A retrospective analysis of honey bee (Apis mellifera) pesticide toxicity data

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Abstract

Current USEPA ecological risk assessments for pesticide registration include a determination of potential risks to bees. Toxicity data are submitted to support these assessments and the USEPA maintains a large database containing acute and chronic toxicity data on adult and larval honey bees (Apis mellifera), which USEPA considers a surrogate for Apis and non-Apis bees. We compared these toxicity data to explore possible trends. This analysis indicated a significant correlation between acute contact and oral median lethal dose (LD\textsubscript{50}) values for adult honey bees ($\rho = 0.74$, $p < 0.0001$). Using default EPA modeling assumptions, where exposure for an individual bee is roughly 12x lower through contact than through ingestion, the analysis indicates that the oral LD\textsubscript{50} is similarly if not more protective of the contact LD\textsubscript{50} for the majority of pesticides and modes of action evaluated. The analysis also provided evidence that compounds with a lower acute toxicity for adults through contact and oral exposure pathways may still be acutely toxic for larvae. The acute toxicity of herbicides and fungicides was higher for larvae relative to oral and contact toxicity for adult honey bees for the same compounds and the no observed adverse effect level (NOAEL) from chronic toxicity studies were lower for larvae relative to adults, indicating increased sensitivity of larvae. When comparing 8-day LD\textsubscript{50} values between single dose larval acute studies to those derived from repeat dose 22-day larval chronic toxicity studies, the LD\textsubscript{50} values derived from chronic studies were significantly lower than those from acute toxicity tests ($Z = -37$, $p = 0.03$).

Introduction

Insect pollination is required to produce approximately $30$ billion worth of agricultural products in the US annually and provides a key ecosystem service within many natural environments [1, 2]. The honey bee (Apis mellifera) is frequently used for its pollination services in agriculture [3] and costs associated with pollination services have more than doubled for some
crop species since the 1990s [4]. Additionally, considerable losses to both managed and non-managed insect pollinators have called attention to the factors contributing to pollinator declines [5, 6]. Efforts to examine these environmental stressors have led to a consensus in the scientific community that there are multiple factors affecting declines in bee health including parasites (e.g., *Varroa destructor*), pathogens (multiple viral, bacterial, and fungal diseases), pesticides, lack of adequate nutrition [4], and poor honey bee queen performance [7]. Furthermore, insect pollinators are at risk from habitat loss resulting in declines of floral biodiversity related to conversion of lands to agricultural production and urbanization, bee management practices, lack of genetic diversity and/or changes related to climate [5, 8]. None of these factors have been identified as the single cause of declines; rather, it is the interaction of these factors that appears to be what is most detrimental to bees [8, 9]. Bees can interact with natural and synthetic xenobiotics through a variety of exposure pathways [e.g., 10] and the role of pesticides as a stressor has been a primary focus of research examining the challenges faced by bees [11].

Organizations charged with determining the potential for ecological risks from pesticides including the USEPA (U.S. Environmental Protection Agency) and the EFSA (European Food Safety Agency) have identified pollinator protection goals and require the submission of pollinator toxicity data when registering pesticides. When examining risks towards pollinators, honey bees are often used as a study species because of their agricultural importance and amenability (i.e., availability of standardized test method and ready availability) for lab and field-based experimentation [12]. Additionally, honey bees can serve as a surrogate study model for examining risks to other bee species [13]. A multi-tiered approach is used by USEPA when examining pesticide risks to bees during an ecological risk assessment [11].

The Guidance for Assessing Pesticide Risks to Bees [13] provides a broad overview of pollinator risk assessment used by the USEPA and outlines the tiered approach used for risk assessment. The initial tier (Tier 1) serves as a screen and consists of laboratory-based acute and chronic toxicity studies with adult and larval honey bees conducted in accord with the Organization for Economic Cooperation and Development (OECD) and/or USEPA Office of Chemical Safety and Pollution Prevention (OCSPP) standardized test guidelines (TG) or guidance documents (GD). Tier 1 toxicity studies include: adult acute contact (AAC; OCSPP 850.3020 [14] and OECD TG 214 [15]); adult acute oral (AAO; OECD TG 213 [16]); the adult chronic oral (ACO; OECD TG 245 [17]), larval acute oral (LAO; OECD TG 237 [18]), and larval chronic oral (LAO; OECD GD 239 [19]). Collectively, these data serve to inform the need for higher-tier colony-level exposure and effect studies intended to provide increased realism at the semi-field (Tier 2; e.g., OECD GD 75) [20] and full-field (Tier 3; OCSPP 850.3040) [21] level.

Tier 1 studies quantify effects at the individual-level and include measurements for mortality and sublethal effects. During laboratory-based testing, point estimates of acute (i.e., lethal dose for 50% of the bees tested; LD₉₀) and chronic toxicity (i.e., no-observed adverse effect level; NOAEL) values are calculated for adult and larval worker honey bees for a given pesticide. The toxicity endpoints generated from Tier 1 toxicity studies can then be compared to model estimates of contact/oral exposure to estimate risk to individual bees (as detailed in [13]).

Within the field of toxicology, there is a general interest in reducing, replacing, and refining the current reliance on animal testing [22, 23]. While these efforts have historically focused on vertebrates, new approach methodologies such as high-throughput *in vitro* assays and predictive models (computational toxicology) are emerging that leverage existing data and show promise toward more efficient yet equally protective means of evaluating risk across a broader range of taxa (i.e., vertebrate and invertebrate). While reduced reliance on whole animal
testing is an important benefit of such efforts, the development of predictive models and in vitro assays or possible reductions in data requirements must be based on sound science demonstrating that such tools are relevant and reliable or that other lines of evidence are suitably predictive if they are to gain regulatory acceptance [24]. Over the last several years USEPA has received hundreds of Tier 1 honey bee studies through the pesticide registration and reregistration processes under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U. S.C. §136 et seq.). This analysis is intended to examine patterns among these data and assess whether efficiencies can be gained in targeting the specific studies needed for risk assessment.

This analysis utilizes EPA’s honey bee toxicity database to compare the relative toxicity of insecticides, fungicides and herbicides across acute (single dose) and chronic (repeat dose) tests with adult and larval worker bees. The objective of this analysis was to explore the relative sensitivity of larval bees versus adults and to determine the predictive utility of: acute contact toxicity data relative to acute oral toxicity in adults; of acute toxicity data for chronic toxicity; and of chronic (repeat dose) larval toxicity data for acute (single dose) larval toxicity.

**Methods**

This retrospective analysis includes toxicity data from Tier I honey bee toxicity studies conducted in accordance with OECD and/or OCSPP test guidelines/guidance documents including: the adult acute contact (AAC; OCSPP 850.3020 [14] and OECD TG 214 [15]); adult acute oral (AAO; OECD TG 213 [16]); the adult chronic oral (ACO; OECD TG 245 [17]), larval acute oral (LAO; OECD TG 237 [18]), and larval chronic oral (LCO; OECD GD 239 [19]) honey bee toxicity studies. While the larval toxicity tests focus primarily on the ingestion of pesticide residues in diet, exposure in these studies is not limited to oral ingestion but includes contact exposure as well. Also, while the chronic toxicity studies with larval bees typically report exposure in terms of cumulative dose (i.e., μg ai/larva), USEPA calculates exposure from these studies in terms of daily dose (i.e., μg ai/larva/day). USEPA recognizes that it is not possible to readily determine the actual volume of diet consumed by the developing larvae each day the organism is dosed. Therefore, daily dose is calculated by dividing the cumulative dose by 4 (i.e., the number of days over which the larvae are dosed).

**Pesticide classifications**

**Chemical classes and modes of action (MOA).** For some comparisons, we organized pesticides into categories by chemical class (e.g., organophosphates), pesticide class (e.g., insecticides), and chemical mode of action (MOA) (S1 Table). When comparing across adult acute oral and contact toxicity data, we categorized pesticides into classes (e.g., fungicide, herbicide, insecticide) using multiple reference databases (See pesticide classification sources in S2 Table). Additionally, we further sub-classified insecticides using general MOAs established by the Insecticide Resistance Action Committee (IRAC; https://irac-online.org/) and the Pesticide Properties Database (See S3 Table). For all other comparisons, we categorized pesticides using a general MOA, and a brief description MOA [25] to further characterize each compound (See sources of information in S2 Table). Pesticide classifications and MOAs are summarized in S1 Table.

