Chronic exposure to thiamethoxam can promote *Chronic bee paralysis virus* infections in honeybees

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Abstract

Co-exposure to pesticides and viruses is likely to occur in honeybee colonies. Pesticides can be present in pollen, nectar, and persist in stored food (honey and bee-bread). Viruses can spread between honeybees by contact or trophallaxis, or may be vectorised by the mite *Varroa destructor*. Here, we studied the effect of chronic co-exposure to thiamethoxam and *Chronic bee paralysis virus* (CBPV) on honeybee health. No synergistic effect of co-exposure was observed on bee survival, nor on the ability of bees to metabolise the pesticide to

clothianidin. However, we found that co-exposure caused an increase in CBPV loads that reached the viral levels usually found in overt infections. The effect of co-exposure on CBPV replication was associated with down-regulation of vitellogenin and dorsal-1a gene transcription. These results could explain CBPV-related mortality peaks in single colonies or whole apiaries exposed to both stress factors.

1.Introduction

Many studies have provided concurring evidence that several stress factors, acting individually or in combination, are responsible for honeybee and other pollinator losses: loss of habitats, global warming, decreased availability of food sources, pesticide use in agriculture, and spread of parasites and pathogens (Goulson et al., 2015; S. G. Potts et al., 2010a).

Viruses are increasingly being investigated as potential causes of honeybee loss (Brutscher et al., 2016; Chen and Siede, 2007; Genersch and Aubert, 2010; Gisder and Genersch, 2015). They generally persist in honeybee populations at low viral levels without clinical signs until the emergence of overt infections. These sudden viral outbreaks are caused by unknown factors, apart from viruses that can be transmitted by the *Varroa destructor* parasites, which multiply and transmit large quantities of viruses and diminish immune barriers (de Miranda and Genersch, 2010; Di Prisco et al., 2016). At these high levels, viral infections can cause high worker mortality and/or colony losses (Chevin et al., 2012; Dainat et al., 2012a; Garrido-Bailon et al., 2010; Highfield et al., 2009).

Another factor possibly causing these viral outbreaks could be exposure to pesticides. Some pesticides have been found to impair honeybee immune response and to be linked to honeybee diseases (Sánchez-Bayo et al., 2016). This last decade, pesticides, and among them neonicotinoids, have been increasingly incriminated as one of the most dangerous factors in honeybee colony losses (Efsa, 2013; Pisa et al., 2014; Sanchez-Bayo and Goka, 2014). Some interactions between viruses and neonicotinoids have already been reported. Increased mortality in honeybee larvae with high viral loads of *Black queen cell virus* (BQCV) has been found when these virus-infected bees were co-exposed to sublethal doses of thiacloprid (Doublet et al., 2015a). Significant increases in *Deformed wing virus* (DWV) loads have also

been found in honeybees co-exposed to the virus and clothianidin (Di Prisco et al., 2013). The authors of this paper also discovered that the honeybees could no longer control the viral replication because the transcription of the dorsal-1a gene, an NF-κB effector protein involved in the Toll pathway (Brutscher et al., 2015), was inhibited by the effect of clothianidin on another effector of this pathway (leucine-rich repeat - LRR).

In order to study the factors triggering the transition from covert to overt viral infections in honeybees, we performed co-exposure experiments with the *Chronic bee paralysis virus* (CBPV) and the neonicotinoid thiamethoxam.

CBPV, which causes chronic bee paralysis (Bailey et al., 1963), is not yet classified, but shows similarities to the *Nodaviridae* and *Tombusviridae* families (Olivier et al., 2008). Its genome is made up of two segments of single-stranded RNA in a non-enveloped anisometric capsid (Youssef et al., 2015). This virus has been found to be transmitted horizontally by contact between healthy and sick bees in the hive (Ribière et al., 2007). Aside from the hairless black body symptom known since Antiquity, tremors, paralysis, and inability to fly are the most frequent and characteristic symptoms, with a further characteristic sign being piles of dead or paralysed individuals in front of the hives (Aubert et al., 2008; Ribière et al., 2010). However, confusion with symptoms of pesticide intoxications (Johansen, 1977) could occur (Ribière et al., 2010). CBPV has been shown to have neurotropism, and was observed to be present in the mushroom bodies of infected honeybees, which could cause the specific nervous symptoms (Olivier et al., 2008). In one study, CBPV was detected in 28% of apiaries in France (Tentcheva et al., 2004), but only 2% of colonies were reported to show clinical signs of the disease (Laurent et al., 2015). CBPV has different prevalence rates across Europe, with a prevalence of 10% of tested colonies in Austria (Berényi et al., 2006) and none in Hungary (Forgách et al., 2008). Natural outbreaks occur sporadically, more frequently in the spring and summer (Ribière et al., 2010). Viral loads found in bees from symptomatic colonies are significantly higher than in bees from asymptomatic colonies (over 10^8 genome equivalent per bee) (Blanchard et al., 2007). Bailey et al. (Bailey et al., 1963) suggested that overt disease occurs when bee resistance is diminished.

Thiamethoxam is a neonicotinoid insecticide commonly used around the world on oil-seed rape, a crop that is widespread and attractive to honeybees (Simon-Delso et al., 2015; van der

Sluijs et al., 2013). This pesticide has been reported to be present at various doses in the honeybee environment, with for example concentrations reaching 13.3 ng/g in nectar from oil-seed rape, and 86 ng/g in pollen from field margin plants (Botías et al., 2015). It has also been detected in hive matrices, at a maximum of 20.2 ng/g in honey (Barganska et al., 2013), and 53.3 ng/g in stored pollen (C. A. Mullin et al., 2010c). Neonicotinoids bind with high affinity to acetylcholine receptors, altering neuronal signals, which leads to paralysis and death of the insects (Nauen et al., 2003). At sublethal doses, it can have negative effects on homing flights in foragers (Henry et al., 2012), and olfactory memory and learning (Yassine Aliouane et al., 2009). Chronic exposure has been shown to damage the brain and gut of Africanized honeybees (Catae et al., 2014; Oliveira et al., 2014). Thiamethoxam is quickly and readily metabolised to clothianidin, which is also marketed as an insecticide (Nauen et al., 2003). Clothianidin shows a similar effect to thiamethoxam on insect acetylcholine receptors, and has also been found to inhibit the honeybee immune system, which in turn can promote the replication of DWV (Brandt et al., 2016; Di Prisco et al., 2013).

In order to study the effect of co-exposure to CBPV and thiamethoxam, which is likely to occur in the field, we examined the mechanisms of the interaction in honeybees. We monitored the impact of both stressors separately and in combination on honeybee survival and viral loads, after 10 days of chronic exposure to thiamethoxam (oral exposure) and to CBPV-infected honeybees (contact exposure). We also selected three genes that are part of immune pathways: dorsal-1a, an effector of the NF-κB pathway which can lead to the production of antimicrobial peptides (AMPs); apidaecin (also involved in AMP production); and prophenoloxidase (ppo) from the melanisation pathway (Boncristiani et al., 2013; Di Prisco et al., 2013; Simone et al., 2016). Finally, we selected three genes that play a role in detoxification processes: glutathione-S-transferase 3 (GstS3), catalase and CYP6AS14 (Boncristiani et al., 2012; Mao et al., 2011). Based on the results obtained concerning these genes, we also investigated whether CBPV infection could impair thiamethoxam metabolisation to clothianidin, by carrying out a kinetic analysis.

