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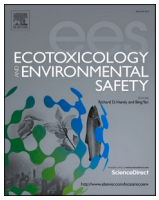

Chronic larval and adult honey bee laboratory testing: Which dietary additive should be considered when a test substance is not solubilized in acetone?

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ABSTRACT

Chronic toxicity tests on adult and larval honey bees (*Apis mellifera*) can require the use of dietary additives (solvents, emulsifiers, adjuvants and viscosifier agents) when the active ingredient of plant protection products cannot be dissolved or does not remain stable and homogeneous within the test diets. Acetone is the widely used and accepted solvent allowed within the international regulatory guidelines, but it can be ineffective in keeping certain compounds in solution and can cause toxicity to adults and larvae. In this publication, we present an evaluation of alternative additives in adult and larval diets. Six dietary additives including five solvents (ethanol, isopropanol, n-propanol, propylene glycol and triethylene glycol) and a viscosifier agent (xanthan gum) at five concentrations along with a negative control and a solvent control (acetone) were investigated at seven laboratories. The safe levels for bees were determined for each of the additives used in the 10-day chronic adult and 22-day chronic larval tests. In the 10-day chronic adult study, ethanol and isopropanol were found to be safe at concentrations $\leq$ 5.0 %, while xanthan gum can be reliably used at concentrations $\leq$ 0.1 %. Greater variability across laboratories was observed for N-propanol, propylene glycol, and triethylene glycol and these agents may cause mortality when added to diets at concentrations above 0.25–0.5 %. The safe levels of additives to larval diet in the 22-day chronic larval test had a greater variability and were generally lower than what were observed for adult diet. Our results do not recommend the inclusion of ethanol or n-propanol into the larval diet, and isopropanol, propylene glycol, and triethylene glycol may cause mortality at concentrations above 0.25–0.5 %. Safe levels for xanthan gum were more variable than what was observed for adults, but it can be used reliably at concentrations $\leq$ 0.05 %. Our analyses conclude that several additives can be integrated successfully in honey bee laboratory bioassays at levels that cause low mortality to adults and larvae.

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1. Introduction

There is an increasing demand globally for chronic adult and larval honey bee (*Apis mellifera*) toxicity testing with plant protection products. To comply with the environmental risk assessment requirements from countries in which these compounds will be used to control crop pests, weeds or plant diseases, agricultural companies need to provide ecotoxicity data for product registration submissions. The US EPA and other regulatory agencies across the globe rely on laboratory bioassays with honey bees to evaluate the potential risks of plant protection products on bee colonies. The 10-day chronic test using adults (OECD guideline No. 245, Honey Bee (*Apis mellifera* L.), Chronic Oral Toxicity Test (10-day Feeding), 2017) and 22-day chronic test on larvae (OECD guidance No. 239, Guidance Document on Honey Bee Larval Toxicity Test following Repeated Exposure, 2016) evaluate the oral toxicity of plant protection products via exposure of *A. mellifera* to treated sucrose solution and royal jelly-based artificial larval diet, respectively. The data generated in these two chronic studies are important because, unlike the acute studies which only address acute contact and oral exposure scenarios, chronic studies are representative of the exposure of in-hive honey bees to compounds which may persist in nectar and pollen.

Most of these tests are performed using high purity active ingredients that may have low solubility in aqueous solutions, such as sucrose solution and artificial larval diet. The limit of solubility of these active ingredients may result in no or minimal effects and result in a misleading indeterminate toxicological endpoint (e.g., LD_{50} > highest test level). Thus, in many situations, the use of solvents, adjuvants and/or viscosifier agents may be required to increase the solubility and maintain the stability and homogeneity of the material within the test diets (Bluhm et al., 2017). Both the 10-day chronic adult and 22-day chronic larval tests require analytical verification of concentrations in diet (OECD 2016, 2017a, 2017b) so maintenance of solubility and homogeneity in the test diets is crucial to the success and validity of the studies for regulatory authorities. In the 10-day chronic adult test, the bees are chronically exposed via ingestion of treated diet throughout the test. The treated diets are usually prepared and stored for up to four days under refrigerated conditions in the dark (ca. 6 ± 2 °C) and used as needed during that period (OECD, 2017a, 2017b), but diets may also be prepared daily for compounds that have low stability or solubility in sucrose solution. In the 22-day chronic larval test, bees are exposed for four days during the larval stage, the last three days of exposure being with treated larval diet C (OECD, 2016). The treated larval diet C is usually prepared on Day 4 and, in some situations, stored refrigerated to be used on subsequent days (i.e., Days 5 and 6). In both honey bee chronic tests, the technical material may come out of solution during the 24-hour interval feeding period, which may compromise the analytical verification of test concentrations in diet and consequently the reliability of the studies due to potential inconsistencies in dosing. Thus, the use of dietary additives is a useful tool to keep the material homogeneously distributed and suspended in diet during the tests.