**Toxicity classifications.** When comparing the relative acute oral toxicity across pesticides for both adults and larvae, we relied upon the following USEPA acute toxicity classification categories [26]:

- Practically Non-toxic (LD₅₀ ≥ 11 μg a.i./bee);
- Moderately Toxic (2 μg a.i. < LD₅₀ < 11 μg a.i./bee); and,
• Highly Toxic (LD$_{50}$ ≤ 2 μg a.i./bee).

Non-definitive toxicity endpoints include values which are greater than (>) the highest tested dose or are less than (<) the lowest dose level. When classifying toxicity values, we categorized non-definitive endpoints, which had “>” values as if they were definitive. Because of their non-definitive nature, we distinguished these values from the definitive endpoints when representing them visually. If an endpoint value was < 2 μg a.i./bee, we classified it as “highly toxic non-definitive.” If an endpoint had either < or > signs expressed within the range of LD$_{50}$ = 2–11 μg a.i./bee, we classified it as “Moderately Toxic non-definitive.” Lastly, if a compound had a non-definitive LD$_{50}$ > 11 μg a.i./bee, we classified it as “Practically Non-toxic” with no distinction between definitive and non-definitive.

Source of endpoint data
For data curation and screening, within the USEPA Office of Pesticide Programs (OPP), the Environmental Fate and Effects Division (EFED) routinely produces Data Evaluation Records (DERs), which represent the agency’s independent review, evaluation, and endpoint selection for registrant-submitted (unpublished) and open literature pesticide toxicity studies. The toxicity endpoints and primary study information from the honey bee toxicity studies are available through EPA’s ECOTOXicology Knowledgebase (https://cfpub.epa.gov/ecotox/). All mortality-based honey bee toxicity endpoints included in this retrospective analysis are extracted from DERs that were completed prior to August 2019. Due to this cut-off date, some chemicals and endpoints were not included in the analysis. During USEPA’s review process, each DER is assigned a specific study classification based on quality and adherence to established guidelines. Studies classified as “acceptable” fully adhere to the standardized testing guidelines while “supplemental” studies deviate somewhat from the standardized testing conditions, but the deviations are not considered serious enough to compromise scientific integrity of the results. In this analysis, we only included studies and their endpoints if they were classified as either “acceptable” or “supplemental.” Studies classified as “invalid” are not scientifically sound and were excluded from further analysis. Additionally, we only included endpoints from studies testing technical grade active ingredients (TGAI; active ingredient alone); therefore, data on typical end-use products (TEP; active and inert ingredients) were not included in the analysis. See S4 Table for a complete listing of studies that were considered after applying the screening criteria.

Study and endpoint selection
When performing an analysis of adult bee acute oral and contact toxicity we compared the lethal dose to 50% of the organisms tested (i.e., LD$_{50}$) from AAO and AAC studies. We excluded pesticides not currently registered for use in the U.S. and toxicity endpoints reported in non-standard units that couldn’t be converted to μg a.i./bee. Available endpoints from these acute adult studies are often non-definitive (i.e., the LD$_{50}$ > maximum tested concentration) because of maximum dose requirements for testing under the guidelines. Despite the uncertainty associated with non-definitive endpoints, they can provide meaningful information for risk assessment [27] and their exclusion would have limited the dataset to compounds with a relatively high acute toxicity towards honey bees (e.g., insecticides). The analysis included non-definitive values in comparisons where there was one definitive value and one non-definitive value (no comparisons were made between two non-definitive endpoints). This analysis also utilized larval LD$_{50}$ values generated from LAO and LCO study guidelines. For pesticides with multiple toxicity endpoints derived from independent studies, the analysis utilized the
lowest or most definitive LD\textsubscript{50} value. For example, if two studies resulted in LD\textsubscript{50} values of 0.5 and 0.1 µg a.i./bee, we used 0.1 µg a.i./bee as the LD\textsubscript{50} value. When both non-definitive values and definitive value(s) were present, the analysis utilized the definitive value. The analysis also excluded acute toxicity data extending beyond the 48-hour time interval to ensure the endpoints represented the same study duration. S1 Table lists all included acute adult endpoints.

When comparing oral acute and chronic toxicities, we included mortality data from honey bee adult and larval toxicity studies that adhered to four OECD test guidelines: AAO; ACO; LAO and LCO. This included the estimated LD\textsubscript{50} (µg a.i./bee or µg a.i./larva) from both adult and larval acute (single dose) toxicity study guidelines (AAO and LAO) as well as the Day 8 (D8) LD\textsubscript{50} estimates from larval chronic (repeat dose) toxicity studies (LCO). Endpoints from chronic studies can be expressed as the cumulative dose consumed during the exposure period (µg a.i./bee) or as the daily dose consumed per individual (µg a.i./bee/day). We compared all endpoints in common units (i.e. µg a.i./bee/day or µg a.i./larva/day) because daily dose is used by the current USEPA exposure model for honey bees during ecological risk assessments [13] and is the basis for understanding relative toxicity at USEPA. EPA relies upon daily dose-based exposures for both acute and chronic based exposures because it is important to review the comparable relationships between acute and chronic endpoints using the same units. This approach is utilized because risk to developing honey bee larvae is assessed based on daily exposures via food consumption not cumulative food consumption during the entire larval stage.

We excluded endpoints based on dietary concentrations such as median lethal concentration (LC\textsubscript{50}) and no/lower observed adverse effect concentration (NOAEC and LOAEC; i.e. mg a.i./kg diet). Although typically reported, we did not use NOAEL and LOAEL values from the acute studies (AAO and LAO) in any analysis. Additionally, we excluded any studies on chemical degradates, studies containing multiple active ingredients, and TEP-based exposures from the analysis. As noted earlier, while ingestion of treated diet (i.e., oral exposure) is the primary focus of the larval toxicity studies, it is important to note that larvae are also in direct contact during the bioassay. However, measurement endpoints from the larval studies are evaluated in risk assessment assuming the oral route of exposure is dominant [18, 19].

**Adult oral and contact endpoint sensitivity ratio**

When comparing the relative toxicities between oral and contact endpoints for a given pesticide, we employed a relative sensitivity ratio (R), using LD\textsubscript{50} values standardized by µg a.i./bee.

\[
R = \frac{\text{Acute adult oral LD}_{50} \text{ (µg a.i./bee/day)}}{\text{Acute adult contact LD}_{50} \text{ (µg a.i./bee/day)}}
\]

A value of 1 signifies no difference between oral and contact toxicity; whereas values less than 1 represents higher oral toxicity and values greater than 1 represent higher contact toxicity. The absence of sample variance data corresponding to the reported LD\textsubscript{50} values precluded statistical comparisons for individual ratio differences. Instead, we relied on nominal thresholds at five and ten-fold differences above and below the 1:1 relationship benchmark to evaluate the strength of any identified relationships. We required at least one definitive endpoint to perform the analysis. All non-definitive endpoints included in this comparison were greater than (> ) values. Ratios calculated using both a non-definitive and a definitive endpoint provide a conservative estimate because a non-definitive (i.e., greater than) endpoint is an under-estimation of the actual LD\textsubscript{50} and is typically derived from the highest tested dose in an individual study as the actual LD\textsubscript{50} is higher than what was actually tested.
Adult and larval chronic and acute comparisons

Acute-to-chronic toxicity ratio (ACR) calculation. Acute-to-chronic toxicity ratios (ACR) serve as a means to estimate the acute or chronic toxicity of a chemical towards an organism when only acute or chronic toxicity data are available [28]. We calculated ACRs for honey bee mortality endpoints as the ratio of the LD$_{50}$ value from the acute oral toxicity studies (either AAO or LAO) to the most sensitive mortality NOAEL from the chronic toxicity study (either ACO or LCO):

$$\text{ACR} = \frac{\text{Median Lethal Dose (LD}_{50})}{\text{No Observed Adverse Effect Level (NOAEL)}}$$

The ACO (adult) studies provide only a single mortality NOAEL endpoint; however, the LCO (larval) studies, multiple mortality NOAEL values (i.e., larval mortality at Day 8; pupal mortality at Day 15; and adult mortality or emergence at Day 22). In this analysis, we focused on the most sensitive mortality NOAEL endpoint when calculating larval ACRs. Additional measurement endpoints (e.g., food consumption; adult bee weight) are also provided in these studies and occasionally may represent a more sensitive response than mortality. However, to minimize potential confounding factors, these endpoints are not included in this comparison.