2.Materials and Methods

2.1. Winter-born honeybees

Experiments were performed with honeybees (*Apis mellifera*) obtained from three colonies previously tested negative for CBPV, *Acute bee paralysis virus* (ABPV), *Sacbrood virus* (SBV) and DWV, located at the ANSES Sophia Antipolis laboratory winter apiary (Youssef et al., 2015), in February 2016 and March 2017 (Experiments 1 and 2, respectively). In 2016, colonies in the winter apiary were fed with 50% sucrose syrup prepared in our laboratory from pure sucrose ($D^{(+)}$-sucrose, Acros Organics, Fisher Scientific, USA) and water, and protein paste, also prepared in our laboratory from candy sugar (Apifonda, Südzucker AG, Germany), fructose syrup (Fructoplus, Icko-Apiculture, France), *Saccharomyces cerevisiae*, and a commercial mix of supplemental *S. cerevisiae*, ascorbic acid, and various proteins and minerals (Apifeed, SINTAL, Italy). Both experiments were based on the same protocol in order to study the influence of thiamethoxam and CBPV co-exposure on bee survival, virus loads and physiology (Experiment 1), and on pesticide metabolisation (Experiment 2). Frames containing late-stage pupae were collected and placed in an incubator overnight at 34°C. Emerging bees were pooled to minimise colony-born bias and distributed into cages: 30 bees per cage of about 780 cm^3 (Pain, 1966), with a capacity of 100 bees. Cages were maintained at 34°C in incubators with saturated humidity, and bees were fed *ad libitum* with one feeder containing 50% sucrose syrup, a second feeder containing 50% sucrose syrup supplemented with 1% protein (Provita'Bee, ATZ Diététics, France), and a third containing crystallised sugar paste. All feeders were available continuously for 9 days after emergence. Once the experiment began, feeders containing candy and syrup with protein were removed. Only the 50% sucrose syrup feeders, supplemented with thiamethoxam or not depending on the conditions, were provided to the honeybees.

2.2. Honeybee CBPV exposure

In order to reproduce natural transmission of CBPV, viral exposure was performed by contact between experimental honeybees and previously CBPV-inoculated honeybees. Five-day-old bees were inoculated with the CBPV strain A-79P (accession numbers: EU122229.1 and EU122230.1), according to the previously described protocol (Youssef et al., 2015). After 4 days of incubation, 5 to 9 CBPV-infected bees (depending on the difficulty of obtaining a

high number of bees of the same age) were marked and used to propagate the virus to nine-day-old honeybees (30 healthy honeybees per cage). A preliminary experiment had shown that both proportions of infected bees per cage had comparable effects on survival and viral transmission (p>0.05; data not shown). The injected bees (marked bees) died within the first three days, but were not removed, to continue propagating the virus.

2.3. Honeybee thiamethoxam exposure

To carry out pesticide exposure, a standard solution of thiamethoxam at 100 mg/L (prepared in water) was diluted in 50% sucrose as previously described (Coulon et al., n.d.), 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. These doses were considered to be field-relevant, on the basis of the previously cited thiamethoxam concentrations found, and considering that it is very difficult to predict or measure a repeated amount of pesticide in nectar or pollen, due to the way in which biotic and abiotic factors act on the production of the flower (Aston and Bucknall, 2009).

2.4. Experiment 1: Influence of thiamethoxam and CBPV co-exposure on bee survival, virus loads and physiology

After nine days of growth for emerging honeybees, feeders were removed and replaced by only one feeder containing either 50% sugar syrup or 50% sugar syrup supplemented with thiamethoxam, according to the following six conditions (eight cages per condition): *i*. Control bees (bees not exposed to CBPV nor to thiamethoxam); *ii*. Bees in contact with CBPV-infected bees (five CBPV-infected bees as inoculum per cage); *iii*. Bees fed with 10 µg/L thiamethoxam-contaminated syrup (about 0.25 ng/bee/day); *iv*. Bees fed with 200 µg/L thiamethoxam-contaminated syrup (about 5.0 ng/bee/day); *v*. Bees co-exposed to both CBPV-infected bees and 0.25 ng/bee/day of thiamethoxam; and *vi*. Bees co-exposed to both CBPV-infected bees and 5.0 ng/bee/day of thiamethoxam.

Feeders were changed and weighed and survival was monitored daily (unmarked dead bees were removed from the cages). The volume consumed per bee was estimated taking into account the number of surviving honeybees per cage. At days 5 and 10 post-exposure, bees

from four cages per condition were sampled in liquid nitrogen and stored at -80°C for testing of gene transcription levels and viral load analysis.

2.5. Experiment 2: Influence of thiamethoxam and CBPV co-exposure on pesticide metabolisation

Nine-day-old bees were exposed or co-exposed to CBPV and/or thiamethoxam in the same way as previously described but in the following conditions for Experiment 2: *i*. Control bees; *ii*. Bees in contact with CBPV-infected bees (nine CBPV-infected bees as inoculum per cage); *iii*. Bees fed 2.5 ng/bee/day of thiamethoxam; and *iv*. Bees co-exposed to both CBPV-infected bees and 2.5 ng/bee/day of thiamethoxam. Feeders were changed and weighed and survival was monitored daily. After 1, 5, 10, 12, 15, and 18 days post-exposure, bees from each condition were sacrificed to analyse pesticide residues over time: bees from one cage for Control and CBPV conditions, and bees from three cages for the 2.5 ng/bee/day of thiamethoxam and the 2.5 ng/bee/day of thiamethoxam/CBPV conditions. At day 18 post-exposure, bees from three additional cages were sacrificed for each condition containing pesticide exposure. These bees were anesthetised using CO_2 gas, and then dissected to remove their rectum, where the pesticide residues might accumulate (Coulon et al., n.d.). Samples were stored at -20°C until chemical analysis.

2.6. Quantification of thiamethoxam and clothianidin

Neonicotinoid residues in samples (one sample: 20 whole bees or 20 dissected bees) were quantified using liquid chromatography with electrospray tandem mass spectrometry (LC-MS/MS), according to the protocol described by Martel *et al*. (Martel and Lair, 2011). 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.

2.7. Quantification of virus and gene expression levels

Eight bees were randomly selected from each experimental condition (sampling of two bees per cage) at day 10 after the beginning of the treatments. Viral RNA was extracted and CBPV

loads were measured in each individual honeybee by quantitative PCR following the protocol described by Schurr *et al.* (Schurr et al., 2017). The viral loads were expressed in decimal-logarithm (log_{10}) of CBPV genome equivalent per bee (copies/bee).

Eight additional honeybees were randomly selected from each experimental condition, and total RNA was isolated from each individual bee using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. The concentration and purity of total RNA were assessed by spectrophotometry (Varioskan Flash Spectral Scanning Multimode Reader; Thermo Fisher Scientific, USA) before being adjusted to the final concentration of 500 ng/µL of RNA.

The expression levels of immune genes (vitellogenin, dorsal-1-a, apidaecin, prophenoloxidase [ppo]) and detoxification genes (glutathione-S-transferase 3 [gst3], catalase and cyp6as14) were assessed using a StepOne Real-Time PCR System (Life Technologies, USA) based on a SYBR green detection method. The cycle threshold values of selected genes were normalised to the geometric mean of the housekeeping genes β-actin and RpL32. Relative gene transcription data were analysed using the $2^{\Delta\Delta Ct}$ method. To verify that the amplification efficiencies of the target and reference genes (β-actin and RpL32) (Reim et al., 2015) were approximately equal, amplifications of five 10-fold dilutions of the total RNA sample (from 1,000 to 0.1 ng per reaction) were analysed in triplicate. The efficiency plot for Log input total RNA vs. ΔCt curve had a slope lower than ± 0.1.

Amplifications were performed with the following thermal cycling profiles: one cycle at 48°C for 15 min for reverse transcription, one cycle at 95°C for 10 min, 40 cycles at 95°C for 15s and 60°C for 1 min, and one cycle at 68°C for 7 min, using the Power SYBR Green RNA-to-Ct 1-Step Kit (Thermo Fisher Scientific, USA). All primer pairs were designed using PrimerExpress 3.0 software (Life Technologies, USA) following the standard procedure. Negative (H_2O) and positive controls (previously identified positive samples) were included in each qRT-PCR run.

2.8. Statistics

Survival was established using a Kaplan-Meier estimation (Efron, 1988; Pepe and Fleming, 1989), and curves compared with log-rank tests (Bland and Altman, 2004). Synergistic

interactions were tested using a χ^2 of compliance test comparing survival measurements obtained for each day with the corresponding calculated expected measurements (Aufauvre et al., 2012; Mcvay et al., 1977). Log_{10}-transformed viral loads were analysed using a one-way ANOVA test followed by post-hoc *t*-tests or Tukey HSD tests (Miranda et al., 2013) or Kruskal-Wallis tests, followed by Wilcoxon pairwise tests with Bonferroni correction if data were not normally distributed (significant Shapiro-Wilk test). For gene expression analysis, the fold change in ΔCt was calculated using the $2^{\Delta\Delta Ct}$ method using control conditions as the basic reference. Transcription differences were compared using the ΔCt obtained and ANOVAs followed by Fisher's LSD post-hocs or Kruskal-Wallis tests, followed by Wilcoxon pairwise tests with Bonferroni correction if data were not normally distributed (significant Shapiro-Wilk test).

