Draft Sept 2019

Guidance for Conducting Large Scale Honey Bee Colony Feeding Studies for Evaluating Pesticide Risks to Bees

International Commission on Plant Pollinator Relations (ICPPR)

Large Scale Colony Feeding Study Testing Workgroup

1 Introduction

1.1 Current Problem and Need for Additional Guidance

Within the last five years, regulatory authorities in Europe, North America, and elsewhere have greatly expanded their procedures for quantifying pesticide risks to bees (*e.g.,* EFSA 2013; USEPA 2014). These risk assessment schemes generally rely on a tiered approach in which pesticide toxicity testing is first conducted using individual honey bees in the laboratory (Tier 1). If necessary, subsequent testing is conducted using honey bee colonies under semi-field/feeding (Tier 2) and/or full field (Tier 3) conditions. Multiple designs of Tier 2 semi-field and colony feeding studies have been developed and used to varying degrees by regulatory authorities which differ in their scope, strengths and limitations (Attachment 1). Recently, regulatory authorities in North America have used the "Large Scale Colony Feeding Study" (LSCFS) design to quantify chronic exposure via sucrose solution/colony response relationships of neonicotinoid insecticides on honey bees (USEPA 2016, 2017) and other active ingredients. The LSCFS quantifies a NOAEC (no observed adverse effect concentration) and LOAEC (lowest observed adverse effect concentration) endpoint considering colony level impact. These endpoints are then compared to residue levels in crop (typically pollen and nectar) following application in the field to determine which applications present a potential risk. Residues in pollen and nectar can be derived from various application methods including soil and seed treatment, tree injection and foliar applications. For additional information on the residue study guidance, please refer to XXX. The LSCFS design involves a relatively large number of replicates (*e.g.,* 12 sites/colonies per treatment), treatment levels (5 treatments + controls), and colony condition assessments (*e.g.,* 8-9 assessments over 12+ months, including overwintering). Despite its high cost and continued use in regulatory risk assessments, no formal regulatory protocol exists for conducting the LSCFS.

Commented [BMB1]: Might be good to link

1.2 Goals and Objectives

The goal of this project is to develop guidance for the design and conduct of honey bee tests using the LSCFS design. As such, this project has the following broad objectives:

- Compile information gained from past LSCFS designs and identify key protocol elements
- Improve the utility of honey bee LSCFS for use by regulatory authorities in pesticide risk assessment
- Foster consistency of LSCFS results interpretation within and across regulatory authorities

This project is being conducted by the "Semi-Field and Field Testing" work group (SFFTWG) of the ICPPR's Bee Protection Group. The SFFTWG contains experts on honey bee field testing from government, industry, academia and contract research organizations (CRO) who have amassed a wealth of experience in the design and conduct of bee semi-field effects studies.

An important aspect of this forthcoming guidance is that it is not intended to supersede specific recommendations made by a regulatory authority for the design and conduct of a given LSCFS. Rather, it is intended to provide a common starting point from which a study protocol can be appropriately tailored to meet the specific objectives required by a regulatory authority

1.3 Document Scope

The primary focus of this project is on providing information and guidance on how to design LSCFS for assessing pesticide effects on honey bee colonies. This guidance is built on recent experience by contract research organizations (CROs) and academic researchers who have conducted this or similar studies.

The list of specific design elements to be addressed in this guidance include the following topics:

- Number of replicates and treatments to address regulatory objectives for discerning a treatment level effect?;
- Methods used to reduce variation in colony strength prior to testing;
- Dosing volume/frequency and media (sucrose vs pollen, typically sucrose);
- Frequency of colony condition assessments;
- Digital imaging vs. visual inspection of frames
- Residue sampling of hive matrices
- Control performance acceptability
- Treating for pests/pathogens
- Statistical analysis of results

1.4 Intended Audience

The guidance presented in this document is intended to be used as a resource by both the regulated and regulator communities who may be involved in designing, conducting and interpreting bee field feeding studies for use in pesticide risk assessment.

1.5 General Methods and Approach

The guidance will be informed by a significant body of existing LSCFS that have been generated to date and expertise of the work group members. Some key steps in the guidance development process include:

1. Compilation of key regulatory objectives for the LSCFS and protection goals
2. Synthesis of study design elements from existing LSCFS and 'lessons learned'
3. Identification of key issues requiring additional guidance and/or research
4. Evaluation of statistical methods considered most appropriate for the LSCFS
5. Drafting / internal review of guidance document.

Once drafted, some type of peer and stakeholder review will be conducted of the draft guidance.

The anticipated product is a guidance document that informs risk assessors and stakeholders on how to design and conduct LSCFS for satisfying common regulatory objectives for use in assessing pesticide risk to bees. It is anticipated that such a document might be used as a basis for developing OECD guidance

on the topic. A published manuscript in the scientific literature may also constitute a secondary work product.

2 Summary and Review of Bee-Relevant Semi-Field Studies

2.1 Current Guidance Available on Semi-Field Study Designs

Commented [SK2]: So I'm not sure if you want to include the tunnel studies; as they involve different routes of exposure vis a vis the CFS design. Unless this section intends to guide users to the most appropriate SF study design, (and I don't think it should), I suggest it only be focused on canvasing the existing feeding study designs (e.g., last 3 columns).
Also, text is obviously needed to accompany the table.

Commented [BMB3R2]: [redacted]

Attachment 1. Crosswalk of Common Semi-Field and Field Honey Bee Test Designs Considered in Regulatory Risk Assessment of Plant Protection Products (PPP)

Attribute	~~Tunnel Study (EPPO 170)~~	~~Tunnel Study (CEB 230)~~	~~Tunnel study (OECD 75)~~	Colony Feeding Study (Oomen)	Colony Feeding Study (Oomen+EFSA recommendations)	Large Se Colony Feeding (EPA)
Guideline, Reference	~~EPPO 170~~	~~CEB 230~~	~~OECD 75~~	Oomen (1992) and its review by Lückmann &Schmitzer (2019)	EFSA (2013) Appendix O	No form protocol, but referenced in USEPA 2016 Appendix 4
Study Purpose	~~Evaluation of side-effects of PPP on honey bees~~	~~Evaluation of side-effects of PPP on honey bees~~	~~Assess the impact of a PPP on development of honey bee brood based on crop-applied product. Similar to EPPO 170.~~	Assess side effects of PPP on honey bee brood based on sucrose feeding	Assess side effects of PPP on honey bee brood based on sucrose feeding	Develop colony-level NOAEC and LOAEC based in dietary exposure (spiked sucrose and/or pollen)
Primary Measurement Endpoints	~~Mortality, colony assessment, foraging level~~	~~Mortality, colony assessment, foraging level~~	~~Brood development: Brood termination rate, Brood index, Compensation index (from at least 100 cells containing eggs at BFD0)~~	Brood development: Brood termination rate, Brood index, Compensation index (from at least 100 cells containing eggs, at least 100 cells containing young larvae, at least 100 cells	Brood development: Brood termination rate, Brood index, Compensation index (from at least 200 cells containing eggs, at least 200 cells containing young larvae, at least 200 cells containing old larvae at BFD0)	Colony strength (adult bees), comb area with eggs, larvae, pupae, and food, hive weight, overwintering survival

