Chemosphere 287 (2022) 132214

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

An *in vitro* model for assessing the toxicity of pesticides in beeswax on honey bee larvae

Christina Kast *, Verena Kilchenmann

Agroscope, Swiss Bee Research Centre, Schwarzenburgstrasse 161, Bern, 3003, Switzerland

HIGHLIGHTS

- Model evaluating the toxicity of residues in beeswax on honey bee larvae.
- Model for the migration of pesticides from beeswax into the larval diet.
- Coumaphos levels in beeswax up to 20 mg/kg were non-lethal.
- LC_{50} = 55.9 mg/kg for chronic exposure of larvae to coumaphos in beeswax.
- LC_{50} = 12.5 mg/kg for dietary exposure of larvae to coumaphos.

GRAPHICAL ABSTRACT

ARTICLE INFO

Handling Editor: A. Gies

Keywords:
Apis mellifera
Larval rearing
Larval toxicity
Pesticide
Coumaphos
Beeswax
Varroa destructor

ABSTRACT

While many studies have examined residue levels in beeswax, little is known about the levels that pose a risk for honey bee development. In an *in vitro* study, we aimed to assess the toxicity of pesticides in wax for worker larvae using coumaphos as a model substance. First, we reared larvae in beeswax with the aim to correlate the larval toxicity to the corresponding levels of coumaphos in beeswax. In a second step, we tested to which extent coumaphos migrates from the beeswax into the larval diet and if such dietary levels are toxic to larvae.

We observed dose-related toxicity when larvae were exposed to coumaphos concentrations in beeswax from 30 to 100 mg/kg. The lethal concentration in 50% of the individuals (LC_{50}) was calculated to be 55.9 mg/kg, while the no observed effect concentration (NOEC) for exposure of larvae to coumaphos in wax was 20 mg/kg. Additional test series without larvae allowed to assess the migration of coumaphos from the beeswax into the diet. The resulting dietary coumaphos concentrations were four to five times lower than the initial concentrations in wax. In accordance, the LC_{50} for chronic exposure of larvae to coumaphos in the diet was 12.5 mg/kg, which was 4.5 times lower than the LC_{50} obtained for wax exposure. Finally, a coumaphos level of 20 mg/kg in wax led to a dietary concentration of 3.9 mg/kg that was close to the NOEC of 3 mg/kg obtained in the diet.

In conclusion, both experimental approaches suggest that coumaphos concentrations of up to 20 mg/kg in wax are non-lethal.

* Corresponding author.
E-mail address: christina.kast@agroscope.admin.ch (C. Kast).

https://doi.org/10.1016/j.chemosphere.2021.132214
Received 5 July 2021; Received in revised form 3 September 2021; Accepted 7 September 2021
Available online 8 September 2021

1. Introduction

Losses in honey bee (*Apis mellifera*) colonies are associated with many causes, including poor nutrition, queen quality, parasites and brood diseases, and exposure to pesticides. In particular, *Varroa destructor* and its associated viruses are a major threat to honey bees (Dahle, 2010; Guzmán-Novoa et al., 2010; Rosenkranz et al., 2010). To fight the *Varroa* mite, beekeepers use veterinary drugs that can accumulate in the hive. Additionally, bees may bring pesticides into the colonies when they forage for pollen and nectar. Lipophilic pesticides, which include veterinary drugs and plant protection products, mainly accumulate in beeswax. The most important contaminants quantitatively in beeswax are acaricides that beekeepers use on a regular basis to treat their colonies against *V. destructor* (Bogdanov et al., 2002; Calatayud-Vernich et al., 2017).

According to good beekeeping practices, wax combs are exchanged on a regular basis. Beekeepers melt the old combs as well as capping wax to produce wax blocks. Subsequently, these blocks are used for the production of new foundation sheets, which serve as templates for the bees to build new combs. When wax is recycled, contaminants such as coumaphos remain in the wax and are still present in the newly produced wax foundation sheets (Bogdanov et al., 1998; Martel et al., 2007), potentially affecting honey bee health.

To date, experimental studies of the continuous exposure of honey bees to contaminants in beeswax are limited. The highly sensitive honey bee larvae are exposed to contaminants if these substances migrate from the beeswax into the larval jelly or if larvae come into direct contact with the beeswax (Wilmart et al., 2021). We chose coumaphos as a model substance to study the effect of residues in beeswax, since coumaphos is one of the most frequently detected residues in beeswax. Coumaphos displays a high prevalence in beeswax of European origin, such as from Belgium (Ravoet et al., 2015; Wilmart et al., 2016; El Agrebi et al., 2020), France (Chauzat et al., 2011), Germany (Wallner, 1999, 2014; Spiewok, 2017; Shimshoni et al., 2019; Alkassab et al., 2020), Italy (Boi et al., 2016; Porrini et al., 2016; Perugini et al., 2018), Spain (Orantes-Bermejo et al., 2010; Calatayud-Vernich et al., 2017; Lozano et al., 2019; Murcia Morales et al., 2020), and Switzerland (Bogdanov, 2006; Kast et al., 2021), as well as in beeswax of North American origin (Mullin et al., 2010; Wu et al., 2011; Ostiguy et al., 2019; Fulton et al., 2019) and in wax from Uruguay (Harriet et al., 2017). Maximal concentrations of up to 26.9 mg/kg and 91.9 mg/kg, respectively, have been reported for European and North American comb wax (Calatayud-Vernich et al., 2017; Mullin et al., 2010). Previous studies have shown that coumaphos residues in beeswax can negatively affect queen larvae and queen quality. Beeswax containing coumaphos at 100 mg/kg used for queen rearing resulted in a rejection rate of queen cells above 50% (Pettis et al., 2004), and the surviving queens weighed less and showed reduced performance (Collins et al., 2004). Furthermore, exposure to a combination of coumaphos and *tau*-fluvalinate in beeswax during development affected adult queens' egg-laying rate (Walsh et al., 2020). Other studies have reported that this combination of pesticides in beeswax affected drone sperm viability (Fischer and Rangel, 2018). A recent study also showed the transfer of coumaphos from the beeswax to the brood (Murcia Morales et al., 2020), thus potentially affecting larval development.

In this study, we aimed to establish an experimental model for testing the risk of pesticide residues in beeswax for worker larvae using coumaphos as a test substance. We were particularly interested in the levels of coumaphos in beeswax that do not result in increased larval mortality. For this purpose, we reared larvae in coumaphos-containing beeswax and assessed mortality rates up to the adult stage. Further, we investigated the extent to which coumaphos migrates from the beeswax into the larval diet and whether the resulting dietary coumaphos levels affected mortality rates.

