001~~)~~, Moncharmont et al. ~~(~~2003~~)~~, Aliouane et al. ~~(~~2009~~)~~ ~~and the EPA Guideline OPPTS 850.3020~~ (USEPA, 2012a). ~~Participants of the Workshop identified several gross factors that should be considered when considering an adult chronic toxicity test, which are listed below. A more detailed list of chronic study design elements and considerations and proposed design elements can be found in Appendix 1.~~

- There is no standardized duration for the study considering that the longevity of honey bees differs between summer and winter. However, if the study aims at representing the typical exposure period of a forager on plants, then a 10-day period will cover most of the cases. Indeed, these bees will have already reached 14 days of age prior to being recruited as foragers, that is, the last activity of female worker bees. For summer bees, with their shorter life span and greater likelihood of being in the immediate vicinity of a treated crop, it is unlikely that their lifespan would last any longer than 10 days on the treated crop. Should the treated crops not be in their immediate vicinity, then it is likely that exposure will take place over a more limited period as the number of possible foraging trips per day declines as the distance increases. It is currently recommended that the study be performed over a10-day duration to ensure the most likely constant exposure period as well as high control survival (longer study durations may result in reduced control survival that can limit the ability of the study to detect treatment effects).
- To achieve a 10-day study duration, a mixed pollen (protein source) and sucrose (carbohydrate source) diet may be required.
- Some pesticides may induce reduced food intake due to repellency (e.g., pyrethroids) and the longevity of the bees may be affected by the reduced food intake due to repellency rather than reflecting a toxic effect of the pesticide. Therefore, food intake has to be assessed in parallel with mortality on a daily basis. The pattern of exposure may affect the observed toxicity for example, a single dose per day versus continuous exposure. Continuous exposure could mean: 1) dosed diet *ad libitum* or, 2) a fixed amount of dosed diet daily (e.g., 2 hours plus untreated diet during the rest of the time). Research is still underway to determine which approach is most appropriate.

8.6 HONEY BEE BROOD TESTS IN THE LABORATORY

The *in vitro* honey bee brood test provides quantitative oral/contact toxicity data on larvae for active ingredients or formulated products. These data should be used in an appropriate brood risk assessment scheme. *In vitro* larvae tests have been developed by Rembold and Lackner (1981) and used for the assessment of pesticides by Wittmann (1981). Some years later, Aupinel et al. (2005) improved this method in several aspects. Participants of the Workshop discussed brood tests, specifically the study design by Aupinel et al. (2005), and ~~considered~~ further design considerations and improvements ~~to this test~~. A detailed list of suggested modifications to the Aupinel et al. study design can be found in Appendix 2.

8.7 ADULT TOXICITY TESTING WITH NON-*APIS* BEES

As discussed previously, there is always an uncertainty regarding the extent to which a surrogate test species, such as the honey bee, is a sensitive indictor of the many other species it represents. Data currently available suggest that adult non-*Apis* bees are similar in pesticide sensitivity to *A. mellifera* when bodyweight is taken into account. This conclusion is based on the analysis of a data set composed mainly of test results for pesticides of older chemistries, so some caution may be in order when considering compounds of new chemical classes. Figure 8.1 shows the relative toxicity (contact LD50 normalized to 1 g body weight) of 21 pesticides to bumble bees and solitary bees in comparison to the honey bee. Figure 8.2 depicts the decline in toxicity of residues on foliage for honey bee adults compared to the solitary alfalfa leafcutter bee (*Megachile rotundata*) and the alkali bee (*Nomia melanderi*). Figure 8.3 depicts the median lethal doses of sprayed residues of four pesticides (clothianidin, imidacloprid, lambda cyhalothrin, and spinosad) to *A. mellifera*, *M. rotundata*, and *Osmia lignaria*. These data suggest that the toxicity of these pesticides falls within an order of magnitude of the values for *A. mellifera*. This indicates that an assessment factor of 10 may be adequate to account for interspecies differences in sensitivity when acute toxicity values for honey bees are used in risk assessments.

