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Concentration- and time-dependent toxicity of commonly encountered pesticides and pesticide mixtures to honeybees (*Apis mellifera* L.)

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HIGHLIGHTS

- Time-dependent toxicities of common pesticides in Israeli beehives were determined.
- Thiacloprid complied with the Haber's rule.
- DMPF, dimethoate and imidacloprid displayed time-diminished-toxicities.
- DMF and acetamiprid revealed time-reinforced-toxicities.
- DMPF and imidacloprid were found to constitute the highest risk to honeybees.

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ABSTRACT

Honeybees are exposed to a wide range of pesticides for long periods via contaminated water, pollen and nectar. Some of those pesticides might constitute health hazards in a time- and dose-dependent manner. Time-dependent toxicity profiles for many applied pesticides are lacking, despite the fact that such profiles are crucial for toxicological evaluations. Therefore, we sought to determine the time-dependent toxicities of pesticides/pesticide metabolites frequently found in Israeli beehives, namely, amitraz metabolites, N'-(2,4-dimethylphenyl)-N-methylformamidine (DMPF) and N-(2,4-dimethylphenyl)-formamide (DMF), coumaphos, imidacloprid, thiacloprid, acetamiprid and dimethoate (toxic reference). By applying accepted methodological approaches such as the modified Haber's rule (product of concentration and exposure duration leads to a constant effect) and comparisons between cumulative doses at different time points, we determined the time-dependent toxicities of these pesticides. We also studied the mixture toxicities of frequently occurring pesticide combinations and estimated their potential contributions to the overall toxicities of neonicotinoids. Thiacloprid was the only pesticide that complied with Haber's rule. DMPF, dimethoate and imidacloprid exhibited time-diminished -toxicities. In contrast, DMF and acetamiprid exhibited time-reinforced toxicities. Neither the binary mixtures nor the tertiary mixtures of DMF, DMPF and coumaphos at 10 times their environmentally relevant concentrations potentiated the neonicotinoids' toxicities. DMPF and imidacloprid were found to present the greatest hazard to honeybees, based on their 50% lethal cumulative dose and 50% lethal time. Amitraz's instability, its low detection frequency and high toxicity profile of its metabolite, DMPF, lead us to the conclusion that DMPF constitutes the actual toxic entity responsible for amitraz's toxic effect.

1. Introduction

Insect-mediated pollination is crucial for sustainable agriculture and for maintaining floral biodiversity (Klein et al., 2007). Honeybees (*Apis mellifera*) are major pollinators, responsible for the

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pollination of about 35% of all food crops worldwide (Aizen et al., 2008). In recent years, a steady decline of honeybee colonies (termed *colony-collapse disorder*) has been reported worldwide. This phenomenon has been attributed to multiple factors such as the parasitic mite *Varroa destructor*, various microbial and viral pathogens, malnutrition, habitat loss, migratory stress and increased pesticide applications (Calatayud-Vernich et al., 2016; Goulson et al., 2015; Ravoet et al., 2015). Certain pesticides/pesticide mixtures are considered to constitute a major threat to the well-being of honeybees, specifically certain neonicotinoid insecticides (imidacloprid, clothianidin and thiamethoxam), fipronil, pyrethroids and mixtures of those insecticides with fungicides capable of inhibiting P450 mono-oxygenases (Blacquière et al., 2012; Codling et al., 2016; Kiljanek et al., 2017). The latter have been found to be responsible for detoxification of many pesticides (Iwasa et al., 2004; Manjon et al., 2018). Honeybees may be exposed to multiple pesticides for extended periods of time and these pesticides may interact additively, synergistically or antagonistically. It has been suggested that these pesticides and their interactions may be a major driver of colony-collapse disorder (Goulson et al., 2015).

Honeybees may be directly exposed to a wide range of pesticides that are applied in agricultural fields during their foraging. They then carry contaminated pollen and nectar back to their hives (Beekman and Ratnieks, 2000; Bromenshenk and Preston, 1986; Porrini et al., 2003; Raeymaekers, 2006). Numerous studies have clearly demonstrated that the combination of accumulated pesticides in beeswax and residual pesticide levels in honey and pollen/beebread may contribute to enhanced mortality and morbidity of honeybees (Bommuraj et al., 2019; Chauzat et al., 2011; Kasiotis et al., 2014; Lambert et al., 2013; Mullin et al., 2010).

The direct application of acaricides within the beehive such as coumaphos and amitraz, intended to mitigate *Varroa* infestations, constitutes an additional stress factor with potential sublethal effects on the honeybees (Boncristiani et al., 2012; Desneux et al., 2007; Johnson, 2015). According to the publically available online records since 2015 up to 2020, coumaphos and amitraz are the only acaricides approved for in-hive application against *Varroa destructor* (Veterinary Product Records). Notwithstanding, in the last ten years coumaphos was not used as varrocide by Israeli beekeepers, due to widespread mite resistance (Shimshoni et al., 2019).

Consequently, coumaphos residues found in Israeli beehive products in recent years, reflect coumaphos tendency to persist and accumulate in beeswax rather than its current use (Kochansky et al., 2001; Shimshoni et al., 2019).

The acaricide coumaphos and the amitraz metabolites N-(2,4-dimethylphenyl)-formamide (DMF) and N'-(2,4-dimethylphenyl)-N-methylformamidine (DMPF) are among the most abundant contaminants in beehive products, due to their high persistence, reflected in a prolonged half-life, and consequently marked accumulation, mostly in beeswax (Bogdanov, 2004; Calatayud-Vernich et al., 2019; Chiesa et al., 2016; Fulton et al., 2019; Mullin et al., 2010; Perugini et al., 2018; Ravoet et al., 2015). In contrast, amitraz is rarely detected in beehive products, due to its rapid hydrolysis/degradation into two major metabolites: DMF and DMPF (Bommuraj et al., 2019). Many agricultural pesticides and acaricides are lipophilic (LogP > 2) and not very volatile, which allows them to easily accumulate in beeswax (Shimshoni et al., 2019). However, the neonicotinoid insecticides are highly hydrophilic, nonvolatile compounds (LogP < 1) and, therefore, are found almost exclusively in honey at low residual levels (below their corresponding maximum residue levels).

Due to their high toxicity to honeybees, the neonicotinoids clothianidin, thiamethoxam and imidacloprid have been banned in the European Union from further use in open agricultural fields, while the US Environmental Protection Agency (US-EPA) has recently enforced restrictions regarding the application of neonicotinoids to certain blooming crops (European Commission; US-EPA). In contrast, in Israel, there is still intensive agricultural use of neonicotinoids, with those chemicals comprising about 25% of the total insecticides used in local agriculture (Shimshoni et al., 2019).