Attribute	~~Tunnel Study (EPPO 170)~~	~~Tunnel Study (CEB 230)~~	~~Tunnel study (OECD 75)~~	Colony Feeding Study (Oomen)	Colony Feeding Study (Oomen+EFSA recommendations)	Large Scale Colony Feeding Study (EPA)
				containing old larvae at BFD0)		
Secondary Measurement Endpoints	~~Behaviour~~	~~Behavior in foraging activity~~	~~Colony strength, forager mortality, flight activity, behavior abnormalities, comb area as eggs, larvae, pupae, food, presence of queen~~	Adult mortality, behavior	Adult mortality, behavior, morphological abnormalities	Pathogen and parasite rates, in-hive residues, queen replacement, food consumption
Colony restrictions	~~In tunnel (+ free flying on monitoring site)~~	~~In tunnels~~	~~In tunnel (+ free flying on monitoring site)~~	Free flying	Free flying	Free flying
Exposure routes	~~contact (direct, foliar) and/or oral~~	~~contact (direct, foliar) and/or oral~~	~~contact (direct, foliar) and/or oral~~	Oral	Oral	Oral
Exposure mechanism	~~Application to crop (or surrogate) per label instructions (e.g., foliar spray, seed treatment, soil treatment)~~	~~Application to crop (or surrogate) per label instructions (e.g., foliar spray, seed treatment, soil treatment)~~	~~Application to crop (or surrogate) per label instructions (e.g., foliar spray, seed treatment, soil treatment)~~	Sucrose solution	Sucrose solution	Sucrose solution or pollen patties. Typically sucrose solution.
Exposure units	~~Application rate (g a.i./ha)~~	~~Application rate (g a.i./ha)~~	~~Application rate (g a.i./ha)~~	Concentration (µg a.i./L or kg) recommended for high volume field use	Concentration (µg a.i./L or kg) recommended for high volume field use	Concentration (µg a.i./L or kg)

Attribute	~~Tunnel Study (EPPO 170)~~	~~Tunnel Study (CEB 230)~~	~~Tunnel study (OECD 75)~~	Colony Feeding Study (Oomen)	Colony Feeding Study (Oomen+EFSA recommendations)	Large Scale Colony Feeding Study (EPA)
Exposure/ Dosing Duration	~~Typically 7 days in tunnel + observation period outside of tunnels for 1 brood cycle~~	~~Typically 11 days in tunnels (2-4 days before application to 5-7 days after application)~~	~~Typically 7 days in tunnel + observation period outside of tunnels for 1 brood cycle~~	7-9 days after feeding (1 brood cycle)	At least 28 days after start of feeding	6 weeks feeding, but exposure may last until end of over-wintering via food stores
# Treatments	~~Test item + control + reference~~	~~Test item + control + reference~~	~~Test item + control + reference (IGR~~	Variable, + IGR reference	Variable, + IGR reference	5 + control + residue monitoring
# Reps/ Treatment	~~Sufficient to allow appropriate risk assessment, normally ≥ 3 tunnels~~	~~≥ 3 tunnels~~	~~≥ 3 tunnels~~	≥3 colonies	≥3 colonies	12 separate sites (apiaries), 24 colonies in the control group, 12 colonies per treatment level
Total # Colonies	~~≥ 9 (1/tunnel)~~	~~≥ 9 (1/tunnel)~~	~~≥ 9 (1/tunnel)~~	Variable (dose-response)	Variable (dose-response)	~96
Colony starting size	~~3000-5000 adults (at least 3 brood+food combs)~~	~~10,000-15,000, 10 frames, queen right~~	~~Ca. 6000 adults (for crop area of 40m²), ration brood to food not higher than 4:1, queen right~~	10,000-15,000, 10 frames, queen right	10,000-15,000, 10 frames, queen right	roughly 12,000 adults, 4-8 brood combs, 6-10 food combs, queen right
Test Acceptability Criteria	~~Unacceptable if excessive control mortality or response to reference toxicant is "low." (EPPO 170)~~	~~Min number forager bees/m² at application time: 5 on flowers, 3 on cereals. Mortality increase at 1DAA max 50% in control, min~~	~~Not given; (mostly in the study plans: BTR<50% in control and >50% in reference item)~~	Repeat tests if control mortality > 15% or mortality in reference toxicant treatment is low	Not given in EFSA Document	No numerical criteria, but repeat if control colony loss is excessive

Attribute	Tunnel Study (EPPO 170)	Tunnel Study (CEB 230)	Tunnel study (OECD 75)	Colony Feeding Study (Oomen)	Colony Feeding Study (Oomen+EFSA recommendations)	Large Scale Colony Feeding Study (EPA)
		500% in toxic reference				
Use in Risk Assessment	Tier 2 risk based on application rate	Tier 2 risk based on application rate	Required if a product is likely to affect bee brood development.	Unclear, but possibly simpler study design to screen brood effects. Higher tier option to refine risk for brood.	Potential effect on larvae	Tier 2 risk based on nectar and pollen residues conc.
Strengths	-Controlled (high end) exposure (100% of diet contaminated) -Integrates multiple exposure routes -Evaluates actual application conditions/ methods	-Controlled (high end) exposure (100% of diet contaminated) -Integrates multiple exposure routes - -Evaluates actual application conditions/ methods	-Controlled (high end) exposure (100% of diet contaminated) -Integrates multiple exposure routes -Quantitative assessment of brood development -Evaluates actual application conditions/ methods	- lower resource/ space requirement - Quantitative assessment of brood development - BTR (brood termination rate) not confounded by tunnel stress (bee are free flying) - workers forced to forage for pollen, additional nectar	- longer exposure period (9 days) - Quantitative assessment of brood development - BTR not confounded by tunnel stress (bee are free flying) - workers forced to forage for pollen, additional nectar	- long-term exposure - direct linkage to pollen/nectar residue studies - robust statistical power - colony-level dose response relationship
Limitations	-short-term exposure in tunnels -some endpoints (daily mortality) are highly variable	-short-term exposure in tunnels -enclosure stress on bees	-short-term exposure in tunnels -enclosure stress on bees -high variability in brood termination rate between	- short exposure, - exposure to other pesticides possible - exposure limited to dietary, only	- exposure to other pesticides possible - exposure limited to dietary, only partially controlled	- exposure limited to dietary, exposure to other pesticides possible - resource intensive

