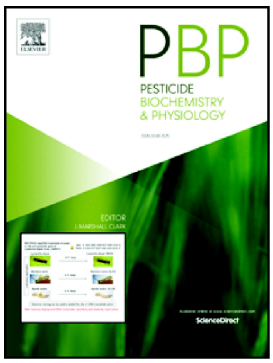


Metabolisation of thiamethoxam (a neonicotinoid pesticide) and interaction with the chronic bee paralysis virus in honeybees

M. Coulon, F. Schurr, A.-C. Martel, N. Cougoule, A. Bégaud, P. Mangoni, A. Dalmon, C. Alaux, Y. Le Conte, R. Thiéry, M. Ribière-Chabert, E. Dubois

PII: S0048-3575(17)30163-3
DOI: doi:10.1016/j.pestbp.2017.10.009
Reference: YPEST 4133

To appear in: *Pesticide Biochemistry and Physiology*

Received date: 12 April 2017
Revised date: 16 October 2017
Accepted date: 21 October 2017

Please cite this article as: M. Coulon, F. Schurr, A.-C. Martel, N. Cougoule, A. Bégaud, P. Mangoni, A. Dalmon, C. Alaux, Y. Le Conte, R. Thiéry, M. Ribière-Chabert, E. Dubois , Metabolisation of thiamethoxam (a neonicotinoid pesticide) and interaction with the chronic bee paralysis virus in honeybees. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Ypest(2017), doi:10.1016/j.pestbp.2017.10.009

Metabolisation of thiamethoxam (a neonicotinoid pesticide) and interaction with the Chronic bee paralysis virus in honeybees

M. Coulon[a, b], F. Schurr[a], A.-C. Martel[a], N. Cougoule[a], A. Bégaud[a], P. Mangoni[a], A. Dalmon[b], C. Alaux[b], Y. Le Conte[b], R. Thiéry[a], M. Ribière-Chabert[a], E. Dubois[a]

[a]ANSES Sophia Antipolis, Unit of Honeybee Pathology, 105, route des Chappes, 06902 Sophia-Antipolis, France

[b]INRA PACA, UR 406 Abeilles et Environnement, Site Agroparc, 84914 Avignon, France

Contact: eric.dubois@anses.fr

Abstract

Pathogens and pesticides are likely to co-occur in honeybee hives, but much remains to be investigated regarding their potential interactions. Here, we first investigated the metabolisation kinetics of thiamethoxam in chronically fed honeybees. We show that thiamethoxam, at a dose of 0.25 ng/bee/day, is quickly and effectively metabolised into clothianidin, throughout a 20 day exposure period. Using a similar chronic exposure to pesticide, we then studied, in a separate experiment, the impact of thiamethoxam and *Chronic bee paralysis virus* (CBPV) co-exposure in honeybees. The honeybees were exposed to the virus by contact, mimicking the natural transmission route in the hive. We demonstrate that a high dose of thiamethoxam (5.0 ng/bee/day) can cause a synergistic increase in mortality in co-exposed honeybees after 8 to 10 days of exposure, with no increase in viral loads. At a lower dose (2.5 ng/bee/day), there was no synergistic increase of mortality, but viral loads were significantly higher in naturally dead honeybees, compared with sacrificed honeybees exposed to the same conditions. These results show that the interactions between pathogens and pesticides in honeybees can be complex: increasing pesticide doses may not necessarily be linked to a rise in viral loads, suggesting that honeybee tolerance to the viral infection might change with pesticide exposure.

Keywords: Thiamethoxam, pathogen, synergistic interaction, co-exposure, survival, tolerance

1. Introduction

The Western honeybee, *Apis mellifera* sp., is a key ecological species, both for the pollination services it procures [1], and for its role as a bio-indicator of environmental pollution (i.e. in-hive concentration of xenobiotics fortuitously collected in the field) [2,3]. During the last decades, an alarming amount of honeybee colony losses has been reported in the Northern Hemisphere [4], and the number of colonies is decreasing whereas demand for crop pollination is growing [1,5,6]. In Europe, overwintering losses ranged from 2.4% to 15.4% and from 0.04% to 11.1% of seasonal losses (i.e. during the foraging season), in 2014 [7]. Average losses reached 51.1% over the whole year in the USA for the same period [8]. These losses can be caused by various factors, including anthropogenic changes (growing use of pesticides, landscape alteration – decrease in resource availability), and introduction of exotic parasites, such as the mite *Varroa destructor*, the microsporidian parasite *Nosema ceranae*, and viruses [1]. In addition, there is growing evidence that these factors may interact and exacerbate bee mortality [9].

Neonicotinoids, a family of acetylcholine receptor inhibitors, which represented more than 25% of the insecticides on the market in 2010 [10], are increasingly blamed for causing high mortalities in laboratory conditions, as well as colony losses, either alone [11,12] or in conjunction with other stress factors [13–16]. By binding with high affinity to acetylcholine receptors, they effectively block the binding of acetylcholine and overstimulate cells, leading to paralysis and death of cells and/or of individuals [17]. Thiamethoxam, a nitro-substituted neonicotinoid, is one of the most commonly used insecticides worldwide, either as a systemic insecticide in seed coatings, or directly sprayed on crops [10,17]. Recent studies have shown sub-lethal effects of low-dose exposure to thiamethoxam during homing flights in honeybees

[11]. In addition, thiamethoxam is known to be converted into its main metabolite, clothianidin, in plants and insects [17,18]. However, the metabolisation kinetics has not been described for honeybees. Clothianidin, which is also commercialised as an insecticide on its own, is reportedly slightly more toxic than thiamethoxam (clothianidin 48 h oral median lethal dose 50% [LD50]: 2.69 ng/bee ; thiamethoxam 48 h oral LD50: 4.41 ng/bee [19]).Thiamethoxam is one of the most used pesticides, on crops attracting honeybees (like oilseed rape [17]), and its potential interaction with pathogens should be investigated.

Indeed, pathogens, and notably viruses, are also frequently incriminated in the decline of domestic honeybees. About 26 honeybee viruses have been described, but only some cause visible symptoms, such as brood and or adult bee mortalities, paralysis or deformed wings, and have been correlated with colony losses [20–23]. Among these viruses, the seven most prevalent in the Northern Hemisphere are: *Acute bee paralysis virus* (ABPV) or its related viruses *Israel acute paralysis virus* and *Kashmir bee virus* (forming together the AKI viral complex), *Deformed wing virus* (DWV), *Sacbrood virus* (SBV), *Black queen cell virus* (BQCV), and *Chronic bee paralysis virus* (CBPV) [7,24–26]. In USA and France, their prevalence was described to be respectively about 14% to 22% and 17% to 58% for AKI viral complex , 85% and 97% for DWV, 86% for SBV (prevalence in France), 90% and 86% for BQCV, and 16% and 28% for CBPV [7,24,26,27]. CBPV is different in many ways from the other honeybee viruses belonging to the *Picornavirales* order (such as DWV [*Iflaviridae*] or ABPV [*Dicistroviridae*]), which only contains positive RNA strand viruses that translate directly into one polyprotein [25]. Not yet classified, CBPV is close to *Nodaviridae* and *Tombusviridae* virus families, possesses an anisometric structure, and is composed of two separate positive RNA strands [28–32]. Despite the virus being present in one quarter of tested hives in France [26], only 2% of specific clinical signs (see below) were observed during the French EPILOBEE surveillance programme [7]. Most honeybee viruses often

cause covert infections: they can be present in a great number of hives and yet not trigger any observable clinical symptoms [24]. The onset of an overt infection can be caused by the transmission of the virus by *Varroa destructor,* for DWV, ABPV, IAPV, and KBV, for example [33,34]. There is no evidence of CBPV transmission by *Varroa* mite. However, covert infections can evolve into overt infections (with clear clinical symptoms such as piles of dead bees in front of the hives and bees that are unable to fly, with tremors, paralysis, and sometimes a loss of hair and a darker colour of individuals) when CBPV-infected bees have been confined in populous hives for a long period of time [32]. The authors suggested that this could be due to bad weather during spring and to the capacity of CBPV to be transmitted by contact among bees, especially when close contact causes abrasion of the cuticle. Individuals developing clinical signs has been measured at 10^8 to up to 10^{10} copies of CBPV per honeybee [30,35]. The properties of the CBPV, and the relatively high numbers of infected apiaries, make this virus interesting in co-exposure studies.

A honeybee colony can potentially concentrate pesticides from nearby (up to 10 km) crops through its foraging and storing activity. The hive is also a good place for pathogen development, due to high population numbers and regulated temperature. Because of these two factors, it is highly probable that co-exposure of pesticides and pathogens will occur in the field [3]. Such co-exposures can lead to interactions, which can be additive, antagonistic or synergistic, between the stressors [36]. Significant effects of co-exposure between pesticides and viruses have already been observed, such as an increase in BQCV loads in nurse bees fed pollen containing a mix of chlorpyrifos, boscalid and pyraclostrobine [37], or an increase in DWV loads in honeybees exposed to clothianidin [38]. Doublet et al., (2014) recorded higher mortalities (and an increase in BQCV loads) in honeybee larvae exposed to thiacloprid. Pesticide exposure, by increasing virus loads, could lead to overt infections. The

co-exposure of honeybees to CBPV and thiamethoxam, while likely to occur in the field, has not yet been investigated.

In the present study, we describe the metabolisation kinetics of thiamethoxam into its main metabolite, clothianidin, in honeybees. Then we chronically co-exposed honeybees, in laboratory conditions, to CBPV and to different concentrations of thiamethoxam, in order to measure the effect of a potential interaction on survival, and on viral loads in individual honeybees.

2. Materials and Methods

1.1. Thiamethoxam-contaminated syrup

A certified pesticide standard for thiamethoxam (99% purity) was obtained from Techlab (Saint-Julien-lès-Metz, France). Pesticide concentration in syrup was adjusted to expose honeybees to the intended daily doses, taking into account a previously measured mean daily consumption of 25 µL of 50% sucrose syrup per bee. A standard working solution of thiamethoxam at 100 mg/L (prepared in water) was diluted in 50% sucrose, to obtain the final concentrations of 10 µg/L, 100 µg/L and 200 µg/L, corresponding to the expected daily doses of 0.25, 2.5 and 5.0 ng/bee, respectively. Thiamethoxam syrup solutions were tested to ensure the concentration and the absence of degradation into clothianidin.

