Experiment 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/day3.1.2. Metabolisation of thiamethoxam into clothianidin

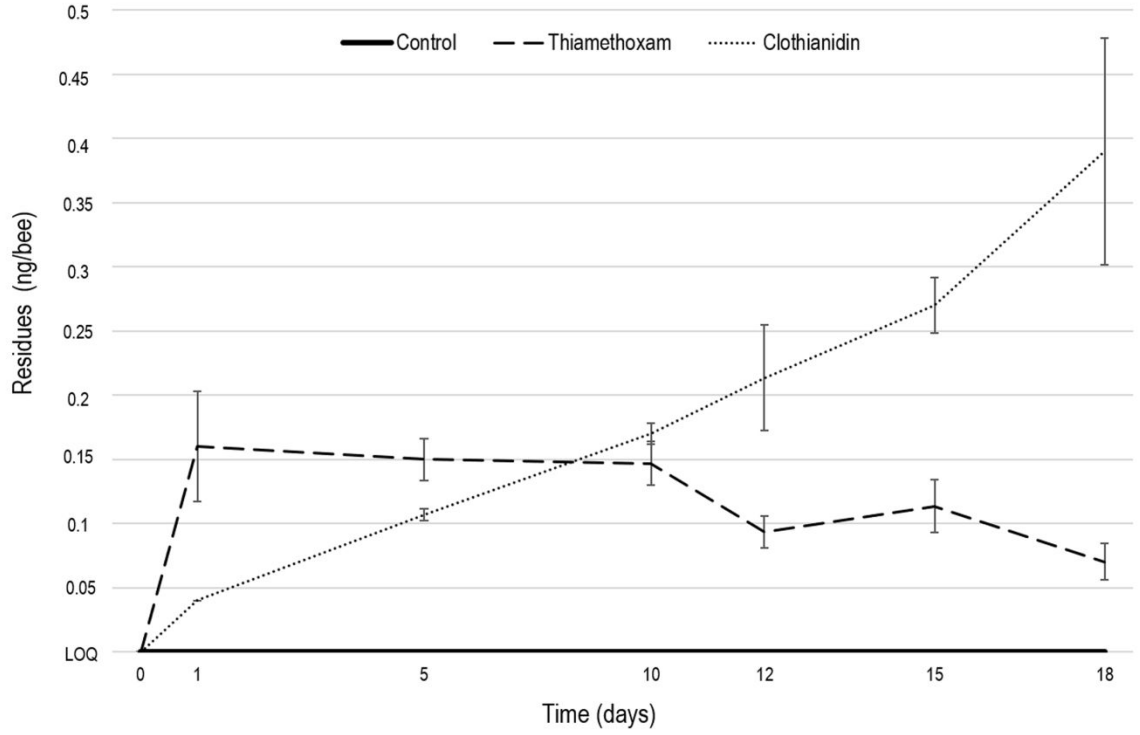

Fig. 2: Metabolisation kinetics of thiamethoxam in chronically exposed honeybees. Solid line: quantity of thiamethoxam in control honeybees (control), dashed line: quantity of thiamethoxam in thiamethoxam-fed honeybees (thiamethoxam), dotted line: quantity of clothianidin in the same thiamethoxam-fed 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.

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.