Attribute	~~Tunnel Study (EPPO-170)~~	~~Tunnel Study (CEB-230)~~	~~Tunnel study (OECD-75)~~	Colony Feeding Study (Oomen)	Colony Feeding Study (Oomen+EFSA recommendations)	Large Scale Colony Feeding Study (EPA)
	~~-endosum stress on bees -influenced by weather conditions~~		~~colonies (within treatment) -influenced by weather conditions~~	partially controlled		

3 Regulatory objectives

Key Regulatory Objectives for Colony Feeding Studies in Relation to Protection Goals

The risk assessment process for evaluating the potential risk of pesticides to bees generally follows a tiered framework that moves from a conservative, screening level risk assessment based on laboratory data using individual bees (i.e., Tier I) to, if necessary, a higher tier refined assessment based on colony level field studies (i.e., Tier II semi-field studies and Tier III full-field studies). As part of the tiered testing process for evaluating potential effects on bees, higher-tier refinements shift the focus from exposure and effects on individual bees to that of the intact colony.

Colony feeding studies are one type of Tier II semi-field study designed to determine potential effects on free foraging whole colonies during and after dietary intake of a known concentration of pesticide. It should be noted that there may be multiple different study designs that include feeding colonies, and different regulatory authorities may prefer one study design over another. The design of the study should consider the questions which arose during the lower tier assessments, and general risk concerns. In general, colony feeding studies are intended to represent a progressively more realistic level of refinement for pollinator studies compared to laboratory based studies since they are intended to reflect the potential effects of a pesticide in the field. Depending on the design, colony feeding studies can allow testing of effects over a longer period of time so that potential chronic effects may be investigated. In addition, feeding studies may be amenable to additional treatment levels and replication, thus facilitating the quantification of a dose-response relationship and determination of a No Observed Adverse Effect Concentration (NOAEC) at the colony level for the specific route of dietary exposure (e.g., spiked pollen, nectar or both). This information may be particularly useful and transferable from the test concentration to other crops where residue concentrations in pollen and nectar are available in conjunction with associated application rates.

Commented [GSIB4]: Do you really want to include the pollen feeding?

Commented [BMB5R4]: I think we should leave in option for pollen. It is just considerations for different matrices of exposure.

Commented [SK6R4]: I agree. There is no getting around pollen route as an issue; the question becomes how to address it. I think this guidance should address the experimental ways to address this exposure route (e.g., pollen feeding) and issues that have been encountered with pollen feeding studies.

The basis of the risk assessment is to ensure that the protection goals set out for bees are met. Below is a table summarizing global protection goals and how the large scale colony feeding study addresses those goals.

Regulatory agency	Protection goals	How the LSCFS addresses regulatory protection goals
PMRA/US EPA	Contribution to bee diversity	At the population level, colony survival, colony strength, overwinter survival, pollen storage area and area with nectar/honey
	Provision of pollination services	
	Production of Hive products	
EU (EFSA 2013)	Effects on colonies/populations are negligible (i.e., not greater than 7% effect currently proposed by EFSA)	Comparison of effects between control and dosed colonies. Number of hives/apiaries and replication can be considered in study design.

Commented [SK7]: [redacted]

Commented [BMB8]: [redacted] – can you confirm, not sure if these have changed – pls edit as you see fit

The general protection goals in turn dictate potential assessment endpoints for which specific measurement endpoints are identified. For colony feeding studies, measurement endpoints depend on the risk hypothesis tested and the nature of uncertainties identified in lower-tier tests. The primary measurement endpoints for colony feeding studies include colony strength, brood pattern and development, food storage and consumption, and queen and colony health. Other potential endpoints include overwintering survival, ability to requeen and other fitness measures such as colony weight. In addition, other study designs may include different endpoints, depending on the risk concerns identified.

It is important that this guideline is interpreted with appropriate flexibility to ensure that the specific requirements are addressed and that the aims and objectives of each colony feeding study are clearly specified. Specific study design attributes in a semi-field feeding study that are considered valuable from a regulatory perspective include for example: incorporation of multiple treatment levels to obtain a dose response and NOAEC; adequate statistical power to detect biologically meaningful effects; minimal influence of confounding anthropogenic stressors (e.g., other pesticides); quantifiable estimates of pesticide exposure; sufficient biological monitoring to quantify short-term and long-term impacts on hive health.

~~Table [SEQ Table * ARABIC]: Protection goals and examples of associated assessment and measurement (population and individual) endpoints for bees for Canada and the United States.~~

~~Protection Goal~~	~~Assessment Endpoints~~	~~Example Measurement Endpoints~~	
		~~Population level and higher~~	~~Individual level~~

Commented [SK9]: I know this table comes from our guidance, but including individual level measurement endpoints kinda confuses the objective of the LSCFS...which is to ID colony level effects.

Commented [BMB10R9]: [redacted]

~~Contribution to Bee Biodiversity~~	~~Species richness[1] and abundance~~	~~Individual bee survival (honey bees) and colony strength and survival (social bees) Species richness and abundance[2]~~	~~Individual worker and larval survival assays; larval emergence; queen fecundity/reproduction~~
~~Provision of Pollination Services~~	~~Population size[2] and stability of native bees and commercially managed bees~~	~~Colony strength and survival; colony development~~	~~Individual worker and larval survival assays; queen fecundity; brood success; worker bee longevity~~
~~Production of Hive Products~~	~~Quantity and quality of hive products~~	~~Quantity and quality of hive products; including pesticide residue levels in honey/wax~~	~~Individual worker and larval survival assays; queen fecundity/reproduction; larval emergence~~

~~[1]Use of honey bees as a surrogate for other insect pollinators has limitations; however, it is assumed that as with all surrogates, data on individual organisms as well as colony level data would provide some relevant information on the potential effects of a pesticide on both solitary bees as well as "eusocial bees. In addition, protection of honey bees would contribute to pollinator diversity indirectly by preserving the pollination and propagation of the many plants species pollinated by honey bees, which also serve as food sources for other pollinating insects.~~

~~[2]For managed honey bees, population size can include numbers of colonies.~~

4 Components of guidance

4.1 Hive Management - Use of standard local beekeeping practices

It is impossible to have a successful large scale colony feeding study without good husbandry. It is further important that the husbandry be conducted with respect to the underlying study goals and interferes minimally with the quality/meaningfulness of the collected data. As such, use of standard local beekeeping practices is recommended as part of the study design. Depending on the location of the study, beekeeping practices may change, and thus this should be a flexible component of the study design.