2. Materials and methods

2.1. Honey bee colonies

The honey bee *(Apis mellifera)* colonies were located at the Swiss Bee Research Centre at Agroscope, Berne, Switzerland (46°55'49"N 7°25'9"E). At least three months before the experiments, all colonies were treated for *Varroa* infestation using organic acids. The colonies tested negative for European foul brood. First instar larvae were obtained from four bee colonies as described earlier (Lucchetti et al., 2018).

2.2. Larval diet

Larval diets A, B, and C contained sugars (D (+)-glucose anhydrous and D (−)-fructose; Merck, Darmstadt, Germany), yeast extract (Becton, Dickinson and Company, Allschwil, Switzerland), and royal jelly previously produced at the Swiss Bee Research Centre. The sugar/yeast solution (10 ml) used for the preparation of diet A consisted of 1.2 g glucose, 1.2 g fructose, 0.2 g yeast extract, and 8.4 g MilliQ water, while 10 ml of sugar/yeast solution for diet B contained 1.5 g glucose, 1.5 g fructose, 0.3 g yeast extract, and 8.0 g water. Finally, 10 ml of sugar/yeast solution for diet C was composed of 1.8 g glucose, 1.8 g fructose, 0.4 g yeast extract, and 7.45 g water. The solutions were filtered through a 0.2-μm mesh cellulose acetate filter (Hahnemuehle, Dassel, Germany) and combined 1 + 1 (v/w) with royal jelly. The final composition of the diets (g/100 g) are listed in Table 1.

2.3. Chronic exposure of honey bee larvae to coumaphos in beeswax or in the diet

Two chronic exposure test series were performed in line with the study of Aupinel et al. (2005) with minor modifications. In the first test series, larvae were exposed to coumaphos through beeswax, and in the second, they were exposed to coumaphos added to the diet. During June and July 2018, at least four independent test series were performed for each concentration in beeswax and at least six independent test series for each concentration in the diet.

For exposure tests with coumaphos in beeswax, we prepared beeswax containing eight concentrations of coumaphos (PESTANAL™; Sigma-Aldrich, Buchs, Switzerland; No. 45403), ranging from 5 to 100 mg/kg. Coumaphos was dissolved in acetone (SupraSolv; Merck, Darmstadt, Germany) and added to our own beeswax containing no coumaphos. The beeswax samples were melted at 80 °C and shaken by hand to obtain homogenous wax samples. The coumaphos concentration in beeswax was verified prior to the experiments using the protocol that was published in Kast et al. (2020).

Melted beeswax (75 μL, which corresponds to 70 mg wax) was pipetted into a Polystyrene grafting cell (code CNE/3; Nicoplast Society,

Table 1
Composition of diets A, B, and C.

	Yeast extract g/100 g	Glucose g/100 g	Fructose g/100 g	Royal Jelly g/100 g	Water g/100 g
Diet A	0.953	5.714	5.714	47.619	40.000
Diet B	1.409	7.042	7.042	46.948	37.559
Diet C	1.865	8.392	8.392	46.620	34.732

Maisod, France). The cell was placed at 85 °C before rotating it at room temperature in such a way that a thin layer of beeswax coated the inner surface of the cell. The coated cells were transferred into 48-well tissue culture plates (Greiner Bio-One; Frickenhausen, Germany) previously filled with a piece of a cotton dental rolls (Ø 8 mm; Hartmann, Neuhausen, Switzerland). The dental rolls were soaked with 500 μL of a solution of 15.5 ml glycerol 85% (Merck, Darmstadt, Germany) filled up to 100 ml with a solution of 0.4 g/100 g methylbenzethonium chloride (Sigma-Aldrich, Steinheim, Germany). On day 1, the first instar larvae were grafted with paint brushes (3/0) and placed in the beeswax-coated cells containing 20 μL of diet A. For the tests series with coumaphos in beeswax, all diets (A, B, and C) were prepared without adding coumaphos.

For the exposure test series with coumaphos in the diet, we prepared diets containing eight concentrations of coumaphos ranging from 1 to 30 mg/kg. Coumaphos was dissolved in acetone and added at equal concentrations to the diets A, B, and C. An equal amount of acetone was supplemented in the diet for the negative controls. On day 1, larvae in the first instar stage were placed in cells containing 10 μL of diet A without coumaphos. After grafting, another 10 μL of diet A containing coumaphos was added to the cells to obtain the desired concentration in diet A.

The rearing experiments were performed according to the protocol previously described (Lucchetti et al., 2018). Briefly, the plates containing the cells were placed at 34.5 °C in a chamber of a relative humidity of 95%. On day 3, larvae were fed 20 μL of diet B, while on days 4, 5, and 6, larvae were fed 30, 40, and 50 μL of diet C, respectively. From days 3–8, we monitored larval mortality daily and discarded dead larvae without replacement. On day 7, the remaining food was removed and the cells were transferred in a chamber of a humidity of 70%. Furthermore, on day 11, we verified whether metamorphosis was complete. We discarded dead pupae on the following days. Finally, on day 15, we placed the culture plates individually in plastic containers until the bees emerged.

To calculate the cumulative coumaphos dose per larva, daily coumaphos doses were adjusted to correct for the density increase in the larval diets from A to C (for details, see Table 2).

2.4. Migration of coumaphos from the beeswax to the diet

To test if coumaphos migrates from beeswax into the larval diet, we performed an additional test series in 2019 on beeswax-coated cells containing diet without larvae. The tested coumaphos concentrations in beeswax were zero, 10, 20, 50, and 100 mg/kg. These concentrations were verified prior to the experiment (Kast et al., 2020). For each concentration, 48 cells were coated with 75 μL beeswax each and placed into 48-well tissue culture plates containing cotton dental rolls soaked with 500 μL of 15.5 ml/100 ml glycerol 85% (no methylbenzethonium chloride). Subsequently, 20 μL of diet A (without coumaphos) was added to each cell. The tissue culture plates containing the cells were then placed in the humidity chamber at 95% RH, and the chamber was placed in an incubator at 34.5 °C. After 48 h, the diet was harvested from the cells. The diet from 16 cells was combined, resulting in three test samples (average weight 250 mg) per 48 well plate. The entire experiment was repeated three times, starting on three different days.

