Chronic toxicity of clothianidin, imidacloprid, chlorpyrifos, and dimethoate to *Apis mellifera L.* larvae reared in *vitro*

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Abstract

BACKGROUND: The effects of chronic exposure to two neonicotinoids (clothianidin and imidacloprid) and two organophosphates (chlorpyrifos and dimethoate) on survival, developmental rate and larval weight of honey bee larvae reared in *vitro* were determined. Diets containing chemicals were fed to larvae with the range of concentrations for each compound based on published acute toxicity experiments and residues found in pollen and nectar, both components of the larval diet.

RESULTS: Four concentrations of each compound and controls were tested: chlorpyrifos: 0.5, 0.8, 1.2, 8 mg/L; clothianidin: 0.1, 0.4, 2, 10 mg/L; dimethoate: 0.02, 1, 6, 45 mg/L; imidacloprid: 0.4, 2, 4, 10 mg/L; positive control: dimethoate (45 mg/L); solvent control: acetone or methanol; and negative control. A significant decrease in survival, relative to the solvent control, occurred in the 0.8, 1.2 and 8 mg/L chlorpyrifos, 0.4, 2 and 10 mg/L clothianidin, and 45 mg/L dimethoate diets, but not the imidacloprid diets. CONCLUSION: The treatment of larval diets with clothianidin, dimethoate and imidacloprid did not affect survival, developmental rate, or weight of immature honey bees; however, treatment with chlorpyrifos did. Overall, our results are valuable for evaluating the chronic toxicity of these pesticides to developing honey bees.

Keywords: *Apis mellifera*; larvae; chlorpyrifos; clothianidin; dimethoate; imidacloprid

1 INTRODUCTION

The western honey bee (*Apis mellifera* L.) is considered one of the most important pollinators, not only for agricultural crops, but also for wild plants worldwide.[1-3] Honey bee populations have been declining in regions of Europe and North America[4,5] and this has caused great alarm among beekeepers and crop producers. Multiple factors have been attributed to this decline, factors including parasites, pathogens, poor nutrition, queen failure, habitat loss, migratory stress, and pesticides.[6-8] Among these factors, the potential impact of pesticides, particularly those applied in agricultural settings, are of particular interest to us.

Neonicotinoid pesticides are among the most used insecticides in the world.[9] Neonicotinoid insecticides, including acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, thiacloprid, and thiamethoxam, act as agonists of the nicotinic acetylcholine receptor (nAChRs) of insects.[10] Neonicotinoids are applied foliarly, as a seed coating or by root drench application,[11,12] and may translocate to pollen and nectar through the xylem in growing plants.[13] Thus, honey bees could be exposed inadvertently to the compounds when foraging for nectar and pollen.[14-16] Moreover, residues of neonicotinoids in water resources could be another potential oral exposure route for honey bees.[17]

Although concentrations of neonicotinoids in bee food sources are typically low,[16] low doses of neonicotinoids can induce sublethal effects on honey bees.[18-21] Studies conducted under laboratory, semi-field and field conditions showed that exposure to imidacloprid can affect learning and memory,[22,23] foraging activity,[18,23-25]

feeding behavior,[26] bee communication by dances,[27] homing behavior,[24,28] hypopharyngeal glands and respiratory function[29] and queen fecundity.[30] Conversely, there are fewer studies reporting the effects of clothianidin on honey bees, though impacts on the survival and behavior of winter bees have been noted.[31]

Organophosphorus insecticides (OPs) are also one of the most widely used classes of insecticides. These compounds act on the nervous system of insects by inhibiting acetylcholinesterase. Residues of OPs have been detected in colony matrices,[32] and their potential hazard to colonies has been noted.[33-35] Chlorpyrifos is an OP pesticide used foliarly in crop management[36] Chlorpyrifos has a relatively high toxicity to bees compared to other pesticides,[37,38] and sublethal doses may threaten the success and survival of honey bees.[39] Dimethoate, another OP, is often used foliarly in the field to control crop pests, and it is most commonly used as a positive control in toxicity tests due to its high toxicity to honey bees.[40]

Worker honey bees can forage up to 12 km around their hive[41] and, therefore, are frequently exposed to a variety of pesticide residues present in water, nectar, pollen, or propolis.[32,37] Additionally, pesticides and corresponding metabolites are brought back to the hive and stored in hive matrices. Honey bee larvae may be exposed to an accumulation of these chemical residues via diet,[42] although residue levels in brood food have not been well studied. That said, pesticide residues have been reported in pollen and nectar, including residues of pesticides we tested in the current study. Pollen and nectar are components of larval diet.

