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Biomarker responses and lethal dietary doses of tau-fluvalinate and coumaphos in honey bees: Implications for chronic acaricide toxicity

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ABSTRACT

Evidence suggests that acaricide residues, such as tau-fluvalinate and coumaphos, are very prevalent in honey bee colonies worldwide. However, the endpoints and effects of chronic oral exposure to these compounds remain poorly understood. In this study, we calculated LC_{50} and LDD_{50} endpoints for coumaphos and tau-fluvalinate, and then evaluated in vivo and in vitro effects on honey bees using different biomarkers. The LDD_{50} values for coumaphos were 0.539, and for tau-fluvalinate, they were 12.742 in the spring trial and 8.844 in the autumn trial. Chronic exposure to tau-fluvalinate and coumaphos resulted in significant changes in key biomarkers, indicating potential neurotoxicity, xenobiotic biotransformation, and oxidative stress. The Integrated Biomarker Response was stronger for coumaphos than for tau-fluvalinate, supporting their relative lethality. This study highlights the chronic toxicity of these acaricides and presents the first LDD_{50} values for tau-fluvalinate and coumaphos in honey bees, providing insights into the risks faced by colonies.

1. Introduction

Due to the significant and often irreversible impacts of human activity on ecosystems, there is a growing need to develop tools to monitor the effects of pollution on organisms. These tools must be able to evaluate the physiological and functional integrity of individuals, particularly focusing on indicators of exposure to environmental stress factors. Biomarkers reveal information about the environment in terms of contamination by xenobiotics and thus, by definition, their biochemical, molecular, cellular and/or physiological levels can be potentially used to reflect exposure to pesticides and heavy metals (Nicewicz et al., 2021; Peakall, 1994). As such, biomonitoring programs are generally based on the study of a set of biomarkers in sentinel species of interest (Caliani et al., 2021; Lambert et al., 2012).

Honey bees are considered suitable species to measure environmental contamination because they are frequently exposed to pesticides through their foraging activity on cultivated and wild plants, from which they collect pollen and nectar that is subsequently stored in the hive (Celli and Maccagnani, 2003; David et al., 2016; Ghini et al., 2004). In addition, the regular application of veterinary drugs by beekeepers to control parasites like *Varroa destructor* leads to the accumulation of acaricide residues in different matrices within the hive. In fact, different studies have indicated that acaricides are the most prevalent pesticides in the wax and pollen stored in honey bee colonies, in particular coumaphos and tau-fluvalinate (Agrebi et al., 2020; Alonso-Prados et al., 2020; Calatayud-Vernich et al., 2018; Chauzat et al., 2011; Mullin et al., 2010; Orantes-Bermejo et al., 2010). Indeed, these compounds can be detected at high concentrations even several years after their application (Benito-Murcia et al., 2021). Therefore, due to the persistence of these lipophilic substances in the colony, leading to more long-term contact of the animals to them, it is essential to determine the effects of such chronic exposure on honey bee health.

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The active substances tau-fluvalinate and coumaphos are stable at the melting temperature of wax, and they are very lipophilic (Bogdanov et al., 1997; Kast et al., 2021; Kochansky et al., 2001; Shimshoni et al., 2019). Therefore, these compounds can accumulate in apolar matrices like stored pollen and wax. Indeed, these residues can be detected at high concentrations in the wax and pollen inside the hive for up to four years after the last application of these treatments (Benito-Murcia et al., 2021; Bogdanov and Liebefeld, 2006; Lodesani et al., 2008; Wallner, 1999). Honey bees are chronically exposed to these active substances, both orally through the consumption of pollen and honey, and topically, for example, through contact with acaricide residues in the wax (Becher et al., 2014; Sponsler and Johnson, 2017). The data available on the toxicity of these compounds in bees are based on the acute, contact and oral LD_{50} values (Chmiel et al., 2020; Sanchez-Bayo and Goka, 2014), and it is extremely variable for both compounds. However, honey bees are usually chronically exposed to these acaricides and thus, it is generally necessary to carry out studies to evaluate the long-term effects of chronic exposure to these two compounds. Indeed, the toxic effects of a particular xenobiotic in bees are likely to depend on physiological factors, including life stage, breed, age, season, temperature, feeding history and current or past exposure to other toxins (Johnson, 2015).

To evaluate the effects of these two acaricides on honey bee physiology, we used a series of biomarkers related to neurotoxicity (i.e acetylcholinesterase), biotransformation (i.e. carboxylesterase and glutathione S-transferase) and oxidative stress (i.e. malondialdehyde formation) in vivo, that were validated as biomarkers of exposure to these substances in a previous study (Benito-Murcia et al., 2022). Acetylcholinesterase (AChE) catalyses the hydrolysis of the neurotransmitter acetylcholine and controls the termination of the nerve impulse in the postsynaptic membrane. This esterase is one of the targets of organophosphate insecticides like coumaphos and dimethoate, and in insects, this enzyme is mainly located in the central nervous system (CNS) neuropil, where it is very abundant (Fournier and Mutero, 1994; Praveena and Sanjayan, 2011). Carboxylesterase (CbE) catalyses the hydrolysis of carboxylic acids to acids and alcohols in phase I of detoxification, and it fulfils many functions like neurotransmitter degradation and hormone or pheromone metabolism. CbE is a component of the defence system against xenobiotic substances, and it is frequently involved in the resistance of insects to organophosphates, carbamates and pyrethroids (Cui et al., 2015; Jackson et al., 2013; Newcomb et al., 1997). Significantly, many compounds interact deleteriously with this enzyme, such as those derived from organophosphates, carbamates, pyrethroids and sulfonamides (Wheelock et al., 2005). The glutathione S-transferase (GST) family of catalysts participates in phase II detoxification of some xenobiotic substances, such as organophosphates or organochlorines, increasing their solubility in water for excretion (Claudianos et al., 2006; Kostaropoulos et al., 2001). Finally, the formation of malondialdehyde (MDA) was measured as it is a commonly used marker of lipid peroxidation generated by Reactive Oxygen Species (ROS). Interestingly, exposure to pathogens and pesticides may drive greater accumulation of this compound (Nair et al., 2008; Olgun et al., 2020).

The aims in this study are I) to optimise the method for experimental exposure of honey bees to coumaphos and tau-fluvalinate in the laboratory, thereby guaranteeing than the honey bees are exposed to the nominal concentration provided through the food using OECD No. 245 (OECD, 2017), and to establish the chronic LC_{50} and LDD_{50} values for these substances in honey bees; II) to evaluate the effect in vivo of exposing adult honey bees to chronic lethal doses of these compounds through the activity of a set of biomarkers and calculate the integrated biomarker response (IBR) index; and III) to evaluate the toxicity of both substances in a range of concentrations, including the LC50, in bee tissues over the same set of biomarkers using in vitro assays.

2. Materials and methods

2.1. Chronic toxicity test

2.1.1. Preparation of the bees and stock solutions for the assays

The chronic toxicity tests were carried out using OECD guideline No. 245 (OECD, 2017) as described previously (Benito-Murcia et al., 2022) for an exposure period of 10 days.

One day before carrying out the tests, the bees were collected from the frames without using anaesthesia and were placed in cages. The cages consisted on 0.6 L plastic glass beaker with two holes in the sides covered with a fine nylon mesh to ensure adequate ventilation. A third opening in the bottom of the beaker enabled a 10 ml syringe to be inserted and used as a feeder. Each cage contained 10 individuals and once the bees were put into the cages, they were left for 24 h with syrup (50% sugar and water, v/w) and fed ad libitum using 5 ml sterile syringes.

The solutions to administer the reference substance (dimethoate) and the test substances (tau-fluvalinate and coumaphos) were prepared as aqueous 50/50 wt/volume sucrose solutions. Syringes (5 ml) were used as feeders. A stock solution of the test substances (tau-fluvalinate and coumaphos) was also prepared with acetone, maintained in the dark, refrigerated at 4 °C and when this was included in the feeding solutions, which were prepared every day. The maximum concentration of acetone to which the bees were exposed never exceeded 5% and was 2% in these trials.

2.1.2. LD_{50} values determination

The effect of chronic exposure to tau-fluvalinate (F: PESTANAL®, CAS No. 102851–06–9, Merck, Germany), coumaphos (CMP: PESTANAL®, CAS No. 56–72–4, Merck, Germany) and dimethoate (DMT, reference substance: PESTANAL®, CAS No. 60–51–5) was determined for 10 days in adult bees. The solutions were administered ad libitum to the animals tested, and the concentrations and replicates used were established according to the statistical requirements to determine the LDD_{50} (μg/bee/day) with a confidence interval of 95% (OCDE No. 54).

A total of 3 tests were performed, two with tau-fluvalinate (spring bees, date-22/06/20 and autumn bees, date-14/09/20) and one with coumaphos (spring date-12/05/20). For each of the concentrations of the test substances, 3 or 4 cages were prepared (Table 1), as recommended in the OECD working guide. In a toxicity test involving 5 concentrations, 4 cages (replicates) were used for each concentration, and when 7 concentrations were used (tau-fluvalinate on autumn: F autumn), 3 cages (replicates) were added for each concentration. An identical number of cages with bees were fed with syrup alone (negative control, expressed as C-) and with syrup to which the solvent was added as a control to correctly evaluate the mortality produced by the substances used in the assays (positive control, expressed as C+). In addition, 3 cages containing syringes with syrup or with syrup and solvent were included in all the studies, in this case without bees, to correct for any possible imbalance caused by evaporation and to take these into account when calculating the daily consumption. Each replicate was examined daily throughout tests to collect dead bees, and to establish

Table 1
Nominal concentrations (ppm) of the different substances used.

