érimentations in situ sur le DWV et développement d'un clone

Ce chapitre décrit les travaux effectués au laboratoire de l'INRA d'Avignon sur la co-exposition entre le thiaméthoxam et le DWV en conditions naturelles, ainsi que le développement d'outils de génétique inverse pour l'étude du DWV-A et DWV-B.

Il a déjà été observé au laboratoire un effet significatif de la molécule fille du thiaméthoxam, la clothianidine, sur le système immunitaire, qui entrainait une hausse des charges en DWV chez les abeilles co-exposées (Di Prisco et al., 2013). Nous avons également vu précédemment que le thiaméthoxam en exposition chronique pouvait dans certaines conditions causer des mortalités synergiques lorsque les abeilles étaient co-exposées au CBPV et au thiaméthoxam à forte dose (Chapitre 1, 2) ou, même à plus faible dose, causer une hausse des charges naturelles (bruit de fond) et expérimentales en CBPV (Chapitre 1, 3). Cependant, aucune expérience n'a à notre connaissance encore étudié si ces observations au laboratoire étaient applicables dans des conditions naturelles au sein de la colonie. L'exposition chronique en colonie étant difficile à réaliser, et les contaminations ponctuelles tout aussi probables, j'ai cette fois ci utilisé des expositions aigues au thiaméthoxam (tout comme l'avaient fait Di Prisco et al., 2013). L'exposition orale à encore une fois été choisie compte tenu de la nature systémique de l'utilisation principale de ce pesticide.

Les questions de recherches auxquelles j'ai cherché à répondre dans ce deuxième chapitre sont les suivantes :

- ✓ Est-il possible de se passer d'injection pour transmettre et étudier l'infection des abeilles par le DWV, en utilisant des infections naturelles déjà présentes ou la voie orale ?
- ✓ Quels sont les effets d'une co-exposition entre le DWV et le thiaméthoxam en doses aigues sublétales ?

- Sur la mortalité des abeilles ?
- Sur les charges virales en DWV et virus qui peuvent être naturellement présents dans les abeilles ?
- Sur certains traits du comportement comme le retour à la ruche après une première sortie ou l'âge des abeilles lors de cette première sortie ?

✓ Les effets observés peuvent-ils être expliqués par un effet de l'un, de l'autre ou de l'interaction entre ces facteurs sur l'expression de gènes de l'immunité ou de la détoxication ?

En premier lieu, j'ai voulu étudier **l'effet d'une co-exposition aigue au thiaméthoxam** sur des abeilles dans leur colonie, tout d'abord infectées de façon naturelle, puis expérimentalement par voie orale ou injection. Lors de la deuxième expérience les entrées et sorties des abeilles étaient suivies par un compteur optique. Ces deux expériences et les résultats obtenus seront décrits sous la forme d'un article scientifique.

Dans un second temps, afin de disposer d'un outil de génétique inverse pour le DWV, **nous avons développé deux clones** correspondants au DWV-A et à un recombinant DWV-A/B en système hétérologue levure. Le développement de ces clones et les résultats préliminaires obtenus lors des tests d'infectivité sont décrits sous la forme d'un article méthodologique rédigé en anglais, bien qu'il ne puisse être publié en l'état car des essais complémentaires sont nécessaires.

1. Etudes de co-expositions thiaméthoxam-DWV en ruche

Pour étudier les effets d'une co-exposition entre le DWV et le thiaméthoxam, j'ai effectué deux expériences complémentaires.

J'ai étudié l'effet de la voie d'infection sur la charge virale en DWV. En effet, dans des conditions naturelles les abeilles peuvent être infectées horizontalement par l'alimentation ou par injection *via* l'acarien vecteur *Varroa destructor*. J'ai donc testé la co-exposition à des doses environnementales de thiaméthoxam tout d'abord en utilisant des cohortes d'abeilles infectées naturellement dans des colonies sélectionnées selon un gradient d'infection au DWV. Cette première expérience visait à tester si les augmentations de charges obtenues par Di Prisco et al., 2013 en cagette avec la clothianidine et des infections expérimentales en DWV, étaient reproductibles dans des conditions les plus naturelles possible (Figure 28).

J'ai ensuite, dans une deuxième expérience, testé une inoculation « contrôlée »c une co-exposition au thiaméthoxam. Une partie des abeilles testées ont donc été infectées par voie orale, l'autre partie par injection. Cette deuxième expérience visait à étudier les possibles effets d'une co-exposition sur des traits comportementaux, en utilisant les compteurs optiques développés par l'INRA d'Avignon et décrits précédemment dans (Alaux et al., 2014; Bordier et al., 2016), et à les relier ces effets potentiels à la transcription des gènes préalablement décrits (Chapitre 1, 3) correspondants aux voies de l'immunité ou de la détoxication (figure 29). Les résultats obtenus lors de ces deux expériences complémentaires sont présentés sous forme d'article en prévision d'une valorisation scientifique.

Des analyses complémentaires permettront d'évaluer l'impact des traitements effectués sur d'autres traits de vie, la durée et le nombre de vol effectués par les abeilles testées, par exemple. Des études ont déjà testé l'impact du DWV sur ces traits de vie, mais les résultats sont au premier abord contradictoires. D'un côté une première étude n'a pas permis observer d'effets du DWV seul sur la durée ni sur les vols d'orientations (Wolf et al., 2016). De l'autre, Toutefois des durées de vol de butinage plus courtes ont été observées chez des

Effet du thiamethoxam sur les charges naturelles en DWV

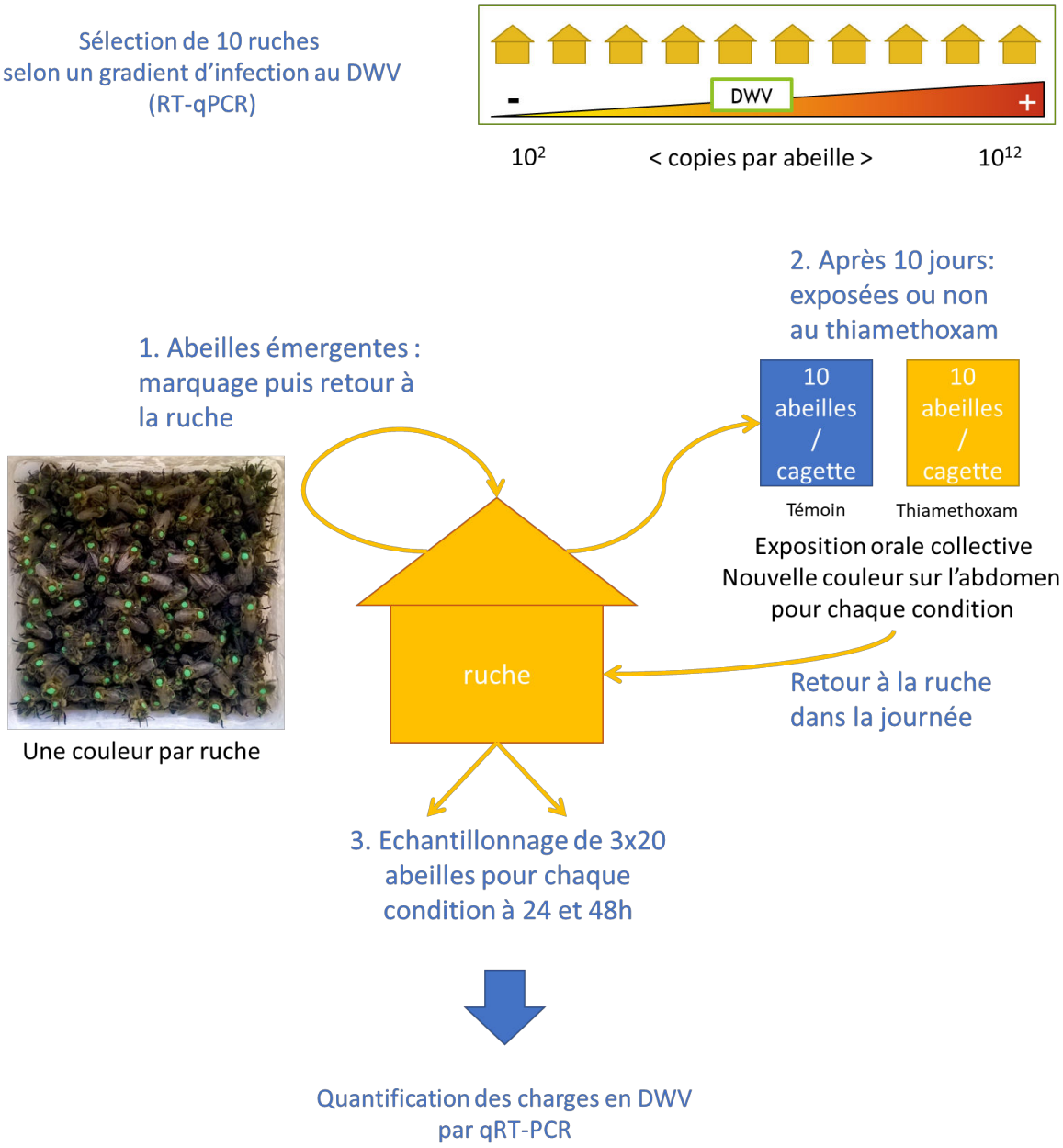

Figure 28 : Schéma récapitulant la méthode utilisée afin de tester l'effet d'une exposition aigue au thiaméthoxam sur des abeilles provenant de colonies infectées selon un gradient de la plus faible charge en DWV à la plus élevée.