Comparison of larval LD$_{50}$ estimates derived from acute and chronic studies

Larval chronic oral toxicity studies (LCO) are primarily aimed at determining NOAEL and LOAEL values; however, an LD$_{50}$ value on Day 8 can also be calculated. Despite being able to derive the same endpoint as larval acute (single dose) oral toxicity studies, LCO studies use a repeat daily dosing (typically four consecutive days) while LAO studies evaluate the effects of a single dose. USEPA recognizes that the likelihood of acute (single dose) exposure for larval honey bees may be limited under natural conditions; however, the LD$_{50}$ value is useful in characterizing the acute toxicity of a pesticide and is consistent with what is done for other taxa (e.g., birds, mammals, fish, aquatic invertebrates). When both acute and chronic larval toxicity studies were available for a chemical, we compared the single dose larval acute LD$_{50}$ from the LAO study to the day eight (D8) LD$_{50}$ estimates from the LCO study. If multiple LD$_{50}$ values were available for a single active ingredient under either study guideline, we selected the most sensitive LD$_{50}$ for comparisons. The analysis excludes non-definitive LD$_{50}$ values. We compared endpoints using a ratio of the LAO to LCO LD$_{50}$ values:

$$\frac{\text{LAO based Larval } \text{LD}_{50} (\mu g \text{ a.i./lara} \text{v/day})}{\text{LCO based Larval } \text{LD}_{50} (\mu g \text{ a.i./lara} \text{v/day})}$$

Statistical analysis, data visualization, and risk comparisons

The analysis utilized Microsoft Excel to perform calculations and visualizations for sensitivity comparison ratios across study types and pesticide classifications. We used linear regression and a Spearman’s correlation analysis when investigating the relationship between log-transformed definitive AAO and AAC LD$_{50}$ endpoints in JMP® Pro 14 (SAS Institute inc., Cary, NC). We produced additional visualizations using JMP® Pro 14. Lastly, a Wilcoxon Signed Rank Test on matched pairs ($\alpha = 0.05$) was used to compare the LAO and LCO based LD$_{50}$ estimates. When comparing experimental endpoints to estimated exposures through food consumption and contact for adults and larvae, we relied upon default exposure factors included...
in BeeREX v.1.0 (https://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/models-pesticide-risk-assessment#beerex) and as described in the USEPA 2014 [13].

Results

In total, 1,888 studies in the database were considered for analysis prior to data screening. Then screening criteria were implemented to ensure only the most robust comparisons were generated (see Methods). As a result of the screening criteria, the analysis excluded 60% of the total dataset. For the studies that passed the initial screen, the adult acute contact toxicity studies (AAC; [14, 15]) represented the greatest percentage (412; 52%) of the total followed by the adult acute oral toxicity studies (AAO; [16]) (136; 18%). The other three toxicity studies adult chronic oral (ACO; [17]), larval acute oral (LAO; [18]) and larval chronic oral (LCO; [19]), comprised a similar and much smaller proportion of the included studies with 9% (76), 7% (58) and 13% (106) respectively. After the most sensitive endpoints were identified from each study, and duplicate studies for each chemical were removed, a total of 195 chemicals, representing 115 chemical classes and 24 general mechanisms of action, had at least one study representing two or more of the test guidelines (S1 Table).

While there were often more chemicals with honey bee studies available, comparisons were limited based upon the study pairs within each chemical and the nature of the endpoint data (i.e., definitive vs non-definitive; Table 1). For example, following data curation and screening, we identified 146 chemicals which had both acute oral and contact toxicity values for adult bees (S1 Table), but a large portion of these had non-definitive values for both studies and therefore were excluded from analyses. Of these chemicals, fungicides and herbicides had a high number of chemicals with both studies being non-definitive toxicity values (i.e., 33 of 40 fungicides and 50 of 60 herbicides; S1 Table). Whereas only 10 of 41 insecticides had non-definitive LD$_{50}$ values for both studies. Based on the requirement of at least one endpoint being definitive, there were 49 chemicals that had endpoints that could be compared. The final dataset for comparing the results of oral and contact studies for the same pesticide, was therefore limited to 31 insecticides, 10 herbicides, 7 fungicides and one nematicide (S1 Table).

The primary toxicity endpoint from the adult (AAO) and larval (LAO) acute oral toxicity studies is the LD$_{50}$; whereas, the chronic studies (ACO and LCO) are used to calculate multiple endpoints. In the available dataset, AAO studies had a relatively low proportion (31%) of

| Target Chemistry | Adult acute contact (AAC; 850.3020/OECD TG 214) | Adult acute oral (AAO; OECD TG 213) | Adult chronic oral (ACO; OECD TG 245) | Larval acute oral (LAO; OECD TG 237) | Larval chronic oral (LCO; OECD GD 239) |
|------------------|-----------------------------------------------|-------------------------------------|---------------------------------------|-------------------------------------|---------------------------------------|
| Number of studies in database$^1$ | 1222 | 347 | 112 | 75 | 132 |
| Number of Chemicals with each study$^1, 2$ | 167 (41) | 152 (40) | 69 (69) | 58 (34) | 96 (32; 95)$^4$ |
| Herbicides$^1, 2$ | 69 (8) | 62 (7) | 32 (32) | 26 (14) | 46 (13; 45) |
| Fungicides$^1, 2$ | 46 (3) | 43 (5) | 16 (16) | 15 (5) | 30 (8; 30) |
| Insecticides$^1, 2$ | 46 (29) | 42 (28) | 19 (19) | 16 (14) | 19 (11; 19) |
| Other$^1, 3$ | 6 (1) | 5 (0) | 2 (2) | 1 (1) | 1 (0; 1) |

$^1$ These numbers represent total available studies, however not all studies could be used in all comparative analyses.

$^2$ Numbers in parentheses represent the number of chemicals with definitive adult acute LD$_{50}$ endpoints. Chronic and larval toxicity endpoints were all definitive.

$^3$ Other includes: nematicides, molluscicides, miticides, and microbicides.

$^4$ LCO studies provide both LC50 and NOAEL estimates, the numbers in parenthesis reflect the number of definitive endpoints for each respectively.

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definitive toxicity endpoints; however, NOAEL values from ACO tests were all definitive (Table 1). For the larval toxicity studies, most of the NOAEL endpoints included in the retrospective were set at the highest tested concentration because no-effects were detected (i.e., no LOAELs); however, 65% of the larval LD$_{50}$ endpoints were definitive (S1 Table). The analyses between adult and larval chronic and acute toxicity studies utilized only the definitive endpoints.

**Relative toxicity patterns**

All available LD$_{50}$ data, provided in S1 Table, were classified as either highly toxic (LD$_{50}$ ≤ 2 μg/bee), moderately toxic (2 < LD$_{50}$ < 11 μg/bee) or practically non-toxic (LD$_{50}$ >11 μg/bee) on an acute exposure basis. Fig 1 illustrates that based on the adult acute LD$_{50}$, insecticides are primarily classified as highly- or moderately toxic on an acute exposure basis; however, herbicides and fungicides are predominately classified as practically non-toxic to bees on an acute exposure basis. Non-definitive (ND) endpoints for highly and moderately toxic categories are provided separately; these are combined with definitive endpoints for the practically non-toxic category (defined as LD$_{50}$ ≥11ug/bee). Larval LD$_{50}$ values generated from LAO and LCO study guidelines are combined, the most sensitive estimate was plotted for chemicals that had estimates for both. For the larval LD$_{50}$ values the patterns are similar; however, a greater proportion of herbicides and fungicides were classified as highly to moderately toxic.