Acetone is the most commonly used solvent in in-vitro honey bee studies, with the guidelines allowing for up to 5 % of the final concentration in the adult diet and up to 2 % of the final concentration in the artificial larval diet (OECD 2016, OECD 2017). However, acetone can cause honey bee mortality at concentrations of 1 % and 2 % in both 22-day larval and 10-day chronic adult tests, respectively (Schmehl et al., 2018). In addition, acetone at these concentrations can also be ineffective in keeping some technical materials in solution for a full 24-hour period. The poor solubility of some technical active ingredients combined with acetone toxicity on bees can limit the plant protection products concentrations that must be evaluated in the honey bee risk assessments. Thus, the ideal solvent is one that keeps the active ingredient stable and dispersed homogeneously in the diet while not adversely impacting the survival of honey bee larvae and adults.

Bluhm et al. (2017) reported the findings of a working group of five independent laboratories which collaborated in an investigation of the toxicity thresholds of alternative solvents (or mixtures with solubilizers and viscosifier agents) commonly used by contract research organizations (CROs) as percent of the dietary additives for use in the 10-day chronic adult toxicity test. However, information is still not available on how dietary additives may influence study measurements other than mortality, such as food consumption in the 10-day chronic adult test. Additionally, only a few publications have demonstrated the potential effects of acetone on honey bee larvae (Aupinel et al., 2007, 2009; Wilkins et al., 2013; Pacific EcoRisk, 2017; Schmehl et al., 2018). Therefore, the objective of the present study was to investigate the potential use of six alternative dietary additives (five solvents and one viscosifier agent) that may be considered as surrogates for acetone in both the 10-day chronic adult and the 22-day chronic larval toxicity tests with honey bees, and to identify "safe" working levels for these alternative dietary additives. We operably define "safe" as any tested concentration of additive that did not show a statistically significant effect in toxicity tests, otherwise known as the No Observed Effect Concentration (NOEC). Where variability in the statistically determined NOEC was observed across participating laboratories, we discuss safe levels in term of the range of NOECs observed, where use of an additive at a concentration within that range includes increased probability, or chance, of additive-based testing interference. At the end, the lowest safe levels (the maximum concentration that does not cause toxicity to honey bee larvae and adults) for the six dietary additives are reported to be used in honey bee chronic assays.

2. Materials and methods

Seven laboratories generated honey bee (*Apis mellifera* L.) toxicity data on diet additives, including Bayer CropScience LP (Chesterfield, MO, USA), Biochem Agrar (Germany), Eurofins Trialcamp Europe (Spain), Eurofins US (Alachua, FL, USA), Innovative Environmental Services (IES, Switzerland), Smithers (Wareham, MA, USA), and Syntech Research Group (France). Based on preliminary data generated during a 2018 pilot study often using a shortened chronic adult oral test, the following diet additives were selected for subsequent testing: 1) ethanol, an alcohol miscible in water; 2) isopropanol, an alcohol miscible in water; 3) n-propanol, an alcohol miscible in water; 4) propylene glycol, a diol miscible in water; 5) triethylene glycol, a dihydroxy alcohol miscible in water; and 6) xanthan gum (viscosifier agent), a polysaccharide soluble in water and forming viscous solutions. The alternative dietary additives evaluated in the study were selected based on laboratory feedback on which additives are considered for regulatory bee testing when active ingredients are incompatible with acetone or diet alone.

The laboratories performed the 10-day chronic adult and the 22-day chronic larval toxicity tests based on OECD guideline No. 245, 2017 and OECD guidance document No. 239, 2016, respectively. The studies fulfilled the minimum requirements related to the study design and methodology according to the OECD documents but were not conducted according to Good Laboratory Practice (OECD, 1998). There were some variations in methodology between the laboratories such as number of replicates, type and dimension of cages and type of feeders. Even with these differences in the methods, all the tests were considered reliable based on each laboratory's in-house standard operating procedures.

This paper compiled and analyzed results of 16 data sets from five laboratories in 2019 and 22 data sets from six laboratories in 2020 (Table 1). The studies were conducted using five concentration levels for each alternative diet additive, including 0.25 %, 0.5 %, 1.0 %, 2.5 % and 5.0 % for ethanol, isopropanol, n-propanol, propylene glycol, and triethylene glycol and 0.05 %, 0.1 %, 0.2 %, 0.4 %, and 0.8 % for xanthan gum. A solvent control (acetone) was included for 21/22 of the 10-day chronic adult tests and 14/16 of the 22-day chronic larval tests. The acetone concentration levels were 0.5 % (4/21 tests) or 2.0 % (17/21 tests) in the 10-day chronic adult tests and 0.5 % (13/14 tests) or 1.0 % (1/14 tests) in the 22-day larval chronic tests that included a solvent

Table 1
Number of studies conducted for six alternative diet additives in 2019 and 2020 across six participating laboratories.