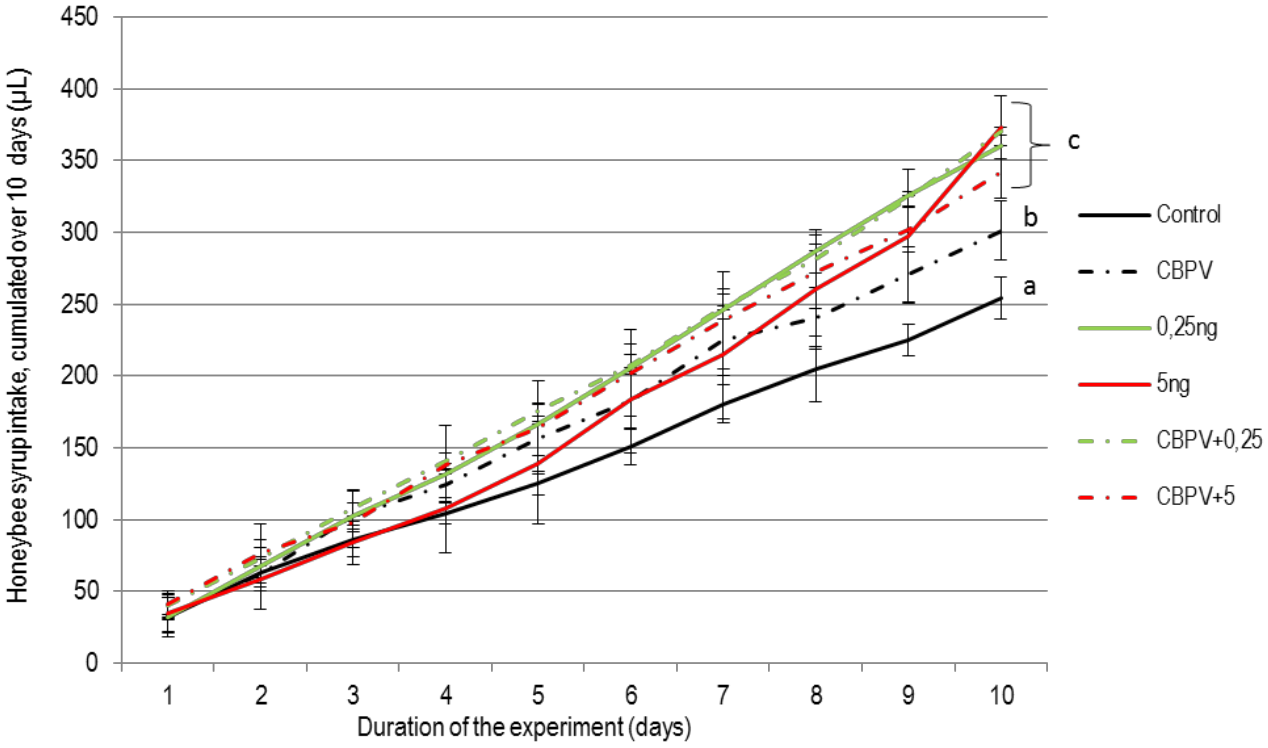

Fig. 1: Cumulated syrup intake (µL/bee) of bees exposed or co-exposed to CBPV and/or thiamethoxam. Syrup consumption is shown for each condition: Control bees, CBPV-exposed bees, Thiamethoxam-exposed bees (0.25 or 5.0 ng per bee), and bees co-exposed to CBPV and Thiamethoxam (0.25 or 5.0 ng per bee) (syrup intake is normalised considering the sampled cages and honeybee survival). Means and standard deviations of cumulated intakes are shown. The letters indicate significant differences (p<0.05) found at Day 10 post-exposure or co-exposure.

3.Results

3.1. Experiment 1: Influence of thiamethoxam and CBPV co-exposure on bee survival, virus loads and physiology

a) Syrup intake

CBPV infected bees consumed slightly more sugar syrup than control bees over 10 days (at the limit of significance, p=0.05; Fig. 1). However, a clear increase in syrup intake was observed in bees exposed to thiamethoxam in combination or not with CBPV and regardless of the dose (0.25 or 5.0 ng) as compared to control bees (p<0.01 for each condition; Fig. 1). The syrup intake in thiamethoxam-exposed bees (co-exposed or not to CBPV) was also higher than in CBPV-infected bees (p<0.01) except for bees co-exposed to CBPV and 5.0 ng/bee/day of thiamethoxam (p=0.14).

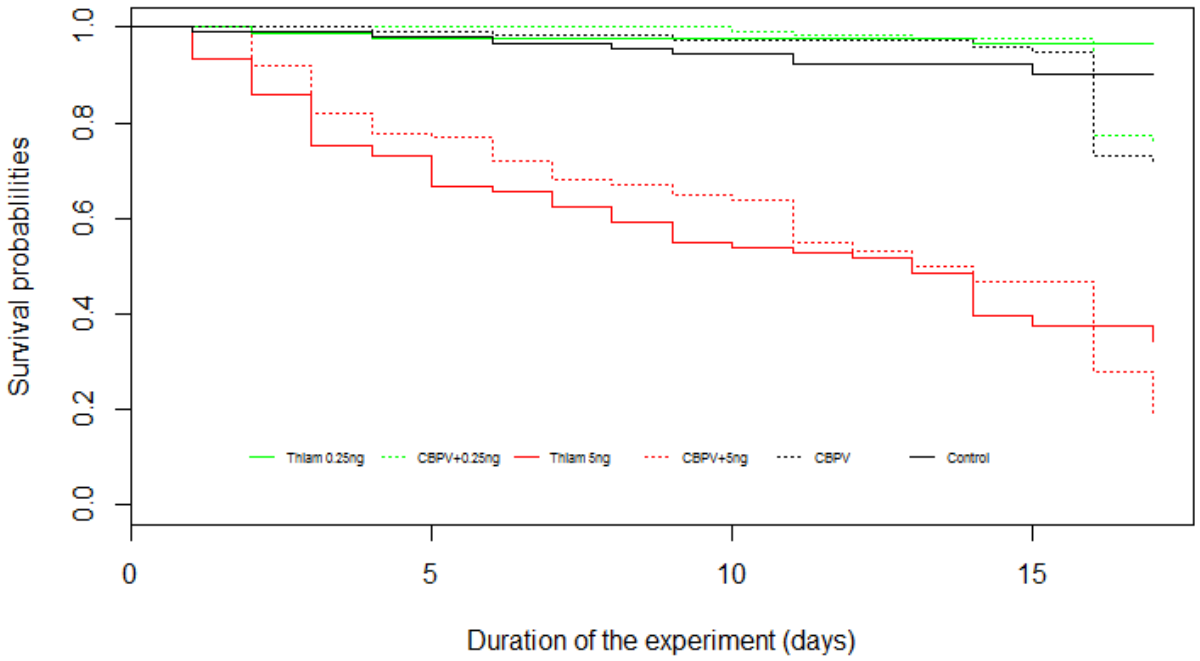

Fig. 2: Survival of bees exposed or co-exposed to CBPV and/or thiamethoxam. The survival rate is shown for each condition: Control bees, CBPV-exposed bees, Thiamethoxam-exposed bees (0.25 or 5.0 ng per bee), and bees co-exposed to CBPV and Thiamethoxam (0.25 or 5.0 ng per bee) (survival is normalised considering the sampled cages as Kaplan-Meier allows for censoring data). Letters show statistical differences between conditions (log-rank test). Three significantly different groups emerged from the statistical analysis: a) control honeybees and honeybees exposed to 0.25 ng/bee/day of thiamethoxam, b) honeybees exposed to CBPV alone, and co-exposed to 0.25 ng/bee/day of thiamethoxam and CBPV, c) honeybees exposed to 5.0 ng/bee/day of thiamethoxam and co-exposed to 5.0 ng/bee/day of thiamethoxam and CBPV.

b) Survival

The statistical analysis of the survival rates separated the honeybees into three different groups (Fig. 2). The survival rate of bees exposed to the lower dose of thiamethoxam (0.25 ng/bee/day) was not different from that of control honeybees (p=0.09; Fig. 2). However, bees from both groups exhibited a better survival rate than CBPV-infected honeybees (p<0.01 for both conditions) and bees co-exposed to CBPV and 0.25 ng/bee/day of thiamethoxam (p<0.01 for both conditions). Finally, bees exposed to the highest dose of thiamethoxam in combination or not with CBPV exhibited the lowest survival rate (p<0.01 when compared to the other conditions).