CMP	F (spring test)	F (autumn test)
30	77.5	425
62.4	157.5	500
125	310	600
250	625	725
500	750	875
		1050
		1250

The coumaphos (CMP) and tau-fluvalinate (over spring or autumn bees) concentrations.

the mortality and consumption rates of each group.

The cages were placed in an incubator (Memmert HCP240; precision: ± 0.1 °C and ± 0.5% relative humidity) at 33 °C, and with a relative humidity of 65% (as recommended by the OECD TG245 and Buendía-Abad et al., 2021; Higes et al., 2010; Martín-Hernández et al., 2017). To estimate the syrup consumption of each treatment group during the 10 days of testing, the syringes were weighed three times each day on a high-precision balance (Sartorius CP225D), always at the same time (with deviations of ≤ 2 h), calculating the average of the three weights. The dead bees and the density of the syrup (1.23 g/ml) were taken into account to estimate μg/bee/day for the LDD_{50} calculation. The syrup consumption was corrected for the weight loss of the feeders obtained from the bee-free cages and divided by the number of bees that were alive in each cage. Cages in which consumption was lower than the evaporation or in which liquid had been spilled were eliminated from the analysis. The dead individuals from each cage were collected, counted and preserved at − 80 °C, and surviving animals were anesthetized with CO_2 and frozen for posterior analysis.

2.1.3. Validity criteria to determine the test endpoints (LC_{50} and LDD_{50})

According to the criteria established in the OECD guideline No. 245, chronic toxicity tests are valid as long as mortality of those test animals receiving only syrup or syrup with the solvent don't exceed 15%. For the dimethoate (reference substance) exposed animals, the mortality must be at least 50% at the end of the 10 days of exposure. All of these criteria were met in the three tests performed.

2.2. Biomarkers

2.2.1. In vivo assays

The methods for tissue preparation, biomarkers of neurotoxicity (AChE), metabolism (CbE, GST) and oxidative damage (MDA) measurements and the quantification of integrated biomarker response (IBR) index are detailed in a previous study (Benito-Murcia et al., 2022).

2.2.2. In vitro assays

To analyse the in vitro sensitivity of biomarkers, in a set of experiments, tissue extracts of bees were incubated during 1 h at 25 °C in a presence of different concentrations of dimethoate, coumaphos and tau-fluvalinate. The in vitro evaluation of the biomarkers was performed on 30 bees extract using the same preparation of tissue for enzyme extraction used for the in vivo assays. The activity of each biomarker was measured in extracts of honey bees collected from untreated colonies before exposing them to different ranges of final concentrations of dimethoate (1.3–2.6 $\times 10^{-3}$ ppm), coumaphos (20.92–0.08 ppm) or tau-fluvalinate (309–1.2 ppm), employing three replicates for each of the doses specified. The solutions were prepared with a stock solution in acetone that was serially diluted in water to set the different concentrations to be tested in tissue extracts. The range of concentrations tested for each compound included the LC_{50} values calculated for these pesticides using OECD No. 245 test. Enzymatic activities were expressed as % of basal enzyme activity without drug (mean ± SD) and were fitted using a dose-response curve to determine de IC_{50} (half maximal inhibitory concentration) of the dimethoate, coumaphos and tau-fluvalinate.

2.3. Statistical analysis

A probit test was performed on the data to determine the LC_{50} and LDD_{50} values. To establish whether there are dose-response dependence effects on the biomarkers after exposure to the substances, Spearman's coefficients were calculated. Moreover, to evaluate differences between the consumption in each experiment, a Shapiro-Wilk test was performed first. If the significance level was greater than 0.05, it was assumed that the variables complied with the principle of normality, subsequently performing an ANOVA at 95% confidence interval (CI). A Bonferroni's post-hoc test was used for all the two-by-two comparisons of equality of the means. The level of significance in all tests was set to ≤ 0.05. All statistical analyses were performed using the SPSS V. 28.0.1 software. IC_{50} values and inhibition parameters were calculated and adjusted using the models provided by GraphPad Prism software.

3. Results

3.1. Chronic toxicity test

3.1.1. Estimated syrup consumption

The syrup consumption recorded in each experiment varied depending on the substance in which they were carried out. After carrying out a test to compare all groups, significant differences were evident between the mean consumption among the treatments (F 6.010, DF= 2, $p = 0.008$: Fig. 1). However, the Bonferroni post-hoc test indicated that only the group exposed to coumaphos consumed significantly less syrup than the group exposed to tau-fluvalinate on the first trial (mean difference −0.114, standard error 0.33, $p = 0.007$). By contrast, there were no significant differences among groups for the mean consumption in the first and second tau-fluvalinate trials (mean difference 0.046, standard error 0.031, $p = 0.483$).

3.1.2. Honey bee mortality

The mortality rate in the tau-fluvalinate (F) (spring test) assay was 67.5% for dimethoate (DIM) at the end of the study, while the mortality rate in the controls was ≤ 15% (0% for the C+ group (syrup with acetone) and 2.5% for the C- group (syrup)). In the F (autumn test), the mortality rate for DIM was 100% and 3.33% for C- and 13.3% for C+ . Finally, in the CMP assay, the mortality rate for DIM was 100%, 5% for C-, and 0% for C+ .

3.1.3. Determination LC_{50} and LDD_{50}

The chronic toxicity LC_{50} and LDD_{50} values are shown in Table 2. When studying exposure to coumaphos, an LDD_{50} of 0.539 μg/bee/day ($\chi 2 = 32.166$, $p = 0.021$) was obtained. Similarly, of the two tests carried out with tau-fluvalinate, the first gave a LDD_{50} of 12.74 μg/bee/day ($\chi 2 = 42.163$, $p = 0.001$), while the second resulted on a LDD_{50} of 8.84 μg/bee/day ($\chi 2 = 59.950$, $p < 0.001$).

3.2. Biomarker responses

3.2.1. In vivo approach: biomarker values and Integrated Biomarker Response (IBR) in exposed honey bees

Considering the values of each biomarker for all the treatments applied (results showed as supplementary material (SM Tables 1, 2 and 3)), DMT treatment led to a significant inhibition of AChE and CbE activities, and increased GST and MDA values relative to the syrup control (C-) bees. Also, esterases AChE and CbE activities were significantly inhibited by the different concentrations of coumaphos administered. Conversely, MDA formation was slightly enhanced in bees exposed to all the concentrations of coumaphos used, except in the group exposed to 60 ppm of coumaphos.

Curiously, in the first tau-fluvalinate assay, only the MDA biomarker was significantly affected, showing a reduction with respect to the control groups in all the tau-fluvalinate concentrations. In the autumn test, some concentrations of fluvalinate (425 and 1050 ppm) produced a significant inhibition of CbE activity when 4-NPA and 4-NPB substrates were used.

The dose-response dependence effects on the biomarkers after exposure to the substances to the biomarkers in the different in vivo tests carried out are summarised in Table 3.

The IBRv2 values for each treatment and concentration are reflected in the spider plots in Supplementary Material Fig. 1. The IBRv2 value associated with the induction of greater stress in the animals was higher for coumaphos (IBRv2 = 10.65), and it was lower in the first tau-fluvalinate assay (IBRv2 = 4.27), than in the second (IBRv2 = 5.59).