Diet Additive	Class	Larval	Adult
Propylene Glycol	Solvent	3	5
Triethylene Glycol	Solvent	1	3
Ethanol	Solvent	4	4
N-propanol	Solvent	3	3
Isopropanol	Solvent	2	3
Xanthan Gum	Viscosifier agent	3	4
Total number of studies		16	22

control based upon past laboratory experiences and feedback. A negative control was included within all tests. A total of thirty-six first instar larvae (3 replicates of 12 larvae) was tested per treatment group and the control in the chronic larval test. A total of thirty (<2 day old) adult honey bees was tested per treatment group and control in the chronic adult tests. Each 10-day chronic adult test recorded mortality, while diet consumption was recorded for 16/22 of the tests. Each 22-day chronic larval test recorded mortality and emergence data with emergence being the endpoint analyzed.

The laboratory data sets were anonymized using alphabetic identifiers A through G and populated into a consistent tabular format (Supplementary Tables 1–3). The mortality data at test termination for both study designs and the consumption data for the 10-day chronic adult study design were then analyzed using the Comprehensive Environmental Toxicity Information System (CETIS) software version 2.1.3.8 (Tidepool Scientific Software, McKinleyville, CA). A pair wise comparison of the negative control to the acetone solvent control was performed. Data normality and equal variances were determined using the Shapiro-Wilk W Normality Test and the Bartlett Equality of Variance test, respectively. For the 10-day mortality and 22-day emergence endpoints, the concentration for each diet additive were compared to the negative control using the Jonckheere-Terpstra Step-Down Test for normally distributed data and equal variances. The Mann-Whitney U test was used when these assumptions were not met. Safe levels for consumption were generated for the 10-day consumption endpoint using the Dunnett's multiple comparison test. The safe level for mortality for each additive was based upon meeting validity criteria (≥85 % survival in 10-day adult test, ≥70 % emergence in 22-day larval test) and being statistically similar to the negative control, while the safe level for consumption is the highest concentration that is statistically similar to the negative control. A statistical comparison between and among laboratories were not conducted given the study objective to identify the lowest safe concentration (NOEC) of the evaluated additives to inform additive selection when conducting laboratory-based toxicity bioassays.

3. Results and discussion

3.1. Acetone toxicity

In the 10-day chronic adult test, 0.5 % acetone did not result in mortality or changes in consumption (Fig. 1A, B). Statistically significant effects on mortality were observed for 2.0 % acetone in 3 of 17 tests (18 %) and effects on diet consumption were observed in 1 of 15 tests (7 %) (Fig. 1A, B). Though most tests showed no effects at 2.0 % acetone, some tests resulted in significantly higher mortality or lower food consumption when compared to the control group. Although not recorded during the tests, incomplete acetone evaporation after diet preparation could be a possible factor contributing to bee mortality. There is anecdotal evidence of lower toxicity to acetone when bees are fed on diets which are adequately evaporated after preparation compared to higher toxicity when bees feed on diets immediately after preparation. Since the interval between diet preparation and feeding was not recorded in the tests, it is uncertain whether the observed adverse effects at 2.0 % acetone were due to variation in bee sensitivity or evaporation of the acetone during diet preparation. Variations in the response to acetone