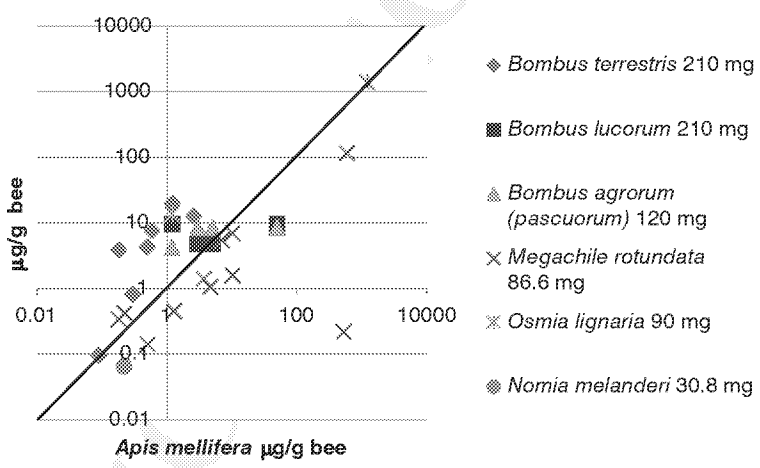

FIGURE 8.1 Comparison of the contact toxicity (LD50) of 21 pesticides to adults of *Apis mellifera*, three species of the social bee Bombus and three species of solitary bees (Osmia, Megachilidae, and Nomia). Points below the diagonal line indicate greater sensitivity than *Apis mellifera*, while points above the diagonal line represent lower sensitivity than *Apis mellifera* (Johansen et al., 1983). (For a color version, see the color plate section.)

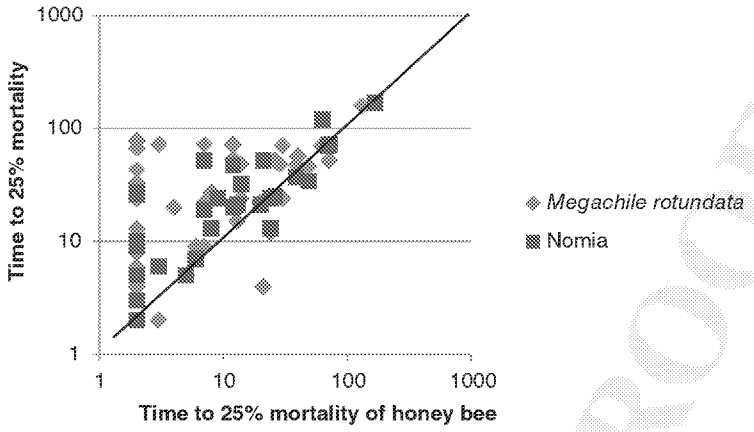

FIGURE 8.2 Comparison of the toxicity of pesticides to adults of *Apis mellifera* with the solitary bees *Megachile rotundata* and *Nomia melanderi* based on time for sprayed residues to decline to a concentration causing 25% or less mortality. Points below the diagonal line indicate greater sensitivity than *Apis mellifera*, while points above the diagonal line represent lower sensitivity than *A. mellifera* (Johansen et al., 1983). (For a color version, see the color plate section.)

As part of the problem formulation for an ecological risk assessment, risk assessors and risk managers can consider whether testing should include non-*Apis* species, such as when evidence or information suggests that the honey bee is not likely to be a reasonable surrogate for a crop, landscape, or region owing primarily to concerns regarding marked differences in potential exposure rather than in toxicity per se, that is, susceptibility rather than sensitivity. When selecting species to be used in the laboratory, it is important to consider their availability, ease of ~~handing~~ and survival under controlled laboratory conditions. Therefore, it is recommended that both relevance (to a risk assessment and attendant protection goals) and sensitivity and susceptibility are considered when determining whether to employ non-*Apis* species in an assessment.