To date, only a few studies have been conducted to assess the effect of

pesticides on honey bee larvae.[43-46] Chronic exposure to pesticides impact the survival and development of bee brood.[47] Conceivably, impacts on the larval phase could lead to weakening of the colony structure over time. The toxicity of neonicotinoids and organophosphorus on adult workers is well investigated, but little is known about their toxicity to larvae.

A difficult challenge facing those investigating honey bee toxicology lies in determining if sublethal effects of pesticides on brood play a role in colony loss in field conditions. Risk assessments contributing to this determination should be based on the probability of exposure to actual residue levels. We used various concentrations of our test pesticides (chlorpyrifos, dimethoate, imidacloprid, clothianidin) chosen based on published acute toxicity experiments and/or found in pollen and nectar. The latter were chosen in order to mimic realistic exposure scenarios of honey bee larvae to diet containing treated pollen and/or nectar. In a previous study, we determined as a first step the acute toxicity of these pesticides in treated food to larvae reared *in vitro*.[45] The objective of the present study was to assess the chronic effects of two neonicotinoids and two organophosphorus pesticides to honey bee brood reared *in vitro* at different exposure concentrations according to residue levels found in pollen/nectar (see Table 1) and from our previous acute toxicity test.[45]

2 MATERIALS AND METHODS

2.1 Insecticides

All test substances were purchased from Chem Service, Inc. (660 Tower Lane

West Chester, PA 19380, United States). The name, product number, purity, and expiration of each test substance were as follows: 1) chlorpyrifos: N-11459-250MG, purity 99.5%, expiration 8/31/2018; 2) clothianidin: N-11493-100MG, purity 99.5%, expiration 1/31/2020; 3) dimethoate: N-11758-100MG, purity 99.4%, expiration 7/31/2017; 4) imidacloprid: N-12206-500MG, purity 99.5%, expiration 2/28/2020.

2.2 Honey bee larvae reared *in vitro*

All honey bees were obtained from five healthy colonies at an apiary managed at the Honey Bee Research and Extension Laboratory, Entomology and Nematology Department, University of Florida (Gainesville, FL, USA) during May − July, 2016. The colonies were of mixed race, European-derived stock, housed in standard Langstroth-style equipment, and managed per common best management pratices for the region (including feeding when necessary, managing diseases/pests, etc.). Honey bee larvae were reared *in vitro* according to Schmehl et al.[48]

Our discussion of the *in vitro* timeline corresponds to Schmehl et al.'s Table 3, column 3, where we discuss all timepoints from grafting as day D = 0 or D0 (grafted larvae are 87 ± 12 h old at this timepoint, and this includes the egg stage). Honey bee queens were caged on a wax comb (D-4) for 24 hours to lay eggs. At D0 (75 h after the queens were released), the resulting larvae were transported to a sterile laboratory environment for grafting. The larvae were transferred from the comb to sterile, 48-well tissue culture plates (STCPs) with 20 μL of diet A (royal jelly 44.25%, glucose 5.3%, fructose 5.3%, yeast extract 0.9% and water 44.25%) prepared in each well. The STCPs then were placed horizontally in a larval growth chamber maintained

at 94% R.H. and 35°C. On D2 (48 h after grafting), each larva was fed 20 µL of diet B (royal jelly 42.95%, glucose 6.4%, fructose 6.4%, yeast extract 1.3 % and water 42.95%). On D3, 4 and 5, each larva was fed 30 µL, 40 µL and 50 µL, respectively, of diet C (royal jelly 50%, glucose 9%, fructose 9%, yeast extract 2% and water 30%). Larvae were transferred from the larval STCP to the prepared pupal STCP when all available diet had been consumed (as early as D6). Pupal STCPs were maintained at 75% R.H. and 35°C. Adult worker bees began to eclose as soon 18 days after grafting. Emerging adults were collected at least twice daily and were maintained in hoarding cages with *ad libitum* access to pollen and 50% sugar water solution (w/v).[48]

2.3 Experimental design

The reported residue levels of the pesticides of interest found in pollen and nectar in honey bee colonies are incredibly broad. Therefore, we tested a wide range of concentrations for each compound based on residues found in pollen and nectar[32,38] and the acute toxicity data from our previous study.[45] The concentrations of each substance are listed in Table 1. Chlorpyrifos, dimethoate and imidacloprid were dissolved in methanol to prepare stock solution and clothianidin was dissolved in acetone. The solvent accounted for 0.5% of the volume in final diets.