**Comparison of AAC and AAO LD$_{50}$**. Overall, there was a significant positive correlation between AAO and AAC log transformed endpoints (Spearman’s $\rho = 0.74$, p <0.0001, n = 49

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Fig 1. Proportional relationships of toxicity categories across adult and larval life stages and exposure based on contact and oral LD$_{50}$ values for insecticides, herbicides, and fungicides. AAC- Adult Acute Contact (n = 149), AAO- Adult Acute Oral (n = 146), LAO Larval Acute Oral & LCO—Larval Chronic Oral LD$_{50}$ (n = 110).

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study pairs, Fig 2). For the included chemicals, 83% had less than a five-fold difference between the two endpoints, while 86% had less than a ten-fold difference.

When comparing ratios ("R values") between oral and contact LD_{50} values there is a generalized pattern that suggests the acute oral LD_{50} is as sensitive if not more sensitive than the acute contact LD_{50}, as illustrated in Fig 3 when log_{10} R ≤ 0. Nine pesticides, 7 neurotoxicant insecticides (i.e., clothianidin, flupyradifurone, imidacloprid, metaflumizone, oxamyl, tetrani-liprole, and thiodicarb), one fungicide (captan) and 1 herbicide (bromoxynil), had R values ≤ 0.1 (R = 0.08, 0.012, 0.05, 0.02, 0.04, 0.008, 0.06, 0.005, and 0.02, respectively) and are depicted in Fig 3 when log_{10} R ≤ -1 which signifies a higher degree of oral toxicity (10-fold or more) relative to contact toxicity. Comparatively, eight pesticides had R values ≥ 1, indicating relatively higher acute contact toxicity for these pairs (10-fold or more). These nine pesticides included 6 neurotoxicant insecticides (i.e., tefluthrin, transfluthrin, lambda-cyhalothrin, momfluorothrin, carbaryl and deltamethrin; R = 10, 21, 24, 25, 34 and 53 respectively), one respiration inhibitor/regulator insecticide (i.e., pyridaben; R = 110), and two biosynthesis inhibiting fungicides (i.e., fenhexamid and spiroxamine; R = 465 and 24 respectively). These are depicted in Fig 3 as log_{10} R values ≥ 1.

The USEPA’s pesticide risk assessment process for bees assumes that the estimated environmental concentrations (EECs) for adult contact and oral routes of exposure are approximately
12x different (i.e., oral EEC > contact EEC; see [13]). This is because pesticide exposure though ingestion of residues in diet is expected to occur at a higher rate (12x) than would occur from contact exposure (absorption through the integument); therefore, the contact toxicity of a chemical would need to be 12X more toxic to represent an equivalent level of risk. Sixteen percent (8/49) of the chemicals had adult acute contact LD<sub>50</sub> values 12x or more toxic than the adult acute oral LD<sub>50</sub>. These exceptions included 6 contact insecticides (i.e., pyridaben, deltamethrin, momfluorothrin, lambda-cyhalothrin, carbaryl, and transfluthrin) and 2 fungicides (i.e., fenhexamid, spirotetramat) with non-definitive oral LD<sub>50</sub> values.

**Comparison of LAO and LCO based LD<sub>50</sub>s.** Comparisons were made (Table 2) between the LD<sub>50</sub> values from the larval acute (single dose) oral toxicity studies (LAO; OECD TG 237) and the Day 8 LD<sub>50</sub> estimates from the larval chronic (repeat dose) studies (LCO; OECD GD 239). The LD<sub>50</sub> values from the LAO studies and those derived from LCO studies were found to be significantly different (Wilcoxon Signed Rank Test on matched pairs, n = 15 Z = -37, p = 0.03) with LD<sub>50</sub> values derived from chronic tests being significantly lower than those from single exposure larval testing. The LD<sub>50</sub> comparisons were made directly between 15 individual pesticide active ingredients derived from 15 different chemical classes spanning 6 different MOAs with several studies representing neurotoxicants and biosynthesis inhibitors. The comparison between the LAO and LCO resulted in only two chemicals (i.e., acetamiprid and MCPA) where the single dose LD<sub>50</sub> value was more sensitive than the repeat dose LD<sub>50</sub> value.

**Acute toxicity endpoints: Adult (AAO) and larval (LAO, LCO) LD<sub>50</sub> values.** The acute toxicity of various pesticides to adult and larval honey bees were compared using the LD<sub>50</sub> values determined in their respective studies. The AAO toxicity data were available for 152 pesticides. Since the LAO studies were more limited in number than any other guideline (Table 1),
the LCO studies were also relied upon for estimating the larval LD$_{50}$ values which expanded the total number of pesticides to 112 (Table 3). When both LAO and LCO values were available, and only one was definitive, the definitive endpoint was selected. If both LAO and LCO endpoints were available and were definitive, the lower (more sensitive) LD$_{50}$ value was selected. Table 3 provides the distribution of the definitive and non-definitive LD$_{50}$ data for adults and larvae across the types of pesticides. The non-definitive data were provided here to illustrate that very few fungicides and herbicides had definitive values from the AAO test.

Direct chemical comparisons across adult and larval LD$_{50}$ values. Only 11 pesticides had both adult and larval acute oral LD$_{50}$ values which were definitive (Table 4). The adult LD$_{50}$ was compared to either or both of the larval acute LD$_{50}$ estimates from the LAO or LCO (8-day LD$_{50}$s) studies. The 11 pesticides span 10 different chemical classes, but only include insecticides and one herbicide, and eight of the insecticides are neurotoxicants. Notably, this dataset only includes one neonicotinoid, due to the cut off in timing for DERs and endpoints.

Table 2. Comparison of larval honey bee (Apis mellifera) acute (LAO; single dose; OECD TG 237) and chronic (LCO; repeat dose; OECD GD 239) LD$_{50}$ values (N = 15) expressed in terms of μg ai larva$^{-1}$ day$^{-1}$.

| Pesticide       | Type       | Chemical Class | General MOA          | LAO LD$_{50}$ | LCO LD$_{50}$ | Ratio (Single: Repeat dose) | LD$_{50}$ |
|-----------------|------------|----------------|----------------------|---------------|---------------|-----------------------------|-----------|
| Ipconazole      | Fungicide  | Triazoles      | Biosynthesis Inhib.  | 22            | 14            | 1.6                         |           |
| MCPA            | Herbicide  | Chlorophenoxy acid/ester | Growth Regulator | 34.6          | 93.9          | 0.4                         |           |
| Cycloate        | Herbicide  | Thiocarbamate  | Biosynthesis Inhib.  | 10            | 8.4           | 1.2                         |           |
| Prosulfuron     | Herbicide  | Sulfonylureas  | Biosynthesis Inhib.  | 26            | 21            | 1.2                         |           |
| Acetochlor      | Herbicide  | Chloroacetanilide | Biosynthesis Inhib. | 26.5          | 11.8          | 2.2                         |           |
| Forchlorfenuron | Herbicide  | Phenylureas    | Growth Regulator     | 15            | 4.23          | 3.5                         |           |
| Pinoxaden       | Herbicide  | Phenylpyrazoline | Biosynthesis Inhib. | 5.2           | 1.4           | 3.7                         |           |
| Amicarbazone    | Herbicide  | Triazolinones  | Photosynthesis       | 45            | 5.2           | 8.7                         |           |
| Acetamiprid     | Insecticide| Neonicotinoids | Neurotoxicant        | 1.16          | 5.43          | 0.2                         |           |
| Abamectin       | Insecticide| Glycosides     | Neurotoxicant        | 0.0011        | 0.0005        | 2.2                         |           |
| Tetranilprole   | Insecticide| Diamide        | Neurotoxicant        | 0.013         | 0.0046        | 2.8                         |           |
| Tolfenpyrad     | Insecticide| Pyrazole       | Respiration Inhib./Reg. | 0.044       | 0.014         | 3.1                         |           |
| Propargite      | Insecticide| Sulfite ester  | Respiration Inhib./Reg. | 25.31      | 4.8           | 5.3                         |           |
| Fenpyroximate   | Insecticide| Phenoxy pyrazoles | Respiration Inhib./Reg. | 0.2        | 0.029         | 6.9                         |           |
| Sulfoxaflor     | Insecticide| Sulfoximine    | Neurotoxicant        | 2.65          | 0.0494        | 53.6                        |           |