2.5. Sample preparation for extraction of coumaphos from the diet

The samples were prepared according to a modified QuEChERS method, based on a procedure previously described by Zheng et al. (2018). The diet (0.25 g) was weighed in a 50-ml polypropylene conical tube. Subsequently, 2.5 ml of water was added and the tube was shaken by hand for 1 min. Next, 6 ml of acetonitrile (SupraSolv; Merck, Darmstadt, Germany) was added, and the tube was mixed for 5 min at maximum speed on an orbital shaker (Heidolph UNIMAX 2010). After adding 2 g of magnesium sulphate anhydrous (Puriss. p. a.; Honeywell, Seelze, Germany; No. 63136), 0.5 g sodium chloride (EMSURE®; Merck, Darmstadt, Germany; No. 1.06404), 0.5 g tri-sodium citrate dihydrate (EMSURE®; Merck, Darmstadt, Germany; No 1.06448), and 0.25 g sodium hydrogen citrate sesquihydrate (ReagentPlus®; Sigma-Aldrich, Steinheim, Germany; No. 359084), the tube was shaken again for 10 min. The sample was centrifuged at 4000 *g* (Eppendorf Centrifuge 5804) at 4 °C for 15 min. Subsequently, the supernatant was added to a 15-ml tube containing 75 mg of primary-secondary amine (PSA) (Bondesil-PSA 40 μm; Agilent Technologies, USA; No. 12213024), 75 mg of C18 sorbent (Bondesil-C18 40 μm, Agilent Technologies, USA; No. 12213012), and 450 mg of magnesium sulphate. The tube was mixed twice for 1 min on a vortex and then centrifuged at 4500 *g* (Multifuge 1 S-R Heraeus) at 4 °C for 20 min. The supernatant was transferred to a new tube and dried with a rotary vacuum evaporator. Subsequently, the sample was reconstituted in 1 ml acetonitrile by mixing for 1 min on a vortex, a tip of a spatula of sodium sulphate (EMSURE®; Merck, Darmstadt, Germany; No. 1.06639) was added, and the mixing was repeated one more time. Lastly, the sample was filtered with nylon membrane filter (Chromafil AO-45/15 MS 0.45 μm; Macherey-Nagel, Düren, Germany; No. 729049) for analysis.

2.6. Gas chromatography analysis of coumaphos in the diet

Gas chromatography analysis was performed on a Thermo Trace Ultra 2000 gas chromatograph coupled with an MS/MS triple quadrupole (Thermo Quantum), as previously described (Kast et al., 2020) using a retention capillary column deactivated with OV-1701-OH (0.53 mm ID) of 50 cm and a DB-1 analytical capillary column (J + W, 0.25 mm ID, 0.25 μm film thickness) of 30 m. Briefly, 1 μl of the final extract of the diet was injected on the column. The gas chromatograph temperature program was 2.0 min at 75 °C, 75°–250 °C at 5 °C/min, 250°–300 °C at 3 °C/min, where it was held for 50 min. The source temperature (TSQ Quantum) was 250 °C and the ionisation energy I was 70eV. For identification of coumaphos, the transitions of *m/z* 362 to 334

Table 2
Cumulative coumaphos dose per larva.

		Coumaphos (μg/larva)					
		Diet A	Diet B	Diet C	Diet C	Diet C	cumulative over 7 days
Volume (μL)		20	20	30	40	50	160
Coumaphos conc. in the diets (mg/kg)	1	0.02	0.02	0.03	0.04	0.06	0.2
	3	0.06	0.07	0.10	0.13	0.17	0.5
	5	0.11	0.11	0.17	0.22	0.28	0.9
	10	0.21	0.22	0.33	0.44	0.56	1.8
	15	0.32	0.33	0.50	0.67	0.83	2.6
	20	0.43	0.44	0.67	0.89	1.11	3.5
	25	0.54	0.55	0.83	1.11	1.39	4.4
	30	0.64	0.66	1.00	1.33	1.67	5.3

(CE10), *m/z* 362 to 109 (CE25), and *m/z* 226 to 163 (CE18) were used, and for quantification the transition *m/z* 226 to 163.

Quantification was achieved through matrix matched external calibration with coumaphos PESTANAL™. The limit of quantification (LOQ) was 0.008 mg/L, which corresponds to 0.032 mg/kg in diet A. The recoveries were performed in triplicate in 0.25 g royal jelly (Allwex Food Trading, Bremen, Germany) at spiking levels of 0.04, 0.4, 4, and 40 mg/kg and were between 80% and 106% on average.

2.7. Statistics

Kaplan-Meier curves of survival probabilities were estimated and depicted using functions of the R (R Core Team, 2018) package survival (Therneau, 2015; Therneau and Grambsch, 2000). Stratified logrank tests as well as pairwise comparisons (Tukey contrasts, Hothorn et al., 2008) as post hoc tests in Cox regressions showed that the factors experiment and colony cannot be neglected in the estimation of LC_{50}, LOEC, and NOEC values of coumaphos. Therefore, common dose-response approaches (log-logistic functions) were not applicable. Instead, binomial logistic regression models, including terms for experiment and colony and their interaction with treatment (with and without control mortality correction, according to Schneider-Orelli), were applied to the mortality estimates. The latter were calculated as proportions of the number of dead larvae and the initial number of larvae for each combination of experiment/treatment/colony. Two outlying mortality observations were identified and excluded for diet and one for wax on the basis of diagnostic plots and Bonferroni outlier tests available in the package Rcmdr (Fox and Bouchet-Valat, 2018; Fox, 2005, Fox, 2017); negative mortality values induced by the Schneider-Orelli correction of some low-concentration treatments had to be excluded from the regression models of the corrected mortalities.

Intercept and slope estimates of the fitted logistic models were used to estimate the median lethal concentration (LC_{50}) values with the function dose. p () of the MASS package (Venables and Ripley, 2002); consistent lowest observed effect concentration (LOEC) and no observed effect concentration (NOEC) values were obtained by Dunnett contrasts in Cox regression and logistic regression applying the function glht () of the R package multcomp (Hothorn et al., 2008). Compact letter displays for the survival graphs based on Tukey contrasts were prepared by the function cld () of the same package applied to the respective Cox regression models. Statistical significance was assumed for p values < 0.01.

3. Results

3.1. Exposure of honey bee larvae to coumaphos in the beeswax

A chronic exposure test series on honey bee larvae was performed in wax supplemented with eight different concentrations of coumaphos ranging from 5 to 100 mg/kg. No significant differences in survival up to the adult stage were observed between the control and coumaphos concentrations in beeswax up to 20 mg/kg ($p = 0.72$). The emergence rates were 74% (control), 68% (5 mg/kg), 65% (10 mg/kg), and 66% (20 mg/kg). However, significant differences as compared to the control were obtained at coumaphos levels of 30 mg/kg and above (30 mg/kg, $p < 0.01$; 50 mg/kg and above, $p < 0.001$). The emergence rate was 56% for a coumaphos concentration of 30 mg/kg and 42%, 33%, and 35% for 50 mg/kg, 60 mg/kg and 70 mg/kg, respectively. Coumaphos at a concentration of 100 mg/kg induced a mortality rate of 84% up to the adult stage, since only 16% of the larvae fed with coumaphos at a concentration of 100 mg/kg completed metamorphosis and subsequently emerged as adults (Fig. 1).