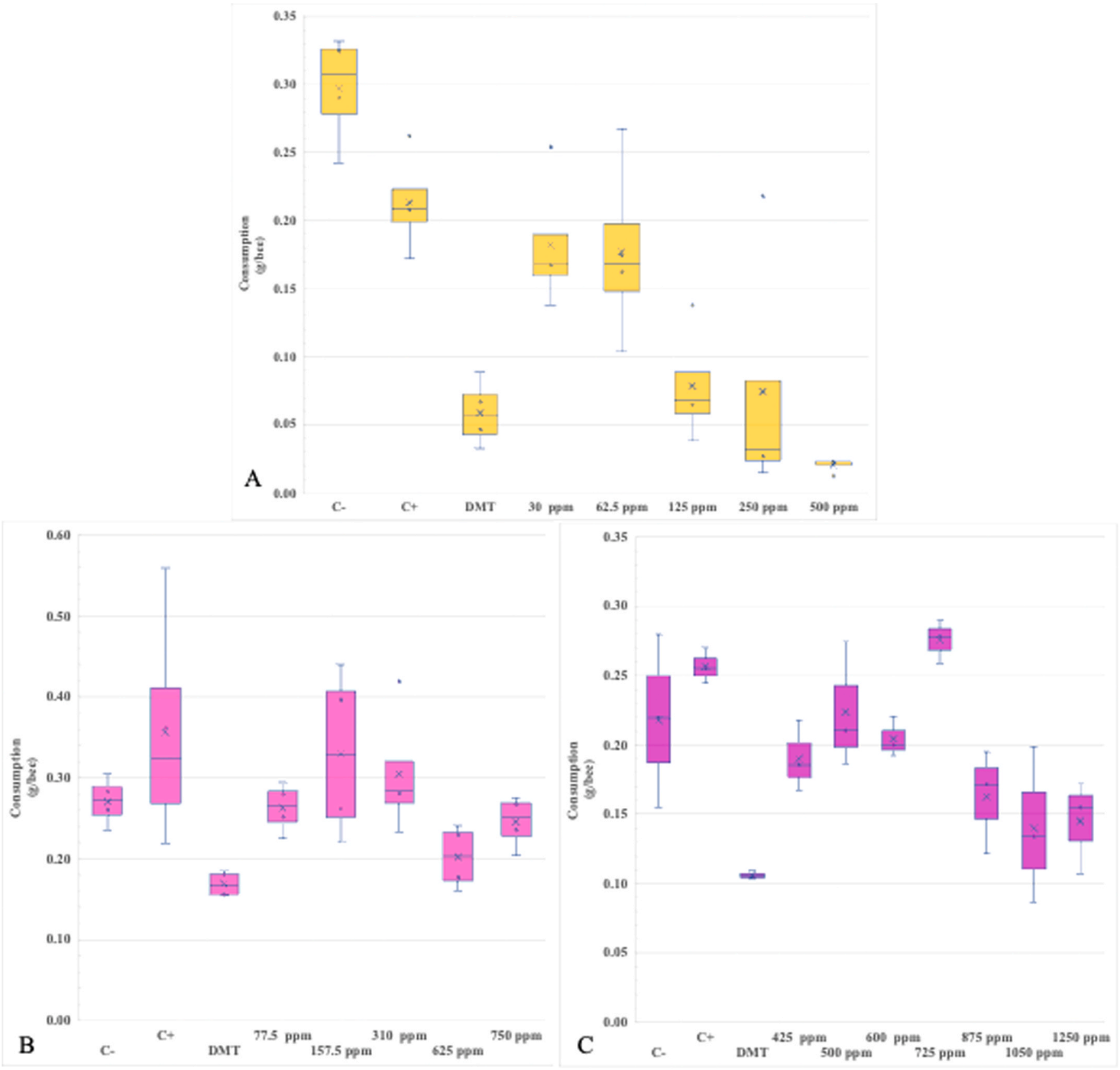

Fig. 1. Accumulated syrup consumption (g/bee) in each assay for the different treatments: coumaphos (A); tau-fluvalinate assay on spring (B) and tau-fluvalinate assay on autumn (C). Control- syrup; Control+ , syrup and acetone; DMT, dimethoate; concentrations were expressed as ppm.

Table 2
Chronic LC_{50} and LDD_{50} values for each of the trials.

Assay	LC_{50} (ppm)			LDD_{50} (µg/bee/day)		
	endpoint	95% Confidence Interval		endpoint	95% Confidence Interval	
		Lower	Upper		Lower	Upper
CMP	48.587	30.310	92.499	0.539	0.447	0.645
F (spring)	623.068	501.955	850.084	12.742	10.146	18.342
F (autumn)	530.357	471.067	583.270	8.844	7.110	10.018

Chronic test endpoints values and lower and upper limits generated by the model from the 95% Confidence Interval. CMP, coumaphos; F, tau-fluvalinate.

Clear inhibition of esterases (AChE and CbE) was evident in coumaphos assay, while increased values of neurotoxicity and biotransformation biomarkers (AChE and CbE, GST activities, respectively) and oxidative damage (MDA changes) were analysed in fluvalinate assays. A clear positively dose-dependence in IBR values were quantified for coumaphos and fluvalinate treatments (see IBR values in Fig. 2).

3.2.2. *In vitro approach: effect on biomarkers of honey bees extracts*

In this set of assays, we were interested in evaluating if the biomarkers used were also affected in vitro exposures. The main changes detected in the in vitro approach are summarized in Table 4.

IC_{50} values achieved and the goodness of fit to a sigmoidal standard curve (R^2) are showed in Table 5 and represented in Figs. 2, 3 and 4. For tau-fluvalinate, not clear effects were evident, although there was a moderate activation of AChE and CbE (Fig. 1C). No significant changes

Table 3
Summary of the main changes to the biomarkers in the different in vivo tests carried out on bees.

Substance/assay	AChE	CbE				GST	MDA
		1-NA	1-NB	4-NPA	4-NPB		
CMP	↘	↘	↘	↘	↘	↘	↔
F (spring bees)	↗	↗	↔	↔	↔	↘	↗
F (autumn bees)	↗	↔	↔	↔	↔	↗	↔

Neurotoxicity: acetylcholinesterase (AChE); Biotransformation enzyme: carboxylesterase (CbE): 1-naphthyl acetate (1-NA), 1-naphthyl butyrate (1-NB), 4-nitrophenyl acetate (4-NPA), 4-nitrophenyl butyrate (4-NPB) substrates; glutathione *S* transferase (GST) activities; Oxidative damage product, malondialdehyde (MDA). ↘: a decrease or inhibition; activation; ↗: increase or activation; ↔: no change; *Statistically significant dose-dependent differences (Spearman's correlation coefficient $p < 0.05$)

were evidence for GST or MDA biomarkers in order reach an IC_{50}.

To compare the OECD No. 245 LC_{50} values (Table 2) and the results obtained through in vitro exposures, a line has been marked on the graphs in Figs. 1, 2 and 3.

4. Discussion

The selection of the tau-fluvalinate (F) concentrations tested in the present study was based on relevant data from the literature (van Buren et al., 1992; Johnson et al., 2013), as well as a previous investigation (Benito-Murcia et al., 2022), where a concentration equivalent to the acute oral dose reported in the literature was used, combined with an estimated syrup consumption of 16.2 mg/bee/day. This concentration resulted in a mortality rate of 13.1%, serving as the baseline for our experiments. Additionally, the concentrations tested in our study fell within the range of levels detected in wax and beebread samples from Spanish apiaries (Calatayud-Vernich et al., 2018; Alonso-Prados et al., 2020), making them relevant to real-life field conditions. These considerations ensured that our study was conducted with meaningful concentrations that reflect the potential exposure of honey bees to these acaricides in their natural environment. The lethal doses calculated here are also consistent with the NOEC (No Observed Effect Concentration) of 750 μg a.i. (active ingredient)/kg (0.75 ppm) calculated previously (Sabová et al., 2022) in acute toxicity tests. In contrast, the Lethal Concentration (ppm) calculated for tau fluvalinate was 13,700 ppm (Aljedani, 2022), although this measurement is difficult to extrapolate to the actual amount of a substance that bees may consume in the field. In the latter study, Abbott's formula was used to correct for mortality during the experiment. This method was first proposed when most statistical analyses were performed manually (Abbott, 1925), and there was a need for simple numerical calculations. This has subsequently been adapted to continuous responses, in which the objective is to estimate the concentration at which a specific effect occurs relative to the mean control response. In the latter situation, there is no need to adjust for the background, and the primary purpose of normalizing to the control is to modify the data such that a probit analysis (which assumes quantal data) can be used to fit a model. Anyhow, the use of this practice violates the statistical theory of independence of the data (Green et al., 2018). In this study, it was shown that tau-fluvalinate is more toxic in autumn bees than in spring bees, and the ability of a toxicant to accumulate within an exposed organism can be estimated by its bioconcentration factor (BCF), calculated from its octanol-water partition coefficient (Kow value) (LU et al., 2014), which reflects its ability to accumulate in fat within organisms. Both tau-fluvalinate (log Kow= 7.02) and coumaphos (log Kow= 4.13) are extremely lipophilic substances, which could be explained by their greater accumulation in the fatty tissues of bees emerging in autumn than in spring, which defines it as a toxin that can accumulate at its target site and irreversibly bind to the target molecules (Holder, 2016). However, if each molecule causes only one unit of injury, then the cumulative injury is still proportional to the exposure time, which is greater in autumn bees probably due to the larger amount of these substances in fat body tissues.

In the case of tau-fluvalinte, the acute contact LD_{50} in adults ranges from 0.448 to 18.8 μg/bee and the acute oral LD_{50} is 9.20 μg/bee, while those of coumaphos by contact are 6.2–31.2 μg/bee and acute oral 26 μg/bee (reviewed by Chmiel et al., 2020), In the case of tau-fluvalinate, the chronic oral lethal doses were similar to those reported by Johnson et al. (2013), but in the case of coumaphos, it was approximately 26 times more toxic. Moreover, coumaphos showed to be about 10 times more toxic than tau-fluvalinate in the different trials of this study. This outcome was not expected considering the acute toxicities reported in the literature, and it may be explained by toxic reinforcement toxicity (TRT), which results from over prolonged exposures, causing injury from exposure to even small residue levels, as they build up to lethal levels with time. Therefore, TRT toxicants have the potential to cause much greater injury than may be predicted from the exposure concentration and therefore pose a greater risk to exposed organisms (Holder, 2016). It could be hypothesized that this process occurs in the case of coumaphos, and likewise, with other acaricides detected at high frequencies, such as chlorfenvinphos (Orantes-Bermejo et al., 2010; Calatayud- Vernich et al., 2018, Alonso-Prados et al., 2020). In addition, other substances, such as neonicotinoids, the phenylpyrazole fipronil, the fungicide boscalid, and some molecules produced in the decomposition of simple sugars have been also shown to display a time-cumulative toxicity in honey bees (EFSA, 2022; Holder et al., 2018; Sánchez-Bayo, Tennekes, 2020; Simon-Delso et al., 2018; Rondeau et al., 2014). Therefore, in order to evaluate the possible bioaccumulation and TRT effects, there is a need for long-term toxicity studies with the most commonly used acaricides in colonies.