The survival rates found in the co-exposure conditions did not differ from the mortalities expected from an additive effect of CBPV and thiamethoxam exposure (for both doses; p>0.05).

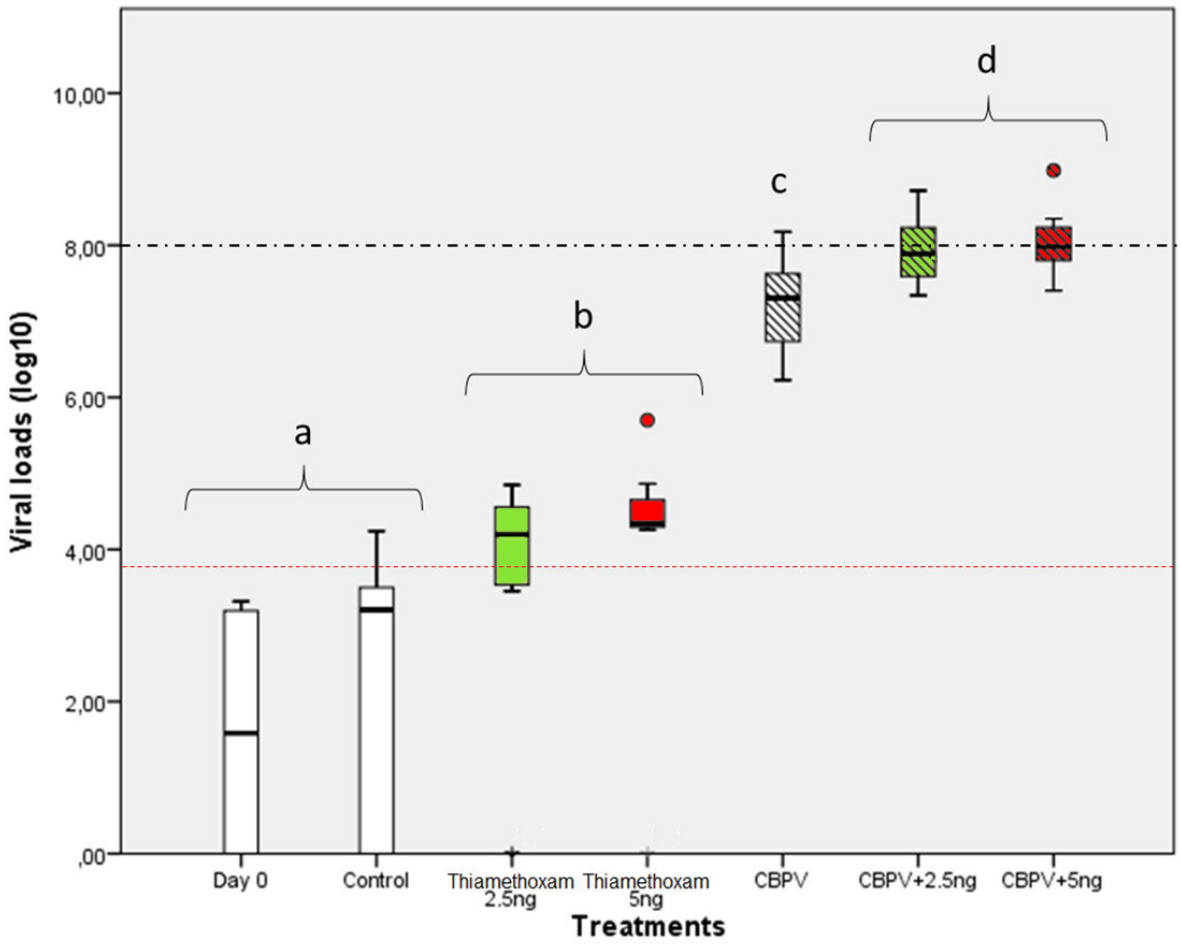

Fig. 3: Viral loads (log10 of the number of copies/bee) quantified in honeybees exposed or co-exposed to CBPV and/or thiamethoxam. Day 0 corresponds to newly emerged bees, whereas other measures were performed after 10 days of exposure. The CBPV infection level is shown for each condition: Control bees, CBPV-exposed bees, Thiamethoxam-exposed bees (0.25 or 5.0 ng per bee), and bees co-exposed to CBPV and Thiamethoxam (0.25 or 5.0 ng per bee) (n = 8 bees per condition). The dashed-dotted black line represents the "infection threshold" (10^8 copies/individual) above which infected honeybees are known to develop clinical signs of CBPV disease (Blanchard et al., 2007). Different letters show statistical differences between experimental conditions (Mann-Whitney test). Box-plots show the distribution of populations, with first quartile (25%), median (50%), third quartile (75%) (boxes), minimum and maximum (whiskers) and outliers (circles). The dotted red line shows the limit of quantification of the method (Blanchard et al., 2007).

c) Viral loads

As expected, control bees had low levels of CBPV, which were not significantly different from the levels observed in newly-emerged bees (p=0.38, Fig. 3). The CBPV levels of both groups were significantly lower than those encountered in bees exposed to both doses of thiamethoxam (p<0.03 for both groups). Exposure to CBPV-infected bees induced a significant increase of CPBV in nestmate bees, when compared to control bees (p<0.01), as well as bees exposed to thiamethoxam (p<0.02 for both doses).

Finally, the bees co-exposed to the virus and the pesticide exhibited the highest viral loads, which were significantly different from all the other conditions (p<0.05). In addition, for these co-exposure conditions, the CBPV levels at 0.25 and 5.0 ng of thiamethoxam reached a mean of 1.42×10^8 and 2.08×10^8 copies/bee, respectively, which was above the "infection threshold" leading to clinical signs of CBPV disease.