The adverse effects of pesticides on honeybees may vary substantially depending upon the daily dosage, time of exposure, honeybee age (developmental stage) and the concomitant pathogen load (Gerig, 1975; Poquet et al., 2016; Tahori et al., 1969; Vance et al., 2009; Wahl and Ulm, 1983). Most studies of the chronic toxicity of pesticides to honeybees have been conducted on newly emerging honeybees according to OECD guidelines, although within the beehive, the honeybee population is heterogeneous in terms of age and developmental stage (OECD, 2017). Insects and other animals poisoned with lower cumulative doses of certain pesticides such as neonicotinoids do not die immediately, but rather only after prolonged exposure, as opposed to the acute lethality induced by higher cumulative doses over shorter periods of time (Sánchez-Bayo and Tennekes, 2020). The latter phenomenon has been described as time-reinforced cumulative toxicity, occurring after the animal has surpassed a critical level of cellular/tissue damage. Adverse effects are typically reported as lethal doses (LD) in acute- and chronic-exposure tests (e.g., 2-day LD_{50} and 10-day LD_{50}), while the time to various degrees of mortality, such as time to 50% mortality (LT_{50}) at different pesticide concentrations, is rarely reported. Since the duration of exposure is no less important than the dose and, as stated above, might reinforce the toxicity at constant doses, it is of utmost importance to fully characterize the time-dependent toxicity of each pesticide over a wide range of concentrations as clearly stated in EFSA 2013.

According to Haber's rule, the product of the initial pesticide exposure concentration (C) and the duration of exposure (t) leads to a constant toxic effect, which was expanded mathematically to the Druckrey and Küpfmüller equation (see Methods and Materials; Druckrey and Küpfmüller, 1949; Haber, 1924). Numerous studies have evaluated the time-reinforced toxicity of imidacloprid, pyrethroids and fipronil (Holder et al., 2018; Sanchez-Bayo and Goka, 2014; Simon-Delso et al., 2018; Tennekes et al., 2011); whereas a substantial knowledge gap exists regarding the time-dependent toxicity profiles of amitraz metabolites, other neonicotinoids, coumaphos and mixtures of those chemicals. The pesticides studied hereby, were among the most commonly occurring pesticide residues found in Israeli honey samples, which were also commonly found in honey and beeswax samples worldwide (Martinello et al., 2020; Mullin et al., 2015; Shimshoni et al., 2019).

This study had two main objectives. The first objective was to evaluate the chronic and acute oral toxicities of the most commonly detected pesticides in Israeli honey (namely, DMF, DMPF, coumaphos, imidacloprid, thiacloprid and acetamiprid; Bommuraj et al., 2019), as well as dimethoate as toxic reference (Medrzycki et al., 2013) and mixtures of those chemicals over a 10-day period by measuring mortality as a function of duration of exposure and the pesticide concentration/cumulative oral dosage. The second objective was to uncover any underlying time-dependent toxicities by characterizing the time-dependent relationships of each pesticide. Evaluations of the acute and chronic oral toxicities of the major metabolites of amitraz to honeybees are presented here for the first time.

2. Materials and methods

2.1. Reagents

All pesticide standards were purchased from Sigma-Aldrich (St. Louis, MO, USA) at a purity grade above 99%. Stock standard solutions of individual pesticides (100 mg/mL) were prepared in acetonitrile (HPLC grade; Sigma-Aldrich, St. Louis, MO, US) and stored at −20 °C. The working solutions were prepared by carrying out appropriate dilutions of the stock solutions. Sucrose was purchased from Sigma-Aldrich (St. Louis, MO, USA). Double-distilled water (DDW) was obtained using a Milli-Q purification apparatus (Millipore Direct-Q UV, Bedford, MA, USA).

2.2. Working solutions of selected pesticides

Insecticide/acaricide residues with an occurrence rate of more than 15% in honey samples collected during 2018 were selected for inclusion in this time-dependent toxicity study (Bommuraj et al., 2019; Shimshoni et al., 2019). Pesticide working solutions were obtained by diluting the stock solutions (100 mg/mL in acetonitrile) with acetonitrile. Subsequently, 0.2 mL of each working solution were spiked into 10 mL 50% aqueous sucrose solution to yield the desired final concentration of pesticide in 50% sucrose. The solvent concentration in all of the sucrose feeding solutions was kept at a constant 2% (v/v). The blank sucrose solution (negative control) and the positive-control sucrose solution (including dimethoate) also contained 2% acetonitrile (v/v).

2.3. Toxicological study design

Honeybees of various ages and life stages (Supplementary Material, Table S1) were collected between June 2019 and September 2019 from three healthy beehives located at the Agricultural Research Organization, Volcani Center, Israel. Time mortality and the relationship between pesticide concentration and mortality were evaluated following chronic oral administration of the pesticides/pesticide mixtures at a range of concentrations, according to the OECD guidelines (OECD, 2017). Honeybees were collected by opening the beehives and shaking the bees from the frames into a plastic container (0.3 m^3). Subsequently, 30 honeybees were gently vacuumed into each ventilated, transparent 900-mL plastic cage, using a modified vacuum cleaner. Drones and dead bees were removed from the cages before commencing the study. Honeybees of different life stages and ages were used since the purpose of the present study was to evaluate the time concentration−mortality relationship among a demographically representative sample of adults.

Honeybees were kept in the dark in an incubator at 34.5 °C ± 2 °C and 65% ± 20% relative humidity for 10 days. Cages of honeybees were randomly assigned to the various treatments and randomly positioned within the incubator. Honeybees were fed ad libitum with 4.5 mL of 50% sucrose solution spiked with a specific concentration of the pesticide/pesticide mixture, provided through two horizontally fitted feeding vials (5 mL). The feeding vials had two 2.5-mm holes, enabling the honeybees to feed freely. Each of the treatment groups and the negative and the toxic reference groups were composed of three replicates each containing 30 honeybees. The sample size was equal to or exceeded the sample sizes used in comparable previously published studies.

Honeybee mortality was recorded every day and dead honeybees were removed at the same time. Vials containing freshly prepared sucrose solution were replaced every day and weighed just before administration and again the following day to estimate the quantity of sucrose solution consumed. This consumption value was divided by the number of living bees to obtain the consumption per bee per day. The evaporation of sucrose solution was also measured for 24 h in cages and subtracted from the total daily change in sucrose consumption, in order to obtain the daily amount of ingested sucrose solution according to the OECD guideline 245 (OECD 2017).

