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Hazard of a neonicotinoid insecticide on the homing flight of the honeybee depends on climatic conditions and *Varroa* infestation

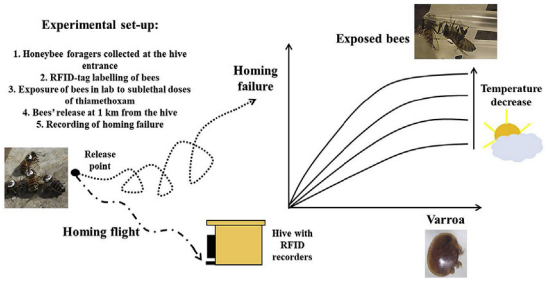
Coline Monchanin [a, b], Mickaël Henry [c, d], Axel Decourtye [a, b, c], Anne Dalmon [c, d], Dominique Fortini [e], Elodie Bœuf [c, d], Ludovic Dubuisson [b], Pierrick Aupinel [e], Colombe Chevallereau [e], Julie Petit [b], Julie Fourrier [b, c, *]

[a] ACTA, Site Agroparc, F 84914, Avignon, France
[b] ITSAP-Institut de l'Abeille, Site Agroparc, F 84914, Avignon, France
[c] UMT Protection des Abeilles dans l'Environnement, Site Agroparc, F 84914, Avignon, France
[d] INRA, UR 406 Abeilles et Environnement, F 84914, Avignon, France
[e] INRA, Unité expérimentale Abeilles, paysages, interactions et systèmes de culture, F 17700, Surgères, France

HIGHLIGHTS

- Temperature and colony health modulate insecticide-induced homing failure in honeybee.
- *Varroa* mites exacerbate homing failure caused by the insecticide thiamethoxam.
- Low temperatures aggravate insecticide-induced homing failure.
- Pesticide risk assessment should include temperature and *Varroa* mites as covariates.

GRAPHICAL ABSTRACT



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ABSTRACT

The paradigm for all toxicological bioassays in the risk assessment of pesticide registration reflects the principle that experimental conditions should be controlled to avoid any other factors that may affect the endpoint measures. As honeybee colonies can be frequently exposed to bio-aggressors in real conditions, often concomitantly with pesticides, co-exposure to pesticide/bio-aggressors is becoming a concern for regulatory authorities. We investigated the effects of the neonicotinoid insecticide thiamethoxam on the homing performances of foragers emerging from colonies differentiated by health status (infestation with *Varroa destructor* mites, microsporidian parasite *Nosema* spp. and Deformed Wing Virus). We designed a homing test that has been recently identified to fill a regulatory gap in the field evaluations of sublethal doses of pesticides before their registration. We also assessed the effect of temperature as an environmental factor. Our results showed that the *Varroa* mite exacerbates homing failure (HF) caused by the insecticide, whereas high temperatures reduce insecticide-induced HF. Through an analytical Effective Dose (ED) approach, predictive modeling results showed that, for instance, ED level of an uninfested colony, can be divided by 3.3 when the colony is infested by 5 *Varroa* mites per 100 bees and at a temperature of 24 °C. Our results suggest that the health status of honeybee colonies and climatic context should be targeted for a thorough risk assessment.

* Corresponding author. ITSAP-Institut de l'Abeille, Site Agroparc, F 84914, Avignon, France.
E-mail address: julie.fourrier@itsap.asso.fr (J. Fourrier).

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1. Introduction

Current risk assessment of plant protection products on honeybees before registration is mainly based on lethal effect measurements in the laboratory, completed by tests performed in semi-field and field conditions, according to the risk assessed. However, sublethal doses of pesticides that do not affect the survival of the bees may induce physiological or behavioral impairments and thus may have an impact on the colony dynamics (Desneux et al., 2007). Recent evidence in free-flying bees has shown that sublethal exposure to neonicotinoid insecticides decreased homing success in forager bees (Henry et al., 2012a, 2014; Schneider et al., 2012; Matsumoto, 2013). These results, obtained after a controlled exposure in the laboratory, have also been confirmed by field exposure for the survivorship of worker bees (Henry et al., 2015). With the current revision of the risk assessment of plant protection products on pollinators before pesticide homologation, new methods have been requested (EFSA, 2013). In its guidance document, the European Food Safety Authority identified the homing flight test, initially designed by Henry et al. (2012a), as a model for assessing the effects of sublethal doses of pesticides. Homing flight performance provides information on a broad spectrum of parameters that may be disrupted by pesticide exposure in free-flying bees and in field conditions. Homing performances incorporate many physiological and cognitive functions like navigation, memory, muscle flight contraction and energetic metabolism.

However, as previously shown by Henry et al. (2014), the assessment of sublethal effects of a pesticide on homing performance in field conditions can be influenced by factors such as temperature and landscape complexity. As colonies can be frequently exposed to bio-aggressors, it is also an open question as to whether the health status of the colony may influence the homing performance of exposed bees and thus the assessment of pesticide's effects. Indeed, some bio-aggressors are important stressors of the honeybee colony, potentially playing roles in colony losses worldwide, such as the ectoparasitic mite *Varroa destructor* (Le Conte et al., 2010; Neumann and Carreck, 2010; Nazzi and Le Conte, 2016), the intestinal fungus *Nosema* spp., common adult honeybee diseases (Higes et al., 2008; Giersch et al., 2009), and viruses. Among the viruses, the Deformed wing virus (DWV) transmitted by the vector *V. destructor* is highly prevalent, as more than 70% of the colonies are often contaminated (Berényi et al., 2006; Forgách et al., 2008; Tentcheva et al., 2004).