Table 1 The concentrations of each test substance relative to the residues reported in pollen/beebread or nectar/honey.

Pesticides	Concentrations (mg/L)	Pollen/Beebread (References)	Nectar/Honey (References)
Chlorpyrifos	0.5	10 × mean residue in pollen[32]	30 × maximum residue in nectar/honey[38]
	0.8	maximum residue in pollen[32]	50 × maximum residue in nectar/honey[38]

	1.2	LC5, 1/10th LC50[45]	80 × maximum residue in nectar/honey[38]
	8	10 × maximum residue in pollen[32]	500 × maximum residue in nectar/honey[38]
Clothianidin	0.1	10 × mean residue in pollen[38]	10 × maximum residue in nectar/honey[38]
	0.4	10 × maximum residue in pollen[38]	40 × maximum residue in nectar/honey[38]
	2	50 × maximum residue in pollen[38]	200 × maximum residue in nectar/honey[38]
	10	250 × maximum residue in pollen[38]	1000 × maximum residue in nectar/honey[38]
Dimethoate	0.02	10 × mean residue in pollen[38]	4 × mean residue in nectar/honey[38]
	1	200 × maximum residue in pollen[38]	115 × maximum residue in nectar/honey[38]
	6	1/10th LC50[49]	690 × maximum residue in nectar/honey[38]
	45	positive control used by OECD[40]	5000 × maximum residue in nectar/honey[38]
Imidacloprid	0.4	10 × mean residue in pollen[32]	5 × maximum residue in nectar/honey[38]
	2	10 × maximum residue in pollen[32]	25 × maximum residue in nectar/honey[38]
	4	LC5[45]	50 × maximum residue in nectar/honey[38]
	10	1/10th LC50[45]	125 × maximum residue in nectar/honey[38]

The following treatments were conducted for each test solution: four concentrations of each pesticide, negative control, solvent control, and 45 mg/L dimethoate (positive control). Five replicates were conducted for each treatment using larvae from five different source colonies. Additional plates of honey bees reared *in vitro* were used at D2 to replace the larvae that died before they had started consuming the diet containing the pesticide (< 5% mortality at this point). On D2, a

minimum of twelve robust larvae per replicate were randomly selected and fed 20 μL of the diet B containing the test substance of their designated treatment group. Larvae were fed 30 μL, 40 μL, and 50 μL of Diet C containing the test solution designated to their assigned treatment group on D3, 4, and 5 respectively.

2.4 Endpoints

Larval survival was assessed daily by viewing larvae under a dissecting microscope. Larvae were considered dead if there was no spiracle movement. Pupal survival was monitored daily by visual inspection of the pupae. Dead pupae were recognized by occasional black or white sub-dermal necrotic stains or visible wilting. Any larvae or pupae determined to be dead were removed from the STCPs. Percent survival rates were calculated as follows:

Larval survival = (# larvae that reached D9/# larvae D2) $\times$ 100

Pupal survival = (# adults that eclosed/#larvae that reached D9) $\times$ 100

Total survival = (# adults that eclosed/#larvae D2) $\times$ 100.

The developmental rate was calculated for each treatment group as follows:

Larval developmental rate = date of pupation initiation – grafting date

Pupal developmental rate = emergence date – date of pupation initiation

Total developmental rate = emergence date – grafting date

Larval weight at D6 was recorded (Mettler Toledo, Model #: AL204, Columbus, OH, USA) for each larva immediately prior to transfer to the pupal STCP. Larval weight was calculated as follows:

Larval weight at D6 = weight of larval cell cup with larva – weight of empty larval

cell cup).