For the chronic toxicity study, honeybees were fed for 10 consecutive days 50% sucrose solutions containing either imidacloprid (dosages: 0, 0.25, 0.5, 1, 1.25, 1.5 mg/L), thiacloprid (0, 10, 25, 50, 100, 150 mg/L), acetamiprid (0, 10, 25, 50, 100, 150 mg/L), DMPF (0, 5, 10, 20, 30, 40 mg/L), DMF (0, 100, 200, 400, 500, 600 mg/L), coumaphos (0, 10, 25, 50, 75, 100 mg/L) or dimethoate (0.05, 0.1, 0.2, 0.4, 0.6, 0.8 mg/L). Each cage received one of the doses in triplicate. The dosage ranges spanned and exceeded 10−100 times the environmentally realistic concentrations (Shimshoni et al., 2019). In addition, binary and tertiary pesticide combinations were tested for 10 days at 10 times their highest environmentally relevant concentrations detected in Israeli honey/beeswax samples (Bommuraj et al., 2019). The binary mixtures were as follows: DMF (1 mg/L) + DMPF (1 mg/L); DMF (1 mg/L) + coumaphos (10 mg/L) and DMPF (1 mg/L) + coumaphos (10 mg/L). The tertiary mixture consisted of DMF (1 mg/L) + DMPF (1 mg/L) + coumaphos (10 mg/L). Subsequently, a potentiation study was carried out for each of the most commonly occurring neonicotinoids (imidacloprid, thiacloprid and acetamiprid) at the same chronic concentration range indicated above in the presence of the tertiary mixture: DMF (1 mg/L) + DMPF (1 mg/L) + coumaphos (10 mg/L).

To determine the acute toxicities of the individual pesticides, we performed the same procedure described above, namely continuous ad libitum feeding over 48 h. The study was terminated after 48 h and we examined slightly higher concentrations, namely: imidacloprid (dosages: 0, 0.5, 1, 1.25, 1.5, 2, 2.5, 3, 4, 5, 10 mg/L), thiacloprid (0, 50, 100, 150, 200, 250, 400, 500 mg/L), acetamiprid (0, 50, 100, 150, 200, 250, 300, 350, 400, 500, 750 mg/L), DMPF (0, 10, 20, 30, 40, 50, 100 mg/L), DMF (0, 1000, 1100, 1200, 1300, 1400, 1500 mg/L) and coumaphos (0, 25, 50, 75, 100 mg/L).

2.4. Toxicological endpoints

We calculated 3 toxicological endpoints (and their 95% confidence intervals): time to 50% mortality (LT_{50}) at different pesticide concentrations, concentration inflicting 50% mortality (LC_{50}) and cumulative dose inflicting 50% mortality (LCD_{50}). The average lethal cumulative dose per honeybee was calculated by adding up the average daily pesticide doses consumed/day for each day, up to 10 days (obtained from the product: average daily sucrose volume consumed/live bee/day multiplied with the corresponding pesticide concentration in the sucrose solution). Subsequently, the average acute and chronic LCD_{50} values were obtained by log-logistic modelling (see below equation A.3) of the % survivability vs. the LCD obtained for each of the tested pesticide concentrations after 48 h (acute) and 10 days (chronic), respectively. We corrected all of the observed mortalities against the results from the negative control group, using Abbot's equation (Abbot, 1925):

$$\%corrected\ mortality = 100* \frac{mortality\ of\ treatment\ group - control\ group\ mortality}{1 - control\ mortality} \quad (1)$$

All of the negative control groups exhibited a mortality rate between 0 and 3.3% over the course of the entire study. The percent corrected mortality was converted to percent corrected survivability as follows:

$$\%\ corrected\ survivability = 100\ -\ \%corrected\ mortality \quad (2)$$

The toxicological endpoints were determined by fitting the experimental data to the following three-parameter log-logistic model:

$$\%corrected\ survivability = c + \left(\frac{100 - c}{1 + 10\hat{(}\log x - \log z)b}\right) \quad (3)$$

The parameters *c*, *z* and *b* were defined as the lower asymptote (in %), the toxicological endpoint at 50% mortality and the Hill coefficient. The independent variable *x* was defined as the pesticide concentration/cumulative dose or time of exposure in days (Gesztelyi et al., 2012). We fixed this three-parameter sigmoid model to the higher asymptote (i.e., the survivability is 100% at time 0) and the lower asymptote (i.e., the mortality at infinite dose equals 100%). The toxicological endpoints were then calculated relative to these two asymptotes. However, since the toxicological test was terminated after 10 days, only non-extrapolated LT_{50} values were provided, as the honeybee mortality in the negative control group after 10 days was not recorded.

2.5. Cumulative toxicity

The relationship between experimentally determined LCD_{50} and exposure time was fitted to the Druckrey-Küpfmüller equation (Druckrey and Küpfmüller, 1949), as follows:

$$c * t^b = \text{constant} \quad (4)$$

This equation states that the product of the initial exposure concentration/dosage (c) and the duration of exposure (t) to the power of b (>0) is the constant toxic effect.

That power-law relationship was fitted on log-transformed axes and the slope of the relationship (-b) was determined by linear regression, to assess the time-dependent toxicity of each of the examined pesticides. The slope -b on the log (concentration) − log (time) linear regression takes the value −1, if the toxicant follows Haber's rule. However, for a slope < −1, the toxicant exerts time-reinforced toxicity (Bunce and Remillard, 2010). For a slope > −1, a time-diminished detoxification occurs, for instance, by metabolic enzyme induction and/or pharmacodynamic desensitivity (Bunce and Remillard, 2010). In this manner, we estimated the slope (and 95% confidence intervals) of only one type of linear regression, namely the log (LCD_{50}) vs. log (time), since the number of available data points for applying additional linear regression types such as log (concentration) vs. log (LT_{50}) and log (LC_{50}) vs. log (time) was small, in light of the 10-day study time frame.

In addition, we compared the results obtained from the Druckrey-Küpfmüller model with the European Food Safety Authority (EFSA) approach, which is based on the direct comparison of the acute LCD_{50} (48-h exposure) and the chronic LCD_{50} (10 days of exposure; EFSA, 2013). If there is no cumulative toxicity, these lethal cumulative doses should be equal. A ratio of a chronic LCD_{50} (10 days exposure) to an acute LCD_{50} that is bigger than 1 implies a time-diminished detoxification pharmacokinetic and/or dynamic mechanism; whereas a ratio smaller than 1 is indicative of time-reinforced toxicity.

2.6. Mixture toxicology study

The chronic toxicity of various mixtures of the most frequently encountered pesticides in Israeli honey (DMF, DMPF, coumaphos), found in more than 85% of all samples, were studied in a mixed honeybee population. The pesticide concentrations tested were within the range of pesticide residues detected in honey, as well as up to 10 times higher. Since all of the three pesticides were found in more than 85% of all analyzed honey samples, we also explored possible potentiation effects of the tertiary mixture on the most frequently encountered neonicotinoids in Israeli honey, namely, acetamiprid, imidacloprid and thiacloprid (Bommuraj et al., 2019; Shimshoni et al., 2019). For that purpose, we studied the dose-response effect of each of the neonicotinoids separately and in the presence and absence of the tertiary mixture at 10 times their highest reside concentration in honey, namely 1 mg/L of DMF and DMF and 10 mg/L of coumaphos.