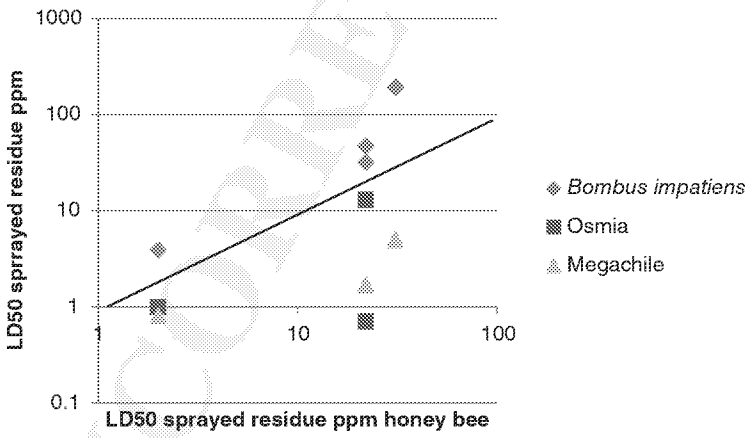

FIGURE 8.3 Comparison of the toxicity (LD50) of sprayed residues of clothianidin, imidacloprid, lambda-cyhalothrin and spinosad to adults of *Apis mellifera, Megachile rotundata*, and *Osmia lignaria* (Scott-Dupree, personal communication). Points below the diagonal line indicate greater sensitivity than *A. mellifera*, while points above the diagonal line represent lower sensitivity than *A. mellifera* ~~(Johansen et al., 1983).~~ (For a color version, see the color plate section.)

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Owing to differences in potential exposure, non-*Apis* bees may provide a means of examining the potential effects of these differences in the susceptibility of a species. For example, honey bees are capable of foraging over long distances and may have a wide range of forage available to them. However, non-*Apis* bees, for example, orchard mason bees (*O. lignaria*), are limited in the area in which they forage and may be confined to a particular treated area where the likelihood of exposure is increased.

8.7.1 Non-*Apis* Bee Testing Methods

As discussed earlier, toxicity tests intended to support regulatory decisions typically involve highly standardized testing protocols and rely on test species that are readily available and lend themselves to testing under laboratory conditions. The test species must be available in large enough numbers and have well-defined husbandry conditions to support replicate testing and thrive under specified test conditions used to examine particular routes of exposure. As with honey bees, the endpoints measured in toxicity tests with non-*Apis* bees have frequently focused on lethality; measures of sublethal effects on non-*Apis* bees would require similar linkages to assessment endpoints as those identified for honey bees. The development of these linkages may be more challenging though, as sublethal effects on individual solitary bees may have a considerably different impact at the population level than similar effects to social bees that form large colonies where the colony may have sufficient redundancy to buffer it from such effects.

The social non-*Apis* bee species most readily manipulated in the laboratory are the genera Bombini and the Meliponini (stingless bees). Some *Bombus* species are also readily available as they are used in commercial pollination of greenhouse crops. Several laboratory studies with non-*Apis* species have been published which reflect a range of methods (Table 8.2). As mentioned earlier, the ability of one non-*Apis* bee species to act as a surrogate for others involves the ready availability, and ability for that species to tolerate testing conditions. This then would indicate that the husbandry needs of that organism are well understood.

8.7.2 Non-*Apis* Larval Testing

Although toxicity testing with some species of adult non-*Apis* bees have been reported with some frequency, published laboratory studies conducted with non-*Apis* larvae are more limited, these are listed below (Table 8.3).