Official toxicological bioassays support the principle that experimental conditions should be maximally controlled to avoid that external factors, other than the tested pesticide, do not influence the measurement endpoint. But in the field, honeybee colonies are frequently exposed to bio-aggressors and co-exposure with other pesticides is common (Neumann and Carreck, 2010; Pettis et al., 2012; Poquet et al., 2016). This co-exposure issue has also become of major concern for regulators concerning pesticide risk assessment before homologation (EFSA, 2012a; Rortais et al., 2017). Guidelines also mention that animals used for the tests must come from healthy colonies, without any visible symptoms of diseases (EPPO, 2010; OECD, 1998a, 1998b). But epidemiologic surveys conducted around the world have shown that honeybee colonies may host numerous pathogens while remaining mostly asymptomatic (Poquet et al., 2016). We expect that an infestation with bio-aggressors, even below the symptomatic threshold, could modulate the effects of the tested pesticide. The inverse, modulation of the response to a bio-aggressor following a pesticide exposure, has already been demonstrated. For example, interactions between *Nosema* microspores and sublethal exposures to a neonicotinoid or a phenylpyrazol (fipronil) significantly weakened honeybees by decreasing their survival (Alaux et al., 2010; Aufauvre et al., 2012; Vidau et al., 2011). Recent studies highlighted that bio-aggressors can increase motor or behavioral impairment of bees exposed to neonicotinoid insecticides (Blanken et al., 2015; Coulon, 2017).

In this study, we tested whether the infestation of the colonies with *V. destructor* mite, *Nosema* spp., and DWV may impact honeybee homing success and modulate the sublethal effect of thiamethoxam. We also addressed the effect of ambient temperatures on the homing performances of exposed bees as Henry et al. (2014) have previously shown the influence of higher temperatures on homing success.

2. Materials and methods

2.1. Honeybees

This study took place in June and July 2016 on two different apiaries (Avignon and Surgères in France, Fig. S1). Experiments were conducted on 18 colonies of *Apis mellifera* (Buckfast) honeybees (15 colonies from Avignon and 3 colonies from Surgères) with proper foraging activity and no visible disease symptoms as called for by published guidelines (EPPO, 2010; OECD, 1998a, 1998b). The colonies did not receive any chemical treatments (e.g. varroacide) for at least 6 weeks prior to the experiments. Colonies used had a fertile queen with a known history that was not older than 2 years. They were prepared to be as homogenous as possible regarding colony strength (at least 10 000 workers), food storage (2–3 frames), number of bees and amount of brood (5–7 frames). Averaged-size colonies were used for the need of our experiment. One to four days before the experiment a colony evaluation was performed using the method described in Maisonnasse et al. (2016). This method is based on the assessment of the percentage of bees, open and capped brood, food stored for each comb's frame. Honeybees were confined to a 10-comb Dadant hive equipped with one food super. The colonies were installed on the field site at least one week before starting the experiment in order to allow honeybees to be familiar with their environment. Colonies were separated every few meters to maximally avoid drift of labelled bees into other colonies. Each experiment was performed with a different colony and the 18 colonies were tested one after the other at different day during the experimental period.

2.2. Determination of the colonies' health status

We worked with a gradient of *V. destructor* infestation of the colonies. To do so, ten of the colonies were infested with *Varroa* four to six weeks before the experiments, by twice receiving at two-week intervals, two worker (or one male) brood combs with capped brood from highly infested colonies. The remaining colonies were not artificially infested, and evolved with their natural infestation of *Varroa* mite. For the two infestation phases, we controlled artificially-infested colonies using sticky boards placed below the bottom boards (Dietemann et al., 2013) after the first and second infestation. *Varroa* load varied from 0 to 3 mites per sticky board after the first week of infestation. For the second week, *Varroa* infestation varied from 0 to 81 mites per sticky board, with a mean infestation of 22 mites. So we considered our infestation as low to moderate. Indeed, a drop of about 3–5 *Varroa* mites per day is considered as a slight increase (FERA, 2010). As we wanted to have a marked gradient for our experiment, we kept only the most infested colonies from our artificial infestation (6 colonies). The other naturally infested colonies from our apiary were more infested than our artificially infested ones as a whole. Three naturally infested colonies (n° 16 to 18) belonged to another apiary (Surgères, Table S4). Finally during the experiment, artificially

infested colonies ranged from 0.3 to 2.1 varroas per 100 bees and naturally infested colonies ranged from 0 to 33.5 varroas per 100 bees (Table S4).