Table 3. Distribution of all available acute toxicity data (LD$_{50}$ values) for honey bee (Apis mellifera) adults and larvae. AAO- Adult Acute Oral, LAO- Larval Acute Oral, LCO- Larval Chronic Oral.

| Endpoint Characteristics and Pesticide Class | Adult (AAO) LD50s | Larval (LAO and LCO) LD50s |
|---------------------------------------------|-------------------|----------------------------|
|                                             | Fungicides | Herbicides | Insecticides | Other$^{1}$ | Fungicides | Herbicides | Insecticides | Other$^{1}$ |
| Definitive                                  | n        | 4          | 7           | 28          | 0           | 11         | 20          | 18         | 1           |
|                                             | min      | 47         | 3.3         | 0.004       | 0.0005      | -          | -           | -          | -           |
|                                             | max      | 455        | 127         | 792.4       | 60          | 87.2       | 63          | 72         | -           |
|                                             | median   | 91.8       | 45.9        | 0.3         | 14.8        | 20.5       | 0.6         | -          | -           |
| Non-definitive                              | n        | 39         | 54          | 14          | 5           | 24         | 31          | 6          | 1           |
|                                             | min      | >0.9       | >11         | >0.1        | >0.4        | >0.004     | >1.1        | >0.005     | -           |
|                                             | max      | >13,443    | >445        | >200        | >200        | >150       | >100        | >14.5      | >1          |
| Total                                       | n        | 43         | 61          | 42          | 5           | 35         | 51          | 24         | 2           |
| Proportion definitive                       | 0.09     | 0.11       | 0.67        | 0.00        | 0.31        | 0.39       | 0.75        | 0.50       |

$^{1}$“Other” group includes nematicides and miticides.
that were available for this analysis. For each of the comparisons (LAO: AAO or LCO:A AO) 8 pesticides could be compared. The AAO LD₅₀ value was more sensitive than the LAO and LCO based LD₅₀ value in 5 and 1 of the pesticides respectively (Table 4).

### Comparison of mortality-based NOAEL endpoints from ACO and LCO studies. The available dataset contains a total of 96 LCO studies, most of which (85%) reported a mortality-based NOAEL and LOAEL from at least one of the time points (i.e., larval Day-8, pupal Day-15 or adult Day-22). There were 69 ACO toxicity studies, with 59% of them having both mortality-based NOAEL and LOAEL values. Fig 4 illustrates the general pattern of increased toxicity (i.e., more sensitive NOAEL values) of fungicides, herbicides and miticides to larvae relative to adults, while for insecticides the average NOAEL for adults and larvae was within one standard error.

To look more closely at this pattern, comparisons of the NOAELs based on mortality were made between the ACO and LCO studies of 57 pesticides that had both studies. In this paired dataset, 28 of the pesticides did not report effects up to the highest concentration tested (i.e., non-definitive LOAELs) in the ACO study; whereas, for the LCO study, only 13 pesticides had non-definitive LOAEL values. For one pesticide (i.e., oxyfluorfen) there was no established NOAEL as the study had statistically significant effects at all test levels. The paired NOAEL endpoint comparisons span a variety of MOAs and chemical classes (Fig 5), but as a whole and across both chemical class and MOAs (other than for neurotoxic insecticides), the larval NOAEL values were generally more sensitive than their corresponding adult endpoints (log₁₀ < 0), similar to the pattern observed across all available data (Fig 4). However, to understand this difference in terms of relative potential for risk, the data in Fig 5 are compared to the red dotted line in the figure. The red line represents the ratio under which a risk quotient would be equivalent based on default Bee-REX dietary dose exposure assumptions for larval and adult bees.

In contrast neurotoxic insecticides (n = 4) had a distribution of ratios that predominantly exceeded the red dashed line indicating a generally more sensitive adult NOAEL and a likely larger risk potential for adult bees. The larval NOAEL values were more sensitive than the adult NOAEL counterparts in for 40 (70%) of the 57 chemicals. Under the default exposure assumptions in risk assessment ([13]; based on differential growth stage and caste food

### Table 4. Comparison of honey bee (Apis mellifera) adult acute (AAO; OECD TG 213), larval acute (LAO; OECD TG 237), and Larval Chronic Oral (LCO; OECD GD 239) technical grade active grade ingredients (TGAI) LD₅₀ values (N = 11). Units for the adult and larval LD₅₀ values are μg ai bee⁻¹ and μg ai larva⁻¹, respectively.

| Pesticide         | General Class | Chemical Class | General MOA                              | Adult AAO LD₅₀ | Larval LAO LD₅₀ | Larval LCO Day 8 LD₅₀ | LAO:AAO LD₅₀ Ratio | LCO:AAO LD₅₀ Ratio |
|-------------------|---------------|----------------|------------------------------------------|----------------|----------------|-----------------------|-------------------|-------------------|
| Fenazaquin        | Insecticide   | Quinazoline    | Respiration Inhib./Reg.                   | 5.8            | 0.347          | -                     | 0.06              | -                 |
| Tolfenpyrad       | Insecticide   | Pyrazole       | Respiration Inhib./Reg.                   | 0.63           | 0.044          | 0.014                 | 0.07              | 0.02              |
| Abamectin         | Insecticide   | Glycosides     | Neurotoxicant                            | 0.0044         | 0.0011         | 0.000498              | 0.25              | 0.11              |
| Cyclaniliprole    | Insecticide   | Diamide        | Neurotoxicant                            | 0.702          | -              | 0.05225               | -                 | 0.07              |
| Tetranipliprole   | Insecticide   | Diamide        | Neurotoxicant                            | 0.0103         | 0.013          | 0.0046                | 1.26              | 0.45              |
| Amicarbazone      | Herbicide     | Triazolinones  | Photosynthesis                           | 24.4           | 45             | 5.2                   | 1.84              | 0.21              |
| Oxamyl            | Insecticide   | Carbamate      | Neurotoxicant                            | 0.379          | 0.931          | -                     | 2.46              | -                 |
| Fipronil          | Insecticide   | Phenylpyrazoles| Neurotoxicant                            | 0.00405        | 0.0218         | -                     | 5.38              | -                 |
| Sulfoxaflor       | Insecticide   | Sulfoximine    | Neurotoxicant                            | 0.146          | 2.65           | 0.0494                | 18.15             | 0.34              |
| Formetanate hydrochloride | Insecticide | Carbamate | Neurotoxicant | 0.16 | - | 0.11 | - | 0.69 |
| Thiamethoxam      | Insecticide   | Neonicotinoids | Neurotoxicant                            | 0.005          | -              | 0.78                  | -                 | 156               |

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consumption rates), the instances where the ratio is less than 0.42 indicates that the larval NOAELs are more sensitive than the adult NOAELs, and thus would result in a greater risk given the exposure assumptions. This occurs for 31 (54%) of the 57 chemicals (S1 Table).

**Acute-to-chronic ratio (ACR) analyses**

Acute-to-chronic ratios (ACRs) for adult honey bees were calculated using the AAO LD\(_{50}\) values and the ACO mortality-based NOAEL values. While there were 49 chemicals with both of these studies, only 14 had definitive values for both. In terms of the chemicals that had a non-definitive LD\(_{50}\) from the AAO study, 13 had reported mortality effects in their ACO studies and defined a LOAEL. For the 14 chemicals that had definitive values for both studies, the ACRs ranged from 0.94 to 38. The only pesticide with an ACR less than 1 was the herbicide bromoxynil, which had an ACR of 0.94. The other two herbicide adult ACRs were 22 and 38 (Fig 6).