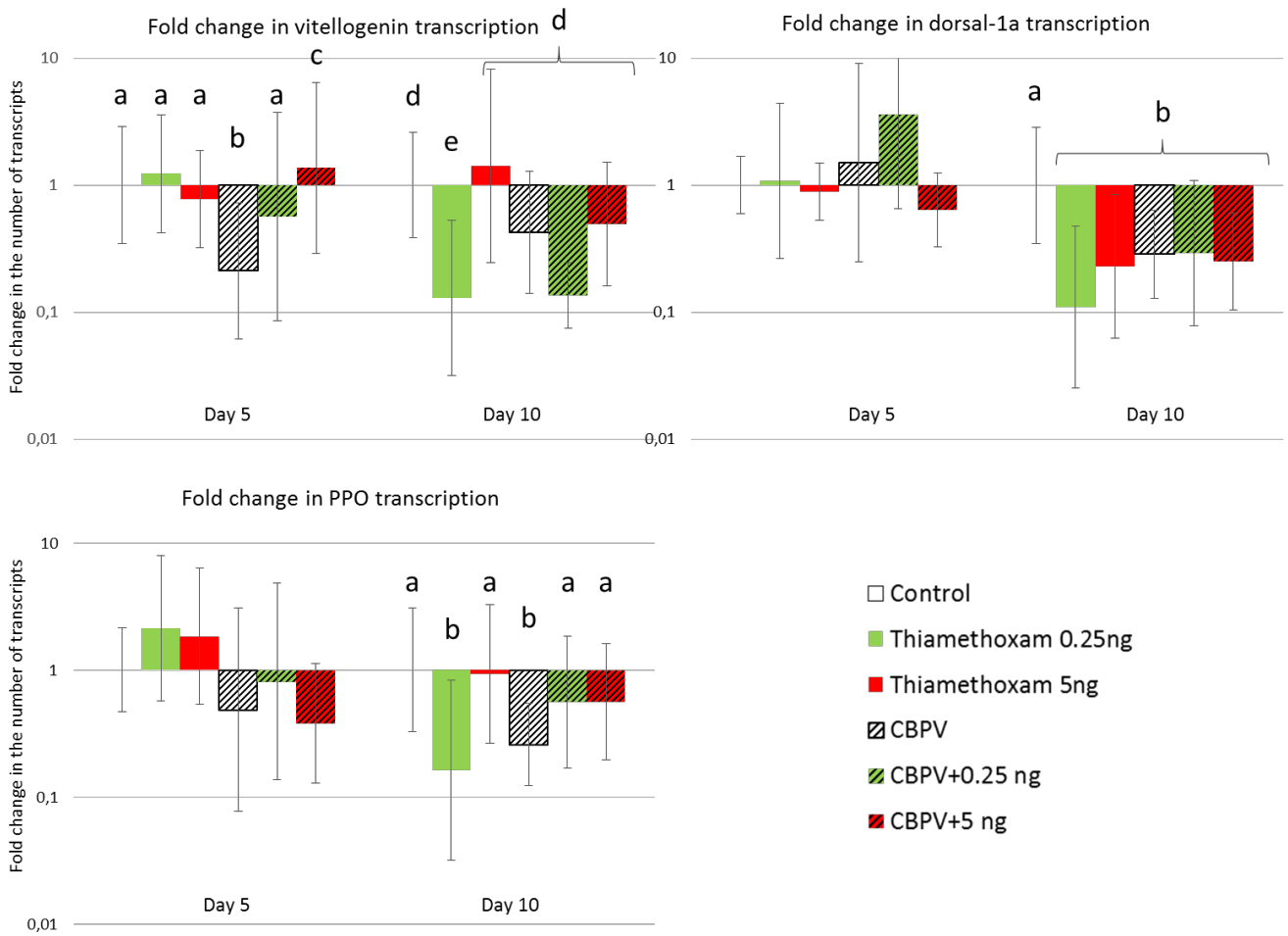

Fig. 4: Graphic representation of the fold changes in transcription for the tested immunity-related genes after exposures. The graph was built using the $2^{-\Delta\Delta Ct}$ method, at Day 5 and Day 10 after the beginning of co-exposure. Letters show statistically different groups, calculated on the basis of the ΔCt. Statistical groups are specific for each gene. No letter indicates that no statistical differences were found between the conditions (p>0.05).

d) Expression level of immune and detoxification genes

The relative changes (compared to the control bees) in transcription of the six selected genes, three immune-related (Fig. 4) and three detoxification-related (Fig. 5), were measured in honeybees after 5 and 10 days of exposure to thiamethoxam, CBPV, or co-exposure to virus and pesticide. For each gene and condition, 8 individual honeybees were tested. The statistical differences between the fold change in transcription between control and exposed (or co-exposed) honeybees were considered significant at $p \leq 0.05$. The transcription level of the apidaecin gene showed no significant differences ($p > 0.05$) in any of the conditions (data not shown).

i) Effect of thiamethoxam

Compared to control conditions, thiamethoxam at the dose of 0.25 ng/bee/day significantly up-regulated transcription of the detoxification gene *gsts* 5 days after the beginning of pesticide exposure (Fig. 5, b; $p=0.044$). It also down-regulated the catalase gene (Fig. 5, b; $p=0.044$). After 10 days, the pesticide significantly down-regulated transcription of the vitellogenin gene (Fig. 4, e; $p<0.01$), of the immune-related genes dorsal-1a (Fig. 4, b, $p<0.01$), ppo (Fig. 4, b; $p<0.01$), and of the detoxification-related genes *gst3* (Fig. 5, d, $p=0.04$), cyp6as14 (Fig. 5, d; $p<0.01$), and catalase (Fig. 5, d; $p<0.01$).

The 5.0 ng/bee/day dose of thiamethoxam significantly down regulated dorsal-1a (Fig. 4, b; p<0. 01) and cyp6as14 (Fig. 5, d; $p=0.01$), but only at 10 days after the beginning of exposure.

i) Effect of CBPV

Compared to control conditions, CBPV down-regulated catalase at 5 days after the beginning of exposure (Fig. 5, b; $p<0.01$). The transcription of the vitellogenin (Fig. 4, b; $p=0.01$) and cyp6as14 (Fig. 5, b; $p=0.03$) genes was also reduced after 5 days of virus exposure, as well as the dorsal-1a (Fig. 4, b; $p<0.01$) and ppo (Fig. 4, b; $p=0.02$) genes after 10 days.

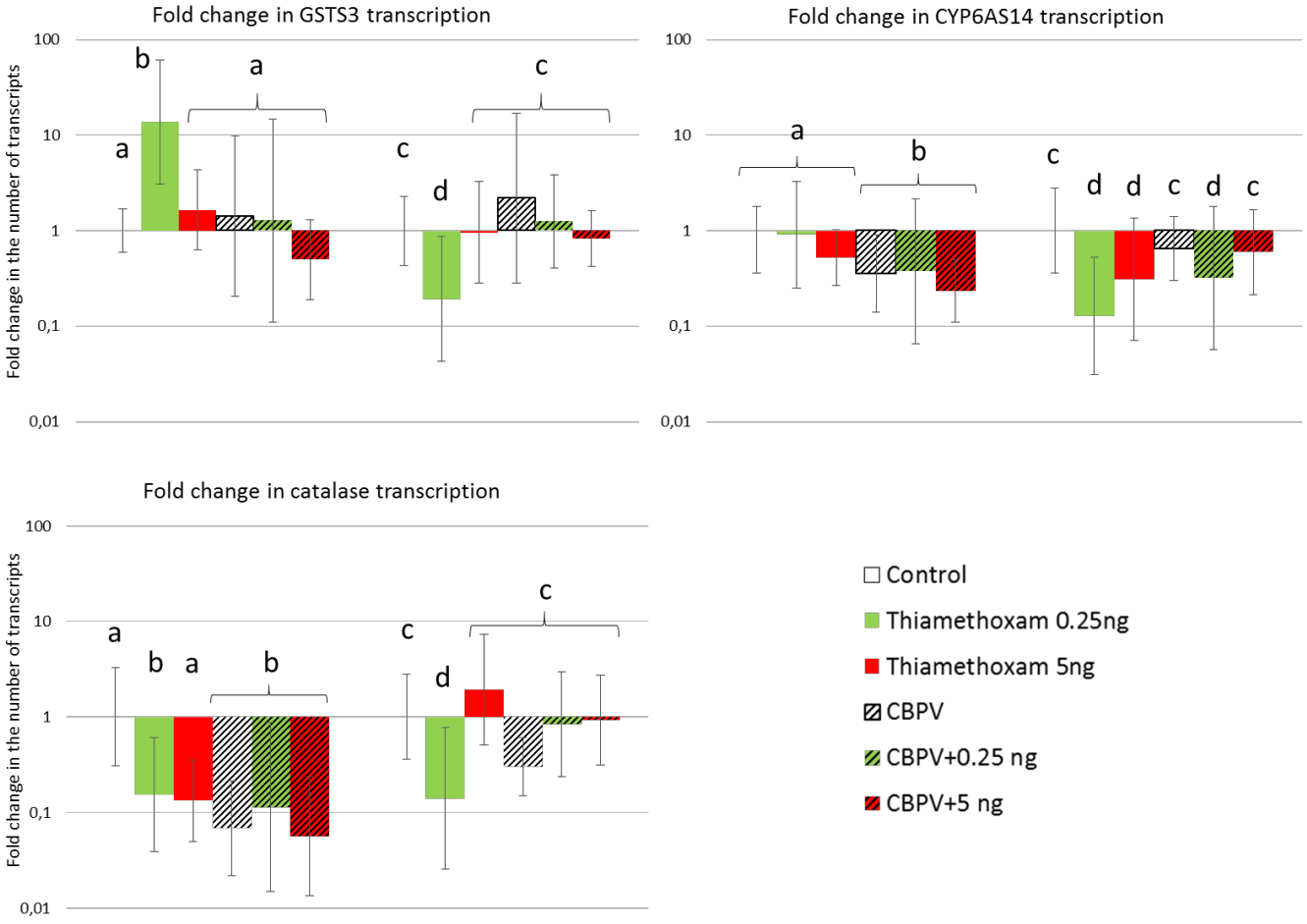

Fig. 5: Graphic representation of the fold changes in transcription for the tested detoxification-related genes after exposures. The graph was built using the $2^{-\Delta\Delta Ct}$ method, at Day 5 and Day 10 after the beginning of co-exposure. Letters show statistically different groups, calculated between the ΔCt. Statistical groups are specific for each gene. No letter indicates that no statistical differences were found between the conditions (p>0.05).

i) Effect of co-exposure

Co-exposure of honeybees to 0.25 ng/bee/day of thiamethoxam and CBPV down-regulated catalase at 5 days after the beginning of co-exposure (Fig. 5, b; $p<0.01$), and dorsal-1a after 10 days (Fig. 4, b, $p<0.01$), compared to control conditions. In this last case, it was also different from the thiamethoxam 0.25 ng dose alone ($p=0.03$).