Interestingly, as reported in the review of Chmiel et al. (2020), there is a lot of variation in the data presented in the literature for oral and contact LD50, which may be due to differences in the methods used. For example, the time of exposure, the excipients present in the commercial substance, as well as the co-solvents used in these experiments, can contribute to this variability. So, in apicultural toxicology, standardized assays like OECD Guideline No. 245 or the EFSA Bee Guidance Document could play pivotal roles. Adherence to internationally recognized guidelines ensures reliable and comparable outcomes, potentially leading to more homogenous and comparable data for oral and contact LD50 and other toxicological endpoints from the literature. This advancement in data consistency can further enhance the understanding and protection of honey bee populations and the environment. The present study provides a comprehensive approach to understand the effect of chronic exposure to two of the most frequently detected acaricides in honey bee hive matrices, encompassing chronic toxicity, sub-lethal effects, and real-world exposure scenarios.

The results of IBR revealed a higher toxicity of coumaphos (10.66), associated with the induction of greater stress in the animals, with respect to tau-fluvalinate in both F assays, and also a higher toxicity of tau-fluvalinate for autumn bees than for spring bees. These results agree with those reported in Benito-Murcia et al. (2022), supporting the suitablity of these biomarkers as key tools to measure the toxicity of these acaricides in honey bees. In the case of the differences observed in the response of in vivo assays to both organophosphates (DMT and CMP), the higher concentration used for coumaphos (30–500 ppm as opposed to 0.9 ppm dimethoate) could be a plausible explanation, since the activity of the biomarkers was in general related to the dose applied. In addition, even though dimethoate was considered a more toxic compound than coumaphos according to previous data (Uhl et al., 2016), the IBR data obtained here revealed that the latter could generate more pronounced toxicity in bees at the higher doses administered.

In terms of the biomarkers analysed and like other organophosphates, coumaphos inhibited AChE and CbE activity in vivo (Yao et al.,

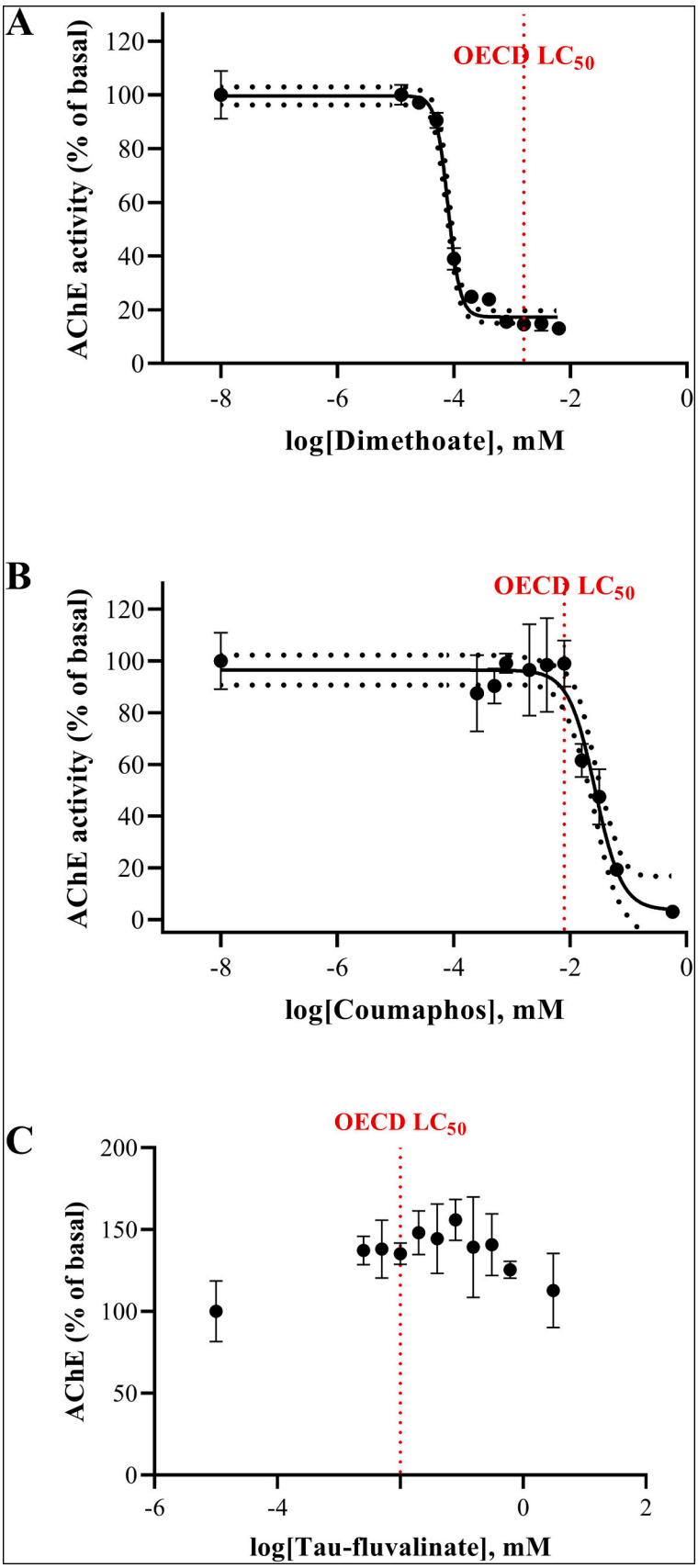

Fig. 2. In vitro effect of dimethoate (A), coumaphos (B) and tau-fluvalinate (C) on acetylcholinesterase (AChE) activity in honey bees extracts. Curves are representative of three independent extract preparations and the graphs show the mean enzyme activity (•) ± SD. In all graphs, the vertical discontinuous line indicates the LC50 obtained in the in vivo assay following OECD TG245 for each pesticide.

Table 4
Summary of the main changes to the biomarkers in the different in vitro tests carried out on bees.

Substance/assay	AChE	CbE				GST	MDA
		1-NA	1-NB	4-NPA	4-NPB		
CMP	↘	↘	↘	↘	↘	↔	↘
DMT	↘	↘	↘	↘	↘	↔	↗
F	↗	↗	↔	↔	↗	↔	↔

Neurotoxicity: acetylcholinesterase (AChE); Biotransformation enzyme: carboxylesterase (CbE): 1-naphthyl acetate (1-NA), 1-naphthyl butyrate (1-NB), 4-nitrophenyl acetate (4-NPA), 4-nitrophenyl butyrate (4-NPB) substrates; glutathione *S* transferase (GST) activities; Oxidative damage product, malondialdehyde (MDA). ↘: a decrease or inhibition; activation; ↗: increase or activation; ↔: no change; *Statistically significant dose-dependent differences (Spearman's correlation coefficient $p < 0.05$).

2018). In the case of AchE, exposure to coumaphos led to a dose-dependent inhibition in assays of both bees and bee tissues, a response that has been observed previously in bees exposed to this organophosphate (Davies and Williamson, 2009). This inhibition is particularly relevant since dampening the activity of this enzyme alters the motor performance of bees (Williamson et al., 2013), which could affect their foraging activity and hive hygiene behaviour (Gashout et al., 2020), and put at risk pollination and bee colony health. The CbE activity in bees exposed to coumaphos is inhibited in a dose dependent manner in vitro for all substrates, an effect that was not evident in the in vivo assays, perhaps due to the high mortality of bees during the first 100 h of the assay. Exposure to coumaphos also resulted in the inhibition of GST, as occurs in bees exposed to another organophosphate insecticide, acephate (Yao et al., 2018). The tendency of coumaphos to inhibit GST activity was also observed in vitro, although the dose-response relationship was not statistically significant.

The exposure of bee tissues to tau-fluvalinate activated of 1-NA increased as the dose of tau-fluvalinate augmented. The frequent use of this acaricide since the late 1980s (Trouiller, 1998) and the persistence of its residues in different hive matrices (Alonso-Prados et al., 2020; Benito-Murcia et al., 2021; Calatayud-Vernich et al., 2018) could have led to bees developing specific detoxification pathways for this acaricide. GST activity increased in the second trial as a function of the concentration of tau-fluvalinate administered, which might also reflect a defence and detoxification mechanism of bees against pyrethroids that would contribute to the resistance to this class of insecticide, as seen in other insects (Kostaropoulos et al., 2001; Ramoutar et al., 2009). In fact, GST plays an important role in the removal of pyrethroid-induced lipid peroxides and in sequestering pyrethroids or their metabolites (Kostaropoulos et al., 2001; Vontas et al., 2001), which could explain the results obtained here. This kind of acaricide is also commonly found in adult honey bee tissues and brood (Fulton et al., 2019; Murcia-Morales et al., 2022), due to its active transfer from lipophilic matrices (wax and stored pollen). It is important to emphasize that it is impossible to obtain bees that have not been exposed to these substances for at least one month before the experiments due to the high prevalence of these compounds in colonies where beekeeping is professionally practised (Alonso-Prados et al., 2020; Calatayud-Vernich et al., 2018; Chauzat et al., 2011; Marti et al., 2022; Mullin et al., 2010; Perugini et al., 2018).