One to four days prior to the experiment, mite infestation of the honeybees from each tested colony was quantified by washing a sample of bees from a brood comb with water and detergent (Dietemann et al., 2013) in order to count the phoretic mites and establish the number of *Varroa* mites per 100 honeybees (Lee et al., 2010). On the day of the experiment, all types of foragers were sampled at the hive entrance for further *Nosema* spp. and virus analysis. One hundred and one hundred twenty foragers per colony were collected alive and immediately frozen at −20 °C for *Nosema* and Deformed Wing Virus (DWV) sampling respectively. The infestation by the intestinal fungus *Nosema* was assessed according to OIE method for counting fungal spores (Fries et al., 2013). For each colony, the number of spores was counted from a pool of 50 bees' abdomen. The abdomen were cut with scissors and transferred in a filter bag with 10 ml of water. They were grinded with a rolling pin and filtered three times (Total of 50 ml water volume). A sample of macerate was collected and placed on a counting cell under a microscope. The number of *Nosema* spores were counted and converted into a number of spores per bee. For DWV, three pools (= biological replicates) of 40 bees were analyzed for each colony. RNA extraction and reverse transcription were performed as described in Dalmon et al. (2017). The number of DWV copies per bee was assessed by quantitative PCR using a StepOne-Plus Real-Time PCR System (Applied Biosystems®) and the SYBR Green detection method. The quantitative PCR method amplifies a highly conserved region coding for the helicase, then detects all DWV variants (Locke et al., 2012). Here we used natural DWV infection of the colonies. Previous studies have shown recombinant variants to be predominant in France (Dalmon et al., 2017). Three microliters of ten-fold diluted cDNA were mixed with 7 µl of SYBR Green master mix (Applied biosystems) containing 10 pmol of primer DWV F8668 and B8757 (Locke et al., 2012). Amplification was performed after 10 min at 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. Absolute reference to the corresponding synthetic gene (136bp) of DWV was used to calculate the mean number of DWV copies per bee, while accounting for RNA extraction yield. Quantitation was replicated twice (= technical replicates). Classical linear regression analysis of cycle quantification (Cq) value onto log10 of virus gene copies were used to calculate the number of copies of virus gene in each reaction, from standards curves (10-fold serial dilution series) of the synthetic gene of known concentration. They were converted into estimated absolute amounts of virus per bee taking into accounts for the reaction and the extraction dilution factors.

2.3. Radio frequency identification (RFID) device

Radio frequency identification (RFID) technology (Streit et al., 2003; Decourtye et al., 2011) depends on the emission of a radio signal by the reader which is received by the tag positioned on the bee. Each time a tag-equipped bee passes near a reader (about 3 mm), the tag obtains its operating power from the reading success and emits a unique identification code. We used RFID tags (13.56 MHz system; Microsensys GmbH, Erfurt, Germany) of approximately 1 mm by 2 mm, weighing about 3 mg (3% of bees' weight), which is less than the weight of pollen loads (between 8 and 29 mg) or nectar (between 40 and 80 mg) carried during a foraging flight (Southwick and Pimentel, 1981; Wells and Giacchino, 1968). Four readers were placed at the hive entrance, each spanned a tunnel of 14 × 21.5 mm (7 mm high). Readers were installed at the hive entrance on a wooden interface and were separated from each other by at least 4 cm to avoid interference during RFID recording. Each time a bee equipped with a tag passed through a reader, the tag identification code and the exact time of the event (date, hour, minutes and seconds) were recorded in the MAJA system host (Microsensys GmbH, Erfurt, Germany) and the data were collected 24 h after release. For each experimental session, one colony was equipped with the RFID system placed at the hive entrance at least three days before the experiments to allow the bees to know the device and circulate well through the readers. To prepare the following experiments, two other colonies were equipped at the same time with a "blank" system in wood that mimics the RFID readers with the interface to allow the bees to get used to the equipped hive entrance.

2.4. Insecticide treatment

Technical grade thiamethoxam (99% pure) was purchased from Cluzeau Info Labo (France). Thiamethoxam was tested at three sublethal doses, 0.11, 0.33 and 1 ng per bee following a geometric progression with a ratio of 3. The highest nominal dose of 1 ng per bee was previously tested by Henry et al. (2012a) and corresponds to the oral median lethal dose (LD50) divided by five (European Commission, 2006). The median nominal dose of 0.33 ng per bee is the closest to the field estimated exposure dose of 0.276 ng per bee for 20% sugar content nectar in field conditions (EFSA, 2012b). Since the main potential exposure route is the ingestion of the contaminated nectar following plant treatment by seed dressing, thiamethoxam was orally administrated. The doses of thiamethoxam were prepared in acetone and diluted into a 30% (w/v, sucrose/demineralized water) feeding sucrose solution. The final volume of acetone in the feeding sucrose solution was 0.1% (v/v). The effects of the exposure to insecticide-added feeding solutions were compared with those of an untreated control sucrose solution containing 0.1% acetone (v/v). Finally, the concentrations of thiamethoxam prepared in sucrose solution were analyzed by the French food safety agency (ANSES) using the technique LC-MS/MS (limit of thiamethoxam quantification = 0.3 ng/ml) to detect real doses. The real doses detected by chemical analysis ranged from 0 (control) to 1.27 ng per bee.