8.8 SUBLETHAL EFFECTS AND TEST DEVELOPMENTS

Sublethal effects are defined as the effects to individual that survive the exposure level eliciting the effect. As discussed, while not specifically designed for such, current acute tests include the recording and measuring of sublethal effects. The laboratory-based (10-day) chronic study, however, is designed (i.e., longer exposure duration) with the intent of providing more specific information on sublethal effect. Beyond these, experimental research published in the open literature has gone further into investigating sublethal effects of pesticides to bees. This research has revealed insights on physiology and behavior (Desneux et al., 2007). Most experimental research regarding the behavioral effects of pesticides on bees has occurred over the last 10 years. While these test methods and results ~~are interesting,~~ further work is needed not only to standardize test methods but also to be able to understand the impact of a sublethal effect in the context of the whole colony. A sublethal effect at the individual level is only relevant to protection goals when it can be linked to a resulting effect at the colony level. This section discusses some of the methods that have been developed to measure the potential sublethal effects of pesticides on honey bees.

TABLE 8.2
Published Laboratory Tests with Non-*Apis* Bees and Associated Methodologies

Species	Oral	Contact	Reference
Megachile rotundata *Osmia lignaria*	Individually housed adult bees with access to plastic ampoule containing pesticide inserted at base of periwinkle flower 87–90% success rate		Ladurner et al., 2003, 2005
M. rotundata	Group feeding of 10 newly emerged bees on 1 mL	1. Direct application— held at 25°C for 20 minutes to reduce activity, 1 μL applied to dorsal thorax 2. Filter paper soaked in pesticide and dried	Huntzinger et al., 2008
Bombus impatiens, *M. rotundata*, *O. lignaria*		Contact with treated filter paper	Scott-Dupree et al., 2009
M. rotundata (4–5-day-old adults); *Nomia melanderi* (2–3 weeks old)		Direct application to mesoscutum	Mayer et al., 1998
O. lignaria	Individually fed using flower (cherry) method For delayed activity fed on fresh sucrose	Cooled to 4°C before dosing, 1 μL applied to thorax	Ladurner et al., 2005
N. melanderi, *M. rotundata*	Placed into tubes inserted in caps of glass vials with individual bees, group-housed after dosing	Direct application to dorsal thorax	Johansen et al., 1983
M. rotundata		1 μL applied to thorax of males and females	Tasei et al., 1988
Bombus terrestris	Individually dosed and then group-housed	1μL applied to ventral thorax	Thompson, 2001

8.8.1 Proboscis Extension Response in Laboratory

When a bee lands on a flower, it extends its proboscis as a reflex stimulated by nectar. This reflex leads to the uptake of nectar and induces the memorization of the floral odors diffusing concomitantly. Thus, the memorization of odors plays a prominent role in flower recognition during subsequent forage trips by the same individual (Menzel et al., 1993). Under laboratory conditions, learning and memory can be analyzed using a bioassay based on the olfactory conditioning of the proboscis extension response (PER) on restrained individuals.

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TABLE 8.3
Larval Test Methods for Non-*Apis* Bee Species

Species	Test Elements	Measurement Endpoints	Reference
Osmia lignaria	Eggs raised on treated pollen in 24-well culture plates; cocoons overwintered and emerged 29°C	Timing and completion of larval development; mortality; emergence, sex and weight	Abbott et al., 2008; Tesoriero et al., 2003; Peach et al., 1995
Megachile rotundata	Eggs collected from leaf tunnels, separated into 96-well plates and dosed pollen; cocoons overwintered and emerged	Timing and completion of larval development; mortality; emergence, sex and weight	Abbott et al., 2008
Osmia cornuta	Eggs placed on provisions in gelatin capsules, 1 μL applied to surface of provisions	Mortality	Tesoriero et al., 2003
M. rotundata	Leaf envelope opened and provision dosed	Weight of emerged adults	Peach et al., 1995
Nomia melanderi, *M. rotundata*	Eggs and young larvae directly dosed	Completion of cocoons	Johansen et al., 1983
M. rotundata	Male immature stages, dosed pollen provision	Number developing, cocoon completion	Tasei et al., 1988
Bombus terrestris	Larvae kept 10/egg cup with three adults 28°C, and 50% relative humidity, tested 1-, 4- and 6-day old larvae, fed treated pollen dough or sucrose 24 hours,	Mortality	Gretenkord and Drescher, 1996