For tau-fluvalinate, our results did not show clear effects on certain biomarkers, although a moderate activation of acetylcholinesterase (AchE) and carboxylesterase (CbE) was observed. However, no significant changes were evidenced for glutathione S-transferase (GST) or malondialdehyde (MDA) biomarkers to reach an IC50 (half-maximal inhibitory concentration). These findings suggest that the chronic effects of tau-fluvalinate on honey bees may not be as pronounced as those observed with coumaphos. While tau-fluvalinate demonstrated a dose-dependent activation of GST in vivo, this effect did not reach a significant threshold for an IC_{50} response. The AchE activity also increased after acute lethal exposure of bees to tau-fluvalinate in previous studies, in which a dose-response effect was also obtained (Gashout et al., 2018). AchE plays a fundamental role in the CNS of bees and shifts in its activity could have negative effects on bees (Galizia et al., 2011). In fact, previous studies showed that fluvalinate exposure alters memory, learning and the response to sucrose in bees (Frost et al., 2013), all processes involving CNS activity. Indeed, the observed effects of tau-fluvalinate on AchE activation and the potential lack of significant oxidative stress in our study align with findings from other neurotoxins, such as neonicotinoid insecticides. Neonicotinoids have been shown to increase AchE expression in adult bees at sub-lethal doses, leading to alterations in memory, learning, and food searching behaviour (Boyle and Sheppard, 2017; Palmer et al., 2013; Siviter et al., 2018). This similarity in AchE activation suggests a common neurotoxic mechanism shared by tau-fluvalinate and neonicotinoids, which may affect bees' central nervous system and related behaviours. However, it is essential to consider that the lack of significant changes in certain biomarkers may be attributed to the dosages applied or exposure duration, both could influence the manifestation of oxidative stress effects in our experiments and could require further investigation. Furthermore, it is worth exploring additional biomarkers, such as cytochrome P450, which may play a relevant role in the detoxification of tau-fluvalinate (Johnson et al., 2009; Mao et al., 2011).

The observed mean Integrated Biomarker Response (IBR) values in bees exposed to tau-fluvalinate showed a significant difference between the first and second trials, consistent with the higher concentrations administered and the higher lethality recorded in the second trial. This suggests that higher doses of tau-fluvalinate may lead to a more pronounced alteration in the biomarkers' activity, indicating a dose-dependent effect. Conversely, the IBR value for coumaphos was higher than that for tau-fluvalinate, indicating that the biomarkers used exhibited greater variability in response to coumaphos exposure, which had a lower LD50 and therefore produced higher toxicity. The elevated IBR value for coumaphos aligns with the previously determined LD50 results for this organophosphate, suggesting a strong correlation between toxicity and biomarker alterations. Moreover, when comparing the IBR values of coumaphos to those of dimethoate or tau-fluvalinate, the IBR for coumaphos was higher, indicating a greater response to this compound. This observation is consistent with the higher toxicity of

Table 5
In vitro IC_{50} values (ppm) for each of the trials.

Assay	AChE		CbE							
	IC_{50}	R^2	1-NA		1-NB		4-NPA		4-NPB	
			IC_{50}	R^2	**IC_{50}**	R^2	**IC_{50}**	R^2	**IC_{50}**	R^2
DMT	**0.015**	*0.98*	**1.81**	*0.83*	**1.44**	*0.79*	**1.50**	*0.55*	**1.33**	*0.84*
CMP	**8.66**	*0.91*	**1.24**	*0.94*	**6.09**	*0.96*	**0.39**	*0.96*	**0.49**	*0.97*

In vitro test endpoints IC_{50} (half maximal inhibitory concentration) achieved for Neurotoxicity: acetylcholinesterase (AChE); Biotransformation enzyme: carboxylesterase (CbE): 1-naphthyl acetate (1-NA), 1-naphthyl butyrate (1-NB), 4-nitrophenyl acetate (4-NPA), 4-nitrophenyl butyrate (4-NPB) substrates. CMP, coumaphos; F, tau-fluvalinate.

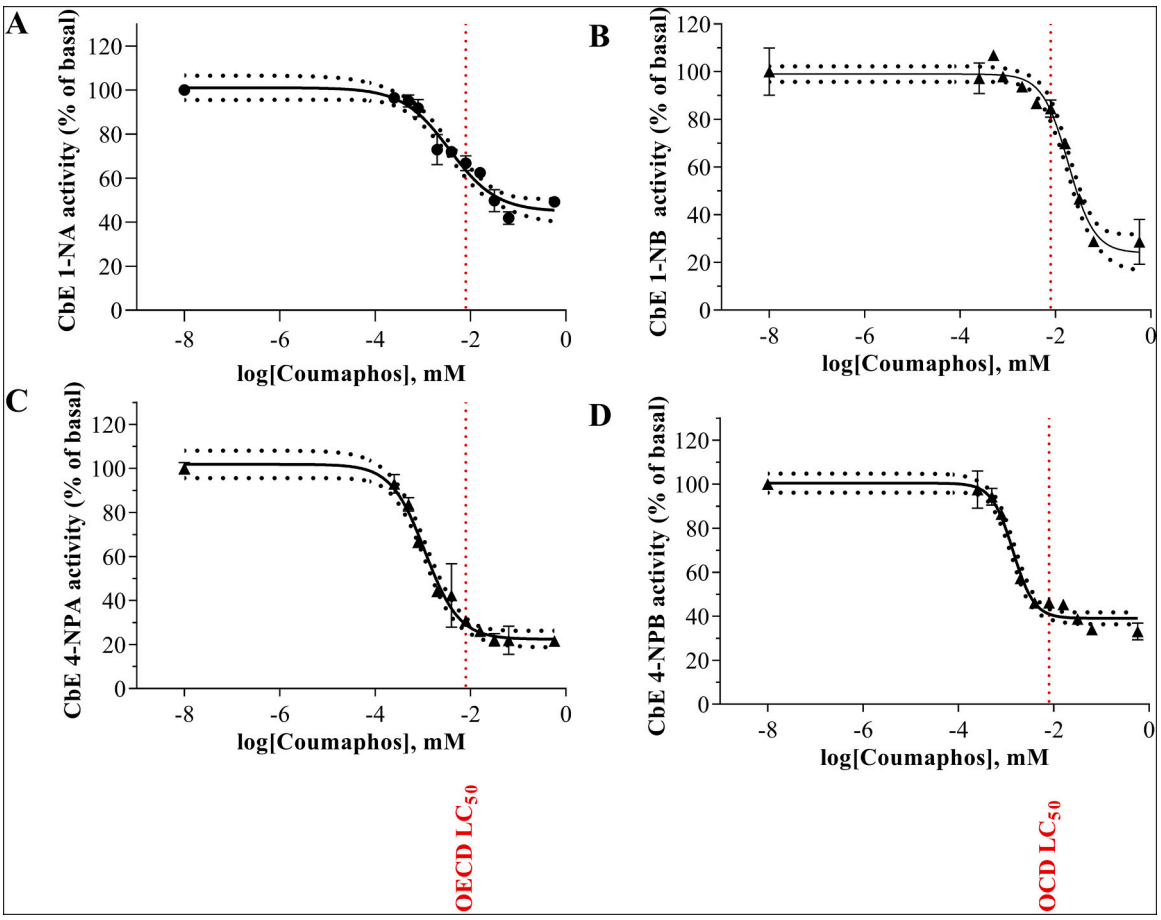

Fig. 3. In vitro effect coumaphos on carboxylesterase (CbE) activity measured in honey bees extracts with different substrates. A: 1-naphtyl acetate (1-NA), B: 1-naphtyl butyrate (1-NB), C: 4-nitrophenyl acetate (4-NPA) and D: 4-nitrophenyl butyrate (4-NPB). Curves are representative of three independent extract preparations and the graphs show the mean enzyme activity (•) ± SD. In all graphs, the vertical discontinuous line indicates the LC50 obtained in the in vivo assay following OECD TG245 for each pesticide.

coumaphos compared to dimethoate or tau-fluvalinate, as evidenced by the LD50 results for the pyrethroid. These findings further highlight the importance of using IBR as an integrated approach to assess the overall impact of pesticides on honey bees and indicate the potential of coumaphos to induce more pronounced toxicity based on the biomarker response. In summary, the IBR values revealed distinct patterns in response to tau-fluvalinate and coumaphos exposures, with higher doses of tau-fluvalinate leading to greater changes in biomarker activity. Coumaphos, on the other hand, demonstrated a higher IBR value, suggesting a more significant impact on the biomarkers studied, consistent with its higher toxicity compared to other pesticides. The IBR approach provides valuable insights into the differential toxicity of pesticides and the alterations they induce in honey bee biomarkers, contributing to a comprehensive understanding of their effects on bee health.

The in vitro analyses conducted in this study revealed varying levels of biomarker inhibition depending on the substrate used. The AchE activity exhibited higher sensitivity to DMT compared to CMP, which is consistent with the known lethality of dimethoate. In contrast, the IC_{50} to CbE activity with different substrates were found to be far from the LC_{50} values calculated in our study. These results emphasize the importance of employing these biomarkers for assessing the initial signs of bee exposure to organophosphate compounds, as they can serve as early indicators of toxicity. The effect of exposure to DMT on the biomarkers measured in bees in vivo was unclear, except for the slight inhibition of CbE activity. This suggests that the in vitro analyses might provide a more sensitive and reliable indication of biomarker responses to dimethoate exposure, although a bioactive compound such as omethoate would need to be included in the experiments to properly determine the effects of dimethoate on AchE, CbE, and MDA, as performed in other species (Albendín et al., 2017; Özkol et al., 2012; Sun et al., 2002).

On the other hand, the in vivo assay revealed a marked response of AChE, CbE, and GST to coumaphos exposure, indicating that honey bees could potentially serve as a sentinel species for detecting contamination by this organophosphate in biomonitoring programs. The in vitro analyses allowed a comparison of biomarker responses to different substrates and highlighted the potential of these biomarkers as sensitive indicators of organophosphate exposure. These findings highlight the importance of considering both in vitro and in vivo analyses to comprehensively assess the impact of pesticides on honey bees. These studies could provide valuable insights into the potential toxicological effects of acaricides and underscore the need for standardized and reliable methodologies in toxicological research.