2.7. Statistical analysis

Descriptive statistics, as well as the log-logistic models and the log-log linear regressions were carried out using the statistical analysis program GraphPad (GraphPad, Prism version 5.00 for Windows, San Diego, CA, USA). A comparison of the mean percentage age distribution of honeybees with the reported literature values was accomplished using two-way, non-repeated measures ANOVA. Significant interactions were explored with Tukey's multiple comparison tests. The amount of food consumption in the different treatments was evaluated by one-way ANOVA at a significance level of $P \leq 0.05$. Sucrose consumption of pesticides with respect to treatment and time as independent variables was evaluated by two-way, repeated measures (mixed model) ANOVA with a Bonferroni post hoc test conducted at a significant level of $p \leq 0.05$. Time-dependent changes in the lethal cumulative dose were determined by a linear regression *F*-test, in which we determined the difference relative to the slope from zero, set as the null hypothesis, at a significance level of $p \leq 0.05$. Comparisons between the LCD_{50}, LC_{50} and LT_{50} values of the examined neonicotinoids in the presence and absence of the tertiary mixture were made using a two-way *t*-test. An unpaired *t*-test was employed to determine whether the mean chronic LCD_{50} and mean acute LCD_{50} were significantly different from 1, by utilizing the statistical analysis program GraphPad.

3. Results and discussion

The age distribution of honeybee samples collected from frames of one-story beehives for toxicological evaluations was determined according to the study design published by van der Steen et al. (2012). The age-distribution profile of the honeybee samples studied here was similar to the age distribution reported by van der Steen et al. (2012) (Table S1).

3.1. Sucrose consumption as a function of treatment and study duration

Surprisingly, peer-reviewed reports of daily oral sucrose consumption of honeybees reared in vitro as a function of chronic pesticide exposure (10 days) are lacking for the majority of compounds tested here and are reported for the first time in this paper. No significant differences in mean daily sucrose consumption were observed between the negative control group (no-treatment group) and the different treatment concentrations of the following pesticides: acetamiprid, thiacloprid, coumaphos, DMF, DMPF and dimethoate (toxic reference; Supplementary Material, Fig. S1). Furthermore, for the aforementioned pesticides, no significant difference was observed in daily sucrose consumption as a function of time (the within-group variable) or pesticide concentration (the between-group variable), according to the repeated two-way mixed ANOVA analysis followed by the Bonfferoni post hoc test (Supplementary Material, Fig. S2).

Imidacloprid was the only pesticide that significantly decreased the daily sucrose consumption of honeybees at concentrations

above 0.5 mg/L on Days 8–10, as compared to the control, resulting in a substantial decline in the mean daily sucrose consumption (Supplementary Material, Fig. S2). The latter observation is in agreement with previous studies that have reported a significant decline in sucrose consumption following exposure to increasing imidacloprid concentrations (Meikle et al., 2016). Consequently, it seems that imidacloprid, at certain concentration levels, exerts an aversive response of honeybees to contaminated sucrose solutions.

3.2. Determination of acute and chronic toxicity

A cumulative dose- or concentration-response curve and a time-response curve were established for each of the tested pesticides among caged honeybees of a representative population age (Fig. 1; Supplementary Material, Fig. S3). The honeybee mortality rate observed over 10 days of daily exposure to 2% acetonitrile in 50% sucrose solution was identical to that observed for the pure 50% sucrose solution (data not shown), indicating that 2% acetonitrile had no negative impact on honeybee mortality.

Dimethoate was included in the present study as a toxic reference, in order to demonstrate the validity and sensitivity of the assay to the toxicological effects of the examined insecticides in compliance with the Standard Methods for Toxicology Research in *Apis mellifera* (Medrzycki et al., 2013). The mortality rates for the negative control and the toxic reference (dimethoate) were in agreement with two validity criteria established by the OECD test guidelines for the evaluation of pesticides' chronic toxicity (10 days), namely the average mortality for the negative control was less than 15% at Day 10 and the average mortality in the toxic standard was higher than 50% at Day 10 (OECD, 2017). The LCD_{50} (0.13 μg/bee) and LC_{50} (0.45 mg/L) values for dimethoate after 10 days of exposure (Table 1) were within the ranges reported in numerous studies, confirming the validity and sensitivity of the present assay (Beran, 1970; Fiedler, 1987; OECD, 2017). However, the dimethoate concentrations tested (0.05–0.8 mg/L) were too low to induce acute mortality within 48 h and, therefore, acute LCD_{50}/LC_{50} could not be calculated (Fig. 1; Table 1). The latter observation is in agreement with the published literature, which reports that a higher concentration (>1 mg/L) is required for LCD_{50}/LC_{50} determination (Gough et al., 1994).

Table 1 shows the calculated chronic (10 days) and acute (2 days) LCD_{50}/LC_{50} values for the remaining compounds tested here, except for coumaphos, which was not toxic even at the highest dose tested (i.e., 100 mg/L). The use of a higher dose of coumaphos was not feasible due to the visible precipitation of coumaphos at concentrations above 100 mg/L in 50% sucrose solution. The lack of oral coumaphos toxicity in our study stands in stark contrast to the oral toxicity reported by Gregorc et al. (2018). Gregorc et al. (2018)