2.5. Homing experiments

On the morning of the test day when foraging was active, healthy-looking foragers without *V. destructor* mites and carrying pellets of pollen or not were captured at the hive entrance equipped with the RFID device using entomological forceps. A total of about 700 bees were captured and placed by groups of 100–150 individuals in boxes. They were transported a first time to a release site at 1000 m (+/- 100 m) away from the experimental colonies. Hydrophobic colored powder (pink fluorescent pigments – T series, COLOREY SAS, France) was added to each box containing captured bees with a proportion of 0.5 mg per bee. A preliminary comparative acute toxicity study showed that the pink hydrophobic powder had no significant or abnormal effects on mortality of bees compared to bees that were not powdered (Table S1). Boxes were gently shaken in order to color the bees. Then, the boxes were opened simultaneously. At the hive entrance of the experimental colony, about 160 colored bees that returned to the hive were collected on the flight board within a maximum of 2 h. Thereby, the captured, powdered bees had at least one homing experience to the hive from the release site. Bees were caged in groups of 40 individuals with candy *ad libitum* and were brought to the laboratory. Bees of all cages stayed with candy *ad libitum* for an additional 30 min to synchronize their dietary state. Then, the test began and the bees underwent a starvation period of 90 min. During this starvation phase, the bees were transferred one by one from cages

to a holding cage where a foam plunger allowed them to be immobilized without damage and they were labelled with an RFID microchip. The microchip was glued on the thorax of the foragers using dental cement (Temposil®, Coltene). For each tested colony, we labelled 30 to 35 bees per treatment (total of 120–140 bees labelled). After labelling, the foragers were transferred in groups of 10 bees into cages according to treatment tested, and placed in the dark before the exposure phase. The honeybees were collectively exposed to one of the four treatments (control, 0.11 ng/bee, 0.33 ng/bee or 1 ng/bee of thiamethoxam) by feeding them with 20 µl per honeybee (200 µl for 10 bees) of the 30% (w/v) sucrose solution containing thiamethoxam at different concentrations or the control solution. The volume of sucrose solution was distributed using a feeder system enabling contact with the food only through the mouth parts. Moreover, we previously showed that homing success did not significantly differ between bees exposed individually or collectively (per group of 10 bees), when comparing bees that received the same thiamethoxam treatment (Fig. S2). The bees in a cage shared the feeding solution by trophallaxis. The exposure phase was completed once the honeybees had consumed all the administered volume.

After feeding with treated or untreated sucrose solution, the bees underwent another one-hour starvation period in the dark. Then, they were transported a second time to the release site they were previously released after powdering, at 1000 m (+/- 100 m) from the hive equipped with the RFID system. Homing events were recorded. Homing experiments were processed within a total of four distinct experimental sessions, each carried out with a distinct apiary or release point. Whenever floral resources were deemed to be scarce in the course of a session, individual foragers were fed for a few minutes before release (candy or 30% w/v sucrose solution). RFID recording of the marked foragers' homing flights to the hive started immediately after release and lasted 24 h. The climatic conditions (temperature (T°C), hygrometry (%)) were recorded during the 24-h period using a data logger placed near the tested hive.

2.6. Data analysis

We analyzed the homing statistics of 2169 honeybees obtained from the 18 colonies.

Data were analyzed using the R software for statistical computing, v. 3.3.1. (R Core Team, 2016). The homing flight was treated as a binary response variable (0 = no return, 1 = return during the 24 h of recording). In order to assess the effects of different factors on the homing performance, we used generalized linear mixed-effects models (GLMM) with a logit link function using the R package lme4 (Bates et al., 2018). To conform the assumption of independent outcomes in the binary homing observations, the identity of experimental colonies and of the experimental sessions were included as random grouping variables (colonies nested within experimental sessions). The exposure dose was introduced as a fixed, quantitative, explanatory variable. The additional explanatory variables were the temperature (mean temperature recorded during the 24-h period), the sanitary variables of the colonies: *Varroa* mite infestation rate ($\log_{10}$-transformed), *Nosema* infection ($\log_{10}$-transformed) and DWV infection (average pathogen load per bee (mean number of gene copies per bee) from the 3 biological replicates). As the DWV variable was correlated with the *Varroa* variable (Spearman test, $r_s = 0.464$, $P < 0.05$), DWV was detrended from *Varroa* by residual values to be used and included in the models.

All the possible two-way interactions among explanatory variables were considered within the frame of a multimodel inference procedure (Burnham and Anderson, 2002) using the R package MuMIn (Barton, 2018). The multimodel inference produces a single global model by averaging coefficients of explanatory variables within a set of simpler models, with respect to each model's relative weight of evidence. The weight of evidence ω_i of a simpler model *i*, based on the Akaikee information criterion (AIC), gives the probability that model *i* is the best one in the model set, considering a parsimony tradeoff between fit and complexity. We restricted the multi-model inference to the sub-set of best models totaling 95% cumulative weight of evidence, i.e. the sub-set of best models with 95% chance of including the most parsimonious combination of explanatory variables (Table S2). Importantly, averaged coefficients are computed along with their standard error, making it possible to derive *P*-values for assessing the significance of each explanatory variable in the global model. Additionally, we computed the relative importance of each explanatory variable in the global model, based on its occurrence and relative contribution to the cumulative weight of evidence within the 95% best model sub-set. The relative importance ranges from 0 (variable absent from the best model sub-set) to 1 (variable present in each of the best models), and increases as it occurs in models with greater weights of evidence.

Each explanatory variable was standardized beforehand to the range [0,1] by subtracting each datum point from the minimum value divided by the maximum value minus the minimum value. Then, variable values were readily interpretable in terms size of effect and were comparable among each other.

Multimodel predictions allowed us to establish a predicted dose-response function according to different pathogen loads and temperature scenarios encountered in our experimental context. To summarize the variation of predicted dose-response functions, we computed the predicted Effective Dose ED_{20}, i.e. the exposure dose predicted to trigger a 0.20 mortality rate due to homing failure (HF) in exposed bees, for a range of pathogen loads and temperature scenarios. As previously developed in Henry et al. (2014), the ED_{20} framework is intended to pinpoint critical pesticide exposure levels liable to push colony dynamics to the brink of collapse. When added to background mortality, a 0.20 HF mortality entails >35% reduction of colony size during an exposure event (Henry et al., 2012b), which is taken to be a "large effect size" threshold in the official arena of risk assessment (EFSA, 2013).