5. Conclusions

This work represents the first approximation to calculate the chronic LC_{50} of coumaphos and tau-fluvalinate, two of the most frequently detected acaricide residues in honey bee matrices.

This study shows that chronic exposure to tau-fluvalinate and coumaphos modulates the activity of enzymes related to neurotoxicity, xenobiotic biotransformation and oxidative stress. Coumaphos has a dose-dependent inhibitory effect on AChE and GST in vivo, while in vitro it has a similar effect on CbE, AChE and MDA. In vivo, GST is activated

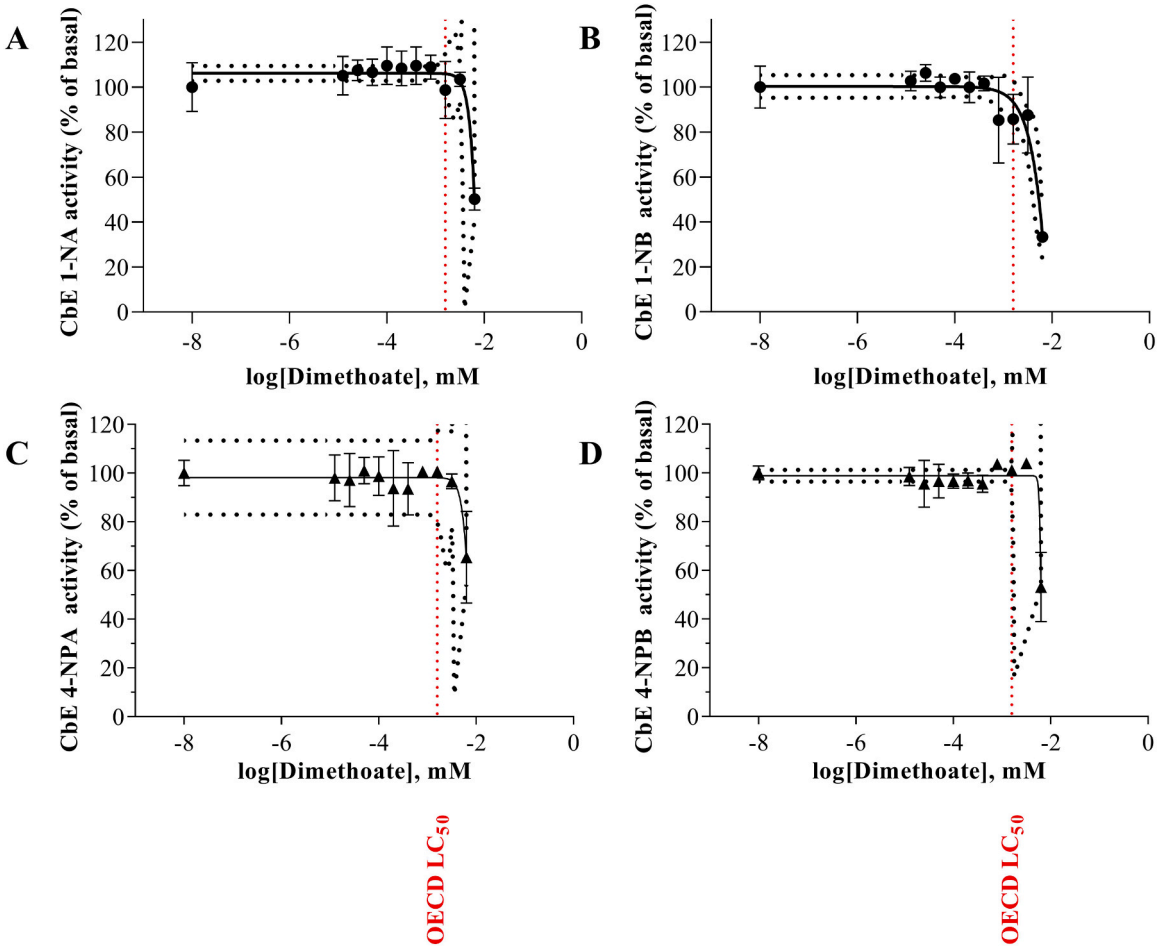

Fig. 4. In vitro effect dimethoate on carboxylesterase (CbE) activity measured in honey bees extracts with different substrates. A: 1-naphtyl acetate (1-NA), B: 1-naphtyl butyrate (1-NB), C: 4-nitrophenyl acetate (4-NPA) and D: 4-nitrophenyl butyrate (4-NPB). Curves are representative of three independent extract preparations and the graphs show the mean enzyme activity (•) ± SD. In all graphs, the vertical discontinuous line indicates the LC50 obtained in the in vivo assay following OECD TG245 for each pesticide.

by tau-fluvalinate in a dose-dependent manner, while the same effect was only seen when the 1-NA substrate was used to assess CbE activity in vitro. The results of the tau-fluvalinate assays suggest that there is some detoxification of tau-fluvalinate by honey bees at low doses. Finally, the IBR was higher in response to coumaphos than to tau-fluvalinate, indicating that this organophosphate is more toxic than the pyrethroid, both in terms of lethality (as determined previously) and of the alterations to the biomarker activity studied. Interestingly, the responses of the biomarkers to the coumaphos exposure are similar in the in vitro and in vivo approaches. Thus, assays and the set of biomarkers used in this study could be a valuable tool in toxicological studies. The development of this research line could provide evidence on the mechanism of action of different substances, such as tau-fluvalinate and/or other pyrethroids, that induce toxicity and that can trigger detoxification processes in honey bees and/or resistance mechanisms in other arthropods as Varroa.

This study highlights the chronic toxicity of tau-fluvalinate and coumaphos on honey bees, with a significant focus on the novel determination of LDD_{50} for tau-fluvalinate. The implications of our findings extend to the evaluation of bee health, pollinator conservation, and the need for comprehensive, long-term studies on commonly used acaricides in colonies. Further research is warranted to optimize chronic exposure testing, explore tissue tropism, and investigate metabolites of tau-fluvalinate to better understand bioaccumulation and toxic reinforcement toxicity dynamics. These findings highlight the importance of considering in vitro and in vivo analyses to comprehensively assess the impact of pesticides on honey bees. Overall, this study provides valuable information on the potential toxicological effects of acaricides and underscores the need for standardized and reliable test and methodologies in toxicological research, which will aid in the development of sustainable strategies for pollinator conservation and ecosystem health.

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.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.etap.2023.104330.

References

Agrebi, N., el, Traynor, K., Wilmart, O., Tosi, S., Leinartz, L., Danneels, E., de Graaf, D.C., Saegerman, C., 2020. Pesticide and veterinary drug residues in Belgian beeswax: occurrence, toxicity, and risk to honey bees. Sci. Total Environ. 745, 141036 https://doi.org/10.1016/j.scitotenv.2020.141036.

Albendín, G., Arellano, J.M., Mánuel-Vez, M.P., Sarasquete, C., Arufe, M.I., 2017. Characterization and in vitro sensitivity of cholinesterases of gilthead seabream (*Sparus aurata*) to organophosphate pesticides. Fish. Physiol. Biochem. 43, 455–464. https://doi.org/10.1007/s10695-016-0299-y.

Aljedani, D.M., 2022. Evaluation of the potential toxicity of tau-fluvalinate on adult honeybees *Apis mellifer*a, under laboratory conditions. Appl. Ecol. Environ. Res. 20, 55–67. https://doi.org/10.15666/AEER/2001_055067.

Alonso-Prados, E., Muñoz, I., de la Rúa, P., Serrano, J., Fernández-Alba, A.R., García-Valcárcel, A.I., Hernando, M.D., Alonso, Á., Alonso-Prados, J.L., Bartolomé, C., Maside, X., Barrios, L., Martín-Hernández, R., Higes, M., 2020. The toxic unit approach as a risk indicator in honey bees surveillance programmes: a case of study in *Apis mellifera iberiensis*. Sci. Total Environ. 698, 134208 https://doi.org/10.1016/j.scitotenv.2019.134208.

Becher, M.A., Grimm, V., Thorbek, P., Horn, J., Kennedy, P.J., Osborne, J.L., 2014. BEEHAVE: a systems model of honeybee colony dynamics and foraging to explore multifactorial causes of colony failure. J. Appl. Ecol. 51, 470–482. https://doi.org/10.1111/1365-2664.12222.

Benito-Murcia, M., Bartolomé, C., Maside, X., Bernal, J., Luis Bernal, J., Jesús Del Nozal, M., Meana, A., Botías, C., Martín-Hernández, R., Higes, M., 2021. Residual tau-fluvalinate in honey bee colonies is coupled with evidence for selection for varroa destructor resistance to pyrethroids. Insects 12, 731. https://doi.org/10.3390/insects12080731.

Benito-Murcia, M., Botías, C., Martín-Hernández, R., Higes, M., Soler, F., Perez-Lopez, M., Míguez-Santiyán, M.P., Martinez-Morcillo, S., 2022. Evaluating the chronic effect of two varroacides using multiple biomarkers and an integrated biological response index. Environ. Toxicol. Pharmacol. 94, 103920 https://doi.org/10.1016/J.ETAP.2022.103920.

Bogdanov, S., Liebefeld, A., 2006. Contaminants of bee products. Apidologie 37, 1–18. https://doi.org/10.1051/APIDO:2005043.