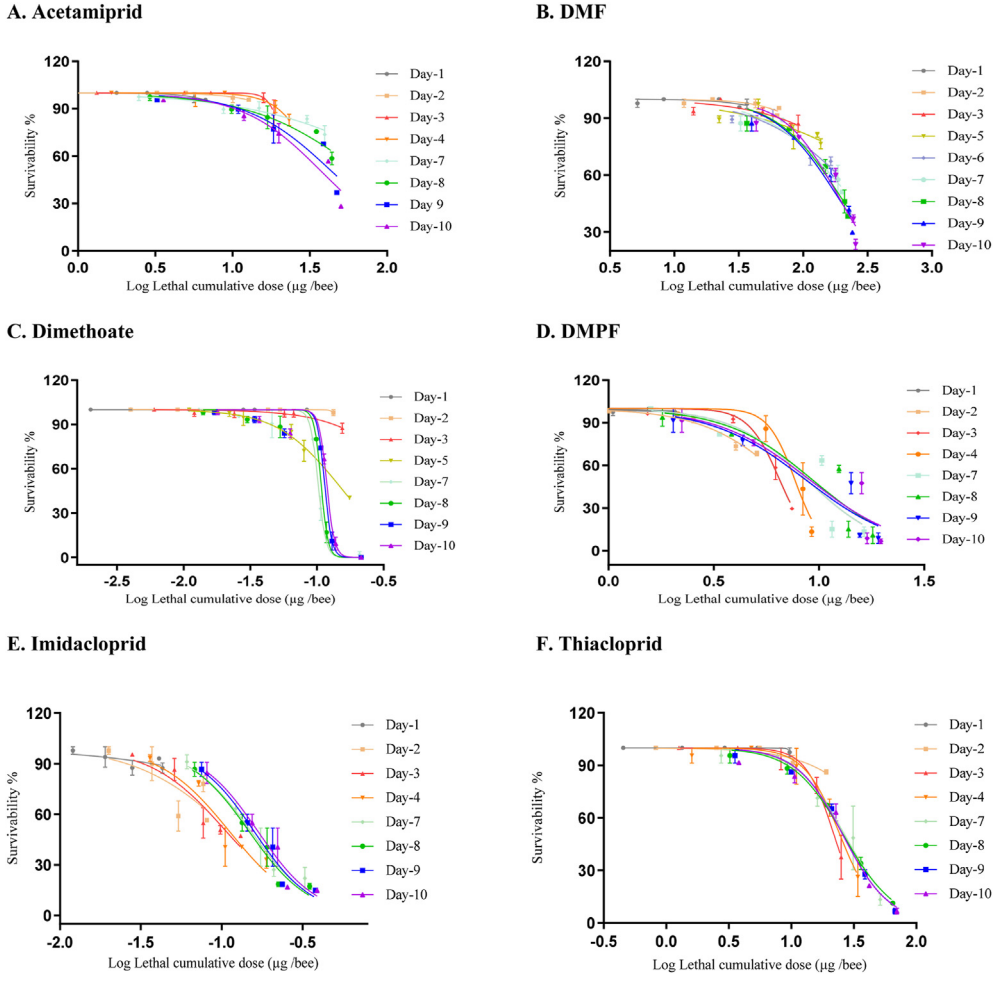

Fig. 1. Honeybee survivability (%) vs. average Log lethal cumulative dose (LCD, μg/bee/day) plotted for each day up to 10 days. The average LCD values were obtained by adding up the average daily pesticide doses consumed/day for each day, up to 10 days (obtained from the product: daily sucrose volume consumed/live bee/day multiplied with the corresponding pesticide concentration in the sucrose solution).

Table 1
Chronic and acute toxicity parameters of each pesticide.

Pesticide	Chronic toxicity after 10 days		Acute toxicity after 2 days		Ratio of chronic LCD_{50} to acute LCD_{50}[d]
	LCD_{50} μg/bee[a] ± SE[b]	LC_{50}[c] in sucrose (mg/L) ± SE	LCD_{50} μg/bee ± SE	LC_{50} in sucrose (mg/L) ± SE	
Acetamiprid	40.6 ± 2.0	98.9 ± 1.1	63.1 ± 3.9	277.3 ± 1.0	0.6 ($P = 0.04$, $t = 5.1$)
Dimethoate	0.13 ± 0.01	0.45 ± 1.1	ND[e]	>0.8	–
DMF[f]	197.6 ± 6.2	479.1 ± 1.0	ND	>1500	–
DMPF[g]	15.9 ± 0.2	16.7 ± 1.1	5.9 ± 0.1	42.1 ± 1.0	2.7 ($P = 0.0005$, $t = 44.7$)
Imidacloprid	0.2 ± 0.01	0.6 ± 1.1	0.08 ± 0.01	2.2 ± 1.0	2.5 ($P = 0.01$, $t = 8.5$)
Thiacloprid	28.6 ± 1.0	58.4 ± 1.1	37.8 ± 2.4	194.3 ± 1.1	0.8 ($P = 0.07$, $t = 3.6$)

[a] LCD_{50}, lethal cumulative dose (μg/bee) resulting in 50% mortality.
[b] SE, standard error.
[c] LC_{50}, lethal concentration (mg/L) resulting in 50% mortality.
[d] Statistical analysis employing unpaired *t*-test; $p \leq 0.05$ was considered a significant difference.
[e] ND, not determined due to a lack of toxicity at the tested range of concentrations.
[f] DMF, N-(2,4-dimethylphenyl)-formamide.
[g] DMPF, N'-(2,4-dimethylphenyl)-N-methylformamidine.

reported 50% mortality after 9 days at concentrations above 92.6 mg/kg. Notwithstanding, it is reasonable to assume that the lack of consistency between both studies is due to differences in the feed provided daily to the honeybees. In our study, coumaphos was administered in sucrose solution, while Gregorc et al. (2018) provided coumaphos orally, spiked into commercial solid patty composed of sugar and small amounts of protein and fat. Hence, a variable bioavailability and mean duration of persistence of coumaphos between the two formulations is to be expected, which could provide a plausible explanation for the lack of agreement between the two studies.

The two most toxic pesticides evaluated, displaying the lowest.

Chronic (10 days) and acute (48 h) LCD_{50} and LC_{50} values, were imidacloprid (chronic: 0.2 μg/bee; 0.6 mg/L and acute: 0.08 μg/bee; 2.2 mg/L) and DMPF (chronic: 15.9 μg/bee; 16.7 mg/L and acute: 5.9 μg/bee; 42.1 mg/L; Table 1). According to numerous studies, amitraz is highly unstable under environmental conditions, especially in beehive matrices and aqueous solutions, which explains its exceptionally low detection level and low detection frequency (Brimecombe and Limson 2006; Shimshoni et al., 2019; van Eeden et al., 2004). Consequently, it is reasonable to assume that the amitraz metabolite DMPF is the actual toxic entity, responsible for amitraz's toxic response in honeybees and possibly other organisms as well (Shimshoni et al., 2019). The neonicotinoids thiacloprid and acetamiprid were significantly less toxic than imidacloprid, with higher chronic and acute LCD_{50}/LC_{50} values; whereas DMF was the least toxic compound with the highest chronic LCD_{50} (198 μg/bee) and LC_{50} (>1500 mg/L) values (Table 1). As stated above, the toxicity of a chemical depends not only on the dose, but also on the duration of exposure and, therefore, determining the time to induce toxic effects at various concentrations is of importance for risk assessors.

Table 2 shows only the experimentally determined (non-extrapolated) time to reach 50% mortality at the highest concentrations for up to 10 days of exposure. As expected, the LT_{50} values for all of the tested pesticides decreased as their concentrations increased (Fig. 2; Table 2). For example, DMPF had the lowest LT_{50} value (2.5 days) at its highest concentration tested (40 mg/L) and the highest LT_{50} value (9 days) was observed for the concentration that was 2 times lower (20 mg/L). On the other hand, the LT_{50} values (7–8 days) of amitraz's less toxic metabolite, DMF, were reached only at concentrations that were 15–20 times higher than those observed for amitraz's more toxic metabolite, DMPF. Hence, the relative toxicities of different pesticides can be determined (see Section 3.5) by comparing the product range of the.

Concentration multiplied by the corresponding LT_{50} value of different pesticides (Table 2). Specifically, the lower the numerical value of the product range within the tested time frame, the greater the toxicity of the tested chemical. The product value can be interpreted as honeybees' exposure to the pesticide concentration over the corresponding LT_{50} time period, which could vary disproportionally to changing pesticide concentrations (Table 2). Dimethoate and imidacloprid displayed the lowest ranges of toxic exposure (i.e., 3.7–3.8 and 4.9–5.5, respectively) and consequently can be considered substantially more toxic than the other studied pesticides. Although the exposure values for DMPF were 25–45 times higher than those observed for dimethoate and imidacloprid, these differences were not statistically significant due to the small number of data points and consequently diminished power of the statistical test, which is the result of the limited study duration (10 days) and/or narrow pesticide concentration range (Table 2). Notwithstanding, the exposure values for DMPF, imidacloprid and dimethoate were significantly lower than those observed for thiacloprid and DMF. Moreover, the thiacloprid exposure product was significantly lower than that of DMF. Since only a single exposure value was experimentally available for acetamiprid, that pesticide was excluded from the statistical comparison. However, based on that single value, acetamiprid's toxicity can be ranked between that of thiacloprid and that of DMF (Table 2). Taken together, the pesticides can be rated toxicologically in the following descending order: dimethoate, imidacloprid ≥ DMPF > thiacloprid ≥ acetamiprid ≥ DMF.