The ACRs for larval honey bees were calculated using the LAO LD\(_{50}\) values relative to the most sensitive larval daily dose mortality or adult emergence-based NOAEL from the LCO study.
study. Larval ACRs ranged from 1.8 to 52 for the 23 chemicals that had definitive acute LD$_{50}$ values. There were another 17 chemicals with non-definitive LD$_{50}$ values from the LAO study. Their LCO counterpart studies also did not define an LD$_{50}$ based on 8-day mortality, and for 9 of them no effects were observed at the highest doses tested. An additional review of the larval ACR based on the LCO-derived LD$_{50}$ allowed for 31 chemical comparisons (S5 Table). As indicated by the direct comparison of LD$_{50}$ values from the LAO and LCO studies, the LCO study typically provided a more sensitive LD$_{50}$ value than the LAO study; therefore, the ACRs for most chemicals are smaller (range from 0.23 to 195, Fig 6). Notably two ACRs were very different from the other estimates; the fungicide valifenalate (ACR = 70) and insecticide thiamethoxam (ACR = 195, indicated with the orange star in Fig 6). Both of these ACRs were generated from definitive LD$_{50}$ values from LCO studies and had NOAECs that were bounded by LOAECs.

**Discussion**

**Toxicity trends between oral and contact tests**

Despite testing different exposure pathways, this retrospective analysis indicates that adult acute contact (AAC) and adult acute oral (AAO) LD$_{50}$ endpoints exhibit a positive relationship (Spearman’s $\rho = 0.74$, $p < 0.0001$, $n = 49$, Fig 2). These results indicate that, while differences in exposure pathway may influence the delivery of a chemical, the difference in tested pathway between AAO and AAC studies did not manifest in differing sensitivities towards a given compound in terms of LD$_{50}$ values. The comparisons between the adult acute contact and oral LD$_{50}$ values suggest that there were relatively similar toxicities reported for most chemicals between study types across pesticide classes, with oral toxicity being slightly more sensitive or as sensitive as the counterpart contact-based endpoint. Based on current assumptions used in USEPA’s pesticide risk assessment framework (See [13]), the difference between exposure to a...
pesticide through food consumption is modeled at higher rate (12x) relative to a contact exposure and as a result the contact toxicity of a compound would need to be 12x more toxic than the acute oral endpoint to elicit an equivalent level of risk. As indicated by the sensitivity ratio values (R values) between contact and oral endpoints, this analysis found that only 16% of chemicals had AAC LD$_{50}$ values $\leq$ 12x AAO LD$_{50}$ and the majority (84%) of compounds had less than a five-fold difference between LD$_{50}$ values (Fig 3). This finding indicates that based on the data and exposure assumptions in USEPA’s risk assessment process, AAO LD$_{50}$ values can be protective of AAC values. However, the value of using acute oral LD$_{50}$ values as a surrogate for AAC studies may be limited when considering the loss of information provided by contact-specific endpoints. While the inclusion of a non-definitive/greater than (>) values introduces some uncertainty into the R values because the true endpoint value is unknown, it permits the inclusion of compounds with vastly different toxicities across exposure pathways (oral vs. contact) in the analysis. For example, half of the six pesticides had an R value $\geq$ 1 (indicating relatively higher contact toxicity) were in the pyrethroid insecticide class. This may indicate differences in the adsorption, distribution, metabolism, and/or excretion between contact and dietary exposure for this class of chemicals and may provide useful information when characterizing risks from these compounds. While two fungicides also had R value $\geq$ 1, they both had non-definitive acute oral LD$_{50}$ values; therefore, caution should be exercised in interpreting the significance of this ratio for these pesticides. Ultimately, the inclusion of some non-definitive values results in more conservative ratios because the non-definitive endpoint value is an overestimation of toxicity.

Fig 6. Larval and adult honey bee (Apis mellifera) acute-to-chronic ratios (ACRs), across pesticide types. The two types of larval ACRs are separated, based on the source of the acute larval LD$_{50}$ value (from either the LAO or LCO study). [sample sizes n>1 are provided below boxes, n of 1 is indicated with an "-x-"; shaded boxes represent first quartile to the third quartile, "x" represents the mean, horizontal bar is the median, whiskers extend out to the 95th percentiles].

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Acute toxicity to adult and larval honey bees

While the primary aim of larval acute oral toxicity studies (LAO) is to establish an LD$_{50}$, larval chronic (repeat dose) oral toxicity studies (LCO) have also been used to derive an LD$_{50}$ value that can be used as a surrogate for a single-dose LAO LD$_{50}$. To account for the difference in exposure between the two study types, the comparison between the LAO and Day 8 LD$_{50}$ from LCO studies values are made using a converted daily dose, where dosing is normalized to essentially an equivalent per day basis (μg/beel/day). We report that LD$_{50}$ endpoints from LCO studies were more sensitive relative to LD$_{50}$ values from LAO studies (Wilcoxon Signed Rank Test on matched pairs, n = 15 Z = -3.7, p = 0.03). The endpoints used in this analysis were all definitive. Despite being primarily used for determining chronic NOAEL values, LCO studies can yield LD$_{50}$ values that are lower and protective of those calculated in LAO studies. It is important to consider that this comparison was limited to only fifteen pesticides, and that two of the chemicals resulted in more sensitive LD$_{50}$ values generated from the LAO study. The inclusion of a greater number of pesticides and MOAs would strengthen the understanding of this relationship. However, based on our current dataset the LCO appears to generate LD$_{50}$ endpoints which can be considered protective relative to the LAO LD$_{50}$ values.

We also compared the distribution of acute toxicities for adults and larvae across pesticide types (i.e., insecticides, herbicides, fungicides, and other) using acute toxicity classifications (Fig 1). The overall distribution of pesticide types was similar between life stages (adults and larvae), but the proportion of toxicity values that were definitive was much greater for larvae than adults, and for insecticides as compared to other types of pesticides (especially in studies of adults). When comparing the number of definitive LD$_{50}$ endpoints classified as "highly toxic", the majority of these values were from studies testing insecticides (Fig 1). However, we report a greater number of herbicides and fungicides classified as moderately toxic to honey bee larvae as compared to adults, potentially because there were more definitive LD$_{50}$ values in these studies. This highlights the importance of larval studies when characterizing the potential toxicities of pesticide types other than insecticides.

When examining differences between acute adult and larval toxicity studies, we only compared chemicals with definitive endpoints and only 11 pesticide active ingredients could be directly compared (Table 4). The analysis indicates that adult LD$_{50}$ values were more sensitive than the larval LD$_{50}$ values calculated in LAO studies (5 out of 8 studies). Conversely, larval chronic (LCO) LD$_{50}$ values were generally more sensitive than the corresponding adult values in 88% of comparisons (7 out of 8 comparisons see Table 4). In ecological risk assessment, differences between larvae and adult diet composition and consumption rates impacts exposures of a pesticide to larvae and adults [13]. These difference in dietary dose-based exposures result in larval toxicity endpoints needing to be 2.36 times lower than the adult toxicity endpoints (ratio = 0.42) to result in the same level of risk [13]. Thus, the ratios less than 0.42 expressed in Table 4 indicate instances where the larval LD$_{50}$ value would result in higher risk estimation based upon the default assumptions of exposure. Depending on the source of the larval LD$_{50}$ (LAO or LCO), either 3/8 or 5/8 of the studies give a ratio below 0.42, indicating a greater risk for larval honey bees. However, the limited dataset will need to be expanded to determine if the larval honey bees are indeed more sensitive than the adults across broader pesticide and MOA groupings. This observation of greater larval sensitivity was also observed in the chronic toxicity studies, discussed below. We also report that thiamethoxam had the largest difference between adult and larval endpoints (see Table 4). This finding is in line with prior work demonstrating that adults are more sensitive to thiamethoxam [29]. No other neonicotinoid endpoints were available for this comparison, limiting our ability to draw conclusions about this effect based on insecticide class.
Chronic toxicity to adult and larval honey bees