Dans chaque colonie, entre 700 et 1000 abeilles ont été échantillonnées et marquées à l'étape 1, pour obtenir environ 200 abeilles à l'étape 2. L'objectif étant d'obtenir un minimum de 3 échantillons de 20 abeilles par condition (témoin ou exposées au thiaméthoxam) et par jour d'échantillonnage (24 ou 48h après exposition).

abeilles infectées par le DWV (Wells et al., 2016). Une réduction d'activité de vol chez des abeilles ayant subi un stress immunitaire a également déjà été observée (Alaux et al., 2014).

Ainsi une analyse de nos résultats selon cet angle pourrait éclaircir ces résultats. L'élaboration de nouveaux modèles statistiques est nécessaire afin de pouvoir tester ces traits comportementaux, et elle est en cours.

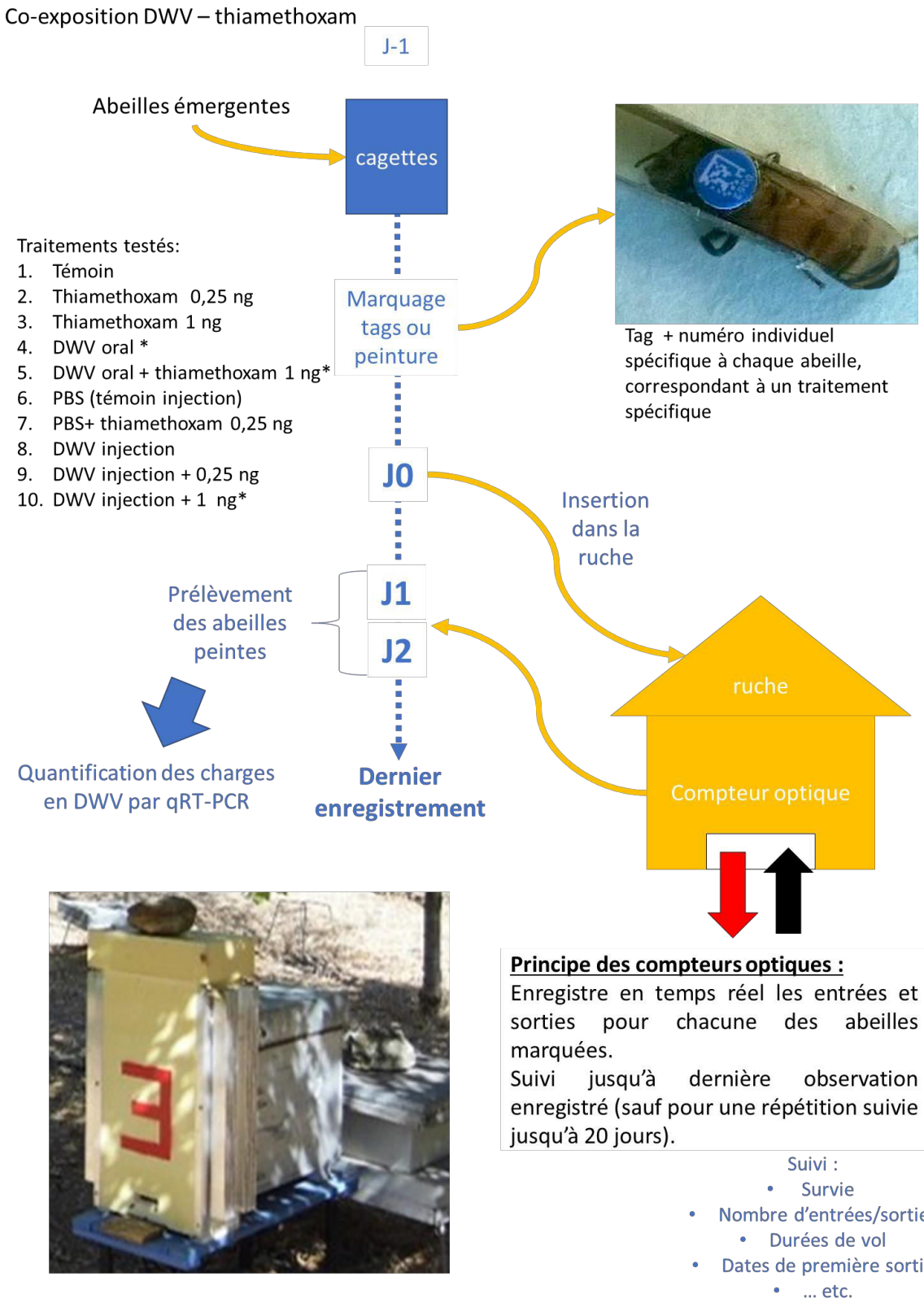

Figure 29 : Schéma récapitulant la méthode utilisée afin de tester l'effet de différentes co-expositions entre le DWV et le thiaméthoxam avec des compteurs optiques.

Article 3:

Interactions between thiamethoxam and *Deformed wing virus* can drastically impair behaviour of free flying honey bees

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Abstract

Honeybee colony declines are believed to be driven by the combination of multiple stress factors. However, little is known about how co-exposure to stress factors can alter bee survival and behavioural performances in natural conditions. We therefore studied the potential interaction between a neonicotinoid, thiamethoxam, and a highly prevalent honeybee pathogen, the *Deformed wing virus,* in colony conditions. First, we found that field-realistic doses of thiamethoxam do not increase DWV loads in infected honeybees. Then, we followed bee flight activity by using optical bee counters. We exposed bees to DWV by feeding or

injection, and/or to field-relevant doses of thiamethoxam. Contrary to oral exposure, injection of DWV resulted in increased DWV loads and reduced bee survival. A precocious onset of foraging was observed in DWV injected bees and was related to a reduction in the vitellogenin expression level. Combined exposure to DWV and thiamethoxam did not result in higher DWV loads compared to bees only exposed to DWV, but induced precocious foraging, increased the risk of not returning to the hive after the first exit, and decreased survival when compared to single stress exposures. This is the first evidence of possible deleterious interaction between DWV and thiamethoxam in natural conditions.

1. Introduction

Heavy losses of honeybee colonies in the Northern hemisphere has been documented since the beginning of the 21st century (Lee et al., 2015). It is crucial to study these losses as it is known that 84% of the 264 most important crops in Europe depend at least to some extent on animal pollination (Williams, 1994). There is now a scientific consensus on the fact that these honeybee colony losses are the result of multifactorial causes, including a decrease in floral resource availability, spread of pathogens and pesticide use (Goulson et al., 2015).

Honeybee colonies are routinely co-exposed to several pesticides and their corresponding metabolites. These pesticides are carried back to the hive via pollen and nectar. In addition, honeybee colonies are very attractive and valuable for pathogens due to the high concentration of individuals and stored food in the colony (Schmid-Hempel, 1998). Therefore, honeybees can concentrate pesticides and pathogens in their colony, and are often co-exposed to stress factors (Cornman et al., 2012; C. a. Mullin et al., 2010; Simon-Delso et al., 2014; vanEngelsdorp et al., 2009). Interactions between pesticides and pathogens have been reviewed by Poquet et al., 2016. Synergistic interactions between the microsporidia *Nosema ceranae* and a neonicotinoid or fipronil (phenylpyrazole) have been found to significantly decrease honeybee survival (Alaux et al., 2010a; Aufauvre et al., 2012; Vidau et al., 2011). *N. ceranae* infection rates increase when brood is exposed to high levels of pesticide residues (Wu et al., 2012), and imidacloprid (a neonicotinoid) has been found to increase *N. ceranae* infections in exposed colonies (Pettis et al., 2012b).

Pesticides can also interact with bee viruses. DeGrandi-Hoffman et al. (2013) showed that the number of nurse bees contaminated with *Black queen cell virus* (BQCV) increased

significantly when they were fed with pollen containing a mix of the insecticide chlorpyrifos (organophosphates) and a commercial fungicide solution (Pristine ® BASF (active ingredients: boscalid and pyraclostrobin)). Combined exposure to sublethal doses of the neonicotinoid thiacloprid and BQCV was also found to significantly increase mortality and BQCV viral loads in honeybee larvae (Doublet et al., 2015a). This latter combined effects could be explained by the immune suppression induced by the pesticide. Indeed, (Di Prisco et al., 2013) reported an inhibition of an immune cascade effector by the neonicotinoid clothianidin, which leads to the bee being unable to control *Deformed wing virus* (DWV) replication.

However, most studies on stress factor co-exposures were performed in laboratory controlled conditions. As a result, little is known about their consequences on bee behaviour, such as foraging activity. We therefore tested the potential effects of a co-exposure between a pathogen (DWV) and a common pesticide (thiamethoxam) in natural conditions. DWV is one of the most prevalent honeybee viruses in Europe; it has for example been detected in 97% of tested French apiaries in 2002 (Tentcheva et al., 2004). It is a single strand positive RNA virus, of the Picornavirales order, which gathers many of the viruses infecting honeybees (Remnant et al., 2017). However, as for many honeybee viruses, DWV mostly causes covert infection in hives (de Miranda and Genersch, 2010). Overt infections often occur when the virus is transmitted to the pupae by the Varroa desctructor mite through injection while the mite feeds (de Miranda and Genersch, 2010; Möckel et al., 2011). Its most visible symptom is the occurrence of deformed wings. In addition, DWV infection is known to impair associative learning and memory formation (Iqbal and Mueller, 2007), cause precocious foraging trips (Benaets et al., 2017) and drastically reduce bee lifespan (Rueppell et al., 2017).

Thiamethoxam is a neonicotinoid insecticide commonly used over the world (Sanchez-Bayo and Goka, 2014), especially on oil seed rape, a crop that is widespread and attractive to honeybees (Simon-Delso et al., 2015). It can be quickly metabolized both in insects and plants into clothianidin, which is also commercialized as an insecticide (Nauen et al., 2003). Neonicotinoids bind with high affinity to the acetylcholine receptors of insects. Sublethal doses of thiamethoxam have been shown to cause negative effects on homing flights in foragers (Henry et al., 2012), and impair orientation performances of foragers in complex mazes (Fourrier et al., 2009). Furthermore, its metabolite clothianidin has been found to have an inhibiting effect on the honeybee immune system (Brandt et al., 2016; Di Prisco et al.,

2013), or to cause a significant reduction of foraging activity and longer foraging trips in exposed foragers (Schneider et al., 2012).