4.2 Study design, site locations and hive placement

The experimental design should detect effects defined by the protection goals, but it also needs to be realistic, manageable, and repeatable to be used for regulatory purposes. The number of hives per apiary needs to be balanced with practicality while addressing regulatory/stakeholder concerns. Replication is an important component of the study design in order to have good statistical power and lower variability. For this particular study design, individual sites (i.e, apiaries) are considered replicates. Each site should contain one or more control colonies (two recommended), one colony for each of the (5) treatments, and one monitoring colony for analysis of pesticide residues and pollen source identification (if part of the study design). Pollen can be collected from monitoring hives at each site for

Commented [AC11]: I think it would be helpful to add a subsection about the establishment, selection and randomization of the study colonies and include recommendations for packages vs existing colonies, selection parameters and (stratified) randomization to the different treatments

Commented [SK12R11]: I strongly agree; the build up, hive culling/selection process is critical to the study execution. Key questions: how many hives to begin with? What time of year? How long to let them grow? How similar is "close enough"? How determine similarity?

Commented [BMB13R11]: [redacted]

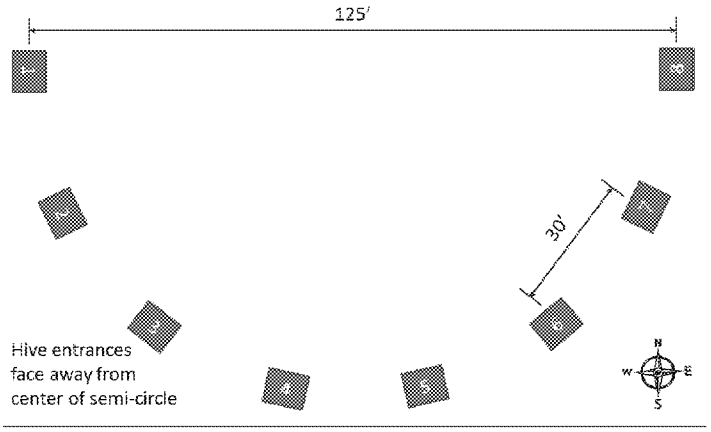
Commented [AC14]: Shouldn't the sites be consider a blocking factor? The colony/hive is the experimental unit.

Commented [SK15R14]: There is room for interpretation on this issue, but since the colony is intimately connected to the surrounding landscape, the site/apiary is viewed as the experimental unit; multiple hives at a site are considered pseudo replicates from the perspective of the landscape, much like multiple organisms in a aquarium. Colony strength is the blocking variable.
That being said, there are no doubt colony studies that evaluate multiple hives at a single site and treat the hive as the experimental unit. In this case, I would argue that the hive/landscape is not replicated and that the colony responses only take into account a portion of the variability that would be expected had multiple apiaries/sites were used. I think this issue needs further discussion /justification in the document, especially since controls tend to be double represented at each apiary and if I recall correctly, 'n' is considered 24 rather than 12--- that suggests to me the opposite is being assumed (hive = experimental unit).

assessment of forage availability and the local pollen flora. Pollen samples can be collected by means of a pollen trap eight times during the study for subsequent pollen source identification.

An example layout is provided in Figure 1 in which the hives are laid out in a semi-circle with a minimum distance of 9.1 metres between hives to prevent drifting of individual bees to adjacent hives.

A total of 12 replicate sites per treatment is recommended and results in a statistical power of 92. It is recommended that each apiary (site) is 3-5 kilometres apart.



4.3 Overwintering / Supplemental feeding (generic)

Depending on the location where overwintering preparation is part of beekeeping standard practices (such as North America), and the scientific question driving the study design, some LSCFS may include an overwintering component. In these cases, hives may require supplemental feeding prior to overwintering in order to build up the winter cluster and increase food stores (see Section 4.7 for recommended cluster size and hive weights). The timing of feeding and preparation of the colonies is important to consider in order to maintain healthy colonies (i.e. healthy control colonies in order to compare against treatment colonies).

It is recommended that typical local beekeeper management be used in the study to determine when and how often to feed. Hives can be given supplemental food prior to overwintering, as needed. The surrounding area should be monitored for availability of food, so that feeding does not occur too early (e.g., while bees are still foraging).

The goal of supplemental feeding is to successfully overwinter control colonies under local environmental conditions. Significant loss of control colonies over winter can negatively impact the study by reducing the power of the statistical tests and complicate the assessment of pesticide effects. Reasons for different outcomes among untreated control colonies were examined by Abi-Akar et al. by

Commented [MAF16]: Can we show the calculations involved in this?

Commented [BMB17R16]: [redacted]

Commented [AC18]: Maybe this was discussed earlier and I missed those discussions, but any considerations here in terms of minimizing the ag area (maybe set a max % of the area in a x km radius)...

Commented [BMB19R18]: We discussed not being too prescriptive given it may be difficult to find an area with specific restrictions depending on the size of the study Any other thoughts?

Commented [SK20R18]: I think the statistical power needs to be referenced/supported. It really depends on the combination of variance and # reps and endpoint being evaluated. I'm not sure if this value is from when we (EPA) first analyzed statistical power or if it has been updated to reflect the current body of LSCFS submitted. I think it might be informative to evaluate the statistical power of a subset of LSCFS at least to provide an indication of the range of power being achieved. Could also talk about MSD. A big question in my mind is whether 12 reps is really needed given the current body of studies? I would rather show a table of power and MSD as a function of variance and replicates rather that prescribe 12 reps a priori.

Commented [BMB21R18]: See comment above.

using data from seven LCFS conducted in North Carolina between 2013 and 2017 (manuscript in preparation). Applying the mechanistic honey bee colony model BEEHAVE, the authors systematically examined the factors leading to weaker or stronger colonies at this indicative fall time point. The model was previously calibrated and validated with data available for control colonies from the seven colony feeding studies (Schmolke et al., in preparation). Results from these systematic BEEHAVE simulations showed that feeding schedules are very important to fall colony conditions and consequently, overwintering survival. With systematic simulations, the authors derived the following recommendations for study designs that could lead to higher likelihood of overwinter survival of control colonies:

Commented [SH22]: We expect to submit the two related papers in a peer review journal before end of this year, likely between Oct-Nov 2019. The reference should be updated in due course

- Provide more supplemental sugar to increase honey store and colony size before the winter.
- Begin supplemental feeding no later than August or September. Beginning later (September) can increase fall honey stores; beginning earlier (August) can increase fall colony sizes. For either option, beginning before the winter is important.

4.4 Varroa/Nosema treatment

Hives can be treated/controlled for Varroa mites, small hive beetle, and Nosema, however, treatment should not be given immediately prior to and during exposure since this may disrupt brood development. It is recommended that treatment occurs equally to all hives (treatment and control).