A risk quotient (RQ) is the ratio of the potential exposure to a substance and the level at which no adverse effects are expected. The US EPA created a tool called "BeeREX" to determine the risk a compound poses to honey bees based on the no observable adverse effect dose (NOAED) (https://www.epa.gov/sites/production/files/2015-11/beerexv1.0.xlsx). RQ values are then compared to levels of concern (LOCs). The LOC for chronic exposure is 1.0. Any number in the results table that is under 1.0 means that the compound poses little or no risk to bees. If it is ≥1, then the compound may require higher-tiered testing (*e.g.* semi-field tunnel studies) or label mitigation.[50] Larval NOAED was calculated as follows:

Larval NOAED = NOAEC (no observed adverse effect concentration) × cumulative consumption of diet

2.5 Statistics analysis

Statistical analyses were performed using the SAS 9.2 software program (USA).[51] Overall survival curves were compared for each compound using a Kaplan-Meier survival analysis. Survival endpoints (larval survival, pupal survival, and total survial) were determined using ANOVA and Tukey's HSD tests. The survival data were normalized with an arcsine-square root transformation of proportions prior to the ANOVA analysis. ANOVA and Tukey's HSD tests were used to compare developmental rates (larval, pupal and total) and larval weight among the experimental groups. We used the U.S. Environmental Protection Agency's BeeREX

model to calculate risk quotients (RQs) for all test compounds (https://www.epa.gov/sites/production/files/2015-11/beerexv1.0.xlsx).

3 RESULTS

3.1 Chlorpyrifos

Overall survival of larvae fed 0.5 mg/L chlorpyrifos was not significantly different from that of larvae fed diet containing the solvent control, but was significantly lower from those fed the negative control diet (Table S1, Fig. 1A). Overall survivals of larvae fed 0.8, 1.2 or 8 mg/L chlorpyrifos were all significantly lower than those of larvae fed the negative and solvent control diets (Table S1, Fig. 1A). A one-way ANOVA was conducted to evaluate for differences between treatment groups in the chlorpyrifos study for larval survival ($F_{34} = 52.45$, $p < 0.0001$), pupal survival ($F_{34} = 18.15$, $p < 0.0001$), and total survival ($F_{34} = 45.15$, $p < 0.0001$). Larval survival was unaffected in individuals fed 0.5 mg/L chlorpyrifos though it was significantly lower than that of the negative and solvent controls for individuals fed 0.8, 1.2 and 8 mg/L chlorpyrifos (Fig. 2A). The highest chlorpyrifos concentration (8 mg/L) affected pupal survival compared to that of the solvent and negative controls (Fig. 2A). Total survival of larvae fed 0.8, 1.2 and 8 mg/L chlorpyrifos was significantly lower than that of the negative or solvent controls (Table S1, Fig. 2A). NOAEC (no observed adverse effect concentration) of chlorpyrifos was 0.5 mg/L.

A one-way ANOVA indicated differences between treatment groups in the chlorpyrifos study for larval developmental rate ($F_{187} = 4.651$, $p = 0.0005$) and pupal rate ($F_{187} = 2.993$, $p = 0.0127$), but not total rate ($F_{187} = 1.617$, $p = 0.1570$). Despite

this, no meaningful patterns (i.e. no predictable dose responses) are discernable (Fig. S1). Larval weight was not affected by chlorpyrifos relative to any of the other treatment groups ($F_{193} = 1.52$, $p = 0.1847$, Fig. S2).

3.2 Clothianidin

Overall survival was lower for larvae fed a diet with 0.4, 2 or 10 mg/L clothianidin than for larvae fed negative and solvent control diets, and for those fed a diet with 0.1 mg/L compared to those fed the negative control, but not solvent control, diet (Table S2, Fig. 1B). A one-way ANOVA indicated differences between treatment groups in the clothianidin study for larval survival ($F_{34} = 14.14$, $p < 0.0001$) and total survival ($F_{34} = 13.13$, $p < 0.0001$), but not pupal survival ($F_{34} = 2.48$, $p = 0.0565$). Larval and total survival was significantly lower for larvae fed a diet with 10 mg/L clothianidin than for larvae fed negative and solvent control diets (Fig. 2B). That effect was not seen for pupal survival (Fig. 2B). NOAEC of clothianidin was 0.1 mg/L.