For coumaphos in wax, the LC_{50} recorded on day 22 (adult emergence) was estimated at 33.7 mg/kg without correction for the control mortality and at 55.9 mg/kg with correction for the control mortality (Table 3). The LOEC with a statistically significant difference from the control group was estimated at 30 mg/kg (Dunnett contrasts in Cox regression $p < 0.01$), while the NOEC was 20 mg/kg (Table 3). The same LOEC and NOEC values were obtained using a logistic regression model for survival proportions. In conclusion, coumaphos concentrations from 30 to 100 mg/kg in beeswax showed significant dose-related toxicity in honey bee larvae, while concentrations of 20 mg/kg and below were non-lethal.

3.2. Migration of coumaphos from beeswax into the diet

In the second step, we investigated the extent to which coumaphos present in beeswax migrates into the diet. We chose experimental conditions in accordance with the previously performed test series. A sample of 20 µl of diet A was exposed to beeswax-coated cells for two days, thus reflecting conditions for the exposure of early stage larvae. Coumaphos in beeswax at concentrations of 10, 20, 50, and 100 mg/kg resulted in dietary concentrations of 2.7, 3.9, 11.4, and 19.8 mg/kg, respectively (Table 4). Hence, coumaphos migrated from beeswax into the diet, in which concentrations were between one-fourth and one-fifth of the initial concentrations in beeswax.

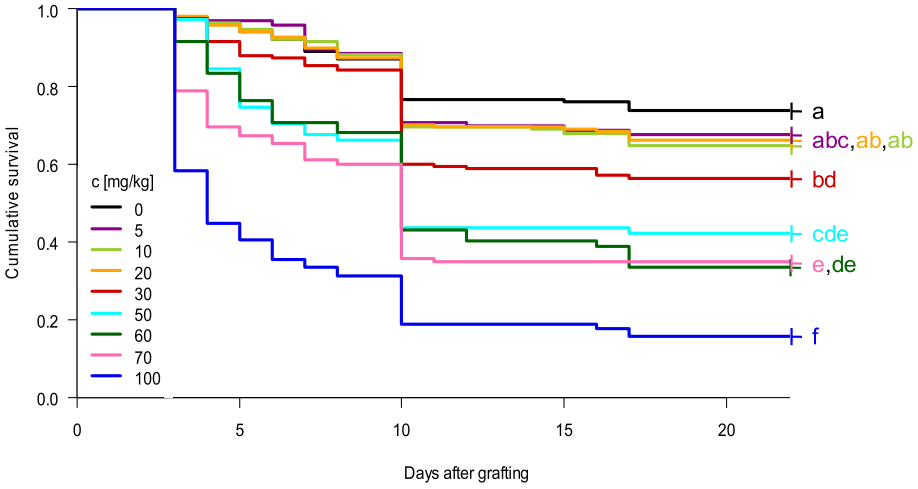

Fig. 1. Toxicity of coumaphos in wax: Survival curves represent the control larvae ($n = 192$), larvae exposed to wax containing coumaphos at a concentration of 5 mg/kg ($n = 96$), 10 mg/kg ($n = 168$), 20 mg/kg ($n = 168$), 30 mg/kg ($n = 192$), 50 mg/kg ($n = 71$), 60 mg/kg ($n = 72$), 70 mg/kg ($n = 95$), and 100 mg/kg ($n = 96$). Bioassays were terminated on day 22, after the bees emerged as adults. Letters at the end of the curves designate significant differences between treatment groups, based on Tukey contrasts (p values < 0.01). At least four independent test series were performed for each concentration and survival curves show the median values.

Table 3
LC_{50}, LOEC, and NOEC, with standard errors in brackets, for larvae exposed to coumaphos in wax and in diet.

	LC_{50} (mg/kg)		LOEC	NOEC
	without correction for control mortality	with correction for control mortality	(mg/kg)	(mg/kg)
coumaphos in wax	33.7 (3.4)	55.9 (6.0)	30 (0.2)	20 (0.2)
coumaphos in diet	7.2 (0.7)	12.5 (0.7)	5 (0.2)	3 (0.2)

Table 4
Migration of coumaphos from beeswax into the diet.

Coumaphos in wax (mg/kg)	Coumaphos in diet (mg/kg)		
	mean	SD	*n*
10	2.7	0.9	8
20	3.9	0.9	8
50	11.4	2.5	9
100	19.8	4.2	9

3.3. Exposure of honey bee larvae to coumaphos in the diet

A chronic exposure test series was repeated on honey bee larvae using diets supplemented with eight different concentrations of coumaphos, ranging from 1 to 30 mg/kg. Little mortality was observed within the first two days for all the tested concentrations. No significant differences in survival up to the adult stage were observed between the control and dietary coumaphos concentrations of 1 mg/kg and 3 mg/kg ($p = 0.99$). Emergence rates were 74% (control), 64% (1 mg/kg), and 67% (3 mg/kg). However, significant differences compared to the control were obtained for higher dietary concentrations (5 mg/kg, $p < 0.01$; 10 mg/kg, $p < 0.001$). The emergence rate was 54% for a coumaphos concentration of 5 mg/kg and 36% and 25% for concentrations of 10 mg/kg and 15 mg/kg. Coumaphos at a concentrations of 20 and 25 mg/kg induced a mortality rate of 89% and 86% up to the adult stage, while less than 1% of the larvae fed with coumaphos at a concentration of 30 mg/kg completed metamorphosis and subsequently emerged as adults (Fig. 2). The LC_{50} recorded on day 22 was 7.2 mg/kg without correction and 12.5 mg/kg with correction for the control mortality (Table 3). The LC_{50} was a factor of 4.5 lower for coumaphos in the diet as compared to exposing the larvae to coumaphos in beeswax (Table 3). The LOEC was 5 mg/kg (Dunnett contrasts in Cox regression $p < 0.01$), while the NOEC was 3 mg/kg (Table 3). The same LOEC and NOEC values were obtained using a logistic regression model for survival proportions. Taken together, coumaphos dietary concentrations from 5 to 30 mg/kg showed significant dose-related toxicity in honey bee larvae, while concentrations of 3 mg/kg and below were non-lethal.

3.4. Comparison of the migrated portion of coumaphos to dietary NOEC, LOEC, and LC_{50}

A coumaphos concentration of 20 mg/kg in wax resulted in a dietary coumaphos concentration of 3.9 mg/kg, which was close to the NOEC (3 mg/kg) and below the LOEC (5 mg/kg) for larvae chronically exposed to dietary coumaphos (Fig. 3), thus further supporting that such a concentration in wax is non-lethal. On the other hand, a beeswax concentration of 50 mg/kg resulted in a dietary coumaphos concentration of 11.4 mg/kg, which was close to the LC_{50} (12.5 mg/kg), while a beeswax concentration of 100 mg/kg led to a dietary coumaphos concentration clearly above the LC_{50} (Fig. 3).