Treatment for Varroa mite and other pests/pathogens can be flexible. For example, for Varroa treatment there may be environmental conditions that impact efficacy of certain treatments (i.e. formic acid should not be applied above certain ambient temperature) and new products may emerge on the market in the future that may be more effective. Thresholds for treatment can be considered when applicable.

In order to begin the experiment with 'healthy hives', free of disease and unintentional contamination, new equipment and bee packages should be used when possible. It is recognized that not all areas have this option. For example, currently the EU (European Union) and Brazil do not use packaged bees. When new equipment/packages are not feasible, hives being used in the study should be sourced from areas with no to minimal pesticide use.

4.5 Swarm control

Swarming (which typically occurs in the spring) is the reproductive process by which a new honey bee colony is formed when the queen bee leaves the colony with a large group of worker bees. Swarming significantly impacts colony size, population structure, and much of the food stores are depleted. While in spring the colonies often recover, this could introduce variability sources during a critical time (i.e. during the exposure period) that can confound results later. Similarly, and while it is less frequent, swarming or absconding can negatively impact the study late in the season when having smaller hives

before overwintering may impact their survival. Therefore, the study should aim to avoid swarming by employing standard beekeeping practices, and should be documented when it occurs.

Some of the potential methods of preventing swarming could include increasing available space in the hive by adding more hive "brood" boxes.

Commented [GSIB23]: Do you want to allow this also after start of feeding?? If yes, highly appreciated!

Commented [BMB24R23]: [redacted]

4.6 Queen excluder

A queen excluder is a barrier inside the hive that allows worker bees but not larger queens and drones to cross the barrier. The benefit of a queen excluder is that it helps keep the brood nest in a certain area, and it helps to conduct CCAs (colony condition assessments) more quickly. However, it removes space in the hives, and depending on the bee strain this may be a benefit or hindrance. It is recommended to not use a queen excluder in North American studies because the bees are likely to swarm if limited to a single brood box.

4.7 Colony Size and Condition

A. Initial colony size

The initial size of the colony matters in the study design, and it is currently recommended for most field studies that the number of bees range from >10 000 to 15 000. However, the dose of solution should scale to colony size, and the study design may also influence this number. In studies with an overwintering component, higher bee numbers (i.e., colony strength) are recommended in order to help sustain the colony through the winter. However, larger colony size may also result in dilution of exposure. Therefore, the initial size of the colony in areas or study designs with no overwintering, can be smaller.

B. Colony growth

As the colonies grow, it is important to provide adequate space for additional growth so that the queen has space to lay eggs and for the workers to store nectar. Two or more hive bodies are recommended in order to reduce swarm pressure and to give room to store treated food. Care should be used in providing adequate space at the appropriate time while the colonies are growing and not necessarily at the start of the study. For example, one hive body during the initial setup and additional bodies added when space is adequately used up in the first box. This will prevent colonies from building up, rather than out, too quickly and will also prevent the colony to limit small hive beetle infestation. It is noted that all colonies (treatment and control) should be treated equally.

C. Overwintering considerations

4.7.1.1 Colony size

Colony size requirements is also a crucial consideration prior to overwintering. However, the minimum number of bees required for a successful overwinter is likely dependent on the area in which study is conducted. In general, colonies should contain a minimum of 5,000 bees prior to winter for mild climates (and adequate honey stores for the area) while other regions may require at least 10,000 to 12,0000 bees to ensure adequate survival rates. Other factors such as disease and available food stores also impact overwintering success.

Commented [SK25]: This seems like an absolute minimum in warm climates; I think in temperate climates, these colonies would have a significant probability of failing.

Commented [BMB26R25]: [redacted]

4.7.1.2 Supplemental feeding

Supplemental feeding prior to winter (if available Autumn forage is not available in sufficient quality/quantity) not only increases honey stores but also allows the colony to grow to an optimal cluster size prior to winter. Supplemental feeding is a common beekeeping management practice and one that may be required to allow for successful overwintering of control colonies. Therefore, supplemental feeding is acceptable in regulatory studies. Local floral resources in the area, past experiences and hive assessments can provide critical information to determine the amount and frequency of the supplement food needs prior to winter. It is recommended then to monitor the hive bee numbers (i.e., colony strength) and honey stores periodically and use these two parameters as determining factors for supplemental feeding.

4.7.1.3 Honey stores

The amount of honey stores is equally important to cluster size in determining likelihood of overwintering success. Similar to cluster size (minimum number of bees), the amount of honey stores needed is based on the location of the study sites. For example, based on advice from a master beekeeper in Orange County, North Carolina, USA (Austin 2014), hives require 18 to 27 kg (a 10-frame medium or deep box, respectively) of honey to last from the first hard frost in October until the plants/trees bloom in April.

Analysis by Abi-Akar et al. (manuscript in preparation) of seven previous LSCFSs conducted in North Carolina, USA, suggest that colonies' initial conditions at the time of arrival in the study apiaries may affect fall colony conditions. The dominant factor was honey: colonies beginning with more initial honey led to considerably larger honey stores and adult bee populations before winter. Though more initial adult bees also increased fall colony metrics, more so when initial honey was low, the effect was smaller. With systematic simulations of the BEEHAVE model, the authors derived the following

recommendations for study designs that could lead to higher likelihood of overwinter survival of control colonies:

- Choice of colonies to include in LCFS as well as their organization within apiaries, should be mainly based on size of initial honey stores. Colonies with large honey stores are likely the strongest, regardless of number of bees. Colonies with small initial honey stores (e.g., approximately 4 kg or less) are at risk of early death during the summer, particularly if they have comparatively more adult bees (more mouths to feed), and should be excluded from studies.
- As a secondary criterion at the initial placement stage, avoid colonies that have a combination of medium-to-low honey and low adults. Colonies with honey stores approximately 14 kg or less (but greater than 4 kg) can grow prior to the winter if they have more initial adults.

It should be noted that these fall parameters are not predictive of overwinter success, but may aid in reducing hive loss, and should be documented in the study in order to help interpret data.

4.8 Genetic Strain

The typical strain in the country/area where the study is conducted should be used in the study.

4.9 Start Date of Study and Length of Exposure

It is recommended that the study and dosing start when minimal forage is available (during dearth), to maximize the treatment solutions are taken into the study hives. Starting dosing during the dearth gives the advantage of preventing dilution of the treatment solutions and contamination from other sources. The dearth period (and subsequently dosing regimen) will depend on the study location.

However, the timing is important in that the study should avoid starting too late or too early in the season. Starting the study too early may result in swarming, which should be avoided in the study. In addition, it is preferable the exposure period does not start in the spring when overwintering is part of the study design. On the other hand, starting the study later in the season can result in CCAs occurring later in the fall, which coincides with the period that colonies are preparing for overwintering making it difficult to compare treated and control hives owing to an overall decline in bee numbers for all hives during that time. In addition, hives may not have sufficient time to build honey stores prior to winter, and CCAs later in the fall may introduce stress and sources of variation to the colony during overwinter preparation.