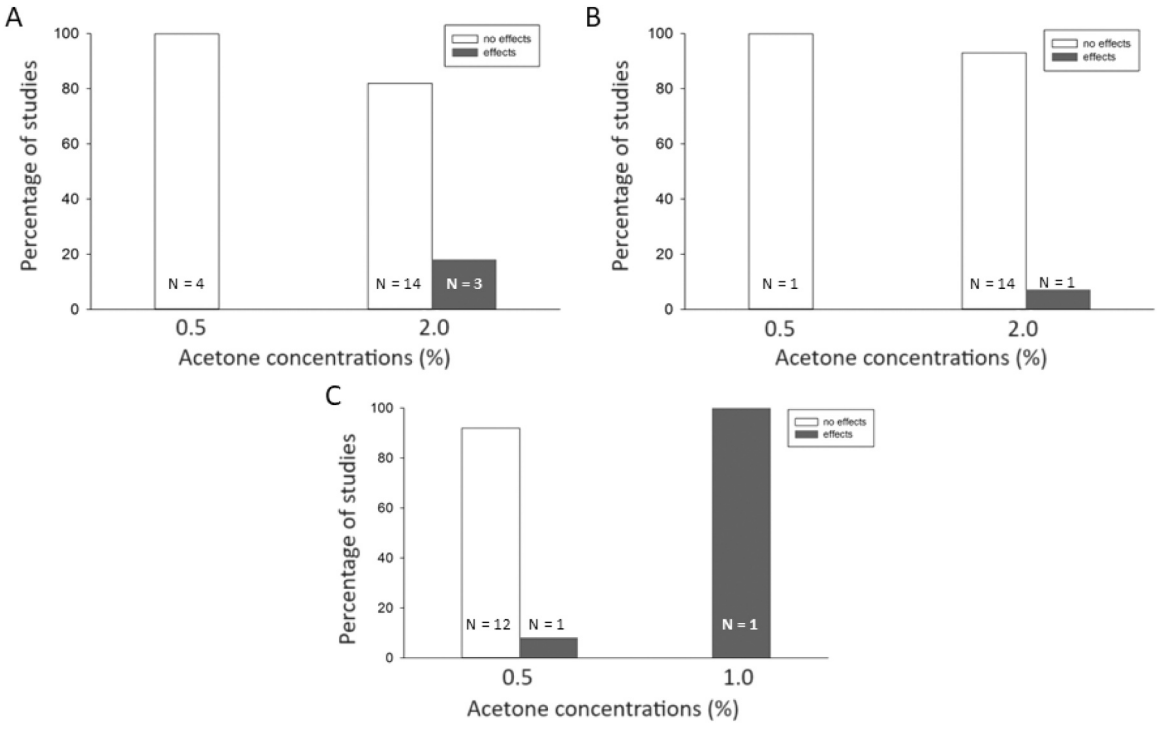

Fig. 1. Effect of acetone on adult mortality (A), adult diet consumption (B) and larval mortality (C). There were multiple concentrations of acetone included in the study that were dependent upon common practices of the participating laboratories. The number of studies is defined by "N" within the figure, with the white bars representing studies where no statistically significant effects from acetone were observed relative to the non-solvent control, and where the black bars represent the number of studies where effects were observed relative to the non-solvent control.

between laboratories has been previously demonstrated. Four tests evaluated by Bluhm et al. (2017) demonstrated no effects at 5.0 % acetone. In contrast, Schmehl et al. (2018) reported acetone toxicity even at 2.0 % acetone in diet. Therefore, we emphasize that laboratories may observe innate variation in additive sensitivity.

In the 22-day chronic larval test, effects on emergence were observed with 0.5 % acetone in 2 of 13 tests (15 %), and no effects were observed on emergence in the single test that used 1.0 % acetone (Fig. 1C). It is largely agreed upon by contract testing laboratories that 0.5 % acetone is the highest concentration to use in the 22-day chronic larval study before adverse effects from the acetone may be observed. However, the viscosity of the larval diet may enable the acetone carrier solvent to be evaporated when placed in the diet prior to the test item being integrated into the diet, and therefore allowing higher concentrations of acetone to be used without adversely affecting the organism (Krueger et al., 2021). Cornement et al. (2017) demonstrated that larval and pupal survival, and adult hatching were significantly increased when acetone was evaporated from larval diets. In addition, it was demonstrated that evaporation of acetone does not negatively affect the solubility and stability of the active ingredient solubilized in the diet.

3.2. 10-day chronic adult test – additive safe levels for bees based on mortality and consumption

Xanthan gum did not result in an effect on mortality across any of the laboratories at concentrations at or below 0.1 % (Fig. 2A). Across the testing laboratories, no effect on mortality was demonstrated at 0.1 % (4/4 tests), 0.2 % (1/4 tests) and 0.4 % (2/4 tests), which is similar to the safe levels published by Bluhm et al. (2017); however higher concentrations of xanthan gum led to a highly viscous adult diet and will reduce consumption of the adult diet and may result in crystals within the aperture of the feeder. Consumption in our study was negatively impacted at concentrations as low as 0.05 % (Fig. 2B), and it is expected that this reduction is more related to the viscosity of the diet, rather than to any physiological effect of the xanthan gum on the feeding behavior of the bee. Many laboratories use xanthan gum concentrations between