1.2. Emerging honeybees

Emerging honeybees were obtained from previously tested healthy colonies selected in ANSES Sophia Antipolis laboratory apiary. For winter experiment, six colonies from this apiary were kept in an indoor apiary (winter apiary) maintained at 25°C, with access to the outdoors for cleansing flights, fed with 50% sucrose syrup, with water, and with paste composed of frozen pollen mixed with Fructoplus syrup (Icko Apiculture, Bollène, France).

Emerging honeybees were pooled to minimise colony-born bias and distributed in cages (30 bees per cage of about 780 cm^3, with a capacity of 100 bees, built from [39]). The cages were maintained in incubators (34°C, with saturated humidity). Caged bees were fed *ad libitum* with 50% sucrose syrup, and 50% sucrose syrup supplemented with 1% protein (Provita'Bee, ATZ Diététics, France), and crystallised sugar paste for 5 to 9 days after emergence. After this "growth period", the sugar paste and the 50% sucrose syrup supplemented with protein were removed.

1.3. Experiment 1: metabolisation kinetics of thiamethoxam

Twenty-eight cages were prepared in January 2016 (overall 840 emerging bees) from four healthy-colonies (winter apiary). After a 5 days "growth period", twenty-one cages were chronically exposed to 0.25 ng/bee/day of thiamethoxam *via* the sole remaining feeder filled with thiamethoxam-contaminated syrup (10µg/L). Seven control honeybee cages were fed with 50% sucrose syrup without thiamethoxam. Feeders were changed and weighed for syrup intake measurement every day. Survival in all cages was monitored daily.

One control cage was sacrificed (by freezing the bees at -18°C) on day 0 after beginning of the experiment. Then, on day 1, 5, 10, 12, 15, and day 18, one control cage and three exposed cages were sacrificed (for a total of 25 cages). In order to study if thiamethoxam or its metabolite would be excreted by bees in the field, the three remaining cages, fed with thiamethoxam, were sampled on day 18. The bees from these cages were anesthetised using CO_2 gas and the rectum of each bee was excised by pulling on the last tergite and stinger. All bee samples (whole bees and dissected bees) were stored at -18°C until chemical analysis.

1.4. Experiment 2: CBPV-thiamethoxam co-exposure

Emerging honeybees from three previously tested CBPV-negative colonies were sampled in early July 2015. One group of emerging bees were used to prepare CBPV-infected bees to

vector the virus. These bees (n=243) were distributed in 5 cages of 50 honeybees and after a 5 days of the "growth period", they were anesthetised using CO_2 gas, paint marked, injected in the thorax with 4.0 x 10^4 copies of purified CBPV, and then left to develop the disease for 4 days in a separate incubator, at 34°C with saturated humidity. At the same time, 54 cages (overall 1620 emerging bees) were prepared from the same colonies. At the end of a 9 days growth period, the feeders were removed and the CBPV and/or thiamethoxam exposure started according to the following six conditions (nine cages per condition):1. Control bees (bees not exposed to CBPV nor to thiamethoxam); 2. Bees in contact with CBPV-infected bees (introduction of nine symptomatic bees in each cage, which died from the disease within the first 1 to 3 days (24,69% were dead 1 day after introduction in the cage; 82,72% after 2 days, and 91,98% after 3 days), but weren't removed to continue to act as an inoculum); 3. Bees fed 2.5 ng/bee/day of thiamethoxam (100 µg/L thiamethoxam-contaminated syrup); 4. Bees fed with 5.0 ng/bee/day (200 µg/L thiamethoxam-contaminated syrup); 5. Bees co-exposed to both CBPV-infected bees and 2.5 ng/bee/day of thiamethoxam; 6. Bees co-exposed to both CBPV-infected bees and 5.0 ng/bee/day of thiamethoxam. Three cages per condition were sampled on day 1, 5 and day 10 after starting the virus and/or pesticide exposure. Survival was monitored daily (unmarked dead bees were removed from the cages, labelled and kept at -18°C). Sampled bees were stored at -18°C until viral analysis.

1.5.Quantification of thiamethoxam and clothianidin

The neonicotinoid residues were quantified from a pool of 20 honeybees from the same cage (whole bees or dissected bees), using liquid chromatography with electrospray tandem mass spectrometry (LC-MS/MS), according to the protocol described in [40]. Briefly, the pesticides were extracted using acetonitrile and liquid partitioning with *n*-hexane. One clean-up was then performed on a florisil cartridge (1 g, 6 mL) and the extract was analysed by LC-MS/MS.

1.6.Quantification of viral loads

CBPV load was measured in nine unmarked (i.e. not injected) individual honeybees sampled from the three cages sacrificed at each sampling date (three bees from each cage), or in nine (or as many as possible) dead bees (unmarked) collected at the same sampling date. Each honeybee was crushed in 1 mL 0.01 M phosphate buffer using three tungsten 0.5 mm beads and a TissueLyser (Qiagen) for 30 s at 30 Hz, and repeated three times. The resulting homogenate was then clarified by two successive centrifugations of 10 min, at 8000 x *g* and 4°C. Viral RNA was extracted from 200 µL of supernatant using High Pure Viral RNA kits from Roche Diagnostics, according to the manufacturer's instructions. RNA was recovered in 50 µL of 10 mM Tris-HCL, pH 8.5, supplied in the kit. Retro-transcription into cDNA was performed using random primers and SuperScript's SSRT II kit (Invitrogen) from 12.5 µL of purified RNA [41]. Quantitative PCR was carried out on 5 µL of complementary DNA using protocol and primers from [30] (limit of quantification, LOQ: 3.9 $\log_{10}$ copies per individual).

1.7.Statistics

Survival was established using a Kaplan-Meier estimation [42,43] which allows taking into account the sampled bees in whole cages as censored data (individuals removed from the experiment, but not dead), and curves compared with log-rank tests [44]. Log-transformed viral loads were analysed using a one-way ANOVA test followed by post-hoc *t*-tests or Tukey HSD tests [45]. Synergistic interactions were tested using a χ^2 of compliance test comparing obtained survival measurements for each day with the corresponding calculated expected measurements [46,47].

3. Results

1.8. Experiment 1: metabolisation kinetics of thiamethoxam

3.1.1. Effects of thiamethoxam on survival and syrup intake

Survival of the honeybees was not affected by pesticide exposure. Kaplan-Meier curves of control and of 0.25 ng/bee/day thiamethoxam-fed bees did not differ (p=1; day 10 mortality rates: respectively 2.9% and 2.0%, data not shown).

Mean daily syrup intake over the course of the experiment was significantly higher (p<0.01; Fig. 1) for thiamethoxam fed bees (mean: 36 µL/bee; standard deviation <0.01) than for control bees (mean: 28 µL/bee; standard deviation <0.01). According to the intake volume mean, the bees were exposed to 0.36 ng/bee/day instead of the expected 0.25 ng/bee/day.

3.1.2. Metabolisation of thiamethoxam into clothianidin

Levels for both neonicotinoids (thiamethoxam and clothianidin) were under the limit of detection (LOD, 0.015 ng/bee) in control bees over the course of the experiment (Fig. 2). Thiamethoxam levels reached 0.15 ng/bee one day after the beginning of exposure and remained stable until day 10. Then, this level dropped to around 0.10 ng/bee on day 12, 15 and 18. In contrast, clothianidin levels increased steadily throughout the experiment, from under 0.05 ng/bee after one day of exposure to almost 0.40 ng/bee after 18 days (Fig. 2). Clothianidin seems to accumulate in the tested honeybees.

3.1.3 Comparison of thiamethoxam levels in whole and dissected bees

Thiamethoxam levels between whole bees and dissected bees (without the rectum) after 18 days of chronic exposure did not differ significantly (p=0.23), but clothianidin levels were significantly higher in whole bees than in dissected bees (p=0.04; Fig. 3), suggesting accumulation in the rectum.

1.9.Experiment 2: CBPV-thiamethoxam co-exposure

3.2.1. Effect of co-exposure on mortality

Survival rates were determined using the Kaplan-Meier estimate (Fig. 4). These rates were distributed in four significantly different groups. The first group, (a), with the highest survival rates (>80% live bees after 10 days of experiment) was composed of control bees and bees fed with 2.5 ng/bee/day of thiamethoxam. The second group, (b), with lower survival rates, was composed of bees that were only in contact with CBPV-infected bees (69% live bees after 10 days), and bees that were co-exposed to CBPV-infected bees and 2.5 ng/bee/day of thiamethoxam (58% live bees after 10 days). The third group, (c), with yet a lower survival rate, was composed of bees that were fed with 5.0 ng/bee/day of thiamethoxam (45% live bees after 10 days). Finally, the group with the lowest survival rate, (d) (4.6% live bees after 10 days) was composed of bees that were co-exposed to both CBPV-infected bees and 5.0 ng/bee/day of thiamethoxam.

A significant effect of co-exposure on mortality was found only at the highest dose of thiamethoxam after up to 8 days of co-exposure ($p<0.01$; Fig. 5). The survival rate of bees co-exposed to both CBPV and 2.5 ng/bee/day of thiamethoxam was not significantly different from bees that were only exposed to CBPV-infected bees ($p>0.05$).

The mortality in the co-exposure condition exceeded what would be expected from an additive effect between CBPV and 5.0 ng/bee/day of thiamethoxam (i.e., the effect was higher than the sum of the effects observed in groups exposed to each of the stressors alone). The observed mortality (day 8: $p<0.01$ [$a=10.85$]; day 9: $p<0.01$ [$a=11.49$]; day 10: $p=0.01$ [$a=10.58$]) was much higher than the expected mortality (χ^2 table with 1 df, $a=6.635$ [$p=0.01$] or $a=10.828$ [$p<0.01$]). This demonstrating that there was a synergistic effect between the CBPV and thiamethoxam at the dose of 5.0 ng/bee/day.