Compared to control conditions, co-exposure to 5.0 ng/bee/day of thiamethoxam and CBPV down-regulated the transcription of vitellogenin (Fig. 4, c; $p<0.01$), catalase (Fig. 5, b; $p=0.01$), and cyp6as14 (Fig. 5, b; $p<0.01$) at 5 days after the beginning of co-exposure, and dorsal-1a (Fig. 4, b, $p<0.01$) at 10 days. After 5 days, this co-exposure also significantly reduced transcription of vitellogenin compared to thiamethoxam at 5 ng alone ($p<0.01$) and the co-exposure between CBPV and thiamethoxam at 2.5 ng ($p=0.03$).

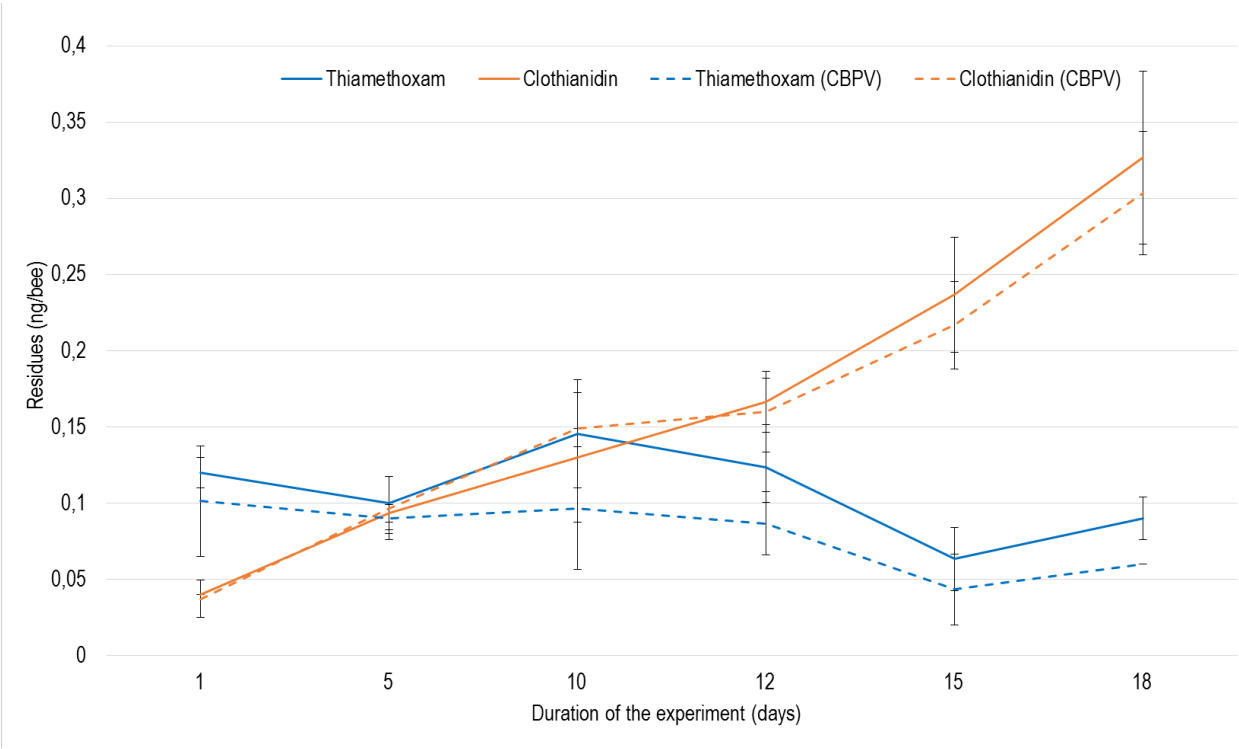

Fig. 6: Metabolisation kinetics of thiamethoxam in bees exposed to the pesticide and co-exposed to pesticide and CBPV. The metabolisation of thiamethoxam to clothianidin is shown over time in bees exposed to thiamethoxam (0.25 ng/bee/day) and exposed or not to CBPV (n=20 bees per condition). Means and standard deviations are shown.

3.2. Experiment 2: Influence of thiamethoxam and CBPV co-exposure on pesticide metabolisation

a) Thiamethoxam metabolisation into clothianidin

The quantity of thiamethoxam and clothianidin in honeybees exposed chronically to 0.25 ng/bee/day was assessed (Fig. 6). Levels for both neonicotinoids were under the limit of detection (LOD = 0.015 ng/bee) in control and CBPV-exposed bees over the course of the experiment (data not shown). In bees exposed to 0.25 ng/bee/day of thiamethoxam, the pesticide level remained stable over the course of the experiment and under 0.15 ng/bee. In contrast, clothianidin levels increased steadily throughout the experiment, from under 0.05 ng/bee after one day of exposure to almost 0.35 ng/bee after 18 days.

No significant difference was found at any time and for any residue between the bees exposed only to thiamethoxam and bees co-exposed to thiamethoxam and CBPV.