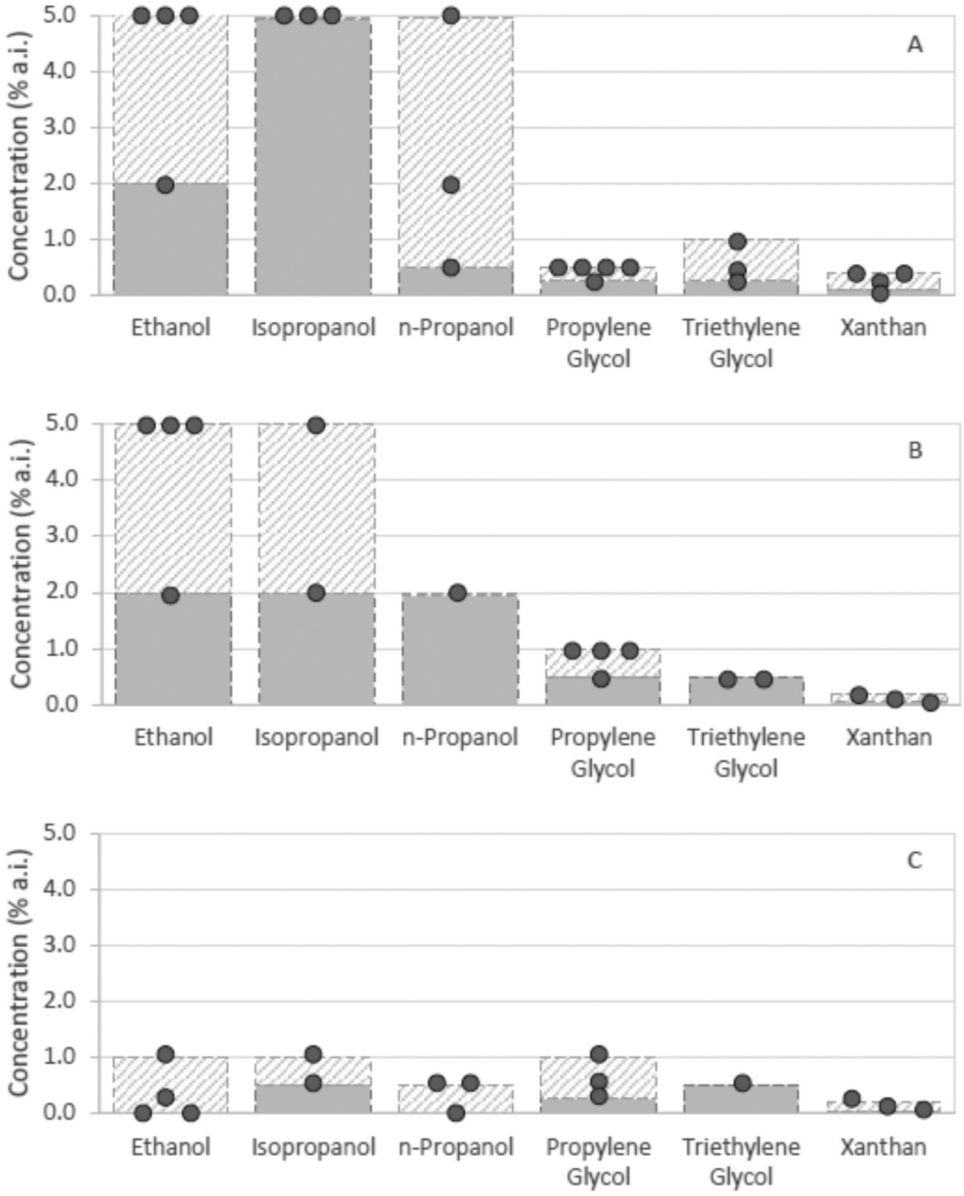

Fig. 2. Range of safe levels (i.e., range of No Observed Effect Concentration - NOEC) for bees using dietary additives tested for mortality (A), diet consumption (B), and adult emergence (C). Hatched area of bar represents the range of uncertainty in NOECs given variability in individual laboratory results represented by symbols. Symbols (•) indicate results from each individual laboratory test.

0.05 % and 0.1 %, which is confirmed as a safe range to adult honey bees as demonstrated from our mortality data. Xanthan gum is an essential tool as a dietary additive across testing laboratories to create homogeneous suspension, enable accurate analytical verification, and prevent clogged feeders from a test substance in the diet when minimal solubility is possible.

Isopropanol did not result in an effect on mortality up to concentrations of 5 % for all three tests within this study and ethanol only resulted in a slight effect in one lab (<10 % mortality at the 2 % concentration) across the four tests (Fig. 2A). These results demonstrate that these two solvents are good dietary additives when technical materials with low solubility in acetone are tested. Consumption was minimally reduced by the addition of ethanol in the diet, with three of the four tests resulting in no differences in consumption up to concentrations of 5 % (Fig. 2B). Of the two labs that measured consumption of isopropanol, neither observed any reduction in diet consumption at or above 2 %. Of the six additives included in our study, our data suggests that ethanol and isopropanol will result in the most consistent performance (i.e., low mortality, minimal effects on consumption) when included in adult diet for honey bee laboratory bioassays.

N-propanol was another alcohol evaluated, yet there were highly variable results (Fig. 2A) with one of the three tests yielding effects above 0.5 %, one above 2 %, and one resulting in no mortality across all concentrations (i.e., ≤ 5 %). Only one of the three tests with n-propanol measured consumption and observed no effects at concentrations at or above 2 %, however the performance of n-propanol is inconclusive at concentrations above 0.5 % based upon the available data.