4. Discussion

Here we propose an experimental model for estimating the risk of exposure to residues in beeswax. Additionally, our study can serve as a model for assessing the migration of a pesticide from beeswax into the larval jelly. Our results of the test series exposing larvae directly to contaminated beeswax were in line with the test series on migration and dietary exposure. Hence, migration assays in combination with chronic oral toxicity assays may be sufficient for risk assessment of pesticides in beeswax.

We aimed to define the level of coumaphos in beeswax that can be tolerated by larvae as well as the critical concentration that affects larval development. *In vitro* larval rearing in wax containing coumaphos revealed that coumaphos concentrations in beeswax up to 20 mg/kg were non-lethal, while higher concentrations led to increased mortality rates. Furthermore, a coumaphos concentration of 20 mg/kg in wax resulted in a dietary coumaphos concentration that was below the LOEC of 5 mg/kg obtained for larvae chronically exposed to coumaphos in the diet. Hence, tests on wax exposure as well as tests on migration in combination with oral exposure showed that coumaphos levels up to 20 mg/kg in wax are non-lethal, with lethal effects starting at concentrations of 30 mg/kg in wax.

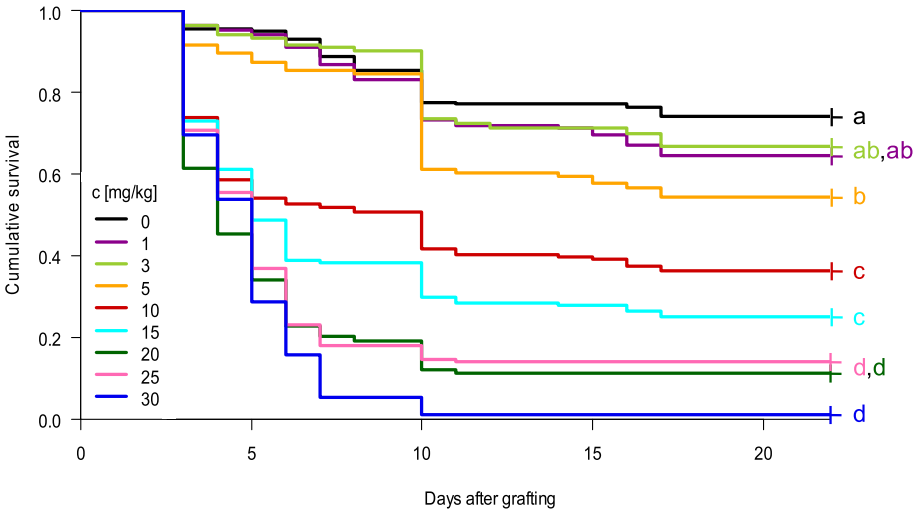

Fig. 2. Toxicity of coumaphos in the diet: Survival curves represent the control larvae ($n = 240$), larvae fed with diet containing coumaphos at a concentration of 1 mg/kg (n = 191), 3 mg/kg ($n = 192$), 5 mg/kg ($n = 239$), 10 mg/kg ($n = 264$), 15 mg/kg ($n = 144$), 20 mg/kg ($n = 168$), 25 mg/kg ($n = 144$), and 30 mg/kg ($n = 115$). Bioassays were terminated on day 22, after the bees emerged as adults. Letters at the end of the curves designate significant differences between treatment groups, based on Tukey contrasts (p values < 0.01). At least six independent test series were performed for each concentration and survival curves show the median values.

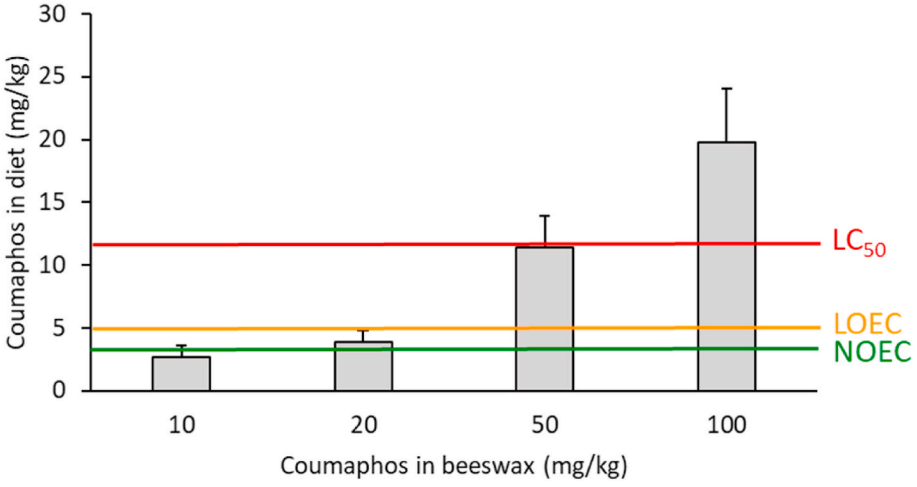

Fig. 3. Comparison of dietary coumaphos concentrations from exposure of diet to coumaphos-containing beeswax to the toxicity values obtained in larval rearing. The LC_{50} (red), LOEC (orange) and NOEC (green) were obtained by exposing honey bee larvae to various dietary concentration of coumaphos. Beeswax containing coumaphos at 50 mg/kg led to a dietary coumaphos concentration of 11.4 mg/kg, which is just below the LC_{50} of 12.5 mg/kg, while beeswax with coumaphos at 20 mg/kg led to a dietary coumaphos concentration below the LOEC of 5 mg/kg and close to the NOEC of 3 mg/kg. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

In our study, the dietary coumaphos concentrations were between a fourth and a fifth of the initial concentrations in beeswax, which corresponded well with a 4.5-fold lower LC_{50} for dietary exposure as compared to exposure through beeswax. Hence, the lethal effects observed at coumaphos levels in wax starting at 30 mg/kg can be explained by the oral exposure of larvae to coumaphos, since coumaphos migrates into the larval jelly. Indeed, previous *in vitro* studies have revealed the oral toxicity of coumaphos to honey bee larvae (Zhu et al., 2014; Dai et al., 2017, 2018; Tomé et al., 2020). An LC_{50} of 90 mg/L corresponding to an LD_{50} of 2.7 μg/larva was obtained for acute toxicity when coumaphos was given as a single dose on day 4 (Dai et al., 2017). On the other hand, we obtained an LC_{50} of 12.5 mg/kg corresponding to an LD_{50} of 2.2 μg/larva for chronic exposure of honey bee larvae to coumaphos in the diet starting on day 1 (L1-larvae). Furthermore, survival was significantly decreased as compared to the control when coumaphos was given at a concentration of 25 mg/L continuously starting at day 3 (cumulative dose of 3.5 μg/larvae; Dai et al., 2018). In contrast, in our study exposure started at day 1. Young larvae are usually more sensitive to toxins than at a later stage (Lucchetti et al., 2018), which might explain the moderately higher toxicity in our study (mortality rate of 89% for a dose of 3.5 μg/larvae; Fig. 2, Table 2). Besides oral exposure, honey bee larvae might also be exposed to coumaphos in beeswax by contact, especially when the larvae become larger and fill out the cells.