3.2.2. Viral loads

The distribution of the CBPV loads is shown in Figure 6 and 7. Viral loads in the unmarked honeybees that were alive and sacrificed on sampling (here named "live-sampled") differed significantly between two experimental groups: exposed and not exposed to the virus ($p<0.01$), indicating the success of our transmission method (Fig. 6). As expected, groups composed of control bees and bees exposed to the pesticide showed the lowest viral loads, mostly under or around the real-time PCR LOQ. Contact with CBPV-infected bees induced virus contamination in the tested bees (unmarked bees), regardless of pesticide exposure. There was no significant effect of co-exposure on CBPV viral loads in live-sampled honeybees ($p=0.13$), but a tendency can be seen for the honeybees co-exposed to 5 ng of thiamethoxam and CBPV. Our transmission method doesn't allow us to control the virus doses, and this exacerbates individual variability.

The only significant effect that was observable in dead bees was on day 5 and 10, where dead bees co-exposed to CBPV and 2.5 ng/bee/day of thiamethoxam had higher CBPV loads than live-sampled bees in the same condition ($p<0.01$; Fig. 7).

4. Discussion

In this study, we first showed that thiamethoxam is quickly and effectively metabolised into clothianidin and would be excreted by honeybees. In addition, we demonstrated that chronic co-exposure between thiamethoxam at 5 ng/bee/day and CBPV can cause a significant increase in mortality in honeybees, compared to single-factor exposure. Moreover, the pesticide and the virus showed a synergistic effect on mortality, after 8 days of exposure and beyond, at the highest tested pesticide dose (5.0 ng/bee/day). Because CBPV is often detected in apiaries [7,26], and thiamethoxam is used on common crops that are very attractive to honeybees, such as oilseed rape [10,48], this type of co-exposure to two stress factors is likely to occur in the field.

Before investigating the stress factor interactions, we assessed the metabolisation of the thiamethoxam in honeybees (Fig. 2). Thiamethoxam is known to be readily metabolised by plants and *Lepidoptera* into its main metabolite, clothianidin [17]. To our knowledge, there was no data on the fate of thiamethoxam in the case of a chronic, long-term exposure in honeybees, such as could happen if pesticide contaminated pollen or nectar is stored in the hive. Our results not only demonstrated that thiamethoxam is rapidly metabolised into clothianidin, but also revealed that metabolisation accelerates over time, as the thiamethoxam levels remained stable over 10 days but then decreased slightly even though bees were continuously fed with 10 µg/L solutions of thiamethoxam-contaminated syrup. Syrup intake is steady through the experiment, even slightly increasing over time, which further underlines this acceleration. Honeybees may have been investing more of their resources in the metabolisation process as time went by, mobilising more energy on this task [49]. In addition, the syrup intake did not decrease after 10 days, but was significantly higher in the thiamethoxam groups compared to controls (Fig. 1). This finding concurs with previous results [50,51] that showed that honeybees are not repelled by thiamethoxam and even

consume higher amounts of syrup contaminated with the pesticide. This increased intake could also be explained by the aforementioned increased mobilisation of energy resources [49].

We also observed that clothianidin accumulated in honeybees over the course of the experiment, reaching up to four times the daily amount of ingested thiamethoxam (Fig. 2). The purpose of metabolisation is to make xenobiotic molecules less toxic, as well as easier to excrete [52]. However, honeybees kept in cages cannot carry out cleansing flights. If clothianidin accumulated in the rectum, this metabolite would normally be excreted during cleansing flights [49,53,54]. We thus hypothesised that this growing amount of clothianidin was not accumulating in the body of the honeybees but in the rectum. The amount of clothianidin found in the dissected samples was significantly (about five-fold) lower than in the whole honeybees (Fig. 3). This result shows that the clothianidin produced as a result of metabolisation of thiamethoxam would mostly be excreted, in field conditions. Moreover, the metabolite probably cannot be reabsorbed through the rectum, which is cuticle lined, making it impermeable to nicotine and nicotine-derived metabolites [53]. Our results on thiamethoxam metabolisation suggest that the interaction we observed in the following experiment was not caused by clothianidin; and either comes from the thiamethoxam itself or from the pesticide metabolisation process.

To minimise the amount of stress applied to our tested honeybees, and mimic insofar as possible natural conditions, we developed a CBPV-transmission process based on the direct contact route [32]. A preliminary experiment showed that this transmission process was effective, because previously healthy honeybees showed CBPV viral loads that were significantly higher than viral loads from control bees (data not shown). In the case of CBPV, this viral-transmission process can replace the usual technique of injecting a purified virus solution into CO_2-anasthetised bees. Such anaesthesia exacerbates handling stress and impacts

long term survival [55]. In order to reduce the natural viral-contamination of honeybees but with the aim to reproduce field conditions, we experimented on 9-days old honeybees. Ten days after the beginning of our experiment of co-exposure, the tested honeybees reached the age at which the honeybees are or become foragers in natural conditions, and thus are on the front line of thiamethoxam exposition through nectar and pollen collection [54].

The synergistic effect on bee survival observed between CBPV and thiamethoxam (Fig. 4 and 5) occurred only at the highest pesticide dose (5.0 ng/bee/day of thiamethoxam). As 5.0 ng/bee equates to a concentration of 200 ng/g in syrup in our experimental design, this dose is higher than what is usually found in the field. Previously studies found a maximum of 13.3 ng/g of thiamethoxam in nectar from oilseed rape, and 86 ng/g in pollen from plants on field edges [56]; 53.3 ppb (ng/g) in stored pollen [57]; and a maximum of 20.2 ± 0.4 ng/g in honey [58]. However, the potential concentration of a given pesticide in nectar or pollen, considering the growing use of commercial thiamethoxam-coasted seeds, is very difficult to predict from flowers in the field. For example, pesticide concentration in nectar and the presence of the nectar itself, can vary with multiple factors such as the position of the flower on the plant, intrinsic differences between species, varieties, or even flower to flower, meteorological conditions, time of day, soil structure and previous exposure to pesticides, differences between pulverisation and systemic pesticides, rapidity of metabolisation by the plant, etc. [59]. Thus, our experiment, even if not in the average range of previously found thiamethoxam concentrations, is still realistic in the current state of thiamethoxam field studies.

The 5.0 ng thiamethoxam concentration is equal to the oral 48 h LD50 concentration reported by the European Union and EFSA [19,60]. In our study, the mortality of exposed honeybees only reached 50% after 9 days of chronic exposure, and after 7 days of virus co-exposure. This difference from the reported acute LD50 may arise from differences in bee genetic

background between the studies [61–63]. In addition, detoxification mechanisms or efficiency may differ between chronic and acute exposure [63,64].

Our first working hypothesis to explain the observed synergy on mortality was that thiamethoxam has a negative impact on the immune system of our tested honeybees [38,65,66], leading to an increase in CBPV viral loads, which in turn would explain the increased mortality. However, viral load varied with treatment (Fig. 6). Groups of honeybees not in contact with infected honeybees presented viral loads that were around or equal to the PCR LOQ, which represents the CBPV-load found in non-symptomatic hives in natural conditions [28,30]. Nevertheless, exposure to thiamethoxam alone tended to increase the median of the natural CBPV load slightly in live-sampled honeybees after 10 days for both thiamethoxam doses (2.5 and 5.0 ng/bee/day). Interestingly, exposure to thiamethoxam in these two groups appeared to decrease the variability between samples in both groups. Live-sampled honeybees from the CBPV co-exposed conditions showed a similar trend.

Interestingly, the synergistic effect of the co-exposure (Fig. 5) was not associated with a significant increase in CBPV viral loads in live-sampled honeybees (Fig. 6). However, high viral loads were detected in dead honeybees co-exposed to CBPV and 2.5 ng/bee/day of thiamethoxam, although the mortality rate in this group was not different from CBPV-only exposed honeybees. This viral load increase concurs with previous findings [38], whereby clothianidin exposure caused an increase in DWV viral loads in co-exposed honeybees, through negative regulation of the NF-κB factor, which is part of the honeybee immunity and seems to regulate viral loads. We therefore infer that thiamethoxam has an effect on this immune factor and that, although CBPV does not belong to the *Iflavirus* genus, CBPV multiplication may also be regulated by NF-κB. In contrast, the co-exposure between 5.0 ng/bee/day of thiamethoxam and CBPV caused significantly higher mortality in honeybees (Fig. 5), but did not increase the viral loads compared with the CBPV-only exposed

honeybees. This decoupling between viral load and survival in honeybees can be attributed either to the fact that the high dose of thiamethoxam killed the honeybees before the virus had time to replicate, that the detoxification processes used resources that would have been used for the CBPV to replicate, or, more hypothetically, that the presence of CBPV had a negative impact on the detoxification of the pesticide. However, this response may also be explained by a negative effect of the 5.0 ng/bee/day dose of thiamethoxam on the tolerance of individuals to the virus. Tolerance, which was initially described in plant-pathogen relationship studies and later animal models, describes mechanisms that are not directly aimed at decreasing pathogen intrusion or multiplication, but rather compensate for the energetic costs or tissue damage caused by either the pathogen or the individual's own immune response [67–69]. This immune response can allow individuals to remain healthy and/or maintain good fitness even with high pathogen loads. Such alternative responses to a pathogen burden have been observed in different lineages of *Drosophila melanogaster* infected with the same strain of pathogenic bacterium (*Pseudomonas aeruginosa*), suggesting that genetic background plays a role [70]. In our study, because the honeybees were strictly homogenised in all experiments, the genetic background of both honeybee groups (co-exposed to CBPV and 2.5 or 5.0 ng/bee/day of thiamethoxam) could not be associated with genetic variations in tolerance. Nonetheless, exposure to the various pesticide doses may have had an impact on some physiological responses, resulting in a decrease in tolerance to the viral infection.

We demonstrated that chronic co-exposure to both CBPV and thiamethoxam, which can very possibly occur in the field, leads to a synergistic effect on mortality at a high pesticide dose (5.0 ng/bee/day). However, this synergistic effect was not reflected by an increase in CBPV viral loads, but could be explained by a negative effect of the pesticide on the honeybee's tolerance to the virus. We also highlighted the metabolisation kinetics of thiamethoxam in

chronic, sub-lethal doses exposed honeybees, showing that thiamethoxam is indeed converted into clothianidin in honeybees and that this clothianidin is likely to be quickly excreted in field conditions. The metabolisation kinetics suggest that alternative metabolism pathways might be set up after 10 days of exposure. Further investigations on the effect of co-exposure at the transcriptional level on selected immune and detoxification-related genes will help shed more light on these novel results.