The maintenance of stable conditions within colonies relies on a division of labor, with bees spending the first 2–3 weeks of their adult life working in the hive (feeding and taking care of the brood, building comb), and then the rest of their life outside of the hive (foraging for nectar and pollen to supply the colony growth) (Seeley, 1995). However, a significant loss of foragers for the colony will accelerate the behavioral maturation of young bees to replace them (Robinson, 1992). Similarly, parasites also lead to precocious foragers (Dussaubat et al., 2013; Goblirsch et al., 2013; Natsopoulou et al., 2015), which may not be optimally adapted to foraging tasks (Vance et al., 2009)(Schippers et al., 2006). Altogether, loss of bees and poor performance of precocious foragers may affect the colony homeostasis and development, and ultimately lead to colony failure (loss of resilience) (Perry et al., 2015). Such parameters need then to be taken into account when assessing the influence of stress factors on bees.

We assessed in this study the influence of DWV/thiamethoxam co-exposure on bee survival and onset of foraging, as well as on the expression levels of genes involved in immunity and detoxication.We selected four genes that are part of immune pathways: *vitellogenin*, a glycolipoprotein that takes part in ageing processes as well (Amdam et al., 2004b), *dorsal-1a*, an effector of the NF-κB pathway which can lead to the production of AMPs, the AMP *apidaecin*; and *ppo* from the melanisation pathway (Boncristiani et al., 2013; Di Prisco et al., 2013; Simone et al., 2016). We also selected three genes which take part in detoxication processes: *gsts3*, *catalase* and *CYP6AS14* (Boncristiani et al., 2012; Mao et al., 2011). For this purpose we used optical bee counters tracking individual flight activity of tagged bees (Alaux et al., 2014; Bordier et al., 2016; Dussaubat et al., 2013) and analysed the viral loads and gene expression of painted bees that had been through the same treatments.

2. Materials and methods

2.1. Experiment 1: Influence of thiamethoxam on virus loads in naturally infected bees

a) Colony screening for DWV infection

To establish a gradient of colonies infected by DWV from low (<10^7copies/bee) to high (>10^8 copies/bee) infections (de Miranda and Genersch, 2010), we screened 24 colonies distributed in four apiaries (screening of a pool of 40 bees/colony), in mid-April 2016, through quantitative PCR. Ten colonies were selected to establish a gradient of DWV infection level, which included 5 colonies being under the "overt infection" threshold, and 5 being above this threshold. Colonies with too high a number of copies of *Acute paralysis virus* (ABPV) and/or *Chronic bee paralysis* virus (CBPV) were not selected (>10^3 copies/bee (Amiri et al., 2015)). The 10 selected colonies were moved in a single apiary located at INRA, Avignon, France.

b) Experimental set up

Experiments were performed in June and August 2016. To obtain cohorts of bees with the same age, brood frames were collected from the hives and put in an incubator overnight at 34°C. The next day (day 0), emerging bees were collected, marked with a dot of paint on the thorax (one colour per colony), and then returned, with the brood frames, back to their own colony.

To apply thiamethoxam at an age susceptible to be linked to the first, early exposures that can occur in natural conditions, ten days later marked bees were re-collected and placed in cages, in groups of 10 bees to be exposed to thiamethoxam (early exposure that can occur in natural conditions via pollen or stored nectar (Efsa, 2013)). After 2-4 hr of starvation, bees were collectively fed with 20 µL of 30% sucrose solution containing or not thiamethoxam (99% purity, Techlab, Saint-Julien-lès-Metz, France) at the following doses: 0.25ng/bee (June) or 1ng/bee (August). A previous study showed that the solution is evenly distributed by trophallaxis between individuals (Fourrier et al., 2014). Bees were paint marked on the abdomen with a

different colour according to their treatment. One to two hours after they had eaten all the sugar solution, bees were once again returned to their colony. At day 11 (24 h post-exposure) and Day 12 (48 h post-exposure), 3samples of 15 to 20 bees per group and colony were sampled and immediately placed into liquid nitrogen for DWV quantification.

2.2. Experiment 2: Influence of thiamethoxam and DWV co-exposure on survival, onset of foraging, and physiology

a) Experimental set up

The influence of thiamethoxam/DWV co-exposure on bee survival and onset of foraging was determined by using optical bee counters. Two colonies with 5 frames selected among the previously tested low DWV colonies (< 2.5 x 10^6 copies DWV/bee) were equipped with optical counters at the hive entrance in early May 2016. Emerging bees were collected from brood frames originating from the same "DWV-low" colonies and incubated overnight at 34°C. Newly-emerged bees were distributed in cages and marked with either a 3-mm wide bar code printed on laminated paper and glued (Sader®) onto the thorax (66 bees per treatment and replicate in 2016 and 60 bees in 2017, supplementary material Table S1) or a paint mark on the abdomen (one colour per treatment, 80-100 bees per treatment and replicate, supplementary material Table S1). After marking, bees were kept overnight in an incubator at 34 °C with a saturated humidity and 50% sucrose syrup. On the next day, they were assigned to the following treatments: 1. Control bees; 2. Bees individually fed with 0.25 ng of thiamethoxam in 5 µl of syrup; 3. Bees injected with PBS (Phosphate Buffer Saline solution); 4. Bees injected with PBS and individually fed with 0.25 ng of thiamethoxam; 5. Bees injected with DWV (~10^4 copies/bee), and 6. Bees injected with DWV (~10^4 copies/bee) and individually fed with 0.25 ng of thiamethoxam. The experiment was replicated 5 times (in May and July 2016, and 3 between April and May 2017). The PBS injection treatments were performed as a control for the effects of the injection, as it has been shown that piercing honeybee cuticle can challenge their immunity (Alaux et al., 2014; Evans et al., 2006; Siede et al., 2012).

For the 2017 replicates (supplementary material Table S1), additional experimental groups were included: 7. Bees individually fed with an inoculum of ~10^8 copies of DWV, 8. Bees

individually fed with 1 ng of thiamethoxam, 9. Bees injected with DWV ~10^4 copies/bee and individually fed with 1.00 ng of thiamethoxam, and 10. Bees individually fed with ~10^8 copies of DWV and with 0.25 ng of thiamethoxam.

b) Thiamethoxam and DWV exposures

To obtain the DWV inoculum, an archived environmental sample of DWV-A already described (KX373899) (Dalmon et al., 2017) was injected in pupae for multiplication. After 24h at 34°C and saturated humidity, five pupae were crushed into 500 µl of PBS then centrifuged twice for 10 min at 8,000 *g* and 4°C, each time collecting the supernatant, to eliminate most tissues and cell debris. Bees were injected with supernatant from the second centrifugation, and an aliquot of supernatant was later quantified using real-time RT-PCR to retrospectively assess the exact number of viral copies injected. Dilution was calculated as 2.75 x 10^4 copies of DWV in the 46nl of the inoculum that was injected to each bee. Injections were performed using a Nanoject (Drummond Scientific, Broomall, PA, USA) and heat elongated glass microcapillary tubes, between the third and fourth tergites of bees previously anesthetized with CO2 and maintained on ice.

Exposure to thiamethoxam was realised as follows. After 2h of starvation, bees were individually fed with 5 µL of syrup. The 0.25 ng (2016) or 1ng doses of thiamethoxam (2017) in 5 µl of syrup solution were obtained from successive dilutions, first in water then in 30% syrup, of a 1 mg/ml solution of thiamethoxam. Syrup was prepared by mixing 30% p/v powdered sugar in water.

The same technique was used to feed the 10^8 copies/bee of DWV for the *per os* treatments.

After treatment bees were introduced into the hives. No immediate rejection from the hive colonies was witnessed. For viruses and gene expression analysis, three samples of 3 bees per treatment (identified from their paint mark on the abdomen) were sampled in dry ice at 24 and 48 h after their re-introduction to the colony, and stored at -80°C. Bees tagged with barcodes were followed using optical counters, as previously described in Alaux et al., 2014 and Bordier et al., 2016.

c) Onset of foraging and survival

Between 60 and 66 bees per experimental group and replicate were followed with optical bee counters (for more details see Alaux et al., 2014). Briefly, the bee counter is composed of a modified entrance with eight tunnels f, a camera monitoring the entrance, a computer for image acquisition and software that analyses the images and record the in-and-out activity of bees. For each detected bee, we obtained its ID, direction (in or out of the hive) and the time of activity (day, hour, minute, and second). From these raw data, we retrieved the time spent out of the hive at each exit, and identified the first day of foraging for each individual, defined as a trip lasting longer than 10 min (Benaets et al., 2017; Marco Antonio et al., 2008; Woyciechowski and Moron, 2009). All tagged bees were followed until no bee could be detected (up to 51 days ; for each bee the last detection being considered as time of death).

2.3. Virus and gene transcription quantification

The number of DWV copies was determined by quantitative PCR using a StepOne-Plus Real-Time PCR Systems (Applied Biosystems®, Life Technologies) and the SYBR Green detection method. For the experiment 1, pools of 15 bees from each treatment and replicate were crushed in a bag with 1.5mL of PBS, and then total RNA was extracted from 500 µL of homogenate in 900 mL of Qiazol. For Experiment 2, a pool of 3 bees per treatment and replicate was crushed directly in 900 mL of Qiazol with a 0.8 cm-diam. bead and a TissueLyser (Qiagen) (4 times 30 seconds at 30 Hz). The homogenate was then centrifuged for 2 min at 12,000 g and 4°C, and the supernatant was collected into a new tube to be processed for RNA extraction. Then, total RNA was extracted using Qiagen's RNeasy Universal Plus Mini Kit, following the manufacturer's instructions (QIAGEN, Hilden, Germany). RNA was quantified using a spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific) then diluted to obtain a 500 ng/µl RNA concentration. Samples were stored at -80°C. Reverse transcription was performed with the High capacity RNA to cDNA kit (Applied Biosystems, Saint Aubin, France) according to the manufacturer's protocol.