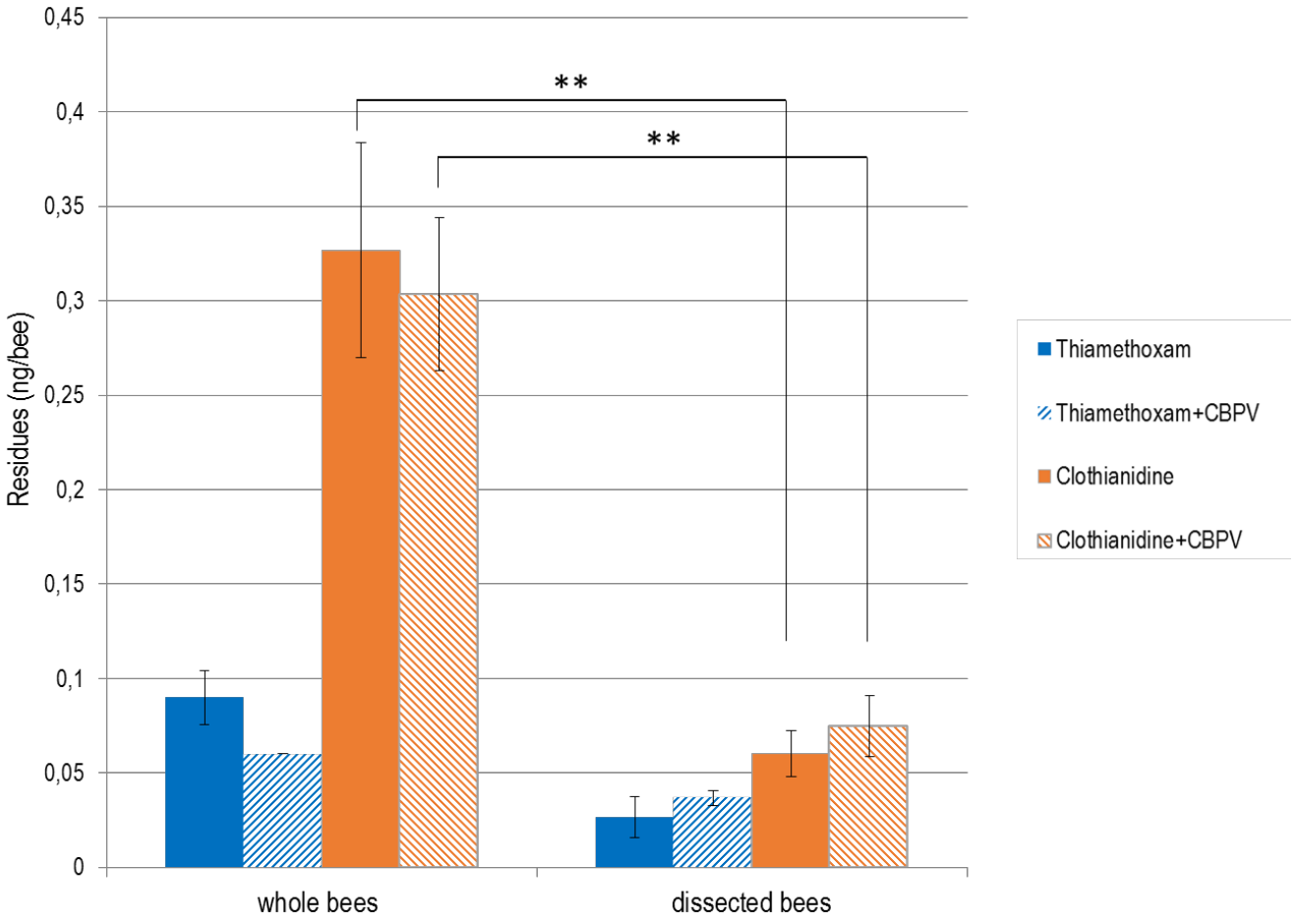

Fig. 7: Thiamethoxam and clothianidin levels in whole bees and dissected bees (n=20 bees). The levels of the pesticide (thiamethoxam) and its metabolite (clothianidin) are shown in whole bees and dissected bees (rectum excised); (**) denotes a significant difference ($p<0.01$) between the clothianidin measurements in whole and dissected bees. Means and standard deviations are shown.

a) Pesticide residue levels in whole bees and dissected bees

Thiamethoxam levels between whole bees and dissected bees (rectum excised) did not differ significantly in bees exposed to thiamethoxam and infected or not with CBPV (p=0.35 and p=0.45, respectively; Fig. 7). Clothianidin levels were significantly higher in whole bees than in dissected bees, for both treatments (p<0.01). However, there was no significant difference in the thiamethoxam or the clothianidin levels between treatments, either in whole (p=0.33 and p=0.59, respectively) or dissected honeybees (p=0.19 and p=0.28, respectively).

4.Discussion

Several viruses in bees cause silent or covert infections until they progress to levels associated with more obvious pathological symptoms (Amiri et al., 2015; Aubert et al., 2008). Aside from the role of vectors such as *Varroa destructor* (Gisder et al., 2009; B Locke et al., 2012; Nazzi et al., 2012), the mechanisms underlying this transition are not well known. Here we found that, when exposed to thiamethoxam, honey bees infected with CBPV levels mimicking covert infection can develop viral infection levels known to be related to clinical signs of CBPV disease. We discuss here how co-exposure to both stress factors might affect bee health.

In this study, we observed a significant effect of thiamethoxam on syrup intake (Fig. 1), which could be explained by the honeybees attraction for the pesticide (Kessler et al., 2015). We observed the same phenomenon in a previous experiment (Coulon et al., n.d.). This phenomenon could contribute to higher exposure levels of bees to the pesticide. A similar effect was observed in CBPV-infected bees alone, but to a lesser extent. The increase in syrup consumption could be explained by a higher sugar requirement due to the energy invested in fighting the disease (Alaux et al., 2010b; Evans and Spivak, 2010) and/or in detoxification processes (Rand et al., 2015). The co-exposed honeybees (to both virus and pesticide) did not show syrup consumption higher than bees exposed solely to thiamethoxam, underlining the absence of interaction between the virus and the pesticide on this physiological trait, and diminishing the bias of higher exposure to the pesticide.

We assume that the relatively low impact of CBPV on survival (Fig. 2), lower compared to our previously reported results (Coulon et al., n.d.), could be explained by variability in reproducibility of experimental results. This variability could have three various explanations. First, the honeybees and their colonies in this experiment were kept in controlled conditions in the winter apiary. Not only were temperature and humidity controlled by keeping the colonies inside (Youssef et al., 2015), but their sources of food were also controlled. Winter apiary colonies were fed with only syrup and protein paste prepared in our laboratory which do not contain pollen or nectar. Our previous experiment was carried out in summer, with colonies kept outside and which fed by foraging for pollen and nectar (Coulon et al., n.d.). The optimal nutrition intake provided by pollen and nectar depends on weather and availability on the surrounding floral composition (Aston and Bucknall, 2009). Nutrition can have an impact on immune resistance (Alaux et al., 2010b), and it has been shown that different pollens have different effects on nurse bee physiology and resistance to the parasite *Nosema ceranae* (Di Pasquale et al., 2013) and pesticide resistance (Huang, 2012). Pathogens have also been shown to be transmitted through pollen (Higes et al., 2008b; Singh et al., 2010). A retrospective analysis of the honeybees used in our previous experiment showed that honeybees co-exposed to thiamethoxam and CBPV were also contaminated with about 10^5 copies per bees of SBV, and between 10^4 and 10^6 copies per bees of BQCV (data not shown). In the present study, the colonies kept in the winter apiary and fed with artificial food were free of SBV and BQCV (in addition, ABPV and DWV were not detected, data not shown). While the SBV and BQCV viruses are known to cause symptoms on brood (Aubert et al., 2008), little is known about the potential sublethal effects in adult honeybees. Moreover, pollen and nectar are part of the sources via which honeybees can be exposed to pesticides (Botías et al., 2015; Chauzat et al., 2006; Johnson et al., 2010; C. A. Mullin et al., 2010a), especially early in life when larvae are being fed (Desneux et al., 2007; Rortais et al., 2005). Rearing larvae in contaminated brood frames has, for example, been shown to impair emerging honeybee resistance to *N. ceranae* (Wu et al., 2012). This type of natural exposure to biotic and abiotic contaminants could not be controlled in emerging bees coming from colonies kept outside in summer.

Second, the two experiments were performed in different seasons (in winter - albeit in a winter apiary with controlled temperature for the present study, and in summer for the previous one (Coulon et al., n.d.)). Consequently, physiological differences due to developmental influences cannot be ruled out. Winter honeybees notably have larger fat

bodies and possess more vitellogenin and phenoloxidase than summer bees (Amdam, G.V., Omholt, 2002; Behrends and Scheiner, 2010; Erban et al., 2013; Seehuus et al., 2006); both proteins are known to support a stronger immune system when at high levels (Amdam et al., 2004b). Further experiments comparing for example vitellogenin transcription levels and fat body size between summer-born, winter-born and true winter honeybees could confirm this hypothesis.

Third and last, this difference between the two experiments may also arise from differences in bee genetic background between the studies (Laurino et al., 2013; Rinkevich et al., 2015; Suchail et al., 2001).

Nevertheless, viral loads in the tested honeybees increased gradually with the presence of pesticide at any dose, after exposure to CBPV, and after co-exposure to both stressors (Fig. 3). Therefore, viral loads in the co-exposure conditions could result from an interaction between thiamethoxam and CBPV, regardless of the dose. We cannot conclude whether this interaction is synergistic or additive, since overt infections are threshold-dependent (Blanchard et al., 2007). The CBPV levels in co-exposed honeybees were the only ones to exceed the number of viral copies per bee that is usually related to the development of clinical signs (10^8 CBPV genome copies per bee). This specific effect of co-exposure on bees could explain the appearance of peaks in CBPV mortality in spring, when hives are highly populated. Importantly, covert CBPV infection (Aubert et al., 2008; Tentcheva et al., 2004) associated with chronic exposure to lipophilic pesticides accumulated in hive matrices (Chauzat and Faucon, 2007; C. A. Mullin et al., 2010b), or brought in from the field (Botías et al., 2015; Chauzat et al., 2006; Johnson et al., 2010; C. A. Mullin et al., 2010a), might cause an overt infection leading to massive death. However, despite the significant increase in CBPV levels that was found in this study, we did not observe a synergistic effect of the virus and pesticide on short-term bee mortality.