Conflict of interest statement

The authors confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Acknowledgements

We are grateful to Carolyn Engel-Gautier, professional translator and native English speaker, for revising the paper.

Funding

This study was supported by "Phyto-Pharmaco vigilance" scheme (ANSES, France).

References

[1] S.G. Potts, J.C. Biesmeijer, C. Kremen, P. Neumann, O. Schweiger, W.E. Kunin, Global pollinator declines : Trends , impacts and drivers Global pollinator declines : trends , impacts and drivers, (2010). doi:10.1016/j.tree.2010.01.007.

[2] G. Celli, B. Maccagnani, Honey bees as bioindicators of environmental pollution, Bull. Insectology. 56 (2003) 137–139.

[3] Y. Poquet, C. Vidau, C. Alaux, Modulation of pesticide response in honeybees, Apidologie. 47 (2016) 412–426. doi:10.1007/s13592-016-0429-7.

[4] P. Neumann, P. Carreck, Honey bee colony losses, J. Apic. Res. 49 (2010) 1–6. doi:10.3896/IBRA.1.49.1.01.

[5] M.A. Aizen, L.D. Harder, The Global Stock of Domesticated Honey Bees Is Growing Slower Than Agricultural Demand for Pollination, Curr. Biol. 19 (2009) 915–918. doi:10.1016/j.cub.2009.03.071.

[6] T.D. Breeze, B.E. Vaissière, R. Bommarco, T. Petanidou, N. Seraphides, L. Kozák, J. Scheper, J.C. Biesmeijer, D. Kleijn, S. Gyldenkærne, M. Moretti, A. Holzschuh, I. Steffan-Dewenter, J.C. Stout, M. Pärtel, M. Zobel, S.G. Potts, Agricultural policies exacerbate honeybee pollination service supply-demand mismatches across Europe, PLoS One. 9 (2014). doi:10.1371/journal.pone.0082996.

[7] M. Laurent, P. Hendrikx, M. Ribière-Chabert, M.-P. Chauzat, EPILOBEE: A pan-European epidemiological study on honeybee colony losses, 2015. https://ec.europa.eu/food/sites/food/files/animals/docs/la_bees_epilobee-report_2012-2014.pdf.

[8] K. V. Lee, N. Steinhauer, K. Rennich, M.E. Wilson, D.R. Tarpy, D.M. Caron, R. Rose, K.S. Delaplane, K. Baylis, E.J. Lengerich, J. Pettis, J.A. Skinner, J.T. Wilkes, R. Sagili, D. vanEngelsdorp, A national survey of managed honey bee 2013???2014 annual colony losses in the USA, Apidologie. 46 (2015) 292–305. doi:10.1007/s13592-015-0356-z.

[9] D. Goulson, E. Nicholls, C. Botías, E.L. Rotheray, Bee declines driven by combined stress from parasites, pesticides, and lack of flowers, SciencExpress. (2015) 1–16. doi:10.1126/science.1255957.

[10] J.P. van der Sluijs, N. Simon-Delso, D. Goulson, L. Maxim, J.M. Bonmatin, L.P. Belzunces, Neonicotinoids, bee disorders and the sustainability of pollinator services, Curr. Opin. Environ. Sustain. 5 (2013) 293–305. doi:10.1016/j.cosust.2013.05.007.

[11] M. Henry, M. Beguin, F. Requier, O. Rollin, J.-F. Odoux, P. Aupinel, J. Aptel, S. Tchamitchian, A. Decourtye, A Common Pesticide Decreases Foraging Success and Survival in Honey Bees, Science (80-.). 337 (2012) 1453–1453. doi:10.1126/science.1224930.

[12] L. Tison, M.-L. Hahn, S. Holtz, A. Rössner, U. Greggers, G. Bischoff, R. Menzel, Honey Bees â€™ Behavior Is Impaired by Chronic Exposure to the Neonicotinoid Thiacloprid in the, (2016). doi:10.1021/acs.est.6b02658.

[13] F. Nazzi, F. Pennacchio, Disentangling multiple interactions in the hive ecosystem, Trends Parasitol. 30 (2014) 556–561. doi:10.1016/j.pt.2014.09.006.

[14] C. Alaux, J.-L. Brunet, C. Dussaubat, F. Mondet, S. Tchamitchan, M. Cousin, J. Brillard, A. Baldy, L.P. Belzunces, Y. Le Conte, Interactions between Nosema microspores and a neonicotinoid weaken honeybees (Apis mellifera), Environ. Microbiol. 12 (2010) 774–782. doi:10.1111/j.1462-2920.2009.02123.x.

[15] C. Vidau, M. Diogon, J. Aufauvre, R. Fontbonne, B. Viguès, J.-L. Brunet, C. Texier, D.G. Biron, N. Blot, H. El Alaoui, L.P. Belzunces, F. Delbac, Exposure to Sublethal Doses of Fipronil and Thiacloprid Highly Increases Mortality of Honeybees Previously Infected by Nosema ceranae, PLoS One. 6 (2011) e21550. doi:10.1371/journal.pone.0021550.

[16] V. Doublet, M. Labarussias, J.R. de Miranda, R.F.A. Moritz, R.J. Paxton, Bees under stress: sublethal doses of a neonicotinoid pesticide and pathogens interact to elevate honey bee mortality across the life cycle, Environ. Microbiol. 17 (2015) 969–983. doi:10.1111/1462-2920.12426.

[17] R. Nauen, U. Ebbinghaus-Kintscher, V.L. Salgado, M. Kaussmann, Thiamethoxam is a neonicotinoid precursor converted to clothianidin in insects and plants, Pestic. Biochem. Physiol. 76 (2003) 55–69. doi:10.1016/S0048-3575(03)00065-8.

[18] Y. Benzidane, S. Touinsi, E. Motte, A. Jadas-Hécart, P.Y. Communal, L. Leduc, S.H. Thany, Effect of thiamethoxam on cockroach locomotor activity is associated with its metabolite clothianidin, Pest Manag. Sci. 66 (2010) 1351–1359. doi:10.1002/ps.2022.

[19] D. Laurino, M. Porporato, A. Patetta, A. Manino, Toxicity of neonicotinoid insecticides to honey bees: Laboratory tests, Bull. Insectology. 64 (2011) 107–113.

[20] B. Dainat, J.D. Evans, Y.P. Chen, L. Gauthier, P. Neumann, Dead or Alive: Deformed Wing Virus and Varroa destructor Reduce the Life Span of Winter Honeybees, Appl. Environ. Microbiol. 78 (2012) 981–987. doi:10.1128/AEM.06537-11.

[21] S.D. Desai, R.W. Currie, Effects of Wintering Environment and Parasite – Pathogen Interactions on Honey Bee Colony Loss in North Temperate Regions, (2016) 1–24. doi:10.1371/journal.pone.0159615.

[22] E. Genersch, W. von der Ohe, H. Kaatz, A. Schroeder, C. Otten, R. Buechler, S. Berg, W. Ritter, W. Muehlen, S. Gisder, M. Meixner, G. Liebig, P. Rosenkranz, The German bee monitoring project: a long term study to understand periodically high winter losses of honey bee colonies, Apidologie. 41 (2010) 332–352. doi:10.1051/apido/2010014.

[23] D. VanEngelsdorp, D.R. Tarpy, E.J. Lengerich, J.S. Pettis, Idiopathic brood disease syndrome and queen events as precursors of colony mortality in migratory beekeeping operations in the eastern United States, Prev. Vet. Med. 108 (2013) 225–233. doi:10.1016/j.prevetmed.2012.08.004.

[24] M. Aubert, B. Ball, I. Fries, R. Moritz, N. Milani, I. Bernardinelli, Virology and the Honey Bee, European C, European Commission publications office, 2008. http://www.eurosfaire.prd.fr/7pc/doc/1262083725_virology_and_the_honey_bee.pdf.

[25] E. Genersch, M. Aubert, Emerging and re-emerging viruses of the honey bee (Apis mellifera L.), Vet. Res. 41 (2010) 54. doi:10.1051/vetres/2010027.

[26] D. Tentcheva, L. Gauthier, N. Zappulla, B. Dainat, F. Cousserans, M.E. Colin, M. Bergoin, Prevalence and Seasonal Variations of Six Bee Viruses in Apis mellifera L . and Varroa destructor Mite Populations in France, Appl. Environ. Microbiol. 70 (2004) 7185–7191. doi:10.1128/AEM.70.12.7185.

[27] K.S. Traynor, K. Rennich, E. Forsgren, R. Rose, J. Pettis, G. Kunkel, S. Madella, J. Evans, D. Lopez, D. VanEngelsdorp, Multiyear survey targeting disease incidence in US honey bees, Apidologie. 47 (2016) 325–347. doi:10.1007/s13592-016-0431-0.

[28] M. Ribière, P. Lallemand, a. L. Iscache, F. Schurr, O. Celle, P. Blanchard, V. Olivier, J.P. Faucon, Spread of infectious chronic bee paralysis virus by honeybee (Apis mellifera L.) feces, Appl. Environ. Microbiol. 73 (2007) 7711–7716. doi:10.1128/AEM.01053-07.

[29] V. Olivier, I. Massou, O. Celle, P. Blanchard, F. Schurr, M. Ribiere, M. Gauthier, In situ hybridization assays for localization of the chronic bee paralysis virus in the honey bee (Apis mellifera) brain, J. Virol. Methods. 153 (2008) 232–237. doi:10.1016/j.jviromet.2008.06.027.

[30] P. Blanchard, J. Regnault, F. Schurr, E. Dubois, M. Ribiere, Intra-laboratory validation of chronic bee paralysis virus quantitation using an accredited standardised real-time quantitative RT-PCR method, J. Virol. Methods. 180 (2012) 26–31. doi:10.1016/j.jviromet.2011.12.005.

[31] I. Youssef, F. Schurr, A. Goulet, N. Cougoule, M. Ribière-Chabert, H. Darbon, R. Thiéry, E. Dubois, RNA 1 and RNA 2 Genomic Segments of Chronic Bee Paralysis Virus Are Infectious and Induce Chronic Bee Paralysis Disease, J. Immunol. Res. 2015 (2015) 1–8. doi:10.1155/2015/423493.