3.3. Potentiation study

Within the context of this study, the term *potentiation* is used to refer to the phenomenon observed when a chemical or mixture of chemicals that had no toxic effect per se at the dosage employed was able to significantly enhance the toxicity of another chemical with which it was combined. The concomitant occurrence of several.

Pesticides has been demonstrated in Israeli honey and beeswax samples. In particular, three pesticides (referred to as the tertiary mixture) were simultaneously prevalent in more than 85% of all analyzed honey and beeswax samples, namely, DMF, DMPF and coumaphos (Bommuraj et al., 2019; Shimshoni et al., 2019). Consequently, this study was concerned with the potentiation of the chronic toxicity of the most commonly found neonicotinoids (imidacloprid, acetamiprid and thiacloprid) in Israeli honey by the aforementioned tertiary mixture.

First, we investigated the toxicological effects of binary and tertiary mixtures of DMF, DMPF and coumaphos at 10 times the highest concentrations at which they were found in real honey and beeswax samples. None of the binary and tertiary mixtures were

Table 2
Time (days) to reach 50% mortality, as determined by non-linear regression analysis of mortality vs. time (days), calculated for each concentration separately.

Pesticide	Pesticide concentration (mg/L), inducing 50% mortality at the indicated lethal time (LT_{50})	LT_{50}[a] + SE[b] (days)	Product of pesticide concentration and time to cause 50% mortality (exposure) in mg/L*days	Statistical comparison of the exposure ranges of the pesticides[c]
Acetamiprid	150	8.3 ± 0.3	1245	B
Dimethoate	0.6	6.2 ± 0.2	3.7	E
	0.8	4.7 ± 0.1	3.8	
DMF[d]	500	8.1 ± 0.2	4050	A
	600	7.1 ± 0.1	4260	
DMPF[e]	20	9.0 ± 0.3	180	D E
	30	4.0 ± 0.3	120	
	40	2.5 ± 0.3	100	
Imidacloprid	1	5.5 ± 1.1	5.5	E
	1.25	4.3 ± 0.5	5.4	
	1.5	3.5 ± 0.4	5.3	
Thiacloprid	100	6.4 ± 0.3	640	C
	150	2.9 ± 0.2	435	
Tertiary mixture[f] + acetamiprid	100	9.7 ± 0.7	970	B
	150	7.7 ± 0.5	1155	
Tertiary mixture + imidacloprid	1.0	7.1 ± 0.8	7.1	E
	1.25	5.9 ± 0.7	7.3	
	1.5	5.5 ± 0.9	8.2	
Tertiary mixture + thiacloprid	100	5.5 ± 0.6	550	C D
	150	2.0 ± 0.5	300	

[a] LT_{50}, lethal time (days) to reach 50% mortality.
[b] SE, standard error.
[c] One-way ANOVA with Tukey test at a significance level of $p < 0.05$. Levels not connected by same letter are significantly different.
[d] DMF, N-(2,4-dimethylphenyl)-formamide.
[e] DMPF, N'-(2,4-dimethylphenyl)-N-methylformamidine.
[f] Tertiary mixture consisting of 1 mg/L DMF, DMPF and 10 mg/L of coumaphos in sucrose solution.

found to induce mortality after 10 days of exposure, as compared to the control. Therefore, we proceeded to investigate whether the same nontoxic tertiary mixture tested above might potentiate the toxicity of the neonicotinoids acetamiprid, thiacloprid and imidacloprid, in the same concentration ranges used to establish their chronic-toxicity profiles (Fig. 2; Table 3). As can be seen from Table 3, no statistically significant differences were observed between the toxicological chronic endpoints LCD_{50}, LC_{50} and LT_{50} of the neonicotinoids with or without the presence of the tertiary mixture. Hence, a potentiation effect of the tertiary mixture on the honeybees' mortality was ruled out. The lack of mortality potentiation of the tertiary mixture is in agreement with field observations by local beekeepers, who usually report no extraordinary mortality events of honeybees outside their beehives, except for when a foliar application of organophosphate and/or neonicotinoid pesticides is carried out in nearby agricultural fields.

3.4. Time-dependent toxicities

In the present study, we determined the log-log relationships between the pesticide cumulative dose vs. time and the ratio of chronic to acute LCD_{50}. Among the pesticides tested, thiacloprid was the only chemical to comply with Haber's rule, as can be deduced from the zero slope of its linear regression log (LCD_{50})-log (time) plot over the entire duration of the study (Fig. 3). The latter observation was confirmed by the EFSA approach, according to which the ratio of chronic LCD_{50}/acute LCD_{50} was not significantly different from 1 (Table 1). Previous studies have demonstrated that mostly non-bio-accumulative toxicants with a short persistence period follow Haber's rule (Gesztelyi et al., 2012).

On the other hand, we found clear evidence for the time-reinforced toxicity of acetamiprid and DMF based on the significant decline of the negative regression curve log (LCD_{50}) over log (time), yielding a negative slope that is significantly different from zero (Fig. 3; Table 4). The observed time-reinforced toxicities of DMF and acetamiprid are reported here for the first time. The lethal cumulative dosage of these ingested pesticide declined as the duration of exposure increased. This pattern is characteristic of bio-accumulative toxicants, which tend to be retained in the honeybee's body. Upon prolonged exposure, these chemicals trigger lethal effects at increasingly lower cumulative doses. Various investigators have postulated that the time-reinforced toxicity could also be a result of accumulated physiological damage/physiological wear-out of the honeybee. In contrast, DMPF, dimethoate and imidacloprid displayed time-diminished toxicity; that is, the LCD_{50} values for those chemicals increased as the duration of the exposure increased (Fig. 3; Table 4).

This time-diminished toxicity was confirmed using two statistical approaches. First, the slopes of the log-log regressions of LCD_{50} vs. time were positive and significantly greater than zero. Second, in compliance with the EFSA protocol, the ratio of chronic to acute LCD_{50} was positive and significantly greater than 1 (Table 1).