For larval rearing in beeswax, the wax quality is of special importance. At our centre, we use organic acids for *Varroa* treatment, which do not accumulate in beeswax. Since we recycle our own wax and monitor its acaricides residues (Kast et al., 2021), the presence of significant acaricide residues can be ruled out. Thus, we expect little or no effect on the mortality rates related to the wax used in this study. Standard protocols with specific requirements for control mortality are available for dietary exposure (Crailsheim et al., 2013; OECD GD 239 Guidance Document, 2016). In our test series, where coumaphos was added to the diet, the control mortality rate was 26% on day 22 (Fig. 2), which is well in line with the requirements of the OECD Guidance document (the adult emergence rate on day 22 should be equal or above 70%, which corresponds to a mortality rate of 30%). On the other hand, our control series narrowly meet the criteria given in the publication of Crailsheim et al. (2013), where levels up to 25% are tolerated, but ideally should not exceed 20%. The main reason might be the fact that mortalities related to grafting were not excluded in our study. Furthermore, the solvent acetone and hot temperatures during the experiments might have additional minor effects. Since the control mortality rate influences the calculation of the LC50 values, we have also calculated the LC_{50} values without correction of the control mortality (Table 3) for an estimation of a minimal LC_{50} level.

To date, there are no legal regulations in beekeeping on maximal levels of pesticides in beeswax. Based on our results, we suggest that coumaphos levels in beeswax should not exceed 20 mg/kg, since higher levels were associated with increased mortality. In Switzerland, commercial beeswax most likely fulfils this criterion. Since 1991, we have monitored acaricides in commercial Swiss beeswax ((Bogdanov, 2004; Kast et al., 2021). We studied annual samples representing the average values of the entire yearly production of all major manufacturers of beeswax foundations in Switzerland. In 2015, we observed an increase in coumaphos residues as compared to previous years, up to an annual value of 3.3 mg/kg for the country's entire production (Kast et al., 2021). In 2015, 2017, and 2019, maximal annual values per manufacturer were 4.5 mg/kg, 6.2 mg/kg, and 3.2 mg/kg, respectively (Kast et al., 2021). This means that the average coumaphos level in beeswax per producer is well below 20 mg/kg and thus should not affect mortality rates. However, to prevent further increase in coumaphos residue levels in beeswax, we started an information campaign in 2016 for beekeepers to advise against using this lipophilic acaricide that accumulates in beeswax. Instead, organic acid, such as formic and oxalic acids, are recommended for mite control since they do not contaminate beeswax. These measures contributed to reduced coumaphos levels in beeswax in more recent years (in 2019: annual value of 0.4 mg/kg for Switzerland; Kast et al., 2021).

Most international studies on residues in beeswax report coumaphos levels below 20 mg/kg. For example, coumaphos levels in foundation wax up to 1.0 mg/kg have been measured in Italy (mean value 0.1 mg/kg; Perugini et al., 2018) and up to 17.4 mg in Spain (mean value 9.4 mg/kg; Calatayud-Vernich et al., 2017), while beeswax from Germany contained coumaphos up to 10.9 mg/kg (mean value 0.7 mg/kg; Shimshoni et al., 2019). North American studies report coumaphos levels in brood comb wax up to 91.9 mg/kg and more recently up to 15.5 mg/kg (mean value 3.3 mg/kg or a median concentration of 0.05 mg/kg; Mullin et al., 2010, and Fulton et al., 2019, respectively). Hence, higher values were measured in brood comb wax compared to recycled beeswax. Since beeswax from various apiaries is usually combined during the recycling process, the residues of a specific compound can be diluted if beekeepers use products with different active ingredients for treatment. The highest values measured in brood combs could be related to measurements being taken shortly after treatment with a coumaphos-containing product or at locations where frames were in close contact with the product. Indeed, we measured coumaphos residues ranging from 36 to 159 mg/kg in central brood combs seven months after a single application of CheckMite®, a coumaphos-containing product authorized in various countries, including Switzerland (Kast et al., 2020). Levels were especially high at positions close to the treatment strips. These residue levels were well

above 30 mg/kg, thus potentially causing elevated mortality in worker larvae.

In the next step, studies on bee colonies should complement our *in vitro* results. While an *in vitro* study permits following each larva under standardized conditions from an early age to emergence, it must be noted that *in vitro* studies do not entirely reflect the conditions in a bee hive. Toxicity could be underestimated in our study, since exposure to coumaphos was initiated at the larval stage L1, while eggs were not exposed. On the other hand, we may overestimate toxicity. Bees add new wax when they build the combs from the foundation so the coumaphos concentration in the comb is below that of the foundation, resulting in lower exposure of the larvae. The levels of coumaphos in wax may also decline in the course of several brood cycles, and the migration of coumaphos from wax into the larval diet may diminish due to honey bee cocoons acting as a barrier (Fries et al., 1998).

An in-hive study showing that wax foundations containing coumaphos, *tau*-fluvalinate, and thymol at concentrations of 10 mg/kg each did not affect brood development (Alkassab et al., 2020). Thus, coumaphos levels up to 10 mg/kg were non-lethal, even in combination with other pesticides (Alkassab et al., 2020). On the other hand, high levels of coumaphos residues in beeswax, in combination with other contaminants, delayed larval development and affected adult longevity (Wu et al., 2011). In a recent study, a maximally tolerable coumaphos concentration of 78 mg/kg in beeswax was calculated for oral exposure of worker larvae, taking into account 10% of the LD_{50} of 2.7 μg/larva and a transfer rate of 69% (Wilmart et al., 2021). In our *in vitro* system, this coumaphos level is above the LC_{50} of 55.9 mg/kg. As suggested by Wilmart et al. (2021), laboratory experiments on migration rates between hive matrices are needed to improve the accuracy of the mathematical modelling. As discussed above, additional in-hive experiments will also be necessary to confirm the proposed maximal level in wax that is still acceptable for developing bees. Additionally, studies should include sub-lethal effects, such as longevity as adults or altered foraging behaviours, to obtain a more complete picture of the effects of coumaphos residues in beeswax.