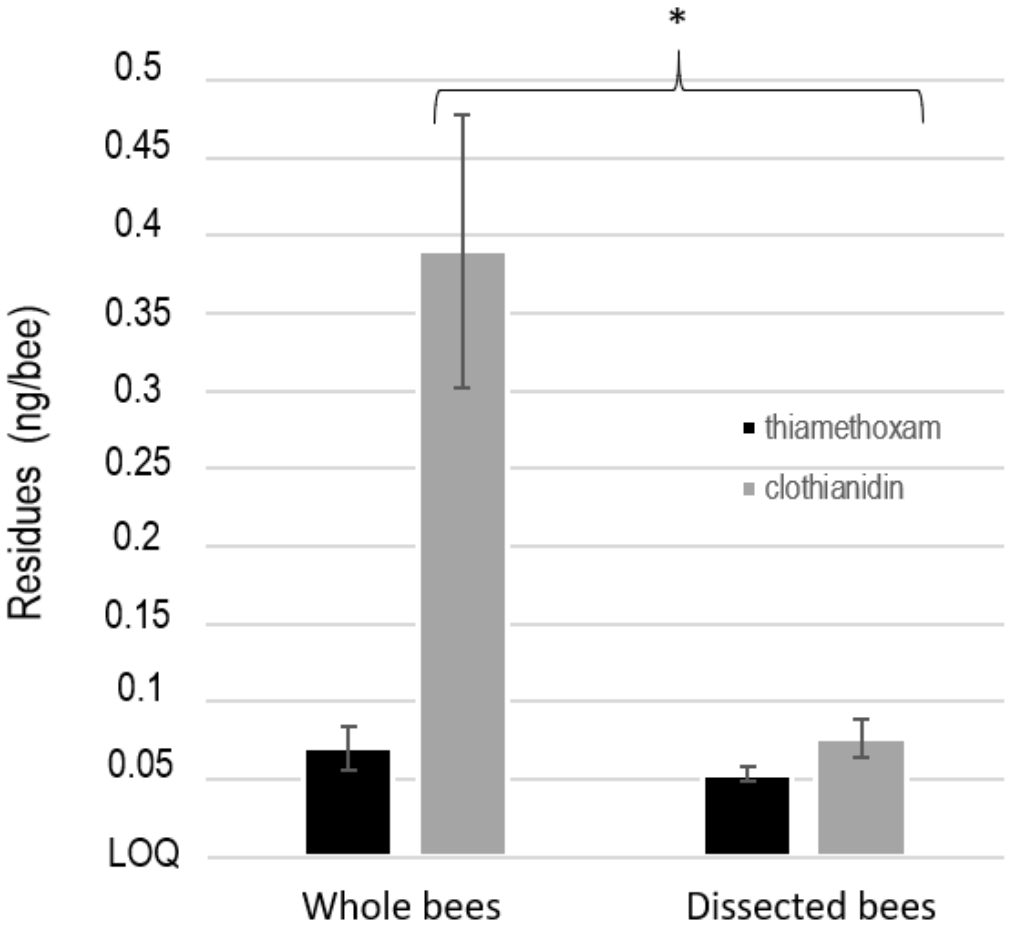

Fig. 3: Comparison of thiamethoxam and clothianidin levels between whole and dissected (rectum excised) honeybees (n=20x3 replicate cages = 60 bees in each group (whole or dissected bees)). Black bars: thiamethoxam measurements; grey bars: clothianidin measurements. Star shows significant difference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

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.