The PER assay is based on the temporal paired association of a conditioned stimulus (CS) and an unconditioned stimulus (US). During conditioning, the PER is elicited by contacting the gustatory receptors of the antennae with a sucrose solution (US) while an odor (CS) is simultaneously released. The proboscis extension is immediately rewarded (Reward R) by the uptake of the sucrose solution. Bees can develop the PER as a conditioned response (CR) to the odor alone after even a single pairing of the odor with a sucrose reward.

The PER assay with restrained workers has been used to investigate the behavioral effects of a number of pesticides (Decourtye et al., 2002; Weick and Thorn, 2002; Abramson et al., 2004; Decourtye et al., 2004). An acute exposure to a test compound can be applied before, during, or after the PER conditioning, and long-term scenarios may be explored with this method for compounds that are expressed in the pollen and nectar. The PER assay has been used to investigate how a chemical treatment can interfere with medium-term (Decourtye et al., 2004) or long-term olfactory memory (El Hassani et al., 2008). PER tests have recorded reduced learning performances for bees after 11 days of treatment with insecticides administered orally (Decourtye et al., 2003) and topically (Aliouane et al., 2009).

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PER assays can provide useful information that can be related to the memory and olfactory discrimination abilities of free-flying foragers. However, there is uncertainty regarding the extent to which the PER assay reflects what would occur under more typical settings (e.g., the bees are not restrained, or the exposure is not constant). PER testing that results in statistically significant effects on olfactory learning should be followed up with additional testing, for example, semi-field testing using intact colonies and tests such as those described in Chapter 9.

8.8.2 Artificial Flowers in Semi-Field Cage

Olfactory processing can be investigated using free-flying foragers visiting artificial flower feeders. The use of artificial flower feeders simulates a natural foraging situation more closely than does the laboratory tests on restrained worker bees using the conditioned PER procedure.

In artificial flower experiments, a nucleus colony (about 4000 workers and a fertile queen) is placed in an outdoor flight cage. Each artificial flower feeder is a plastic Petri dish containing glass balls (allowing landing of foragers on the feeding sites) and filled with a sucrose solution that is or is not treated with the test chemical. To limit the influence of visual or spatial cues, the artificial feeder is rotated slowly (e.g., rpm), and an odorant (e.g., pure linalool) is allowed to diffuse. The device is placed in front of the hive entrance. The conditioning (pairing odor/sucrose reward) is conducted for 2 hours on the first day. Testing is then carried out on the following days. For each observation event, the number of forager visits on either the scented sites or the unscented artificial flowers, is recorded. (For a more detailed list of design elements for the artificial flower experiment, please see Appendix 3.)

The comparison of responses of honey bees before and after exposure to the test chemical on the same colony is probably the main limit of this device. Moreover, there are many unknown points, such as reliability, and sensitivity to large panel of pesticides with various modes of action. Another uncertainty is the actual exposure to individual bees, as bees are not restricted in the length of time they feed at the artificial flowers. Therefore, it is very difficult to characterize the concentration–response relationship.

8.8.3 Visual Learning Performance in a Maze

Orientation performance of bees in a complex maze relies on associative learning between a visual mark and a reward of sugar solution. In a visual learning performance maze, bees fly through a sequence of boxes to reach a feeder containing a reward of sugar solution. The path through the maze spans a number of boxes, including decision boxes (i.e, a box with three holes, each in a different wall, where the bee enters through one hole and is then expected to choose between the two other holes), and non-decision boxes (i.e., a box with two holes, each in a different wall, where the bee entered through one hole and is then expected to leave through the other hole) (Figure 8.4).