A one-way ANOVA indicated differences between treatment groups in the clothianidin study for larval developmental rate ($F_{198} = 7.673$, $p < 0.0001$) and total developmental rate ($F_{198} = 7.760$, $p < 0.0001$), but not pupal developmental rate ($F_{198} = 1.436$, $p = 0.20270$). Larval and total developmental rates were significantly longer for individuals fed 2 or 10 mg/L clothianidin diets than for larvae fed negative and solvent control diets. The total developemental rate was significantly longer for individuals fed 2 mg/L clothianidin diet than for larvae fed negative and solvent control diets (Fig. S3). Larval weight was not affected by clothianidin relative to any

of the other treatment groups ($F_{197} = 1.40$, $p = 0.2163$, Fig. S2).

3.3 Dimethoate

Larvae fed the lowest concentration of dimethoate (0.02 mg/L) had significantly lower overall survival than those fed the negative control but not solvent control (Table S3, Fig. 1C) diets. There were no significant differences between the overall survival of larvae fed 1 mg/L or 6 mg/L dimethoate and those fed negative control or solvent control diets (Table S3, Fig. 1C). Conversely, larvae that were fed the highest concentration of dimethoate (45 mg/L) had significantly lower overall survival than those fed the negative control and solvent control diets (Table S3, Fig. 1C). A one-way ANOVA indicated differences between treatment groups in the dimethoate study for larval survival ($F_{29} = 32.22$, $p < 0.0001$), pupal survival ($F_{34} = 735.38$, $p < 0.0001$) and total survival ($F_{34} = 29.07$, $p < 0.0001$), but all were due to higher mortality in the positive controls than in any other treatment group (Fig. 2C). NOAEC of dimethoate was 6 mg/L.

A one-way ANOVA indicated differences between treatment groups in the dimethoate study for larval developmental rate ($F_{244} = 4.829$, $p = 0.0003$) and pupal developmental rate ($F_{244} = 3.1570$, $p = 0.0088$), but not total developmental rate ($F_{244} = 1.061$, $p = 0.3830$). Despite this, no meaningful patterns are discernable (Fig. S4). Larval weight was not affected by dimethoate relative to any of the other treatment groups ($F_{254} = 1.49$, P = 0.1935, Fig. S2).

3.4 Imidacloprid

Larvae fed 0.4 mg/L and 10 mg/L imidacloprid diet did not have different overall

survival curves than did larvae fed the negative or solvent control diets (Table S4). However, the overall survival of larvae fed a diet containing 2 mg/L or 4 mg/L imidacloprid was significantly lower than that of the larvae fed the negative but not solvent control diets (Table S4). NOAEC of imidacloprid was 10 mg/L.

A one-way ANOVA indicated differences between treatment groups in the imidacloprid study for larval survival (F_{34} = 14.61, $p < 0.0001$) and total survival (F_{34} = 14.72, $p < 0.0001$), but not pupal survival (F_{34} = 1.50, $p = 0.2253$). These differences were a result of mortality in the positive control group (Fig. 2D).

A one-way ANOVA indicated differences between treatment groups in the imidacloprid study for larval developmental rate (F_{228} = 11.312, $p < 0.0001$), pupal developmental rate (F_{228} = 2.253, $p = 0.0393$) and total developmental rate (F_{228} = 1.061, $p = 0.0011$). Larvae fed 10 mg/L imidacloprid took longer to develop than did larvae fed negative and solvent control diets (Fig. S5A). No discernable patterns were seen for the other groups (Fig. S5B, S5C). Larval weight was not affected by imidacloprid relative to any of the other treatment groups (F_{239} = 0.96, $p = 0.4549$, Fig. S2).

3.5 Calculating Risk Quotients

We used our data to determine no observed adverse effect doses (NOAED) and then the RQs for each compound (Table 2). The RQs for each compound were below the RQ of 1.0, the accepted Level of Concern (LOC) for chronic tests.

Table 2 Risk Quotient (RQ) analysis. The RQs were calculated using BeeREX from the United States Environmental Protection Agency

(https://www.epa.gov/sites/production/files/2015-11/beerexv1.0.xlsx)[1]. Maximum

residues in pollen/bee bread or in nectar are derived from the literature.[32,38] Larval

NOAED values are derived from our data.

	Maximum residue reported in pollen/bee bread (mg a.i./kg[2])	Maximum residue reported in nectar (mg a.i./kg)	NOAEC[3] (µg/mL)	Cumulative consumption of diet D2 – D5 (mL)	Larval NOAED[4] (µg a.i./larva)	RQs (chronic dietary)
chlorpyrifos	0.83	0.015	0.5	0.14	0.07	0.10
clothianidin	0.0412	0.01	0.1	0.14	0.014	0.14
dimethoate	0.0042	0.0087	6	0.14	0.84	0
imidacloprid	0.912	0.0728	10	0.14	1.4	0.01

[1]The BeeREX model we used was modified in order to calculate the RQ based upon cumulative dose. The

RQs were calculated based upon the cumulative amounts of nectar and pollen that are consumed during the

worker larval phase of development according to BeeREX.