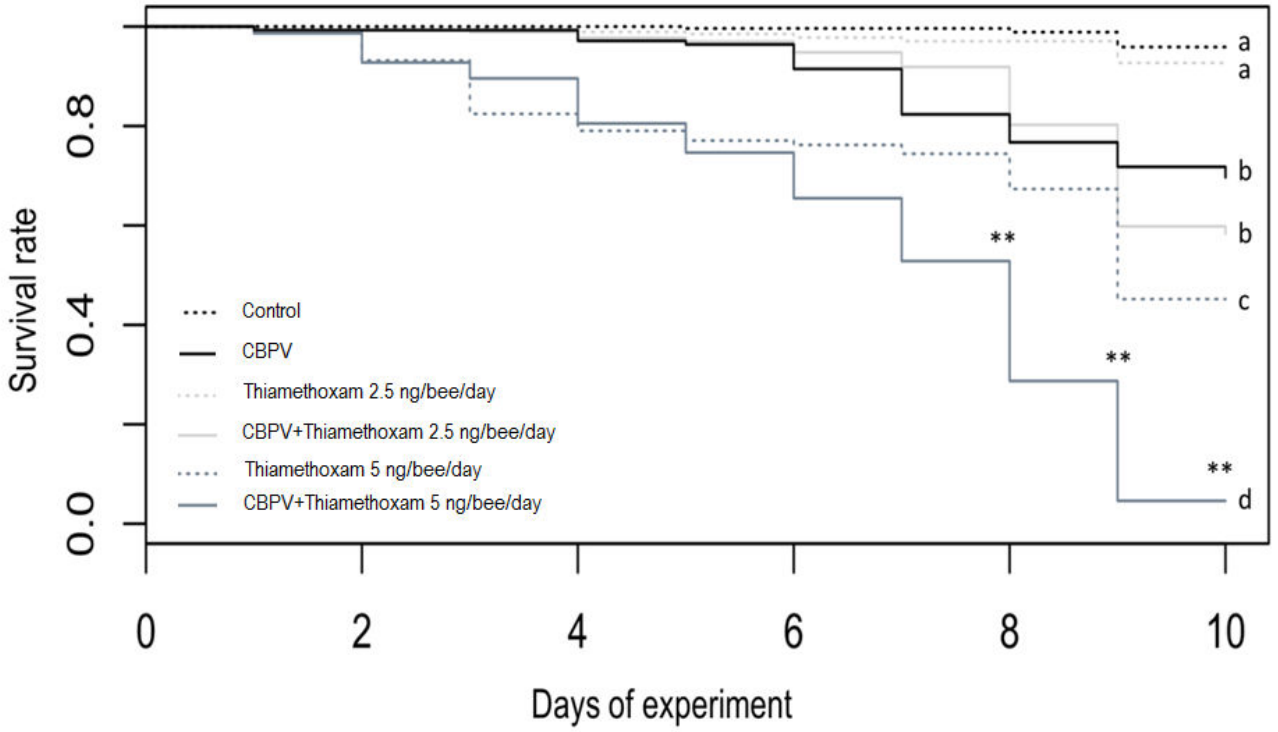

Fig. 4: Survival curves according to Kaplan-Meier estimation. Letters show statistical differences between curves (log-rank test). Dotted black line: control, solid black line: CBPV, dotted light grey line: 2.5 ng/bee/day of thiamethoxam, solid light grey line: co-exposure between 2.5 ng/bee/day of thiamethoxam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

3.2. 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.