For virus quantification, 3 microliters of 10-fold diluted cDNA were mixed with 7 µl of SYBR Green master mix (Applied Biosystems) containing 10 pmol of primers. DWV, but also ABPV, CBPV, BQCV and *Sacbrood bee virus* (SBV) loads were quantified using a

qPCR absolute quantification. A standard curve was obtained for each virus from serial dilutions of viral synthetic fragments of known concentration (MWG, Germany) and the log graph was used to calculate viral loads from Ct values in the samples. Amplification was performed with the following program: 10 min 95°C, then 40 cycles of 15 s at 95 °C, then 1 min at 60 °C. A melting curve was generated from 60 °C to 95 °C. Quantitation was replicated twice. Sequence primers and viral sequences used as reference are shown in Table S2.

Expression level of immune (vitellogenin, dorsal-1-a, apidaecin, pro-phenoloxydase (PPO)) and detoxication genes (glutathione-S-transferase 3, catalase and CYP6AS11) were assessed using the primer pairs reported in Table S2. Relative gene expression data were analysed using β-actin and RpL32 and the geometric mean of both as a reference (Reim et al., 2015). To verify that the amplification efficiencies of the target and reference genes were approximately equal, the amplification of five 10-fold dilutions of the total RNA sample (from 1.0 to 0.1 ng per reaction) in triplicate were analysed. The efficiency plot for Log input total RNA vs. ΔCt had a slope lower than ± 0.1. Amplifications for genes were performed using the StepOne Real-Time PCR System (Applied Biosystems®, Life Technologies) 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 15 s 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). qRT-PCR mix for one sample was done as follow: 10 µL of RT-PCR SYBR Green mix, 0.2 µL of 10 µM forward and reverse primer each, 0.16 µL of Retro-transcriptase enzyme from the kit, 8.44 µL of H_2O, and finally 1µL of RNA sample. All primer pairs were designed using PrimerExpress 3.0 software (Life Technologies) following the standard procedure. Negative (H2O) and positive controls (previously identified positive samples) were included in each qRT-PCR run.

2.4. Statistics

Bee activity was analysed separately for bees exposed to DWV *per os* or via injection. *Per os* treatments included Control, 0.25 ng thiamethoxam, 1 ng thiamethoxam, DWV *per os,* and co-exposure to DWV *per os* and 1 ng thiamethoxam. Injected treatments included PBS injection, co-exposure to PBS and 0.25 ng thiamethoxam, DWV injection, co-exposure to

DWV and 0.25 ng thiamethoxam, and co-exposure to DWV and 1 ng thiamethoxam; in this group, PBS injection was used as control.

a) Bee survival and onset of foraging

The age at death was calculated using the last registered exit for each bee and a Kaplan-Meier estimation (Efron, 1988; Pepe and Fleming, 1989), and survival rates were calculated using the Cox proportional hazards model (Cox, 1972). Variations in the age at which bees performed their first foraging trip was analysed via a general linear mixed model fit by maximum likelihood (Laplace Approximation) using the Poisson probability distribution function. Treatment and month (April as basal level) were considered as fixed factors and the source colony as a random factor. The nature of the interaction was analysed using the χ^2 of compliance with 1 degree of freedom.

The proportions of bees lost after their first exit of the hive was analysed by comparing observed and expected proportions. χ^2 table was used first to compare all treatments with 9 degrees of freedom, then separately for each pairwise combination of treatments with 1 degree of freedom.

b) Virus and gene expression levels

For genes, analyses were carried out on the ΔCt (log 2). For viruses, they were carried on the log10 of the obtained number of copies per bee. Analyses were carried out using either ANOVA followed by Tukey HSD when data followed a normal distribution (non-significant Shapiro-Wilk test) or a pairwise Wilcoxon test with Bonferroni correction when data did not follow a normal distribution (significant Shapiro-Wilk test). All statistical analyses were run with the software R (Version 1.0.143 – © 2009-2016 RStudio).

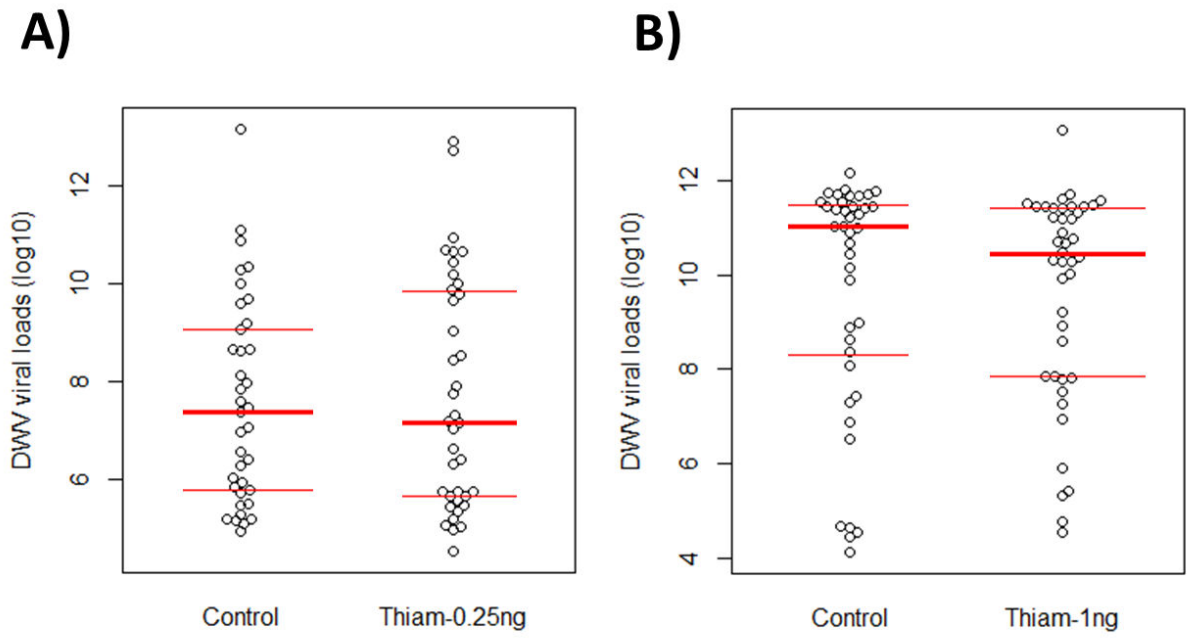

Figure 1: DWV infection level in control and thiametoxam-exposed bees (Experiment 1). A) Level of DWV loads in untreated (control) bees or bees exposed to 0.25 ng of thiamethoxam- (Thiam-0.25 ng)(replicate 1, June 2016), B) Level of DWV loads in untreated (control) bees or bees exposed to 1 ng of thiamethoxam- (Thiam-0.25 ng) (replicate 2 August 2016). Number of copies per bee are shown in $\log_{10}$. In boxplots, red lines represent first quartile (25%), median (50%) and third quartile (75%).

3. Results

3.1. Thiamethoxam influence on viral loads

a) Thiamethoxam influence on natural DWV loads

We first checked whether thiamethoxam exposure impacts virus infection levels in bees originating from DWV naturally-infected colonies (Experiment 1). Control bees exhibited higher DWV loads in the first replicate (month) when compared to the second replicate (month) (Mann-Whitney's U test, $p<0.01$, Fig. 1A and 1B). However, bees exposed to either 0.25 ng or 1 ng of thiamethoxam did not exhibit higher loads of DWV than control bees ($p = 0.945$ and $p = 0.474$, respectively; Fig. 1A and 1B)