Based on a retrospective analysis of recent LSCFS, Abi-Akar et al. (manuscript in preparation) recommends providing more treatment sugar to increase fall colony size and honey stores. Beginning treatment feeding shortly after placement in colonies can help prevent early colony losses for those starting with smaller honey reserves.

The recommended period of dosing is 6 weeks which represents a relatively long blooming period for a highly attractive crop, but can be altered to represent an exposure (dosing period) representative of an actual crop bloom period and pesticide use pattern.

4.10 Exposure route (nectar versus pollen)

This guidance does not prescribe whether dosing should be done with sucrose or pollen, but rather lays out considerations for each method of exposure. It is recommended that the dosing matrix consider the crops for intended use of the test item (i.e. crops that produce pollen and nectar, or pollen only), and properties of the chemical and potential sensitivity of the caste (i.e. larval toxicity versus forager bee toxicity).

The majority of LSCFS conducted to date have focused on dosing bees through sucrose solution (nectar substitute). Nectar is considered the major exposure route for forager bees, but also to thein-hive bees and larvae, which consume both nectar and pollen. However, the exposure through nectar may be diluted or indirect to some life stages or castes of bees that feed on glandular secretions from nurse bees (i.e. early larvae, queen bee). Pollen is mostly consumed by nurse bees and larvae. While exclusion of the pollen route is expected to reduce overall exposure, the impact of this exclusion on the study results is uncertain and will likely depend on the life stage/caste of bee. However, it is notable that in addition to the nectar exposure route and subsequently through honey storage, bees would also be exposed (albeit in lower doses) in bee bread, as bee bread would incorporate both supplied and foraged nectar/sucrose and foraged pollen.

It is suggested that the results of the Tier I laboratory studies are used to determine which type of dosing study would be required. If effects are observed for adults, then a nectar dosing CFS may be more appropriate for determination of effects, versus, if the effects are observed for larvae which may require a pollen dosing study.

If spiked pollen is to be used as the route of exposure in a LSCFS, this may require some consideration of potential concerns including:

- Whether pollen or sucrose is considered as a dosing matrix, the consumption needs to be documented in order to determine colony intake.
- It may be difficult to ensure bees are consuming enough pollen patties when supplied ad libitum,
- There may be an added disruption to the hive if patties are added in small amounts, many times a week,
- ~~Pollen traps are required to limit outside pollen from being stored and consumed by the colony effectively diluting the overall colony intake.~~
- ~~The pollen patties should meet the dietary (protein) needs of the colony and not adversely impact brood production (the primary stage that require pollen consumption for growth).~~

Commented [SK27]: I think another consideration is the difference we see between residues in pollen vs. nectar. Also, regardless of which route is chosen, a potentially significant route of exposure is not being addressed. There are ways to try to account for differential exposure via nectar vs. pollen; but this is not the focus of this document. A separate issue is how we would interpret a CFS that used BOTH sucrose and pollen exposure. I'm not sure how I would interpret those results.

Commented [BMB28R27]: I am not sure how to address you comment in this document – [redacted] would you like to add in a few words in this section?

Commented [SK29]: We don't limit outside nectar foraging and consumption in sucrose studies; not sure why we would limit outside pollen consumption, especially since pollen patties are not an ideal nutritional replacement for natural pollen

Commented [GSIB30]: Are you sure that we want this??? I would like to limit the trap closure to maybe 3 weeks. If the bees really want to avoid the fed patty, they will certainly find a way to bring pollen in via smaller pollen loads. The disadvantages may be bigger than the advantages.

Commented [BMB31R30]: [redacted]?

Commented [GSIB32]: Nice, but how are we doing this?

Commented [BMB33R32]: [redacted]

4.11 Quantity of spiked sucrose solution provided to hives

Commented [SK34]: Agree, 2 L x 2 was done in more recent studies. Can we come up with a consistent recommendation based on colony size and energetic requirements and/or standard bee keeping practices?

Balance is needed between fulfilling the complete carbohydrate needs of the colony while guarantying exposure through the spiked diet. If spiked sucrose solution is used for dosing colonies, then it is difficult to measure true consumption since much of it is stored and not immediately consumed. As well, free foraging bees bring back nectar from outside sources which may affect consumption of the treatment solutions.

Regarding the amount of L/bee is needed to meet bioenergetics, we may need to describe what is found in the open literature. **Action item** [person to be determined].

Commented [BBMB35]: Note from our meeting, but no further action. Do we want to include something?

Commented [MAF36R35]: The PRTF has put together a paper on consumption rate of foraging bees in relation to what is used in BeeREX. I can try to add something her. [redacted]

For energetic requirements, the volume and quantity of sugar is important. However, the volume depends on the hive size. Based on an initial hive size of 10 000 to 15 000 bees, it is recommended that hives are fed 2 times a week with 1000 mL of sugar solution (and increased to 1500 mL or more if colony strength is increasing during the dosing period).

Commented [BBMB37]: I included this, but also see that Amelie's presentation suggests the following:

From the levels tested, feeding 2L rather than 1L of 1:1 sugar solution per feeding prevented losses and improved October metrics.

After the exposure period, any provisioning of sucrose solutions is suspended for a short period until assessments provide the necessary information to initiate a supplemental feeding program with untreated sucrose solution in preparation for winter. However, the exposure continues in the hive months after the exposure period finalizes, evidenced by residues in hive matrices collected after overwintering in past studies. If a break in supplemental feeding program is part of the study design, it should be based on the number of available food stores estimated during colony assessments or beekeeper checks after exposure.

Commented [GSIB38R37]: We fed even 2 L in the more recent studies.

Commented [MAF39R37]: Is that for the 6 weeks treatment period or supplemental feeding?

Based on a retrospective analysis of recent LSCFS, bees need an intensive feeding program throughout the study (see recommendations on supplemental and treatment feedings in points 4.3 and 4.9).

Commented [BMB40R37]: [redacted]

Results from recent LSCFS, which dosed hives with spiked sucrose solutions, showed that residues of the molecules tested were detected and quantified in both hive nectar and hive pollen (beebread), at concentrations lower than the feeding solutions. Dilution of the treatment feeding solution is expected since bees also forage on outside nectar and pollen sources. , Beebread contains a low amount of nectar mixed with untreated pollen sourced from the surrounding flora thus resulting in low residue levels of the test compound in this matrix. Consequently, exposure occurs through both hive pollen and nectar when exposure is via a sucrose feeding solution. How this exposure pattern compares to exposure via contaminated pollen directly is not known, and this is an uncertainty inherent in any semi-field or full-field study design.