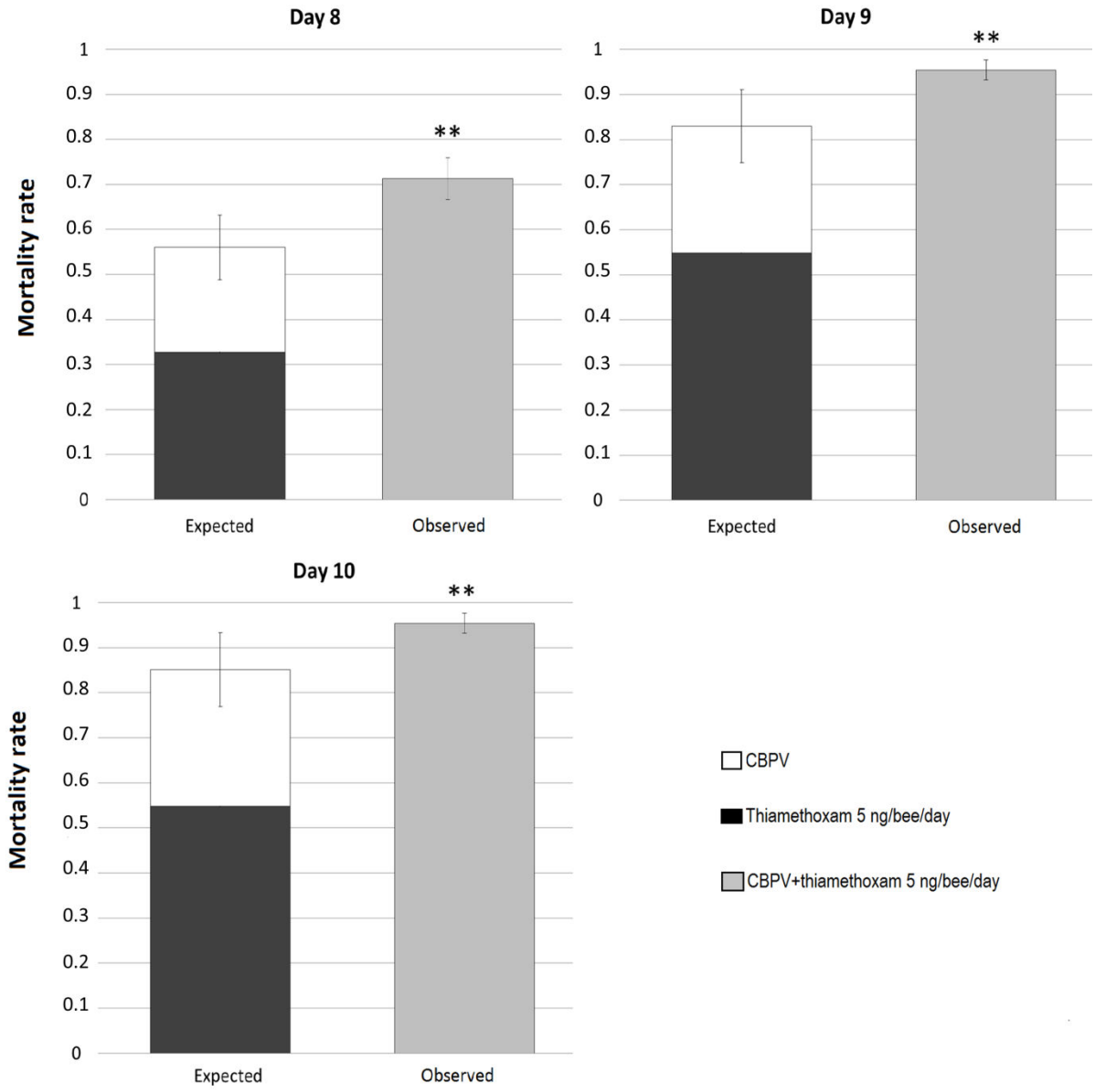

Fig. 5: Comparison between expected mortality rates (additive interactions), and observed mortality rates of honeybees after 8 to 10 days of co-exposure to CBPV and 5.0 ng of thiamethoxam/bee/day. Black bars: mortality rate in honeybees fed thiamethoxam at 5.0 ng/bee/day; white bars: mortality rate in 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).

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.