In order to examine differences in chronic toxicity between honey bee lifestages, adult chronic (ACO) and larval chronic oral (LCO) NOAEL values were compared. The NOAEL is defined as the highest tested dose level which does not yield a significant effect relative to the control group and consequently, both the biological response and selected test concentrations can influence this endpoint. A variety of considerations are made when selecting dose level in a chronic toxicity study such as the estimated environmental concentrations of a pesticide or the inclusion of a “limit dose.” The impact of these factors on test concentration selection undoubtedly influenced the endpoints included in our dataset such that many chemicals reported NOAELs without having a definitive LOAEL. For these reasons, selected dose-levels may not always identify the exposures at which a chronic effect may begin to occur but are useful when establishing exposures which are unlikely to elicit an effect in risk assessment. Ultimately, dosage-level selection may have impacted the interpretation of 12 of the 29 compared ACO and LCO pairs as one or both of the studies had reported no observed effects at the maximum doses tested. Given this assumption, the analysis indicates that larval chronic mortality NOAELs were generally more sensitive (lower) than the corresponding adult values (Fig 4). Research has shown that thermal, nutritional, and chemical stress during honey bee larval development can have latent impacts on the health of adult life stages [30–32]. The LCO study has been designed with the sensitivity of developmental stages in mind and is carried through to adult emergence (eclosion) to capture this potential increase in sensitivity across vulnerable developmental stages. Temporal trends among larval chronic toxicity data, where Day 22 NOAELs were more sensitive than Day 8 (larval) or Day 15 (pupal) NOAELs were observed across all pesticide types (Fig 4). As a result, the increased sensitivity observed at Day 22 may not always be detectable at earlier time points. Despite the cessation of larval feeding (and test substance exposure) just prior to pupation at Day 8, effects on survival during pupation and until adult emergence can be observed at dose levels far lower than those which elicited significant effects during earlier stages of development. This underscores the importance of continuing the chronic assay until eclosion as an adult bee.

Chronic mortality endpoints followed the same pattern as the acute toxicity studies regarding differences among types of pesticides. Insecticides, herbicides, and fungicides (Fig 4) were more toxic to larval bees than to adults. Insecticides have modes of action intended to cause adverse outcomes in insects. Not surprisingly, insecticides had the lowest NOAELs, with greater chronic toxicity for both adults and larvae relative to fungicides and herbicides. The chronic mortality endpoints can also be compared across larval and adult studies using the daily EEC ratio of 0.42, the point where despite differences in the default assumptions of exposure the toxicity endpoints would result in the same magnitude of risk. Roughly half of the pesticides had ratios below 0.42; 31 (54%) out of 57 chemicals had larval LD₅₀ values more sensitive than the adult values. Therefore, in a risk assessment the likely result would be a greater estimation of risk for larvae (Fig 5, S1 Table). Four neurotoxic insecticides had a distribution of ratios that exceeded a ratio of 1 indicating a generally more sensitive adult chronic oral NOAEL (Fig 5), but the small sample size makes patterns across MOAs difficult to conclude. Our finding in this retrospective highlights the overall increased sensitivity of honey bee larvae relative to adults in response to acute exposure, but also indicated that the increased larval sensitivity may not translate to increased risk because of differential exposure potential related to the conservative assumptions that the risk assessment makes for daily consumption rates of adult (i.e., nectar foragers) versus larval honey bees.
Acute-to-chronic (ACR) comparisons for adult and larval honey bees

The ACR is used in ecotoxicology to estimate chronic toxicity when well-defined (definitive) acute toxicity data exist (i.e., LD$_{50}$ data) and the chronic data are unavailable ([28]; the acute toxicity endpoint may also be estimated from the chronic endpoint using an ACR). While this is not typically relied upon for honey bee toxicity evaluations (because of limited data), we conducted this analysis to provide insight of the acute to chronic toxicity relationships for honey bees. We compared ACRs for both adult and larval studies using the LD$_{50}$ from the acute toxicity test guidelines and a defined NOAEL value from the chronic toxicity testing.

Larval ACRs were calculated for 23 and 31 pesticides for LAO and LCO-derived LD$_{50}$ endpoints, respectively, and adult ACRs were calculated for 14 pesticides (Table S5 and Fig 6). Larval ACRs ranged from 0.23–195 (average ACR = 13.4) based on either the LAO or LCO sourced LD$_{50}$ values. This was similar to adult ACRs which ranged from 0.943 to 38.28 (average ACR = 12.3). In comparison to the NOAEL from the same study, the NOAEL was 195 times more sensitive than the repeat dose LD$_{50}$. The adult ACR was 2, which also indicates a more sensitive endpoint in the chronic study, but the difference was far less extreme.

Importance of life stage, study duration and pesticide risk evaluation

Insect pollinator testing is required to support risk assessment for the registration and re-registration of conventional pesticides in the United States (See FIFRA and 40CFR158). As a result, USEPA maintains one of the most comprehensive honey bee toxicity databases, containing toxicity endpoint data on both adult and larval life stages (ECOTOX- https://cfpub.epa.gov/ecotox/). Despite having a large honey bee toxicity database with relevant endpoints, the ability to generate direct comparisons across specific chemicals in this retrospective was limited by the extent of non-definitive values. Non-definitive values are useful for identifying dosages which are unlikely to elicit an effect for risk assessment purposes, but non-definitive values have limited utility in statistical analyses and direct comparisons across life stages. Another limitation of the available data for use in comparisons across tests and life stages is that resulting from studies that were conducted with a TEP as opposed to TGAI. The TEP studies represent approximately one third of all available adult contact and adult oral acute toxicity data. Spruill et al. [33] previously investigated the differences in relative toxicity classifications between TGAI and TEP, across both contact and oral exposure toxicity studies and found that the toxicity estimates for TEP data were similar to TGAI. A high-level comparison of the data available to USEPA also came to a similar conclusion. While the exclusion of studies and toxicity endpoints from the analyses presented here significantly reduced the dataset for comparisons, this ensured a more robust and scientifically defensible analysis.

The evaluation of data submitted subsequent to the release of USEPA’s Guidance for Assessing Pesticide Risks to Bees [13] and the development of the adult chronic oral, larval acute oral, and larval chronic oral test guidelines, illustrates the importance of these newer toxicity studies. These data demonstrate that there is a differential sensitivity to pesticides between adult and larval life stages. The data also show that both herbicides and fungicides are frequently identified as being toxic to larval bees and these toxicities are manifested more in the larval and chronic studies as compared to the acute adult studies. The data also show that the repeated dietary exposure to a pesticide can increase its toxicity to adult bees. This evaluation underscores the importance for pesticide studies on adult and larval honey bees despite their intended target pest (e.g., herbicides and fungicides), as well as the importance of having captured both acute and chronic responses to a pesticide exposure. Moreover, the relationships between study endpoints identified in these analyses may help inform future efforts to streamline pollinator toxicity data needs and reduce future animal testing.
Supporting information

S1 Table. Compendium of chemicals and available most sensitive endpoints for each guideline study after applying screening criteria described in the methods. References for each endpoint are provided in S4 Table.

S2 Table. Online sources of information related to mode of action (MOA) of pesticide to target species.

S3 Table. Insecticide classifications with corresponding Insecticide Resistance Action Committee (IRAC) Mode of Action (MoA) classifications.

S4 Table. Cross reference table and list of references (MRIDs) for each of the endpoints provided in S1 Table.

S5 Table. Endpoints and calculated acute-to-chronic ratios (ACRs) for adult and larval honey bee (Apis mellifera) toxicity studies.

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References

1. Jordan A, Harland PM, Grozinger CM, Khanna V. Economic dependence and vulnerability of United States agricultural sector on insect-mediated pollination service. Environmental Science & Technology 2021; 55 (4), 2243–2253. https://doi.org/10.1021/acs.est.0c04786 PMID: 33496588

2. Calderone NW. Insect Pollinated Crops, Insect Pollinators and US Agriculture: Trend Analysis of Aggregate Data for the Period 1992–2009. PLOS ONE. 2012; 7(5). https://doi.org/10.1371/journal.pone.0037235 PMID: 22629374

3. Klein AM, Vaisiere BE, Cane JH, Steffan-Dewenter I, Cunningham SA, Kremen C, et al. Importance of pollinators in changing landscapes for world crops. Proc Roy Soc B. 2007; 274(1608):303–313. https://doi.org/10.1098/rspb.2006.3721 PMID: 17164193

4. USDA. 2013. Report on the National Stakeholders Conference on Honey Bee Health. National Honey Bee Health Stakeholder Conference Steering Committee.