During conditioning, bees are collectively trained to associate a mark (designating the correct hole/path) with food. To that end, an identical mark is fixed in front of the correct hole/path as well as the sucrose solution feeder outside the maze for 1 hour. After conditioning, the capacity of an individual bee to negotiate a path through the maze is tested. An observer notes the number of correct and incorrect decisions, and then number of turns back. Finally, the bees are captured and placed in rearing cages equipped with a water supply and sugar syrup. Oral delivery of the treatment chemical is via the sucrose solution (50% w/w) available to the bees. After consumption of the treated sugar solution, and a starvation period, the bees are released at the test maze entrance. The effect of the treatment solution on performance is then compared with that of an untreated sucrose solution.

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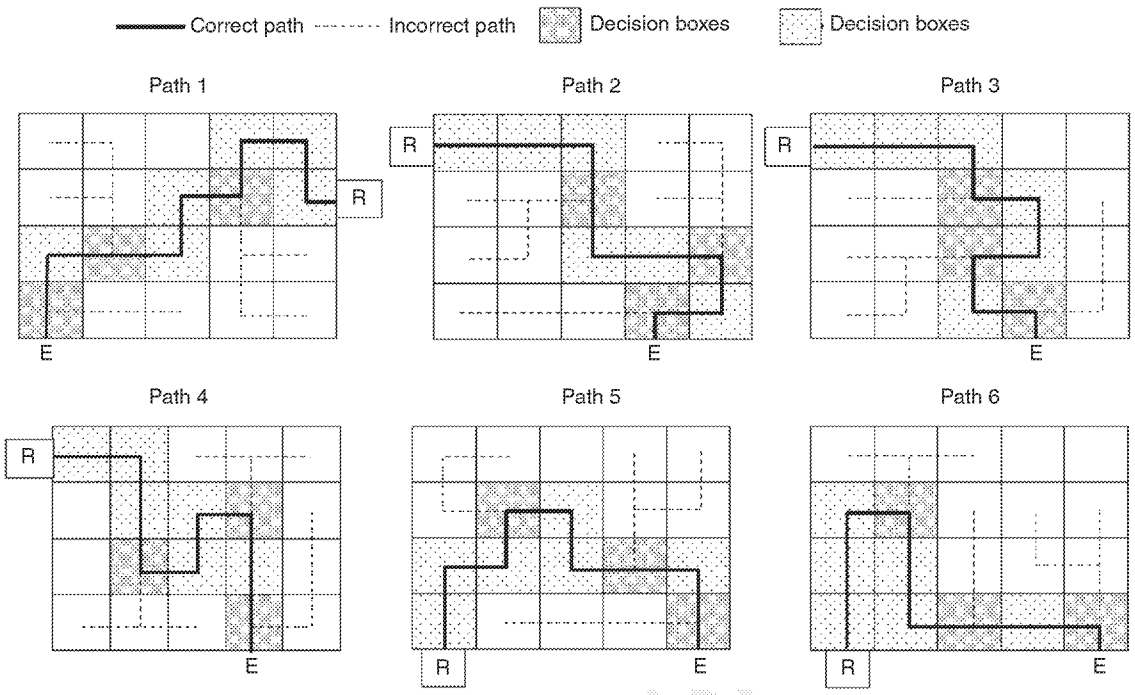

FIGURE 8.4 Maze paths used before, during, and after treatment. Path 1 was used for the conditioning procedure and other paths were used for the retrieval tests. Each path started with the entrance (E), contained three decision boxes, six no-decision boxes, and finished with the reward box (R).