3. Results

3.1. Homing flight success decreases with the highest dose of thiamethoxam

Insecticide dose had a significant effect on homing flight success (GLMM, $n = 2169$, $Z = 2.136$, $P < 0.05$; Table 1). The bees exposed to the highest dose of 1 ng per bee returned to the hive at a significantly lower proportion (42%) compared to non-exposed bees (63%) and bees exposed to 0.11 ng (62%) or 0.33 ng doses (61%) (Fig. 1). In our study, the bees returned to the hive at the average duration of 97.942 ± 221.308 min. Details of the homing success per group of bees and per test are given in Table S3. Infestation with the *Varroa* mite, *Nosema*, or DWV and temperature alone did not affect the homing flight success (Table 1).

3.2. The dose-response function depends on health status and temperature

Among the 18 colonies we studied, infestation by *V. destructor* was measured as between 0 and 33.5 mites per 100 bees. High *Varroa* load were especially recorded for colonies 14 and 15 with 33.5 and 15.8 mites per 100 bees respectively (Table S4). Spore counts of *Nosema* showed an infection gradient ranging from 2 to

Table 1
Summary of the generalized linear mixed models (GLMM) performed to assess the effect of thiamethoxam dose, health and temperature parameters as well as their interactions on honeybee homing success.

Model parameter	Multimodel averaged estimate ± s.e.	*Z*	P-*value*	Relative importance[a]
Intercept	−0.757 ± 1.530	0.495	0.621	
Dose	−2.140 ± 1.001	2.136	**0.0327**	100%
Temperature	0.062 ± 2.209	0.028	0.978	98%
Varroa	0.230 ± 1.354	0.170	0.865	100%
Nosema	2.345 ± 1.356	1.728	0.084	89%
DWV	0.205 ± 0.963	0.213	0.831	36%
Dose x Temperature	2.051 ± 0.744	2.755	**0.0059**	98%
Dose x *Varroa*	−2.864 ± 0.761	3.764	**0.0002**	100%
Dose x *Nosema*	1.534 ± 0.951	1.613	0.107	50%
Dose x DWV	−0.566 ± 0.607	0.931	0.351	13%

[a] Results are given for the multimodel inference procedure. The relative importance measures each variable's occurrence frequency within the best candidate models (n = 10), weighted by the model's respective statistical support. A relative importance of 100% indicates that the variable appears in each of the best models, and therefore receives maximal support as a potential explanatory factor of homing failure.

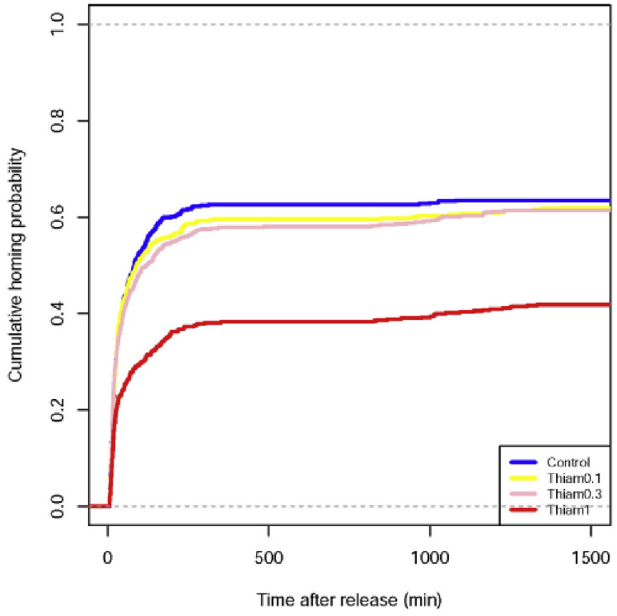

Fig. 1. Cumulative homing probability of groups of foragers during 24 h period after release. Curves were obtained from raw data. The yellow curve represents homing performances for foragers exposed to 0.11 ng per bee of thiamethoxam (544 bees), the pink curve for the 0.33 ng thiamethoxam per bee (541 bees), the red curve for the 1 ng thiamethoxam per bee (528 bees) and the blue curve for the control group of bees (556 bees). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3000×10^4 spores per bee and all the colonies were above the detection limit for the DWV load with viral levels per bee ranging from 1.60×10^7 to 1.00×10^{13}. Details of the colonies' health status are provided in Table S4.

No significant effects were found when insecticide dose was combined with *Nosema* infection or DWV load. Conversely *Varroa* infestation and temperature parameters interacted significantly with thiamethoxam exposure (Table 1, Fig. 2).

Homing success of exposed bees increases with increasing temperatures as revealed by the positive Dose × Temperature interaction (Table 1). On the other hand, homing success of exposed bees decreases with increasing *Varroa* infestation as revealed by the negative Dose × *Varroa* interaction. The significant interactions (Table 1) are represented by curves of homing success as a function of time after release (Fig. 2). Above temperatures of 25 °C and *Varroa* infestation levels of 4.8 mites per 100 bees, homing success of exposed bees is reduced and the homing gap between control and exposed bees increases (Fig. 2). *Varroa* and temperature are important explanatory factors of the variation in dose-response function because of the great relative importance (%) in the set of best candidate models (Table 1). In this study, none of the best candidate models of homing success reached a predominant weight of evidence (all weight values (ω_i) < 90%, Table S2) indicating that it is advisable to turn to multimodel averaging to derive more conservative model predictions (Burnham and Anderson, 2002).