Time-diminished toxicity is apparent for chemicals that tend to up-regulate detoxifying metabolic enzymes, resulting in an enhanced rate of elimination of the pesticide. The major enzyme super-families responsible for the metabolism or detoxification of toxins in honeybees are the cytochrome P450 mono-oxygenases (P450s), glutathione transferases and carboxylesterases (Manjon et al., 2018; Mao et al., 2011). For instance, the P450 enzymes CYP9Q1, CYP9Q2 and CYP9Q3 have been shown to efficiently metabolize and thereby detoxify the pesticides tau-fluvalinate and coumaphos (Mao et al., 2011). Recently, Manjon et al. (2018) demonstrated that P450s are major determinants of variable neonicotinoid sensitivity in honeybees and bumblebees. Specifically, CYP9Q3 was identified as being able to metabolize thiacloprid very efficiently but displayed comparatively little metabolic activity against imidacloprid. This observation provides a piece of crucial evidence for the major impact of the biotransformation pathways on honeybees' sensitivity to various pesticides.

The results of our chronic-toxicity study support the general notion that cyano-substituted neonicotinoids (thiacloprid, acetamiprid) are significantly less toxic to honeybees than nitro-

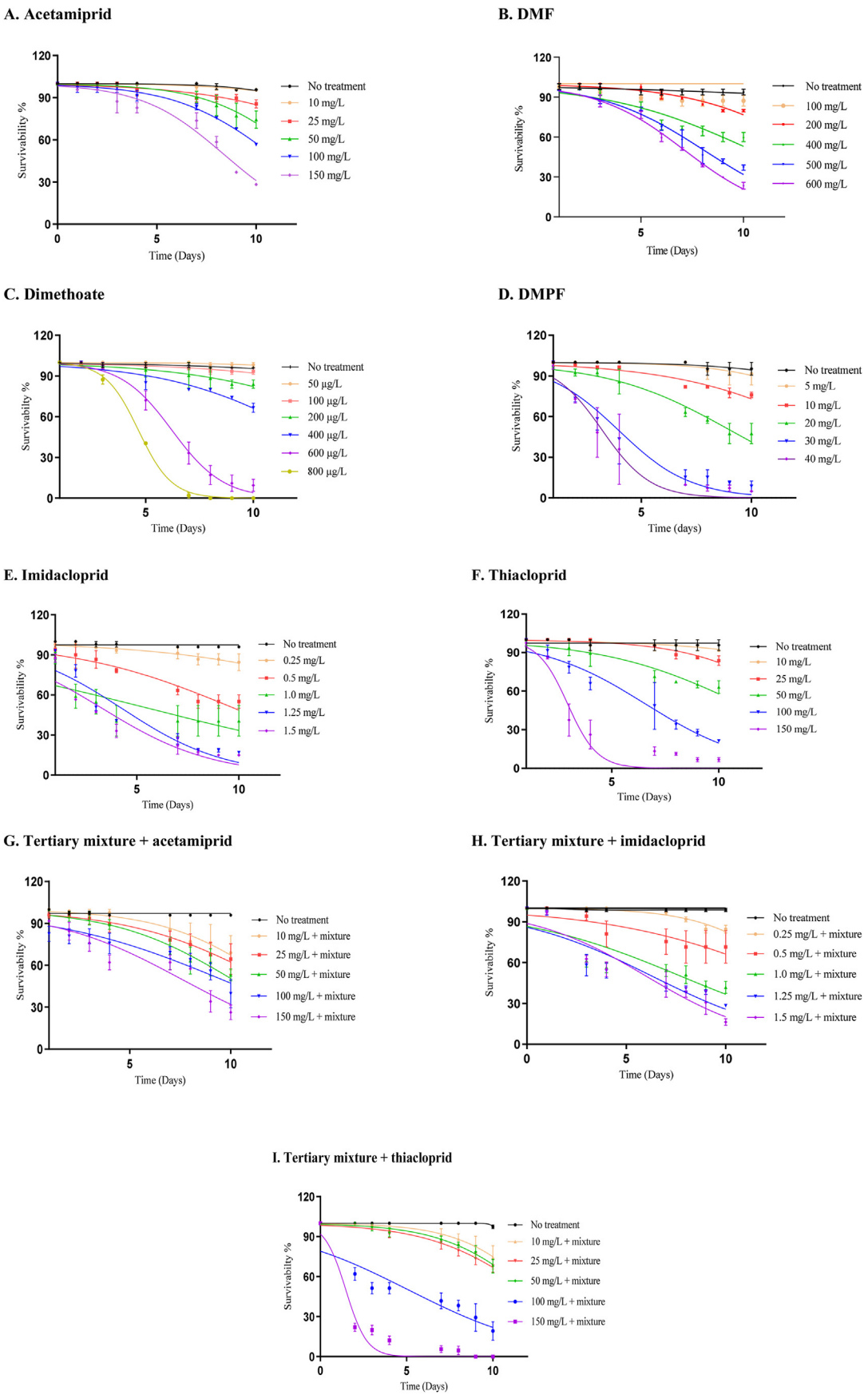

Fig. 2. Honeybee survival (%) vs. time (in days) following oral exposure to different concentrations of pesticide (pesticide concentration is provided in the legend) in the presence of the potentiation mixture (indicated as mixture in the legend) composed of DMF (1 mg/L), DMPF (1 mg/L) and coumaphos (10 mg/L).

Table 3
Statistical comparison between 50% chronic lethal toxicity endpoints (LCD, LC and LT) of exposure to individual pesticides (10 days) and corresponding values in the presence of the tertiary mixture.[a].

Pesticide		Chronic lethal toxicity endpoints + SE[b]		
		Single pesticide	Tertiary mixture + pesticide	Statistical analysis[c]
Acetamiprid	LCD_{50}[d]	40.6 ± 2.0	38.9 ± 3.2	$p = 0.07$ ($t = 3.2$)
	LC_{50}[e]	98.9 ± 1.1	92.8 ± 1.2	$p = 0.07$ ($t = 3.1$)
	LT_{50}[f]	8.3 ± 0.2	7.7 ± 0.4	$p = 0.3$ ($t = 1.3$)
Imidacloprid	LCD_{50}	0.2 ± 0.1	0.2 ± 0.05	$p = 0.7$ ($t = 0.5$)
	LC_{50}	0.6 ± 1.1	0.8 ± 1.1	$p = 0.37$ ($t = 1.2$)
	LT_{50}	3.5 ± 0.4	5.5 ± 0.9	$p = 0.1$ ($t = 2.0$)
Thiacloprid	LCD_{50}	28.6 ± 1.0	19.4 ± 2.2	$p = 0.1$ ($t = 2.4$)
	LC_{50}	58.4 ± 1.1	51.5 ± 1.3	$p = 0.7$ ($t = 0.4$)
	LT_{50}	2.9 ± 0.2	1.5 ± 0.2	$p = 0.2$ ($t = 1.7$)

[a] The tertiary mixture of three acaricides: DMF (0.1 mg/L), DMPF (0.1 mg/L) and coumaphos (10 mg/L).
[b] SE, standard error.
[c] *t*-test (unpaired) at a significance level of $p \leq 0.05$.
[d] LCD_{50}, lethal cumulative dose (μg/bee) that causes 50% mortality.
[e] LC_{50}, lethal concentration (mg/L) that causes 50% mortality.
[f] LT_{50}, lethal time (days) to 50% mortality at the highest tested concentration.