The toxicity of propylene glycol and triethylene glycol was variable between the two additives and among the eight tests, resulting in mortality in concentrations above 0.25 % (1/5 tests) and 0.5 % (4/5 tests) for propylene glycol and in concentrations above 0.25 % (1/3 tests), above 0.5 % (1/3 tests) and 1 % (1/3 tests) for triethylene glycol (Fig. 2A). Although the negative control for one of the triethylene glycol tests had poor survival (i.e., 33 % mortality), we still included the data in our evaluation given the low mortality in the three lowest test concentrations. Food consumption was not reduced for either propylene glycol or triethylene glycol at concentrations below 0.5 % (Fig. 2B). Our data supports the use of propylene glycol and triethylene glycol at concentrations of 0.25 % or below in the 10-day adult test.

3.3. 22-day chronic larval test – Additive safe levels based on emergence

Larvae demonstrated a greater toxicity to additives than what was observed in the 10-day chronic adult toxicity test, but fewer datasets (16 total) were generated for larvae than what were generated for adults (22 total). Xanthan gum did not result in an effect on emergence across any of the laboratories at a concentration of 0.05 %, but was not consistent across the tests, with safe levels ranging from 0.05 % to 0.2 % (Fig. 2C). It is important to highlight that the higher concentrations of xanthan gum were practically inedible due to the extremely high viscosity of larval diet and likely resulted in the starvation of the larvae due to uneaten diet rather than direct toxicity. An emulsifier like xanthan gum may not be as necessary to suspend a given test item within the larval diet as with the adult diet, given that the larval diet has a greater viscosity compared to 50 % sucrose solution. Furthermore, larvae rest directly within their diet, preventing any concern of an insoluble test item clogging a feeder.

The three alcohols included in our study resulted in observed toxicity to larvae at all concentrations above 1 %. Ethanol resulted in no safe levels (<0.25 % ethanol) in two of the four laboratories, with the other two laboratories observing effects above 0.25 % and 1.0 %. The two laboratories that evaluated isopropanol observed effects above 0.5 % and 1.0 %. N-propanol was highly variable, with two laboratories observing effects above 0.5 % and the third laboratory observing effects at all test concentrations. The safe levels for alcohols were all lower for larvae than what was observed for adults, yet isopropanol appears to be suitable at low concentrations (≤ 0.5 %) when a particular test item is highly compatible (i.e., more soluble) with the use of an alcohol as a carrier within the larval diet.

Observed effects from propylene glycol were highly variable across the three testing laboratories, with one laboratory observing mortality above 0.25 %, one laboratory above 0.5 %, and one laboratory above 1.0 % (Fig. 2C). Only one dataset was generated for triethylene glycol, resulting in observed mortality above 0.5 %. There is uncertainty in the safe level of triethylene glycol, given that there was only a single test to evaluate.

Based on data obtained across 38 studies, our recommended dietary alternative agents for each laboratory honey bee study design are listed in Table 2. The proposed safe levels were not based upon food consumption data.

3.4. Points to be considered before selecting a dietary additive

According to the regulatory guideline OECD 245 for 10-day adult toxicity, diet consumption data are used to calculate the mean uptake of a test chemical to the bee over the test period, rather than to calculate an effect threshold. The OECD test guidance 239 for 22-day larval toxicity calculates the toxicity endpoint based upon adult emergence. However, there are some regulatory authorities (e.g., United States Environmental Protection Agency) that consider food consumption data from a 10-day adult test when estimating chronic risks to individual bees (USEPA/PMRA/CDPR, 2014), so we advise the careful selection of additives used in combination with evaluating the toxicity of a test substance so that the additive does not reduce consumption for a study that may be part of a regulatory submission.

Technical procedures should be considered before selecting a dietary additive to be used in a laboratory bioassay with honey bees. Prior to examining the honey bee safety levels for additives presented in this study, experimenters should conduct mixing trials with acetone to confirm if the test substance is effectively dissolved, stable and homogeneously distributed in both sucrose solution and larval diet for a period of 24 h. If acetone is effective in keeping the material in solution at concentrations that cover the risk assessment requirements, we recommend conducting the tests with acetone since it is widely used solvent in honey bee laboratory studies and well accepted by the global regulatory authorities. If acetone is ineffective or incompatible with the test material (e.g., certain microbial-based products), mixing trials should be prepared with more than one alternative additive because some of the solvents may have molecular properties similar to acetone (Borges et al., 2021). In other words, test substances not miscible in acetone may also not be miscible in alcohols and other solvents with similar physicochemical properties such as polarity, density, etc. Although, most of our historical database is with acetone, our findings also suggest that there are suitable alternatives with different physicochemical properties that can be used for honey bee laboratory testing. Therefore, the bee safety data should be consulted only after the selection of suitable additives based on mixing trial results.