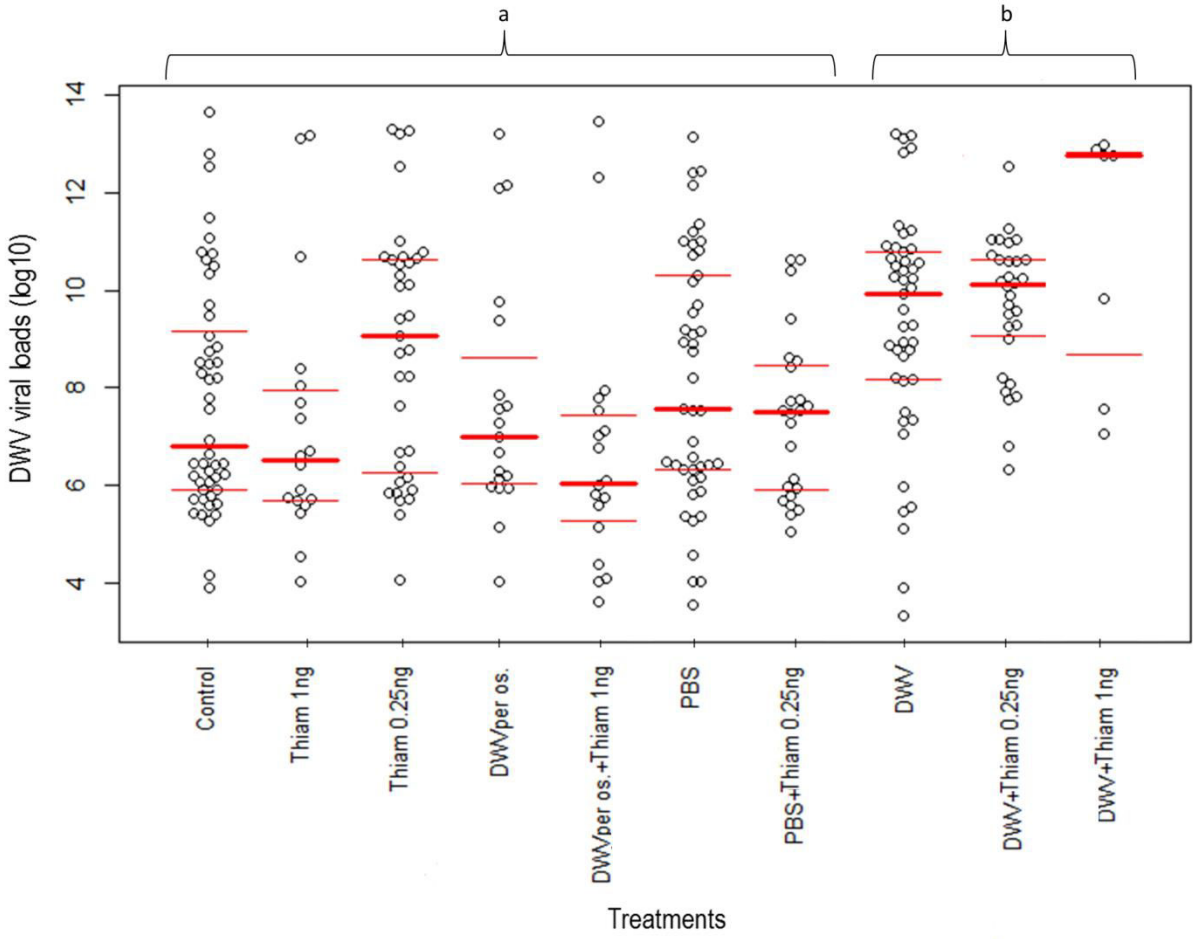

Figure 2: DWV infection level in bees exposed to thiametoxam and/or DWV (Experiment 2). The different treatments are: Control bees, bees injected with PBS (PBS), bees injected with PBS and exposed to 0.25 ng/bee of thiamethoxam (PBS+0.25ng), bees exposed to 0.25 or 1 ng/bee of thiamethoxam (Thiam 0.25ng and Thiam 1ng, respectively), bees infected with DWV (DWV *per os* and DWV injection, respectively), and bees co-exposed to DWV and thiamethoxam (DWV *per os* and Thiam 1ng, DWV and Thiam 0.25ng, DWV and Thiam 1ng). Viral loads are shown in $\log_{10}$. In boxplots, red lines represent first quartile (25%), median (50%) and third quartile (75%). Different letters show statistical differences between groups.

b) Thiamethoxam influence on DWV experimental infections and other natural infections

We also assessed whether exposure to thiamethoxam modified the virus infection levels in experimentally infected bees (Experiment 2). If *per os* infection with DWV did not induce a higher level of DWV as compared to control bees (p=1, Fig. 2), exposure by injection triggered a significant increase of DWV level in bees (p=0.049 for DWV alone and p=0.003 for the co-exposure to DWV and Thiam 0.25ng). Thiamethoxam exposure combined or not with DWV infection (*per os*), as well as PBS injection, did not affect DWV levels in bees (p=1 for all treatments compared to control). Bees co-exposed to DWV injection and 1 ng of thiamethoxam are showed on Fig. 2, but due to their low number of sample they were not significant (seven samples only could be retrieved, Table S1).

For all other tested viruses (ABPV, BQCV, CBPV, SBV) no significant changes between treatments was detected (Supplementary Fig. S1). However significant differences in their levels were observed between replicates (months), likely following a seasonal evolution (Aubert et al., 2008).

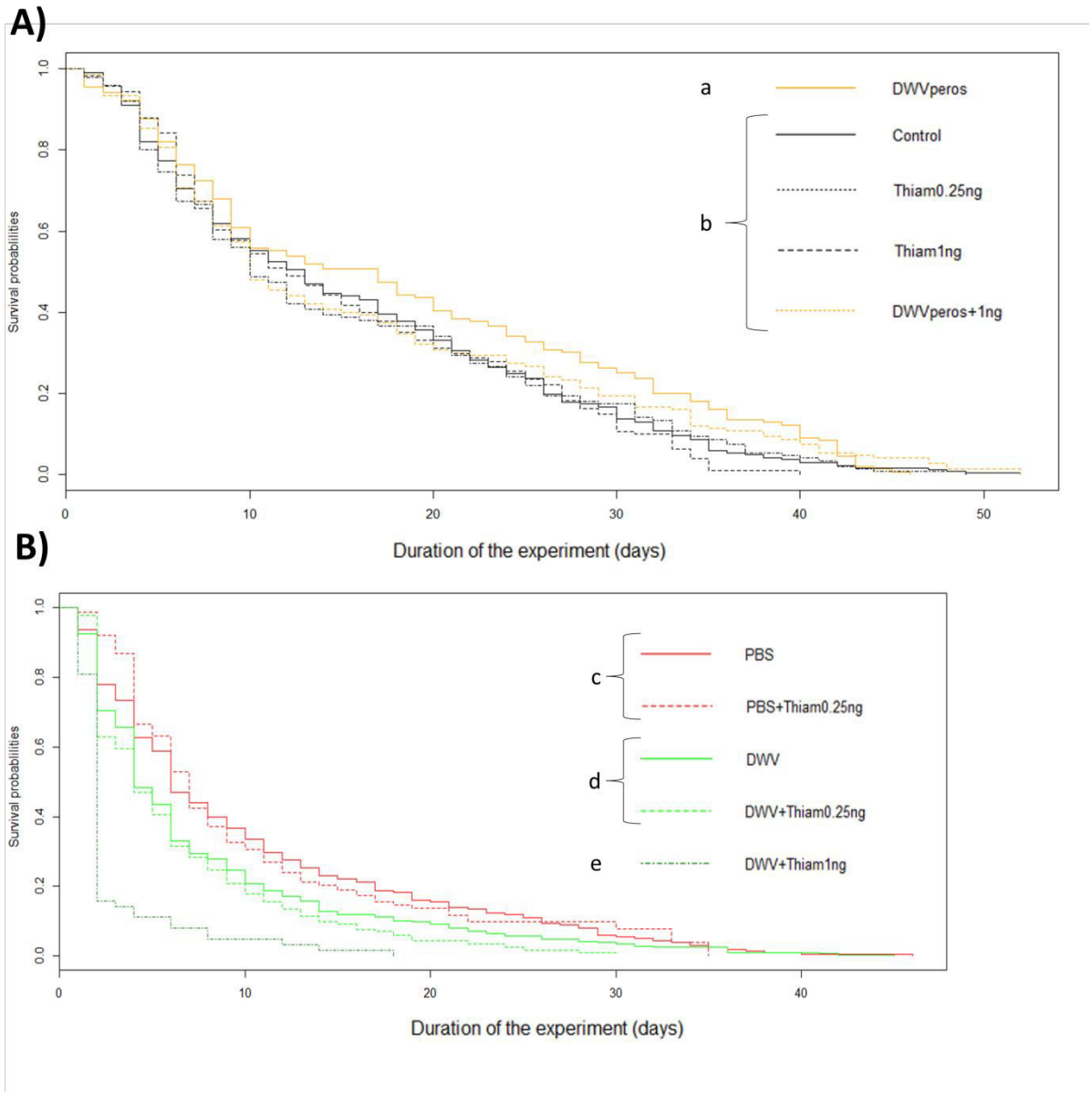

Figure 3: Survival curves of bees exposed to thiamethoxam and/or DWV.

A) Treatments *per os*: untreated bees (Control , n= 339 bees), Thiamethoxam 0.25 ng/bee (Thiam 0.25ng, n=302), Thiamethoxam 1 ng/bee (Thiam 1ng, n=150), DWV*per os* (n=153), DWV*per os* co-exposed with Thiamethoxam 1ng (DWV per os + Thiam 1ng, n=150); B) Treatments with injection: bees injected with PBS (PBS, used here as control, n=311 bees), PBS co-exposed with Thiamethoxam 0.25 ng (PBS+Thiam0.25ng, n=209), DWV (n=368), DWV co-exposed with thiamethoxam 0.25 ng/bee (DWV+Thiam0.25ng, n=253), DWV co-exposed with thiamethoxam 1 ng/bee (DWV+Thiam1ng, n=63), Different letters show statistical differences between groups..

3.2. Bee survival and onset of foraging

a) Survival

Regardless the dose, thiamethoxam did not affect bee survival as compared to control bees (0.25ng: p=0.384 and 1ng: p=0.836, Fig 3A), which was expected from sublethal doses. Furthermore, co-exposure to 1 ng thiamethoxam and DWV (*per os*) did not influence bee survival as compared to control and 1 ng thiamethoxam-exposed bees (p=0.212 and p=0.276, respectively). *per os* infection with DWV significantly increases survival probability as compared to control bees (p=0.018). The injection of DWV significantly decreases survival when compared to bees injected with PBS (p<0.001, Fig 3B). The lowest dose of thiamethoxam (0.25 ng) did not affect the survival of DWV- or PBS-injected bees when compared to bees only exposed to DWV or PBS (p=0.122 and p= 0.876, respectively). However, the highest dose of thiamethoxam (1ng) decreased abruptly the survival of DWV-injected bees (p<0.001 for all treatment comparisons). Only ~10% of bees were alive 4 days after the co-exposure as compared to the 66% and 80% of bees alive in the DWV (injection) and 1 ng thiamethoxam groups.