[32] M. Ribière, V. Olivier, P. Blanchard, Chronic bee paralysis: A disease and a virus like no other?, J. Invertebr. Pathol. 103 (2010) S120–S131. doi:10.1016/j.jip.2009.06.013.

[33] N.L. Carreck, B. V Ball, S. Martin, Honey bee colony collapse and changes in viral prevalence associated with Varroa destructor, J. Apic. Res. 49 (2010) 93–94. doi:10.3896/IBRA.1.49.1.13.

[34] N. Möckel, S. Gisder, E. Genersch, Horizontal transmission of deformed wing virus : pathological consequences in adult bees (Apis mellifera) depend on the transmission route, (2011) 370–377. doi:10.1099/vir.0.025940-0.

[35] A. Chevin, F. Schurr, P. Blanchard, R. Thiery, M. Ribiere, Experimental infection of the honeybee (Apis mellifera L.) with the chronic bee paralysis virus (CBPV): infectivity of naked CBPV RNAs, Virus Res. 167 (2012) 173–178. doi:10.1016/j.virusres.2012.04.012.

[36] M. Holmstrup, A.M. Bindesbøl, G.J. Oostingh, A. Duschl, V. Scheil, H.R. Köhler, S. Loureiro, A.M.V.M. Soares, A.L.G. Ferreira, C. Kienle, A. Gerhardt, R. Laskowski, P.E. Kramarz, M. Bayley, C. Svendsen, D.J. Spurgeon, Interactions between effects of environmental chemicals and natural stressors: A review, Sci. Total Environ. 408 (2010) 3746–3762. doi:10.1016/j.scitotenv.2009.10.067.

[37] G. DeGrandi-Hoffman, Y. Chen, R. Simonds, The Effects of Pesticides on Queen Rearing and Virus Titers in Honey Bees (Apis mellifera L.), Insects. 4 (2013) 71–89. doi:10.3390/insects4010071.

[38] G. Di Prisco, V. Cavaliere, D. Annoscia, P. Varricchio, E. Caprio, F. Nazzi, G. Gargiulo, F. Pennacchio, Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 18466–18471. doi:10.1073/pnas.1314923110.

[39] J. Pain, NOTE TECHNIQUE NOUVEAU MODELE MAINTIEN D ' ABEILLES EN CAPTIVITE, Les Ann. l'Abeille, INRA Ed. 9 (1966) 71–76.

[40] A.-C. Martel, C. Lair, Validation of a highly sensitive method for the determination of neonicotinoid insecticides residues in honeybees by liquid chromatography with electrospray tandem mass spectrometry, Int. J. Environ. Anal. Chem. 91 (2011) 978–988. doi:10.1080/03067310903524822.

[41] M. Ribière, C. Triboulot, L. Mathieu, C. Aurières, J.-P. Faucon, M. Pépin, Molecular diagnosis of chronic bee paralysis virus infection, Apidologie. 33 (2002) 339–351. doi:10.1051/apido:2002020.

[42] B. Efron, Logistic regression, survival analysis, and the Kaplan-Meier curve, (1988).

[43] M.S. Pepe, T.R. Fleming, Weighted Kaplan-Meier statistics: a class of distance tests

for censored survival data., Biometrics. 45 (1989) 497–507. doi:10.1111/1467-9868.00068.

[44] J.M. Bland, D.G. Altman, The logrank test., BMJ. 328 (2004) 1073. doi:10.1136/bmj.328.7447.1073.

[45] J.R. de Miranda, L. Bailey, B. V Ball, P. Blanchard, G.E. Budge, N. Chejanovsky, Y.-P. Chen, L. Gauthier, E. Genersch, D.C. de Graaf, M. Ribière, E. Ryabov, L. De Smet, J.J.M. van der Steen, Standard methods for virus research in Apis mellifera, J. Apic. Res. 52 (2013) 1–56. doi:10.3896/IBRA.1.52.4.22.

[46] J. Aufauvre, D.G. Biron, C. Vidau, R. Fontbonne, M. Roudel, M. Diogon, B. Viguès, L.P. Belzunces, F. Delbac, N. Blot, Parasite-insecticide interactions: a case study of Nosema ceranae and fipronil synergy on honeybee, Sci. Rep. 2 (2012) 1–7. doi:10.1038/srep00326.

[47] J.R. Mcvay, R.T. Gudauskas, J.D. Harper, Effects of Bacillus thuringiensis Nuclear-Polyhedrosis Virus Mixtures on Trichoplusia ni Larvae, J. Invertebr. Pathol. 29 (1977) 367–372. doi:10.1016/S0022-2011(77)80045-1.

[48] N. Simon-Delso, V. Amaral-Rogers, L.P. Belzunces, J.M. Bonmatin, M. Chagnon, C. Downs, L. Furlan, D.W. Gibbons, C. Giorio, V. Girolami, D. Goulson, D.P. Kreutzweiser, C.H. Krupke, M. Liess, E. Long, M. McField, P. Mineau, E. a D. Mitchell, C. a Morrissey, D. a Noome, L. Pisa, J. Settele, J.D. Stark, a Tapparo, H. Van Dyck, J. Van Praagh, J.P. Van der Sluijs, P.R. Whitehorn, M. Wiemers, Systemic insecticides (neonicotinoids and fipronil): trends, uses, mode of action and metabolites., Environ. Sci. Pollut. Res. Int. 22 (2015) 5–34. doi:10.1007/s11356-014-3470-y.

[49] E.E. Du Rand, S. Smit, M. Beukes, Z. Apostolides, C.W.W. Pirk, S.W. Nicolson,

Detoxification mechanisms of honey bees (Apis mellifera) resulting in tolerance of dietary nicotine, Sci. Rep. 5 (2015) 11779. doi:10.1038/srep11779.

[50] S.C. Kessler, E.J. Tiedeken, K.L. Simcock, S. Derveau, J. Mitchell, S. Softley, J.C. Stout, G. a. Wright, Bees prefer foods containing neonicotinoid pesticides, Nature. 521 (2015) 74–76. doi:10.1038/nature14414.

[51] H.M. Thompson, S. Wilkins, S. Harkin, S. Milner, K.F.A. Walters, Neonicotinoids and bumblebees (Bombus terrestris): Effects on nectar consumption in individual workers, Pest Manag. Sci. 71 (2015) 946–950. doi:10.1002/ps.3868.

[52] C. Xu, C.Y. Li, A.T. Kong, Induction of Phase I , II and III Drug Metabolism / Transport by Xenobiotics, 28 (2005) 249–268.

[53] E.E. du Rand, C.W.W. Pirk, S.W. Nicolson, Z. Apostolides, The metabolic fate of nectar nicotine in worker honey bees, J. Insect Physiol. 98 (2017) 14–22. doi:10.1016/j.jinsphys.2016.10.017.

[54] M.L. Winston, The biology of the honey bee, Harvard University Press, Cambridge, 1987.

[55] O. Rueppell, B. Youse, J. Collazo, D. Smith, Early life stress affects mortality rate more than social behavior , gene expression or oxidative damage in honey bee workers, 90 (2017) 19–25. doi:10.1016/j.exger.2017.01.015.

[56] C. Botías, A. David, J. Horwood, A. Abdul-Sada, E. Nicholls, E.M. Hill, D. Goulson, Neonicotinoid Residues in Wildflowers, a Potential Route of Chronic Exposure for Bees, Environ. Sci. Technol. (2015) 151006133035001. doi:10.1021/acs.est.5b03459.

[57] C.A. Mullin, M. Frazier, J.L. Frazier, S. Ashcraft, R. Simonds, D. vanEngelsdorp, J.S. Pettis, High levels of miticides and systemic agrochemicals in North American

beehives: Implications for honey bee health, Abstr. Pap. Am. Chem. Soc. 239 (2010).

[58] Z. Barganska, M. Slebioda, J. Namiesnik, Pesticide residues levels in honey from apiaries located of Northern Poland, 31 (2013) 196–201. doi:10.1016/j.foodcont.2012.09.049.

[59] D. Aston, S. Bucknall, Plants and Honey Bees : An Introduction to Their Relationships, Northern Bee Books, 2009.

[60] Efsa, Conclusion on the peer review of the pesticide risk assessment for bees for the active substance thiamethoxam, EFSA J. 11 (2013) 1–68. doi:10.2903/j.efsa.2013.3067.

[61] D. Laurino, A. Manino, A. Patetta, M. Porporato, Toxicity of neonicotinoid insecticides on different honey bee genotypes, Bull. Insectology. 66 (2013) 119–126.

[62] F.D. Rinkevich, J.W. Margotta, J.M. Pittman, R.G. Danka, M.R. Tarver, J. a. Ottea, K.B. Healy, Genetics, Synergists, and Age Affect Insecticide Sensitivity of the Honey Bee, Apis mellifera, PLoS One. 10 (2015) e0139841. doi:10.1371/journal.pone.0139841.

[63] S. Suchail, D. Guez, L.P. Belzunces, Discrepancy between acute and chronic toxicity induced by imidacloprid and its metabolites in Apis mellifera., Environ. Toxicol. Chem. 20 (2001) 2482–6. http://www.ncbi.nlm.nih.gov/pubmed/11699773.

[64] C.S. Stevenson, C. Docx, R. Webster, C. Battram, D. Hynx, J. Giddings, P.R. Cooper, P. Chakravarty, I. Rahman, J.A. Marwick, P.A. Kirkham, C. Charman, D.L. Richardson, N.R. Nirmala, P. Whittaker, K. Butler, S. Cs, C. Docx, R. Webster, C. Battram, D. Hynx, C. Pr, P. Chakravarty, I. Rahman, M. Ja, C. Charman, R. Dl, N. Nr, P. Whittaker, K. Butler, Comprehensive gene expression profiling of rat lung reveals

distinct acute and chronic responses to cigarette smoke inhalation, (2007) 1183–1193. doi:10.1152/ajplung.00105.2007.

[65] R.R. James, J. Xu, Mechanisms by which pesticides affect insect immunity, J. Invertebr. Pathol. 109 (2012) 175–182. doi:10.1016/j.jip.2011.12.005.

[66] F. Sanchez-Bayo, K. Goka, Pesticide Residues and Bees - A Risk Assessment, PLoS One. 9 (2014). doi:10.1371/journal.pone.0094482.