Conditional predictions of the thiamethoxam dose-response function illustrate well that temperature modifies the shape and steepness of the dose-response function of homing failure (HF) (Fig. 3). In our experimental context, the dose-response curve is steeper at lower temperatures (16–20 °C) than at higher temperatures (24–26 °C). As an example, conditional predictions showed that an ambient temperature of 20 °C may increase HF of 1 ng-exposed bees by 29% compared to a temperature of 26 °C.

3.3. The ED addresses bio-aggressor infestation and temperature dependency

Multimodel conditional predictions show that ED_{20} decreases with increasing *Varroa* infestation (Fig. 4). For instance modeling results showed that ED_{20} is expected to be divided by a factor of 3.3 with a colony infestation of 5 *Varroa* mites per 100 bees compared to a non-infested colony at a temperature of 24 °C. At 26 °C, the predicted ED_{20} value strongly decreases when *Varroa* infestation increases. Moreover, the curves show a sharp transition from low-effect conditions at 26 °C to high-effect conditions at 16 or 20 °C.

4. Discussion

A key issue currently unaddressed by risk assessment schemes is whether the sanitary condition of the colonies can modulate the effects of pesticides tested. Our hypothesis was that the negative effect of the chemical product could be underestimated if colony infestation by bio-aggressors was not considered. In our study, exposure to 1 ng of thiamethoxam significantly decreased homing success of bees, as previously shown by Henry et al. (2012a), and this effect was more significant with increasing *V. destructor* infestation of the colonies, suggesting that the parasitic mite aggravated HF of foragers exposed to the insecticide. High *Varroa* loads were recorded for two colonies (15.8 and 33.5 mites per 100 bees, Table S4). But these high infestations levels are not out compared to what can be found in the literature. Meixner et al. (2014) showed that *Varroa* infestation in European countries could range from 0 to more than 70 mites per 10 g of bees at

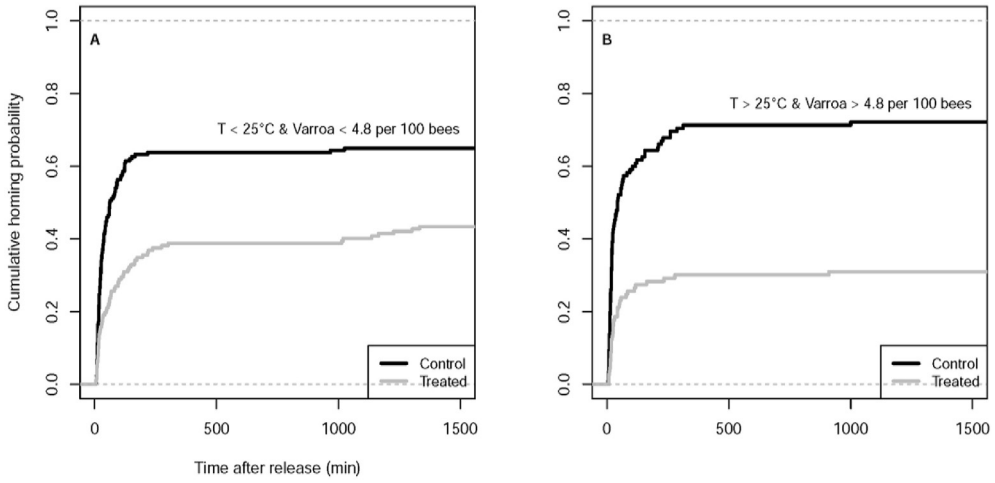

Fig. 2. Observed cumulative homing probabilities during 24 h period after release. Curves were obtained from raw data. The interaction effects between treatment (control vs bees exposed to 1 ng per bee of thiamethoxam) and temperature or *Varroa* infestation are represented by separating data based on average values of temperature (25 °C) and *Varroa* infestation (4.8 mites per 100 bees). Graph A shows homing results for control (174 bees) and exposed bees (152 bees) below average values and graph B shows homing results for control (115 bees) and exposed bees (113 bees) above average values.

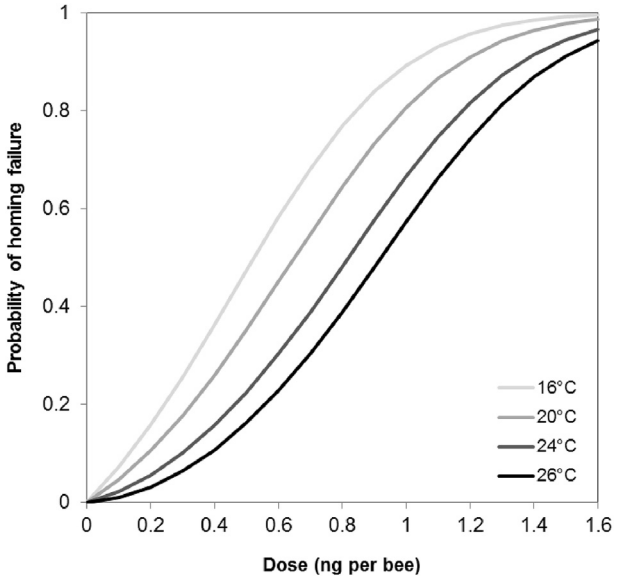

Fig. 3. Conditional predictions of the thiamethoxam dose-response function of honeybee homing failure probability. The predicted dose-response curves (with dose 0 corresponding to control) are presented for differing external temperatures.