A χ^2 of compliance test revealed that the interaction is indeed synergistic for this co-exposure (p<0.05, 1 ddl) but not for the co-exposure to DWV and 0.25 ng.

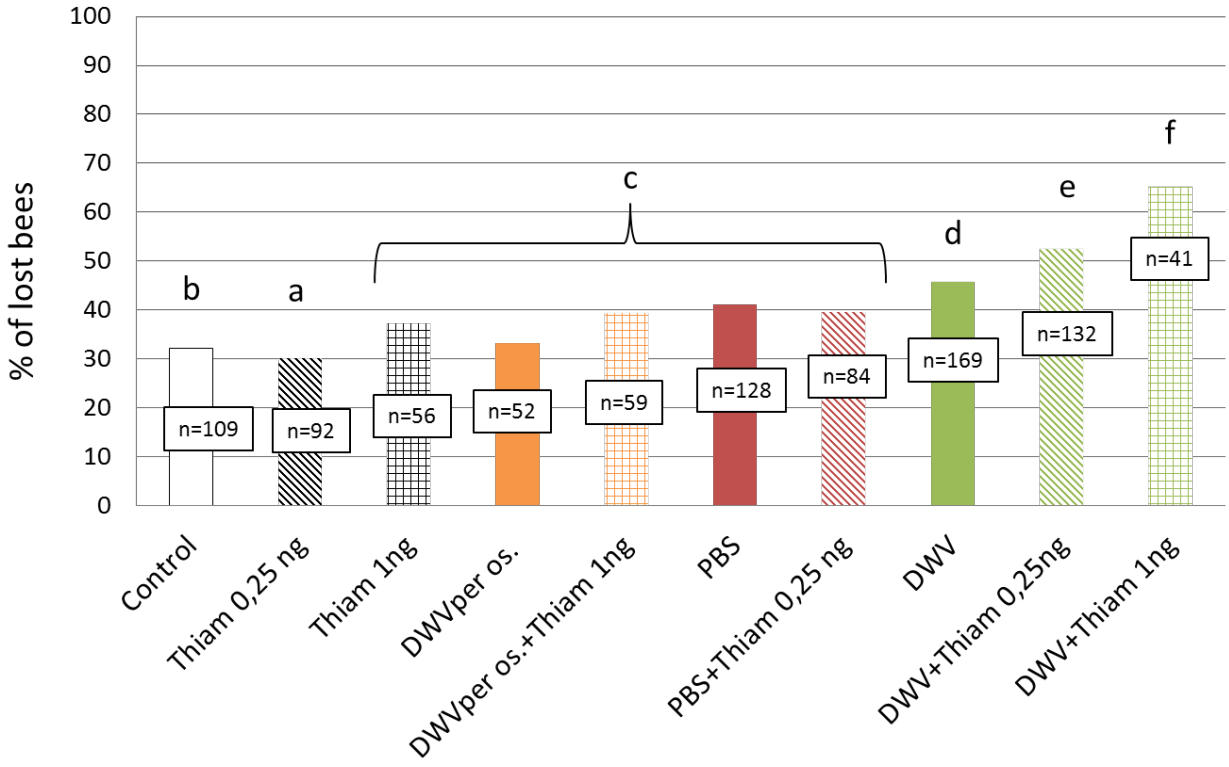

Figure 4: Proportion of bees that were lost at their first exit when exposed to thiametoxam and/or DWV. Number of lost bees are given for each treatment.

Treatments are: untreated control bees, bees fed with 0.25 ng of thiamethoxam, bees fed with 1 ng of thiamethoxam, bees fed with DWV (*per os*), bees co-exposed to thiamethoxam 1 ng and DWV *per* os, bees injected with PBS (PBS, used here as control), PBS co-exposed with 0.25 ng (PBS+Thiam0.25ng), DWV, DWV co-exposed with thiamethoxam 0.25ng/bee (DWV+Thiam0.25ng), DWV co-exposed with thiamethoxam 1 ng/bee (DWV+Thiam1ng). Letters show significantly different between groups (p<0.05) from lowest to highest proportion of lost bees (a<<f).

b) Lost bees

The optical counter allowed us to record the number of bees that never returned to the colony after their first exit from the hive (lost bees). These lost bees were also used in the survival analysis (see above), since we assimilate last exit as a death. However, they were not used to study the precocious onset of foraging, since they left the hive once and never returned.

The lowest proportion of lost bees was found in the bees exposed to 0.25 ng thiamethoxam (30%); (p<0.001 when compared to all others groups). Exposure to thiamethoxam 1 ng (37%), DWV *per os* (33%), DWV *per os* and 1 ng of thiamethoxam (39%), PBS (41%), and PBS and 0.25 ng of thiamethoxam (40%)) induced a higher proportion of lost bees as compared to control bees (32%). The highest proportions of lost bees were observed in the following groups: DWV injection (46%) DWV injection and 0.25 ng of thiamethoxam (52%), and DWV injection and 1 ng of thiamethoxam (65%) (Fig. 2).Finally, co-exposure to DWV injection and 1 ng of thiamethoxam caused a significantly higher proportion of lost bees than DWV alone (p<0.001) and co-exposure to DWV and 0.25 ng of thiamethoxam (p<0.001).

Dependant variable	Covariate	Class	Coefficient	s.e.m.	P-value	Predicted mean age (days)
Age at first foraging flight (>10min) - oral exposures	Intercept (April)	Control	3.083	0.145	<0.001	21.83
	Treatments (April)	DWV *per os*	0.021	0.042	0.625	
		Thiamethoxam 0.25 ng/bee	0.086	0.035	0.015	23.78
		Thiamethoxam 1 ng/bee	-0.072	0.044	0.104	
		DWV *per os* + thiamethoxam 1 ng	-0.066	0.045	0.144	
	Replicates	May	-0.624	0.162	<0,001	
		July	-0.632	0.179	<0.001	
Age at first foraging flight (>10min) - injections	Intercept (April)	PBS	2.510	0.088	<0.001	12.30
	Treatments (April)	PBS+Thiam 0.25 ng	0.066	0.052	0.198	
		DWV	-0.120	0.045	0.007	10.91
		DWV+Thiam 0.25 ng	-0.181	0.059	0.001	10.26
		DWV+Thiam 1ng	-0.806	0.289	0.004	5.50
	Replicates	May	-0.333	0.098	<0.001	
		July	-0.544	0.108	<0.001	

Table 1: Coefficients, standard errors and associated *P*-values, and mean age of first foraging trip is calculated as $e^{(estimate)}$, for the selected models investigating a treatment effect on bee first foraging trips.

Predicted mean ages of first flight are only shown when different from controls (Control or PBS injection).

c) Onset of foraging

Age at the first foraging flight (flights > 10 min) was determined for each treatment group. As for the survival analysis, groups of bees infected with DWV *per os* or via injection were analyzed separately (untreated and PBS-injected bees were assigned as controls in each analysis).

The age at onset of foraging differed between replicates (Fig.4). Indeed, for both treatment categories (*per os* and injected), bees started foraging overall significantly earlier in replicate 1 (May 2016+2017) and 2 (July 2016) than in replicate 3 (April 2017) (Table 1).

Regarding the treatment effects, among the non-injected bees (*per os*) only bees exposed to 0.25 ng of thiamethoxam exhibited a later onset of foraging. Among the injected bees, DWV, with or without co-exposure to the two doses of thiamethoxam, caused an earlier onset of foraging as compared to PBS-injected bees. While bees exposed to DWV and bees co-exposed to DWV and 0.25 ng of thiamethoxam did not start foraging at different ages, bees co-exposed to DWV and 1 ng of thiamethoxam started foraging 5 days earlier.

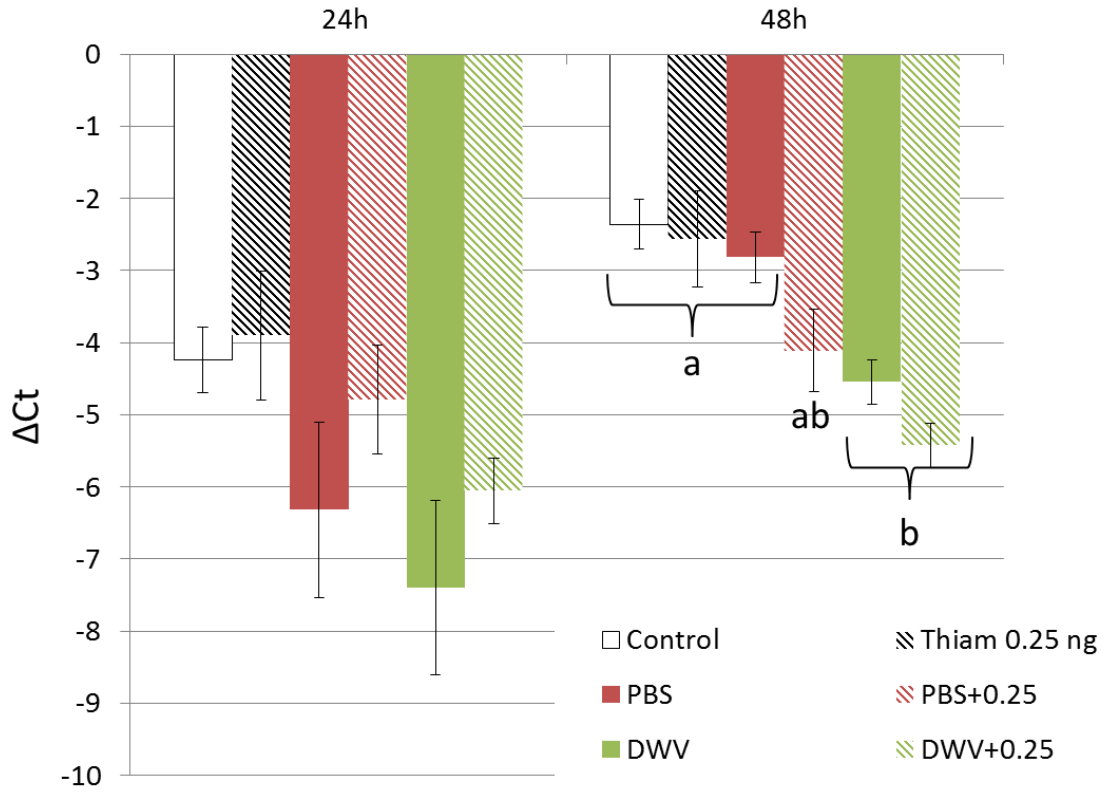

Figure 5: Vitellogenin expression level in bees exposed to DWV and/or thiamethoxam.