The safety of an additive should be considered for both honey bee

Table 2
Proposed safe levels for six dietary additives for the 10-day chronic adult and 22-day chronic larval tests.

10-Day Chronic Adult Test (OECD GL No. 245)	22-Day Chronic Larval Test (OECD GD No. 239)
✓ Ethanol ≤ 5 %	✓ Ethanol = no recommended safe level below 0.25 %
✓ Isopropanol ≤ 5 %	✓ Isopropanol ≤ 0.5 %
✓ N-propanol ≤ 0.5 %	✓ N-propanol = no recommended safe level below 0.25 %
✓ Propylene glycol ≤ 0.25 %	✓ Propylene glycol ≤ 0.25 %
✓ Triethylene glycol ≤ 0.25 %	✓ Triethylene glycol ≤ 0.5 %
✓ Xanthan Gum ≤ 0.1 %	✓ Xanthan Gum ≤ 0.05 %

larvae and adults when planning a study. Laboratories often conduct both chronic larval and chronic adult toxicity tests in the same period when generating data for regulatory submissions. Data intended for regulatory submissions require analytical verification for the test item in the diet, which requires analytical method development and validation prior to the conduct of the study. Different additives between study type (adult vs larval) could require different analytical methods and may result in an increased developmental time and cost. Therefore, it is important to ensure that the additive selected is sufficient for mixing the test substance in both diets and is also safe for both honey bee developmental phases. Otherwise, two different method validations should be considered, one for each diet matrix.

Based on our findings, ethanol and isopropanol demonstrated safety at highest concentrations and can provide versatility in the concentration that may be used to obtain the intended solubility of a test item. Xanthan gum is an effective emulsifier for inclusion within the adult diet, yet should be limited to concentrations at or below 0.1 %. The other additives that were evaluated can be used in bee testing at safe levels at the lower concentrations evaluated in our study (0.25–0.5 %), yet it is critical to determine whether these additives will achieve the necessary solubility and homogeneity of a particular test item in the diet at these lower concentrations. The safe levels of additives for the 22-day chronic larval tests were generally lower than for adults, with some additives – ethanol and n-propanol – not being recommended at any of the concentrations evaluated in our study. The larval diet innately is more viscous, and the provisioned amounts are fully consumed during the larval feeding stage, and therefore may be less likely to require the use of an additive at a high concentration to achieve a homogeneous distribution of a given test item within the diet.

Mixtures of dietary additives were not investigated in this study, but it is known that some combinations of two or more additives are effective and relatively safe to bees. Bluhm et al. (2017) demonstrated that acetone combined with xanthan gum or tween 80 (an ester used as surfactant) may be useful in honey bee adult tests. Some participating laboratories of this current study have often used combinations of acetone + xanthan gum, acetone + tween 80 and even acetone + xanthan gum + tween 80 effectively for the 10-day chronic test (personal communication). However, some of these combinations may lead to unexpected high mortality in the additive control group and/or reduced food consumption due to avoidance or reduced palatability, which may impact the study in some cases and should be evaluated prior to the start of a study.

4. Conclusions

Our analyses conclude that several additives can be integrated successfully in honey bee laboratory bioassays at levels that cause low mortality to adults and larvae, especially when test substances are not solubilized in acetone. Furthermore, certain alternative additives evaluated in this study cause low toxicity to bees at levels equivalent to or even better than acetone. Even though the variability in the laboratory results, the proposed safe levels are based upon the maximum concentration of each dietary additive that did not cause toxicity resulting from a robust dose-response data set from multiple laboratories. There is an increasing demand for pollinator toxicity testing with plant protection products in many countries, and more investigations are necessary with other pollinator groups such as bumble bees (OECD guideline 247 (2017)) and solitary bees. Since the level of sensitivity to dietary additives may differ between bee species, the evaluation of the safe levels of these dietary additives to different species of bees are necessary to improve the quality and reliability of new test methods.

CRediT authorship contribution statement

Hudson V.V. Tomé: Investigation, Writing – original draft, Writing – review & editing, Visualization. **Stephen Clark:** Formal analysis, Visualization, Data curation, Writing – original draft, Writing – review & editing. **Brant C. Jorgenson:** Formal analysis, Visualization, Data curation, Writing – original draft, Writing – review & editing. **Stefan Kimmel:** Investigation, Validation, Resources. **Bettina Wenzel**: Investigation, Validation, Resources. **Carmen Gimeno:** Investigation, Validation, Resources, **John Porch:** Investigation, Validation, Resources. **Michael R. Patnaude:** Investigation, Validation, Resources. **Kristin Schmidt**: Investigation, Validation, Resources. **Line Deslandes:** Investigation, Validation, Resources. **Daniel R. Schmehl:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Daniel Schmehl reports financial support, administrative support, and article publishing charges were provided by Pollinator Research Task Force, LLC.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2023.115718.