Bogdanov, S., Kilchenmann, V., Imdorf, A., 1997. Acaricide residues in beeswax and honey, 239-246 Bee Prod.. *J. Apic. Res* 37, 57–67. https://doi.org/10.1007/978-1-4757-9371-0_29.

Boyle, N.K., Sheppard, W.S., 2017. A scientific note on seasonal levels of pesticide residues in honey bee worker tissues. Apidologie 48, 128–130. https://doi.org/10.1007/s13592-016-0455-5.

Buendía-Abad, M., Higes, M., Martín-Hernández, R., Barrios, L., Meana, A., Fernández Fernández, A., Osuna, A., de Pablos, L.M., 2021. Workflow of *Lotmaria passim* isolation: experimental infection with a low-passage strain causes higher honeybee mortality rates than the PRA-403 reference strain. Int. J. Parasitol. Parasites Wildl. 14, 68–74. https://doi.org/10.1016/J.IJPPAW.2020.12.003.

Calatayud-Vernich, P., Calatayud, F., Simó, E., Picó, Y., 2018. Pesticide residues in honey bees, pollen and beeswax: assessing beehive exposure. Environ. Pollut. 241, 106–114. https://doi.org/10.1016/j.envpol.2018.05.062.

Caliani, I., Campani, T., Conti, B., Cosci, F., Bedini, S., D'Agostino, A., Ammendola, A., di Noi, A., Gori, A., Casini, S., 2021. Multi-biomarker approach and IBR index to evaluate the effects of different contaminants on the ecotoxicological status of *Apis mellifera*. Ecotoxicol. Environ. Saf. 208, 111486 https://doi.org/10.1016/J.ECOENV.2020.111486.

Celli, G., Maccagnani, B., 2003. Honey bees as bioindicators of environmental pollution. Bull. Insect 56, 13–139.

Chauzat, M.P., Martel, A.C., Cougoule, N., Porta, P., Lachaize, J., Zeggane, S., Aubert, M., Carpentier, P., Faucon, J.P., 2011. An assessment of honeybee colony matrices, *Apis mellifera* (Hymenoptera: Apidae) to monitor pesticide presence in continental France. Environ. Toxicol. Chem. 30, 103–111. https://doi.org/10.1002/ETC.361.

Chmiel, J.A., Daisley, B.A., Pitek, A.P., Thompson, G.J., Reid, G., 2020. Understanding the effects of sublethal pesticide exposure on honey bees: a role for probiotics as mediators of environmental stress. Front. Ecol. Evol. 8, 22 https://doi.org/10.3389/fevo.2020.00022.

Claudianos, C., Ranson, H., Johnson, R.M., Biswas, S., Schuler, M.A., Berenbaum, M.R., Feyereisen, R., Oakeshott, J.G., 2006. A deficit of detoxification enzymes: pesticide sensitivity and environmental response in the honeybee. Insect Mol. Biol. 15, 615. https://doi.org/10.1111/J.1365-2583.2006.00672.X.

Cui, F., Li, M.X., Chang, H.J., Mao, Y., Zhang, H.Y., Lu, L.X., Yan, S.G., Lang, M.L., Liu, L., Qiao, C.L., 2015. Carboxylesterase-mediated insecticide resistance: Quantitative increase induces broader metabolic resistance than qualitative change. Pestic. Biochem. Physiol. 121, 88–96. https://doi.org/10.1016/J.PESTBP.2014.12.016.

David, A., Botías, C., Abdul-Sada, A., Nicholls, E., Rotheray, E.L., Hill, E.M., Goulson, D., 2016. Widespread contamination of wildflower and bee-collected pollen with complex mixtures of neonicotinoids and fungicides commonly applied to crops. Environ. Int. 88, 169–178. https://doi.org/10.1016/J.ENVINT.2015.12.011.

Davies, T., Williamson, M., 2009. Interactions of pyrethroids with the voltage-gated sodium channel. Bayer. Crop. J. 62, 159–178.

EFSA, 2022. Evaluation of the risks for animal health related to the presence of hydroxymethylfurfural (HMF) in feed for honey bees. J. efsa 20 (4), e07227. https://doi.org/10.2903/j.efsa.2022.7227.

Fournier, D., Mutero, A., 1994. Modification of acetylcholinesterase as a mechanism of resistance to insecticides. Comp. *Biochem.* Physiol. C. Pharmacol. Toxicol. Endocrinol. 108, 19–31. https://doi.org/10.1016/1367-8280(94)90084-1.

Frost, E.H., Shutler, D., Hillier, N.K., 2013. Effects of fluvalinate on honey bee learning, memory, responsiveness to sucrose, and survival. J. Exp. Biol. 216, 2931–2938. https://doi.org/10.1242/jeb.086538.

Fulton, C.A., Huff Hartz, K.E., Fell, R.D., Brewster, C.C., Reeve, J.D., Lydy, M.J., 2019. An assessment of pesticide exposures and land use of honey bees in Virginia. Chemosphere 222, 489–493. https://doi.org/10.1016/j.chemosphere.2019.01.156.

Galizia, C., Eisenhardt, D., Giurfa, M., 2011. Honeybee Neurobiology and Behavior. ISBN: 978–94-007–2099-2.

Gashout, H.A., Goodwin, P.H., Guzman-Novoa, E., 2018. Lethality of synthetic and natural acaricides to worker honey bees (*Apis mellifera*) and their impact on the expression of health and detoxification-related genes. *Environ. Sci. Pollut. Res.* Int. 25, 34730–34739. https://doi.org/10.1007/S11356-018-3205-6.

Gashout, H.A., Guzman-Novoa, E., Goodwin, P.H., 2020. Synthetic and natural acaricides impair hygienic and foraging behaviors of honey bees. Apidologie 51, 1155–1165.

Ghini, S., Fernández, M., Picó, Y., Marín, R., Fini, F., Mañes, J., Girotti, S., 2004. Occurrence and Distribution of Pesticides in the Province of Bologna, Italy, Using Honeybees as Bioindicators. Arch. Environ. Contam. Toxicol. 47, 479–488. https://doi.org/10.1007/S00244-003-3219-Y.

John W. Green, Timothy A. Springer, Henrik Holbech, 2018, Statistical Analysis of Ecotoxicity Studies. ISBN: 978–1-119–08834-9.

Holder, P. 2016. Little and often makes much: identifying the time reinforced toxicity of pesticides and their impacts on bees. Exeter University.

Holder, P.J., Jones, A., Tyler, C.R., Cresswell, J.E., 2018. Fipronil pesticide as a suspect in historical mass mortalities of honey bees, 13033-12038 PNAS 115. https://doi.org/10.1073/pnas.1804934115.

Jackson, C.J., Liu, J.W., Carr, P.D., Younus, F., Coppin, C., Meirelles, T., Lethier, M., Pandey, G., Ollis, D.L., Russell, R.J., Weik, M., Oakeshott, J.G., 2013. Structure and function of an insect α-carboxylesterase (αesterase7) associated with insecticide resistance. Proc. Natl. Acad. Sci. Usa. 110, 10177–10182. https://doi.org/10.1073/PNAS.1304097110.

Johnson, R.M., 2015. Honey bee toxicology. Annu. Rev. Entomol. 60, 415–434. https://doi.org/10.1146/ANNUREV-ENTO-011613-162005.

Johnson, R.M., Pollock, H.S., Berenbaum, M.R., 2009. Synergistic interactions between in-hive miticides in *Apis mellifera. J. Econ. Entomol.*, 102, 474–479. https://doi.org/10.1603/029.102.0202.

Johnson, R.M., Dahlgren, L., Siegfried, B.D., Ellis, M.D., 2013. Acaricide, fungicide and drug interactions in honey bees (*Apis mellifera*). PLoS One 8, e54092. https://doi.org/10.1371/journal.pone.0054092.

Kast, C., Kilchenmann, V., Charrière, J.D., 2021. Long-term monitoring of lipophilic acaricide residues in commercial Swiss beeswax. Pest. Manag. Sci. 77, 4026–4033. https://doi.org/10.1002/PS.6427.

Kochansky, J., Wilzer, K., Feldlaufer, M., 2001. Comparison of the transfer of coumaphos from beeswax into syrup and honey. Apidologie 32, 119–125. https://doi.org/10.1051/APIDO:2001117.

Kostaropoulos, I., Papadopoulos, A.I., Metaxakis, A., Boukouvala, E., Papadopoulou-Mourkidou, E., 2001. Glutathione S-transferase in the defence against pyrethroids in insects. Insect Biochem. Mol. Biol. 313–319. https://doi.org/10.1016/S0965-1748(00)00123-5.

Lambert, O., Veyrand, B., Durand, S., Marchand, P., Bizec, B. le, Piroux, M., Puyo, S., Thorin, C., Delbac, F., Pouliquen, H., 2012. Polycyclic aromatic hydrocarbons: bees, honey and pollen as sentinels for environmental chemical contaminants. Chemosphere 86, 98–104. https://doi.org/10.1016/J.CHEMOSPHERE.2011.09.025.

Lodesani, M., Costa, C., Serra, G., Colombo, R., Sabatini, A.G., 2008. Acaricide residues in beeswax after conversion to organic beekeeping methods. Apidologie 39, 324–333. https://doi.org/10.1051/apido:2008012.

LU, C.S., Warchol, K.M., Callahan, R.A., 2014. Sub-lethal exposure to neonicotinoids impaired honey bees winterization before proceeding to colony collapse disorder. Bull. Insect 67, 125–130.