Mean ± SD expression levels for untreated bees (control), bees exposed to 0.25 ng of thiamethoxam, PBS-injected bees, PBS-injected bees co-exposed to 0.25 ng of thiamethoxam, DWV-injected bees, and bees co-exposed to an injection of DWV and 0.25 ng of thiamethoxam are shown. Analyses were performed on bees sampled 24 or 48 h post exposure. Letters indicate significant difference (p<0.05).

3.3. Expression level of immune and detoxication genes

Dorsal-1-a, apidaecin, pro-phenoloxydase, glutathione-S-transferase 3, catalase and CYP6AS11 genes were analysed only in bees sampled in replicate 1 and 2 (May and July 2016). Their expression level did not show any significant variation between experimental treatments (p>0.05) (data not shown).

Vitellogenin expression was not tested neither on DWV *per os* groups since no effect was observed on survival and onset of forgaing, nor groups co-exposed to DWV and 1 ng of thiamethoxam since too few bees were collected alive (see "Lost bees", Fig. 4).

There was no significant differences in vitellogenin expression between groups at 24h post exposure (p>0.05). At 48h post exposure, vitellogenin expression in control bees was not significantly different bees injected with PBS (p=0.940), bees exposed to thiamethoxam 0.25 ng (p=0.999), or bees co-exposed to 0.25 ng of thiamethoxam and PBS injection (p=0.081; Fig. 5). Bees injected with DWV or co-exposed to DWV and 0.25 ng of thiamethoxam exhibited a significantly lower vitellogenin expression level than control bees (p<0.001 for both), bees injected with PBS (p=0.011 and p<0.001, respectively), or bees exposed to 0.25 ng of thiamethoxam (p=0.015 and p<0.001, respectively). Vitellogenin expression between bees injected with DWV and bees co-exposed to DWV injection and 0.25 ng of thiamethoxam was not significantly different (p=0.603).

4. Discussion

The combination of stress factors has been suspected to decrease bee survival and therefore lead to colony failure. However, it remains unclear how they might affect bee in natural (colony) conditions. By co-exposing bees to DWV and the neonicotinoid thiamethoxam, we showed that the combination of both can significantly alter behavioural maturation, which causes a steep decrease in survival from premature foraging trips, with a higher failure to return to the colony.

Because other viruses than DWV could have naturally infected the tested bees, and could have introduced bias in our experiments, we tested the viral loads of four other frequently

occurring viruses. Given that the bees had been homogenized before the treatments, and taking into account the absence of significant differences between treatments in other virus levels, we assumed that any observed significant effects in bees were not linked to these background viral infections and could be attributed to our treatments.

To avoid a potential experimental bias due to the stress of injection injury for viral exposure, we carried out an experiment on naturally infected honeybee cohorts. We did not find any effect of thiamethoxam exposure on DWV level, as would be expected from an immunosuppression induced by neonicotinoids (ref). We hypothesise that DWV loads (10^7 to 10^{12} copies/bee) were too high to observe any potential DWV increase. While we selected colonies based on their low DWV-loads, the covert, background infections increased over the season. DWV load is known to increase from spring to autumn, which is intrinsically linked to seasonal population growth of the parasitic mite *Varroa* (Dainat et al., 2012a). Another way for us to avoid injection bias was to perform DWV-infection *per os*, Again, we did not find any significant effect on viral loads, even if we performed exposure with high numbers of DWV (inoculum with 10^8 copies of DWV per bee). This is in line with the findings of Ryabov et al., 2014 who showed that larvae reared in DWV symptomatic hives (thus exposed through alimentation), but not infested by *Varroa,* had DWV loads that stayed close to control (larvae raised in colonies with low DWV levels and no *Varroa*), while *Varroa* parasitized pupae developed very high loads of DWV. We conclude that feeding is not an efficient way to produce a significant experimental infection in honeybees.

Exposure to field-relevant doses of thiamethoxam did not either affect DWV loads in virus-injected bees. Similarly, it is possible that the virus background infection level was already too high to observe an increase in DWV level. However, injection of DWV did increase DWV loads in the tested bees, regardless of their co-exposure to thiamethoxam. Another explanation is that the most infected bees were excluded by nest mates as part of the social immunity (Baracchi et al., 2012; McDonnell et al., 2013), altruistically removed themselves (Rueppell et al., 2010) or simply died and could not be sampled. It should be noted that we previously observed that CBPV viral loads in dead honeybees were significantly different from viral loads in bees that were sacrificed on the same day (Coulon et al.,personal communication).

Regarding survival, the DWV *per os* showed a better survival probability than control bees. This could be explained by the fact that, while bees did not replicate the virus, the ingested DWV could have triggered non-specific defence mechanisms (Alaux et al., 2010b; Evans and Spivak, 2010). However, DWV injection significantly decreased the survival rate of bees. These results are in line with previous studies showing that DWV has a deleterious effect on honeybee lifespan (Benaets et al., 2017; Dainat et al., 2012; Rueppell et al., 2017; Woyciechowski and Moron, 2009). Then, we found that bees co-exposed to DWV and 1 ng of thiamethoxam exhibited the lowest survival rate. Interestingly, exposure to 1ng thiamethoxam alone did not affect bee survival, suggesting an interactive effect between the virus and the pesticide at the highest dose. Such interaction was not observed with the lowest thiamethoxam dose. The survival drop in bees co-exposed to DWV and 1 ng thiamethoxam was related to a higher number of bees that did not return to the colony after their first exit. Indeed, 65% of them never returned after their first exit. One simple explanation is that bees got lost on their return trips, as similarly observed in foragers after a similar dose of thiamethoxam (Henry et al., 2012). However, contrary to Henry et al., 2012, we did not found a significant loss in bees only exposed to thiamethoxam. The main difference is that bees in Henry et al., 2012 were released 1 km away from their colonies, and here bees were not exposed to this challenge. In addition, in this study the bees were exposed at a young age, as opposed to the foragers exposed in Henry et al. An age-dependant pesticide effect could explain the differences observed between both studies (Poquet et al., 2016). This effect was also observed to a lesser extent in bees co-exposed to DWV and 0.25 ng of thiamethoxam or only injected with DWV. At both thiamethoxam doses, the co-exposure effects were additive.

The lowest survival rate and higher rate of lost bees in the DWV and 1 ng thiamethoxam co-exposed group could be explained by their early onset of foraging. Indeed, such precocious foragers may not be optimally adapted to foraging tasks and are usually less resilient than normal foragers (Woyciechowski and Moron, 2009). They are heavier and exhibit lower flight performance than normal-aged foragers (Vance et al., 2009), likely due to different flight metabolic rates and muscle biochemistry (Schippers et al., 2006). Precocious foraging has been previously observed with DWV injections by (Benaets et al., 2017), but also with other stressors, such as *Varroa destructor* (Downey et al., 2000), *Nosema apis* (Wang and Moeller, 1970), or an early life CO_2 anaesthesia (Woyciechowski and Moron, 2009). However, we could not exclude either a self-removal or exclusion by nestmates from the colony (see above).

Considering that there is no effect of 1 ng of thiamethoxam alone, the expected outcome of an additive interaction between DWV injection and exposure to 1 ng of thiamethoxam would be equal to the effect of DWV injection alone. However, honeybees that were co-exposed to DWV and 1 ng of thiamethoxam started foraging at roughly half the age of bees exposed to DWV injection alone. This allows us to conclude that the effect co-exposure between DWV injection and 1 ng of thiamethoxam on this specific trait is of synergistic nature.

The observed effects of DWV injections and DWV co-exposures, highly accentuated by the highest dose of thiamethoxam especially, suggest that these treatments affect physiological traits in bees. Although no change in DWV load was observed when bees were co-exposed to the pesticide, we could not rule out a possible effect of the pesticide on DWV loads due to potential bias in bee sampling (sampling of alive bees, see above). However, it also underlines that the observed synergistic and additive effects must have another origin.

To further explore the influence of both DWV and thiamethoxam on bees, we analysed potential physiological changes, by focusing on immune and detoxication genes. None of the tested genes were affected by the treatments, except vitellogenin, suggesting a lack of effect on the immune and detoxication system. This is somehow in contradiction to a previous study, which shows that *dorsal-1a*, known to take part in the immune response, was down regulated by the thiamethoxam metabolite clothianidin (Di Prisco et al., 2013). However, our first sampling was performed 24 h after an acute exposure, which was probably too late to observe any reaction to the pesticide, as *dorsal-1a* has been shown to react to pesticide exposure then return quickly to normal level, during the very first hours after the honeybees have endured a clothianidine exposure (Di Prisco et al., 2013). The same *dorsal-1a* was also found to be down regulated in bees with high levels of DWV (Nazzi et al., 2012), but a later study reported that peaks in DWV were correlated to peaks in the expression of immune genes, including *dorsal-1a* (Steinmann et al., 2015).. This illustrates that immune responses varies greatly (Doublet et al., 2017) and the timing of sampling accounts a lot for this variation, especially for transcription factors of immune related genes in honeybees.