[2]a.i. = active ingredient

[3]no observed adverse effect concentration

[4]no observed adverse effect dose

4 DISCUSSION

Honey bees are significant contributors to pollination services in many

agricultural systems world-wide.[2] As such, they are inadvertently exposed to

pesticides used in crop production. While the effects of pesticides on adult honey bees has been well studied, the effects on immature bees have been largely overlooked. Honey bee larvae can become exposed to a wide range of pesticides via their diet which includes pollen and honey, both of which have been shown to have pesticide residues. It is possible that exposure to pesticides as immatures can impact colony health and development adversely. Here, we focused on the chronic toxicity of chlorpyrifos, clothianidin, dimethoate and imidacloprid to honey bee larvae reared *in vitro*.

Laboratory bioassays play an important role in assessing the risk of pesticide exposures, as many factors may be different under field conditions. Field-level experiments can be biased by many uncontrolled factors such as weather, pests and management, but the rearing of honey bee larvae *in vitro* allows for more controlled experiments.[51] *In vitro* rearing honey bee larvae protocols useful for toxicity tests have been published and improved through time.[52-54] We used the latest *in vitro* rearing larvae protocol by Schmehl et al.[48] to determine the impacts of our test compounds on developing bees. This methodology has helped to standardize *in vitro* rearing bioassays and facilitate ecotoxicological studies on honey bees, as demonstrated by this study.

Previous studies have demonstrated a high acute oral toxicity of clothianidin to honey bees,[9,11,55] but chronic oral exposure at relatively low levels has induced only slight reductions of sucrose responsiveness.[31] We observed that at $10\times$ the mean residue found in pollen and $10\times$ the maximum residue in nectar/honey (0.1 mg/L),

clothianidin did not affect bee survival or development. However, it did decrease survival at higher concentrations (0.4, 2 or 10 mg/L) of clothianidin. That said, larval survival and total survival were only statistically lower than that for the negative and solvent controls at the highest clothianidin concentration (10 mg/L). Based on the maximum residue level of clothianidin found in pollen (41.2 μg/kg)[37,38] and our calculated RQ for this compound, the contamination of larval diet by field-relevant clothianidin concentrations is unlikely to affect the overall survival of immature honey bees at the residue levels reported in the literature thus far.

There has been a tremendous amount of work that has been conducted to evaluate the toxicity of imidacloprid to bees, especially regarding possible sublethal effects.[9,28,56-58] However, there is still some uncertainty regarding its effect on honey bee health due to inconsistent results[59] or tests including test concentrations higher than those honey bees reasonably can encounter in the field. Field level studies find little convincing evidence that imidacloprid causes a direct mortality to bees at concentrations detected in the environment,[60] but some recent evidence suggests that imidacloprid exposure at 0.25 ppb in nectar for a 6-week duration affects the overwintering survival of colonies.[61] Work conducted by Yang et al. demonstrates that the toxic effect of imidacloprid at low doses may have a harmful affect on larvae[62], but our observations and calculated RQ showed that the four concentrations of imidacloprid we tested did not affect the survival of immature bees within the parameters of our study. Thus, imidacloprid's possible contributions to honey bee colony losses are likely to remain controversial.

The LD$_{50}$ of dimethoate ranges between 1.67–1.9 μg/larva (LD$_{50}$ of 1.9 μg/larva 48 h after oral exposure of larvae to the compound on D4[51]; LD$_{50}$ of 1.67 μg/larva 48 h after oral exposure as second instar larvae[52]). In our study, we exposed larvae to diets with concentrations of 0.02, 1 and 6 mg/L of dimethoate and only the 0.02 dosage had a significant effect. The lack of concentration-dependence indicates an unusual solvent interaction at this dose, or more likely, a spurious result. We did see decreased survival relative to that of the controls at 45 mg/L dimethoate, thus supporting the idea that this concentration is appropriate to use as a positive control in chronic toxicity tests of pesticide impacts on honey bee brood. Based on the maximum residue level of dimethoate found in pollen (4.2 μg/kg) or in honey/nectar (8.7 μg/kg)[38] and our calculated RQ, dimethoate is unlikely to result in the outright mortality of developing honey bees at the residue levels reported in the literature to date.