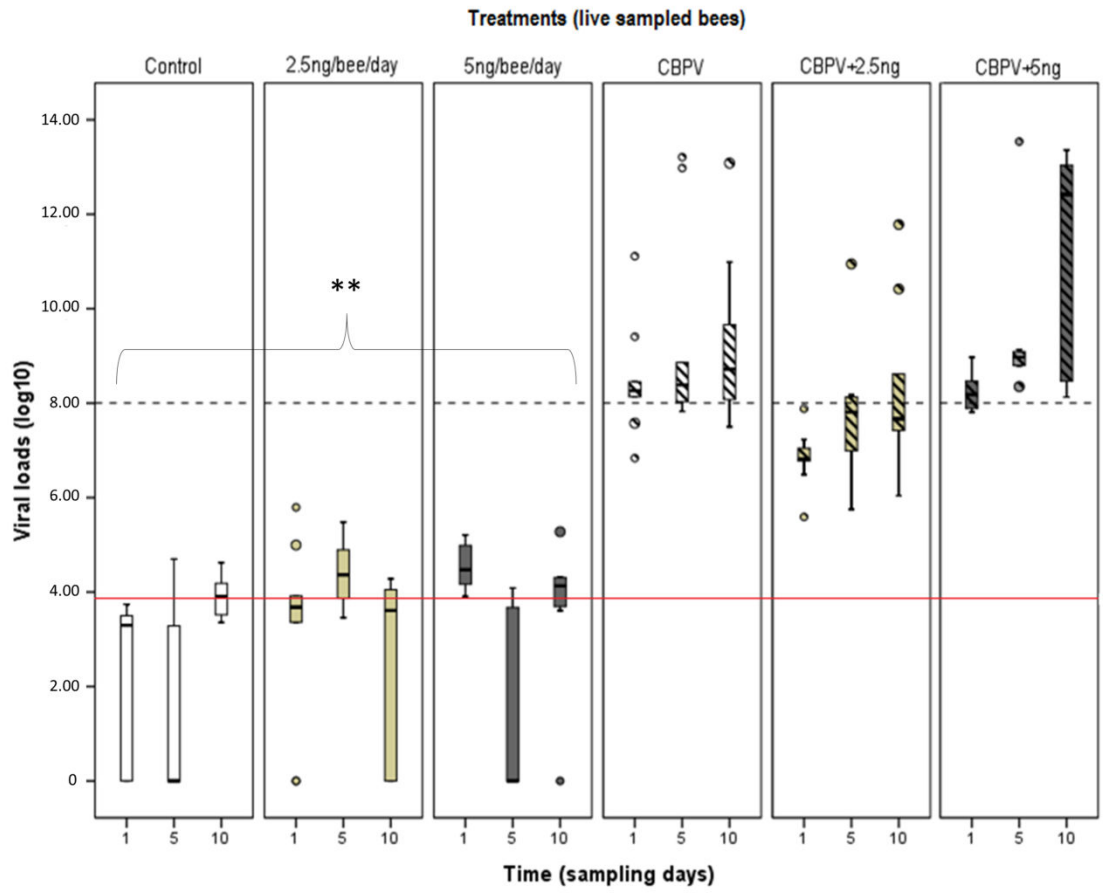

Fig 6: Distribution of the viral loads quantified in live honeybees from the co-exposure experiment (n=9 for each condition and day). White boxes: control bees, light grey boxes: 2.5 ng/bee/day of thiamethoxam, dark grey: 5.0 ng/bee/day of thiamethoxam; hatched: contact with CBPV-sick bees. The dotted 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 (**: $p<0.01$).

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).