Vitellogenin is a protein, produced in the fat body, known to be involved in immunity through the regulation of haemocytes (Amdam et al., 2004b), but also involved in the regulation of division of labour. It is notably a key player in the regulation of behavioural maturation, with

a higher expression in nurses than in foragers (Amdam, 2011). The downregulation of vitellogenin upon DWV injection is consistent with previous studies performed on different strss factors(Boncristiani et al., 2012; Bordier et al., 2017). Antonio et al., 2008 and Nelson et al., 2007 showed that an RNAi mediated inhibition of the transcription of vitellogenin made bees forage (flight>10 min) significantly earlier. We therefore propose that injection of DWV induced a precocious shift towards foraging activity through a down regulation of the transcription of vitellogenin. While we could not test on bees co-exposed to DWV and 1 ng of thiamethoxam, their very low survival and extremely precocious onset of foraging suggest that it could cause a yet steeper decline of vitellogenin.

In conclusion, our results on the pesticide influence on virus loads underlines the difficulty of extrapolating laboratory results to colony conditions. However, by performing behavioural experiments under the same natural conditions, we could identify negative effects of pesticide/virus co-exposure on bee survival and identify some underlying mechanisms (early onset of foraging and first foraging flight failure). Depending on the number of affected bees, this could results in a breakdown of division of labour and in a dramatic colony failure as indicated by Perry et al., 2016. Altogether, our data demonstrates the importance of field relevant conditions for a better understanding of the influence of stress factors in bees and likely colony failure.

Aknowledgments

This study was supported by INRA (Institut National de la Recherche Agronomique – National Institute for Agricultural Research) for the 2016 experiments and by the “Fondation Lune de Miel” for the 2017 experiments.

Supplementary Data

Setting				Treatments									
Experiment	Replicate	Month	Year	Control	Thiam 0,25	Thiam 1	DWV *per os*	DWV *per os* +Thiam 1	PBS	PBS+Thiam 0,25	DWV	DWV+Thiam 0,25	DWV+Thiam 1
Experiment 2 (optical counters)	1	05/05/2016	2016	132 (66x2 colonies)	132 (66x2 colonies)	na	na	na	132 (66x2 colonies)	132 (66x2 colonies)	132 (66x2 colonies)	132 (66x2 colonies)	na
	2	10/07/2016	2016	132 (66x2 colonies)	132 (66x2 colonies)	na	na	na	132 (66x2 colonies)	132 (66x2 colonies)	132 (66x2 colonies)	132 (66x2 colonies)	na
	3	12/04/2017	2017	60	na	60	60	60	60	na	60	na	na
	4	27/04/2017	2017	60	60	60	60	60	60	na	60	60	60
	5	16/05/2017	2017	60	60	60	60	60	60	na	60	60	60
Experiment 2 (painted bees)	1	05/05/2016	2016	100	100	na	na	na	100	100	100	100	na
	2	10/07/2016	2016	100	100	na	na	na	100	100	100	100	na
	3	12/04/2017	2017	80	na	80	80	80	80	na	80	na	na
	4	27/04/2017	2017	80	80	80	80	80	83	na	79	80	83
	5	16/05/2017	2017	80	80	80	80	80	80	na	80	80	80

Table S1: Sampling size for survival and onset of foraging experiment (bee counter).

Name	Sequence (5'-3')	bp	Reference
CYP6AS14			
F	TGACATTGAGTTGACGGACGAT	64	this work
R	GAAACCTGCCGCGAAGAA		
GSTS3			
F	AAACCGATAGCGCAGAGTAACG	87	this work
R	CATCATTGCCTCCCATTCGT		
Catalase			
F	TTTGGTGGGCCTAGAGAATGTC	92	this work
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 work
R	TATCGTAGAGAACCTCGCATTTCC		
PPOAct			
F	CGTTGAAAAGTCGAAGCAGATTAA	112	this work
R	AGGACGCCACCGCAGTATT		
β-actin			
F	GATTTGTATGCCAACACTGTCCTT	69	(Di Prisco et al., 2016)
R	TTGCATTCTATCTGCGATTCCA		
RpL32			
F	CGTCATATGTTGCCAACTGGTTT	107	this work
R	CCATGAGCAATTTCAGCACAA		
DWV			
DWV-F8688	GGTAAGCGATGGTTGTTTG	143	(Locke et al., 2012)
DWV-B8794	CCGTGAATATAGTGTGAGG		
ABPV			
ABPV-F6548	TCATACCTGCCGATCAAG	197	(Locke et al., 2012)
KIABPV-B6707	CTGAATAATACTGTGCGTATC		
SBV			
SBV-qF3164	TTGGAACTACGCATTCTCTG	226	(Locke et al., 2012)
SBV-qB3461	CTCTAACCTCGCATCAAC		
BQCV			
BQCV-qF7893	AGTGGCGGAGATGTATGC	294	(Locke et al., 2012)
BQCV-qB8150	GGAGGTGAAGTGGCTATATC		
CBPV			
CBPV1-qF1818	CAACCTGCCTCAACACAG	296	(Locke et al., 2012)
CBPV1-qB2077	AATCTGGCAAGGTTGACTGG		

Table S2: Primers used for the quantification of selected honeybee genes and viruses.

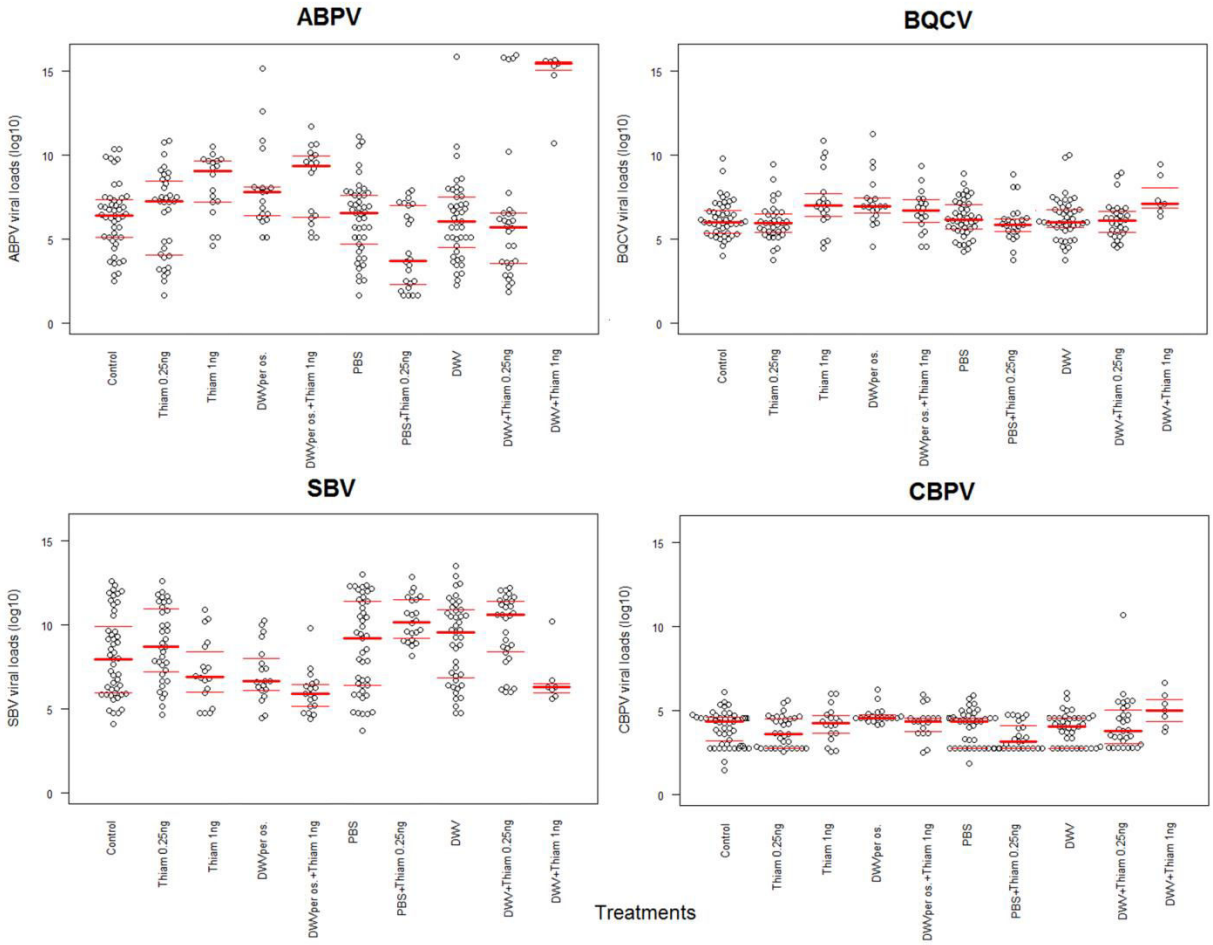

Figure S1: Viral loads distributions for the four additionally tested viruses according to treatments.

Viral loads are shown in $\log_{10}$. The different treatments are: Control bees, bees injected with PBS (PBS), bees injected with PBS and exposed to 0.25 ng/bee of thiamethoxam (PBS+0.25ng), bees exposed to 0.25 or 1 ng/bee of thiamethoxam (Thiam 0.25ng and Thiam 1ng, respectively), bees infected with DWV (DWV *per os* and DWV injection, respectively), and bees co-exposed to DWV and thiamethoxam (DWV *per os* and Thiam 1ng, DWV and Thiam 0.25ng, DWV and Thiam 1ng). In boxplots, red lines represent first quartile (25%), median (50%) and third quartile (75%). Different letters show statistical differences between groups. Swarms show the distribution of populations, representing each sample.