References

Aupinel, P., Fortini, D., Michaud, B., Marolleau, F., Taséi, J., Odoux, J., 2007. Toxicity of dimethoate and fenoxycarb to honey bee brood (*Apis mellifera*), using a new in vitro standardized feeding method. Pest Manag. Sci. 63, 1090–1094. https://doi.org/10.1002/ps.1446.

Aupinel, P., Fortini, D., Michaud, B., Medrzyck, P., Padovani, E., Przygoda, D., Maus, C., Charriere, J.-D., Kilchenmann, V., Riessberger-Galle, U., Vollmann, J.J., Jeker, L., Janke, M., Odoux, J.-F., Tasei, J.-N., 2009. Honey bee brood ring-test: method for testing pesticide toxicity on honeybee brood in laboratory conditions. Julius-Kuhn. Arch 423, 96–101.

Bluhm, W., Gladbach, D., Kling, A., Ruhland, S., Sack, D., et al., 2017. Limited solubility of test items in regulatory honey bee (*Apis mellifera*) testing: potential use of solvents, solubilizers and viscosifiers with aqueous sucrose solution. SETAC. https://doi.org/10.13140/RG.2.2.22562.22726.

Borges, S., Alkassab, A.T., Collison, E., Hinarejos, S., Jones, B., McVey, E., Roessink, I., Steeger, T., Sultan, M., Wassenberg, J., 2021. Overview of the testing and assessment of effects of microbial pesticides on bees: strengths, challenges and perspectives. Apidologie 52, 1256–1277. https://doi.org/10.1007/s13592-021-00900-7.

Comprehensive Environmental Toxicity Information System (CETIS) software version 1.9.7.7. Tidepool Scientific Software, McKinleyville, CA.

Cornement, M., Hodapp, B., Saar, B., Pardo, P., Schmidt, T., Kimmel, S., 2017. OECD GD 239 – Honey bee larvae in vitro testing and solvents: How to handle toxic solvents. International Commission For Plant-Pollinator Relationships (ICP-PR). 13th International Symposium Hazards Of Pesticides To Bees. (PDF) OECD GD 239 – Honey bee larvae in vitro testing and solvents: How to handle toxic solvents (researchgate.net).

Krueger, A.J., Early, T.M., Ripperger, R.J., Cabrera, A.R., Schmehl, D.R., 2021. The utility of a bumble bee (*Bombus* spp.[Hymenoptera: Apidae]) brood test for evaluating the effects of pesticides. Environ. Entomol. 50 (5), 1105–1117. https://doi.org/10.1093/ee/nvab072.

Organization for Economic Cooperation and Development, 1998. OECD 1. Series On Principles Of Good Laboratory Practice And Compliance Monitoring. OECD Principles on Good Laboratory Practice. (as revised in 1997). ENV/MC/CHEM(98) 17. Dist.: 26-Jan-1998.

Organization for Economic Cooperation and Development, 2016. OECD 239 Guidance Document on Honey Bee (*Apis mellifera*) Larval Toxicity Test following Repeated Exposure (Adopted 15 July 2016).

Organization for Economic Cooperation and Development, 2017. OECD 245 Guideline for the Testing of Chemicals: Honey Bee (*Apis mellifera* L.), Chronic Oral Toxicity Test (10-Day Feeding) (Adopted 9 October 2017).

Organization for Economic Cooperation and Development, 2017. OECD 247 Guideline for the Testing of Chemicals: Bumblebee, Acute Oral Toxicity Test (Adopted 9 October 2017).

Pacific EcoRisk, 2017. Results of the Honey Bee (*Apis mellifera* L.) Toxicity Following Chronic (Repeated) Exposure International Ring Test Performance. ⟨http://pollinatorresearchtaskforce.com/wp-content/uploads/2019/03/PRTF-Projects-Ring-Study-Report-Feb-2017.pdf⟩.

Schmehl, D.R., Cabrera, A.R., Tomé, H.V.V., Porch, J., 2018. Novel approaches for integrating a compound with poor solubility into honey bee diets for laboratory testing. Proceedings of the 2018 American Bee Research Conference. Bee World 95: 2, 47–72. DOI:10.1080/0005772X.2018.1450208.

USEPA/PMRA/CDPR, 2014. Guidance for Assessing Pesticide Risks to Bees. Microsoft Word - Pollinator Risk Assessment Guidance 06_19_14 (epa.gov).

Wilkins, S., Jarrat, N., Harkin, S., Thompson, H., Coulson, M., 2013. Effects of solvent on the toxicity of dimethoate in a honey bee in vitro larval study. Pest Manag. Sci. 69 (4) https://doi.org/10.1002/ps.3465, 462-3.