Mao, W., Schuler, M.A., Berenbaum, M.R., 2011. CYP9Q-mediated detoxification of acaricides in the honey bee (*Apis mellifera*). Proc. Natl. Acad. Sci. U. S. A. 108, 12657–12662. https://doi.org/10.1073/pnas.1109535108.

Marti, J.N.G., Kilchenmann, V., Kast, C., 2022. Evaluation of pesticide residues in commercial Swiss beeswax collected in 2019 using ultra-high performance liquid chromatographic analysis. Environ. Sci. Pollut. Res. 29, 32054–32064. https://doi.org/10.1007/S11356-021-18363-9.

Martín-Hernández, R., Higes, M., Sagastume, S., Juarranz, Á., Dias-Almeida, J., Budge, G. E., Meana, A., Boonham, N., 2017. Microsporidia infection impacts the host cell's cycle and reduces host cell apoptosis. PLoS One 12, e0170183. https://doi.org/10.1371/JOURNAL.PONE.0170183.

Mullin, Frazier, M., Frazier, J.L., Ashcraft, S., Simonds, R., Vanengelsdorp, D., Pettis, J.S., 2010. High levels of miticides and agrochemicals in north american apiaries: implications for honey bee health. PLoS ONE 5, e9754. https://doi.org/10.1371/journal.pone.0009754.

Murcia-Morales, M., Heinzen, H., Parrilla-Vázquez, P., Gómez-Ramos, M., del, M., Fernández-Alba, A.R., 2022. Presence and distribution of pesticides in apicultural products: a critical appraisal. Trends Anal. Chem. 146, 116506 https://doi.org/10.1016/J.TRAC.2021.116506.

Nair, V., O'Neil, C.L., Wang, P.G., 2008. Malondialdehyde. Encyclopedia of Reagents for Organic Synthesis. ISBN 13: 9780470842898.

Newcomb, R.D., Campbell, P.M., Ollis, D.L., Cheah, E., Russell, R.J., Oakeshott, J.G., 1997. A single amino acid substitution converts a carboxylesterase to an organophosphorus hydrolase and confers insecticide resistance on a blowfly. Proc. Natl. Acad. Sci. U. S. A. 94, 7464–7468. https://doi.org/10.1073/PNAS.94.14.7464.

Nicewicz, Ł., Nicewicz, A.W., Kafel, A., Nakonieczny, M., 2021. Set of stress biomarkers as a practical tool in the assessment of multistress effect using honeybees from urban and rural areas as a model organism: a pilot study. Environ. Sci. Pollut. Res. 28, 9084–9096. https://doi.org/10.1007/S11356-020-11338-2.

Olgun, T., Dayioğlu, M., Özsoy-Tağkiran, N., 2020. Pesticide and pathogen induced oxidative stress in honey bees (*Apis mellifera* L.). Mellifera 20, 32–52.

Orantes-Bermejo, F.J., Pajuelo, A.G., Megías, M.M., Fernández-Píñar, C.T., 2010. Pesticide residues in beeswax and beebread samples collected from honey bee colonies (*Apis mellifera* L.) in Spain. Possible implications for bee losses. J. Apic. Res. 49, 243–250. https://doi.org/10.3896/IBRA.1.49.3.03.

Özkol, H., Tuluce, Y., Celik, I., Isik, I., 2012. Omethoate modulates some oxidant/antioxidant parameters in frogs (*Rana ridibunda Pallas*). Toxicol. Ind. Health 28, 320–326. https://doi.org/10.1177/0748233711410917.

Palmer, M.J., Moffat, C., Saranzewa, N., Harvey, J., Wright, G.A., Connolly, C.N., 2013. Cholinergic pesticides cause mushroom body neuronal inactivation in honeybees. Nat. Commun. 4, 1–8. https://doi.org/10.1038/ncomms2648.

Peakall, D.B., 1994. The role of biomarkers in environmental assessment. Introduction. Ecotoxicology 3, 157–160. https://doi.org/10.1007/BF00117080.

Perugini, M., Tulini, S.M.R., Zezza, D., Fenucci, S., Conte, A., Amorena, M., 2018. Occurrence of agrochemical residues in beeswax samples collected in Italy during 2013-2015. Sci. Total Environ. 625, 470–476. https://doi.org/10.1016/J.SCITOTENV.2017.12.321.

Praveena, A., Sanjayan, K.P., 2011. inhibition of acetylcholinesterase in three insects of economic importance by linalool, a monoterpene phytochemical. Int. J. Pest. Manag 240–345.

Ramoutar, D., Cowles, R.S., Alm, S.R., 2009. Pyrethroid resistance mediated by enzyme detoxification in *Listronotus maculicollis* (Coleoptera: Curculionidae) from Connecticut. J. Econ. Entomol. 102, 1203–1208. https://doi.org/10.1603/029.102.0345.

Rondeau, G., Sánchez-Bayo, F., Tennekes, H. a, Decourtye, A., Ramírez-Romero, R., Desneux, N., 2014. Delayed and time-cumulative toxicity of imidacloprid in bees, ants and termites. Sci. Rep. 4, 5566 https://doi.org/10.1038/srep05566.

Sabová, L., Cingeľová Maruščáková, I., Koleničová, S., Mudroňová, D., Holečková, B., Sabo, R., Sobeková, A., Majchrák, T., Ratvaj, M., 2022. The adverse effects of synthetic acaricide tau-fluvalinate (tech.) on winter adult honey bees. Environ. Toxicol. Pharmacol. 92, 103861 https://doi.org/10.1016/J.ETAP.2022.103861.

Sanchez-Bayo, F., Goka, K., 2014. Pesticide residues and bees-a risk assessment. PLoS One 9, e094482. https://doi.org/10.1371/journal.pone.0094482.

Sánchez-Bayo, F., Tennekes, H.A., 2020. Time-cumulative toxicity of neonicotinoids: experimental evidence and implications for environmental risk assessments. Int J. Environ. Res. Public. Health 171629. https://doi.org/10.3390/ijerph17051629.

Shimshoni, J.A., Sperling, R., Massarwa, M., Chen, Y., Bommuraj, V., Borisover, M., Barel, S., 2019. Pesticide distribution and depletion kinetic determination in honey and beeswax: Model for pesticide occurrence and distribution in beehive products. PLoS One 14, e0212631. https://doi.org/10.1371/JOURNAL.PONE.0212631.

Simon-Delso, N., Martin, G.S., Bruneau, E., Hautier, L., 2018. Time-to-death approach to reveal chronic and cumulative toxicity of a fungicide for honeybees not revealed with the standard ten-day test. Sci. Rep. 8, 7241 https://doi.org/10.1038/s41598-018-24746-9.

Siviter, H., Koricheva, J., Brown, M.J.F., Leadbeater, E., 2018. Quantifying the impact of pesticides on learning and memory in bees. J. Appl. Ecol. 55, 2812–2821. https://doi.org/10.1111/1365-2664.13193.

Sponsler, D.B., Johnson, R.M., 2017. Mechanistic modeling of pesticide exposure: the missing keystone of honey bee toxicology. Environ. Toxicol. Chem. 36, 871–881. https://doi.org/10.1002/ETC.3661.

Sun, L., Gao, X., Sinica, B.Z., 2002. Characteristics of carboxylesterase in omethoate-resistant and susceptible strains of cotton aphid. *Aphis gossypii*. *J. Econ. Entomol.* 98, 1325–1332.

Trouiller, J., 1998. Monitoring *Varroa jacobsoni* resistance to pyrethroids in western Europe. Apidologie 29, 537–546. https://doi.org/10.1051/apido:19980606.

Uhl, P., Franke, L.A., Rehberg, C., Wollmann, C., Stahlschmidt, P., Jeker, L., Brühl, C.A., 2016. Interspecific sensitivity of bees towards dimethoate and implications for environmental risk assessment. Sci. Rep. 6 (1), 7. https://doi.org/10.1038/srep34439.

Van Buren, N.W.M., Marien, A.G.H., Oudejans, R.C.H.M., Velthuis, H.H.W., 1992. Perizin, an acaricide to combat the mite *Varroa jacobsoni*: its distribution in and influence on the honeybee *Apis mellifera*. Physiol. Entomol. 17, 288–296. https://doi.org/10.1111/J.1365-3032.1992.TB01024.X.

Vontas, J.G., Small, G.J., Hemingway, J., 2001. Glutathione S-transferases as antioxidant defence agents confer pyrethroid resistance in *Nilaparvata lugens*. Biochem. J. 357, 65–72. https://doi.org/10.1042/0264-6021:3570065.

Wallner, K., 1999. Varroacides and their residues in bee products. Apidologie 30, 235–248.

Wheelock, C.E., Shan, G., Ottea, J., 2005. Overview of carboxylesterases and their role in the metabolism of insecticides. J. Pestic. Sci. 30, 75–83. https://doi.org/10.1584/JPESTICS.30.75.

Williamson, S.M., Moffat, C., Gomersall, M.A.E., Saranzewa, N., Connolly, C.N., Wright, G.A., 2013. Exposure to acetylcholinesterase inhibitors alters the physiology and motor function of honeybees. Front. Physiol. 4, 13. https://doi.org/10.3389/FPHYS.2013.00013.

Yao, J., Zhu, Y.C., Adamczyk, J., Luttrell, R., 2018. Influences of acephate and mixtures with other commonly used pesticides on honey bee (*Apis mellifera*) survival and detoxification enzyme activities. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 209, 9–17. https://doi.org/10.1016/J.CBPC.2018.03.005.