Chlorpyrifos has been observed to have profound sublethal effects on the specificity of appetitive olfactory-mediated memory[39,63] and navigation[28] as well as cause a significant increase in larval mortality[44] in treated honey bees. Similarly, we observed a decrease in survival to adulthood of larvae fed 0.8, 1.2 and 8 mg/L chlorpyrifos diets. Chlorpyrifos at 0.8 mg/L is similar to the maximum concentration found in pollen and is 50× higher than the maximum residue in nectar/honey.[32,37,38] Thus, low concentrations of chlorpyrifos in the hive could threaten the health of honey bee colonies. Nevertheless, our study likely represents a worst-case scenario given that our calculated RQ suggests that this compound does not impact

developing bee survival at reported exposure levels.

We observed interesting differences between the chronic and acute exposures found by Dai et al.[45] and in our current work. Chlorpyrifos at 1.2 mg/L, dimethoate at 6mg/L and imidacloprid at 10 mg/L are all similar to $1/10^{th}$ the LC_{50} reported for each by Dai et al.[45] At these concentrations, chlorpyrifos affected survival while imidicloprid only delayed larval development. Interestingly, 6 mg/L of dimethoate did not affect bee survival or development. It is, therefore, plausible that differences between acute and chronic intoxication could vary depending on the variations in the structure and efficacy of pesticides.[31] Furthermore, we saw a lack of dose-response effects for some of the componds at the lower test concentrations [i.e. 0.1 mg/L clothianidin, 1 mg/L dimethoate, and 10 mg/L imidacloprid - Figure 1]. However, this can happen when the test concentrations are lower than the NOAEC values.

Our data collectively suggest that most of the death due to pesticide exposure would happen in the larval stage rather than in the pupal stage for the compounds we tested. This was the case for each compound that impacted larval survival in our study. None of the test compounds affected any developmental rate or larval weight predictably at the test concentrations. In our study, weight and developmental rate were uninformative end-points and failed to provide any toxicological insight beyond that provided by survival.

Our data show that the survivorship (survival to eclosion as adults) was reduced for larvae fed 0.8 mg/L, 1.2 mg/L, or 8 mg/L chlorpyrifos; 0.4 mg/L, 2 mg/L or 10 mg/L clothianidin; and 45 mg/L dimethoate diets. Our tests represent a likely

worse-case scenario exposure of larvae to these compounds around residue levels seen in pollen or honey/nectar which compose part of the total volume of brood food. It seems unlikely that the pesticides levels found in brood food will approach the maximum residues found in pollen or honey/nectar under normal environmental conditions. The RQs we calculated were below the LOCs and suggest that none of the test compounds affect immature bee survival to adulthood at the NOAEDs we determined using our data. This does not preclude the possible existence of any sublethal impacts these compounds may have on developing bees. Furthermore, our discussion on the toxicity of these compounds rests on the assumption that the label will always be followed when the compounds are used. However, off-label uses of the compounds, such as applying them to flowers when in bloom, can present a unique risk to bees and other pollinators. Nevertheless, our data highlight the importance of considering risk when estimating the impact of a compound on developing honey bees.