[67] D.S. Schneider, J.S. Ayres, Two ways to survive infection: what resistance and tolerance can teach us about treating infectious diseases, Nat. Rev. Immunol. 8 (2008) 889–895. doi:10.1038/nri2432.

[68] R. Medzhitov, D.S. Schneider, M.P. Soares, Disease Tolerance as a Defense Strategy, Science (80-.). 335 (2012) 936–941. doi:10.1126/science.1214935.

[69] J.D. Evans, M. Spivak, Socialized medicine: Individual and communal disease barriers in honey bees, J. Invertebr. Pathol. 103 (2010) S62–S72. doi:10.1016/j.jip.2009.06.019.

[70] V. Corby-Harris, K.E. Habel, F.G. Ali, D.E.L. Promislow, Alternative measures of response to Pseudomonas aeruginosa infection in Drosophila melanogaster., J. Evol. Biol. 20 (2007) 526–533. doi:10.1111/j.1420-9101.2006.01267.x.

Figure captions

Fig. 1: Syrup intake (µL/bee) for control and thiamethoxam groups, over the course of the metabolisation experiment. Solid line: Control bees fed with 50% sugar syrup, dashed line: Bees exposed to 0.25 ng/bee/day of thiamethoxam in 50% sugar syrup (n=1 cage of 30 bees for control and 3 cages of 30 bees each for thiamethoxam group). Means and standard deviations are shown.

Fig. 2: Metabolisation kinetics of thiamethoxam in chronically exposed honeybees. Solid line: quantity of thiamethoxam in control honeybees (control), dashed line: quantity of thiamethoxam in thiamethoxam-fed honeybees (thiamethoxam), dotted line: quantity of clothianidin in the same thiamethoxam-fed honeybees (clothianidin) (n=1 sample of 20 bees for control and 3 samples of 20 bees each for the thiamethoxam fed bees). Means and standard deviations are shown.

Fig. 3: Comparison of thiamethoxam and clothianidin levels between whole and dissected (rectum excised) honeybees (n=20x3 replicate cages = 60 bees in each group (whole or dissected bees)). Black bars: thiamethoxam measurements; grey bars: clothianidin measurements. Star shows significant difference between the two clothianidin measurements (*: p =0.04). Means and standard deviations are shown.

Fig. 4: Survival curves according to Kaplan-Meier estimation. Letters show statistical differences between curves (log-rank test). Dotted black line: control, solid black line: CBPV, dotted light grey line: 2.5 ng/bee/day of thiamethoxam, solid light grey line: co-exposure between 2.5 ng/bee/day of thiamethoxam and CBPV, dotted dark grey line: 5.0

ng/bee/day of thiamethoxam, solid dark grey line: co-exposure between 5.0 ng/bee/day of thiamethoxam and CBPV. Three significantly different groups emerged from the statistical analysis: a) control bees and bees exposed to 2.5 ng/bee/day of thiamethoxam, b) CBPV and co-exposure to 2.5 ng/bee/day of thiamethoxam and CBPV, c) honeybees exposed to 5.0 ng/bee/day of thiamethoxam and d) co-exposure to 5.0 ng/bee/day of thiamethoxam and CBPV. Stars denote significance of the χ^2 compliance test and show the synergistic effect of co-exposure on survival (**: $p<0.01$). At day 0, the honeybees are already 9 days old.

Fig. 5: Comparison between expected mortality rates (additive interactions), and observed mortality rates of honeybees after 8 to 10 days of co-exposure to CBPV and 5.0 ng of thiamethoxam/bee/day. Black bars: mortality rate in honeybees fed thiamethoxam at 5.0 ng/bee/day; white bars: mortality rate in honeybees exposed to CBPV-infected honeybees; grey bars: mortality rate in honeybees co-exposed to CBPV-infected bees and thiamethoxam at 5 ng/bee/day. Stars show significant difference between expected and observed mortality rates (**: $p<0.01$). Means and standard deviations are shown (n=3 cages for each measurement and day).

Fig 6: Distribution of the viral loads quantified in live honeybees from the co-exposure experiment (n=9 for each condition and day). White boxes: control bees, light grey boxes: 2.5 ng/bee/day of thiamethoxam, dark grey: 5.0 ng/bee/day of thiamethoxam; hatched: contact with CBPV-sick bees. The dotted line represents "infection threshold" (10^8 copies/individual) over which infected honeybees are likely to develop clinical signs of the CBPV disease (Chevin et al., 2012) and the full red line represents the PCR LOQ ($10^{3.9}$ copies per individual). Box-plots show the distribution of populations, with first quartile (25%), median (50%), and third quartile (75%) (boxes), minimum and maximum (whiskers) and outliers

(circles). Stars show significant difference in viral-load between no CBPV and CBPV exposed honeybees (**: $p<0.01$).

Fig. 7: Distribution of the viral loads quantified in the dead honeybees at each sampling day from the co-exposure experiment. Nine honeybees were sampled for each condition and day except otherwise stated on the graph. White boxes, crossed: contact with CBPV-sick bees; light grey boxes: CBPV and 2.5 ng/bee/day of thiamethoxam, dark grey: CBPV and 5.0 ng/bee/day of thiamethoxam (only those in contact with CBPV-infected bees are shown; there was not enough mortality in the other conditions). The dotted line represents "infection threshold" (10^8 copies/individual) over which infected honeybees are likely to develop clinical signs of the CBPV disease (Chevin et al., 2012) and the full red line represents the PCR LOQ ($10^{3.9}$ copies per individual). Box-plots show the distribution of populations, with first quartile (25%), median (50%), and third quartile (75%) (boxes), minimum and maximum (whiskers) and outliers (circles). Stars show significant difference in viral-load found in CBPV exposed honeybees between day 1 and both days 5 and 10 (**: $p<0.01$).