In order to decipher the underlying mechanisms of this increase in CBPV levels after thiamethoxam exposure, we studied the expression of several genes involved in immunity and detoxification (Figs. 4 and 5). Exposure of honeybees to CBPV alone has a down-regulating effect on immune-related genes transcription, which is contrary to what could have been expected from a reaction to a pathogenic infection (Fig. 4). However, a similar effect on

vitellogenin has been observed in previous studies, caused by various stresses, including but not limited to viruses (Amdam et al., 2004a; Boncristiani et al., 2012; Bordier et al., 2017; Koywiwattrakul et al., 2005). Other viruses, such as DWV, have been found to have a down-regulating effect on dorsal-1a (Nazzi et al., 2012). We also found that the transcription regulations after five days of co-exposure to 5.0 ng/bee/day and CBPV were similar to the regulations (up or down-regulated genes) associated with CBPV alone and to the pesticides alone. After ten days, however, the responses after co-exposure corresponded only to the response found after exposure to the pesticide alone. It has been shown that, as bees age, some immune response gene transcriptions fade, and sometimes even stop completely (Bull et al., 2012; Jefferson et al., 2013). The amount of vitellogenin is also linked to ageing itself (Amdam et al., 2004b; Guidugli et al., 2005).

The experiments performed with honeybees exposed only to thiamethoxam did not show dose-dependent variations in gene transcription, but different, sometimes opposite variations (Fig. 4). For example, the lowest dose of 0.25 ng/bee/day had a significant down-regulating effect on all tested genes after 10 days of exposure, when 5.0 ng/bee/day had this effect only on a few (dorsal-1a and CYP6AS14). This differential response between two very different doses could be explained by a phenomenon known as hormesis. Hormesis can be defined as a biphasic dose-response whereby exposure to low doses of a stressor can stimulate biological processes (Cutler and Rix, 2015). However, no effect of this different response was observed on honeybee survival, nor on their CBPV viral loads. Notably, no up-regulation in vitellogenin transcription was observed, contrary to what had been found in a previous study after 72 h of exposure (Christen and Fent, 2017). However, the authors of this publication showed lower transcription of vitellogenin after 72 h compared to 48 h, which should be consistent with a possible decrease and return to the original values we observed in our measurements performed 5 and 10 days after chronic exposure to thiamethoxam.

We found that thiamethoxam down-regulated dorsal-1a and catalase (Fig. 4). The down-regulation of dorsal-1a concurs with what had already been described by Di Prisco et al. [18] with clothianidin. Here, we infer that this down-regulation occurs later compared to the experiment performed by these authors, because we used comparatively lower doses of pesticides. Moreover, thiamethoxam toxicity is slightly lower than clothianidin (Efsa, 2013; Laurino et al., 2011). The thiamethoxam 48 h oral lethal dose (LD50) has been estimated to be between 1.65 ng/bee and 9.07 ng/bee depending on the European subspecies (Laurino et

al., 2013) and up to 10.86 ng/bee for Saharan honeybees (*Apis mellifera sahariensis*). Even though metabolisation of thiamethoxam to clothianidin is high (Fig. 6), we showed that most of the resulting clothianidin is excreted in the rectum (Fig. 7). In addition, this down-regulation of dorsal-1a could be linked to the rise in viral loads, and explain the higher CBPV loads in honeybees exposed to the pesticides alone than in control bees. Since we found that our collected bees were not totally free of CBPV before the experiment, the down-regulation of this immune-related gene would have allowed for this covert infection to develop into higher viral loads. Keeping in mind that CBPV is a different virus than DWV, we could still refer to Nazzi et al. (Nazzi et al., 2012), who showed that DWV down-regulated dorsal-1a, but not to the point of no transcription, allowing the remaining transcription to control the viral infection sufficiently. However, this down-regulation leaves a fragile balance that can easily be disrupted by another stress affecting the NF-κB effector, thus allowing DWV to replicate uncontrollably and reach high infection levels. Here, we hypothesise that this could be what underlies the significant increase in background CBPV infection in bees exposed to thiamethoxam alone, and the other highly significant increase in experimental CBPV infection in co-exposed honeybees.

While we have considered catalase to be detoxification-related because of literature data (Boncristiani et al., 2012; Mao et al., 2011), its role is to protect cells from a dangerous and ubiquitous metabolic byproduct, H_2O_2 (Calabrese and Canada, 1989). Indeed, in addition to being a byproduct of xenobiotic metabolisation (Calabrese and Canada, 1989), H_2O_2 is known to be produced as an innate response to viral infections in most vertebrates, including insects (Akaike, 2001; Nappi and Christensen, 2005). Therefore, the down-regulation of catalase by both stresses (pesticide and virus) was surprizing (Fig. 5). Nevertheless, we found that the down-regulated transcription of the detoxification-related genes catalase and CYP6AS14 by co-exposure, has no impact on the metabolisation kinetics of thiamethoxam. This underlines the fact that, in these experimental conditions of exposure, the detoxification system might be relatively more robust than the immune system. The metabolisation of thiamethoxam could be effected by different enzymes or pathways. CYP450, for example, is a large family of detoxification-related enzymes, of which a number are present in the honeybee (Berenbaum and Johnson, 2015), but not all could be tested here. Further studies are needed to uncover specifically which detoxification pathways or enzymes play a key role in the detoxification of this specific neonicotinoid in bees. In this context, the use of an overall method to study gene

transcriptions such as RNA sequencing would help to develop a broader picture to shed light on the honeybee's metabolic responses to both neonicotinoid and virus treatments.

In conclusion, we showed experimentally that low doses of thiamethoxam can trigger chronic bee paralysis symptoms in covert CBPV-infected bees. This finding could contribute to a better understanding of the occurrence of colony decline or severe losses of bees.

Supplementary material

Name	Sequence (5'-3')	Amplicon length (bp)	Reference
CYP6AS14			
F	TGACATTGAGTTGACGGACGAT	64	this study
R	GAAACCTGCCGCGAAGAA		
GSTS3			
F	AAACCGATAGCGCAGAGTAACG	87	this study
R	CATCATTGCCTCCCATTCGT		
Catalase			
F	TTTGGTGGGCCTAGAGAATGTC	92	this study
R	TCCTCCTTTGGGTCTACATCATAAC		
Apidaecin			
F	TTTTGCCTTAGCAATTCTTGTTG	81	(Di Prisco et al., 2016)
R	GTAGGTCGAGTAGGCGGATCT		
Dorsal-1A			
F	TCGGATGGTGCTACGAGCGA	153	(Di Prisco et al., 2016)
R	AGCATGCTTCTCAGCTTCTGCCT		
Vitellogenin			
F	AACGCCGTGAAGGTGAACAG	109	this study
R	TATCGTAGAGAACCTCGCATTTCC		
PPOAct			
F	CGTTGAAAAGTCGAAGCAGATTAA	112	this study
R	AGGACGCCACCGCAGTATT		
β-actin			
F	GATTTGTATGCCAACACTGTCCTT	69	(Di Prisco et al., 2016)
R	TTGCATTCTATCTGCGATTCCA		
RpL32			
F	CGTCATATGTTGCCAACTGGTTT	107	this study
R	CCATGAGCAATTTCAGCACAA		
CBPV			
qCBPV9	CGCAAGTACGCCTTGATAAAGAAC		
qCBPV10	ACTACTAGAAACTCGTCGCTTCG	101	(Blanchard et al., 2007)
CBPV S2	(6-Fam)TCAAGAACGAGACCACCGCCAAGTTC (Tamra)		

Supplementary Table 1: Primers used for the quantification of selected honeybee genes and CBPV.

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Author contributions

Marianne Coulon, Eric Dubois and Anne Dalmon designed and developed the experiments. Nicolas Cougoule provided the honeybees. Marianne Coulon performed the experiments with technical help from Frank Schurr. Anne-Claire Martel, Adrien Bégaud and Patrick Mangoni performed the LC-MS/MS neonicotinoid residue quantifications and Marianne Coulon analysed the data. Marianne Coulon and Gennaro Di Prisco performed the gene transcript quantifications and analysed the data. Marianne Coulon and Eric Dubois co-wrote the paper and Anne Dalmon, Cédric Alaux, Gennaro Di Prisco, Magali Ribière-Chabert, Richard Thiéry and Yves Le Conte reviewed and edited the writing.