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Figure legends
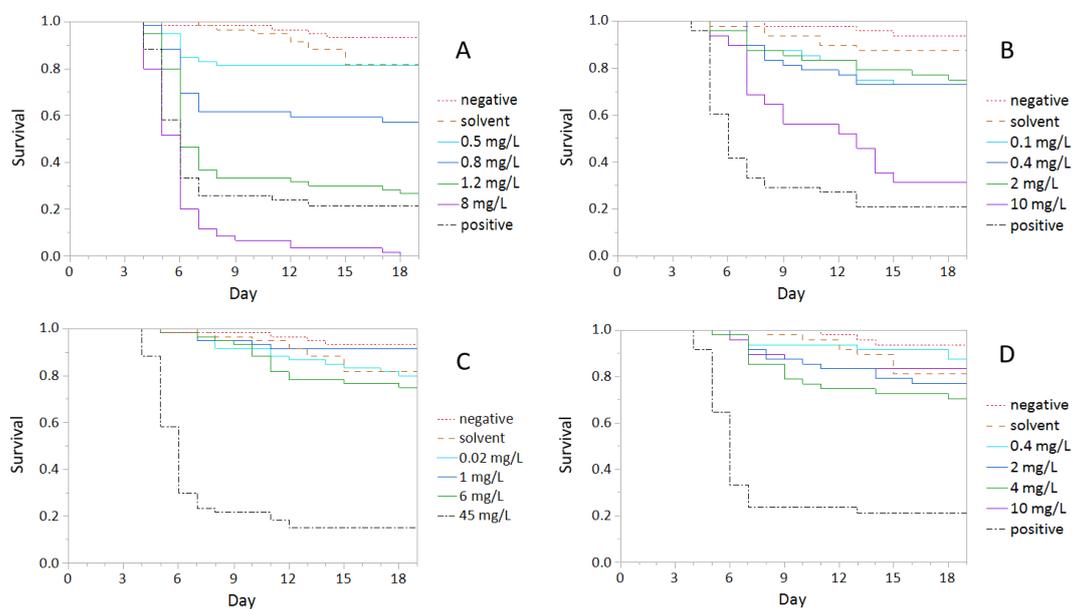
Fig. 1. Overall survival of *Apis mellifera* exposed to concentrations of chlorpyrifos (A), clothianidin (B), dimethoate (C) or imidacloprid (D) during larval development on D2 thru D5 after grafting (N = 5 replicates of 12 larvae/replicate, or 60 larvae, per test substance). Larvae were fed a dimethoate-contaminated diet (45 mg/L) as a positive control, methanol- or acetone-contaminated diet as a solvent control, and no contaminated diet as a negative control. D18 on the figures corresponds to D21 from egg laying to adult emergence for the honey bee (see Schmehl et al. 2016 Table 3, columns 1 and 3. Data analysis corresponds to Table S1-S4.
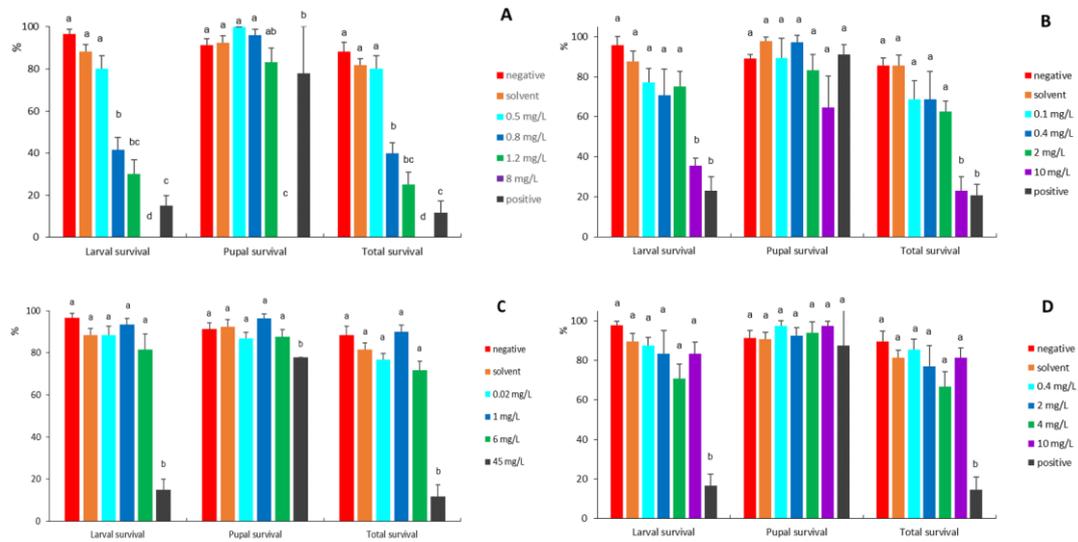
Fig. 2. Percent larval survival, pupal survival, and total survival (mean ± SE) of honey bees exposed to chlorpyrifos (A), clothianidin (B), dimethoate (C) or imidacloprid (D) at D2, D3, D4 and D5 after grafting. Larvae were fed a dimethoate-contaminated diet (45 mg/L) as a positive control, methanol-contaminated diet as a solvent control, and no contaminated diet as a negative control. Bars with the same letter are not different at α≤0.05.