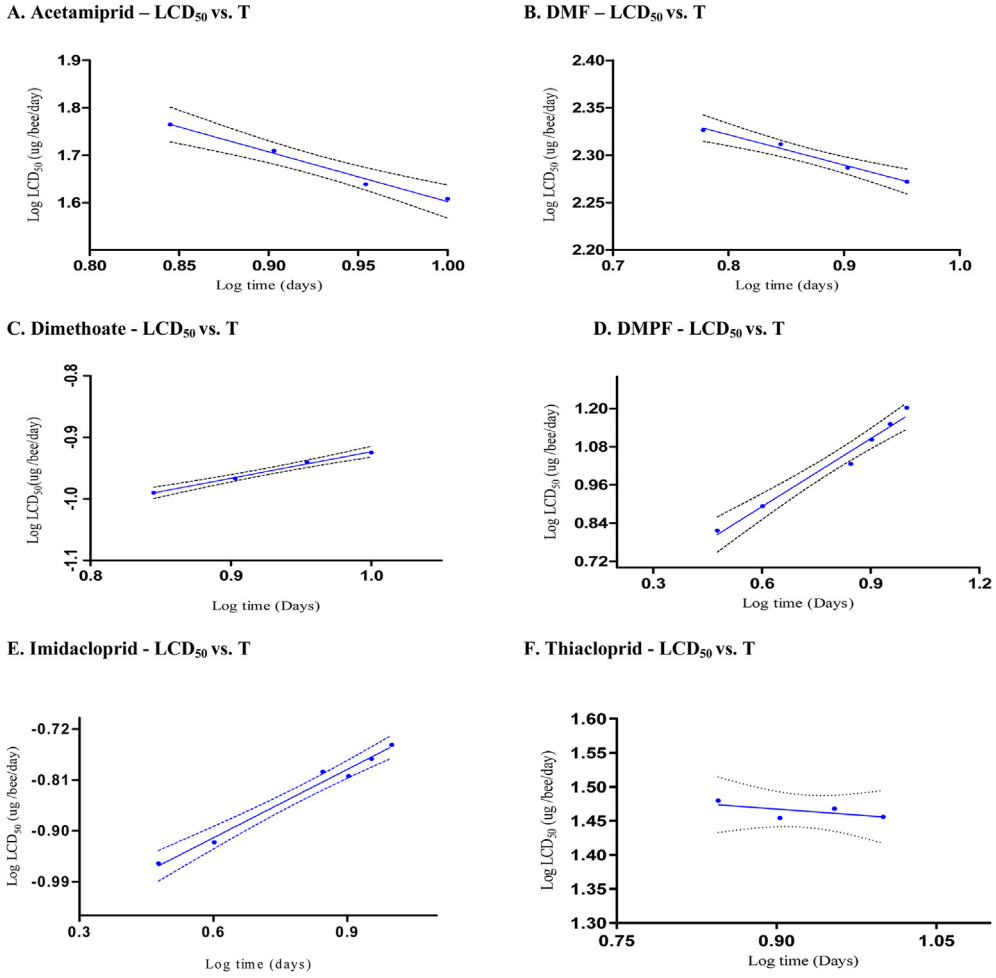

Fig. 3. Log-log linear regression of lethal cumulative dose in μg/bee/day vs. time (in days).

substituted neonicotinoids (imidacloprid) at environmentally relevant concentrations (Hirata et al., 2017). Moreover, our finding of the time-diminished toxicity exerted by imidacloprid is supported by the results published by Holder et al. (2018), which provide compelling evidence for the lack of time-reinforced toxicity. The observed phenomenon might be the result of specific P450 enzyme up-regulation responsible for its biotransformation, as has been demonstrated for several other insect species (Liu et al., 2015).

Table 4
Linear regression *F*-test analysis of log (time) in days vs. log (lethal cumulative dose) expressed in terms of μg/bee/day.[a].

Pesticide	Slope value for LCD_{50}[b] + SE[c]	Statistical analysis		
		R^2[d]	Critical *F*-value[e]	*P*-value[f]
Acetamiprid	−1.1 ± 0.08	0.98	148.7	0.006
Dimethoate	0.4 ± 0.02	0.99	400.3	0.002
DMF[g]	−0.3 ± 0.03	0.98	125.7	0.007
DMPF[h]	0.7 ± 0.05	0.98	175.8	0.0002
Imidacloprid	0.4 ± 0.03	0.98	236.9	0.0001
Thiacloprid	−0.1 ± 0.08	0.41	1.4	0.35

[a] The null hypothesis states that the linear slope of time (days) vs. lethal cumulative dose (μg/bee/day) equals zero. The *F*-test enables us to determine whether the slope is significantly different from zero. For calculated *p*-values > 0.05, the linear slope was statistically defined as zero.
[b] Linear slope.
[c] SE, standard error.
[d] R^2, coefficient of determination, defined as the proportion of the variance in the dependent variable (dose) that is predictable from the independent variable (time).
[e] *F*-value, test statistic for testing the statistical significance of the model.
[f] *P*-value; when the calculated *p*-value is above the significance level of 0.05, then the null hypothesis is accepted as true, hence, the linear slope equals to zero.
[g] DMF, N-(2,4-dimethylphenyl)-formamide.
[h] DMPF, N'-(2,4-dimethylphenyl)-N-methylformamidine.

4. Conclusions

To reduce honeybees' continuous hazardous exposure to various pesticides, time-dependent toxicity studies are of the utmost importance. For the majority of pesticides applied year-round in agricultural fields, data regarding time-dependent toxicities are largely lacking. Therefore, we have determined time-dependent toxicities of common pesticides and pesticide metabolites that are frequently present in Israeli beehives, as well as in honey samples analyzed worldwide, namely the major amitraz metabolites DMF and DMPF, the organophosphate coumaphos and the neonicotinoids imidacloprid, thiacloprid and acetamiprid. Dimethoate was included in the study as the relevant toxic standard.

Thiacloprid was the only one of the studied pesticides to comply with Haber's rule; whereas DMPF, dimethoate and imidacloprid displayed time-diminished toxicities. DMF and acetamiprid revealed time-reinforced toxicities. In addition, we studied the mixture toxicities of the most frequently occurring pesticide combinations and estimated their potential contributions (potentiation) to the overall toxicities of the neonicotinoids. Our findings indicate that neither the binary nor the tertiary mixtures of DMF, DMPF and coumaphos at 10 times their environmentally relevant concentrations potentiate the neonicotinoids' toxicities. DMPF and imidacloprid were found to present the greatest risk to honeybees, based on their $LCD_{50}/LC_{50}/LT_{50}$ values. Based on the instability of amitraz under the prevailing environmental conditions and its consequently low detection frequency, as well as DMPF's high toxicity profile, it is reasonable to assume that DMPF is the actual toxic entity responsible for the toxic response that amitraz elicits in honeybees.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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Credit author statement

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Conflicts of interest

The authors declare no conflicts of interest.

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