4.12 Sampling Scheme for Exposure Characterization

Sampling of in-hive matrices for chemical analysis to quantify residues of the parent test compound and its metabolites (i.e. if the parent chemical degrades quickly) can provide useful information, such as:

- ~~Give an estimate of the proportion of nectar that is brought from outside floral resources~~
- Help interpret monitoring hive data by comparing residues of the test compound in the study hives to residues of the test compound (if already registered in the country) or other compounds in the monitoring hives,
- Help characterize exposure to the study colonies,
- Help determine how the chemical is distributed through the hive, and how loading is distributed among bees and through time.
- Residue levels in capped/uncapped nectar samples before and after overwintering provide an indication of exposure after the dosing period ends. This may depend on the physicochemical properties of the test compound, movement and persistence in the in-hive matrices.

Commented [GSIB41]: How? We fed during package build up, the we feed during exposure, then we feed for overwintering. How are we estimating the nectar brought in from plants?

Commented [BMB42R41]: [redacted]

Commented [SK43R41]: I've heard of pollen grain analysis in nectar, but not sure if that provides an accurate accounting of nectar source. I think the best we can do is estimate the source of pollen brought to the hive. It would be nice to have some studies that use color sucrose and see how much comes from outside the hive when supplemental feeding is done.

It is recommended that samples be taken before, during, and after exposure, the later including sampling before and after overwintering (if part of the study design). The number of samples may vary with the study design, but it is recommended that in-hive samples such as uncapped/capped nectar and beebread be a composite from several frame sides (e.g. recent LSCFS have composited samples from 5 frame sides) to aim for a more representative sample.

The matrix sampled may depend on the caste or life stage that is expected to be affected from exposure, which is information that a series of laboratory tests provide. For example, if forager bees are expected to be affected, then nectar exposure may be most relevant. In contrast, if brood are expected to be affected, then pollen/bee bread may be most appropriate.

4.13 Residue analysis for metabolites

The parent molecule and its metabolites should be analyzed on a case by case basis, if there is either a toxicity concern, and/or fate/exposure concern (i.e. if we need to know how the chemical degrades and moves in the hive). The metabolite(s) should be relevant to bee toxicity, and also to the environment (e.g, formed in plants).

4.14 Pesticides from food sources other than the artificial feeding

The need for screening for other pesticides may depend on the test site. If the test site has surrounding agriculture or there is potential use of pesticides, then this information is integrated and provides evidence to associate potential effects attributed to the exposure of other pesticides rather than those test chemical in the dosing study.

4.15 Robbing and Control Contamination

Robbing behavior is especially strong when there is little nectar in the field, and strong colonies with the largest stores are the most apt to prey upon weaker colonies. In addition, the strain of the bee may predict behaviour, with some bees being more inclined to visit hives (i.e., drift) other than their own. Therefore, to avoid foraging drift, robbing and control contamination, it is recommended that robbing screens be used on all hives. If robbing does occur, it should be recorded in the study report. The following can be considered in order to establish robbing has occurred:

- residues of the test chemical are detected in the control study hives,
- in the field, if there are swarms/"clouds" of bees around the hive – which should not be confused with orientation, or
- hive weight (i.e. a sudden decrease in hive weight may indicate robbing has occurred)

Placement of hives and assignment of treatments at apiaries have to be standardized and randomized. A space of ≥9 m between hives and positioning the study hives within a semi-circle is recommended to reduce drift of returning forager bees.

In recent LSCFS, colonies in the highest treatment levels showing sub-lethal dose effects have been relocated to a separate apiary long or more to prevent contamination of control colonies and colonies in lower test levels.

Commented [GSIB44]: I cannot remember this. The definition for a dead hive is rather related to absence of queen than a minimum number of bees.

Commented [BMB45]: [redacted]

Commented [GSIB46]: This is not always the case and I want to question this. What is the reasoning for excluding the higher dose levels from statistical analysis?

Commented [BMB47R46]: [redacted]

4.16 Observer Bias

Colony condition assessments (CCAs) can be visual or digital. Visual CCAs require trained observers typically assessing the area of a frame side covered with adult bees, brood and food stores. Comparing measurement of the same colonies by different trained observers, at least at the study initiation, is recommended.

Digital CCAs require special equipment and software, which requires calibration and validation with the local or regional honey bee strain used for the study. Some considerations for digital observations is that it is time consuming, and there is more disruption to the colony compared to the visual assessment. Comparing digital to observer results before the experiment could be useful. If possible, one method of observation (either visual or digital) should be consistently used throughout the study.

4.17 Endpoints

D. Colony condition assessments (CCAs)

Commented [SK48]: We've had a lot of discussion in the past about marking queens and tracking if queens are replaced in between CCA's. I think keeping track of queen replacement is a good idea; but others feel that marking queens is too disruptive.

Commented [BMB49R48]: [redacted]

CCAs are conducted to observe overall colony health and growth throughout several months. CCAs typically yield an estimate of the percentage of a frame side with by honey/nectar, bee bread/pollen, capped brood, larvae, and eggs. An estimate of adult bees is also derived by the percent of the frame side covered with bees, which are converted into absolute cell numbers. If the CCA is visual, those observations are recorded in paper datasheets for each side of each frame of the hive. Historically, CCAs have included the following parameters:

- Colony strength (number of honey bees, estimation adapted from IMDORF &GERIG, 1999, and IMDORF et al., 1987).
- Presence of a healthy queen (such as presence of eggs)
- Pollen storage area and area with nectar or honey (estimation adapted from IMDORF & GERIG, 1999, and IMDORF et al., 1987)
- Area containing cells with eggs, larvae, and capped cells (estimation adapted from IMDORF [redacted]
- [redacted]

The following is an example of a typical CCA schedule:

Colony Condition Assessment	Timing[1]	Comments
CCA 1	Start of study (time 7 WBE (weeks before exposure)	First colony assessment for hive selection
CCA 2	Depending on study length, a few weeks after first assessment and before dosing (4 WBE)	Final assessment for hive selection and assignment of hives to apiaries and treatments (stratification)
CCA 3	About a week before dosing (1 WBE)	Assessment before start of feeding
CCA 4	Half way into dosing (3 WAE (weeks after exposure), based on a 6 week feeding schedule)	Assessment during feeding period
CCA 5	About 1 week after dosing has ended (6-7 WAE)	Assessment shortly after feeding period
CCA 6	About 4 weeks after dosing has ended (10-11 WAE)	Assessment after feeding period
CCA 7	In the fall (about 13 WAE)	Last assessment before over-wintering
CCA 8	After overwinter (if part of the study design) March (or when warm enough to open hives)	First assessment after over-wintering

CCA 9	After overwinter (during spring build up) April-May	Last assessment of the study

[1]WAE: Weeks after exposure; WBE: Weeks before exposure

E. Overwintering survival (if part of the study design)

Recent LSCFS conducted for regulatory purposes have included two CCAs after overwintering. This scheme is currently supported by regulatory agencies and is recommended for studies with an overwintering component. The first post-winter CCA typically occurs in early spring, when the temperature is warm enough to open the hive without major impact to brood. The second CCA typically occurs about 4-5 weeks later, during spring build up to ensure colonies are growing as expected, and there are no latent effects of the chemical on colony development.