Authors' contributions

CK and VK designed the experiments. VK performed larval rearing for exposure through beeswax. CK performed larval rearing for the dietary exposure. VK performed migration assays and chemical analysis. CK and VK interpreted the data. CK wrote the paper and VK revised it. Both authors approved the final manuscript.

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.

Acknowledgments

We thank Werner Luginbühl for the statistical analysis and the calculation of the EC_{50}, NOEC, and LOEC. We also thank Benoit Droz for helping as a beekeeper and providing the honey bee larvae.

References

Alkassab, A.T., Thorbahn, D., Frommberger, M., Bischoff, G., Pistorius, J., 2020. Effect of contamination and adulteration of wax foundations on the brood development of honey bees. Apidologie 51, 642–651. https://doi.org/10.1007/s13592-020-00749-2.

Aupinel, P., Fortini, D., Dufour, H., Tasei, J.N., Michaud, B., Odoux, J.F., Pham-Delègue, M.H., 2005. Improvement of artificial feeding in a standard *in vitro* method for rearing *Apis mellifera* larvae. Bull. Insectol. 58, 107–111. https://doi.org/10.1002/ps.1446.

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

Bogdanov, S., Imdorf, A., Charrière, J.D., Fluri, P., Kilchenmann, V., 2002. Qualität der Bienenprodukte und die Verschmutzungsquellen. Teil 1: belastung aus der Landwirtschaft und Umwelt. Schweiz. Bienen-Zeitung 125, 19–21.

Bogdanov, S., 2004. Beeswax: quality issues today. Bee World 85 (3), 46–50. https://doi.org/10.1080/0005772X.2004.11099623.

Bogdanov, S., Kilchenmann, V., Imdorf, A., 1998. Acaricide residues in some bee products. J. Apicult. Res. 37 (2), 57–67. https://doi.org/10.1080/00218839.1998.11100956.

Boi, M., Serra, G., Colombo, R., Lodesani, M., Massi, S., Costa, C., 2016. A 10-year survey of acaricide residues in beeswax analysed in Italy. Pest Manag. Sci. 72, 1366–1372. https://doi.org/10.1002/ps.4161.

Calatayud-Vernich, P., Calatayud, F., Simó, E., Picó, Y., 2017. Occurrence of pesticide residues in Spanish beeswax. Sci. Total Environ. 605–606, 745–754. https://doi.org/10.1016/j.scitotenv.2017.06.174.

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 honey bee colony matrices, *Apis mellifera* (*Hymenoptera apidae*) to monitor pesticide presence in continental France. Environ. Toxicol. Chem. 30 (1), 103–111. https://doi.org/10.1002/etc.361.

Collins, A.M., Pettis, J.S., Wilbanks, R., Feldlaufer, M.F., 2004. Performance of honey bee (*Apis mellifera*) queens reared in beeswax cells impregnated with coumaphos. J. Apicult. Res. 43 (3), 128–134. https://doi.org/10.1080/00218839.2004.11101123.

Crailsheim, K., Brodschneider, R., Aupinel, P., Behrens, D., Genersch, E., Vollmann, J., Riessberger-Gallé, U., 2013. Standard methods for artificial rearing of *Apis mellifera* larvae. J. Apicult. Res. 52 (1), 1–16. https://doi.org/10.3896/IBRA.1.52.1.05.

Dai, P., Jack, C.J., Mortensen, A.N., Bustamante, T.A., Ellis, J.D., 2018. Chronic toxicity of amitraz, coumaphos and fluvalinate to *Apis mellifera* L. larvae reared *in vitro*. Sci. Rep. 8, 5635. https://doi.org/10.1038/s41598-018-24045-3.

Dai, P., Jack, C.J., Mortensen, A.N., Ellis, J.D., 2017. Acute toxicity of five pesticides to *Apis mellifera* larvae reared *in vitro*. Pest Manag. Sci. 73, 2282–2286. https://doi.org/10.1002/ps.4608.

Dahle, B., 2010. The role of *Varroa destructor* for honey bee colony losses in Norway. J. Apicult. Res. 49 (1), 124–125. https://doi.org/10.3896/IBRA.1.49.1.26.

El Agrebi, N., 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.

Fisher, A., Rangel, J., 2018. Exposure to pesticides during development negatively affects honey bee (*Apis mellifera*) drone sperm viability. PloS One 13 (12), 208630. https://doi.org/10.1371/journal.pone.0208630.

Fox, J., Bouchet-Valat, M., 2018. Rcmdr: R Commander. R package version 2.5–1.

Fox, J., 2017. Using the R Commander: A Point-And-Click Interface for R. Chapman & Hall/CRC Press.

Fox, J., 2005. The R Commander: a basic statistics graphical user interface to R. J. Stat. Software 14 (9), 1–42.

Fries, I., Wallner, K., Rosenkranz, P., 1998. Effects on *Varroa jacobsoni* from acaricides in beeswax. J. Apicult. Res. 37 (2), 85–90. https://doi.org/10.1080/00218839.1998.11100959.

Fulton, C.A., Hartz, K.E.H., 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.

Guzmán-Novoa, E., Eccles, L., Calvete, Y., et al., 2010. *Varroa destructor* is the main culprit for the death and reduced populations of overwintered honey bee (*Apis mellifera*) colonies in Ontario, Canada. Apidologie 41, 443–450. https://doi.org/10.1051/apido/2009076.

Harriet, J., Campá, J.P., Grajales, M., Lhéritier, C., Gómez Pajuelo, A., Mendoza-Spina, Y., Carrasco-Letelier, L., 2017. Agricultural pesticides and veterinary substances in Uruguayan beeswax. Chemosphere 177, 77–83. https://doi.org/10.1016/j.chemosphere.2017.02.131.

Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous inference in general parametric models. Biom. J. 50 (3), 346–363.

Kast, C., Kilchenmann, V., Charrière, J.D., 2021. Long-term monitoring of lipophilic acaricide residues in commercial Swiss beeswax. Pest Manag. Sci. 77 (9), 4026–4033. https://doi.org/10.1002/ps.6427.

Kast, C., Kilchenmann, V., Droz, B., 2020. Distribution of coumaphos in beeswax after treatment of honey bee colonies with CheckMite® against the parasitical mite *Varroa destructor*. Apidologie 51, 112–122. https://doi.org/10.1007/s13592-019-00724-6.

Lozano, A., Hernando, M.D., Uclés, S., Hakme, E., Fernández-Alba, A.R., 2019. Identification and measurement of veterinary drug residues in beehive products. Food Chem. 274, 61–70. https://doi.org/10.1016/j.foodchem.2018.08.055.

Lucchetti, M.A., Kilchenmann, V., Glauser, G., Praz, C., Kast, C., 2018. Nursing protects honeybee larvae from secondary metabolites of pollen. Proc. R. Soc. B 285, 20172849. https://doi.org/10.1098/rspb.2017.2849.