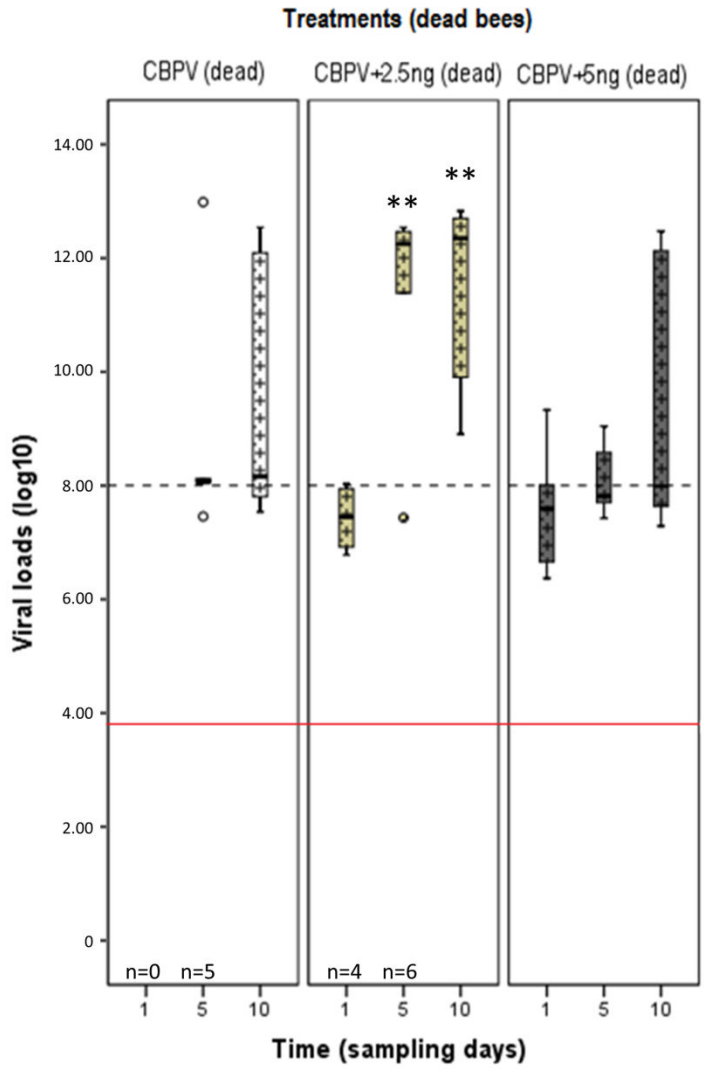

Fig. 7: Distribution of the viral loads quantified in the dead honeybees at each sampling day from the co-exposure experiment. Nine honeybees were sampled for each condition and day except otherwise stated on the graph. White boxes, crossed: contact with CBPV-sick bees; light grey boxes: CBPV and 2.5 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 (**: $p<0.01$).

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 (Laurent et al., 2015; Tentcheva et al., 2004), and thiamethoxam is used on common crops that are very attractive to honeybees, such as oilseed rape (Simon-Delso et al., 2015; van der Sluijs et al., 2013), 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 (Nauen et al., 2003). 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 (du Rand et al., 2015). 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 (Kessler et al., 2015; Thompson et al., 2015) 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 (Rand et al., 2015).

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 (Xu et al., 2005). 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 (du Rand et al., 2015; du Rand et al., 2017; Winston, 1987). 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 (du Rand et al., 2017). 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 (Ribière et al., 2010). 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 (Rueppell et al., 2017). 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 (Clément et al., 2011).

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 (Botías et al., 2015); 53.3 ppb (ng/g) in stored pollen (C. A. Mullin et al., 2010c); and a maximum of 20.2 ± 0.4 ng/g in honey (Barganska et al., 2013). 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. (Aston and Bucknall, 2009). 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 (EFSA, 2013; Laurino et al., 2011). 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 (Laurino et al., 2013; Rinkevich et al., 2015; Suchail et al., 2001). In addition, detoxification mechanisms or efficiency may differ between chronic and acute exposure (Stevenson et al., 2007; Suchail et al., 2001).

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 (Di Prisco et al., 2013; James and Xu, 2012; Sanchez-Bayo and Goka, 2014), 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 (Blanchard et al., 2012; Ribière et al., 2007). 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 (Di Prisco et al., 2013), 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 (Evans and Spivak, 2010; Medzhitov et al., 2012; Schneider and Ayres, 2008). 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 (Corby-Harris et al., 2007). 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

This study was supported by "Phyto-Pharmaco vigilance" scheme (ANSES, France). We are also grateful to Carolyn Engel-Gautier, professional translator and native English speaker, for revising the paper.