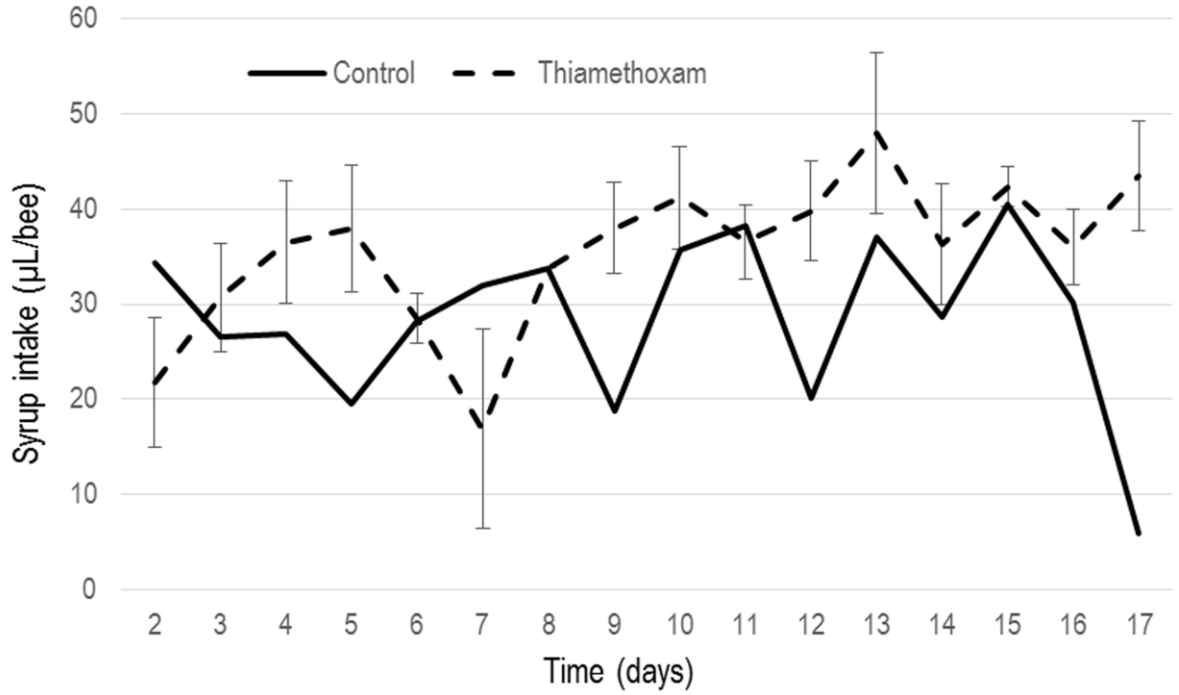


Fig 1

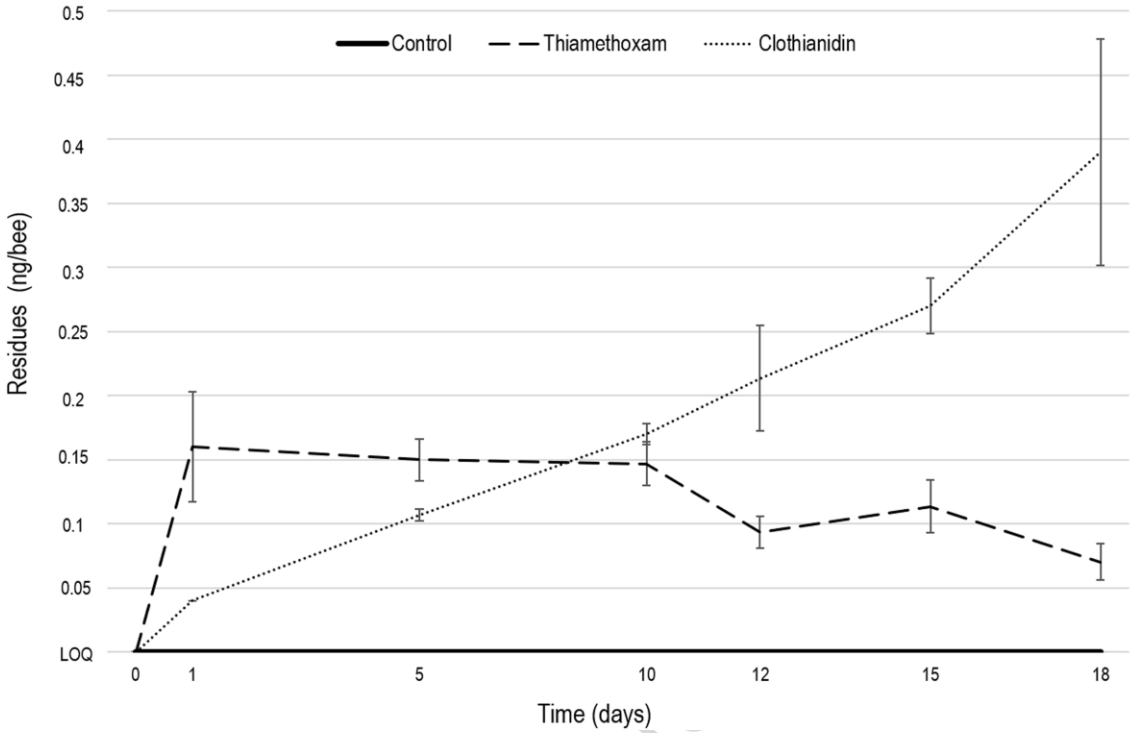


Fig 2

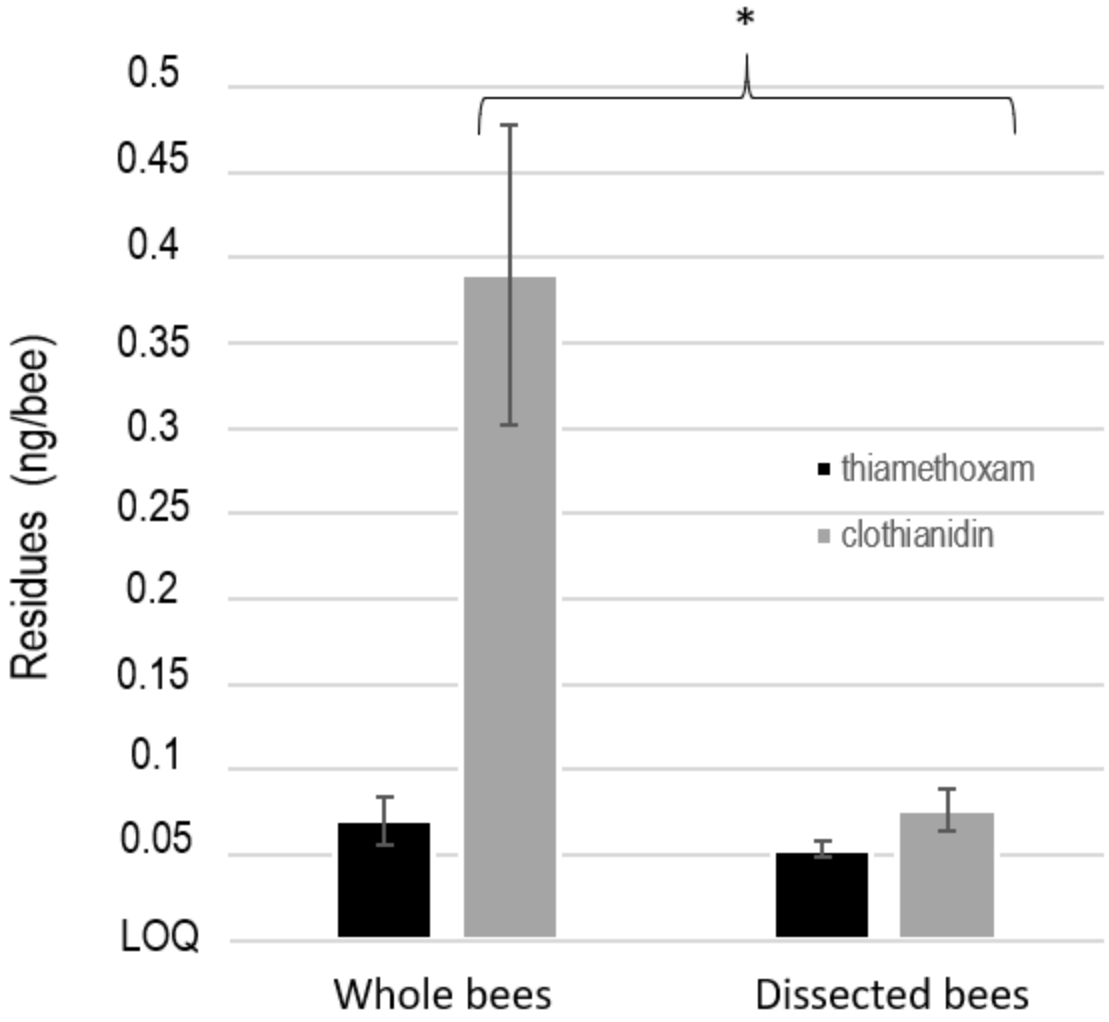


Fig 3

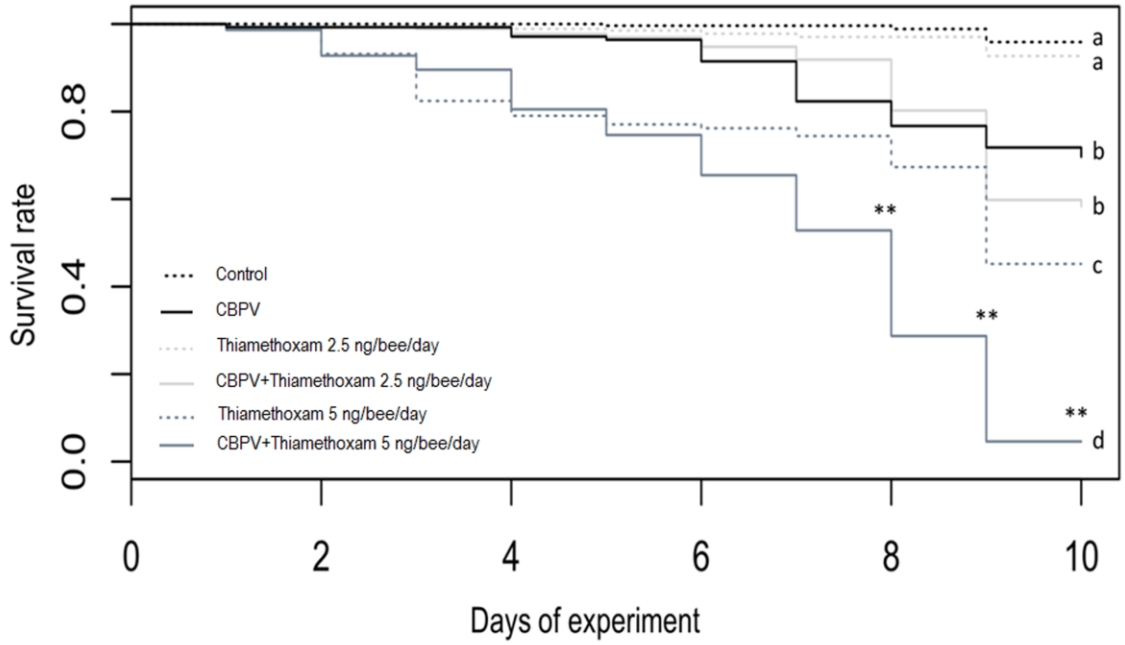


Fig 4

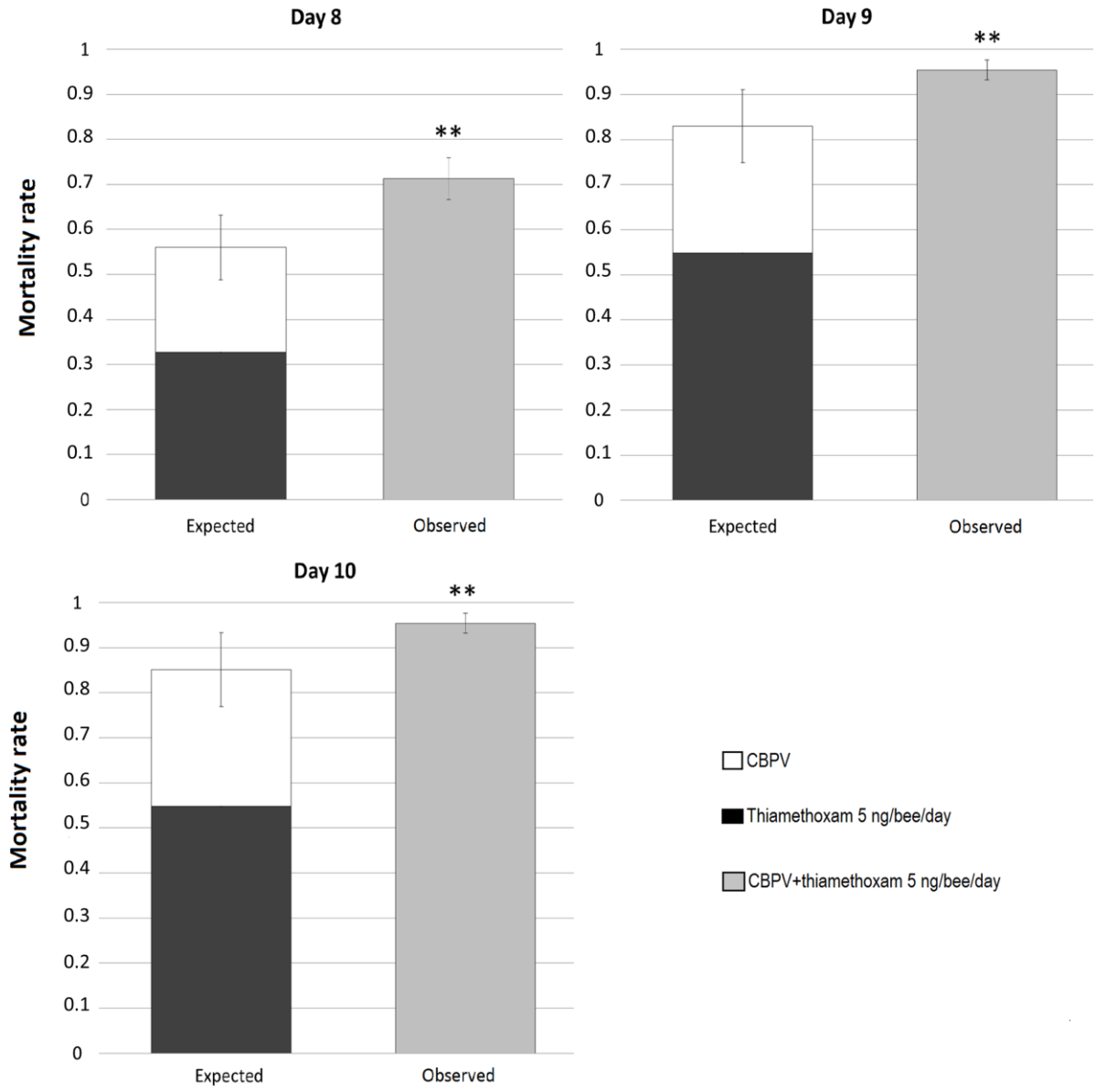


Fig 5

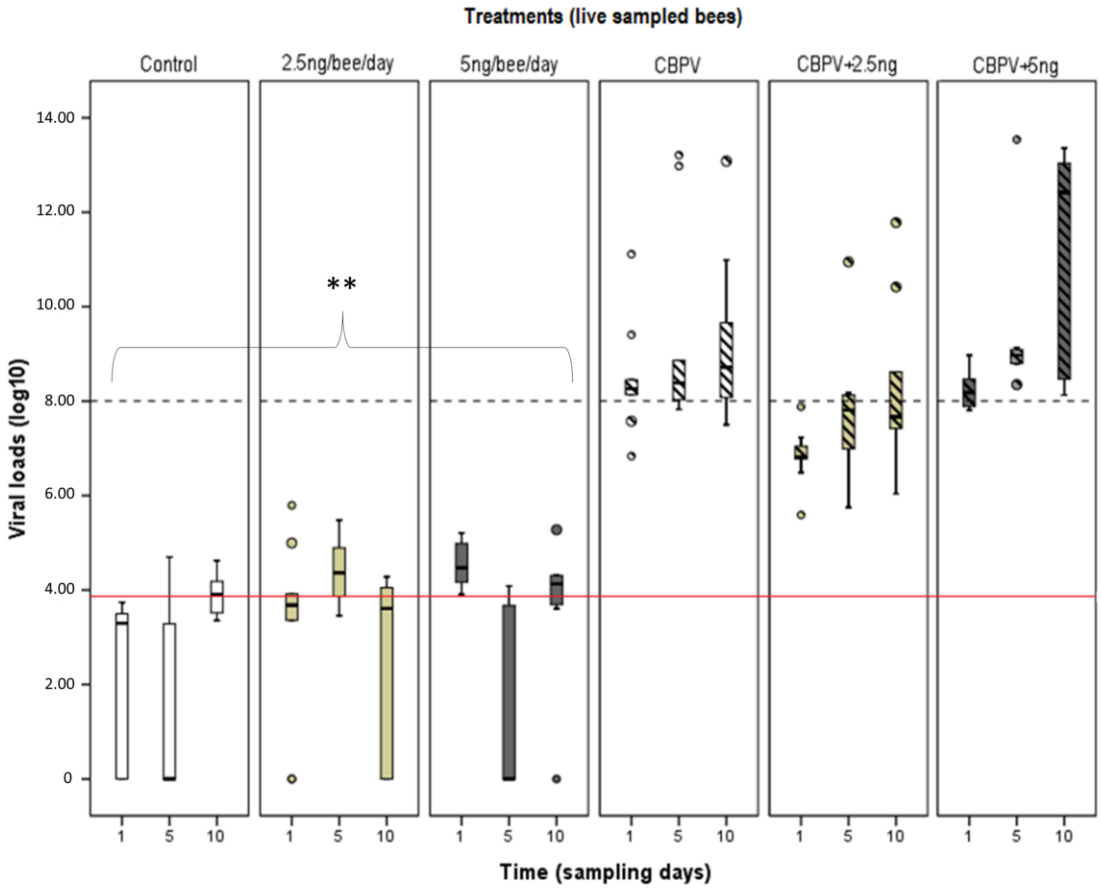


Fig 6

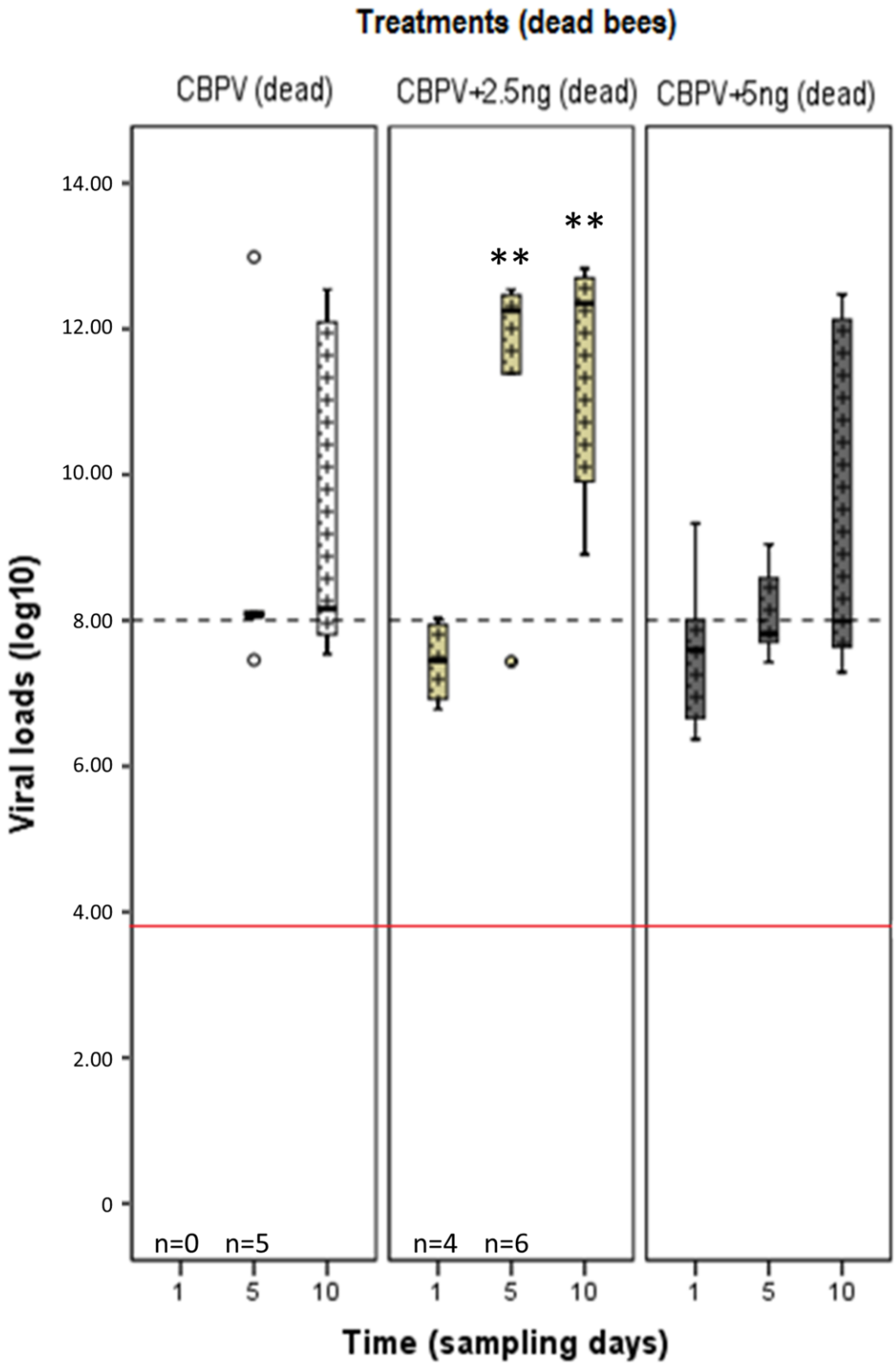
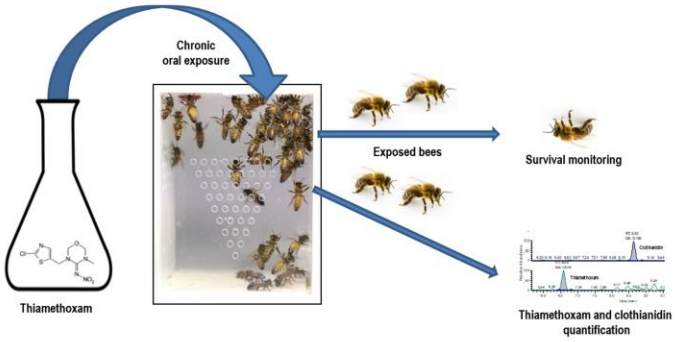


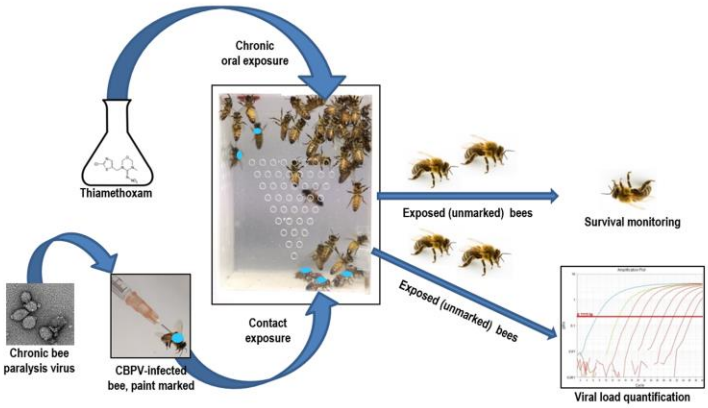
Fig 7

Experiment 1: Metabolisation kinetics



Chronic exposure to 0.25ng/bee/day	Control	Thiamethoxam
Survival	→	→
Quantification	**Thiamethoxam (ng/bee)**	**Clothianidin (ng/bee)**
Concentration	→	↗

Experiment 2: CBPV – thiamethoxam co-exposure



Co-exposure effects	Thiamethoxam (ng/bee/day)	
	2.5	5.0
Survival	→	↘
Viral load	↗	→
Tolerance	→	↘

Graphical abstract

Highlights

- Caged honeybees were chronically exposed to thiamethoxam and CBPV
- Thiamethoxam is metabolized into clothianidin in the honeybee
- Clothianidin accumulates in caged honeybees' rectum
- Thiamethoxam at 5.0 ng/bee/day and CBPV cause a synergistic effect on mortality
- Synergy could be explained by a decrease in the honeybee tolerance to the virus