Menzel et al. (1974) demonstrated that honey bees in flight can associate a visual mark to a reward and this associative learning is used by bees to negotiate a path in a complex maze (Zhang et al., 1996). After treatment with a sublethal dose of a chemical, the ability of bees to perform the task can be impaired compared to untreated control bees (Decourtye et al., 2009). The maze test relies on the visual learning of foragers in relation to navigation. However, while the maze test has demonstrated neurotoxic effects with pesticides, there are insufficient data at this time to determine whether the test will provide useful information for chemicals with other modes of action. Additionally, bee navigation in the field relies upon several guidance mechanisms, (e.g., position of sun, magnetism, etc.), whereas in the maze test, performance is based on the use of a limited number of pertinent cues. Additional experiments are needed to establish whether effects on maze performance reflect what may actually occur when foragers are exposed to pesticides in the field and are then confronted with complex environmental cues. (For a more detailed discussion of Visual Learning Test, please see Appendix 4.)

8.8.4 Radio-Frequency-Identification-Tagged Bees to Measure Foraging Behavior

Experimental test situations have been designed in relation to feeding behavior and social communication (Schricker and Stephen, 1970; Cox and Wilson, 1984; Bortolotti et al., 2003; Yang et al., 2008). Initial experiments that looked at field-level navigation were limited by the number of individual bees that could be simultaneously monitored (using bees marked with paint or colored number tags). To address this limitation,

automated tracking and identification systems have been developed using radio frequency (RF) transponder technology. The use of transponders has the potential to revolutionize the study of insect life-history traits, especially in behavioral ecotoxicology.

Different transponder devices have been employed on honey bees: harmonic radar (~~e.g.,~~ Riley and Smith, 2002) and radio frequency identification (RFID; Streit et al., 2003). Currently, the RFID tags seem to be the technology offering the most advantages. Advantages of RFID include:

- the large number of individual insects that can be tracked;
- the number of detections which can be monitored rapidly and simultaneously (milliseconds);
- limited transpondence interference from matrices such as propolis, glue, plastic, or wood;
- absence of the need for time-consuming visual observations; and,
- reduced disruption to bee behavior given the small size of the RFID tags compared to what is needed for harmonic radar tracking.

Using this test technology, the experimental colony is maintained in an outdoor tunnel. A feeder~~,~~ placed away from a hive can deliver sucrose solution. A tag-equipped bee passing underneath the reader is identified by the reader and is sent to a database with real-time recording. By passing underneath the reader, both at the hive and at the feeder, the foraging bee is monitored twice, thus determining the direction of target and the travel time between the two recording points. The reader software records the identification code and the exact time of the detection in a database for later analysis of spatial and temporal information. Such analyses may include time spent within the hive, the time spent at the feeder, the time spent between the feeder and the hive, the number of entries into and exits from the hive, and the number of entries into and exits from the feeder.

RFID devices allow the study of both the behavioral traits and the lifespan of bees, especially under biotic and/or abiotic stress. However, the large quantity of data obtained with this technique requires an interface for analyzing the data and providing the life-history traits of individual bees. Under semi-field conditions, RFID microchips have provided detectable effects due to exposure to an insecticide (Decourtye et al., 2011). (For a more detailed discussion of the RFID experimental test design, please see Appendix 5.)

8.9 CONCLUSIONS

Although laboratory toxicity tests are currently available for evaluating the potential effects of chemicals on bees, there is no single consistent approach used by different regulatory authorities and, therefore, the design and scope of these tests vary. For the purposes of screening-level risk assessments, many regulatory authorities rely on acute tests using young adult honey bees to evaluate toxicity through contact and oral exposure routes. While guidelines are becoming available that include acute toxicity tests with honey bee larvae, there is also a need to expand these laboratory test methods to examine the effects of chemicals from subacute and chronic exposure durations. Laboratory-based studies will likely continue to focus on individual test organisms; and, although laboratory-based toxicity testing has historically focused on mortality, tests are evolving to provide insight on sublethal effects such as impaired behavior. As the range of measurement endpoints continues to expand, there is a need to provide both qualitative and quantitative linkages between measurement endpoints and assessment endpoints on which regulatory authorities typically base decisions. Efforts are also underway to expand the range of test species to address concerns that *A. mellifera* may not be an adequate surrogate for non-*Apis* bees with considerably different life cycles.

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