Martel, A.C., Zeggane, S., Aurières, C., Drajnudel, P., Faucon, J.P., Aubert, M., 2007. Acaricide residues in honey and wax after treatment of honey bee colonies with Apivar® or Asuntol®50. Apidologie 38 (6), 534–544. https://doi.org/10.1051/apido:2007038.

Mullin, C.A., Frazier, M., Frazier, J.L., Ashcraft, S., Simonds, R., van Engelsdorp, D., Pettis, J.S., 2010. High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. PloS One 5 (3), 9754. http://dx.plos.org/10.1371/journal.pone.0009754.

Murcia Morales, M., Gómez Ramos, M.J., Parrilla Vázquez, P., Díaz Galiano, F.J., García Valverde, M., Gámiz López, V., Manuel Flores, J., Fernández-Alba, A.R., 2020. Distribution of chemical residues in the beehive compartments and their transfer to the honey bee brood. Sci. Total Environ. 710, 136288. https://doi.org/10.1016/j.scitotenv.2019.136288.

OECD GD 239 Guidance Document, 2016. Guidance Document on Honey Bee Larval Toxicity Test Following Repeated Exposure. Environment Directorate Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology. https://one.oecd.org/document/ENV/JM/MONO(2016)34/en/pdf.

Orantes-Bermejo, F.J., Gomez Pajuelo, A., Megias Megias, M., Torres Fernandez-Pinar, C., 2010. Pesticide residues in beeswax and beebread samples collected from honey bee colonies (*Apis mellifera* L.) in Spain: possible implications for bee losses. J. Apicult. Res. 49 (3), 243–250. https://doi.org/10.3896/IBRA.1.49.3.03.

Ostiguy, N., Drummond, F.A., Aronstein, K., Eitzer, B., Ellis, J.D., Spivak, M., Sheppard, W.S., 2019. Honey bee exposure to pesticides: a four-year nationwide study. Insects 10 (13), 1–35. https://doi.org/10.3390/insects10010013.

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.

Pettis, J.S., Collins, A.M., Wilbanks, R., Feldlaufer, M.F., 2004. Effects of coumaphos on queen rearing in the honey bee, *Apis mellifera*. Apidologie 35, 605–610. https://doi.org/10.1051/apido:2004056.

Porrini, C., Mutinelli, F., Bortolotti, L., Granato, A., Laurenson, L., Roberts, K., Gallina, A., Silvester, N., Medrzycki, P., Renzi, T., Sgolastra, F., Lodesani, M., 2016. The status of honey bee health in Italy: results from the nationwide bee monitoring network. PloS One 11 (5), 155411. https://doi.org/10.1371/journal.pone.0155411.

Ravoet, J., Reybroeck, W., de Graaf, D.C., 2015. Pesticides for apicultural and/or agricultural application found in Belgian honey bee wax combs. Bull. Environ. Contam. Toxicol. 94, 543–548. https://doi.org/10.1007/s00128-015-1511-y.

R Core Team, 2018. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. URL. https://www.R-project.org.

Rosenkranz, P., Aumeier, P., Ziegelmann, B., 2010. Biology and control of *Varroa destructor*. J. Invertebr. Pathol. 103 (1), S96–S119. https://doi.org/10.1016/j.jip.2009.07.016.

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 (2), 212631. https://doi.org/10.1371/journal.pone.0212631.

Spiewok, S., 2017. Mittelwände im Test. Deutsches Bienen-Journal 9, 6–9.

Therneau, T., 2015. A Package for Survival Analysis in S version 2.38, URL: https://CRAN.R-project.org/package=survival.

Therneau, T.M., Grambsch, P.M., 2000. Modeling Survival Data: Extending the Cox Model. Springer.

Tomé, H.V.V., Schmehl, D.R., Wedde, A.E., Godoy, R.S.M., Ravaiano, S.V., Guedes, R.N.C., Martins, G.F., Ellis, J.D., 2020. Frequently encountered pesticides can cause multiple disorders in developing worker honey bees. Environ. Pollut. 256, 113420. https://doi.org/10.1016/j.envpol.2019.113420.

Venables, W.N., Ripley, B.D., 2002. Modern Applied Statistics with S, fourth ed. Springer.

Wallner, K., 1999. Varroacides and their residues in bee products. Apidologie 30, 235–248. https://doi.org/10.1051/apido:19990212.

Wallner, K., 2014. Wachs vergisst nicht. ADIZ 12, 10–12.

Walsh, E.M., Sweet, S., Knap, A., Ing, N., Rangel, J., 2020. Queen honey bee (*Apis mellifera*) pheromone and reproductive behavior are affected by pesticide exposure during development. Behav. Ecol. Sociobiol. 74, 33. https://doi.org/10.1007/s00265-020-2810-9.

Wilmart, O., Legrève, A., Scippo, M.L., Reybroeck, W., Urbain, B., de Graaf, D.C., Steurbaut, W., Delahaut, P., Gustin, P., Nguyen, B.K., Saegerman, C., 2016. Residues in beeswax: a health risk for the consumer of honey and beeswax? J. Agric. Food Chem. 64 (44), 8425–8434. https://doi.org/10.1021/acs.jafc.6b02813.

Wilmart, O., Legrève, A., Scippo, M.L., Reybroeck, W., Urbain, B., de Graaf, D.C., Spanoghe, P., Delahaut, P., Saegerman, C., 2021. Honey bee exposure scenarios to selected residues through contaminated beeswax. Sci. Total Environ. 772, 145533. https://doi.org/10.1016/j.scitotenv.2021.145533.

Wu, J.Y., Anelli, C.M., Sheppard, W.S., 2011. Sub-lethal effects of pesticide residues in brood comb on worker honey bee (*Apis mellifera*) development and longevity. PloS One 6 (2), 14720. https://doi.org/10.1371/journal.pone.0014720.

Zheng, W., Park, J.A., Abd El-Aty, A.M., Kim, S.K., Cho, S.H., Choi, J., Yi, H., Cho, S.M., Ramadan, A., Jeong, J.H., Shim, J.H., Shin, H.C., 2018. Development and validation of modified QuEChERS method coupled with LC–MS/MS for simultaneous determination of cymiazole, fipronil, coumaphos, fluvalinate, amitraz, and its metabolite in various types of honey and royal jelly. J. Chromatogr. B 1072 (1), 60–69. https://doi.org/10.1016/j.jchromb.2017.11.011.

Zhu, W., Schmehl, D.R., Mullin, C.A., Frazier, J.L., 2014. Four common pesticides, their mixtures and a formulation solvent in the hive environment have high oral toxicity to honey bee larvae. PloS One 9 (1), 77547. https://doi.org/10.1371/journal.pone.0077547.