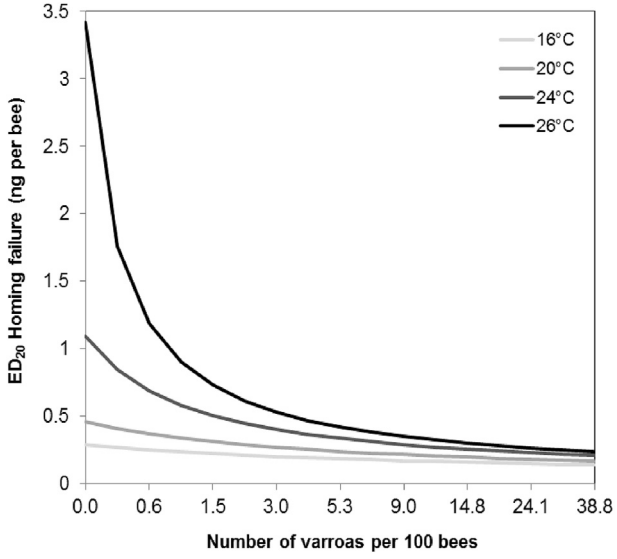

Fig. 4. Honeybee experimental homing failure delineated by the ED_{20} (effective dose (ng/bee) that leads to a 0.20 mortality rate due to homing failure compared to control bees) according to *Varroa* infestation of the colonies. Conditional predictions are presented for differing external temperatures.

autumnal period, largely including our high values. By converting our data, the *Varroa* infestation of our colonies would range from 0 to 24.1 mites per 10 g of bees. In area where the study takes place (south east of France), Kretschmar et al. (2016) recorded colonies with *Varroa* load ranging from 0 to more than 20 *Varroa* mites per 100 bees before a lavender honey flow during summer period (June to August). Ambient temperature also modulates HF of exposed bees. Moreover, greater treatment effect is expected with high *Varroa* infestation of the colonies and low ambient temperatures (Fig. 4).

Current guidelines mention that animals used for the tests must come from healthy colonies, without any visible symptoms of disease (EPPO, 2010; OECD, 1998a; OECD, 1998b). However, they fail to mention a specific threshold of *V. destructor* load for official standard tests, and *Varroa* is a major and highly prevalent parasite in honeybee colonies (Le Conte et al., 2010; Neumann and Carreck, 2010; Nazzi and Le Conte, 2016; van Dooremalen et al., 2012) that can modulate the effect of pesticides. A previous study of Blanken et al. (2015) has shown that co-exposure of the bees to *V. destructor* and the neonicotinoid imidacloprid increased negative effects on flight capacities of foragers compared to exposure to the insecticide alone.

It is known that neonicotinoid insecticides can affect motor capacity and ability to navigate, both of which are important components of the homing flight. Exposure to thiamethoxam can alter honeybee flight ability (Tosi et al., 2017). In addition, acute exposure (1.34 ng/bee) causes hyperactivity and increased flight ability while chronic exposure (1.96–2.90 ng/bee/day) produces hypoactivity and reduced flight ability. Sensory and cognitive abilities of individuals may also be affected. Bees orient themselves using previously learnt local visual cues and celestial information,

such as the position of solar azimuth and polarized light (Menzel et al., 1998; Wehner and Rossel, 1985). Thus, exposure to thiamethoxam could have negative effects on the restoration of memorized learning and as a consequence on the orientation ability of honeybees in flight. Honeybees would then have difficulties in recognizing landmarks, key elements of spatial orientation, in a well-known environment for returning to the hive (Collett et al., 1993; Fischer et al., 2014).

Our results did not show any effects of *V. destructor*, DWV or *Nosema* alone on homing success. These results are not in agreement with previous findings concerning the effect of *Varroa* on homing success (Kralj and Fuchs, 2006), the effect of DWV infection on flight performance (Wells et al., 2016) and on long term foraging behaviour under natural conditions (Benaets et al., 2017), or the effect of *Nosema* on homing success (Kralj and Fuchs, 2010), foraging behaviour (Mayack and Naug, 2010; Alaux et al., 2014; Dosselli et al., 2016), and flying capacities (Mayack and Naug, 2010). As for DWV, all colonies exhibited high loads (1.60×10^7 to 1.00×10^{13}) of the virus. These infection levels were similar to or higher than those of Wells et al. (2016) who found an effect of DWV on bee flight abilities. In our study, high DWV loads in all of the colonies may have prevented us from discriminating any potential effects on homing success. Still, it is important to keep in mind that raw DWV loads were detrended from *Varroa* mite infestation with which it co-varied positively. This means that the observed effect of *Varroa* may actually represent a combined *Varroa* and DWV synergistic effect. Moreover insecticide dose, being a prevalent factor, could mask the effects of sanitary factors as a whole.