Commented [GSIB50]: The problem of the last CCA is, that it often falls in the swarming period. So a lot of colonies might just be queenless or lack brood stage due to swarming which makes any interpretation of test item effects questionable.

F. Varro and nosema ~~Parasite, Nosema and viral loads~~

Monitoring for varroa and, ~~N~~nosema ~~and viruses~~ is recommended as a component of the LSCFS test design. This information is important 1) to determine when to intervene with control measures, and 2) to consider the levels of parasites and pathogens in the interpretation of results. However, it is noted there is currently no health threshold of effects for Nosema and viruses. Varroa thresholds can vary with the time of year and region, but typically range from 1-3 mites/100 bees One common method for varroa monitoring is alcohol wash, which has been used in recent LSCFS. However, other methods could be considered if is demonstrated it has the same level of accuracy to the alcohol wash (e.g. soapy water).

The Varroa level is not an endpoint on its own, but varroa is associated to the population growth of the honey bee colony, and high varroa/virus loads frequently have severe effects on colony health, which can affect the estimation of other endpoints.

Viral loads are not typically assessed in LSCFSs. However, if thresholds are established in the future, they can be considered in the study design.

Commented [BMB51R50]: Do you have an alternate suggestion? Or do we want to add that into this guidance as a consideration?
From a regulatory perspective we just want to see how the colonies are doing in the spring when they begin to forage again...
Other thoughts?

Commented [AC52]: To my knowledge, viral loads have not been part of LSCFS so far. There are limitations with analysis and interpretation of results depending on the method (e.g. relative vs absolute qPRC)

Sigrun: right, we just looked for Varroa and Nosema.

Commented [BMB53R52]: Delete? Or have as an option?

Commented [SK54R52]: One issue with Nosema is no one can tell me what a reasonable threshold is, unlike Varroa. As for viruses, we can indicate that at this time it is not standard practice, but might become relevant in the future.

G. Hive weights

Hive weights are typically measured with a manual balance coinciding with a CCA, or with a designated electronic balance that reports the weight as frequently as every 15 minutes. This information is integrated during to evaluation of the study results, but is not a required endpoint for regulatory purposes. As technology for digital scales advance, the utility of this measurement can become required for regulatory purposes.

Commented [BMB55R52]: [redacted]

Commented [SK56]: With proper equipment, I like this endpoint. There are also devices that record internal colony conditions (temperature, humidity?). Not sure how to interpret these however, but they could be considered as a line of evidence in addressing colony loss (particularly when hive strength is on the boarder of healthy).

The following are some considerations/issues for interpretation of hive weight:

- Timing of weighing (if done during the day during forager activity, then the weight may not be reflective of forager force of the colony). It is recommended that measurements are taken at night when most bees are in the hive.
- Scale failure of electronic balances has been reported.
- Weather also may affect hive weight. For example, during periods of high rainfall, the weights typically increase due to the wood frames soaking up water.

The information that hive weight provides can be derived from other endpoints during CCAs, such as estimates of pollen and honey stores.

All in all, hive weight can be useful to understand other observations, such as:

1. Determining when a swarm occurred (with other hive information, and if the queen is marked),
2. Determining if robbing occurred.

4.18 Experimental Design, Statistical Analysis and Power of the Statistical Tests

Commented [SK57]: So I think there needs to be much more discussion here. EPA did comment on an early draft of statistical analysis; was this ever received/considered? In my mind, the two basic approaches are ANOAVA/multiple comparisons and Repeated Measures ANOVA. In theory, we are dealing with repeated measures and ANOVA/dunnetts ignores this. But repeated measures is more complicated and it is not clear to me of the 'value added' by this complexity. This would be an excellent opportunity to analyze a bunch of our data sets both ways to evaluate this issue.

While the experimental design and statistical analysis of the data will depend on the objective of the study, LSCFS conducted for regulatory purposes have shared some common experimental design elements:

Experimental unit: the colony is the experimental unit, housed in a hive consisting of one or more brood boxes and added honey boxes as needed for space.

Replication: two control and one study colony for each treatment level per apiary, with 12 apiaries, for a total of 24 colonies for the control group and 12 colonies for each treatment level.

Commented [BMB58R57]: [redacted]

Commented [BMB59]: To distribute to statisticians at PMRA and EPA?

Commented [AC60]: Allen can probably formulate better than me the rationale to have 2x the control colonies.

Blocking factor: the apiary is the blocking factor, which accounts for sources of variation such as floral resources, environmental conditions, observer, and colony strength at study initiation.

Treatments/independent variable: an untreated control, 5 treatment levels of the test compound.

Dependent variables: adult bees, eggs, larvae, pupae, nectar/honey and beebread stores, hive weight.

Statistical analysis will depend on a series of assumptions regarding the dependent variables which in this case can be classified as continuous, including: independence of the observation, normal distribution, and equal variances among treatment groups. It is recommended first to analyze the data for normality and equal variances with classic tests such as Shapiro-Wilk and Levene test, respectively. If these assumptions are met, the next step is to compare the treatment levels with the control group with a parametric test such as Dunnett or Williams Test. Both tests can be suitable to determine the Lowest Observable Adverse Effect Concentration (LOAEC) and the No Observable Adverse Effect Concentration

(NOAEC). If assumption of normality and/or equal variance are not met, then non-parametric tests can be used for analysis.

Commented [AC61]: Allen to add more about power of the tests.

5 Research Needs

Commented [BBMB62]: Just some initial thoughts from our discussions–

Others to add more

- Comparison of endpoints and colony level effects of exposure to a compound delivered via pollen patty vs. sucrose solution. Which exposure route results in the worst case exposure scenario? It would be informative to compare the results from a pollen patty CFS conducted concurrently with a sucrose CFS. However, technical and financial limitations may prevent this approach, and thus studies conducted on different years but with the same compound can still provide useful insight. .
- In cases when there is potential for cross contamination from high dose/weak hives to control or lower test level hives it would be useful to set criteria to determine acceptability of the study for regulatory purposes.
- For regulatory purposes, LSCFS conducted with visual CCAs can benefit with the establishment of a standardized procedure to increase the consistency of results from multiple different observers. .
- Method development for the sampling, analysis and interpretation of residue levels in brood food and royal jelly.

6 References

7 Appendices