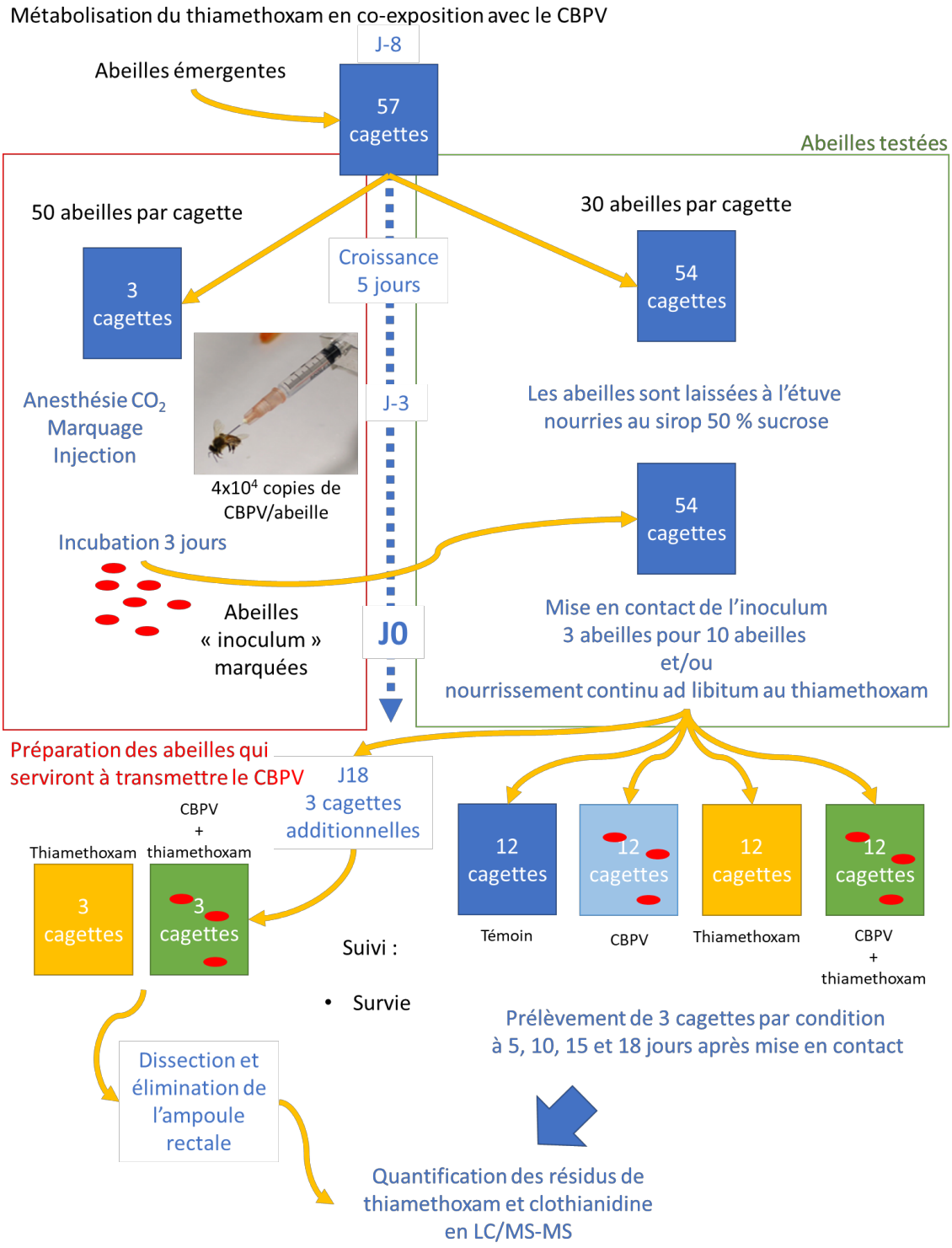

Figure 27 : Schéma récapitulant la méthode utilisée afin de suivre la cinétique de dégradation du thiaméthoxam en clothianidine lors d'une co-exposition avec le CBPV et en conditions contrôlées.

3. Effets d'une co-exposition sur les charges virales, la transcription de gènes et la métabolisation

Afin d'approfondir les résultats obtenus lors de ces premières expériences et de tenter de mettre en lumière les mécanismes qui les causent, nous avons répétées les expériences de co-exposition, et quantifié la transcription d'une sélection de gènes, liés à l'immunité ou à la détoxication des xénobiotiques (figure 25). Pour observer si l'infection virale pourrait avoir un impact sur la cinétique de métabolisation du pesticide, nous avons également répété les dosages du thiaméthoxam et du clothianidine, en rajoutant la condition de co-exposition avec le CBPV (figure 27).

Les résultats obtenus sont présentés sous forme d'article en prévision d'une valorisation scientifique.