In addition, by combining the exposure dose with the *Varroa*, *Nosema* and DWV infestation, only the interaction between the insecticide and *Varroa* infestation significantly affected the homing success of the bees. However, previous studies also showed synergistic negative effects between *Nosema* and insecticides on bees (Alaux et al., 2010; Vidau et al., 2011) and between DWV and thiamethoxam by increasing precocious foraging and HF after the first exit from the hive (Coulon, 2017).

Our present results suggest that *Varroa* mite infestation can modulate the response observed in bees exposed to the neonicotinoid, in particular by increasing the deleterious impact on homing success in the field. Foragers parasitized by *Varroa* mite at the pupal stage have been observed to forage earlier (Janmaat and Winston, 2000). If we hypothesize that a colony, heavily infested by the parasitic mite, includes younger cohorts of foragers (through accelerated age-polyethism), whose cognitive abilities needed for orientation are more fragile and easily impaired by insecticide exposure, then this interaction might further impair the remobilization of memorized information. Blanken et al. (2015) also found that the interaction between *V. destructor* and chronic exposure to imidacloprid at a field representative dose (5.98 ± 0.22 ng ml^{-1}) reduced the flight motor capacities of bees in a flight mill experiment. Moreover, the duration and the flying distance were reduced more by combined stressors than when bees were exposed to a single stressor. Authors suggested that the decreased homing capacity of bees could be explained by the negative effect of a neonicotinoid insecticide in combination with *V. destructor* on flight capacity.

The ED_{20} approach allows us to determine the level at risk of detrimental HF by thiamethoxam exposure, and how HF could be modulated by *Varroa* infestation or by ambient temperatures. Plotting the effective dose predicted to trigger an additional 20% HF compared to control bees (ED_{20}) allows us to observe that higher levels of *Varroa* infestation are associated with lower ED levels (Fig. 4). Lower ED levels are also associated with decreasing temperatures. In the same manner, HF of bees exposed to thiamethoxam was also more pronounced at lower temperatures (Fig. 3).

Our results are in accordance with those of Henry et al. (2014) who found that HF of bees exposed to thiamethoxam increases when temperatures decrease below an average of 28 °C. Recently, Tosi et al. (2016) showed that at low environmental temperature (22 °C), thiamethoxam exposure (1 and 2 ng/bee) reduced bee thorax temperatures in African honeybee foragers from the first hour to at least 24 h after treatment. Foragers use their thoracic flight muscles to produce heat during flight activity under colder environmental conditions (Coelho, 1991). Tosi et al. (2016) suggested that impaired thermoregulation could be the effect of the insecticide on the thoracic muscle activity. Then, this could explain the higher HF of exposed bees at lower environmental temperatures. From our results, the nominal tested dose of 0.33 ng per bee (real dose range: 0.276–0.438 ng per bee) of thiamethoxam was determined to be a No Observed Effect Dose (NOED) on homing success compared to control bees (Fig. 1). This result is comparable to the NOED of 0.42 ng per bee determined from the results of Henry et al. (2014). For risk assessment, tested doses are compared to exposure doses through field-relevant exposure scenarios. The worst-case scenario of residue intakes by foragers for 1 h of flying was estimated to be < 0.276 ng per bee for 20% sugar content nectar (EFSA, 2012b). This exposure scenario is below the determined NOED. But Pierre et al. (1999) reported that sugar content in nectar could vary from 10 to 30% during the blooming of winter oilseed rape. Then, field exposure estimate would vary from 0.184 to 0.552 ng per hour of foraging for 30% or 10% sugar content in nectar respectively (EFSA, 2012b), this showing some uncertainties about exposure estimate. The prior objective of our study was not to give new information about the insecticide thiamethoxam for regulators and risk assessment; this molecule being banned in Europe for outdoor uses (European Union, 2018). However, with the need to assess for possible interactions between the tested chemical and pathogen or parasite loads, our results bring insight. The different results obtained on the effect of pesticide exposure in field studies (Woodcock et al., 2017) could at least be partly explained by the colonies health status, especially *Varroa* infestation, this being under-evaluated. Then in our study, the modulation of the insecticide effects by *Varroa* might change the risk conclusions. From our conditional predictions, critical ED_{20} is, for instance, predicted to be in the range of worst case exposure estimated by EFSA with a value of 0.268 ng per bee for a colony infestation of 3 *Varroa* mites per 100 bees at 20 °C and 0.288 ng per bee for a colony infestation of 9 *Varroa* mites per 100 bees at 24 °C. Our estimated ED levels holds true for our specific experimental context considering colonies' size and should be revised with respect to those parameters whenever colony dynamics is expected to be different. Season is also another possible source of variation of ED_{20} that need to be addressed in further studies.

In conclusion, our results show that *Varroa* infestation of the colonies can modulate the effect of thiamethoxam. The interaction between both stressors can decrease the homing success of foragers. These results lead to new questions on the underlying mechanisms of our results, and in particular, the relationship between *Varroa* mite infestation, precocity of foraging and pesticide effects. Our results also show that weather conditions may influence homing results of exposed bees in which low ambient temperatures coupled with high *Varroa* load in the colonies could reduce homing success. Finally, this study underscores that bioaggressor infestation of the colonies should be targeted for a thorough risk assessment of plant protection products. In addition, the environmental context should be detailed when assessing sublethal effects in field conditions. In that respect, the ED approach could help to predict to what extent *Varroa* infestation and climatic context would need special attention.

Conflicts of interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

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