5. Goulson D, Nicholls E, Botias C, Rotheray EL. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. Science. 2015; 347(6229). https://doi.org/10.1126/science.1255957 PMID: 25721506

6. Reilly JR, Artz DR, Biddinger D, Bobiwash K, Boyle NK, Brittain C, et al. Crop production in the USA is frequently limited by a lack of pollinators. Proc R Soc B. 2020; 287: 20200922. https://doi.org/10.1098/rspb.2020.0922 PMID: 33043867

7. Amiri EM, Strand K, Rueppell O and Tarpy DR. Queen Quality and the Impact of Honey Bee Diseases on Queen Health: Potential for Interactions between Two Major Threats to Colony Health. Insects. 2017. 8(2). https://doi.org/10.3390/insects8020048 PMID: 28481294

8. USDA. 2021. 2021 USDA Annual Strategic Pollinator Priorities and Goals Report. Office of the Chief Scientist, U.S. Department of Agriculture. https://www.usda.gov/sites/default/files/documents/pollinator-priorities-2021R4-508-version.pdf

9. vanEngelsdorp D, Hayes J Jr., Underwood RM, Pettis J. A survey of honey bee losses in the U.S., Fall 2007 to Spring 2008. PLOS ONE. 2008; 3(12): e4071. https://doi.org/10.1371/journal.pone.0004071 PMID: 19115015

10. Johnson RM. Honey Bee Toxicology. Ann Rev of Entomology. 2015; 60(1), 415–434. https://doi.org/10.1146/annurev-ento-011613-162005 PMID: 25341092

11. Benuszak J, Laurent M, Chauzat M-P. The exposure of honey bees (Apis mellifera; Hymenoptera: Apidae) to pesticides: room for improvement in research. Sci Total Environ. 2017; 587–588:423–438. https://doi.org/10.1016/j.scitotenv.2017.02.062 PMID: 28256316

12. USEPA. 2016. Office of Pesticide Programs, U.S. Environmental Protection Agency. Guidance on exposure and effects testing for assessing risks to bees. Washington, D.C. https://www.epa.gov/sites/production/files/2016-07/documents/guidance-exposure-effects-testing-assessing-risks-bees.pdf

13. USEPA. 2014. US Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention Office of Pesticide Programs, Guidance for assessing pesticide risks to bees. United States Environmental Protection Agency, Washington, DC. https://www.epa.gov/sites/production/files/2014-06/documents/pollinator_risk_assessment_guidance_06_19_14.pdf

14. USEPA. 2012. U.S. Environmental Protection Agency, “Honey Bee Acute Contact Toxicity” Ecological Effects Test Guidelines OCSP5 850.3020. EPA 712-C-019 Web: http://www.epa.gov/test-guidelines/pesticides-and-toxic-substances/series-850-ecological-effects-test-guidelines

15. OECD. 1998b. Test No. 214: Honeybees, acute contact toxicity test, OECD guidelines for the testing of chemicals, Section 2. OECD Publishing, Paris, FR.

16. OECD. 1998a. Test No. 213: Honeybees, acute oral toxicity test, OECD guidelines for the testing of chemicals, Section 2. OECD Publishing, Paris, FR.
17. OECD. 2017. Test No. 245: Honey Bee (Apis mellifera) Chronic Oral Toxicity Test (10-Day Feeding), OECD Guidelines for the Testing of Chemicals, Section 2. OECD Publishing, Paris, FR.
18. OECD. 2013. Test No. 237: Honey Bee (Apis mellifera) Larval Toxicity Test, Single Exposure, OECD Guidelines for the Testing of Chemicals, Section 2. OECD Publishing, Paris, FR.
19. OECD. 2016. Guidance Document on Honey Bee (Apis mellifera) Larval Toxicity Test, Repeat Exposure. OECD Publishing, Paris, FR. https://one.oecd.org/document/ENV/JM/MONO(2016)34/en/pdf
20. OECD. 2014. Guidance document on the honey bee (Apis mellifera L.) brood test under semi-field conditions. Series on Testing and Assessment No. 75. ENV/JM/MONO(2007)22.
21. USEPA. 2012c. “Field Testing for Pollinators.” Ecological Effects Test Guidelines OCSPP 850.3040. EPA 712-C-017. Web. http://www.epa.gov/test-guidelines-pesticides-and-toxic-substances/series-850-ecological-effects-test-guidelines
22. Höfer T, Gerner I, Gundert-Remy U, Schulte A, Spielmann H, et al. Animal testing and alternative approaches for the human health assessment under the proposed new European Regulation. Arch. Toxicol. 2004; 78. https://doi.org/10.1007/s00204-004-0577-9 PMID: 15170526
23. Hilton GM, Odenkirchen E, Panger M, Waleko G, Lowit A, Clippinger AJ. Evaluation of the avian acute oral and sub-acute dietary toxicity test for pesticide registration. Regulatory Toxicology and Pharmacology. 2019; 105: 30–35. https://doi.org/10.1016/j.yrtph.2019.03.013 PMID: 30922892
24. OECD. 2005. Guidance document on the validation and international acceptance of new or updated test methods for hazard assessment. Paris, France. http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2005)14&doclanguage=en
25. Russom, CL. USEPA Pesticide Acute MOA Database: Overview of Procedures used in compiling the database and summary of results. 2013. Internal Document. US EPA ORD-Duluth.
26. USEPA. 2017. U.S. Environmental Protection Agency. Technical overview of ecological risk assessment—Analysis phase: Ecological effects characterization. In: USEPA. https://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/technical-overview-ecological-risk-assessment-0
27. Sappington K. Use of unbounded toxicity endpoints in ecological risk assessment. 2013. Integr Environ Assess Manag. 9 (4): 676–677. https://doi.org/10.1002/ieam.1464
28. Raimondo S, Montague BJ, Barron MG. Determinants of variability in acute to chronic toxicity ratios for aquatic invertebrates and fish. Environ Toxicol Chem. 2007; 26(9): 2019–2023. https://doi.org/10.1897/07-068R.1 PMID: 17705662
29. Tavares DA, Rato TC, Carvalho SM, Silva-Zacarin ECM, Malaspina O. In vitro effects of thiamethoxam on larvae of Africanized honey bee Apis mellifera (Hymenoptera: Apidae). Chemosphere. 2015; Vol 135, p 370–378. ISSN 0045-6535, https://doi.org/10.1016/j.chemosphere.2015.04.090 PMID: 25985214
30. Stabentheiner A, Kovac H, Brodshneider R. Honey bee colony thermoregulation—regulatory mechanisms and contribution of individuals in dependence on age, location and thermal stress. PLoS ONE. 2010; 5(1): e9807. https://doi.org/10.1371/journal.pone.0009807 PMID: 20126462
31. Walton A, Dolezal AG, Bakken MA, Toth AL. Hungry for the queen: honey bee nutritional environment affects worker pheromone response in a life-stage dependent manner. Funct Ecol. 2018. https://doi.org/10.1111/1365-2435.12322
32. Wu JY, Anelli CM, Sheppard WS. Sub-lethal effects of pesticide residues in brood comb on worker honey bee (Apis mellifera) development and longevity. PLoS ONE. 2011. https://doi.org/10.1371/journal.pone.0014720 PMID: 21373182
33. Spruill SE, O’Neill BF, Hinarojos S, Cabrera AR. A comparison of acute toxicity endpoints for adult honey bees with technical grade active ingredients and typical end-use products as test substance. Journal of Economic Entomology, 2020; 113(2), 1015–1017. https://doi.org/10.1093/jee/toz305 PMID: 31756247